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CAUSES AND CONTROL OF ANOXIC AEROBIC (AA) (OR LOW F/M) FILAMENT BULKING IN LONG SLUDGE AGE NUTRIENT REMOVAL ACTIVATED SLUDGE SYSTEMS

by

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ABSTRACT

CAUSES AND CONTROL OF LOW F/M BULKING IN LONG SLUDGE AGE NUTRIENT REMOVAL ACTIVATED SLUDGE SYSTEMS

A problem plaguing the greater proportion of nutrient removal activated sludge systems treating municipal sewage, is the prolific growth of filamentous organisms (bulking), which inhibits the rate of sludge settling in the clarifier (secondary settling) stage of treatment and reduces plant throughput.

To find the causes of filament proliferation, a four-stage investigation was conducted: (1) A review of literature indicated that the promoted method for control of bulking in carbonaceous removal systems (the selector reactor) does not control bulking in nutrient removal systems. (2) From a laboratory-scale experimental programme it was concluded that low F/M filaments proliferate when sludge is subjected to alternating anoxic-aerobic conditions with nitrite present. (3) A review of the biochemistry of respiration led to the formulation of a biochemical model for facultative organism respiration, an important aspect of which is a series of mechanisms that give rise to inhibition of aerobic respiration following anoxic conditions. Inhibition of respiration was measured in activated sludge with aerobic batch test procedures. (4) Application of the biochemical model to filaments and floc-formers allowed the formulation of a biochemical/microbiological (bulking) model to describe the prolific growth of filamentous organisms. The model was tested and verified at laboratory-scale.

From the biochemical and bulking models, configuration modifications and operational control procedures were identified and tested, by means of which bulking can be avoided at the design stage or controlled in existing systems. The procedures produced positive results.

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SYNOPSIS

Virtually since the inception of the activated sludge system for wastewater treatment, the presence of filamentous organisms in the mixed liquor has constituted a problem in liquid–organic solids separation in the clarifier (secondary settling tank) stage of the process. High proportions of filaments cause interaction between sludge floc material, reducing the rate of sludge settleability. Increasing sludge settleability would allow an increase in treatment capacity in plants in which sludge bulking restricts throughput and would allow a reduction in secondary settling tank size in future plant designs. These benefits are the motivation for the research conducted in this investigation.

In the 1920s and 1930s bulking did not appear to be widespread and was confined, principally, to plants treating effluents derived from some industries. After the Second World War, from about 1950, bulking became a widespread problem. Causes for this have been variously attributed to (i) greater proportions of industrial waste in the influents, (ii) higher standards for the treated effluents requiring lower organic loading rates per unit of mixed liquor (longer sludge ages, lower F/M ratios), and (iii) changes in plant configuration and mixing mode (in particular, deviation from the plug–flow regime to completely mixed reactors, single or in–series).

Before the Second World War, the approach to solving the bulking problem was to look for factors such as sewage characteristics, and plant configuration and operation that appeared to be associated with filament proliferation and to control bulking by removing the cause. A distinctive feature of the era was that bulking was attributed universally to proliferation of the filament *Sphaerotilus natans.* After the Second World War there was increasing recognition that a variety of filamentous organisms could proliferate in the sludge and give rise to bulking. About 30 filamentous organisms were recognised, of which about 11 were widely present and thus of technical importance. Many of these filaments were previously unknown and hence not included in identification keys. Practical procedures for identification of these organisms in mixed cultures were developed principally by Eikelboom and his

group. Broad classifications were described, based on the specific conditions that were associated with the proliferation of certain filamentous organism types. Important in this regard was the work of Jenkins and his group. They proposed four categories of causative conditions, *viz* (i) low DO, (ii) septic wastewater/sulphides, (iii) nutrient deficiency, and (iv) low food/microorganism ratio (low F/M).

Bulking by filamentous organisms implies that the filaments compete successfully with the floc–formers for a share of the substrate. Chudoba and his group focused attention on competition between filaments and floc–formers in the search to identify causes for bulking situations. It was shown that the Monod function (specific growth rate versus soluble readily biodegradable COD) of the floc–formers and the filaments could differ appreciably. For many filaments the specific growth rate was higher than that of the floc–formers at low COD concentrations and vice versa at higher concentrations. From this it was concluded that in completely mixed reactor systems the soluble readily biodegradable COD (RBCOD) concentration would be low and the filaments would tend to dominate, but in plug–flow systems, at the inlet the soluble RBCOD concentration would be high and the floc–formers would grow faster than the filaments, abstract most of the COD, leaving the filaments with a deficiency in substrate downstream, thereby limiting their growth. They further showed that by manipulation of the plant configuration (installing a selector reactor),or mixing regime (changing to plug–flow) or feeding pattern (implementing intermittent feeding to a completely mixed single reactor), species of floc–formers are selected that have a Monod function with specific growth rates very much higher than those of the filaments. The floc–formers gain a competitive advantage over the filaments, and growth of the filaments is suppressed. The nett increased substrate utilization rate of the floc–formers was demonstrated by increases in maximum specific OUR and rate of soluble COD uptake whenever the selector effect was generated in the floc–formers. The Chudoba group further showed that in nitrifying systems, where anoxic zones were installed at the head of the reactor, filaments such as type 021N and *S. natans* were controlled. This indicated that the floc–formers are at least in part facultative and can take up the substrate in the anoxic zone, whereas the filaments must be aerobic and cannot compete for substrate under anoxic conditions.

Chudoba's hypothesis found general acceptance and application in Europe and the United States where the majority of research into the bulking problem was being

conducted. The hypothesis was widely investigated by many researchers with positive outcomes.

In South Africa, nitrification–denitrification (ND) and nitrification–denitrification biological excess phosphorus removal (NDBEPR) in activated sludge systems requires long sludge ages (15 – 25 days) and additionally the incorporation into aerobic systems of anoxic zones (for ND systems) and anoxic and anaerobic zones (for NDBEPR systems). These plants show a great propensity to bulk, with typical filaments being type 0092 and *Microthrix parvicella*, filaments that were not common in European and North American aerobic plants. In terms of Jenkins categorization these filaments develop in low F/M systems. With the increase in number of ND and NDBEPR systems in Europe and North America, development of bulking sludges with filaments of the low F/M category has become an increasing problem.

The success of Chudoba's selection hypothesis and the paucity of information on its effect in low F/M systems, prompted enquiry into the selector effect in long sludge age aerobic and anoxic–aerobic systems by the Water Research Group of the University of Cape Town. The outcome of this enquiry can be summarized as follows:

In long sludge age systems,

- the selector effect appears to have no significant effect on the proliferation or suppression of low F/M filaments in aerobic or alternating anoxic–aerobic systems,

- low F/M filaments will not proliferate in completely aerobic systems,

- a *necessary* condition for low F/M filament proliferation is alternation between anoxic and aerobic states, however,

- alternation between anoxic and aerobic states is not a *sufficient* condition for bulking because some anoxic–aerobic systems bulk with low F/M filaments whereas others do not.

These findings constituted the state of knowledge on bulking in long sludge age

systems in 1988 when the investigation reported here started.

New research directions

The finding that the selector effect does not control low F/M filament bulking placed research in this field back into an exploratory stage. Accepting that anoxic–aerobic alternation is an essential condition to stimulate low F/M filament proliferation, enquiry was conducted in two directions:

(1) Experimental investigation was directed into the conditions that promote bulking in anoxic–aerobic systems – apart from anoxic–aerobic alternation, other conditions may also be necessary for low F/M filament proliferation. A systematic study of filamentous organism response under separate aerobic and anoxic conditions, and in transition from aerobic to anoxic and from anoxic to aerobic conditions needed to be undertaken.

(2) A review was conducted of the respiratory electron transport pathways of heterotrophic facultative organisms. The observation that both low F/M filaments and floc–formers are sustained in anoxic–aerobic systems implies that both groups of organisms are facultative. Anoxic–aerobic alternation as a necessary requirement for low F/M filament proliferation would indicate that a study of the fundamental biochemical respiratory pathways of facultative organisms operating under alternating anoxic–aerobic conditions would contribute to understanding the causes for the proliferation or reduction of these filaments.

Exploratory experimental investigation

To elucidate the conditions promoting bulking, a wide ranging experimental programme was undertaken using defined artificial substrate and municipal wastewater as influents to long sludge age (≥15 days) laboratory–scale activated sludge systems subjected to a variety of aeration patterns and substrate compositions. The results can be summarised as follows:

- The presence of fats and oils had little influence on proliferation of *M. parvicella.*
- With continuous aeration, sludges with low DSVI values developed.
- With continuous unaerated (with nitrate added – i.e. anoxic) conditions, sludges

with low DSVI values developed.

- Systems developed their highest DSVIs when the aerated mass fraction was between 30 and 40%.

- Systems fed substrate with high TKN/COD ratios (resulting from ammonium addition to the influent) invariably developed sludges with high DSVI values.

- Systems fed substrate with low TKN/COD ratios developed sludges with low DSVI values.

- Nitrate and nitrite addition to the anoxic zone of alternating anoxic–aerobic systems led to an increase in DSVI and with their removal, a decrease in DSVI.

- Increases and decreases in DSVI were accompanied by decreases and increases respectively in VSS.

- Increases in DSVI were accompanied by decreases in COD and nitrogen mass balances and decreases in DSVI were accompanied by increases in COD and nitrogen mass balances.

- Sludge age (F/M ratio) appeared not to influence sludge settleability in alternating anoxic–aerobic systems; the DSVI remained high at sludge ages between 5 and 20 days.

- The filamentous organism types which were present in sludges with high DSVIs were invariably those categorized as low F/M, which dominate in full-scale systems.

- In systems with sludges exposed to alternating anoxic–aerobic conditions which developed a high DSVI, the frequency of alternation (from cycle times of 20 minutes to 3 days) had little or no effect – the DSVI remained high.

From the experimental programme, it was concluded that:
Low F/M filaments proliferate to their greatest extent with exposure of sludge to anoxic–aerobic alternation in which the aerated mass fraction is between 30 and 40 percent of the total and nitrate and/or nitrite is present throughout the unaerated

period or zone.

The fundamental reasons for filament proliferation under these conditions was unclear at the time. Additionally, it was apparent that the low F/M filament categorization was inadequate and the name anoxic–aerobic (AA) filaments was suggested as more appropriate considering the conditions under which this group of filaments proliferate.

Review of literature concerned with facultative organism respiration and formulation of a conceptual biochemical respiratory model for facultative organisms

A literature review was conducted to elucidate the major biochemical /microbiological pathways mediated by facultative organisms under separate anoxic and aerobic conditions and when subjected to frequent changes between the two conditions. This was considered essential for two reasons; first, given that filamentous and floc–forming organisms develop in activated sludge systems in which aerobic and anoxic zones are present, it can be assumed that they are essentially facultative aerobic organisms, and second, the extensive experimental programme had exhausted all obvious avenues of further investigation and a more detailed understanding of the fundamental behaviour of these organisms was required to guide further experiments.

From the literature review a conceptual biochemical model for facultative organism respiration was developed. The major elements of the biochemical model can be considered as the following:

(1) The facultative heterotrophic mass in the activated sludge is conceptualized as a single facultative organism, the respiratory behaviour of which represents the nett respiratory behaviour of the activated sludge. This is possible because the electron transport pathways (ETPs) of facultative organisms are essentially similar in their arrangement of electron transferring complexes, and in the effect of aerated and unaerated conditions on synthesis of the complexes.

(2) Facultative organisms switch from the use of one electron acceptor (e.g. nitrate/nitrite under anoxic conditions) to another electron acceptor (e.g. oxygen under aerobic conditions) when conditions change (e.g. from anoxic to aerobic); the change in use of electron acceptor is effected by the intracellular

diversion of electrons from the anoxic respiration pathway to the aerobic respiration pathway.

(3) Under anoxic conditions (with nitrate/nitrite present) and with slowly biodegradable substrate also present, one of the intermediates produced in the denitrification pathway (i.e. nitric oxide – NO) accumulates, and in switching from anoxic conditions to aerobic conditions, nitric oxide interacts with the aerobic electron transferring complexes the cytochrome oxidases, inhibiting electron transfer to oxygen, thereby inhibiting substrate utilization (determined by measuring oxygen utilization rate – OUR) under aerobic conditions.

(4) Inhibition of OUR under aerobic conditions results in denitrification of nitrite but not nitrate under aerobic conditions.

(5) With an adequate supply of electrons from readily biodegradable substrate, under anoxic conditions, nitric oxide is reduced as rapidly as it is formed; it does not accumulate, and inhibition is not induced under subsequent aerobic conditions.

(6) Under continuous aerobic conditions, the aerobic enzymes (cytochromes *o* and aa_3) are synthesized, and denitrifying enzymes (the reductases) are not synthesized and degrade with time.

(7) Under continuous anoxic conditions the reductases, and the oxidase cytochrome *o* is synthesized, but the principal aerobic enzyme, cytochrome aa_3 is not synthesized and degrades with time.

The biochemical model was examined experimentally.

To examine inhibition of substrate utilization – see (3) above – an aerobic batch test procedure was developed. Sludge from a two–reactor nitrification–denitrification (2RND) system was subjected to anoxic conditions with nitrite present to induce inhibition through accumulation of intracellular nitric oxide. Under aerobic batch test conditions, inhibition of OUR was proportional to the concentration of nitrite present.

To examine inhibition of OUR through aerobic denitrification – see (4) above – sludge from a 2RND system, (from which all nitrite and nitrate was removed), was subjected to aerobic conditions with *nitrite* present and then to an aerobic batch test. The level of inhibition induced was proportional to the length of time the sludge was subjected to aerobic conditions with nitrite present. Inhibition was not induced with *nitrate* present under similar aerobic conditions prior to the aerobic batch test.

To examine the extent of inhibition under aerobic conditions with an adequate supply of electrons to the ETP – see (5) above– an aerobic batch test was conducted on sludge from a 2RND system and nitrite was added *after* the addition of substrate – inhibition was not measured.

To examine the synthesis of aerobic and anoxic enzymes – see (6) and (7) above – a single reactor system was operated for long periods ($\approx$ 3 sludge ages) as either continuous aerobic or continuous anoxic. Aerobic batch tests to measure inhibition were conducted at regular intervals, as were denitrification batch tests to determine the extent of synthesis of the denitrifying enzymes via the denitrification rate. From these tests it was concluded that under aerobic conditions all the denitrifying enzymes degrade, and under anoxic conditions the principal oxidase (cytochrome aa_3) degrades, in agreement with the principles of the biochemical model.

<u>Formulation of a biochemical/microbiological (bulking) model for filamentous and floc-forming organism substrate competition.</u>

The biochemical model for facultative organism respiration was applied to filamentous and floc-forming organisms in activated sludge. For this application it was necessary to assume that both filaments and floc-formers are facultative organisms. A biochemical/microbiological (bulking) model was then formulated for competition between floc-formers and AA filaments for growth-rate limiting substrate under aerobic, anoxic, and under alternating anoxic-aerobic conditions. The bulking model provides an hypothesis for floc-former or AA filament dominance under different conditions and can be stated as follows:

In activated sludge systems, floc–formers and filaments compete for mutually growth–limiting substrate. Under continuous aerobic or continuous anoxic conditions, the floc–formers outcompete the filaments for substrate due to higher

substrate utilization rates and filament growth is restricted. In nitrification–denitrification (ND) and nitrification–denitrification biological excess phosphorus removal (NDBEPR) activated sludge systems, competition between filaments and floc–formers for mutually growth–limiting substrate is influenced by inhibition of the floc–formers substrate utilization under aerobic conditions. Under anoxic conditions, in utilization of substrate, the floc–formers execute the denitrification of nitrate through each of the denitrification intermediates to dinitrogen as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

nitrate → nitrite → nitric oxide → nitrous oxide → dinitrogen

In the absence of, or at low concentrations of readily biodegradable substrate, the intermediate, nitric oxide is accumulated intracellularly in the floc–formers. In the subsequent aerobic zone, intracellular nitric oxide inhibits the utilization of oxygen by floc–forming organisms as a result of the interaction of nitric oxide with the enzymes specific to aerobic respiration, the cytochrome oxidases, cytochrome o and cytochrome aa_3. Under these conditions, floc–forming organisms are inhibited in aerobic respiration. The inhibition of aerobic respiration causes electrons to be redirected to the nitrite–, nitric oxide– and nitrous oxide reductases for the reduction of the nitrogen oxides specific to these reductases (aerobic denitrification); this mechanism continues as long as nitrite remains available. Thus, under aerobic conditions a low concentration of nitrite maintains the intracellular accumulation of nitric oxide (and thereby the inhibitory effect) and higher concentrations of nitrite exacerbate the inhibitory effect. The inhibition of aerobic respiration and the phenomenon of aerobic denitrification in floc–formers results in lower substrate utilization rates and lower nett energy yields.

In contrast to floc–formers, the filamentous organisms are nitrate reducers and execute only part of the denitrification pathway, i.e. the reduction of nitrate to nitrite as follows:

$$NO_3^- \rightarrow NO_2^-$$

They do not accumulate nitric oxide and therefore are not inhibited in aerobic respiration in the subsequent aerobic zone.

Thus, in systems in which sludge is exposed to alternating anoxic–aerobic conditions with nitrite present in the anoxic zone, floc–formers are placed at a disadvantage in the competition for substrate under subsequent aerobic conditions and filaments gain an advantage. Given sufficient advantage, the filaments proliferate with time.

The hypothesis was examined experimentally, firstly by experimental testing of the principal aspects of the model, and secondly, by application of the model to the significant findings of the exploratory experimental investigation, as a means of gauging the general applicability of the model.

With regard to testing the principal aspects of the model: Dissimilar extents of nitrate reduction between filaments and floc–formers under anoxic conditions was tested with a nitrate reduction test. A sludge sample with a high DSVI – containing many filaments ($NO_3^- \rightarrow NO_2^-$) produced NO_2^- only, in 8 out of 10 tests and a sludge containing few filaments ($NO_3^- \rightarrow N_2$) produced N_2 in 8 out of 10 tests, providing support for the assumption that floc–formers are denitrifiers, and filaments are nitrate reducers.

The effect of the presence of nitrite on filament growth in sludge subjected to alternating anoxic–aerobic conditions was tested with continuous nitrite addition to the 2nd anoxic zone of an NDBEPR system. The DSVI increased rapidly, and upon stopping nitrite addition, the DSVI decreased rapidly.

With regard to application of the model to the results of the experimental investigation, the model can explain virtually all the findings listed (1) to (12) earlier.

<u>Measures for control of AA filament proliferation</u>

The principles of the biochemical and bulking models were taken into account in the design of procedures for preventing AA filament bulking at the design stage of activated sludge systems, and in ameliorating AA filament bulking in systems in which it develops.

In design of ND and NDBEPR activated sludge systems, it is essential that the denitrification capacity of the anoxic zone is not exceeded so that nitrite is not present in excess to cause accumulation of nitric oxide and inhibition of floc–former substrate utilization under subsequent aerobic conditions. This can be accomplished

by manipulation of the a– (aerobic–anoxic) recycle to ensure that the nitrate load to the anoxic zone immediately prior to the aerobic zone (i.e. the 2nd anoxic zone in MUCT systems or the primary anoxic zone in modified Bardenpho, UCT and Johannesburg systems) never exceeds the denitrification potential of that zone.

Summary

In closure, this investigation has contributed significantly to knowledge regarding the problem of low F/M filamentous organism bulking. When the investigation commenced, the conventional practice for control of filaments (the selector reactor), although successful for some filament types, had been discredited as a control measure for low F/M filaments. With this as a background, the investigation has:

- Investigated the effect of numerous factors on low F/M filament proliferation through an extensive experimental programme; the specific conditions resulting in low F/M filament proliferation were elucidated and on the basis of these conditions a name change was suggested, from low F/M to AA filaments.

- Formulated a conceptual biochemical model for facultative organism respiration as a consequence of an extensive review of the literature of facultative organism respiration under a variety of conditions.

- Verified the biochemical model with activated sludge subjected to specially developed aerobic batch test procedures.

- Developed a conceptual biochemical/microbiological (bulking) model to explain the proliferation or not of AA filamentous organisms under virtually all conditions encountered in activated sludge systems.

- Verified the bulking model by controlling and inducing bulking incidents in laboratory–scale NDBEPR systems and by applying the model to all significant laboratory–scale experimental results produced during the exploratory experimental investigation.

- Proposed, and tested at laboratory–scale, procedures for control of bulking at the design stage and during operation of full–scale ND and NDBEPR systems.

It is concluded that these developments have established a basic framework which

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LIST OF SYMBOLS

SYMBOL	DESCRIPTION
AA	Anoxic–aerobic
ADP	Adenosine diphosphate
AMO	Ammonium monooxygenase
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
aq	Aqueous
BEPR	Biological excess phosphorus removal
COD	Chemical oxygen demand (mg/ℓ)
cs	Cysteine
Cyt	Cytochrome
Cu	Copper
BT	Batch test
CoA	Coenzyme A
d	Days
h	Hours
hs	Histidine
DBT	Denitrification batch test
DENOX	Denitrification of nitrogen oxides
DO	Dissolved oxygen (mgO/ℓ)
DPNH	Diphosphopyridine nucleotide (reduced)
DSBT	Defined substrate batch test
DSVI	Dilute sludge volume index (mℓ/g)
E_h	Redox potential (mV)
ETP	Electron transport pathway
F	Faradays constant (cal/volt)
$\Delta\mu_H$	Change in proton motive force
$\Delta\psi$	Change in electrical charge (volt)
FAD	Flavin adenine dinucleotide (oxidized)
$FADH_2$	Flavin adenine dinucleotide (reduced)
Fe	Iron
FeS	Iron–sulphur
F/M	Food/microorganism (mgCOD/gVSS)
F_p	Flavoprotein

FMN	Flavomononucleotide (oxidized)
$FMNH_2$	Flavomononucleotide (reduced)
g	Gaseous
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HAO	Hydroxylamine oxidoreductase
IAND	Intermittently aerated nitrification–denitrification
K_s	Half saturation coefficient
k^1	Initial denitrification rate
k^2	Secondary denitrification rate
k^3	Tertiary denitrification rate
mins	Minutes
MLE	Modified Ludzack–Ettinger
MLSS	Mixed liquor suspended solids (mg/ℓ)
Mo	Molybdenum
MUCT	Modified University of Cape Town
n	Number of electrons transferred in the reaction
N	Nitrogen
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NaR	Nitrate reductase
ND	Nitrification–denitrification
NDBEPR	Nitrification–denitrification biological excess phosphorus removal
NiR	Nitrate reductase
NO	Nitric oxide
NOR	Nitric oxide reductase
NO_2^-	Nitrite
NO_3^-	Nitrate
N_2	Dinitrogen
N_2O	Nitrous oxide
N_2OR	Nitrous oxide reductase
OUR	Oxygen utilization rate (mgO/ℓ.h, mgO/gVSS.h)
O/CFCM	Fully aerobic, constant feed, completely mixed
O/CFCM/SEL	Fully aerobic constant feed completely mixed with an aerobic selector
O/IFFD	Fully aerobic intermittently fed fill and draw

ox	Oxidized
p	Phosphorus
polyP	Polyphosphate
PWWF	Peak wet weather flow
Q	Ubiquinone (oxidized)
QH_2	Ubiquinol (reduced)
R	Universal gas constant
RBCOD	Readily biodegradable COD (mg/ℓ)
red	Reduced
R_s	Sludge age (d)
S	Sulphur
s	Seconds
SBCOD	Slowly biodegradable COD
$SSVI_{3,5}$	Stirred settling volume index (g/ℓ)
SVI	Sludge volume index
T	Temperature
TCA	Tricarboxylic acid
TKN	Total Kjeldahl nitrogen concentration (mgN/ℓ)
TMPD	N,N,N'–N'–tetramethyl–ρ–phenylenediamine
UCT	University of Cape Town
μm	Micrometers
μ_{max}	Maximum specific growth rate
VSS	Volatile suspended solids (mg/ℓ)
2RND	Two–reactor nitrification–denitrification
°C	Temperature in degrees Celcius

CHAPTER 1

INTRODUCTION

In the Republic of South Africa, the need for high quality effluents from sewage treatment plants is two-fold. Firstly, the country is rapidly approaching the point of maximum economic exploitation of conventional water resources, and high quality treated effluents are increasingly becoming a resource in their own right to augment conventional resources. Secondly, the country faces a deterioration in quality of the existing conventional water resources from the discharge of inadequately treated wastewaters to rivers, lakes and dams. The quality of effluents from wastewater treatment plants has, in consequence, become a matter of national importance.

The quality demanded of effluents, chemically and bacteriologically, has evolved within the constraints of the treatment technologies available and with due regard for the requirements of the major water users in the country, i.e. the domestic, agricultural, industrial, recreational and conservational sectors. As a consequence of limited water resources, virtually every river and dam is utilized to some degree so that the maintenance of a "healthy" state in all the rivers and storage dams is a matter of vital importance.

The evolution of South African Water Law [1]

The quality of effluents in wastewater treatment is controlled by the principles of South African water law. South African water law has evolved through a fusion of Roman-Dutch law from Dutch settlement of the Cape, and English law from later British rule. Under Dutch rule, the Roman-Dutch principle of *primus fluminus* applied, which vested the State with extensive control over the use of surface waters. Under British rule, the English legal principle of riparian ("on or of the river bank") ownership vested the State with minor control.

With time, unconditional riparian ownership of water came to be inadequate to ensure optimal use of the country's water resources and consequently there was a

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[1] For a more extensive review of this topic, see Chapter 8.2 of Management of the Water Resources of the Republic of South Africa. Department of Water Affairs, 1986. CTP Book Printers, Cape Town.

reversion to State control. Following the report of a commission of enquiry into South African water law in 1952, the Water Act (Act 54 of 1956) was promulgated. In this Act the State assumed greater control over the water resources of the country, although riparian ownership still plays a role in the determination of rights to the use of public water. Engendered in the Water Act was a reversal in philosophy regarding the treatment and disposal of wastewater. Prior to the Water Act, the Union Health Act (1919) required land disposal of effluents, prohibiting local authorities from discharging effluents into watercourses without special permits. In contrast to this, the Water Act made it obligatory to return treated effluents to the river of origin. This requirement focussed attention on adequate treatment of the waste flow before discharge to the receiving watercourse.

Development of technology to meet required effluent standards.

The Water Act is the enabling statute which provides for the promulgation of legislation to control the quality of discharged effluents so as to protect the environment, safeguard the health of the water users and ensure multiple reuse of the water. Three standards have been promulgated in terms of the Water Act: a General Standard applicable to all water bodies, a Special Standard for specified streams, and a Special Standard for Phosphate, applicable only to certain sensitive catchment areas. The General Standard (1962) requires the removal of carbonaceous material to a certain limit and includes a requirement for nitrification of ammonium to nitrate to avoid the effect of ammonium toxicity on aquatic environments. The Special Standard (1962), *inter alia*, places a limitation also on the total nitrogen in the discharge. The Special Standard was promulgated in response to the increase in algal growth in some water bodies in the 1960s and was a consequence of the perception that nitrogen was the limiting nutrient for aquatic plant growth. At this time the most common sewage treatment process was the trickling filter. Although this technology proved adequate for satisfying the General Standard, it was inadequate for meeting the requirements of the Special Standard with regard to the nitrogen effluent quality. To satisfy the nitrogen requirements of the Special Standard, interest developed in the activated sludge process in the late 1960s. To accommodate nitrogen removal in the single sludge aerobic (carbonaceous material removal) activated sludge system, an unaerated zone was included which ensured denitrification, and in 1973 following the research developments of Barnard (1972, 1973), the first biological nitrification–denitrification (ND) treatment plant in South Africa (the four–stage Bardenpho process) was commissioned.

Research into eutrophication during the 1960s had brought about a realization that, for many water bodies, phosphorus rather than nitrogen is more likely to be the growth limiting nutrient. Also, research with ND plants in the 1970s had led to the development of a process modification that facilitated biological phosphorus removal in addition to nitrogen removal, namely the nitrification–denitrification biological excess phosphorus removal (NDBEPR) system. Although this technology was not well developed, in a large measure its availability led to the promulgation of the Special Standard for Phosphate (1980); however the legislation permitted a 5 year period of grace before its enforcement to allow further development and application of the technology. Although phosphorus could be removed with physical–chemical methods, the associated increase in the salt concentration of the treated effluent was considered a grave disadvantage, which limited the reuse value of the water and would exacerbate the mineralization problems already apparent in South Africa's surface water. The biological method had the distinct advantages of lower cost without the higher effluent salt concentrations associated with chemical addition. To achieve maximum P removal by biological means, a number of versions of NDBEPR systems have been developed in South Africa for wastewaters with different influent characteristics, namely the 5–Stage Bardenpho, UCT, Modified UCT, and Johannesburg systems.

<u>A problem encountered in ND and NDBEPR activated sludge systems</u>

The biology, biochemistry, kinetics and stoichiometry of the processes in the ND and NDBEPR plants are now understood to such a level that within the temperature range in South Africa (14 to 24 °C) the system can be designed with confidence to meet the required effluent standards. Indeed, in South Africa the NDBEPR system is given prime consideration whenever a new wastewater treatment plant is planned; this system has come to be looked upon as the standard biological system for medium– and large–scale wastewater treatment plants. In these plants there may be a need for chemical addition, but only in support of, or to augment nutrient removal where the biological process alone cannot achieve the required effluent quality. However, experience with ND and NDBEPR plants in South Africa (and elsewhere) has brought to light a behavioural response that has a significant effect on the economics of design of the system – the systems are prone to produce sludges that settle poorly in the clarifier (secondary settling tank) stage of treatment, where the sludge and treated effluent are separated by gravity sedimentation settling.

The activated sludge biological culture which develops in ND and NDBEPR systems (variously referred to as the ecosystem, bioculture or biocenosis) is diverse in organism type, function and physical characteristic. The organisms in activated sludge can be categorized on the basis of their morphological characteristics, either as ***filamentous*** or ***floc–forming***. The relative proportions of filamentous and floc–forming organisms largely determines the settleability of the sludge in the final stage of treatment, the clarifier. Absence of filamentous organisms gives rise to a sludge that settles rapidly but clarifies poorly, in that small sludge particles remain in the supernatant, a condition termed "pin–point floc" which results in loss of solids with the effluent. Presence of excessive filamentous organisms in a sludge inhibits its settleability in the settling tank and is referred to as a ***bulking sludge***. A bulking sludge may result in gross sludge loss with the effluent i.e. secondary settling tank failure, under normal design operating conditions. For optimum settling tank performance, *some* filaments should be present in sufficient numbers such that their growth within the floc gives the floc structure thereby enhancing the sludge clarification characteristics, but the filaments should not proliferate to the extent that filament bridging between the flocs, with associated diffuse floc–formation, inhibits sludge settleability. The correct proportion of filaments and floc–formers produces a rapidly settling sludge that also clarifies the surrounding mixed liquor.

Bulking sludges are common in nutrient removal activated sludge plants in South Africa. A survey during 1985 of 96 ND and NDBEPR plants showed that 32 percent of the plants had problems with filamentous bulking sludges; a further survey in 1987 of 26 NDBEPR plants showed that 82 percent had bulking problems caused by filamentous organisms.

In many of the existing NDBEPR plants, the low rate of sludge settleability results in a considerable loss of treatment capacity of these plants. Controlling the proliferation of filamentous organisms, thereby maintaining a good settling sludge in the plant would permit significantly higher wastewater flows (50 to 100%) to be treated in existing plants, resulting in huge capital savings. The possibility of achieving such savings is the motivation behind the research into the causes and control of filamentous bulking in ND and NDBEPR systems.

<u>Investigating the bulking phenomena</u>

The discussion above has focussed on bulking problems in ND and NDBEPR

systems in South Africa, but the problem is not confined to these systems and country; it has been encountered worldwide in many different kinds of activated sludge system, including ones that do not have unaerated zones, i.e. completely aerobic systems. Indeed, research into filamentous bulking control outside of South Africa has been focussed principally on the problems associated with completely aerobic systems. In these investigations it has been found that a number of different filamentous organisms contribute to the bulking condition and considerable effort has been directed at identifying the conditions in the system that give rise to proliferation of the different filamentous organism types. The outcome of this research indicated that the filaments which give rise to bulking in aerobic systems are not the same as those in ND and NDBEPR plants. This, and other results led to the categorization of the filaments in terms of the condition that *apparently* gives rise to their proliferation, viz. low dissolved oxygen (DO), low food/microorganism (low F/M) ratio, septic wastewater, nutrient deficiency, and low pH. In terms of this categorization, the filamentous organisms that proliferate in ND and NDBEPR systems belong mainly to the low F/M category. This categorization seemed reasonable because the ND and NDBEPR plants invariably are low F/M (equivalently – long sludge age) plants.

As a consequence of the dominance of low F/M filaments causing the bulking problems in ND and NDBEPR plants, research was commenced to evaluate the strategy that was promoted for the control of the low F/M filaments. This strategy is based on the so–called selector effect.

A selector effect is induced in an activated sludge when a readily biodegradable COD (RBCOD) concentration gradient is established in the system by modifying the mixing regime of the system by introducing plug–flow conditions, compartmentalization, or a selector reactor, the last being a small reactor receiving the sludge return and influent flow before the main aeration reactor. With these modifications, the floc–formers in the activated sludge acquire a high RBCOD uptake rate allowing them to successfully compete for RBCOD substrate against the filamentous organisms. However, when a selector (either aerobic or anoxic) was placed ahead of an intermittently aerated reactor mimicking an Orbal or Carousel nitrification–denitrification system, the selector effect was found to be not effective for controlling the proliferation of the low F/M filaments. Furthermore, anaerobic reactors in the NDBEPR systems, which in effect serve the same function as the selector reactor in that they also allow preferential uptake of RBCOD (or rather its

formation by products such as volatile fatty acids) by floc–formers (in this case the polyP organisms responsible for the BEPR), also were not effective in controlling the low F/M filaments. Curiously, considering that these filaments were the so–called low F/M ones, continuous aeration (i.e. completely aerobic but maintaining the low F/M condition) was found to successfully control them, but the completely aerobic conditions were then not adequate for NDBEPR. It became apparent that the selector effect approach for understanding the low F/M filament bulking problems was no longer productive for guiding experiments into the causes and control of the filaments in this category. This set–back placed research into an exploratory phase requiring new research directions to be identified and pursued, in order to understand the causes and control of low F/M filament bulking.

The failure of the selector effect to control low F/M filament bulking in ND and NDBEPR systems prompted a wide–ranging exploratory programme into the causes of proliferation of these filaments in ND and NDBEPR systems. The programme investigated the problem through two avenues, a laboratory–scale experimental investigation, and a literature review of facultative organism respiration. The results of the experimental investigation are described in Chapter 3 and the literature review is summarized in Chapter 4. From the literature review, a conceptual biochemical model for facultative organism respiration was developed and experimentally tested in Chapter 5. From application of the biochemical model to filamentous and floc–former respiration, a biochemical/microbiological (bulking) model was formulated and is tested experimentally in Chapter 6. Conclusions and recommendations from the investigation are set out in Chapter 7.

It should be noted that not all the information has been gleaned from the experimental results. While the results were critically evaluated and examined insofar as their support or not of the hypothesis is concerned, by virtue of the large number of experimental systems operated, there is still a considerable amount of information that can be abstracted from them. These results will emerge with time as the consequences and implications of the new conceptual structure for understanding AA filament bulking in ND and NDBEPR systems takes root. In the description of the experimental data, all the details are not given in the text; only as much as is required to follow and support the line of reasoning; detailed descriptions of the experimental work are given in Appendices A and B.

During the review of facultative organism respiration a large volume of information

was examined with regard to the electron transport pathways of facultative organisms. The pertinent information has been included in extensive reviews in Appendix C (dealing with the electron transport pathways of facultative organisms) and Appendix D (dealing with the effect of the presence and absence of oxygen on the electron transport pathways).

To complete the review of respiration of organisms in activated sludge from a biochemical standpoint, the biochemical mechanisms of nitrifying organisms are reviewed in Appendix E.

CHAPTER 2

REVIEW OF BULKING

ABSTRACT

The course of change in perception regarding the factors responsible for filamentous organism bulking in activated sludge systems is examined. The conceptual and experimental work responsible for identification of the conditions resulting in the organism classification category "low F/M", are described. The chapter concludes with a summary of work conducted by the UCT Water Research Group, including a discussion of the conditions under which selector reactors are successful or not, elucidation of an experimental artifact present in many laboratory–scale systems which is often the cause of erroneous conclusions regarding low F/M bulking, and identification of one set of conditions which is necessary for low F/M bulking to occur.

2.1 INTRODUCTION

Activated sludge is a biocenosis of microorganisms that includes an extremely large number of species. The variety of species that develop and their concentrations, are essentially governed by four factors: composition of the influent substrate, environmental conditions imposed, species metabolism, and species interaction.

Substrate consists of (1) organic material for heterotrophic organism growth and energy generation, (2) inorganic materials that serve as an energy source for autotrophic growth and, (3) nutrients which are essential for the formation of the metabolic material and the generation of energy. The organic material is present in a variety of forms: chemical – fats, proteins and carbohydrates; physical – soluble and particulate; biodegradable – readily and slowly biodegradable and unbiodegradable. The inorganic materials include the nutrients nitrogen and phosphorus and a variety of trace nutrient elements and growth factors.

The spectrum of environmental conditions are: mixing regime (plug, intermediate or completely mixed); reactor configuration (in–series or single reactors); oxygen present or absent to give anaerobic, anoxic and aerobic states; cyclic loading of substrate and nutrients; organism retention time (sludge age); temperature; pH; and

others.

Factors affecting species metabolism are substrate utilization rate, physiology of species (aerobic, facultative, anaerobic), endogenous mass loss, death and others.

Examples of species interaction are competition, symbiosis, inhibition, commensalism, mutualism, etc..

These four factors influence the population structure so that very different populations may develop with changes in one or more of the factors. Species may attain a relative dominance with one set of conditions and decline to a relative insignificance with another set. Of importance is that the population structure that develops, whether stable or transient, is a natural ecological response.

In the activated sludge system, by imposing specific environmental conditions and substrate supply, the growth of certain species can be promoted so as to fulfill some desired function. Examples are: (1) with a sufficiently long aerobic sludge age the nitrifying organisms develop and produce an effluent low in ammonia; (2) with an appropriate sludge age, and a sequence of in–series reactors operating in anaerobic, anoxic and aerobic states, with inter–reactor recycles, different species of microorganisms develop that (i) nitrify, (ii) denitrify and (iii) store phosphorus in excess of their metabolic requirements; by these means the eutrophic elements, nitrogen and phosphorus are removed from the wastewater. Indeed, much of the later research into the activated sludge process has been concerned with finding the environmental conditions and substrate components that promote the growth of such organisms. However, imposing these conditions may also stimulate the growth of other organisms which may, in some situations, act adversely on the system operation – an important group of organisms which develop and affect plant performance is the filamentous microorganisms.

2.2 FILAMENTOUS BULKING AND SETTLING TANK PERFORMANCE

Quantities of filamentous organisms are found in most activated sludge systems; indeed their presence is essential for effective operation of the plant. The final stage in activated sludge systems is the separation of the organism mass from the stabilized wastewater in the secondary settling tank, and return of the separated mass back to the reactor basin. For efficient liquid–solid separation the organisms must aggregate in compact flocs which settle readily. This aggregation is promoted

if filamentous organisms are present in a "reasonable" concentration. If absent, pin–point flocs form which may lead to inefficient solid–liquid separation with floc escaping in the effluent. However, if present in excess, the filaments bind separate flocs in a web–like structure which impedes the separation, to give rise to the phenomenon known as bulking sludge.

The bulking behaviour of a sludge has come to be defined in terms of the settling properties of the sludge, not in terms of the effluent quality from the settlers; widely used parameters are the sludge volume index (SVI), or the diluted sludge volume index (DSVI) or the stirred settling volume index at 3,5 g/ℓ ($SSVI_{3,5}$). Of these parameters, the DSVI and $SSVI_{3,5}$ give improved correlation with the settling behaviour in the secondary settling tank over that provided by the SVI (Ekama and Marais, 1986a).

Defining bulking in terms of the settling properties of the sludge is necessary in order to eliminate situations where sludge loss to the effluent is due to inadequate hydraulic design of the secondary settling tank or due to pin–point floc formation (good settling but poor flocculation) from toxic materials or very low loaded (very long sludge age) aerobic systems. With some influents (usually industrial) at relatively short sludge ages, bulking may occur due to massive development of zooglea. Consequently microscopic examination of sludge is an essential requirement, along with the settleability test, to establish the nature of the bulking incident.

In design of the secondary settling tank, the requirement to accommodate bulking sludges has significant technical and economic consequences. Sludge settleability governs the daily flow and load that can be treated in an activated sludge plant. For a particular plant the influent peak wet weather flow (PWWF) sets the overflow rate (m/h) in the secondary settling tank, and the daily mass of COD treated and sludge age determines the sludge mass in the biological reactor. Ekama and Marais (1986a) showed that a sludge with a DSVI of 150 mℓ/g can be handled satisfactorily in the settling tank up to a maximum overflow rate of 1 m/h at a mixed liquor suspended solids (MLSS) of 3,5 g/ℓ. Should the DSVI deteriorate to 200 mℓ/g the maximum overflow rate reduces to 0,6 m/h at 3,5 g/ℓ, or to maintain the same overflow rate (at 1 m/h), the reactor MLSS must be reduced to 2,4 g/ℓ – with a DSVI increase from 150 to 200 mℓ/g, about 33 percent less flow and load can be treated in the plant. In contrast, if the DSVI is reduced from 150 to 100 mℓ/g

the overflow rate can be increased to 1,8 m/h at 3,5 g/ℓ, or, the reactor concentration can be increased to 5,4 g/ℓ at an overflow rate of 1 m/h – a 54 percent increase in flow and load can be treated.

Clearly the settling tank can be the bottle–neck in plant performance. Should a plant be designed for a specific load and DSVI and during operation the DSVI is found to be higher, the settling tank will limit the attainable load to less than the design load, or to accommodate the expected load, augmentation of the settling tank capacity will be needed. Controlling, or preferably eliminating bulking therefore holds promise of major financial savings.

2.3 BULKING OCCURRENCE

Subsequent to the development of the activated sludge process by Arden and Lockett (1914), bulking phenomena were reported in the 1920s and early 1930s in the countries where this process was becoming a recognised treatment method. Examples are, in England by Scott (1928), in Holland by Smit (1934), and in the United States by Ruchhoft and Watkins (1928). Doubtless earlier reports can be found. According to the ATV Working Report (1989), up to the 1950s the problem was not a serious one in municipal plants; bulking was encountered mainly in plants treating a high proportion of industrial wastes. However, from 1950, bulking was increasingly encountered in municipal activated sludge plants. In the 1970s and early 1980s, surveys of several hundred wastewater treatment plants in Europe showed that bulking occurred in 50 percent of the plants (Hünerberg *et al.*, 1970; Eikelboom, 1975; Matsché 1977; Wagner, 1982a). In England, Tomlinson (1976) in a survey of 65 plants found that 41 (63 percent) "either experienced serious loss of solids due to bulking or had SVI values greater than 200 mℓ/g".

One important point to note is that these plants were *overtly* aerobic systems mainly designed not to nitrify, that is, the plants were operated at relatively short sludge ages. However, bulking by filamentous organisms has not been limited to aerobic systems: from the early 1970s in South Africa, substantive developments in the activated sludge process have taken place, to remove biologically the eutrophic elements phosphorus and nitrogen. Removal of these substances from the waste flows is particularly urgent in South Africa because the small water resource necessitates long–term storage of water in dams. An appreciable fraction of the flow to these dams may be derived from effluents from the wastewater treatment plants. Influent wastewaters to these treatment plants have relatively high phosphorus and

nitrogen contents and if these are not removed they add to the phosphorus input to the dams and exacerbate the problem of eutrophication of these water bodies.

As mentioned earlier, these nitrogen (N) and phosphorus (P) removal plants have in–series configurations with anaerobic, anoxic and aerobic reactors, inter–reactor recycles and are operated at sludge ages ranging from 15 to 30 days which, from a European perspective, would be considered to be very long sludge ages. Basically, there are two types of biological N and P removal systems, nitrification–denitrification (ND) for N removal only and nitrification–denitrification biological–excess–phosphorus removal (NDBEPR) for both N and P removal, the latter also called a nutrient removal system. In a survey of 111 wastewater treatment plants in South Africa, Blackbeard *et al.* (1986) found that 62 produced bulking sludges. Of the 111 plants surveyed, 33 were nutrient removal and the balance, N removal, either intentionally or inadvertently. In a subsequent survey, Blackbeard *et al.* (1988) focused attention on nutrient removal plants. These plants have become the preferred method for removing N and P from municipal wastewaters in South Africa. Today there are about 45 nutrient removal plants in operation, with sizes ranging from 2 to 150 Mℓ/d, with a total design capacity of about 1200 Mℓ/d. From the survey on bulking and foaming in nutrient removal activated sludge plants in South Africa, Blackbeard *et al.* (1988) found that of 33 nutrient removal plants investigated, 27 produced bulking sludges.

From the review above, clearly, bulking due to filamentous organisms has a long history of occurrence and at present still constitutes a major problem in activated sludge systems. Bulking is encountered over virtually the entire spectrum of systems, from short and long sludge age aerobic systems operating in plug flow, in–series and completely mixed regimes, to long sludge age anoxic–aerobic and anaerobic–anoxic–aerobic nitrogen and phosphorus removal systems.

In earlier reports on bulking some authors appeared to view bulking by filamentous organisms as an invasive sick condition of the sludge, for example, Smit, (1934) speaks of "....the state of bulking in a diseased sludge caused by the development of threadlike organisms." Donaldson (1932) speaks of filamentous organisms as "....the weeds of activated sludge." This point of view is echoed even in recent times when at a conference a participant spoke of bulking as the AIDS of activated sludge.

2.4 CONTROL OF FILAMENTOUS BULKING

There are two approaches to bulking control, (1) non-specific and (2) specific. With non-specific control some toxicant, usually chlorine, although ozone and hydrogen peroxide also can be used, is dosed into the activated sludge system. Because the filamentous organisms causing the bulking extend beyond the flocs into the liquid, they are more exposed to the toxicant and therefore are selectively killed; in contrast the floc-formers are not seriously affected by the toxicant because they find protection inside the sludge flocs. Due to the selective killing of the filaments, their numbers and lengths are reduced and the bulking problem is ameliorated. The toxicant affects all filaments irrespective of type and for this reason this method of ameliorating bulking is termed non-specific.

The procedures for implementation of chlorination is well documented in the literature such as in the bulking control manual of Jenkins *et al.* (1984a). The method has been tested at laboratory-scale for biological N & P removal systems (Lakay *et al.*, 1988) and found to be satisfactory provided the guidelines set down by Jenkins *et al.* (1984a) are followed. However, chlorination has a rather serious shortcoming in that undesirable compounds such as trihalomethanes and chlorinated hydrocarbons tend to form which pose a potential health risk in the event the treated effluents are reclaimed for potable water supplies. To avoid this problem van Leeuwen (1988) and van Leeuwen and Pretorius (1988) investigated the use of ozone for bulking control in an N & P removal pilot plant. They concluded that ozonation successfully controls filamentous bulking and imparts additional benefits i.e. (1) improves the removal of organic substances, (2) aids nitrification and to some degree biological excess P removal (BEPR) and (3) produces an effluent that is more suitable for reuse than effluent from activated sludge treatment plants without ozonation.

The problem with non-specific bulking control is that as soon as toxicant dosing is terminated, the filaments regrow and, inexorably, bulking conditions return. This is because non-specific bulking control deals with the symptoms of bulking, i.e. reduces the filaments, but does not remove the causes of the filament proliferation on a permanent basis.

Specific control of bulking focuses on identifying and eliminating the conditions that promote the proliferation of the specific nuisance filaments causing the bulking problem. Once these conditions are identified, it may be possible to create

environmental conditions in the activated sludge plant which would inhibit or suppress the growth of the filamentous organisms. If successful, the method would provide a permanent solution to the particular bulking situation. Accordingly, this review will focus on the more desirable specific control of bulking.

2.5 CAUSES OF BULKING

In the 1920s and 1930s bulking was widely imputed to be due to *Sphaerotilus natans*. According to Smit (1934), it was Ruchhoft and Watkins (1928) who first succeeded in "....cultivating an organism [*S.natans*] which in pure culture closely resembled the threads present in bulking sludge". Smit (1934) appears to have been the first to show that filamentous species other than *S. natans* could be present in a bulking sludge.

Efforts at identifying the causes of bulking and its possible amelioration has in most instances followed a practical route by attempting to link bulking to some factor in the influent, the design or operation of the plant. Examples are:

Carbohydrate in the influent was implicated in bulking Scott (1928), but Smit (1934) showed that this would apply only in influents high in carbohydrate and could not explain bulking with a normal sewage influent which usually has a low carbohydrate content.

Donaldson (1932) attributed the cause of bulking to the mixing régime in aeration tanks. He suspected that filamentous organism growth in the rectangular diffused aerated tanks was due to back mixing, thereby changing the intended plug–flow regime towards a completely mixed one. As a solution he proposed that the tanks should be baffled.

Tomlinson (1976), from an extensive survey of occurrence of bulking in England, on 65 plants, attempted to identify some of the causes of bulking, *inter alia*, by statistical based analysis. From questionnaires to operators of these plants he could identify no "... overall correlation between the occurrence of bulking and any of the parameters which are normally measured, or specified in design, for example sludge loading, sludge age, retention time, power input, dissolved oxygen concentration, temperature, proportion of industrial waste waters, sewage septicity." He did, however, establish that the mixing regime influenced bulking – plants employing plug flow configurations were less prone to bulking than plants employing complete

mixing, an aspect already noted by Donaldson (1932).

Tomlinson concluded that the survey could not produce more specific information because it dealt with long–term behaviour and bulking often occurred over relatively short periods, and, the range of plants in size and design was very large so that specific causes could not be identified reliably in the statistical analysis.

As to the methods employed by operators to overcome bulking, Tomlinson lists 18 procedures including: increase/decrease of MLSS, hydraulic retention time, sludge recycle ratio, aeration, sludge wasting rate; re–seeding with fresh sludge; control of industrial discharge; ceasing aeration for short periods; aeration of the recycle; and others. Some of these procedures showed success but the *ad hoc* nature in application made it difficult to identify viable procedures for general application.

The ATV Working Report (1989) listed the main factors that gave rise to bulking in the 1950s as:

- Increased proportion of industrial wastewater in municipal plants.
- Increased demands on efficiency and as a result, a tendency to reduce the loading rates
- Operating with completely mixed tanks
- Distributed wastewater feed

In the 1960s identification and classification of filamentous organisms gained increasing attention. Association was sought between the specific filament species and the operational/environmental conditions and influent characteristics. Isolation and identification of the different filamentous species proved a difficult task; many species had not been identified previously and therefore did not appear in reference manual identification keys. Farquhar and Boyle (1971) based identification on morphology and staining and comparing these to a key derived from a number of sources. However, as many of the filaments in activated sludge had not yet been identified and listed, this approach was inadequate. Eikelboom and van Buijsen (1981) recognised that many of the filaments were not yet described taxonomically and that this was unlikely to be done expeditiously. Accordingly, for unidentified filaments they allocated type numbers until such time as the characteristics of the filament were adequately described for inclusion in the standard keys. Their approach was eminently practical, consisting of quick and simple techniques

whereby reasonably reliable identification of filaments could be attained from microscopic studies, staining, etc. Jenkins *et al.* (1984a) produced a manual that built on the work of Eikelboom and van Buijsen. This manual describes in detail the quick practical procedures for identifying filaments and also gives advice on their occurrence and suggestions for their amelioration. This manual has proved of great value in bulking research in South Africa.

About 30 filamentous species have been reported to contribute to bulking in activated sludge systems. Of these, 11 are of importance. Their dominance in activated sludge mixed liquors is dependent in varying degrees on the environmental conditions imposed on the plant by its design and operation, and the influent sewage composition. Bulking surveys in different countries have indicated the sensitivity of the organism population to differences in plant design, plant operation and influent characteristics. Based on 226 samples from 78 plants in the United States, Strom and Jenkins (1984) list the filaments forming the major component in sludges that bulk in decreasing order of occurrence: type 1701, *Nocardia* species, *Haliscomenobacter hydrossis*, types 0041, 021N and 0092, *S. natans*, *Microthrix parvicella*, and others. The ATV Working Group (1989) in Germany lists the following filamentous species in decreasing order of occurrence: Type 021N, *M. parvicella*, type 0041, *S. natans*, *Nocardia* species, *H. hydrossis*, *Nostocoida limicola*, type 1701, and other lesser occurring ones. Blackbeard *et al.* (1986, 1988) in South Africa lists the 6 most frequently dominant filaments in nitrogen and nutrient removal (long sludge age) plants, in decreasing order of occurrence: types 0092, 0675, 0041, *M. parvicella*, types 0914 and 1851. The lists for the United States and Germany are not dissimilar, but both differ substantially from that for South Africa. Plants in Germany and in the United States are principally aerobic with relatively high loading factors (high F/M) or equivalently short sludge ages. Plants in South Africa have sludge ages that can be up to an order of magnitude higher than some plants in the United States and in Germany (i.e. very low F/M) and always incorporate anoxic–aerobic or anaerobic–anoxic–aerobic zones. Whereas the microbiological population structures that develop in the United States and German plants could be expected to differ not too greatly, the significant differences in environmental factors imposed on the organisms in ND and NDBEPR plants in South Africa should give rise to a population structure that differs greatly from those in European and American plants.

With the objective of developing a practical procedure to identify filaments,

considerable effort was expended in Europe and America to find links and associations between the dominant filaments in the bulking sludge and the design, operational and influent aspects of the plants (Mulder *et al.*, 1972; Rensink, 1974; Wagner, 1982a; Richard *et al.*, 1982; Jenkins *et al.*, 1984b; Strom and Jenkins, 1984; Jenkins *et al.*, 1984a).

Jenkins *et al.* (1984a) proposed a categorization of the conditions that give rise to the dominant filament types (see Table 2.1). In regard to using such a categorization, Strom and Jenkins (1984) state "at present most of the filament types can only be categorized to a few broad groupings of associated conditions", implying that at a specific treatment works, other conditions may be present that can change the filament species from that listed in Table 2.1. They illustrated this with several examples.

One category of conditions in Table 2.1 is designated by the term "low F/M" implying a low loading rate of substrate to sludge mass. The category is rather vaguely defined and no explicit published data base for categorization of the low F/M filaments could be traced. However, such a data base must exist or otherwise the category, with associated low F/M filaments, could not have been identified. A problem in defining this category more clearly lies in part in the various meanings attached to the term low F/M, or equivalently load factor. A preferable more consistent parameter would be the sludge age. The load factor can be linked to the sludge age via the yield, endogenous respiration rate, endogenous mass generation, and unbiodegradable solids from the influent. In different countries these are empirically linked in different ways so that for the same sludge age the load factor value in one country does not necessarily correspond with the value in another country. This makes comparison of information presented in the literature in terms of load factor extremely difficult. By using the sludge age instead of the load factor, the differing F/M ratios obtained in different countries for similarly operated plants (all with the same sludge age) will not arise.

Recognising the difficulties described above, even linking the low F/M filament group only to sludge age may be inadequate – this categorization may be unduly broad. With the introduction of nitrogen and nutrient removal plants, the plant configurations and operational modes have changed drastically from the conventional aerobic systems prevalent in Europe and America. In these systems other factors may become as important as the sludge age in causing bulking. If

Table 2.1: Dominant filament types as indicators of conditions causing activated sludge bulking (from Jenkins *et al.*, 1984a).

Suggested causative conditions	Indicative filament types
Low DO	type 1701, *S. natans*, *H. hydrossis*
Low F/M	*M. parvicella*, *H. hydrossis*, *Nocardia* sp., types 021N, 0041, 0675, 0092, 0581, 0961, 0803
Septic Wastewater/Sulfide	*Thiothrix* sp., *Beggiatoa* and type 021N
Nutrient Deficiency	*Thiothrix* sp., *S.natans*, type 021N, and possibly *H. hydrossis* and types 0041 and 0675
Low pH	fungi

indeed this should be found to be so, there would be a case for renaming the low F/M category or creating a new one.

The work reviewed so far attempted to link specific filaments to specific imposed conditions, environmental and substrate, and is basically an empirical approach. The first attempt to provide a more fundamental approach to bulking was due to Chudoba and his group in which the kinetics of growth of filaments and floc-formers serve as a basis for determining whether filament proliferation would take place or not. Chudoba's work has had and still has a definitive effect on European and American perceptions of bulking and has influenced both design and operation of the plants. It is therefore important that the relevance of Chudoba's hypothesis to filamentous bulking should be examined carefully.

Chudoba's Selector Theory – aerobic systems

Chudoba *et al.* (1973a,b) proposed an organism selection criterion as an explanation for the occurrence or non-occurrence of filamentous bulking. This criterion is based on competition between the floc-formers and the filaments for the mutually limiting soluble substrate, as follows: In the Monod formulation for the specific rate of growth of organisms, filamentous organisms have lower values for both the maximum specific growth rate (μ_{max}) and the half saturation coefficient (K_s) than floc-formers. Consequently at low substrate concentrations the filamentous organisms have a higher specific growth rate than floc-formers and at high substrate concentrations, a lower specific growth rate.

Over the past two decades the selection criterion has provided a framework for research into the causes of bulking and its control by specific methods. Results reported by a number of investigators who have measured the Monod constants of various filaments and floc-formers, appear to fit within the structure of the selection criterion: Van den Eynde *et al.* (1982a,b) showed that in general, organisms with high μ_{max} have high K_s values and ones with low μ_{max} have low K_s values. Slijkhuis (1983) measured the μ_{max} of *M. parvicella* (one of the principal filaments causing low F/M bulking) to be 1,66/d; this is considerably lower than a μ_{max} of 4,33/d measured by Richard *et al.* (1982) for a floc-former isolated from activated sludge.

Palm *et al.* (1980) extended the selection criterion to incorporate limiting nutrients: For some filaments (the low DO ones) the limiting nutrient apparently is oxygen, whereas for others the limiting nutrient is the soluble substrate concentration

surrounding the organism, as originally conceived by Chudoba *et al.* (1973a,b). Hao *et al.* (1983) and Lau *et al.* (1984) confirmed the work of Palm *et al.* (1980). From dual species studies they showed that low DO filaments (*S. natans*, type 1701) and floc–formers can be selectively grown by manipulating the DO concentration – if high, the floc–former dominates, if low, the filament dominates.

With regard to bulking in long sludge age (low F/M) systems, Chudoba *et al.* (1973a,b) tested the selection criterion with pure soluble substrates: They controlled the substrate concentration surrounding the organism by having different configurations for the activated sludge system. For example, in a completely mixed single reactor system, the substrate concentration would be low throughout the reactor whereas in a multi–reactor plug–flow system, the substrate concentration would be high in the upstream section and low in the downstream section. They found that in aerobic completely mixed single reactor systems, filamentous organisms proliferated causing bulking, whereas in aerobic multi–reactor plug–flow systems filamentous organisms did not proliferate and a good settling sludge was maintained. From this work, Chudoba *et al.* (1973b) developed the selector reactor for bulking control. The selector reactor is a small aerated reactor upstream of the main aeration reactor and receives the influent and underflow recycle. In the selector reactor, the substrate concentration is high and, in terms of the selection criterion, the floc–formers should grow faster than the filaments and usually will utilize practically all of the soluble substrate. The mass of soluble substrate that passes through the selector unutilized is a very small fraction of that available to the floc–formers in the selector so that filament growth will be restricted and insufficient to cause bulking. The findings of Chudoba were supported by a number of investigators; non–bulking sludges were produced in *aerobic* systems with:

(1) Selectors ahead of the main reactor (Grau *et al.*, 1982; Lee *et al.*, 1982; Jenkins *et al.*, 1983; Ekama and Marais, 1986b; Still *et al.*, 1986; van Niekerk, 1985; van Niekerk *et al.*, 1987);

(2) compartmentalization of the aeration reactor while maintaining continuous feeding of waste water (Chudoba *et al.*, 1974; Rensink *et al.*, 1982; Wu *et al.*, 1984);

(3) batch or intermittent feeding to completely mixed aeration basins (Houtmeyers, 1978; Houtmeyers *et al.*, 1980; Verachtert *et al.*, 1980; van den

Eynde *et al.*, 1982a,b; Eikelboom, 1982; Rensink *et al.*, 1982; Goronszy, 1979; Goronszy and Barnes, 1979; Barnes and Goronszy, 1980; Chiesa and Irvine, 1985; Jenkins *et al.*, 1983; Ekama and Marais, 1986b; Still *et al.*, 1986; van Niekerk, 1985; van Niekerk *et al.*, 1987).

A common feature found in all these investigations, using either selectors, plug-flow reactors or intermittent feeding to single completely mixed reactors is that these systems stimulated in the sludge a high utilization rate for soluble (< 45μm) COD (i.e. readily biodegradable COD, RBCOD). These rates were established in aerobic batch tests by drawing sludge from the system and spiking it with influent and measuring the <45 μm COD reduction with time, or the oxygen utilization rate (OUR) associated with the soluble COD reduction. Where this effect was present, for example, in a single reactor system with a selector upstream of the reactor, the rates of these two parameters are 2–3 times higher than for the system that did not exhibit the effect, a single reactor without a selector, (*inter alia* Houtmeyers, 1978; Houtmeyers *et al.*, 1980; Verachtert *et al.*, 1980; van den Eynde *et al.*, 1982a; Grau *et al.*, 1982; Eikelboom, 1982; Jenkins *et al.*, 1983; Daigger *et al.*, 1985; Ekama and Marais, 1986b; Still *et al.*, 1986, and van Niekerk *et al.*, 1987).

For convenience, we will henceforth describe any system that gives rise to an increase in the soluble COD uptake rate/OUR as exhibiting a selector effect.

From the literature there is strong evidence that where the selector effect is induced, the filamentous organisms *S. natans*, *Thiothrix* sp. and type 021N are controlled. These filaments are frequently encountered in Europe and in the United States, and modification of the plants to induce the selector effect has been accompanied by considerable success in controlling bulking by these filaments. The ATV Working Report (1989) comes out very positively in support of this mechanism: "On the basis of the information available today, the use of a "selector", i.e. a high-load preliminary tank, can be recommended as the most promising measure for preventing and controlling bulking, regardless of the wastewater."

Reviewing the investigations into control of bulking in low F/M systems by the selector effect, where filament identification was performed, bulking was caused not by low F/M filaments but by *S. natans* which, as indicated in Table 2.1, is not a low F/M filament. This raised the question of the appropriateness of the selector system for controlling bulking due to low F/M filaments. It appears that in the

research, controlling bulking in low F/M systems was the focus, rather than controlling bulking by low F/M filaments. These are two distinctly different objectives because bulking in a low F/M system is not necessarily caused by low F/M filaments. It is necessary to distinguish clearly between the two situations: low F/M system bulking is bulking in a low F/M system with the filaments causing the bulking unspecified, i.e. could be *S. natans*; low F/M filament bulking is bulking caused specifically by the filaments grouped into the low F/M category.

Chudoba's Selector Theory – anoxic–aerobic systems

The system modification approach for bulking control in low F/M (long sludge age) systems also was applied by incorporating anoxic selectors in N removal activated sludge systems. The need for this arose from the necessity for denitrification for N removal. If an aerobic selector receiving the influent and underflow recycle streams is placed upstream of the nitrification–denitrification system, the influent RBCOD will be utilized in the aerobic selector. This will result in a significant loss in denitrification – as much as 50 percent – because the influent RBCOD will be utilized in the selector with oxygen rather than with nitrate in the main reactor. If the selector effect can be stimulated in an anoxic selector, the RBCOD will be utilized with nitrate as electron accepter and then the conditions for good N removal and selector bulking control would be met simultaneously.

In evaluating anoxic selectors *per se*, for bulking control in low F/M (long sludge age) activated sludge systems receiving real sewage (with or without acetate or glucose supplementation), Lee *et al.* (1982) reported that incorporation of two anoxic selectors in series, each 1/74th of the total system volume, *did not* control bulking. They sized the selectors in accordance with the volume that would be required to control bulking with aerobic selectors. Based on measurements of soluble COD through the system, they found that not all the soluble biodegradable COD was taken up in the selectors. The leakage of soluble biodegradable COD into the aerobic zone was suggested as the cause for the ineffectiveness of the anoxic selectors. Shao (1986) found that the uptake rate of RBCOD is slower under anoxic conditions than under aerobic conditions so that anoxic selectors should be sized larger than aerobic selectors.

Anoxic selectors normally would be installed upstream of in-series anoxic–aerobic reactors in order to obtain adequate denitrification. This raised the question of whether the amelioration of bulking was due to the selector effect or, perhaps,

simply the removal of RBCOD in the anoxic reactor so that the filaments would in any case not have substrate for growth in the aerobic zone. Wanner *et al.* (1987) investigated this problem. They set up two systems (7 days sludge age), the one with anoxic selector–anoxic–aerobic reactors and the other with anoxic–aerobic reactors. They also set up a single aerobic reactor. They found that the aerobic system bulked badly with type 021N whereas the two systems with the selector–anoxic and anoxic–aerobic reactors did not bulk. They concluded that the removal of the readily biodegradable substrate in the anoxic zone was responsible for the suppression of the bulking organisms. They hypothesized that this was because 021N cannot utilize nitrate as an electron acceptor; they supported this by noting "that severely filamentous mixed cultures had maximum rates of denitrification or nitrate respiration one order of magnitude lower than non–filamentous mixed cultures cultivated under similar conditions". They concluded "on the basis of these findings..... some filamentous organisms cannot use nitrate as an electron acceptor". Wanner *et al.* termed this type of bulking control metabolic selection, to differentiate it from the kinetic selection due to induction of the selector effect.

Irrespective of whether the selector effect operated or not, incorporation of an anoxic zone ahead of the aerobic, at laboratory, pilot and full–scale, widely appeared to have a beneficial effect on bulking (Heide and Pasveer, 1974; Bailey and Thomas, 1975; Tomlinson and Chambers, 1979; Wagner, 1982a; Price, 1982; Cooper and Boon, 1983; Shao, 1986 and Wanner *et al.*, 1987). However, instances are reported where incorporation of an anoxic reactor did not have a beneficial effect. At full–scale Cooper *et al.* (1977) installed a channel type anoxic zone by replacing the surface aerators with stirrers in 25 percent of the aeration basin and compared the settleability with an identical adjacent unit operated without the anoxic zone. Both systems had sludge ages of 18 days. The aerobic unit produced an SSVI of 40 to 50 mℓ/g, the anoxic–aerobic unit 55–75 mℓ/g, that is, the anoxic–aerobic system delivered a worse settling sludge than the aerobic system. The full–scale plant was duplicated at laboratory–scale by two 8 in–series reactor systems. In the aerobic system all the reactors were aerated; in the anoxic–aerobic system the first two reactors were stirred, not aerated. Both systems operated with a sludge age of 16–17 days. The aerobic system bulked with the SSVI increasing to > 300 mℓ/g whereas the anoxic–aerobic system gave an SSVI ranging between 75 and 110 mℓ/g approximately.

In this study the laboratory units gave a bulking behaviour opposite to the full–scale

plants. Furthermore, even the lowest SSVI in the laboratory units is higher than the highest SSVI in the full–scale units. It is not possible to isolate the causes for this disparate behaviour, but a suggested cause is that the laboratory–scale units were seeded with filamentous growth generated in the influent line. Such seeding in a pilot–scale study has been reported by Gabb *et al.* (1985). Unfortunately Cooper *et al.* (1977) did not report on the filamentous organisms found in the laboratory–scale study. It does, however, point to an important factor in utilizing laboratory–scale studies to seek corrective procedures to be applied at full–scale: it is imperative that the laboratory–scale study produces the same filament population as the full–scale study. If this is not achieved the laboratory–scale study loses much of its value as a tool for testing control procedures proposed for the full–scale plant.

In studies on anoxic–aerobic systems, Bailey and Thomas (1975) and Arkley and Marais (1981) found that as the anoxic mass fraction of the upstream anoxic reactor increased, the sludge settleability deteriorated. Arkley and Marais (1981) studied a two in–series reactor system in which the total volume remained constant, the first reactor anoxic and the second aerobic; both completely mixed with a system sludge age of 20 days, and feed continuous. The anoxic zone had sizes ranging from zero (completely aerobic) through 39 and 50 to 70 percent of the total system volume. The underflow recycle was increased as the anoxic volume fraction was increased so that the denitrification potential of the anoxic zone was always exceeded and nitrate always was present in the effluent from the anoxic reactor. As the anoxic reactor volume fraction increased so the sludge settleability deteriorated. These large anoxic volumes could hardly be considered as selectors and very likely did not stimulate a rapid soluble biodegradable COD uptake rate, but they would have removed virtually all the soluble biodegradable COD. In terms of the selector hypothesis, because there was no selector effect, one would expect bulking with each of the anoxic volume fractions. Instead, bulking was absent in the 100 percent aerobic system and bulking increased progressively as the anoxic mass fraction increased. Unfortunately no filament identification was undertaken.

In the anoxic–aerobic work above, with systems that could reasonably be categorized as low F/M, either the filamentous organisms were not identified or where the filaments were identified, e.g. Lee *et al.* (1982), Shao (1986) and Still *et al.* (1986), the dominant filaments were not low F/M ones. Indeed, there appear to be no reports that unequivocally established that anoxic selectors or anoxic reactors control bulking by low F/M filaments. There is no doubt that the selector effect

may be a mechanism of control for certain filamentous organisms, and from the literature it appears that *S. natans*, *Thiothrix* sp. and type 021N can be successfully controlled by selectors, but these are not low F/M filaments. Yet there is a strong perception that low F/M filaments are controlled by the selector effect. For example, Albertson (1991), in reviewing bulking in ND and NDBEPR plants, believed that the conventional designs adopted in these plants, i.e. a small number of large series reactors did not induce the selector effect and this was the reason why bulking was so common in these plants.

In the review, it became increasingly evident that there exists no definitive information on control of low F/M bulking due to the selector/anoxic zone effects in long sludge age nitrification–denitrification plants and that an enquiry should be made into the selector/anoxic zone effects and their implications for bulking in these plants. Accordingly, the Water Research Group at the University of Cape Town in 1985 inaugurated an extensive investigation at laboratory–scale level into the selector effect in aerobic and nitrification–denitrification systems operating at sludge ages of 15 days and longer (Gabb *et al.*, 1989a).

2.6 WATER RESEARCH GROUP INVESTIGATION

As a first phase in the investigation, the Group wished to check if results similar to those reported in the literature for low F/M systems could be obtained under the very long sludge ages, 15 and 30 days (i.e. very low F/M loadings), common in ND plants in South Africa.

Two sets of experiments were done on (1) aerobic and (2) anoxic–aerobic systems. In both sets the systems were operated at long sludge ages, between 15 and 30 days, at 20° C. Each set will be dealt with separately.

Aerobic systems:

The following systems were operated:

(1) Fully aerobic constant feed completely mixed single reactor (O/CFCM) system.

(2) Fully aerobic intermittently fed fill and draw single reactor (O/IFFD) system.

(3) Fully aerobic constant feed completely mixed single reactor system but with

an aerobic selector upstream of the main reactor (O/CFCM/SEL).

In terms of the selector hypothesis, the first (O/CFCM) should bulk, but the second and third (O/IFFD and O/CFCM/SEL) should not.

All systems were fed with unsettled sewage from a residential area. The systems were started with mixed liquor from a nitrification–denitrification plant serving the residential area. This plant produced a bulking sludge, DSVI > 250 mℓ/g with type 0092, *M. parvicella*, types 0675, 0041 and *Nocardia* filaments. The fill and draw system was fed once daily. Dissolved oxygen in the reactors was maintained greater than or equal to 2 mg/ℓ. All systems nitrified.

The fill and draw (O/IFFD) and the single reactor with the selector (O/CFCM/SEL) systems both developed a selector effect, that is, a high RBCOD uptake rate 2–3 times that from the single reactor constant feed (O/CFCM) system. The selector effect could be stimulated or lost over a period of less than one sludge age on changing the system from (1) to (2) or (3), or from (2) or (3) to (1) respectively.

The results were unexpected: *Irrespective of whether the systems developed the selector effect or not, the low F/M filaments present in the parent sludge disappeared and a well settling sludge developed.* In the O/CFCM system sporadic bulking was encountered due to the filament *S. natans*, a filament never observed to cause bulking in South African full–scale N and N & P removal systems. However, it was found that this was caused by seeding of the laboratory–scale system from attached growth on the walls of the feeding tube (Gabb *et al.*, 1989b). Twice weekly cleaning and chlorinating of the tubes resolved this seeding problem and subsequently no bulking due to *S. natans* was encountered. The seeding, therefore, was an artifact arising from laboratory–scale operation. In many of the aerobic laboratory–scale studies incorporating the selector (reviewed above), this artifact very likely was present causing bulking to be due to *S. natans* and not to low F/M filaments. As reported earlier this artifact had been observed previously at pilot–scale by Gabb *et al.* (1985).

The conclusion from this study was that bulking due to low F/M filaments did not occur in long sludge age (> 15 days) completely aerobic systems irrespective of whether a selector effect was present or not.

Anoxic–aerobic systems:
The following anoxic–aerobic systems were set up:

(1) Alternating anoxic–aerobic constant feed single completely mixed reactor (AO/CFCM) system.

(2) Alternating anoxic–aerobic intermittently fed fill and draw reactor (AO/IFFD) system.

These two systems correspond to (1) and (2) in the aerobic system described above except that alternating aerobic–anoxic periods were imposed on the reactors. The AO/IFFD system was fed once daily and for both systems the anoxic–aerobic cycle times were 1h anoxic, 3h aerobic. In the AO/IFFD system, after feeding the anoxic–aerobic cycle time commenced with the aerobic period on one day, and with the anoxic period the following day.

The AO/IFFD system developed the selector effect, the AO/CFCM system did not. (This was established by doing batch tests by drawing samples of sludge from the main reactor, spiking with sewage and measuring the change in <45 μm fraction, or measuring the OUR under aerobic batch conditions and/or the denitrification rate under anoxic batch conditions).

As for the aerobic systems, the results were unexpected: *Irrespective of whether the system developed a selector effect (AO/IFFD) or not (AO/CFCM), these anoxic–aerobic systems did not bulk.* However, there was no doubt that the laboratory–scale nitrification–denitrification (ND) systems of Arkley and Marais (1981) did bulk. Also the surveys of Blackbeard *et al.* (1986; 1988) showed that many of the alternating anoxic–aerobic (nitrification–denitrification) Carousel systems and nitrification–denitrification biological excess phosphorus removal (NDBEPR) systems at full–scale, and all NDBEPR systems at laboratory–scale bulked with low F/M filaments. Evidently the two sets of anoxic–aerobic systems were dissimilar in some respect – factor/s additional to alternating anoxic conditions must play a role in developing bulking sludges.

The Group decided that attention should be focused on the ND systems and not on the NDBEPR systems for the reason that the ND systems were shown to readily produce low F/M filament bulking sludges and the ND system is less complex than

the NDBEPR system. From the survey of Blackbeard *et al.* (1988), the Carousel ND system appeared particularly prone to bulking by low F/M filaments. Accordingly, the following laboratory-scale system was set up to mimic the behaviour of the Carousel.

Two parallel single reactor systems were set up, both continuously fed and completely mixed, and operated at a sludge age of 20 days and at around 20°C. An unaerated-aerated pattern of 6–7 minutes anoxic and 3–4 minutes aerobic in a 10 minute cycle was set up. The large anoxic period was selected by noting that in Arkley and Marais' investigation, the SVI increased with the anoxic mass fraction.

In operating the plants, it was found very difficult to control the oxygen input to give the selected aerobic period of 3–4 minutes. The air on/air off period had to be found by trial and error. The period of aeration depended on the OUR, nitrification rate and MLSS. The air on/air off cycle eventually adopted was 55 seconds air on which increased the DO to approximately 2 mg/ℓ; over the next 2–3 minutes the DO decreased to zero and remained so until the next aeration cycle commenced. It was found that the OUR was dominantly affected by the ammonium concentration in the influent. In order to ensure a high OUR so that the DO would drop rapidly after aeration ceased, additional ammonia was added to the influent to stimulate a high nitrification OUR. The additional nitrate generated also ensured that denitrification did not reduce the nitrate concentration to zero during the anoxic period, (thereby ensuring anoxic, not anaerobic conditions), with about 3–5 mg (NO_3^-–N)/ℓ) remaining at the end of the anoxic period. Throughout the test period, at intervals, anoxic and aerobic batch tests were performed; these showed that both systems did not have a selector effect.

In both systems the DSVI increased from about 110 to between 500 and 600 mℓ/g by day 103. On this day, in one unit intermittent aeration was replaced by continuous aeration – the DSVI decreased rapidly to about 50 mℓ/g. In the other unit with intermittent aeration, the DSVI rose to 600 mℓ/g due to proliferation of *M. parvicella*, and types 1851, 0092, 0914, all low F/M filaments. On day 174, the unit with continuous aeration was changed back to intermittent aeration and slowly the DSVI increased to over 300 mℓ/g by day 304. Meanwhile, from day 140 the unit that had intermittent aeration all the time also showed a decrease in DSVI, from 600 to stabilize at about 300 mℓ/g by day 300, but now the dominant filaments were 0092, 0914, *M. parvicella* and 0041. The two sludges were mixed and

on day 304 the one unit was aerated continuously and the other intermittently. In the system with continuous aeration the DSVI fell rapidly to approx. 100 mℓ/g and thereafter decreased slowly to about 60 mℓ/g; in contrast the unit with intermittent aeration stabilized at a DSVI> 200 mℓ/g.

As noted, the sludges in the intermittently aerated systems above did not exhibit a selector effect. This raised the question as to whether the bulking would be controlled by introducing a selector effect. This was tested as follows: The sludges from the two systems above were mixed to give a DSVI of 190 mℓ/g and returned to the reactors of the two systems. Both systems were then operated with anoxic–aerobic alternation under continuous feed conditions exactly as was done with the one system in the previous experiment. In both systems, after 21 days the DSVI increased from 190 to 340 mℓ/g. Both systems did not exhibit the selector effect and the systems responded virtually identically to the previous experimental system operated under the same set of conditions. After 21 days, in one of the systems two in–series selectors were installed upstream of the reactor, each having 1/32 of the reactor volume. The first selector received the influent and underflow recycle flows. The system with the selectors (selector system) developed a sludge with a soluble (readily biodegradable) COD uptake rate 1,8 times that of the rate of the sludge in the system without a selector. Also, the selector system exhibited zooglea present in the sludge. These two observations confirmed that a selector effect indeed was present. The two systems, one with the selectors and one without, were run for a further 50 days. The DSVI of both remained approximately constant at 340 mℓ/g. Both sludges had essentially the same filaments with the same species dominant, i.e. *M. parvicella*, and types 0092, 0675, 0961 and 0091 in descending order of occurrence.

This experiment prompted two conclusions: In the anoxic–aerobic systems (1) the selector effect did not appear to have any significant effect on the proliferation of low F/M filaments, the system with the selector effect bulked essentially the same as the one without the selector effect; (2) because the readily biodegradable COD (RBCOD) was removed virtually completely in the selectors, it would appear that low F/M filaments do not require RBCOD to proliferate.

The experimental work and the results obtained by the Water Research Group constitutes a significant advance in the understanding of the conditions that give rise to the proliferation of low F/M filaments. These can be summarized as follows:

In long sludge age systems,

(1) the selector effect appears to have no significant effect on the proliferation or suppression of low F/M filaments in both aerobic and alternating anoxic–aerobic systems,

(2) low F/M filaments will not proliferate in 100 percent aerobic systems,

(3) a *necessary* condition for low F/M filament proliferation is alternation between anoxic and aerobic states, however,

(4) alternation between anoxic and aerobic states is not a sufficient condition because some anoxic–aerobic systems bulk with low F/M filaments whereas others do not.

2.7 CLOSURE

The finding that the selector effect did not control low F/M filament bulking placed research in this field back into an exploratory stage. Accepting that anoxic–aerobic alternation is an essential condition to stimulate low F/M filament proliferation, enquiry into the following was considered necessary:

(1) Experimental investigation into the conditions leading to bulking in anoxic–aerobic systems; apart from anoxic–aerobic alternation other conditions may also have to be satisfied for low F/M filament proliferation. A systematic study of the filament response under separate aerobic and anoxic conditions, and in transition from aerobic to anoxic and from anoxic to aerobic conditions needs to be undertaken.

(2) The fact that both low F/M filaments and floc-formers are sustained in anoxic–aerobic systems implies that the species of both groups present in the sludge are facultative. Because anoxic–aerobic alternation is a necessary requirement for low F/M filament proliferation, it would indicate that a study of the behavioural patterns of facultative organisms operating under cyclic aerobic–anoxic changes of state may contribute to the understanding of the causes for the proliferation or reduction of these filaments.

CHAPTER 3

EXPLORATORY EXPERIMENTAL INVESTIGATIONS

ABSTRACT

Experimental work is described which investigates the causes of low F/M filament proliferation in system configurations in which sludge is exposed to alternating anoxic–aerobic conditions and fed defined artificial substrate and municipal sewage. A combination of conditions is identified which results in low F/M filament proliferation, namely, exposure of sludge to alternating anoxic–aerobic conditions with slowly biodegradable COD (SBCOD) present under conditions in which the aerobic mass fraction comprises 30 to 40 percent of the total and nitrite (NO_2^-) is present at a concentration greater than about 1,5 mgN/ℓ when conditions change from anoxic to aerobic.

3.1 INTRODUCTION

From the conclusions of the review into bulking in Chapter 2, eight aspects have been identified which possibly have an influence on proliferation of low F/M filamentous organisms. These eight aspects are as follows:

(1) Readily biodegradable COD (RBCOD) or slowly biodegradable COD (SBCOD) only as influent substrate.

(2) continuous aerobic and continuous anoxic conditions.

(3) Magnitude of the aerobic mass fraction.

(4) Sludge age.

(5) Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations in the anoxic zones.

(6) Differences between alternating anoxic–aerobic conditions caused by intermittent aeration conditions in a single reactor and separate 2–reactor anoxic–aerobic conditions.

(7) Frequency of alternation between anoxic and aerobic conditions.

(8) Dissolved oxygen concentration in continuous aerobic or in the aerobic zone of anoxic–aerobic systems.

The initial task of this investigation was to experimentally determine the influence on filamentous organism proliferation of each of the aspects. The relevance of the experimental programme in the scheme of the overall investigation into bulking is illustrated in Flowchart 3.1. The experimental programme was conducted in two successive parts. In Part I, experiments conducted with defined artificial substrate fed to laboratory–scale activated sludge systems examine some of the eight aspects. In Part II, experiments conducted with municipal sewage fed to laboratory–scale activated sludge systems re–examine many of the same aspects and also examine aspects not tested in Part I.

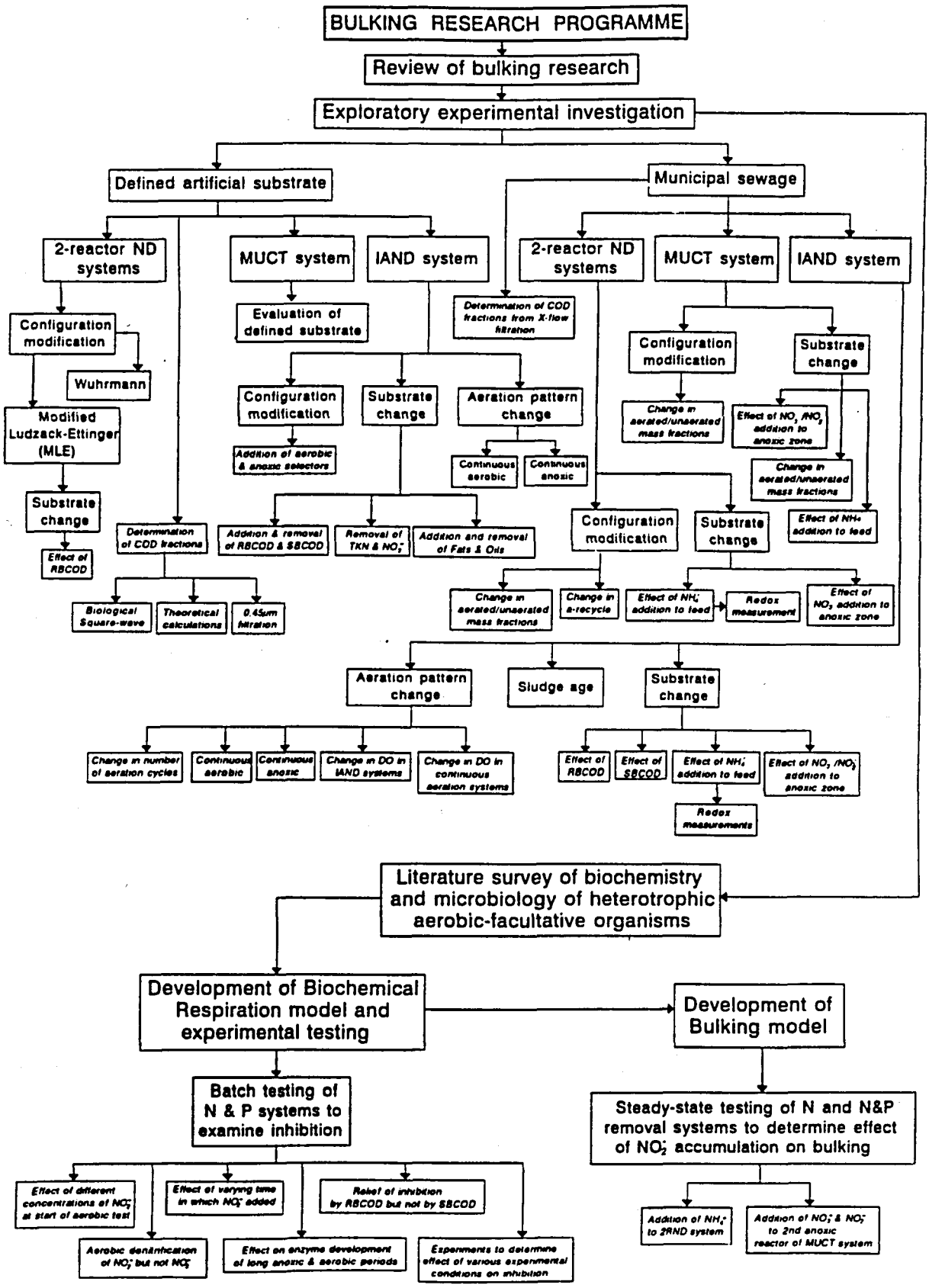
All experimental work described in Parts I and II was conducted at 20° C. In both Parts I and II, investigations were conducted on three types of systems –

(1) single reactor, either continuously aerobic, continuously anoxic, or intermittently aerated nitrification–denitrification (IAND),

(2) two–reactor nitrification–denitrification (2RND) either pre–denitrification (modified Ludzack–Ettinger, MLE), or post–denitrification (Wuhrmann),

(3) multi reactor, nitrification–denitrification biological excess phosphorus removal (NDBEPR) in the modified UCT (MUCT) configuration.

In Part I, the systems were operated at 15 days sludge age, in Part II, the single reactor and two–reactor nitrogen removal systems were operated at 15 days sludge age, and the multi–reactor systems were operated at 20 days sludge age. For details of daily experimental data, COD and nitrogen mass balances, operating parameters and filament identifications, the reader is directed to Appendix A for Systems 1–4 fed defined artificial substrate, and to Appendix B for Systems 5–17 fed municipal sewage.



Flowchart 3.1: Flowchart of the sequence of experimental and literature research conducted during the bulking research programme.

3.2 PART I – DEFINED ARTIFICIAL SUBSTRATE AS INFLUENT

Background

The reasons for using defined artificial substrate as influent for experimental work, as opposed to municipal sewage are described at length in Appendix A. Briefly, a problem associated with the use of municipal sewage in laboratory-scale systems is that the characteristics of each sewage batch may be slightly different and it is uncertain as to whether changes in system response originate from modifications made to system operation or from changes in sewage composition. The use of a defined substrate with a composition similar to municipal sewage would eliminate such experimental artifacts and, additionally, the flexible composition of the defined substrate would allow the effect of changes in composition of the sewage components apparently critical to filament proliferation to be examined. The substrate composition employed is that developed by Gabb *et al.* (1988). The composition of the substrate and a full description of its development and testing are given in Appendix A.

The experimental work conducted with defined substrate investigated the effect on filament proliferation of the following aspects:

- Requirement for fats and oils for growth and proliferation of *Microthrix parvicella (M. parvicella).*
- Readily biodegradable COD (RBCOD) or slowly biodegradable COD (SBCOD) as a requirement for filament proliferation.
- Efficacy of aerobic and anoxic selectors in systems fed RBCOD substrate.
- Continuous aerobic and continuous anoxic conditions.
- Magnitude of aerobic mass fraction.
- NO_3^- and NO_2^- concentrations in the anoxic zone.
- Differences in filament growth response to the alternating conditions in single reactor intermittently aerated (IAND) and 2 reactor (2RND) systems.

In an attempt to maintain brevity during descriptions of the extensive experimental

programme with defined substrate, experiments are described with a minimum of detail, with the exception of results which had a significant influence on the development of the hypothesis (for details see Appendix A).

Experimental results and discussion

Filamentous organisms in MUCT systems

Initial experimental investigations were conducted with an MUCT system (MUCTA) but due to an inability to develop a bulking sludge, experimental work with this configuration was abandoned and a configuration which would lend itself to proliferation of filamentous organisms was sought. (The details of the experiments with MUCTA are described in Appendix A). From the review of bulking it was established that IAND systems tend to produce bulking sludges and this configuration was adopted for the remainder of the experiments with defined substrate.

Effect of inclusion and removal of fats and oils in defined substrate composition on proliferation of M. parvicella

Three of the four IAND systems (Systems 1, 2 and 3) were examined for the effect of fats and oils on the proliferation of *M. parvicella.* In the sludges of the three systems *M. parvicella* was either absent or was present as a tertiary or secondary filament. The three systems were fed defined substrate in which fats and oils were either present or absent. Irrespective of whether fats and oils were present or absent in the substrate or the level at which *M. parvicella* was initially present in the sludge, it did not proliferate to become the dominant filament. Throughout the test periods in all systems, *Haliscomenobacter hydrossis (H. hydrossis)* was most often the dominant filament and types 1851 and 0092 most often the secondary filaments. It was concluded that either *M. parvicella* required a condition additional to fats and oils for proliferation, or that one of the constituents of the defined substrate provided a greater advantage to *H. hydrossis* and type 1851 than to *M. parvicella.* The changes in DSVI and filament type with time as a consequence of changes in substrate composition for Systems 1, 2 and 3 are shown in Figs 3.1, 3.2 and 3.3 respectively.

Inclusion and removal of RBCOD and SBCOD in the artificial substrate

Substrates containing either a large proportion of RBCOD (RBCOD–rich), or a large proportion of SBCOD (SBCOD–rich) were fed to IAND Systems 1 and 3.

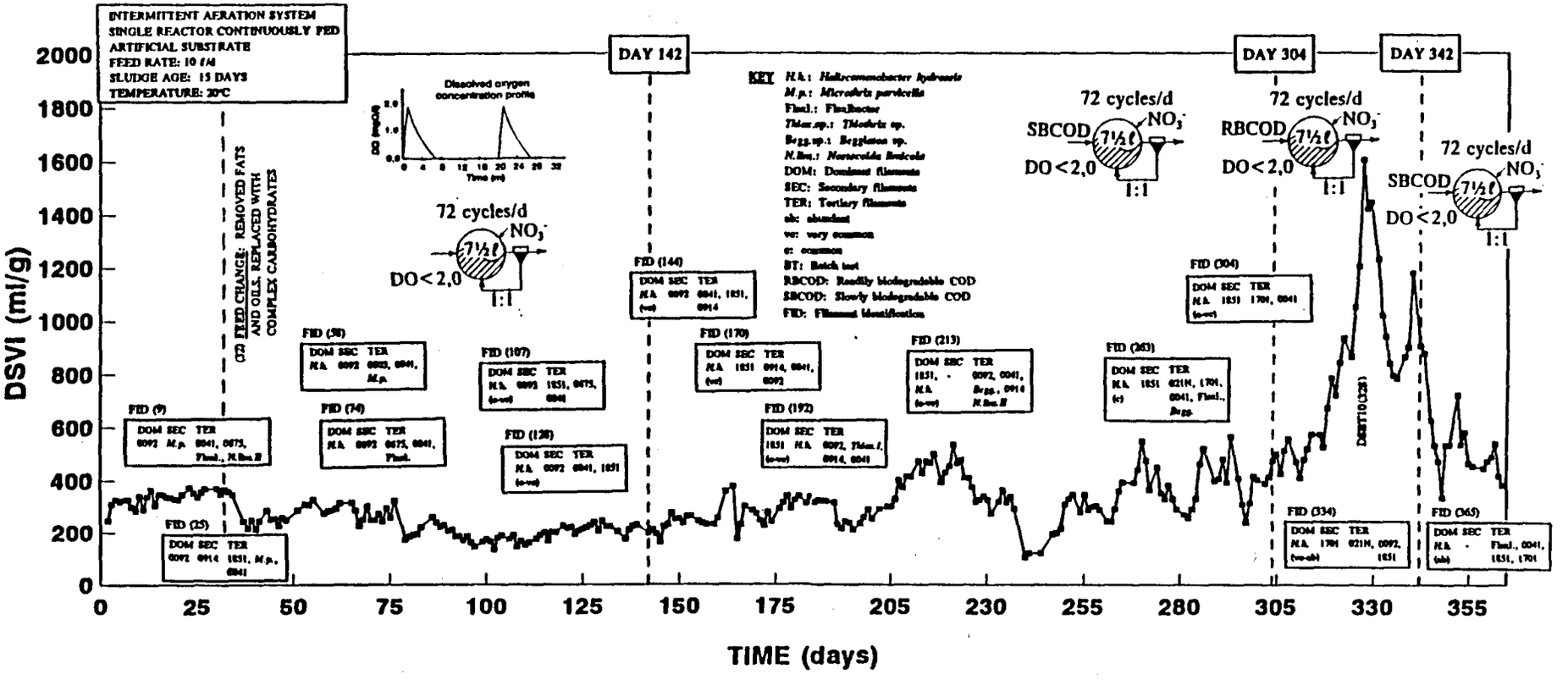


Fig 3.1: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 1 fed defined artificial substrate.

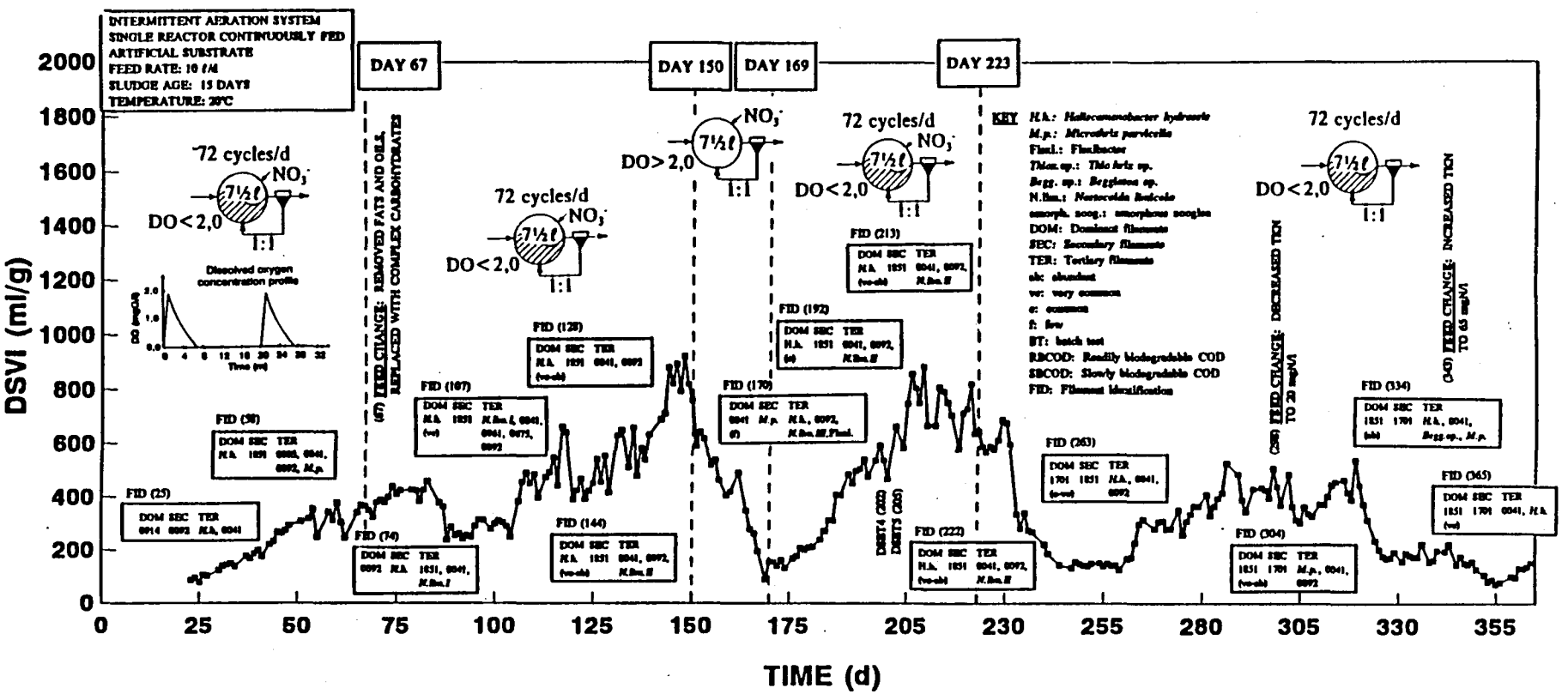


Fig 3.2: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 2 fed defined artificial substrate.

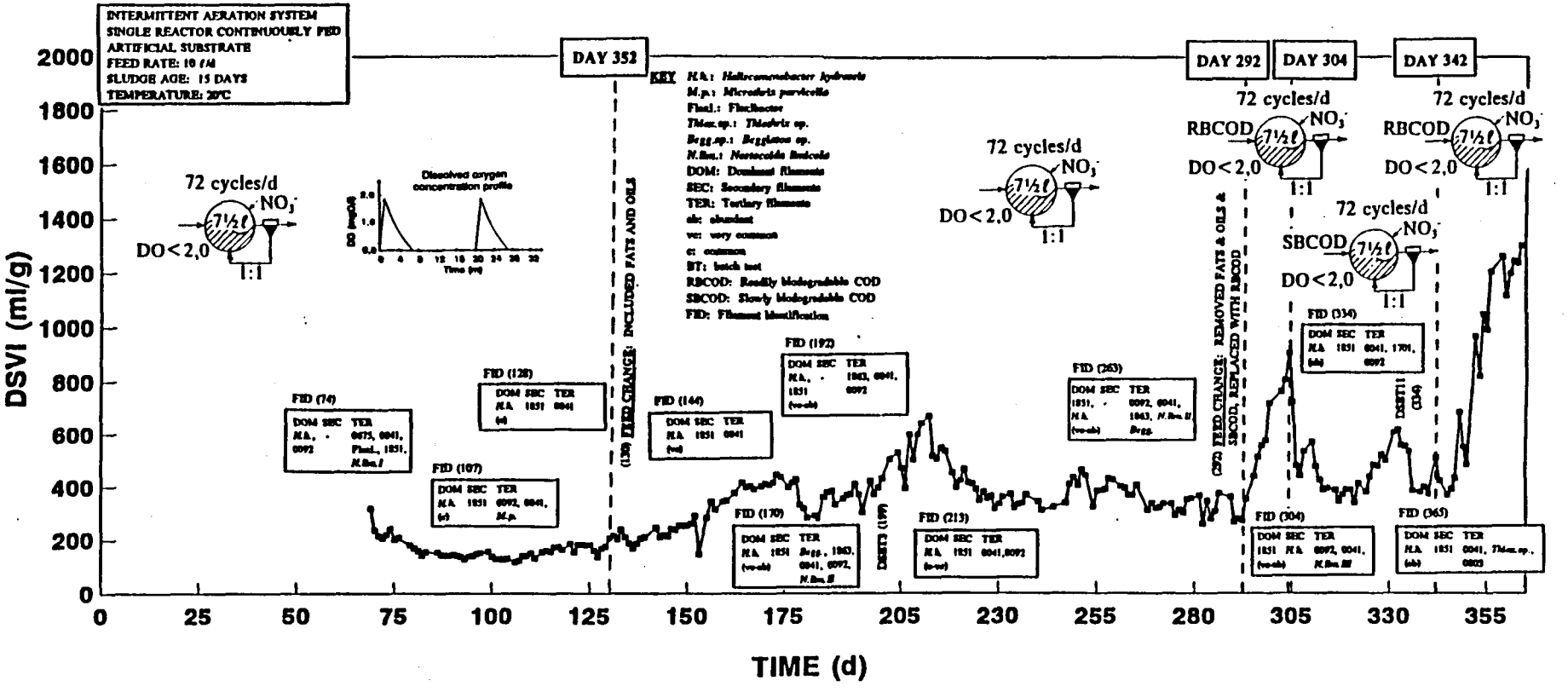


Fig 3.3: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 3 fed defined artificial substrate.

The substrates to each system were changed twice; from SBCOD–rich to RBCOD–rich to SBCOD–rich substrate for System 1 and from RBCOD–rich to SBCOD–rich to RBCOD–rich substrate for System 3. For both systems, the DSVI increased substantially following a change from SBCOD–rich to RBCOD–rich substrate (from ≈ 500 to ≈ 1000 mℓ/g) and decreased similarly following a change from RBCOD–rich to SBCOD–rich substrate, the dominant filaments either *H. hydrossis* or type 1851, or both. The change in DSVI and filament type for Systems 1 and 3 with time during changes in substrate composition are shown in Figs 3.1 and 3.3 respectively.

An interesting result associated with the experiments concerns the change in combined NO_3^- and NO_2^- concentration (NO_{2+3}^-)[1] in the effluent with change in substrate. When the DSVI increased as a consequence of the addition of RBCOD–rich substrate, the effluent NO_{2+3}^- concentration increased, and vice versa. Additionally, small daily changes in DSVI during periods of steady state operation resulted in corresponding small changes in NO_{2+3}^- concentration in the effluent. These changes are shown in Figs 3.4 and 3.5 for Systems 1 and 3 respectively.

Examination of the selector effect

The use of defined substrate of flexible composition provided an opportunity to test Chudoba's selection criteria for filamentous and floc–forming organisms by feeding the system RBCOD–rich substrate. According to the selection criteria, the addition of correctly sized selector reactors to a system with a bulking sludge fed RBCOD will control the proliferation of filamentous organisms – the high concentration of RBCOD in the selectors will favour the growth of floc–forming organisms and the substrate will be completely utilized. The selection criteria was examined with configurations incorporating aerobic and anoxic selector reactors and measurement of the selector effect with aerobic batch tests. Implicit to the selection criteria is that it is high substrate uptake rates (selector effect) induced by the high concentration of RBCOD which control filament proliferation, not the selector reactor configuration *per se*. Therefore, it can be concluded that irrespective of the means by which the selector effect is induced, filament proliferation can be controlled. It follows that the presence of high proportions of RBCOD in influent

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[1] The notation NO_{2+3}^- is used to denote the sum of the concentrations of NO_2^- and NO_3^-, in mgN/ℓ. Because the concentration of NO_2^- was not measured in these experiments, the individual concentrations of NO_3^- and NO_2^- could not be calculated.

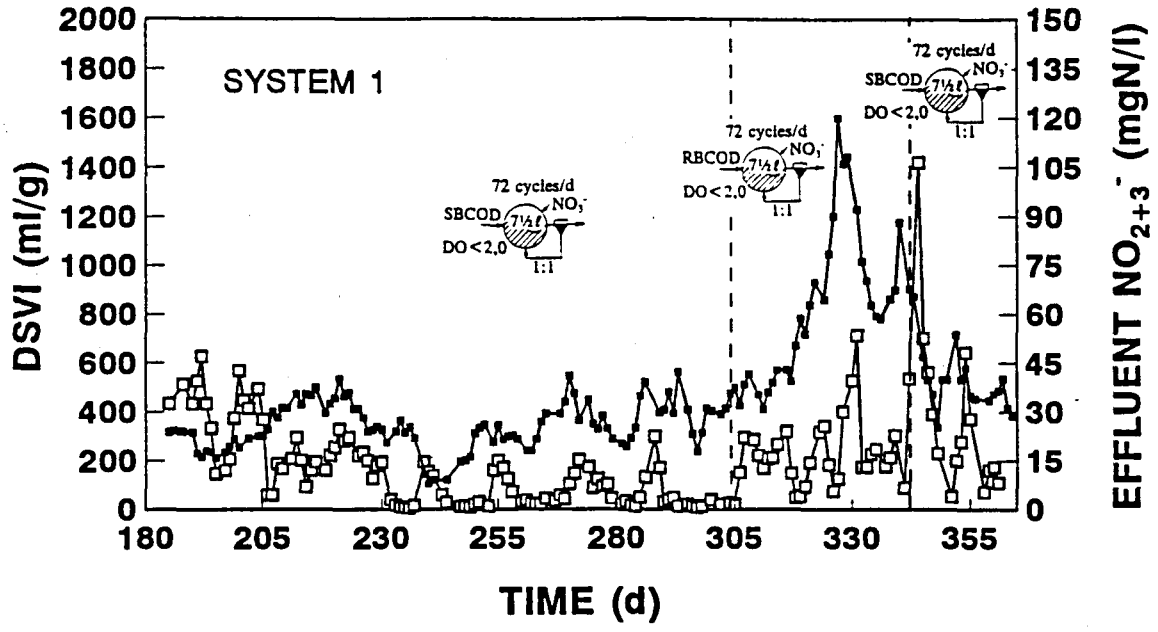

Fig 3.4: Sludge settleability as DSVI (mℓ/g) and effluent nitrate + nitrite concentration (NO_{2+3}^- in mgN/ℓ) with time (d) for System 1.

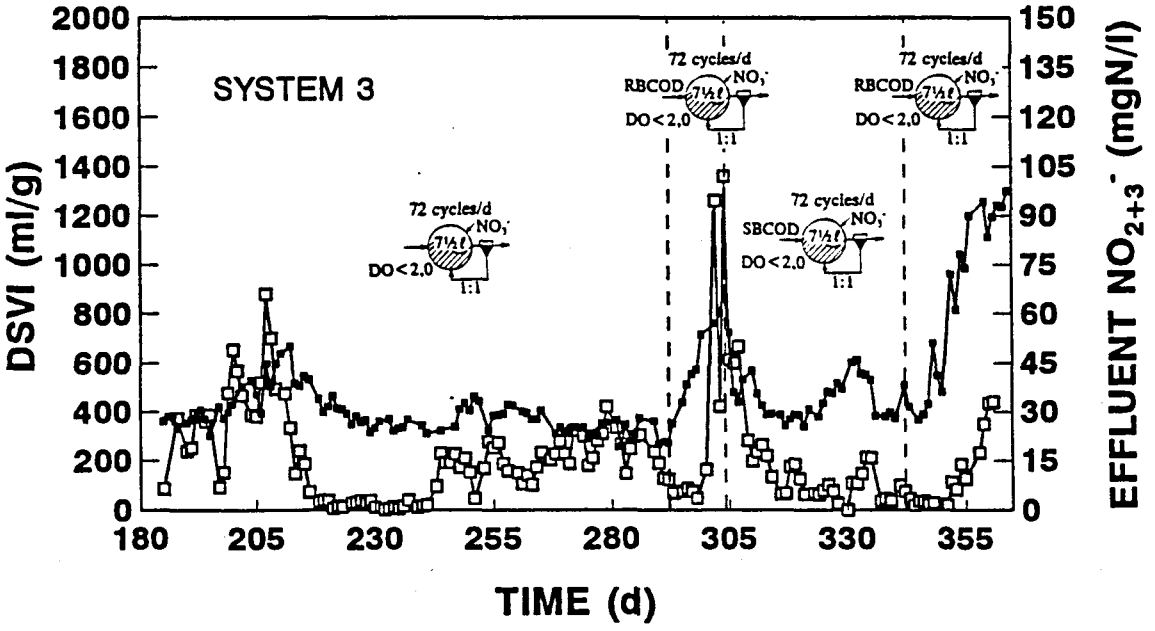

Fig 3.5: Sludge settleability as DSVI (mℓ/g) and effluent nitrate + nitrite concentration (NO_{2+3}^- in mgN/ℓ) with time (d) for System 3.

fed to systems without selector reactors may be sufficient to induce the selector effect and control filament proliferation. Induction of the selector effect was examined with aerobic batch tests on sludge from systems fed substrate with different proportions of RBCOD.

Addition of aerobic and anoxic selectors: The fourth IAND system (System 4) which was fed the RBCOD–rich substrate, developed a DSVI of 1200 mℓ/g; the dominant filaments were *H. hydrossis* and type 1851. Addition to the system of two aerobic selectors comprising in total 12% of the final system volume reduced the DSVI from 1200 to 270 mℓ/g.[2] When the selectors were removed, the DSVI increased rapidly from 270 to more than 1500 mℓ/g; the dominant filament was *H. hydrossis.* Two anoxic selectors comprising in total 12% of the final system volume reduced the DSVI from nearly 1600 to 460 mℓ/g, but the addition of a third anoxic selector did not reduce the DSVI further. COD profiles conducted through the system when the aerobic and anoxic selectors were incorporated indicated that the major fraction of COD from the influent was removed in the 2 aerobic selectors (92%), 2 anoxic selectors (94%), and 3 anoxic selectors (97%). Figure 3.6 illustrates the changes in DSVI with time for System 4.

Comparison of the DSVI values of IAND System 4 fed RBCOD–rich substrate incorporating aerobic selectors (270 mℓ/g) and anoxic selectors (400 mℓ/g) with the DSVI values of IAND systems fed municipal sewage without selectors (250 mℓ/g) (see Warburton *et al.*, 1991) would indicate failure of selector reactors to control filament proliferation. However, as noted above, systems without selector reactors fed average defined substrate and RBCOD–rich defined substrate developed sludges with very high DSVI values (> 1200 mℓ/g) and far in excess of those associated with similar systems fed municipal sewage so on the basis of DSVIs in the 4 defined substrate systems, the relatively low DSVI values observed in System 4 with aerobic and anoxic selectors and fed RBCOD–rich substrate compared with systems fed RBCOD–rich substrate without aerobic and anoxic selectors indicated that the selector reactors controlled the filament proliferation. These results conform to Chudoba's selection criteria in which floc–forming organisms dominate as a result of their higher substrate uptake rates at high concentrations of RBCOD – with the floc–forming organisms taking up more than 92% of the influent substrate, there was

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[2] In order to maintain the percentage aerobic period of the intermittently aerated main reactor between 30 and 35 percent, the aerobic period was decreased upon the addition of the aerobic selectors and increased upon the addition of the anoxic selectors.

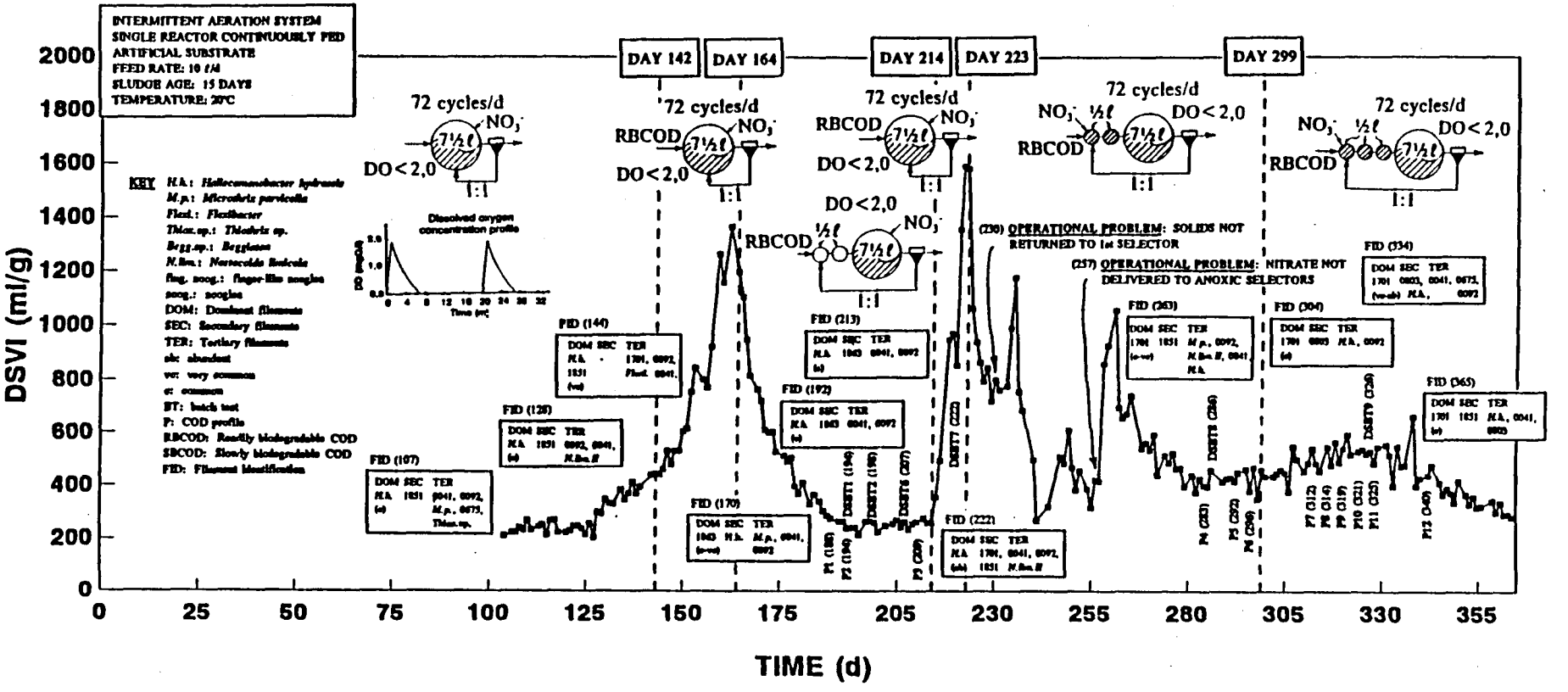


Fig 3.6: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 4 fed defined artificial substrate.

very little left for growth of filamentous organisms in the intermittently aerated main reactor. Aerobic batch tests to examine the selector effect were conducted on sludge from System 4 during periods when it incorporated 2 aerobic selectors, 2 anoxic selectors,and 3 anoxic selectors. For all configurations a selector effect was measured. This finding supports the selection criteria proposal, that high concentrations of substrate under aerobic or anoxic conditions induce high substrate uptake rates. The results and details of the batch tests are given in Appendix A.

RBCOD and SBCOD proportions in the substrate: Aerobic batch tests (DSBT3 – System 3 – Day 199; DSBT4 – System 2 – Day 202; DSBT10 – System 1 – Day 328) were conducted on sludge from three systems without selector reactors. The systems, the substrate fed to the systems, and the DSVIs of the systems were; System 3 (substrate of average RBCOD–SBCOD composition DSVI $\approx$ 300 mℓ/g), System 2 (SBCOD–rich substrate DSVI $\approx$ 600 mℓ/g), System 1 (RBCOD–rich substrate, DSVI $\approx$ 1500 mℓ/g). The substrates fed to the batch tests were all of average composition which contained sufficient RBCOD to measure a selector effect, if present. The results of the batch tests are plotted in Fig 3.7 and details of the batch tests are given in Appendix A.

System 1 with an excessively high DSVI exhibited a selector effect (high specific OUR) but Systems 2 and 3, also with high DSVIs, did not. From these results it can be concluded that high concentrations of substrate under aerobic conditions (irrespective of whether it is utilized in a selector reactor configuration) induce high substrate uptake rates. Further, with reference to the selector effect measured in selector reactor systems, in retrospect it cannot be concluded that the selector reactor configuration and not the RBCOD composition was the cause of the high substrate uptake rates. The results also support the conclusions of Chapter 2 concerning the selector effect and contradict the proposed selection criteria – filaments can proliferate in systems irrespective of whether or not a selector effect is induced.

Continuous aerobic and continuous anoxic conditions

Ketley *et al.* (1991) reported that continuous aerobic and continuous anoxic systems fed either defined substrate or municipal sewage developed low DSVI values (< 150 mℓ/g).

Similar results for continuous aeration were noted for System 2. From Day 67 to

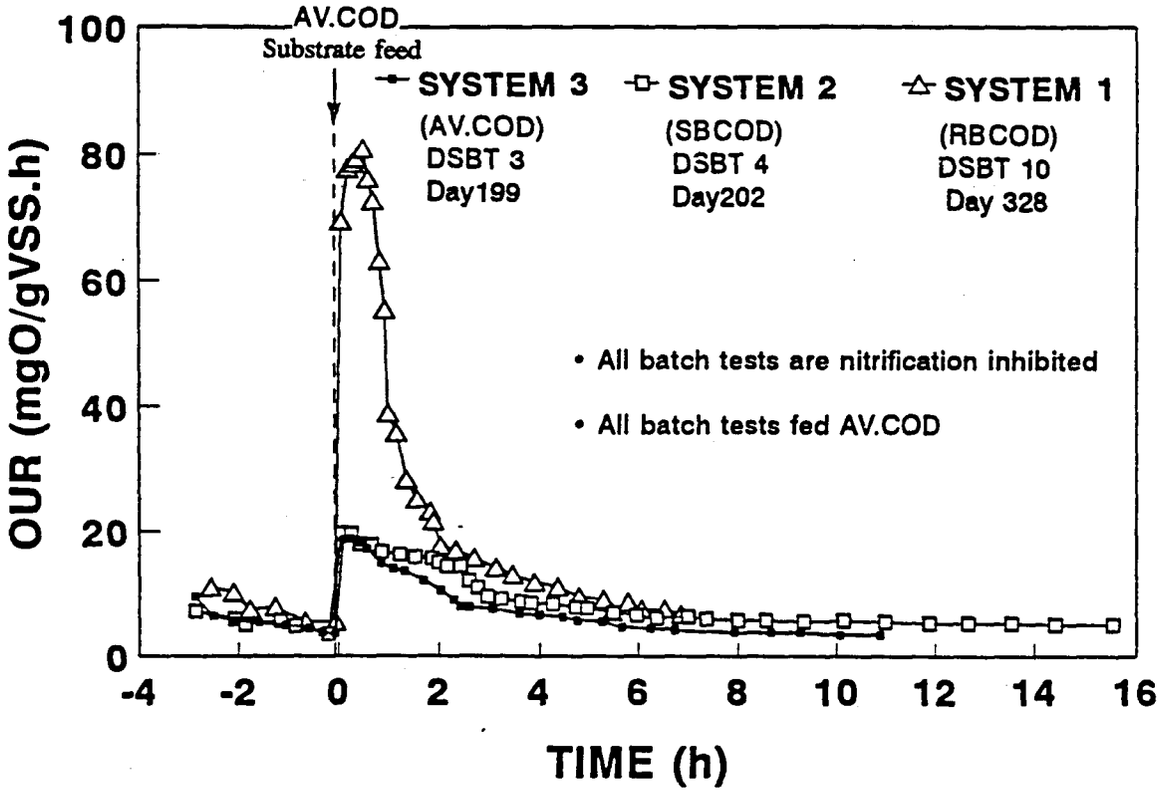


Fig 3.7: Maximum specific oxygen utilization rate (OUR, mgO/gVSS.h) with time for Batch Tests DSBT3, DSBT4, and DSBT10, all fed substrate of average composition (AV.COD) conducted on System 3 on Day 199, System 2 on Day 202, and System 1 on Day 328 respectively, to demonstrate that feeding readily biodegradable substrate (RBCOD) to a parent system induces the selector effect, but feeding slowly biodegradable substrate (SBCOD) or substrate of average composition (AV.COD) does not induce the selector effect.

149, System 2 (fed defined substrate of average composition) was intermittently aerated and high DSVIs developed (> 800 mℓ/g) (Fig 3.2). In order to control the prolific growth of the filamentous organisms (*H. hydrossis* and type 1851) the system was continuously aerated for 20 days from Day 150. The DSVI decreased from 800 to 100 mℓ/g over the 20 day period. Reimposition of intermittent aeration on Day 169 resulted in an increase in DSVI again from 100 to 800 mℓ/g over a period of 35 days as illustrated in Fig 3.2.

Magnitude of aerobic mass fraction

From the work discussed above, it can be concluded that systems which are continuous aerobic or continuous anoxic develop low DSVIs but IAND systems in which sludge alternates between anoxic and aerobic conditions and in which the aerobic period is 30 to 40% of the total, develop high DSVIs. The generality of this result was examined by determining the steady state DSVI values established at different aerobic mass fractions for an IAND system (System 2) fed a substrate of average composition.

The changes in DSVI with time during changes in aeration pattern and nitrate concentration for System 2 are shown in Fig 3.2. Figure 3.8 illustrates the relationship between steady–state DSVI and average percentage aerobic period for periods of steady–state operation. The results indicate that the highest DSVIs develop at 30 to 40% aerobic mass fraction – aerobic mass fractions greater than 40% and less than 30% result in lower DSVI values.

Nitrate and nitrite concentrations in the anoxic zone

A result associated with the investigations into the roles of RBCOD and SBCOD in filament proliferation was that increases and decreases in DSVI were accompanied by increases and decreases in NO_{2+3}^- concentrations in the system. This result focussed attention on the roles of NO_2^- and NO_3^- in filament proliferation although it was uncertain as to whether changes in NO_2^- and NO_3^- concentration caused, or were caused by, changes in filament proliferation.

Experiments with intermittently aerated System 2 with continuous NO_3^- addition (effluent NO_{2+3}^- > 25 mgN/ℓ) and high DSVI (> 600 mℓ/g) indicated that when NO_3^- addition was stopped (effluent NO_{2+3}^- < 5 mgN/ℓ), the DSVI decreased from more than 600 mℓ/g to below 150 mℓ/g in 20 days (see Fig 3.2, Days 223 to 257); the decrease being due to a reduction in *H. hydrossis* growth. However, in the

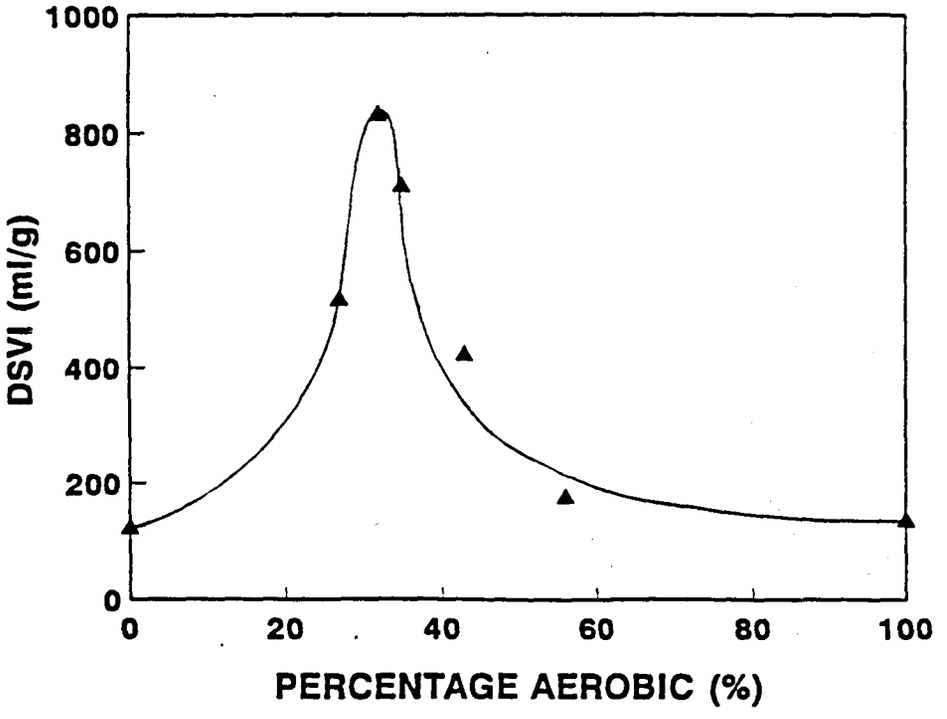


Fig 3.8: Sludge settleability as DSVI (mℓ/g) with percentage aerobic mass (%) for steady–state periods of operation of System 2.

continued absence of added NO_3^-, the DSVI increased, from less than 150 mℓ/g to 400 mℓ/g over a period of 40 days (Days 258 to 298) through the proliferation of filament types 1851 and 1701. Removal of the ammonium (NH_4^+) fraction of the substrate (an organic nitrogen fraction was available for cell growth) on Day 298 in order to restrict nitrification, (effluent $NO_{2+3}^- < 1$ mgN/ℓ) resulted in a decrease in DSVI from 400 to less than 200 mℓ/g over a 44 day period (Days 298 to 342); filament types 1851 and 1701 demonstrating reduced proliferation. The concentration of NO_{2+3}^- in IAND systems appears to have had a significant effect on filament proliferation, but different concentrations of NO_{2+3}^- affect different filament types to different extents; it appears that under intermittent aeration conditions, *H. hydrossis* requires higher NO_{2+3}^- concentrations (> 5 mgN/ℓ) than filament types 1851 and 1701 (< 5 mgN/ℓ) in order to proliferate.

Differences in sludge settleability between IAND and 2RND systems with apparently similar operating conditions

From the work conducted with IAND Systems 1–4 above, it is apparent that filaments proliferate with (1) RBCOD or SBCOD, under (2) alternating anoxic–aerobic conditions, and (3) the presence of NO_3^- and NO_2^- exacerbates the proliferation (DSVI ≈ 400–600 mℓ/g). In contrast to this Hulsman *et al.* (1992) found that for 2RND configurations fed the same defined substrate composition, and with an aerobic mass fraction between 30 and 40% of the total, the usual filaments such as *H. Hydrossis* and 1851 did not proliferate to the same degree (DSVI ≈ 150–200 mℓ/g). The difference in filament proliferation between sludges in the IAND and the 2RND systems, with apparently similar exposure to aerobic and anoxic conditions was noteworthy and is investigated further in Part II below.

Conclusions

From the results of the investigations described above, the following observations and conclusions can be made:

(i) *M. parvicella* did not proliferate under intermittent aeration conditions fed artificial substrate, irrespective of the presence or absence of fats and oils in the substrate.

(ii) *H. hydrossis* and type 1851 proliferated in intermittently aerated systems fed either RBCOD–rich substrate (DSVI ≈ 900 mℓ/g) or SBCOD–rich substrate (DSVI ≈ 500 mℓ/g) or average substrate (30/70 RBCOD/SBCOD

mixture) (DSVI ≈ 600 mℓ/g)..

(iii) In an IAND system fed RBCOD-rich substrate (DSVI ≈ 1200 mℓ/g), two in-series correctly sized aerobic selector reactors controlled proliferation by the filamentous organisms *H. hydrossis* and type 1851 (DSVI ≈ 270 mℓ/g), and three in-series correctly sized anoxic selector reactors controlled proliferation by the same filaments but to a lesser extent (DSVI ≈ 460 mℓ/g).

(iv) Filamentous organisms did not proliferate in fully aerobic (DSVI < 100 mℓ/g) or fully anoxic systems (DSVI < 150 mℓ/g).

(v) In IAND systems the filamentous organisms *H. hydrossis* and type 1851 proliferated to the greatest extent when the aerobic mass comprised between 30 and 40% of the total.

(vi) The filaments *H. hydrossis* and type 1851 proliferated in an IAND system to which NO_3^- in excess was drip fed (DSVI ≈ 800 mℓ/g) but after removal of the NO_3^- and the NH_4^+ fraction of the substrate, the proliferation of the filaments was not sustained (DSVI ≈ 200 mℓ/g).

(vii) The filaments *H. hydrossis* and type 1851 proliferated on artificial substrate in IAND systems (DSVI, 400-800 mℓ/g), to a greater extent than the low F/M filaments *M. parvicella* and type 0914 proliferate on municipal sewage IAND systems (DSVI 200-400 mℓ/g) (see Warburton *et al.*,1991).

(viii) The proliferation of filaments in IAND and 2RND systems seems to be related to system configuration; filaments *H. hydrossis*, type 1851 and type 1701 proliferated in IAND (DSVI ≈ 400-600 mℓ/g) but not in 2RND systems (DSVI ≈ 150-200 mℓ/g), even though the two configurations had apparently similar conditions, i.e. 30% aerobic, 70% anoxic mass fractions.

Aspect (viii) provides scope for investigation in that the condition which allows filaments to proliferate in IAND but not in 2RND systems may be one of the conditions which is responsible for filament bulking in N and N & P removal systems. With this in mind, the feeding patterns and aeration conditions to which sludge is exposed in IAND and 2RND systems were compared and the following

differences were noted:

- The frequency of exposure of sludge to alternating anoxic–aerobic cycles in an IAND system is high (> 30/d) as a result of the large number of intermittent aeration cycles per day, but for a 2RND system is low (< 5/d) as a consequence of the low a– (aerobic–anoxic) and s– (sludge) recycles.

- The RBCOD and SBCOD fractions of the influent are fed into both the aerobic and anoxic periods in IAND systems but in 2RND systems, the influent is all fed into the anoxic zone only (for pre–denitrification MLE systems) or into the aerobic zone only (for post–denitrification Wuhrmann systems).

- The DO concentration in the aerobic period of an IAND system changes progressively from a high value (DO ≈ 2,0 mgO/ℓ) to zero, passing through a period of low DO as a result of biological action, but in a 2RND system the DO concentration remains constant in the aerobic reactor (DO > 2,0 mgO/ℓ) and changes relatively quickly to zero in the anoxic reactor.

- The NO_2^- and NO_3^- concentrations in the anoxic period of an IAND system decrease as a result of denitrification, but in a 2RND system at steady–state, the NO_2^- and NO_3^- concentrations remain constant in the anoxic reactor.

These four differences between IAND and 2RND systems were tested experimentally with municipal sewage as influent and the results are described in Part II of this Chapter.

After operating IAND Systems 1 to 4 for a period of a year (365 days) the use of defined artificial substrate was discontinued for two reasons. The first reason is that the primary objective in its use was fulfilled, i.e. to investigate whether or not filament proliferation can take place on an SBCOD–rich substrate. The second reason is that the filaments which dominated the four laboratory systems fed the defined substrate, *H. hydrossis*, type 1851 and type 1701, were not the same filaments found in full–scale N and N & P removal systems, (*M. parvicella*, type 0092 and type 0914). Real municipal sewage was used in all further experimental work.

Because of the importance of conducting laboratory–scale experiments with the same filamentous organisms as are found in full–scale systems, many of the aspects

investigated with defined substrate described above and found to have a significant effect on filament proliferation were re-examined in Part II with municipal sewage.

3.3 PART II – MUNICIPAL SEWAGE AS INFLUENT

Investigation of the eight aspects listed at the end of Chapter 2 continued with municipal sewage as influent to laboratory-scale systems such that between Parts I and II, all eight aspects were investigated. The experimental work conducted with municipal sewage investigated the effect on filament proliferation of the following aspects:

- Continuous aerobic and continuous anoxic conditions and variation in aerobic mass fraction.
- Influent TKN/COD ratio and NO_3^- and NO_2^- concentrations in the system.
- Sludge age.
- Differences in feeding pattern and aeration conditions between IAND and 2RND systems with apparently similar alternating anoxic-aerobic conditions.
- NO_2^- concentration in the anoxic zone.

The experimental work was conducted on IAND, 2RND and MUCT systems. Nine systems were operated and are named according to their configurations, i.e. Systems 5 to 8: IAND configuration; System 9: 2RND configuration; Systems 10 to 13: MUCT configuration.

Experimental results and discussion

Continuous aerobic, continuous anoxic conditions and variation in aerobic mass fraction

Continuous aerobic conditions: In operation of Systems 5, 6, 7 and 8, the initial conditions imposed were intermittent aeration, in order to develop a high DSVI. For each system the DSVI increased 100 mℓ/g in 35 days as illustrated in Figs 3.9 to 3.12 for Systems 5 to 8 respectively. Each of the systems was then subject to continuous aeration, and for each system the DSVI decreased from around 250 mℓ/g to below 100 mℓ/g in 7 days. With the introduction of intermittent aeration to each system, the DSVI again increased, from below 100 mℓ/g to above 150 mℓ/g in

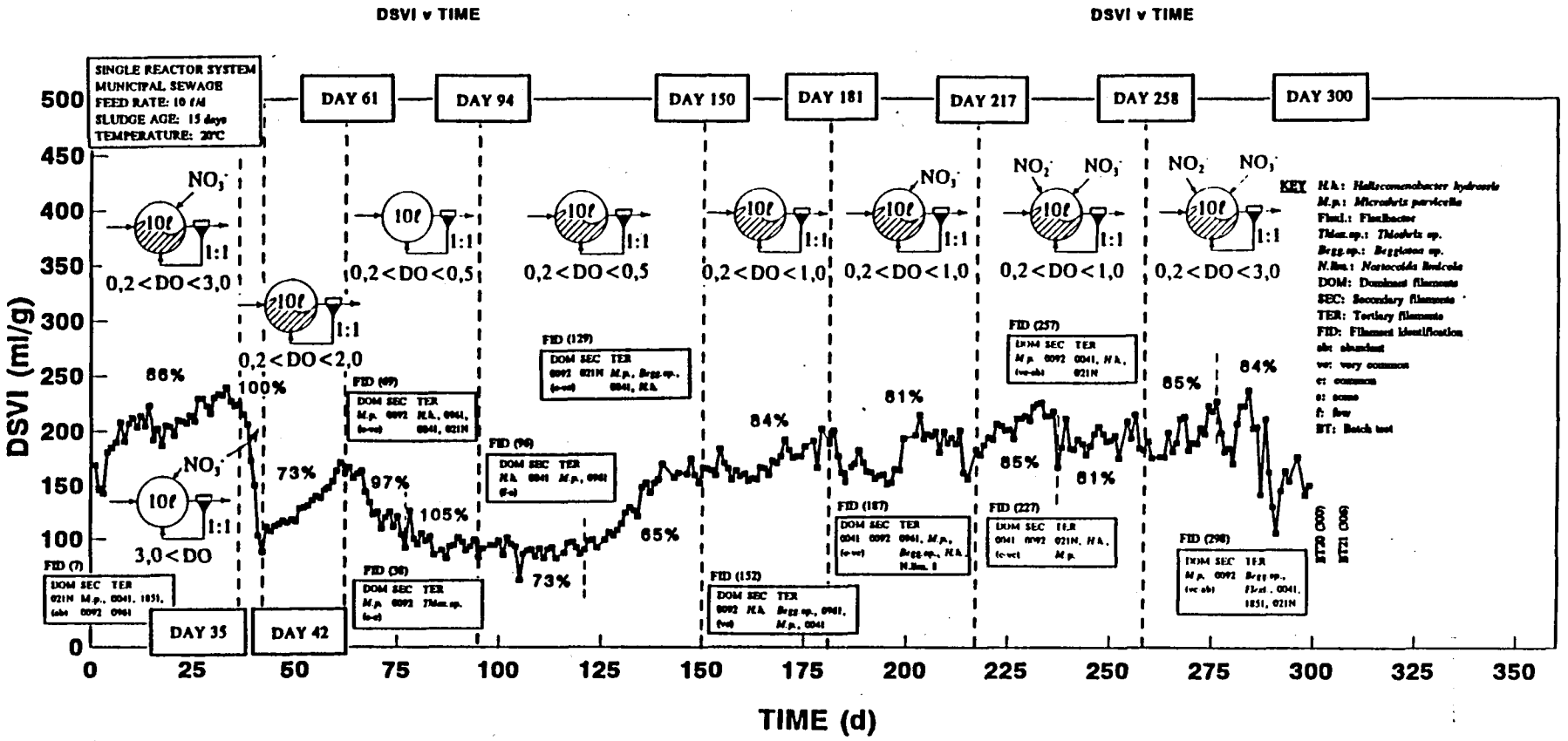


Fig 3.9: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 5. (Percentage values refer to COD mass balances).

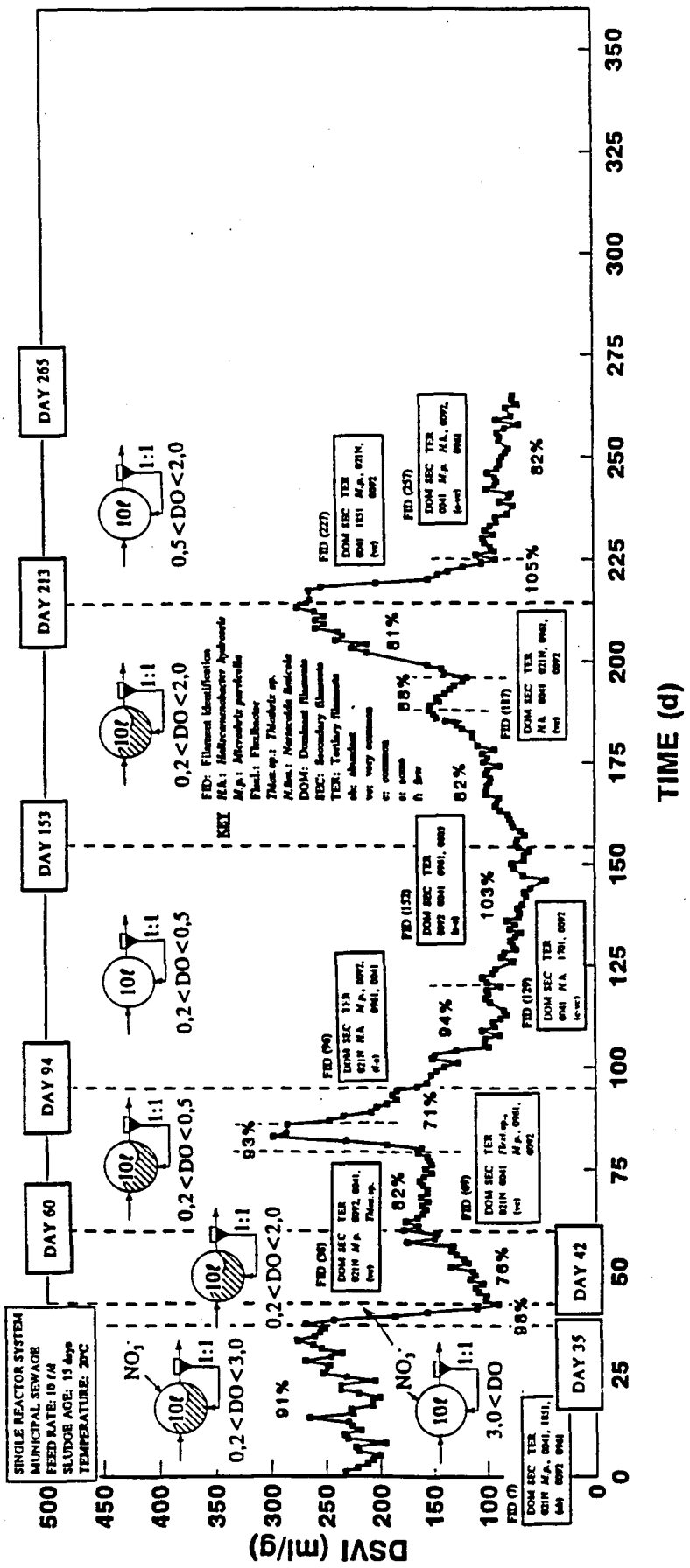


Fig 3.10: Sludge settleability as DSVI (ml/g) and changes in system operation with time (d) for System 6. (Percentage values refer to COD mass balances).

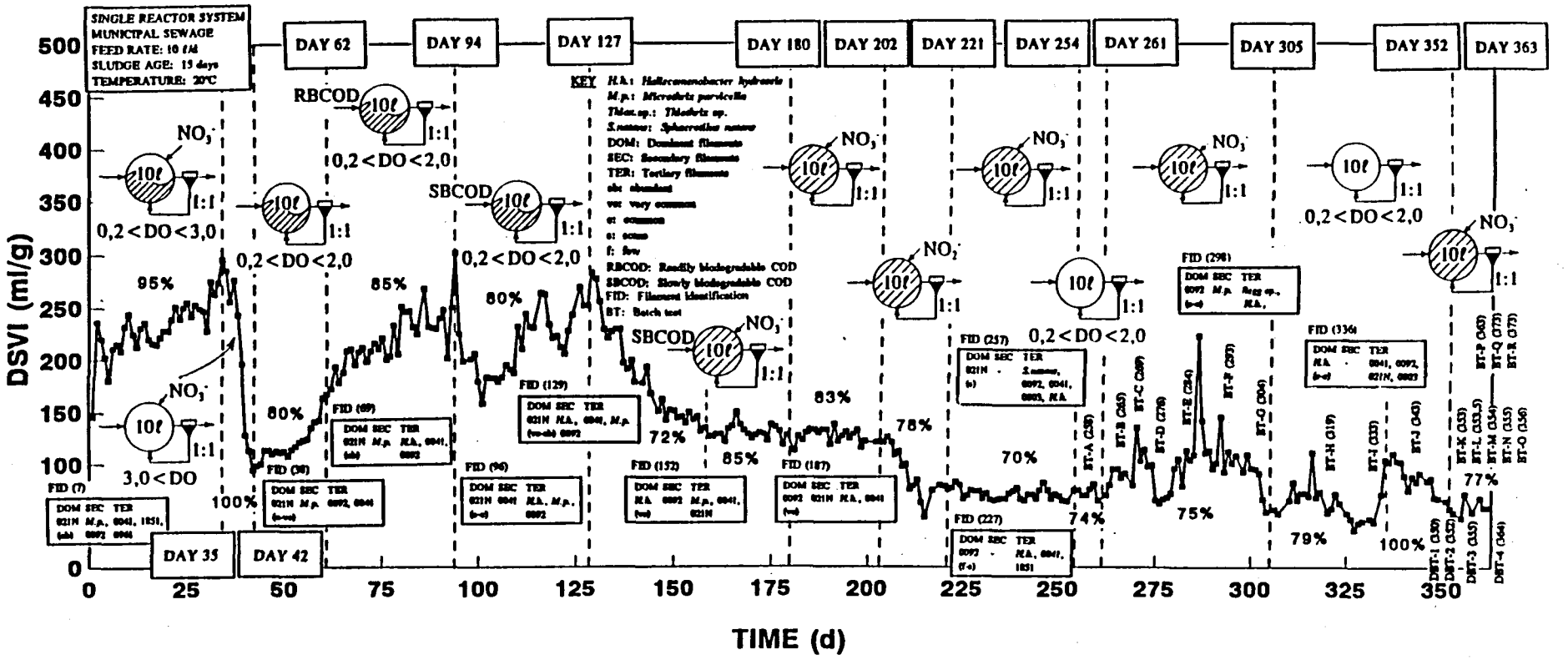


Fig 3.11: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 7. (Percentage values refer to COD mass balances).

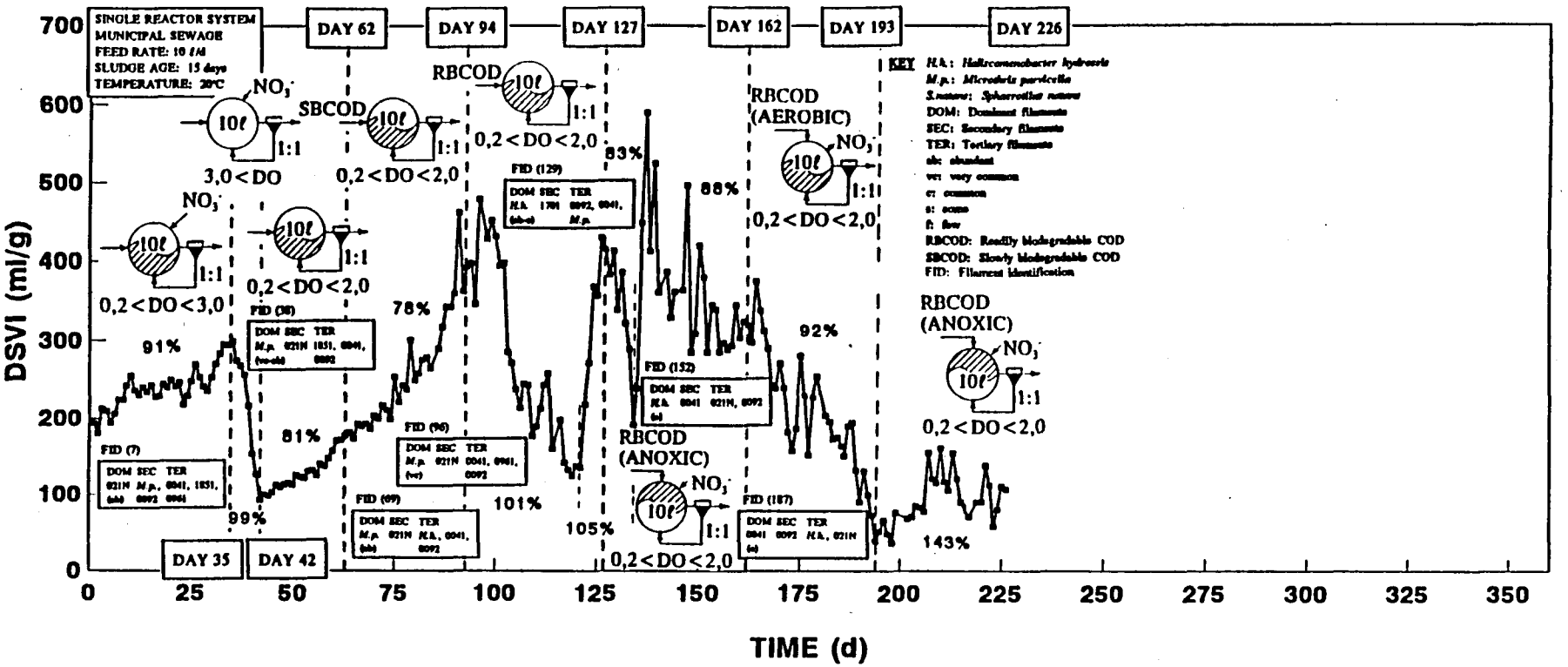


Fig 3.12: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 8. (Percentage values refer to COD mass balances).

just over a sludge age. For each of Systems 5 to 8, the rapid reduction in DSVI with continuous aeration was associated with an increase in the COD balance. Under continuous aeration, the COD balance was between 98 and 100 percent, but with intermittent aeration in which the DO ranged between 0,2 and 3,0 mgO/ℓ, the COD balance was 86 to 95% and with intermittent aeration in which the DO ranged between 0,2 and 3,0 mgO/ℓ, the COD balance was between 73 and 81 percent.

It was found also that other periods during which conditions were changed to continuous aerobic from intermittent aeration (System 5, Days 62 to 94; System 6, Days 95 to 153), the COD balance improved from around 75 percent to between 95 and 105 percent. Reasons for the improvement in COD balance from intermittent aeration conditions (under which a high DSVI developed) to continuous aeration (under which a low DSVI developed) are proposed in Chapter 6 in which a biochemical/microbiological model for bulking is developed. Ketley *et al.* (1991) also investigated the effect of continuous aerobic conditions on bulking sludges developed in three IAND systems. In each system, *M. parvicella* proliferation in the sludge as a consequence of intermittent aeration conditions resulted in a DSVI ≈ 400 mℓ/g, and changing the aeration pattern from intermittent (30–35% aerobic) to continuous caused amelioration of filament proliferation (DSVI ≈ 100 mℓ/g). This result is in agreement with the results of Gabb *et al.* (1989a) in which *M. parvicella* proliferation in IAND systems fed municipal sewage was controlled by continuous aeration.

Continuous anoxic conditions: Ketley *et al.* (1991) investigated the effect of continuous anoxic conditions on bulking sludges developed in three IAND systems. Following the imposition of continuous anoxic conditions with excess NO_3^- supplied as electron acceptor, the DSVI decreased from ≈ 200 to ≈ 89 mℓ/g; the filaments *M. parvicella*, type 1851 and type 0092 each of which was dominant under intermittent aeration conditions, were controlled by continuous anoxic conditions.

Variation in aerobic mass fraction: In nitrification–denitrification (ND) systems, whether IAND, 2RND or ND biological excess phosphorus removal (NDBEPR) systems, the aerobic period serves two functions; (i) to supply oxygen as electron acceptor during heterotrophic organism utilization of organic matter and (ii) to supply oxygen for nitrification of influent NH_4^+ to NO_2^- and NO_3^- by autotrophic organisms as the preliminary step to nitrogen removal through denitrification. Warburton *et al.* (1991) examined the effect on filament proliferation of variation in

the aerobic sludge mass fraction in IAND systems. At a sludge age of 10 days, a 70% aerobic mass fraction system had a much lower DSVI than a 30% aerobic mass fraction system. The effect of aerobic mass fraction on filament proliferation was investigated in two multi-reactor NDBEPR (MUCT) systems. Figure 3.13 illustrates the changes in DSVI over 180 days in two MUCT systems (Systems 10 and 11) with changes in the aerobic mass fractions of the systems. With an aerobic mass fraction of 33% (3rd reactor anoxic), System 10 developed a DSVI $\approx$ 250 mℓ/g which decreased to $\approx$ 150 mℓ/g in 20 days when the aerobic mass fraction was increased to 65% (3rd reactor aerated). With an aerobic mass fraction of 65% (3rd reactor aerated) , System 11 developed a DSVI between 100 and 150 mℓ/g which increased to $\approx$ 200 mℓ/g in 20 days when the aerobic mass fraction was changed to 33% (3rd reactor anoxic). In both systems the dominant filament was 0092. These results are in agreement with those for System 2, fed defined substrate as influent, and described by Fig 3.8 – aerobic mass fractions between 30 and 40 percent result in high DSVI values and, aerobic mass fractions increasingly greater than 40 percent and increasingly less than 30 percent result in increasingly lower DSVI values. During operation of Systems 10 and 11, the parameters DSVI, VSS and effluent NO_{2+3}^{-} changed with time, and are illustrated in Figs 3.14 and 3.15 for Systems 10 and 11 respectively. With increase in aerobic mass fraction from 33% to 65%, the DSVI decreased, but the effluent NO_{2+3}^{-} concentration increased as a consequence of decreased denitrification potential in the system (3rd reactor aerated). Throughout the 180 day period the biological excess phosphorus removal (BEPR) was not affected by the changing effluent nitrate concentration – the second reactor was sufficiently large to fully denitrify the nitrate discharged to it via the sludge underflow, thereby ensuring a nitrate free discharge to the anaerobic zone. A further finding of these experiments was that generally, for high or increasing DSVIs the nitrogen balance was lower than for low or decreasing DSVIs. Values for the nitrogen mass balances and the periods over which they were conducted are shown in Fig 3.13. The reasons for these changes are explained in the bulking model presented in Chapter 6.

Sludge age

In examining the effect of sludge age on filament proliferation, Warburton *et al.* (1991) found that for an IAND system with a 30% aerobic mass fraction and bulking with a low F/M filament sludge, (DSVI > 300 mℓ/g), decreasing the sludge age from 20 days to 10 days had little effect on DSVI, filament proliferation and filament types (i.e. *M. parvicella*, type 0092, type 0675 and type 0041). Decreasing

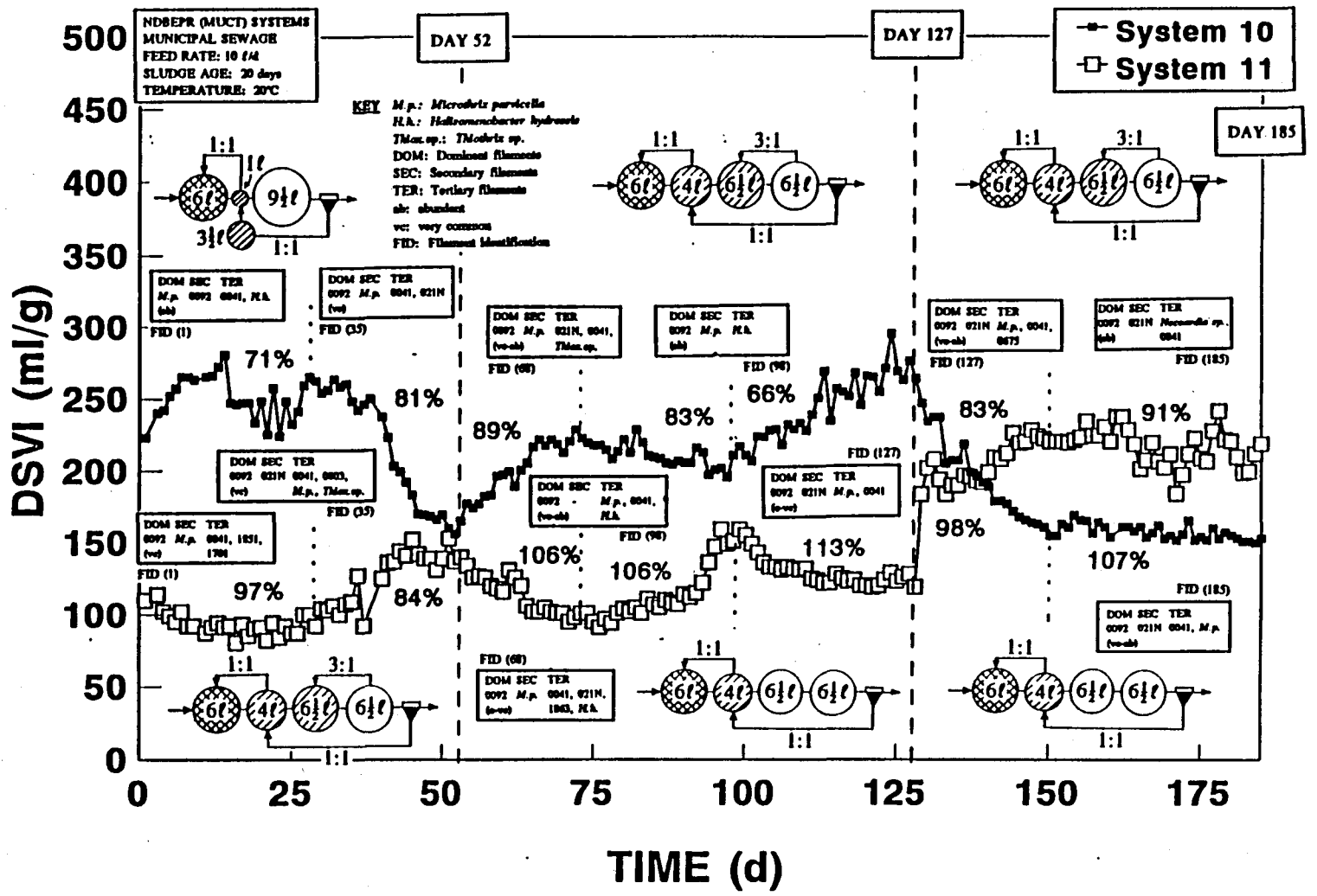


Fig 3.13: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for MUCT Systems 10 and 11. (Percentage values refer to N mass balances).

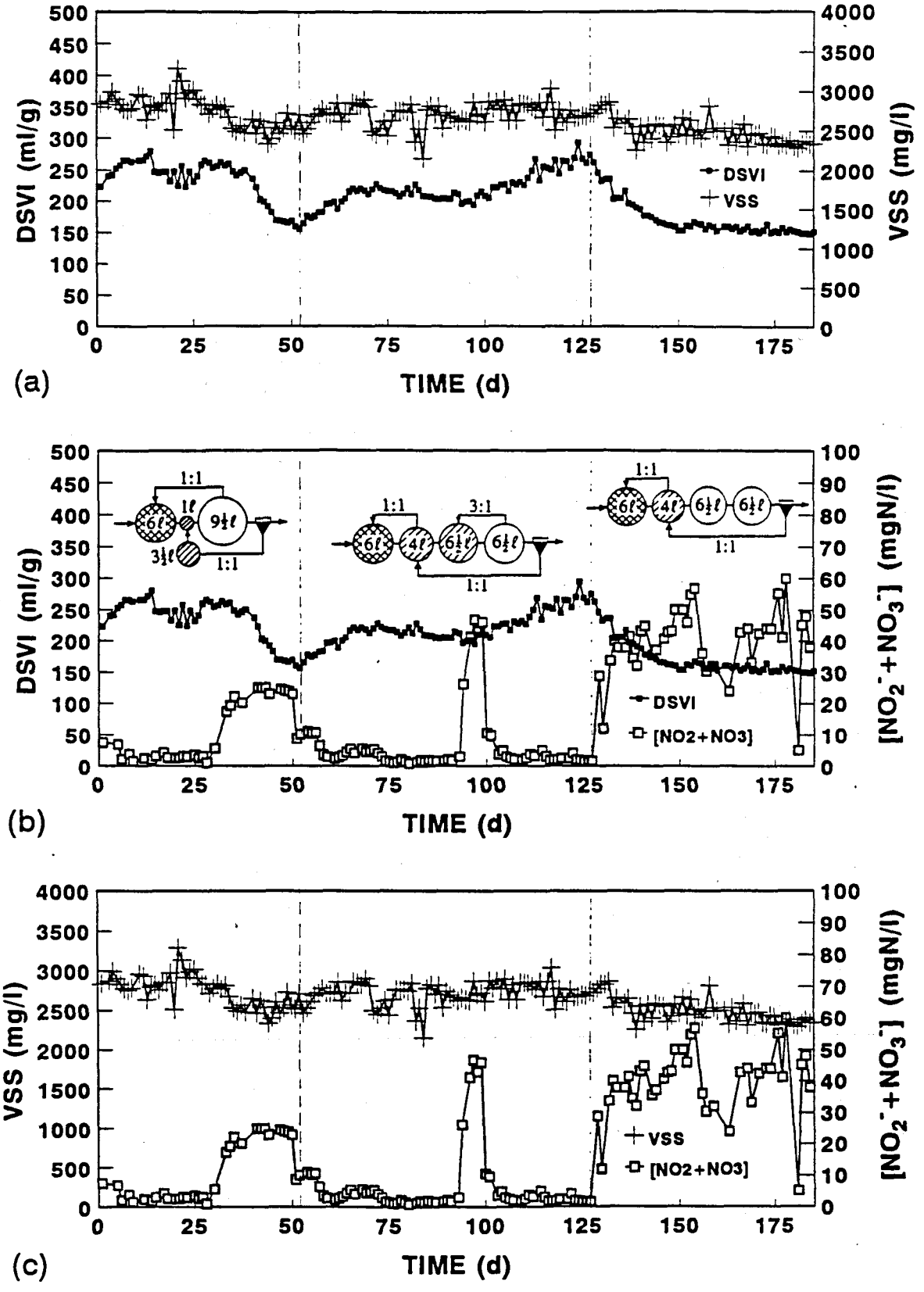
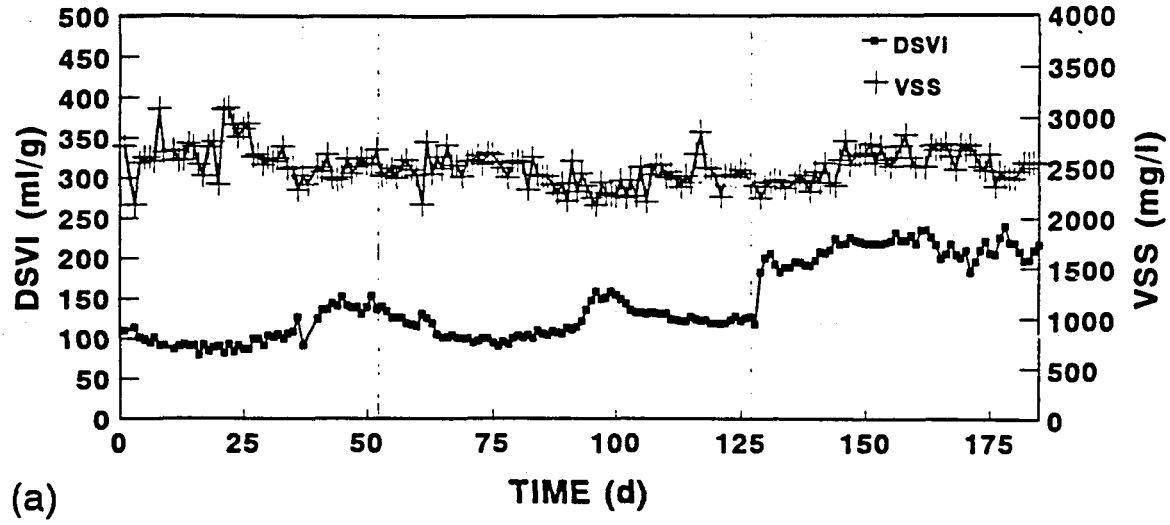
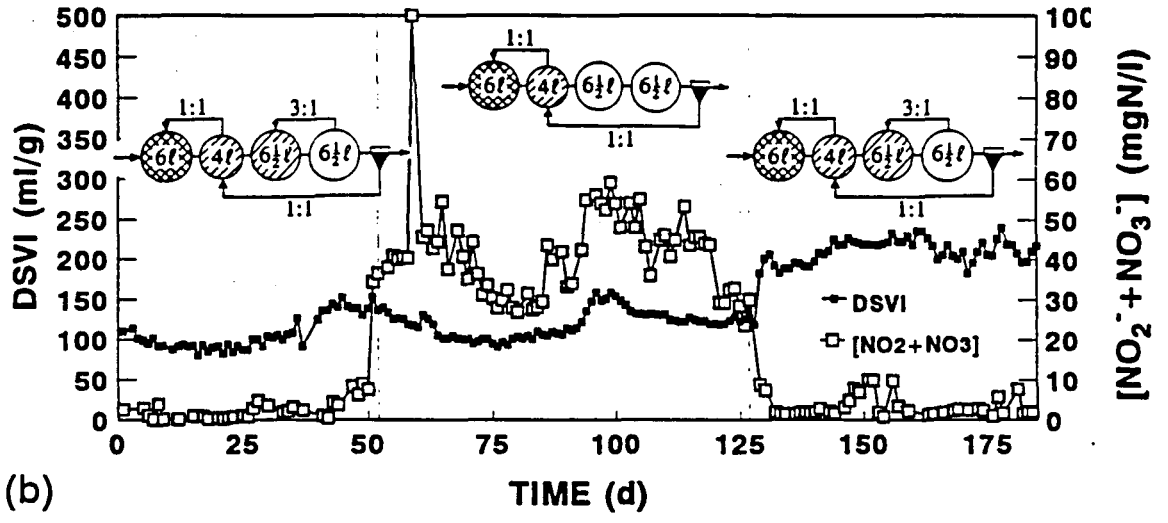


Fig 3.14: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time, and (c) VSS and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time for System 10.





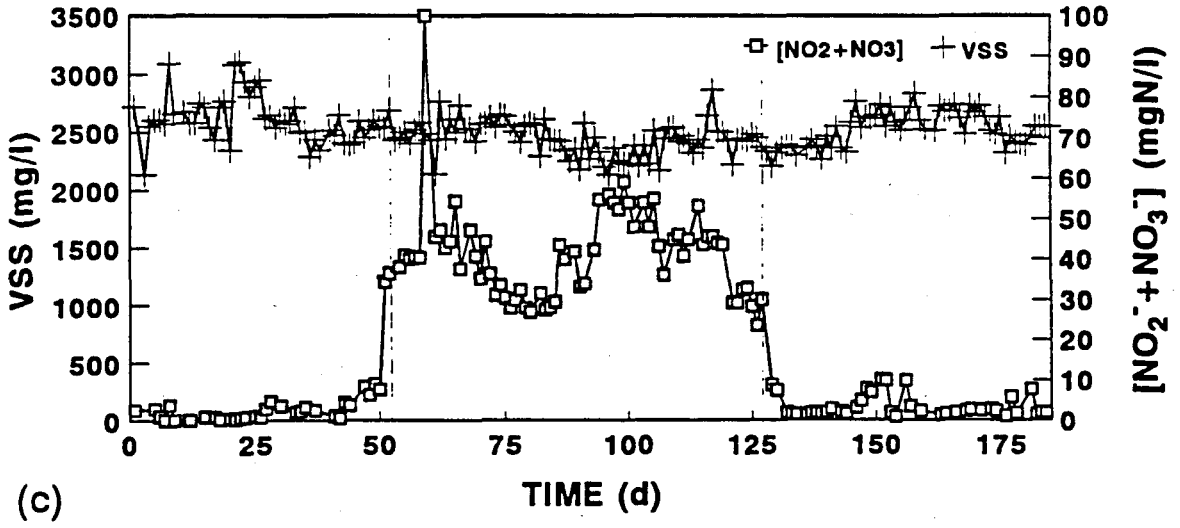


Fig 3.15: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time, and (c) VSS and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time for System 11.

the sludge age further, from 10 to 7 and then to 5 days, also had little effect on filament types, but proliferation was somewhat reduced. The DSVI declined to around 150 mℓ/g. Although with 30% aerobic mass fraction and at sludge ages < 10 days, nitrification was not adversely affected, excess nitrate was dosed to ensure the availability of sufficient nitrate for denitrification.

Influent TKN/COD ratio and NO_3^- and NO_2^- concentrations

The influent TKN/COD ratio governs the degree of nitrogen removal that can be achieved in an N or N & P removal system and hence the effluent nitrate concentration. There are two ways in which the influent TKN/COD ratio can be increased; (1) by addition of ammonium to the influent, and (2) by dosing nitrate to the system. Warburton *et al.* (1991) investigated the effect of nitrate dosing on low F/M filament proliferation in 10 day sludge age IAND systems. The effect of TKN/COD ratio by ammonia dosing to the influent was also examined on two MUCT systems at 20 days sludge age and 33% aerobic mass fraction. Figure 3.16 illustrates the changes in DSVI of two MUCT systems (System 12 and System 13) with changes in the TKN/COD ratio of the influent resulting from the addition and removal of NH_4^+.

With an influent TKN/COD ratio of 0,12–0,14 mgN/mgCOD with ammonium addition to the influent, the DSVI of System 13 increased from ≈ 100 to ≈ 250 mℓ/g over a period of 5 sludge ages (Days 40 to 169) and with an influent TKN/COD ratio of 0,08–0,10 mgN/mgCOD the DSVI decreased in less than two sludge ages to 160 mℓ/g (Days 170 to 198). Similarly, for System 12, with an influent TKN/COD ratio of 0,08–0,10 mgN/mgCOD, the DSVI remained between 100 and 150 mℓ/g (Days 40 to 169) but with an influent TKN/COD ratio of 0,12–0,14 mgN/mgCOD with ammonium addition to the influent, the DSVI increased in less than two sludge ages to nearly 250 mℓ/g (Days 170 to 198). Figures 3.17b and 3.18b illustrate the variation in NO_{2+3}^- concentration with time in the 2nd anoxic reactors of Systems 12 and 13 respectively. With increase in TKN/COD ratio and corresponding increase in DSVI, the concentration of NO_{2+3}^- in the 2nd anoxic reactors increased from ≈ 5 to ≈ 20 mgN/ℓ. An additional finding in these experiments was that as the DSVI increased, the VSS of the sludge decreased, and as the DSVI decreased, the VSS increased. The relationships between DSVI and VSS for Systems 12 and 13 are illustrated in Figs 3.17a and 3.18a respectively. The relationships between DSVI, VSS and NO_{2+3}^- for Systems 12 and 13 are different to those for Systems 10 and 11. Whereas for Systems 10 and 11, the VSS decreased and NO_{2+3}^- increased with

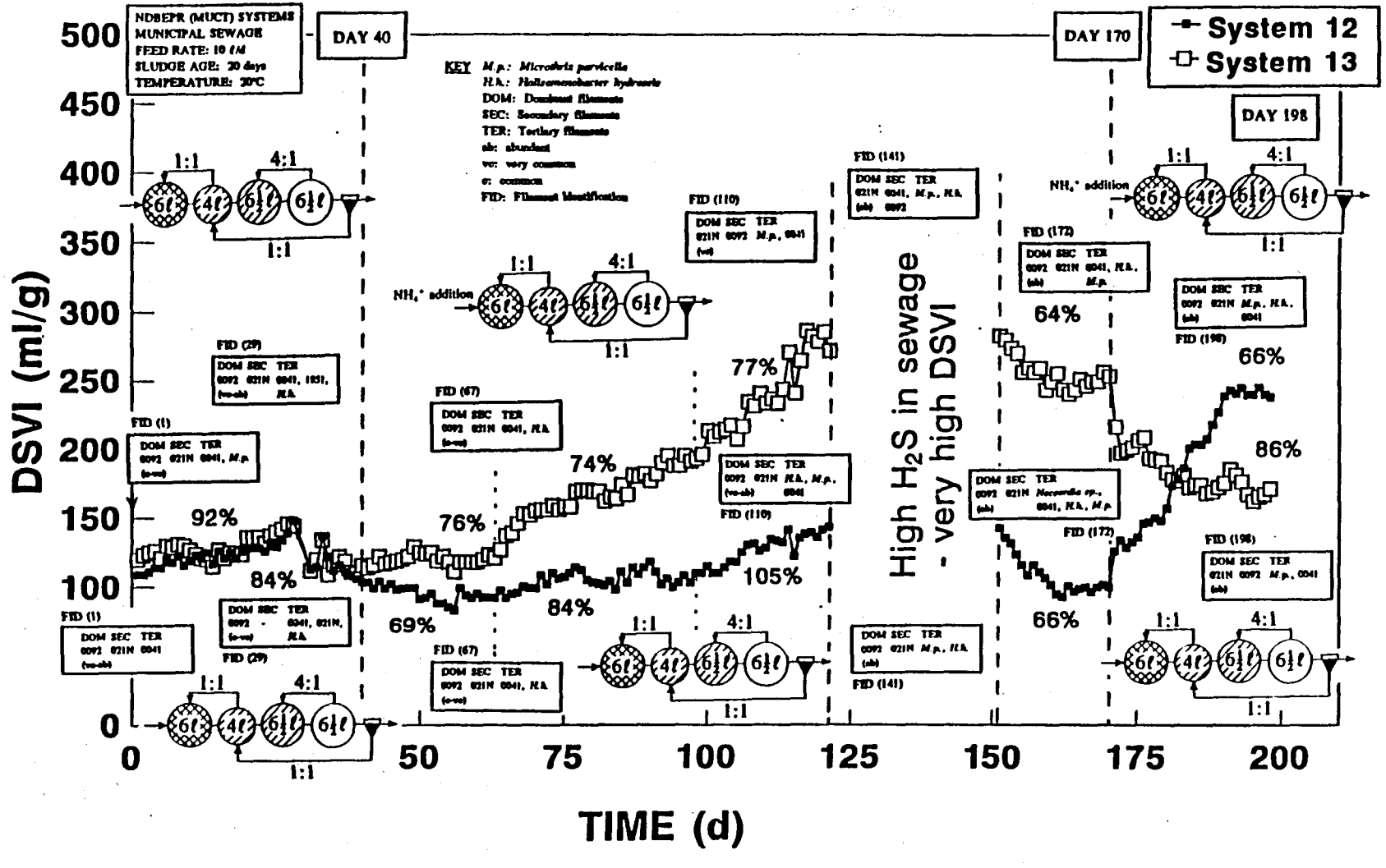


Fig 3.16: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time for MUCT Systems 12 and 13. (Percentage values refer to N mass balances).

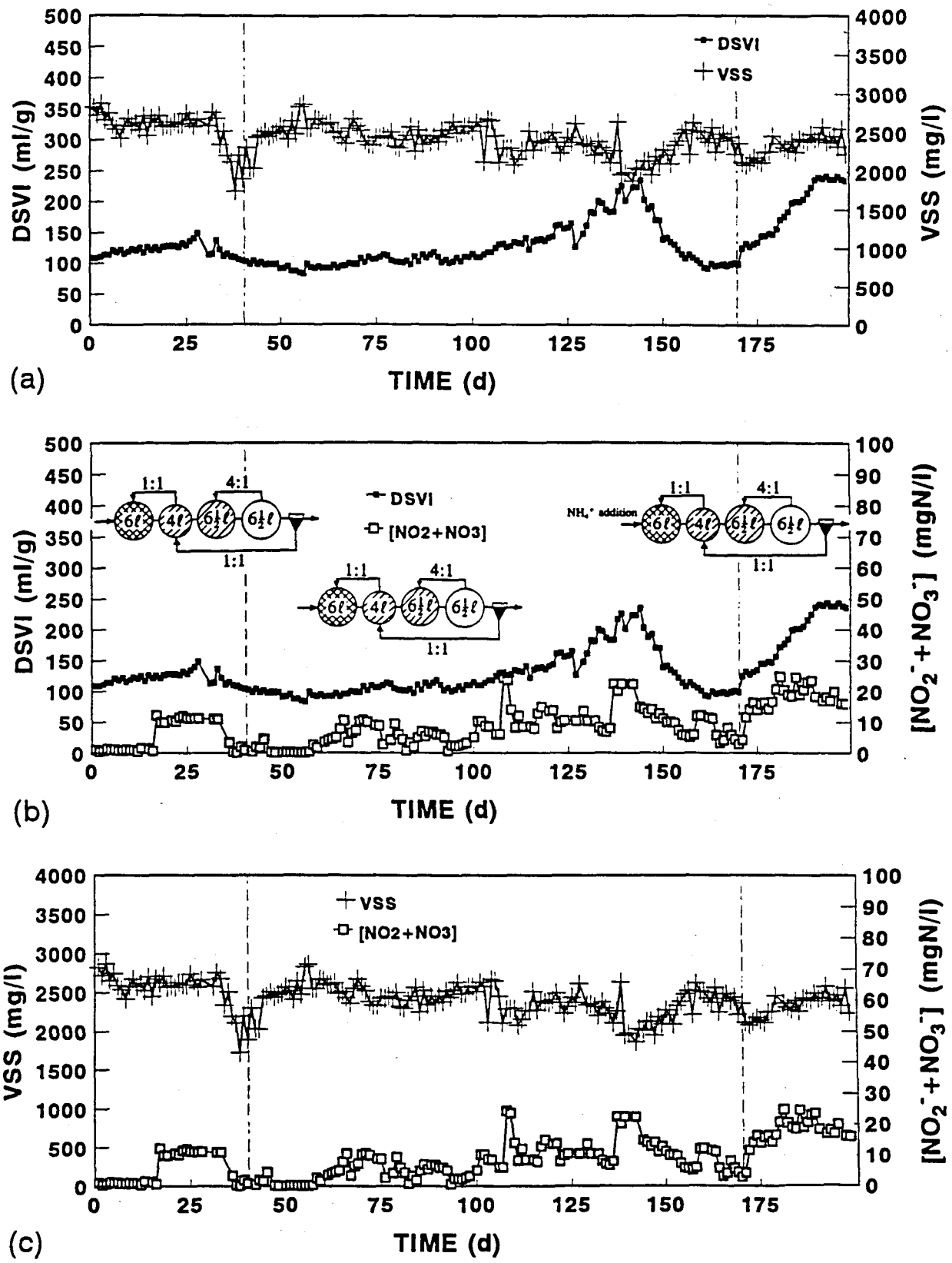
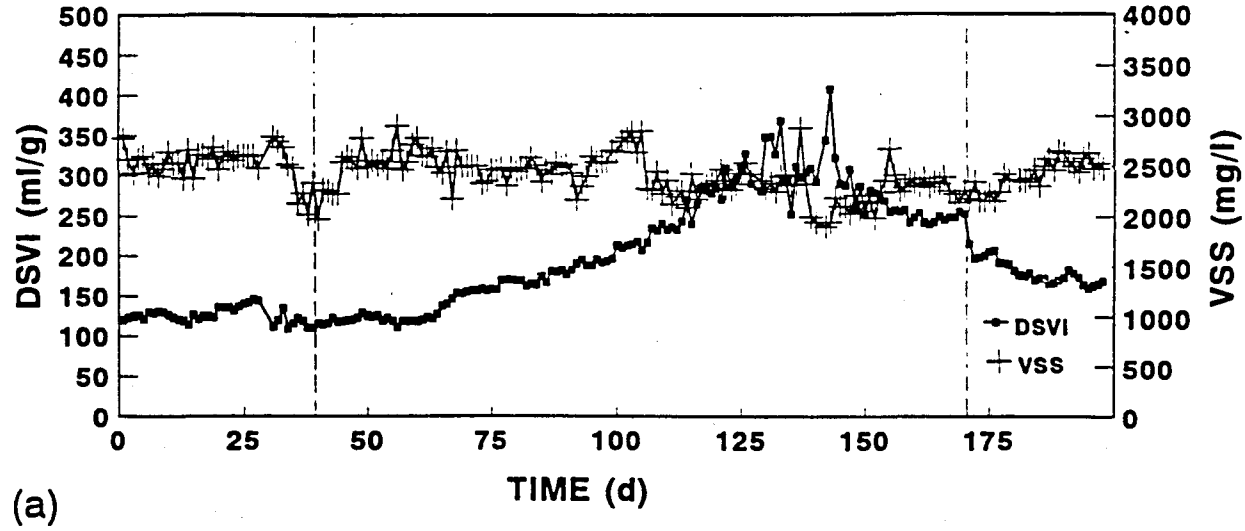
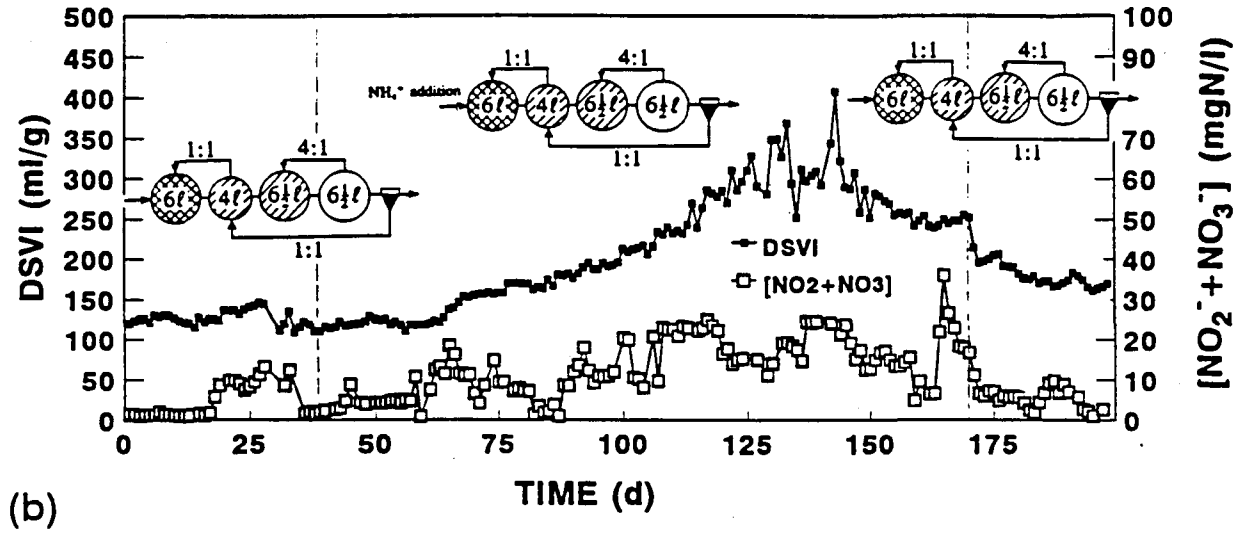


Fig 3.17: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time, and (c) VSS and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time for System 12.





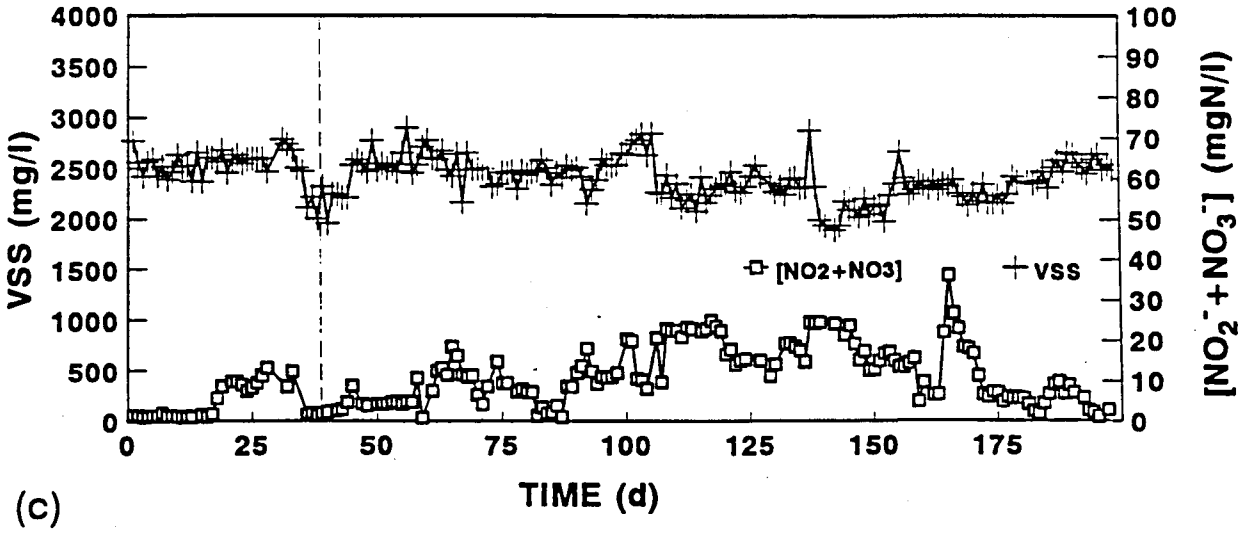


Fig 3.18: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time, and (c) VSS and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time for System 13.

decrease in DSVI, for Systems 12 and 13, the VSS increased and NO_{2+3}^- decreased with decrease in DSVI. Since the aerobic and anoxic mass fractions remained unchanged for Systems 12 and 13, it is likely that the reasons for the changes in parameters in Systems 12 and 13 are different to those in Systems 10 and 11 in which the decrease in DSVI due to increase in aerobic mass fraction resulted in an increase in NO_{2+3}^- due to a reduced denitrification potential. Similarly, as for Systems 10 and 11 described earlier, an interesting finding in these experiments was that generally, for high or increasing DSVIs, the nitrogen balance was lower than for low or decreasing DSVIs. This is illustrated in Fig 3.16. The reasons for these results are explained through description of the bulking model in Chapter 6. The results of the experiments further reinforce the impression that the concentration of NO_{2+3}^- is of particular importance to filament proliferation and this aspect is subject to more intense scrutiny in further work in this Chapter.

Differences between IAND and 2RND configurations with apparently similar operating conditions

In the conclusion to Part I, it was noted that significant differences in filament proliferation occurred in IAND and 2RND systems with apparently similar operating conditions, i.e. similar sludge ages, aerated/unaerated mass fractions and the same influent sewage. At the beginning of Part II, in a more critical assessment of the dissimilarities between IAND and 2RND systems, four differences between the two configurations were noted; namely, frequency of exposure of sludges to anoxic–aerobic conditions, availability of RBCOD under both aerobic and anoxic conditions, concentration of DO in the aerobic period or reactor, and, concentration of NO_3^- in the anoxic period or reactor.

Each of these differences was examined experimentally and with hindsight only one difference had a significant effect on filament proliferation, i.e. the concentrations of NO_3^- and NO_2^- in the anoxic period of an IAND system and in the anoxic reactor of a 2RND system. In order to avoid a lengthy discussion of the experiments relating to differences which did not have a substantial effect on filament proliferation, only the experimental work relating to the concentration of NO_3^- and NO_2^- in the anoxic zone is described in detail. Experiments relating to the other differences between the system configurations (frequency of response to anoxic–aerobic conditions, availability of RBCOD, and concentration of DO) are described only briefly. All experimental work with municipal sewage is described in detail in Appendix B. Although it was realized that the differences between IAND and 2RND

configurations could play a role in filament proliferation, the mechanism by which this could occur was unclear.

(1) *Frequency of exposure of sludges to aerobic/anoxic conditions:*
The first difference concerns the frequency of exposure of sludges in IAND and 2RND configurations to aerobic/anoxic conditions. In IAND systems, each anoxic–aerobic cycle is about 20 to 30 minutes in length, which equates to between 72 and 48 cycles/day. In 2RND systems, the mixed liquor (a–) and underflow sludge (s–) recycle ratios determine the frequency of alternation, and in laboratory–scale systems normal operating conditions would be a = 1 to 3 and s = 1, i.e. the number of anoxic–aerobic cycles varies between 3 and 5/day, which is significantly lower than the 48 to 72 cycles/day in an IAND system. It was proposed that if filaments gain a benefit from exposure to alternating anoxic–aerobic cycles, then that benefit would be proportionally increased by exposure to increased numbers of anoxic–aerobic cycles.

To determine the effect of frequency of exposure of sludge to anoxic conditions, experiments were conducted both with IAND and 2RND systems.

- Ketley *et al.* (1991) examined IAND systems with aerobic periods of approximately 30% and anoxic–aerobic cycle lengths varying between 20 minutes (14 min anoxic, 6 min aerobic) and 3 days (2 days anoxic, 1 day aerobic). All systems had NO_3^- addition during the anoxic period and all systems developed bulking sludges (DSVI > 150 mℓ/g).

- Hulsman *et al.* (1992) examined 2RND systems with aerobic mass fractions comprising 30–40% of the total, one system with a low (3:1) a–recycle ratio, the other system with a high (>30:1) a–recycle ratio. The objective of the high a–recycle ratio in the 2RND system was to provide a frequency of exposure of the sludge to anoxic–aerobic conditions similar to IAND systems. The systems with the low and the high a–recycle ratios both developed non–bulking sludges (DSVI < 150 mℓ/g).

The results of Ketley *et al.* (1991) and Hulsman *et al.* (1992) indicated that the frequency of exposure of sludge to anoxic–aerobic conditions does not influence the proliferation of low F/M filaments; IAND systems bulked irrespective of the number of anoxic–aerobic cycles, and 2RND systems did

not bulk irrespective of the number of anoxic-aerobic cycles the sludge experienced.

(2) *Availability of RBCOD under aerobic and anoxic conditions in a 2RND (MLE) configuration:*

The second difference between IAND and 2RND systems concerns the two fractions of substrate (i.e. RBCOD AND SBCOD) available to the organisms under aerobic and anoxic conditions. In a 2RND system, all the substrate feeds into the anoxic reactor where the RBCOD is utilized completely, the only substrate available in the aerobic reactor is SBCOD. In an IAND system, substrate feeds continuously into the system throughout the intermittent aeration cycle, such that the aerobic and anoxic periods receive both RBCOD and SBCOD, the total amount of substrate received by each of the periods being in proportion to the anoxic and aerobic fractions of the cycle. Three sets of experiments were conducted to examine this aspect:

- A 2RND system (System 9) was operated in which the influent was split between the anoxic and aerobic zones in proportion to their respective mass fractions, in order to approximate the feeding pattern of an IAND system. Figure 3.19 illustrates the changes in DSVI and system configuration with time. The DSVI remained at 150 mℓ/g throughout the experiment, from Days 32 to 88.

- The RBCOD and SBCOD fractions of sewage separated by ultrafiltration (see Appendix B.5) were fed to two different IAND systems (Systems 7 and 8) in which *M. parvicella* was the dominant filament. Figures 3.11 and 3.12 illustrate the changes in DSVI and system feed composition with time for Systems 7 and 8 respectively. For System 7 the DSVI remained higher than 200 mℓ/g for both RBCOD (Days 63 to 94) and SBCOD (Days 95 to 127) as influent. However, the dominant filament was not one of the low F/M filaments, but the septic sewage filament, 021N. For System 8, fed SBCOD substrate (Days 64 to 94), the DSVI increased to ≈ 400 mℓ/g due to the low F/M filament *M. parvicella*, and when fed RBCOD substrate (Days 95 to 127) the DSVI decreased to < 150 mℓ/g, but then increased to ≈ 400 mℓ/g due to the low DO filaments *H. hydrossis* and type 1701. Subsequently the RBCOD fraction of sewage was fed for periods of 1–2 sludge ages during the aerobic period only and then during the anoxic period only of the

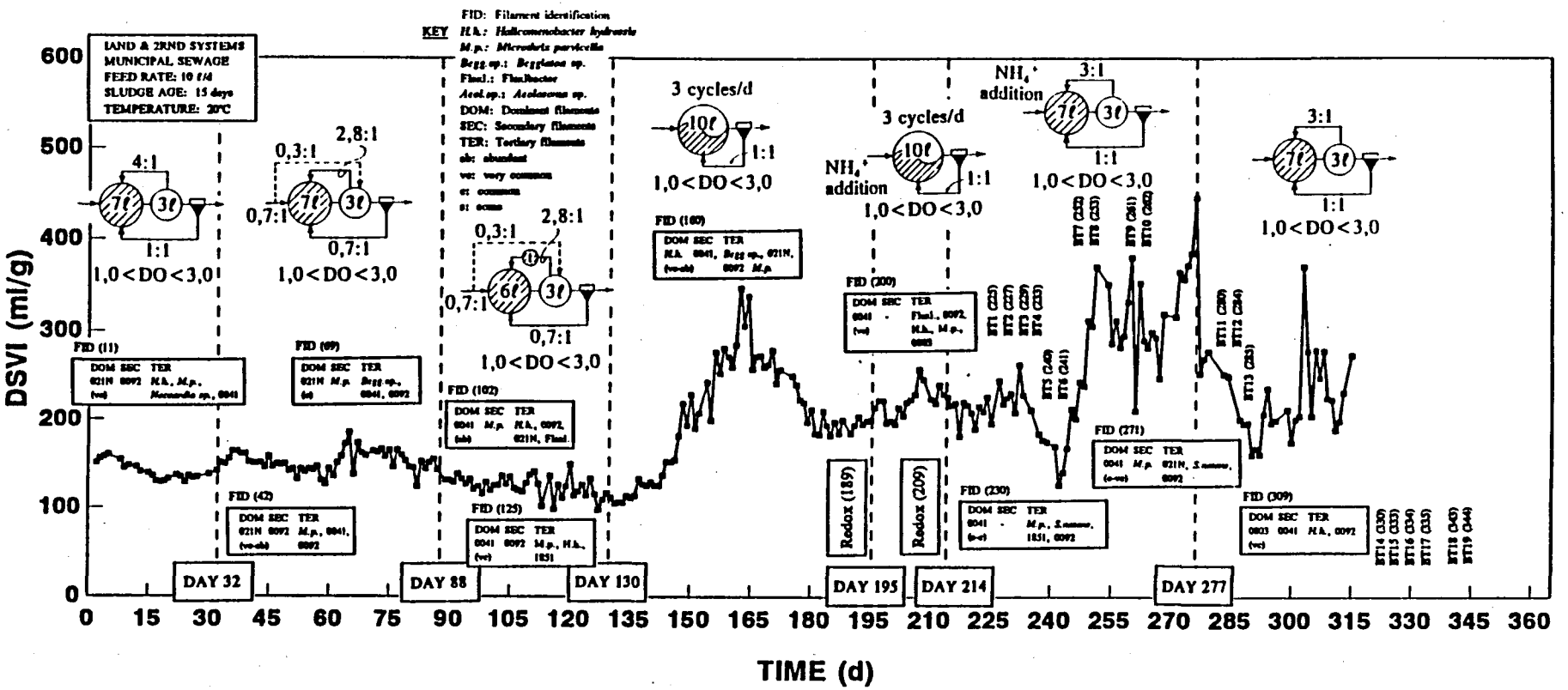


Fig 3.19: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 9.

intermittent aeration cycle of an IAND system (System 8) in an attempt to mimic the pre– and post–denitrification 2RND systems. With RBCOD substrate fed to the anoxic period only (Days 127 to 161, and Days 193 to 225) the DSVI was high or increasing, caused by the filaments *H. hydrossis*, and type 0041. With RBCOD substrate fed to the aerobic zone (Days 162 to 192) the DSVI decreased rapidly.

From the results of the above experiments it was concluded that;

(i) contrary to the proposal that in order to proliferate low F/M filaments require RBCOD in the aerobic zone of an alternating anoxic–aerobic system, the low F/M filaments are able to proliferate with SBCOD sewage only as substrate, and

(ii) with RBCOD as substrate, irrespective of whether it was fed throughout the anoxic–aerobic cycle, or only during the anoxic or aerobic periods, low F/M filaments did not appear to proliferate; the low DO filament type 1701 and the septic sewage filament 021N, somewhat confounded the DSVI results.

The above results with real sewage confirm the earlier experiments described in Part I, which investigated the proliferation of filaments with defined substrate RBCOD and SBCOD fractions; IAND systems fed only RBCOD or only SBCOD fractions developed bulking sludges, with the dominant filaments being *H. hydrossis* and 1851.

(3) *Concentration of DO during aerobic period of IAND and in aerobic reactor of 2RND system:*

The third difference between IAND and 2RND systems concerns the concentration of DO to which the sludges are exposed in the aerobic reactor of a 2RND system or during the aerobic period of an IAND system. In the case of the IAND system, the sludge initially is exposed to a high DO concentration (> 2,0 mgO/ℓ) at the start of the aeration cycle and then to a decreasing concentration of DO as a result of biological action, through a period of very low DO, and finally to a point at which oxygen is absent. In a 2RND system, the sludge is exposed in the aerobic reactor to a constant high DO concentration (> 2,0 mgO/ℓ) for the entire residence time and then in the anoxic reactor the sludge is exposed to conditions in which oxygen is absent. There is no zone in the 2RND system between the aerobic and anoxic

reactors in which the sludge is exposed to a low, or decreasing DO concentration.

To examine the effect of low DO conditions two experiments were conducted:

- Two single–reactor 15 day sludge age systems (Systems 5 and 6) were operated. System 5 had continuous low DO (0,2 < DO < 0,5) for 2 sludge ages (Days 61 to 93), and then intermittent aeration (35% aerobic, 65% anoxic) for 3 sludge ages (Days 94 to 149), the aerobic part of the cycle maintained at low DO (0,2 < DO < 0,5). System 6 had intermittent aeration for 2 sludge ages (Days 60 to 93), and then continuous low DO for 3 sludge ages (Days 94 to 152), the DO concentration the same as for System 5. The changes in DSVI and aeration pattern for Systems 5 and 6 are shown in Figs 3.9 and 3.10 respectively from which it can be seen that with continuous aeration at low DO, the DSVI was low and with intermittent aeration with low DO in the aerobic period the DSVI was high. The filamentous organisms causing the high DSVI were types 0092 and 0041, for System 5 and type 021N for System 6. From the results of the experiments it was concluded:

  (i) Filamentous organisms do not proliferate under continuously aerated conditions irrespective of whether the concentration of DO is low (0,2 < DO < 0,5), or high (0,2 < DO < 1,0–2,0).

  (ii) In order to proliferate, filamentous organisms require the presence of an anoxic period.

  (iii) For intermittent aeration conditions (35% aerobic, 65% anoxic) the higher the peak DO concentration during the aerobic period, the greater the proliferation of filamentous organisms.

  (iv) For continuous aeration conditions, the higher the DO concentration the more rapid the reduction in DSVI.

To examine the effect of a decreasing DO concentration in a 2RND system:

- A 2RND system (System 9) was operated with aerated and unaerated mass fractions of 30 and 70% respectively with the feed split between the aerobic and anoxic periods in proportion to the respective mass fractions of the aerobic and

anoxic zones (i.e. 3 ℓ/d to the aerobic reactor and 7 ℓ/d to the anoxic reactor). The DSVI of the system was between 140 and 170 mℓ/g. A small (1ℓ) unaerated reactor was placed between the aerobic and anoxic reactors receiving sludge from the aerobic reactor via the a-recycle and the concentration of DO in this small reactor was controlled to be very low (< 0,5 mgO/ℓ, thereby avoiding a rapid change in DO concentration between the aerobic and anoxic reactors. (To maintain the same total system volume and aerobic mass fractions, the size of the anoxic reactor was decreased by 1ℓ). It was expected that the DO in the a-recycle would lead to a low DO concentration in the small reactor and closely approximate the period of low DO in the IAND system. During the three sludge ages this system was operated (Days 88 to 129), the DSVI decreased from between 140 and 170 mℓ/g to between 100 and 140 mℓ/g. The changes in DSVI and system configuration with time for this 2RND system (System 9) are shown in Fig 3.19. From the results of these experiments, it was concluded that filament proliferation in IAND configurations is not a consequence of low or decreasing DO concentration

(4) *Changes in the concentration of nitrate in the anoxic period of IAND and 2RND systems:*

The fourth difference between IAND and 2RND systems concerns the concentrations of nitrate in the anoxic period of an IAND system and in the anoxic reactor of a 2RND system. In an IAND system the nitrate concentration at the beginning of the anoxic period is high as a result of nitrate generated by nitrification during the preceding aerobic period. During the anoxic period the nitrate concentration decreases as a result of denitrification and depending on the amount of nitrate generated (or dosed),the nitrate concentration may or may not become zero before the next aerobic period commences. In contrast, in a 2RND system operating at steady state, the nitrate concentration in the anoxic zone remains constant either at zero or some positive value depending on whether or not the nitrate load imposed by the recycles is less or greater than the anoxic reactors denitrification potential.

In the intermittent aeration system changes in the concentration of NO_3^- and NO_2^- in the anoxic period and changes in DO in the aerobic period change the reduction-oxidation (redox) potential (E_h) in those zones. Facultative organisms have a hierarchy for utilization of electron acceptors (i.e. O_2, NO_3^-, NO_2^-), and one means of differentiating between the electron acceptors is via the redox potential of the surrounding liquid which is established by the concentrations of the various

electron acceptors. That is, under aerobic conditions, when O_2, NO_3^- and NO_2^- are present, the redox potential is high, and the first choice electron acceptor, O_2 is utilized. As oxygen is consumed, the redox potential decreases and the organisms switch to the use of the second–, and third–choice electron acceptors, NO_3^- and NO_2^- respectively. As each of these is consumed, the redox potential decreases until anaerobic conditions (no O_2, NO_3^- or NO_2^-) are reached.

With this principle as a gauge, a major difference is apparent in the redox potential of comparable zones in IAND and 2RND systems. For IAND systems, the scenario described above is applicable, but for 2RND systems, organisms experience a different set of redox conditions. A constant redox potential is established in each of the anoxic and aerobic reactors and the organisms, in moving between the two zones, experience a large and sudden change in redox potential.

To examine the differences in redox potential between IAND and 2RND systems the redox potential (E_h) and NO_3^- and NO_2^- concentrations were measured in the anoxic and aerobic reactors of a 2RND system and throughout the anoxic–aerobic cycle of an IAND system.

- Redox potential was measured in the anoxic and aerobic reactors of System 9 (see Fig 3.19) between Days 88 and 130. In the anoxic zone, the average value was –81 mV and in the aerobic zone, +48 mV.

- After establishing the above redox potentials, System 9 (DSVI ≈ 120 mℓ/g) was changed to an IAND system on Day 130. The DSVI increased over an initial period of 17 days to 300 mℓ/g before decreasing to and stabilizing at a DSVI of 180–200 mℓ/g by Day 195. On Day 189, redox measurements were made throughout one 8 hr intermittent aeration cycle and the concentrations of NO_3^- and NO_2^- were measured at intervals of approximately 20 minutes during the same cycle and are illustrated in Fig 3.20.

Following the introduction of oxygen at the start of the aerobic period the value of E_h increased from –130 mV and attained a value of +40 mV at the end of the $2^1/_4$ hrs aerobic period. During the same period the concentration of NO_2^- increased from zero to > 2,0 mgN/ℓ after 80 minutes due to nitrification of NH_4^+ to NO_2^- by *Nitrosomonas* and then decreased to 0,6 mgN/ℓ at the end of the aerobic period as a result of nitrification of NO_2^- to NO_3^- by *Nitrobacter*. The concentration of NO_3^-

increased from 0,5 mgN/ℓ to 9,1 mgN/ℓ during the same period, leading to an increase in E_h by the end of the aerobic period. During the first $2^3/_4$ hrs of the unaerated period, E_h decreased from ≈ +40 mV to ≈ –80 mV, during which time the concentration of NO_3^- decreased from 9,0 mgN/ℓ to less than 1 mgN/ℓ as a result of denitrification of NO_3^- to NO_2^-. During the same period the concentration of NO_2^- increased from 0,5 mgN/ℓ to 0,9 mgN/ℓ. During the remaining 3 hrs the concentrations of NO_3^- and NO_2^- decreased to zero (anaerobic conditions) and the value of E_h decreased to –200 mV. When the nitrate concentration reached low values, nitrite reduction commenced and proceeded simultaneously with nitrate reduction, and E_h began to decrease rapidly, from –80 mV to –180 mV.

As a means of ensuring that the anoxic period had sufficient NO_3^- to avoid anaerobic conditions, NH_4^+ was added to the influent on Day 195 to provide adequate NO_3^- through nitrification during the aerobic period. This increased the TKN/COD ratio from 0,11 to 0,12 mgN/mgCOD and during the following 9 days, the DSVI increased from ≈ 200 to 220 mℓ/g. The TKN/COD ratio was further increased on Day 204, to a value of 0,14 mgN/mgCOD, through the addition of more NH_4^+ to the influent and between Days 204 and 214, the DSVI increased from 220 mℓ/g to 240 mℓ/g. On Day 209 an 8 hr anoxic/aerobic aeration cycle was monitored for E_h, NO_3^- and NO_2^- and the results are illustrated in Fig 3.21. Although the trend in the E_h, NO_3^- and NO_2^- parameters during the aerobic and anoxic cycles are similar to the earlier test conducted on Day 189, the values of the parameters at the end of the unaerated period are markedly different: Whereas in the test conducted on Day 189 the nitrate and nitrite concentrations were zero and E_h was –200 mV, for the test conducted on Day 209, the nitrate and nitrite concentrations were 0,5 and 2,4 mgN/ℓ respectively and the E_h did not decrease below –75 mV.

Although the objective of the experiments was to compare the values of E_h measured for the 2RND system with the values measured for the IAND systems, an apparently more significant aspect was identified in comparing the plots of E_h and DSVI for the two tests conducted on the IAND system, before and after the addition of NH_4^+ to the influent.

During the period in which NH_4^+ was added to the influent (Fig 3.19) the DSVI was high (between 220 and 240 mℓ/g) and increasing and the concentrations of NO_3^- and NO_2^- at the end of the anoxic period were high (0,5 and 2,4 mgN/ℓ respectively). In contrast, during the period in which NH_4^+ was not added to the influent, the DSVI

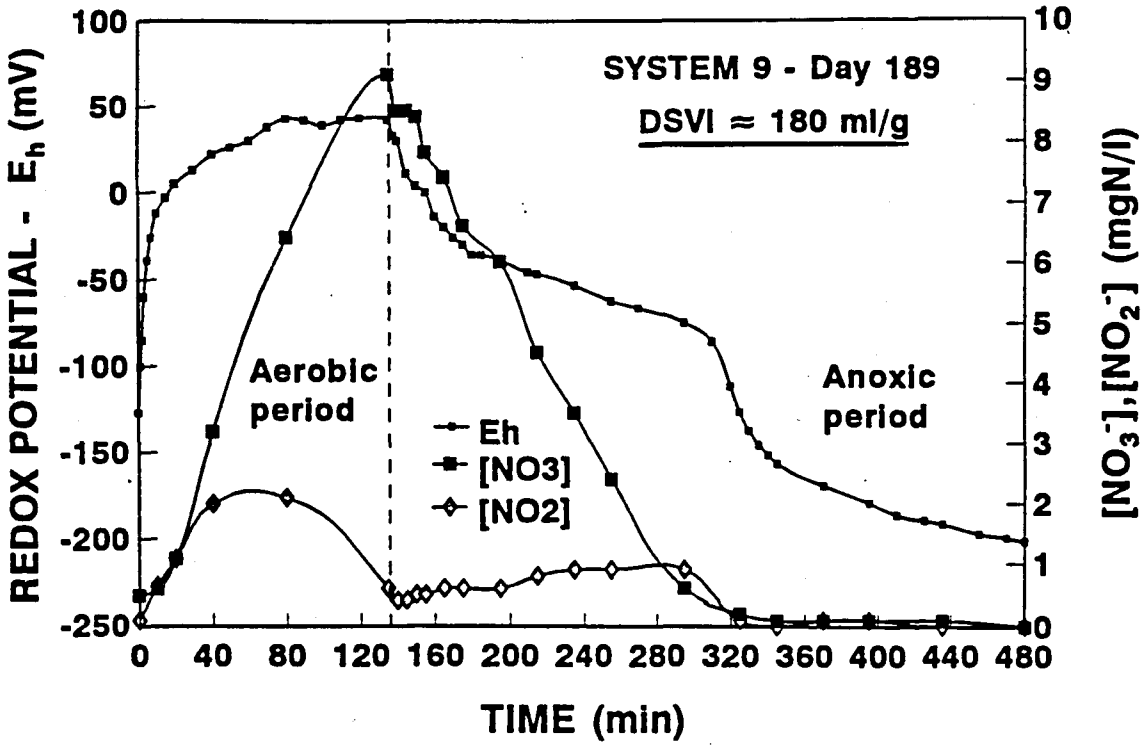


Fig 3.20: Redox potential (E_h in mV) with time (h) for an 8 h aerated-unaerated cycle of System 9 on Day 189.

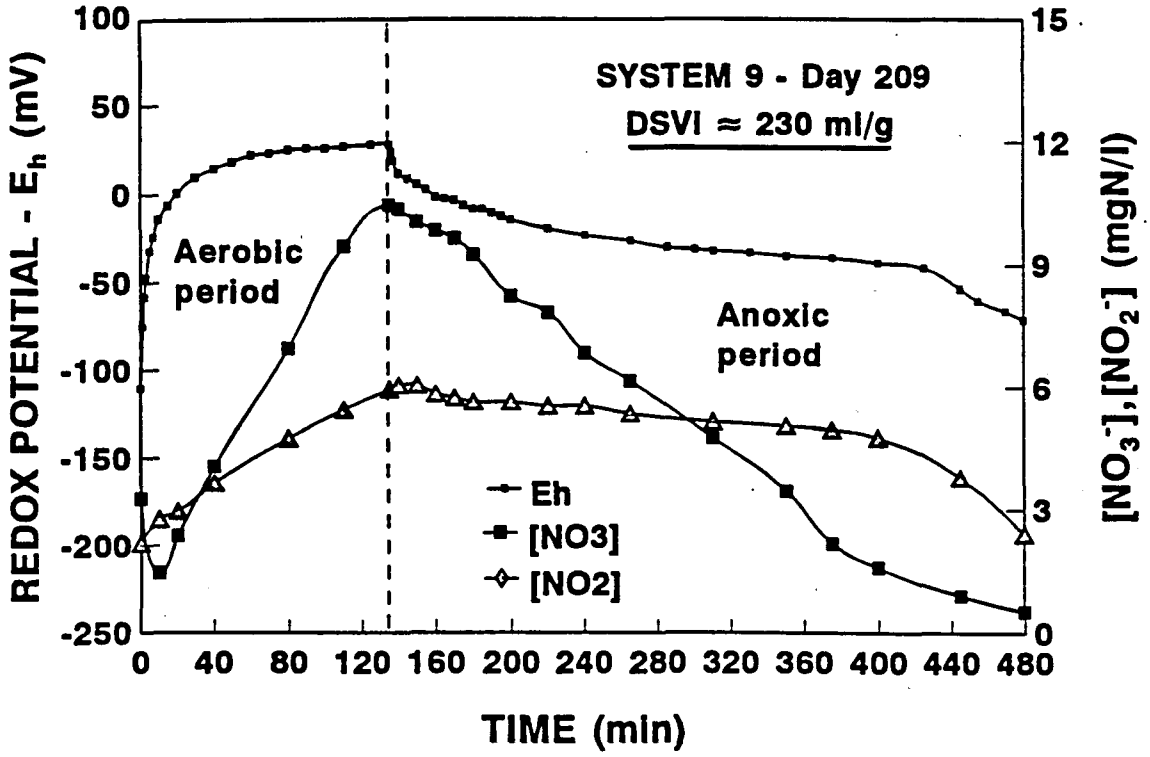


Fig 3.21: Redox potential (E_h in mV) with time (h) for an 8 h aerated-unaerated cycle of System 9 on Day 209.

was lower and constant (180 to 200 mℓ/g) and the concentrations of NO_3^- and NO_2^- were zero at the end of the anoxic period. Although the relationship between NO_3^- and NO_2^- at the end of the anoxic period, and the DSVI of the system appeared to be strong, the mechanism by which the one affected the other was unclear.

It is proposed that the increase in DSVI following the addition of NH_4^+ resulted from an increase in concentration of NO_3^- and NO_2^- during the anoxic period and more importantly, the presence of NO_3^- and NO_2^- throughout the anoxic period. (i.e. no anaerobic period present at the end of the anoxic period).

During operation of System 9 with a TKN/COD ratio of 0,11 mgN/mgCOD between Days 183 and 195, daily samples were taken at the end of the anoxic period of one of the three daily 8 hr cycles; the concentration of NO_2^- in the majority of samples was less than 0,4 mgN/ℓ. Although the TKN/COD ratio was increased to 0,12 mgN/mgCOD between Days 195 and 203, the concentration of NO_2^- in the samples taken at the end of the anoxic period remained less than 0,4 mgN/ℓ. After the TKN/COD ratio was increased to 0,14 mgN/mgCOD on Day 204, the concentration of NO_2^- of the majority of the samples taken at the end of the anoxic period increased to between 2,0 and 4,0 mgN/ℓ between Days 204 and 214.

The finding that the system with NO_3^- and NO_2^- present throughout the anoxic period (DSVI = 230 mℓ/g) had a greater tendency toward filament proliferation than the system in which NO_3^- and NO_2^- had been reduced to zero by the end of the anoxic period (i.e. the system was partially anaerobic, DSVI = 180 mℓ/g) was investigated further. Two reasons prompted this work; (1) all other areas of investigation had been examined, without major indications as to the cause of filament proliferation, and (2) a relationship between NO_3^-/NO_2^- concentration and filament proliferation had considerable inferential support from the earlier experimental work i.e. Warburton *et al.* (1991), and the defined substrate and MUCT experiments described above. In particular, in experiments with defined substrate in Part I, addition of RBCOD and SBCOD to Systems 1 and 3 which resulted in increases and decreases respectively in DSVI were associated with increases and decreases in NO_{2+3}^- in the reactor, and in experiments concerned with the direct addition of NO_3^- to System 2 it was concluded that the concentration of NO_{2+3}^- had a significant effect on the type and extent of filament proliferation. Additionally, in experiments with municipal sewage in Part II, variation in the aerobic mass fractions of two MUCT systems (Systems 10 and 11) which resulted in

variations in DSVI, also resulted in variation in the concentration of NO_{2+3}^- in the 2nd anoxic reactor, the outflow of which enters the aerobic reactor.

The focus of the investigation then changed from examining the relationship between redox potential and filament proliferation to examining the relationship between the NO_3^- and NO_2^- concentrations and filament proliferation. The first task was to determine, *if* the presence of NO_3^- or NO_2^- is the cause of filament proliferation, firstly, which of the two, NO_3^- or NO_2^- is responsible, and secondly, in which zone, anoxic or aerobic, do the filaments proliferate? This is a difficult task since in anoxic–aerobic systems one nitrogen oxide is always present as a result of the other, through either nitrification under aerobic conditions (i.e. $NO_2^- \rightarrow NO_3^-$), or nitrate reduction under anoxic conditions (i.e. $NO_3^- \rightarrow NO_2^-$). Therefore, as a preliminary and more simple task, the effect of NO_3^- and NO_2^- on filament proliferation was examined under fully anoxic conditions, i.e.

- Ketley *et al.* (1991) reported that under continuous anoxic conditions with the addition of excess NO_3^- as electron acceptor, the low F/M filaments did not proliferate. This confirmed that anoxic–aerobic alternation was required. In these experiments NO_2^- and NO_3^- concentrations were not measured individually, but this was not regarded as serious because the filaments did not proliferate.

- System 7 (a continuous anoxic system with excess NO_3^- continuously dosed) was fed only the SBCOD fraction of municipal sewage between Days 128 and 181 (see Fig 3.11). The DSVI at Day 181 was 125 mℓ/g and the system feed was changed from the SBCOD fraction to the complete municipal sewage (i.e. non-fractionated municipal sewage including both the SBCOD and RBCOD fractions). Between Days 181 and 203, the DSVI remained between 120 and 130 mℓ/g. The concentrations of NO_3^- and NO_2^- in the reactor were 30–40 mgN/ℓ and 1–3 mgN/ℓ respectively (NO_2^- was present as a result of the denitrification of NO_3^-). On Day 187 the dominant filament was type 0092. On Day 203, NO_3^- dosing was switched to NO_2^- dosing while the feed remained the complete municipal sewage. The DSVI decreased from 120 mℓ/g to 75 mℓ/g between Days 203 and 222. During this period, the concentration of NO_2^- in the reactor was about 40 mgN/ℓ and no NO_3^- was measured. On Day 222, the continuous NO_2^- dosing was switched to NO_3^- again. The DSVI remained between 60 and 70 mℓ/g between Day 222 and Day 255 and the concentrations of NO_2^- and NO_3^- in the reactor were < 10 and ≈ 50 mgN/ℓ respectively.

The results of these two experiments indicate that filamentous organisms cannot proliferate under fully anoxic conditions irrespective of whether the electron acceptor is NO_3^- or NO_2^-. These experiments confirm that anoxic–aerobic alternation is required for filament proliferation but it is not possible to determine whether filament proliferation is due to the presence of NO_3^- or NO_2^- and further work was conducted to establish this.

- Warburton *et al.* (1991) examined the effect of the concentration of NO_3^- during the anoxic period on filament proliferation in IAND systems operated at a sludge age of 10 days. Two identical IAND systems were operated and an excess NO_3^- dose was alternately switched between them. It was found that dosing of NO_3^- in order to increase its concentration under anoxic and aerobic conditions had a relatively mild influence on DSVI, with filament proliferation generally increasing or high as the NO_{2+3}^- concentration increased above 5 mgN/ℓ.

Nitrite concentration in the anoxic zone preceding the aerobic zone

The effect of the concentrations of NO_2^- and NO_3^- during the anoxic period or reactor on the proliferation of filamentous organisms was investigated by Musvoto *et al.* (1992) who examined separately the effect of continuous addition of NO_2^- and NO_3^- to the second anoxic reactors of two MUCT systems.

It is evident from these experiments that a strong relationship exists between filament proliferation and the concentration of NO_3^- and NO_2^- in the second anoxic reactor of an MUCT system. From the relative rates of increase in DSVI with NO_3^- and NO_2^- addition, it is apparent that NO_2^- has a considerably greater influence than NO_3^- on filament proliferation the reasons for which will become evident in the description of the bulking model in Chapter 6. Similar experiments were conducted with NO_2^- addition and removal to the 2nd anoxic reactor of an MUCT system with similar results, and are described during verification of the bulking model in Chapter 6.

3.4 CLOSURE

During the review of bulking in Chapter 2, eight specific areas were identified which required experimental investigation to identify a new conceptual framework to understand low F/M filament bulking problems. The principal objective of the wide ranging experimental investigation described above was to examine these and other aspects (which arose during the course of the investigation).

At this point it is not the intention to evaluate all this information for its implications regarding the specific conditions required by individual filament types for proliferation, as this was not the intention of the exploratory investigation (such an analysis is conducted in Appendices A and B). Rather, the objective of the investigation was to eliminate the factors which have only a minor or negligible influence on low F/M filament bulking and to clarify the areas that have a major influence, thereby highlighting the direction that the research investigation should take to establish the cause(s) of the low F/M filament bulking problem.

In conformity with this objective the results of the investigation which apparently have a *major* influence on low F/M bulking are as follows:

1) Continuous aerobic conditions control filament proliferation to low DSVI values ($\approx$ 100 mℓ/g).

2) Continuous anoxic conditions control filament proliferation to low DSVI values ($\approx$ 100 mℓ/g).

3) Alternating anoxic–aerobic conditions in which the aerobic mass fraction is between 30 and 40 percent result in maximum proliferation of filaments; aerobic mass fractions increasingly less than 30 percent and increasingly more than 40 percent result in increasingly lower DSVI values.

4) Filamentous organisms proliferate in systems with sludges exposed to alternating anoxic–aerobic condition whether in IAND, 2RND or MUCT systems in which nitrate and/or nitrite are present throughout the anoxic period (IAND) or in the anoxic reactor just prior to the aerobic reactor (2RND, MUCT) i.e. nitrate and/or nitrite are present at concentrations exceeding 5 and/or 1 mgN/ℓ respectively, when the sludge is exposed to aerobic conditions.

5) With regard to the relative importance of nitrate and/or nitrite on filament proliferation in (4) above, nitrite appears to have a greater influence then nitrate on promoting filament growth.

6) Subject to the conditions (3) and (4) above, low F/M filaments proliferated irrespective of the biodegradability (RBCOD or SBCOD) of the substrate available.

Of the eight areas listed for investigation, those that were found to have a minor or no influence on low F/M filament proliferation were; magnitude of sludge age, frequency of alternation between anoxic and aerobic conditions, and concentration of DO in fully aerobic or during the aerobic period or zone of anoxic–aerobic systems.

In the execution and analysis of the experiments discussed in this Chapter, three factors were considered to be *consequences* of the bulking conditions rather than *causes* for it.

- Systems with sludge exposed to alternating anoxic–aerobic conditions demonstrate lower COD mass balances than systems with sludge exposed to continuous aerobic conditions.

- Systems with sludge exposed to alternating anoxic–aerobic conditions which develop a high DSVI produce a reduced sludge mass (VSS), and systems which develop sludges with a low DSVI produce an increased sludge mass.

- Systems with sludge exposed to alternating anoxic–aerobic conditions which develop sludges with a high DSVI generally have lower nitrogen (N) mass balances than alternating anoxic–aerobic systems which develop sludges with a lower DSVI.

An explanation for these observations emerges from the biochemical/microbiological (bulking) model developed in Chapter 6 which evolved from application of the heterotrophic aerobic–facultative respiratory model developed in Chapter 5 to activated sludge systems.

From the results, the primary factor implicated in promoting low F/M filament proliferation was alternating anoxic–aerobic conditions with nitrate and nitrite present during anoxic conditions and at the start of aerobic conditions. The alternating anoxic–aerobic conditions force the facultative heterotrophic organisms to alternatingly switch between DO and nitrate as terminal electron acceptors. It was concluded that the cause for bulking lay in the requirement for the facultative organisms (irrespective of whether filament or floc–former), to continually switch between aerobic and anoxic respiration, this switching either providing an advantage for the filaments or acting as a disadvantage to the floc–formers. This indicated that investigation of the respiratory processes of facultative organisms is required at

a level more fundamental than the externally measured manifestations of activated sludge processes such as COD removal, and oxygen and nitrate uptake rates. This new direction entailed examination of the biochemical mechanisms of transport of protons, electrons, and the electron acceptors, oxygen, and nitrate and nitrite, and the synthesis of enzymes mediating these mechanisms under aerobic, anoxic, and alternating anoxic–aerobic conditions. Investigations in this direction were also prompted through having exhausted all areas of experimental research considered to be influential in filamentous organism bulking. Accordingly, an in–depth review of these biochemical aspects was conducted on obligate and facultative heterotrophic organisms and is presented in the next Chapter.

From the results of the experiments described in this Chapter it is apparent that the so–called low F/M filaments do not proliferate under all low F/M conditions; in particular, under continuous aerobic and continuous anoxic conditions, low F/M filaments do not develop. As a consequence of the finding that this group of filaments invariably proliferate under alternating anoxic–aerobic conditions, it was concluded that the filaments should be renamed anoxic–aerobic (AA) filaments, and this designation is used throughout the remainder of this investigation.

CHAPTER 4

BIOCHEMISTRY OF HETEROTROPHIC RESPIRATORY METABOLISM

ABSTRACT

Biochemical mechanisms for respiration and regulation of respiration for facultative organisms are reviewed in two sequential parts. In Part I, the biochemical reactions involved in the utilization of substrate under aerobic and under anoxic conditions are outlined. In Part II, the mechanisms which initiate, regulate, and terminate respiration under each of aerobic and anoxic conditions are described. A consequence of Part II is identification of a series of conditions and mechanisms in which one of the intermediates of denitrification (nitrite, or nitric oxide) interacts with the aerobic respiration enzymes (cytochrome oxidase), resulting in inhibited aerobic respiration under conditions in which organisms are alternately exposed to anoxic and aerobic conditions. This mechanism is an important aspect of a conceptual biochemical model for aerobic respiration developed in the next Chapter.

PART I: AEROBIC AND ANOXIC RESPIRATION IN FACULTATIVE ORGANISMS

4.1 INTRODUCTION

In the previous Chapter it was concluded that a new research direction is required for filamentous organism bulking research. As a starting point, it was considered that a more fundamental understanding is required of the biochemical mechanisms involved in respiration by aerobic facultative organisms. Consequently a literature review is conducted, the objective of which is to identify the principal electron transport pathways (ETPs) employed by facultative organisms under aerobic, anoxic, and alternating anoxic–aerobic conditions. Although the mechanisms of aerobic ETPs are well established for obligate aerobic organisms, the literature is somewhat piecemeal with regard to the mechanism of anoxic respiration, and considerably incomplete with regard to the mechanisms of respiration of facultative organisms, exposed to alternating anoxic–aerobic conditions. In this review it is the intention to ameliorate this situation by presenting an overview of current knowledge regarding the electron transport complexes and processes associated with facultative heterotrophic organisms.

4.2 CLASSIFICATION OF ORGANISMS

Activated sludge in sewage treatment systems contains a great diversity of organisms. To maintain themselves in the activated sludge biocenosis, organisms require a continuous supply of nutrients and energy. The manner by which they obtain energy and the essential nutrient, carbon, characterizes their niche in the activated sludge bacterial population, and forms the basis for their classification. Figure 4.1 indicates diagrammatically how organisms are classified on the basis of their sources of energy and carbon. Organisms which use light as an energy source are termed *phototrophic*; organisms which derive energy from the breakdown of chemicals are termed *chemotrophic*. Each of these groups is subdivided further by the principal source of carbon used by the organisms for cell synthesis. Organisms which use carbon dioxide (CO_2) as the principal carbon source are defined as *autotrophic;* those which use organic compounds as the principal carbon source are defined as *heterotrophic*. Only the chemoautotrophic and chemoheterotrophic groups of organisms are dealt with in this report and for simplicity are referred to as autotrophic and heterotrophic organisms respectively.

Nitrifying organisms use ammonia (NH_3) as an energy source and CO_2 as a carbon source and, accordingly, are classified as autotrophs; this group of organisms is discussed in Appendix E. The majority of other organisms important in nitrogen and nutrient removal activated sludges use organic compounds both as energy and carbon source, and are classified as heterotrophs; these are discussed below.

The method whereby heterotrophs extract energy and carbon from the breakdown or transformation of organic compounds, generally referred to as *substrate*, is part of the overall process of *metabolism*. Metabolism consists of two main phases, *anabolism* and *catabolism*. *Anabolism* is the enzymatic biosynthesis of the complex molecular components of the organism, such as nucleic acids, proteins, polysaccarides and lipids, from more simple molecules, either formed by the breakdown of complex organic substrate or taken up directly from the surrounding medium. *Catabolism* is the degradative phase of metabolism in which complex organic molecules are enzymatically broken down to smaller molecules (*metabolites*) such that some of the electrons originally present in the organic compound, called the *electron donor*, are removed (oxidation) and transferred through a sequence of enzymatically controlled processes, finally to a compound called a *terminal electron acceptor*. In this sequence of processes there is a release of chemical energy. Some of the energy released is lost as heat, and some is conserved in the formation of the

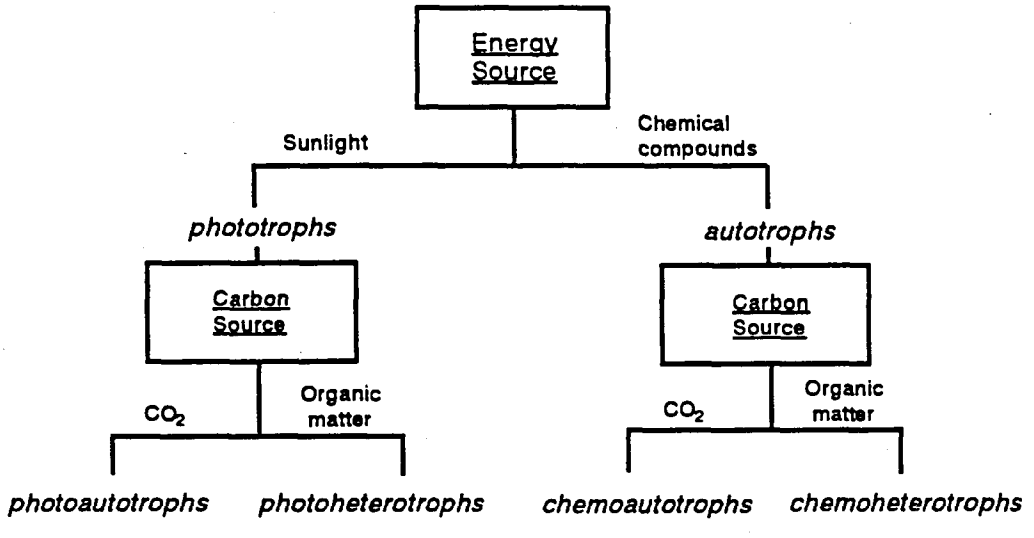


Fig 4.1: Classification of organisms based on their sources of energy and carbon.

energy-rich molecule, adenosine triphosphate (ATP). The energy in the ATP is utilized by the organism for synthesis of new cell material and for maintenance of cell function (e.g. concentration gradients and movement) and structure.

The energy yielding *catabolism* process includes both the processes of *fermentation* and *respiration.* In fermentation, the terminal electron acceptor is internally generated by the organism from the products of organic substrate breakdown; examples of fermentation terminal electron acceptors are acetic acid (reduced to ethanol or lactate), and hydrogen and carbon dioxide (reduced to methane). In contrast, in respiration, a terminal electron acceptor is available external to the organism and is brought into it to accept the electrons. Examples of respiration terminal electron acceptors are oxygen (O_2), nitrate (NO_3^-), nitrite (NO_2^-), and sulphate (SO_4^{2-}). With external electron acceptors, the oxidation of the substrate to CO_2 and H_2O is complete and the biological energy yield is the maximum possible. With internal electron acceptors, the oxidation of substrate is incomplete, because the end product, methane, still contains a relatively large amount of energy. As a consequence, energy liberated by respiratory processes may be 10 times that generated by fermentative processes.

Because this review is devoted exclusively to the process of respiration the process of fermentation will not be addressed any further.

In respiration, when oxygen is present, the conditions are called aerobic and organisms operative under these conditions are undergoing aerobic respiration. Organisms which are able to use only oxygen as terminal electron acceptor are called *obligate aerobes.* When oxygen is absent or present at very low concentrations and nitrate and/or nitrite act as the electron acceptor, the conditions are termed anoxic[1] and organism respiration under these conditions is termed anoxic

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[1]This is the accepted definition of anoxic in the field of sanitary engineering. In the microbiology and biochemistry disciplines, conditions of respiration with nitrate (or nitrite) present, in the absence of oxygen, are referred to as *anaerobic with nitrate (or nitrite) present.* In sanitary engineering, it is standard practice to define *anaerobic* as the condition in which oxygen and nitrate and nitrite are absent, a condition described by bacteriologists as *fermentation.* Conditions in which oxygen is absent and nitrate and/or nitrite is present or in which nitrate and/or nitrite are supplied but are maintained zero by the process of denitrification are called *anoxic.* The term *anoxic* describes a condition under which one or both of nitrate or nitrite act as electron acceptor but the term is not specific as to which. With regard to the operation of wastewater treatment processes, nitrite concentration measurements have until recently, been considered to be of little consequence in process monitoring or kinetic modelling since nitrite concentrations usually are considerably less than

respiration. The process in which organisms use the ionic nitrogen oxides, nitrate and/or nitrite as terminal electron acceptor is called *nitrate* or *nitrite respiration.* Organisms which have the ability to switch between oxygen and nitrate/nitrite as electron acceptors in response to their availability are called *facultative* aerobic organisms and are the group of organisms with which this review is primarily concerned. It is clear that such organisms have highly specialized biochemical pathways and the remainder of this review attempts to elucidate the biochemical mechanisms exhibited by heterotrophic facultative organisms under aerobic and anoxic conditions and in changes between the two conditions.

4.3 STAGES OF RESPIRATORY METABOLISM

In the processes of respiratory metabolism by heterotrophic organisms, organic substrates such as carbohydrate, protein and lipids are oxidized to the end products of CO_2 and H_2O. Through these reactions the organism is able to "capture" energy for utilization. Energy becomes available to the organism through a series of internally mediated redox reactions involving electron and proton transfer from a substrate through a number of intermediate enzyme complexes to the final electron acceptor. To capture the energy from these redox reactions, two types of compounds are coupled into the reactions; energy transport molecules which couple, transport, and release energy, and electron and proton transport molecules. The energy transport molecule of interest to this review is *adenosine triphosphate* (ATP). The mechanisms by which ATP participates in energy capture are described at a later stage. The energy transporting molecule, *guanosine triphosphate* (GTP) is also involved in energy capture but is not of major importance to this review. The electron and proton transport molecules of interest are *nicotinamide adenine dinucleotide* (NADH) and *flavin adenine dinucleotide* ($FADH_2$). The NAD molecule belongs to a family of carriers, known as pyridine nucleotides. The oxidized form of NAD, (i.e. NAD^+), which carries a positive charge on the nitrogen atom[2] is

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nitrate concentrations in such systems. Consequently, little attention has been given to the role of nitrite in activated sludge behaviour or kinetics and the term *anoxic* has been considered sufficient for defining situations in which oxygen is absent and nitrate and/or nitrite is present. With increasing attention being devoted to the role of nitrite in wastewater treatment, the present sanitary engineering terminology may have to be reviewed. Although the majority of work reviewed in this Chapter originates from microbiological sources, the major part of this thesis concerns experimental work conducted in the field of sanitary engineering. As a consequence, the terminology applicable to sanitary engineering is used throughout the thesis, including description of the work gained from a literature search in the bacteriological disciplines.

[2]The positive sign associated with the oxidixed form of NAD (NAD^+) is included to

reduced through reaction with 2 protons and a pair of electrons. NAD^+ accepts one proton and a pair of electrons to form NAD reduced (i.e. NADH + H^+), in accordance with the reversible oxidation–reduction reaction as follows:

$$NAD^+ + 2H^+ + 2e^- \leftrightharpoons (NADH + H^+) \qquad (4.1)$$

NADH is used by the organism in the production of the energy–rich molecule ATP, an aspect discussed in greater detail below.

The FAD molecule is one of the family of enzymes called flavonucleotides. The oxidized form, i.e. FAD, accepts 2 protons and a pair of electrons to form FAD reduced, (i.e. $FADH_2$). The reversible oxidation–reduction reaction is as follows:

$$FAD + 2H^+ + 2e^- \leftrightharpoons FADH_2 \qquad (4.2)$$

To understand how these molecules are used for electron and proton transport, respiratory metabolism can be divided into 4 stages as illustrated in Fig 4.2.

Stage I

Large complex organic molecules are enzymatically degraded (hydrolyzed) to simpler ones; carbohydrates to hexoses and pentoses, proteins to amino acids, and lipids to fatty acids and glycerol.

Stage II

The end products of Stage I are degraded further resulting in the formation of acetyl–Coenzyme A (acetyl–CoA) and carbon dioxide. For the degradation of each group of Stage I end products, i.e. amino acids, hexoses/pentoses, and fatty acids/glycerol, different biochemical pathways are employed.

Figure 4.3 illustrates the association between the degradation pathways for these three groups which result in the formation of acetyl–CoA. Considering each of the pathways in turn:

Amino acid degradation results in the formation of acetyl–CoA, either via the

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show that the molecule ordinarily carries a positive electric charge. The oxidized and reduced forms are designated NAD^+ and NADH respectively and the acronym NAD is used in situations where it is immaterial as to which oxidation state of the molecule is being referred to.

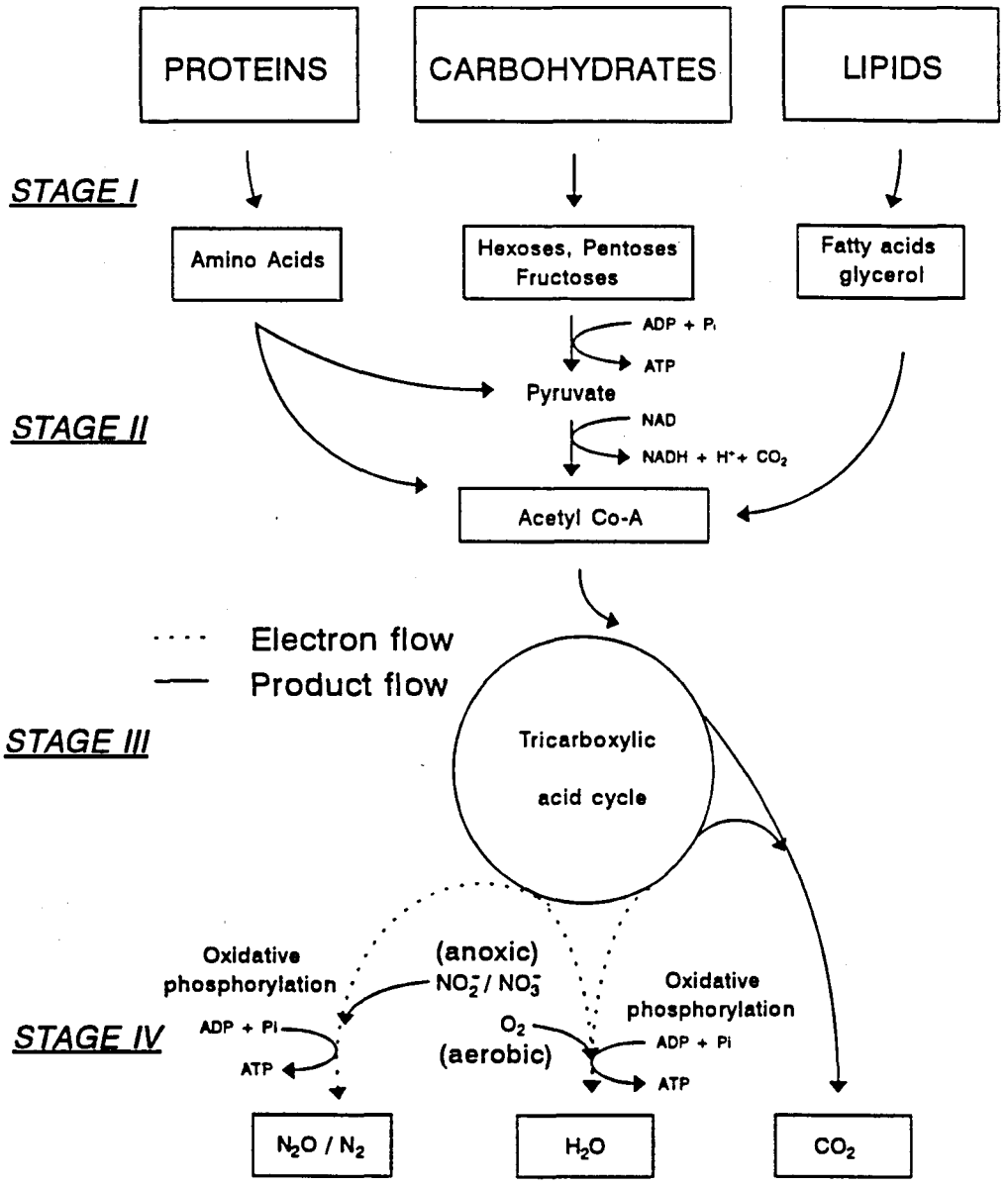

Fig 4.2: Stagewise division of substrate breakdown by facultative heterotrophic organisms, indicating the major metabolic pathways, reactants, and products (from Lehninger, 1975).

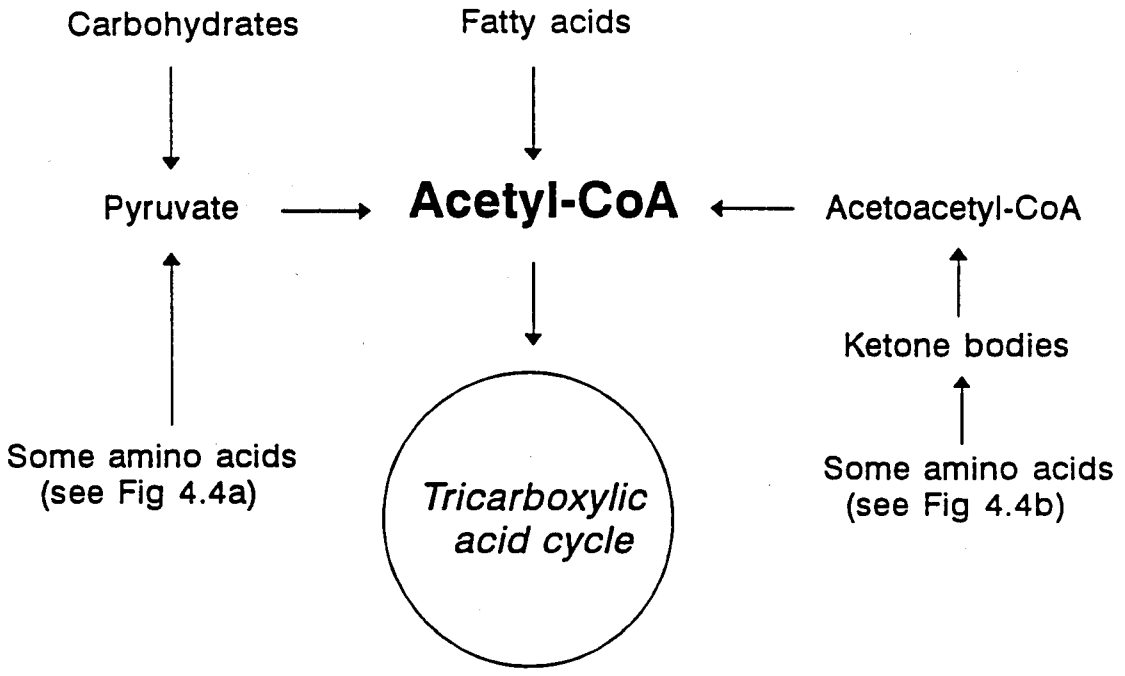

Fig 4.3: Overview of Acetyl–Coenzyme A synthesis indicating the degradation pathways by which acetyl–CoA is formed from Carbohydrates, Fatty Acids, and Amino Acids (redrawn from Martin *et al.*, 1981).

formation of pyruvate (from glycine), or without the formation of pyruvate (from tyrosine). Figure 4.4 shows an example of each type of reaction; the degradation of glycine to acetyl–CoA via pyruvate (Fig 4.4a) and the degradation of tyrosine to acetyl–CoA without the formation of pyruvate (Fig 4.4b). Additionally, some amino acids (e.g. glutamate) are not converted to acetyl–CoA but enter Stage III directly.

Carbohydrate degradation (glycolysis) occurs via a number of pathways for facultative organisms the most common of which is the Embden–Meyerhof pathway. Essentially it can be considered as a 2–stage (10 step) process as illustrated in Fig 4.5. The first stage is an activation stage in which simple sugars (e.g. glucose, fructose) are phosphorylated to a common product, glyceraldehyde 3–phosphate at the expense of ATP. The second stage comprises a series of dehydrogenations (i.e. removal of protons and electrons) by which glyceraldehyde 3–phosphate is converted to pyruvate. Associated with this stage is the formation of NADH and ATP, the formation of the latter a consequence of oxidative phosphorylation.

Hydrolysis of long chain fatty acids occurs by a process termed β–oxidation in which acetyl–CoA, NADH and $FADH_2$ are formed at the expense of ATP via one hydration and two dehydrogenation steps as illustrated in Fig 4.6.

In summary, although many products are generated by the degradation of carbohydrates, proteins and lipids through Stages I and II, those which are of principal interest to this review are, acetyl–CoA, ATP, NADH and $FADH_2$.

Stage III

The end product from Stage II, acetyl–CoA, enters the tricarboxylic acid (TCA) cycle, a cyclic sequence of reactions catalyzed by a series of enzymes. During the cycle the acetyl group of acetyl–CoA is oxidized to form two molecules of CO_2, eight protons (H^+), 4 pairs of electrons (e^-) [3] *and 1 GTP (energetically equivalent to 1 ATP); CoenzymeA is recovered. The TCA cycle (see Fig 4.7), also known as the Krebs or citric acid cycle, is the final catabolic pathway common to all aerobic and facultative organisms.*

Six of the protons and 3 pairs of electrons produced in the cycle are transferred to 3

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[3]The terminology "pairs of electrons" is used to indicate that electrons (e^-) are transported as pairs, unlike protons (H^+) which can be transported individually.

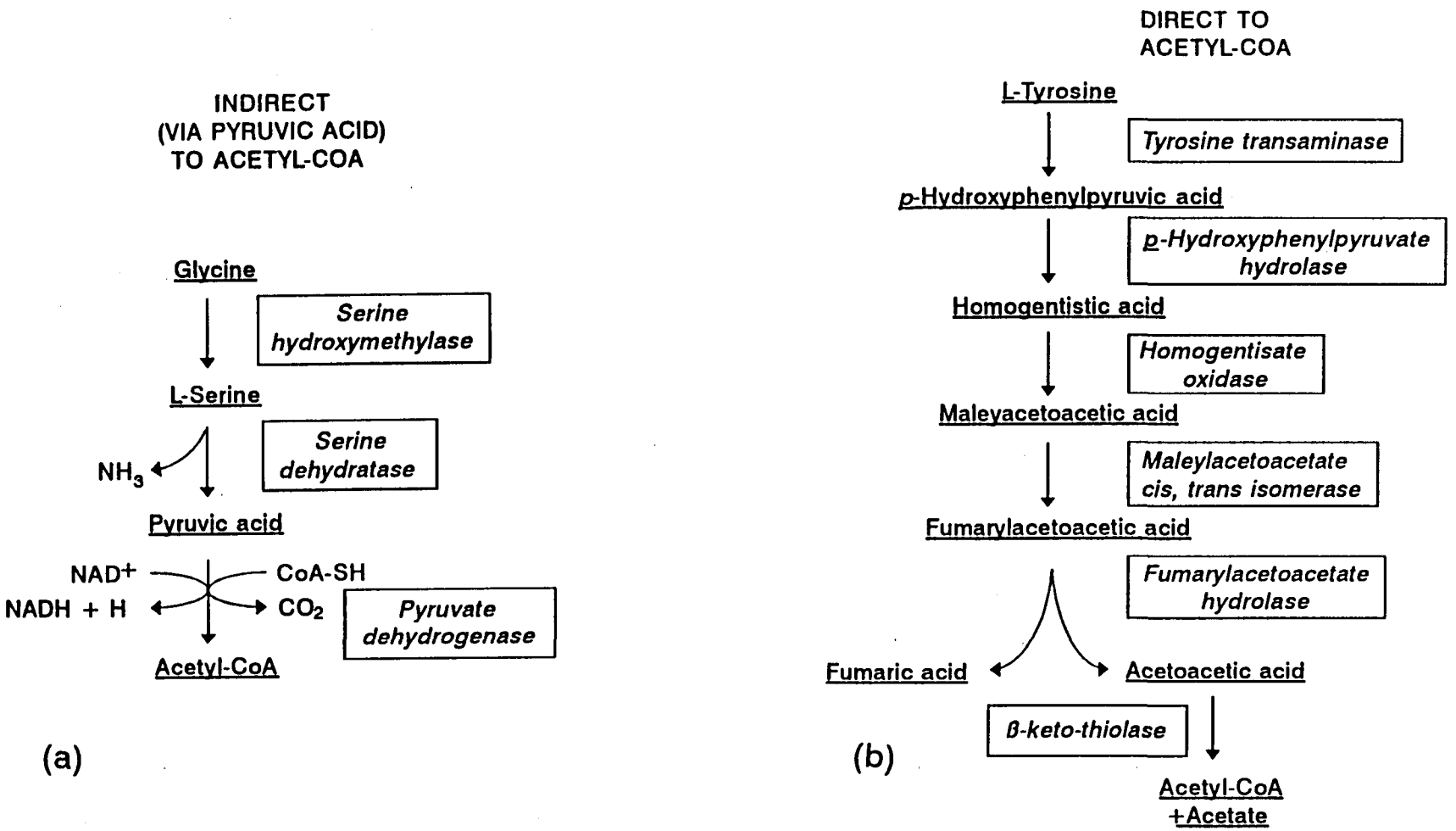

Fig 4.4: Sequential reduction of glycine and L–tyrosine as examples of (a) indirect degradation (pyruvic acid produced) and (b) direct degradation (pyruvic acid not produced) of amino acids to acetyl–CoA (redrawn from Martin *et al.*, 1981).

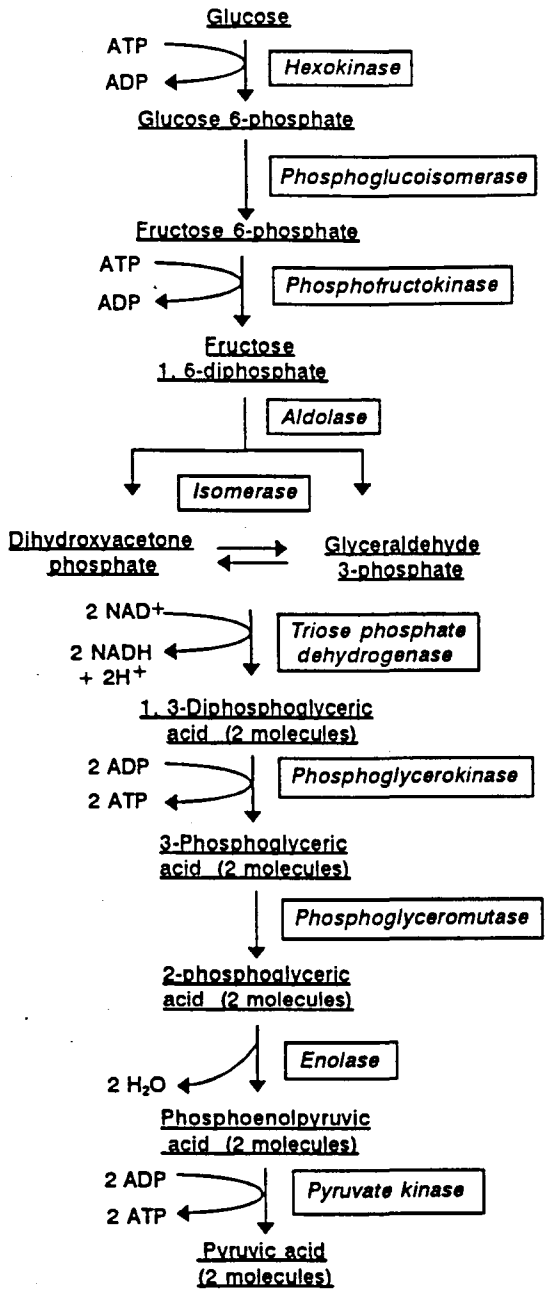

Fig 4.5: Ten step degradation of carbohydrate (glycolysis), demonstrating collection of simple sugars and production of glyceraldehyde 3-phosphate, and conversion of glyceraldehyde 3-phosphate to pyruvic acid with coupled formation of ATP (redrawn from Lehninger, 1975).

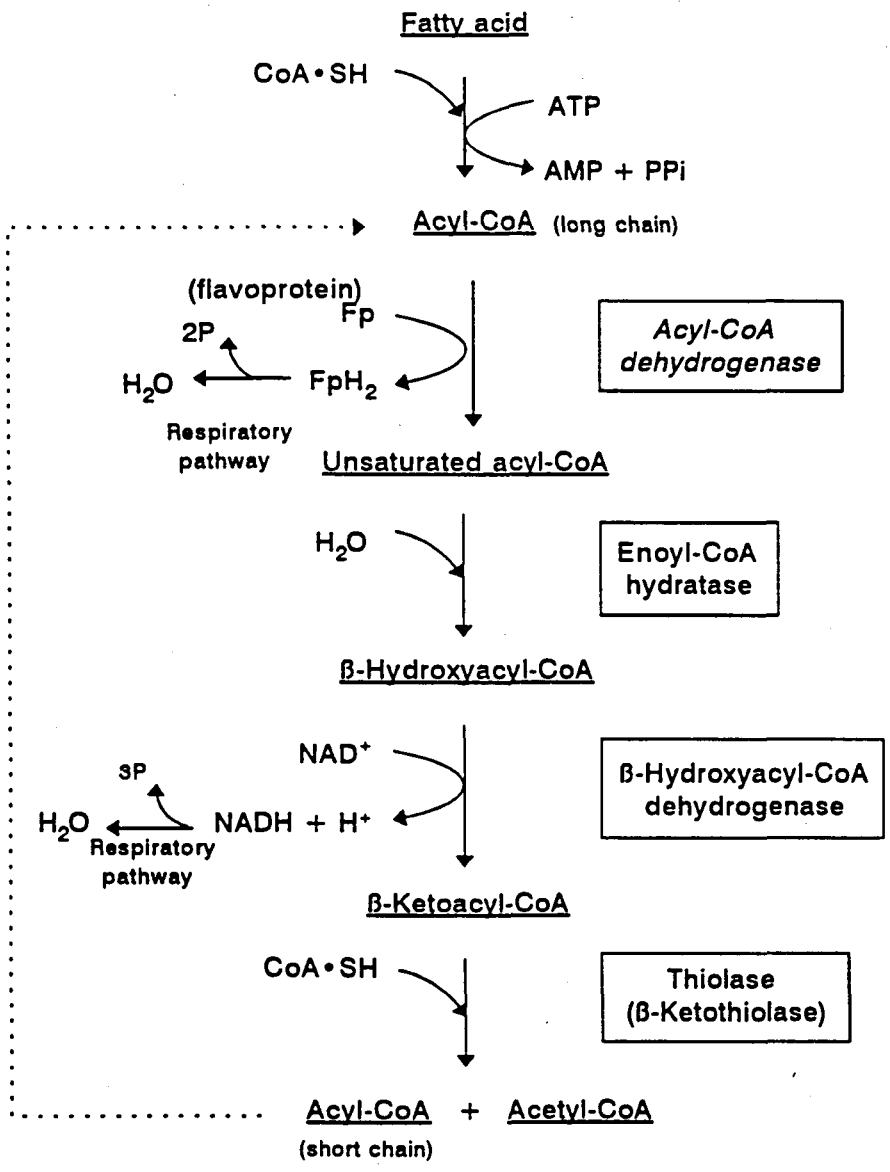

Fig 4.6: Sequential degradation of fatty acid (β–oxidation) to acetyl–CoA (redrawn from Martin *et al.*, 1981).

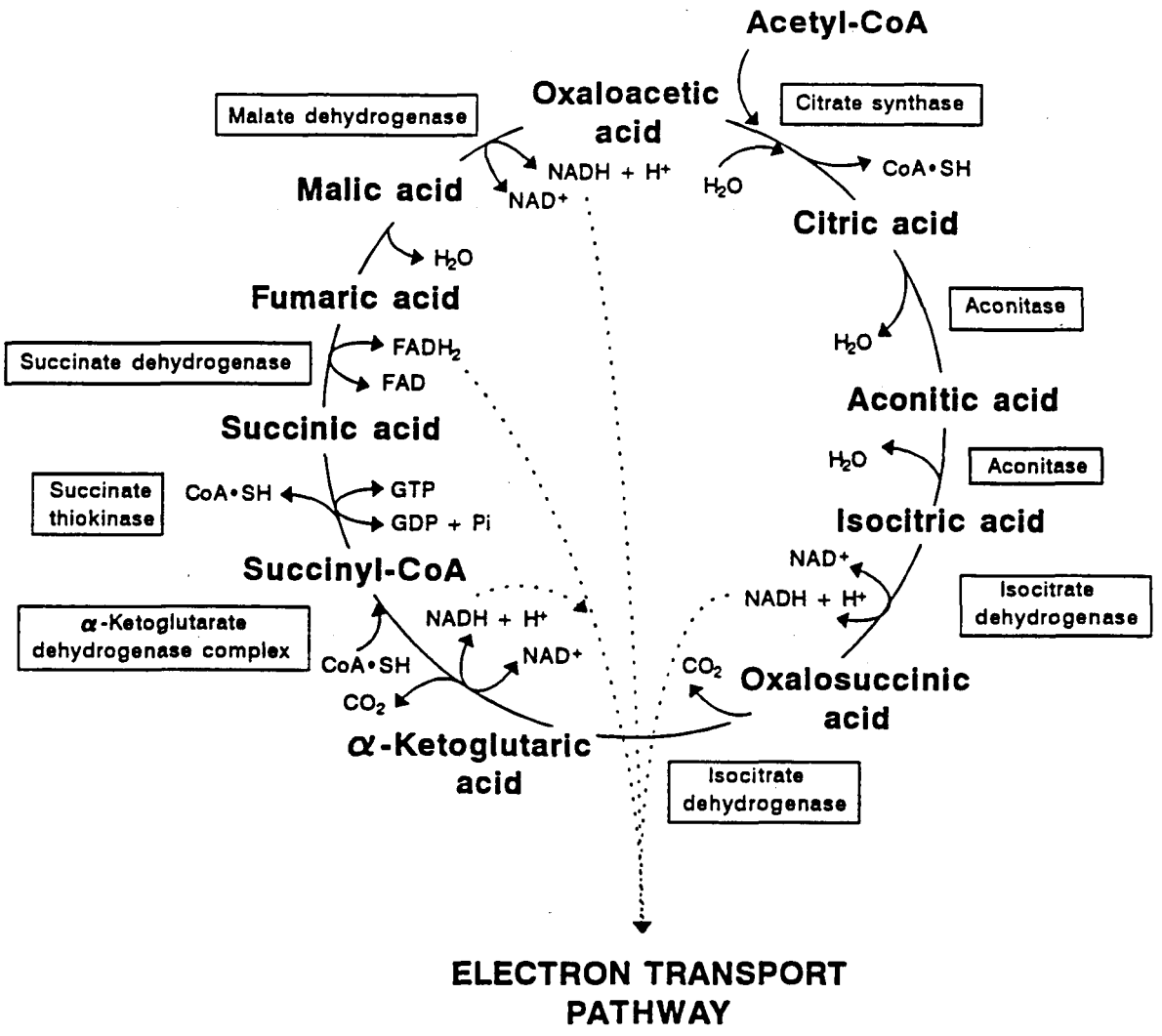

Fig 4.7: Tricarboxylic acid (Krebs) cycle demonstrating the formation of NADH and $FADH_2$ with reference to acetyl-CoA and the electron transport pathway.

NAD molecules to form NADH, and 2 protons and a pair of electrons are transferred to a flavoprotein (Fp) molecule, containing the prosthetic group, flavin adenine dinucleotide (FAD) to form $FADH_2$. The role of the 3 NADH and 1 $FADH_2$ molecules is to translocate the protons and electrons removed from the substrate to Stage IV where the energy associated with them can be released.

Stage IV

In this, the fourth and final stage of oxidation of organic material, the electrons and protons captured by NADH and $FADH_2$ in Stages II and III pass along a pathway of electron and proton carrier enzymes of successively lower energy, to a final (terminal) electron acceptor. During this process much of the free energy of the electrons is conserved, by oxidative phosphorylation, in the form of the energy-rich molecule ATP. The passage of electrons and protons can occur under aerobic and under anoxic conditions. Under aerobic conditions the electrons pass to the final electron acceptor O_2 (with the formation of H_2O) and under anoxic conditions to nitrate (NO_3^-) or nitrite (NO_2^-) (with the formation of H_2O and N_2). With NO_3^- or NO_2^- as external terminal ionic electron acceptors, the gaseous products, nitric oxide (NO) and nitrous oxide (N_2O) are produced in the formation of dinitrogen (N_2); these gaseous nitrogen oxides also act as electron acceptors.

The electron transport pathways (ETPs) for aerobic and anoxic respiration have some common complexes, i.e. some complexes which are utilized in both aerobic and anoxic respiration; other complexes are specific to aerobic or anoxic respiration. Figure 4.8 illustrates the complexes present in both the aerobic and anoxic ETPs of a typical facultative organism. The representation of the electron transport pathway for facultative organisms described by Fig 4.8 is the result of many years of research concerned with establishing the interaction of the anoxic electron-transferring components with the aerobic components. A description of the chronological elucidation of this pathway including details of the experimental investigations is given in Appendix C.

The representation of the respiratory electron transport pathway as a linear array of electron carriers as shown in Fig 4.8 is somewhat misleading in that no recognition is given to the location of each of the complexes with respect to the cytoplasmic membrane, or to the manner by which the location affects the movement of electrons, protons, and intermediates of the aerobic and denitrification pathways. In reality, the complexes are arranged within the membrane in a manner similar to

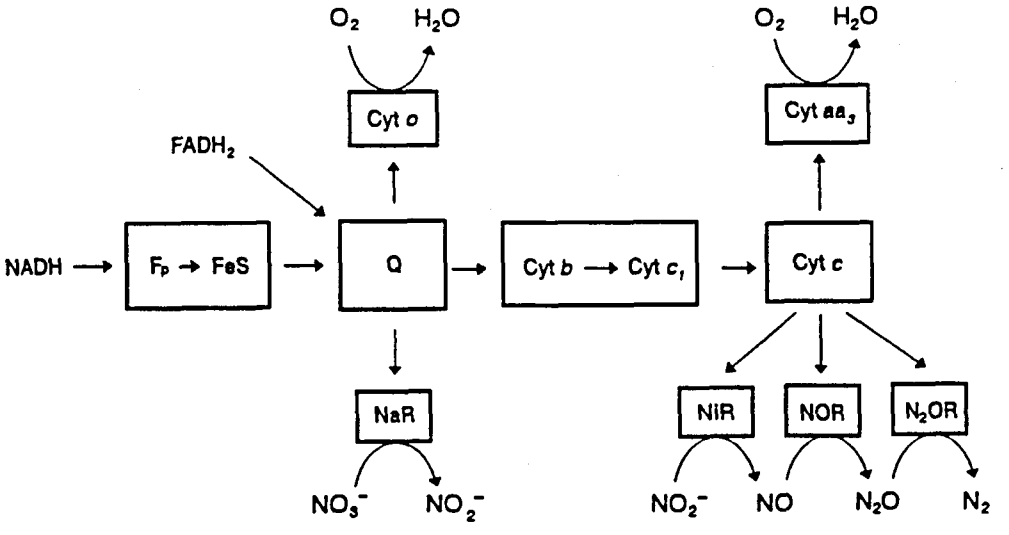

Fig 4.8: Electron transport pathway (ETP) for a typical facultative aerobic heterotrophic organism indicating the points of transfer of electrons to the terminal oxidases and reductases. [Abbreviations: NADH – nicotinamide adenine dinucleotide; Fp – flavoprotein; FeS – iron sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone, Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate]. (Adapted from Ferguson, 1982).

that shown in Fig 4.9 wherein all the complexes that are present under aerobic or anoxic conditions are represented. However, under aerobic or anoxic conditions, not all of the complexes are synthesized and not all the complexes synthesized are active. These aspects will be dealt with in discussion of aerobic and anoxic respiration.

The remainder of this review is devoted to description of the mechanisms of the aerobic and anoxic respiratory processes that occur in Stage IV. The mechanisms of aerobic respiration are described first and then those of anoxic respiration.[4]

4.4 AEROBIC RESPIRATION

The respiratory enzyme complexes involved in the transfer of electrons and protons from NADH and $FADH_2$ to the terminal electron acceptor oxygen are shown in Fig 4.10 which is an adaptation of Fig 4.9 in which only the complexes synthesized under aerobic conditions are illustrated. These are, NADH dehydrogenase, ubiquinone, cytochrome bc_1, cytochrome c and the two cytochrome oxidases, cytochome aa_3 and cytochrome *o*. Each of the complexes, NADH dehydrogenase, ubiquinone, and cytochrome aa_3 is considered as an energy conserving site, at which protons are pumped from the cytoplasmic (inner) to the periplasmic (outer) side of the membrane, this being the first step of the mechanism by which the processes of respiration and oxidative phosphorylation, (i.e. the generation of the high energy molecule, ATP from the low energy molecule, ADP, are linked. Usually the ETPs to cytochrome *o* and cytochrome aa_3 operate simultaneously, with the major portion of electron flow passing to cytochrome aa_3. Cytochrome aa_3 and cytochrome *o* are referred to as the primary and alternative oxidases, respectively. The series of reactions catalyzed by each of the complexes of the ETP with respect to the cytoplasmic membrane are discussed below.

NADH dehydrogenase enzyme complex

The NADH dehydrogenase enzyme complex consists of many polypeptide chains collectively referred to as *flavoprotein (Fp)* and also a series of *iron–sulphur protein complexes* (designated *FeS*). Flavoprotein contains a prosthetic group called *flavin*

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[4]A significant difference in format between the sections dealing with aerobic and anoxic respiration in the presentation of information will be apparent. For aerobic respiration, most of the information is regarded as well established and does not require referencing. Where information is considered not to be well established references are included. For anoxic respiration, the information dealing with reductases is referenced extensively, because it is considered that this information is not well established, and may be open to interpretation.

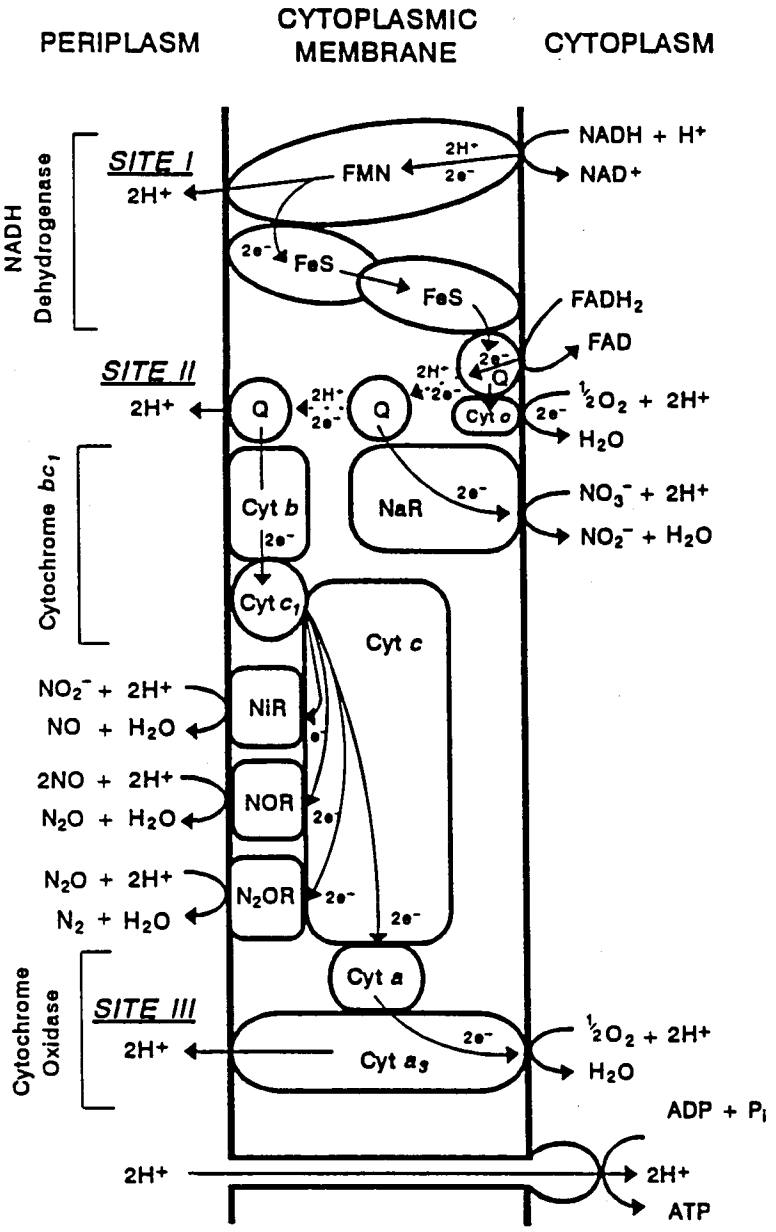

Fig 4.9: Electron transport pathway (ETP) for a typical facultative aerobic heterotrophic organism indicating the active sites of both the aerobic and anoxic electron transferring complexes with respect to the cytoplasmic membrane and the three energy conserving (proton–pumping) sites. [Abbreviations: NAD – nicotinamide adenine dinucleotide; FMN – flavomononucleotide; FeS – iron sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone, Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate].

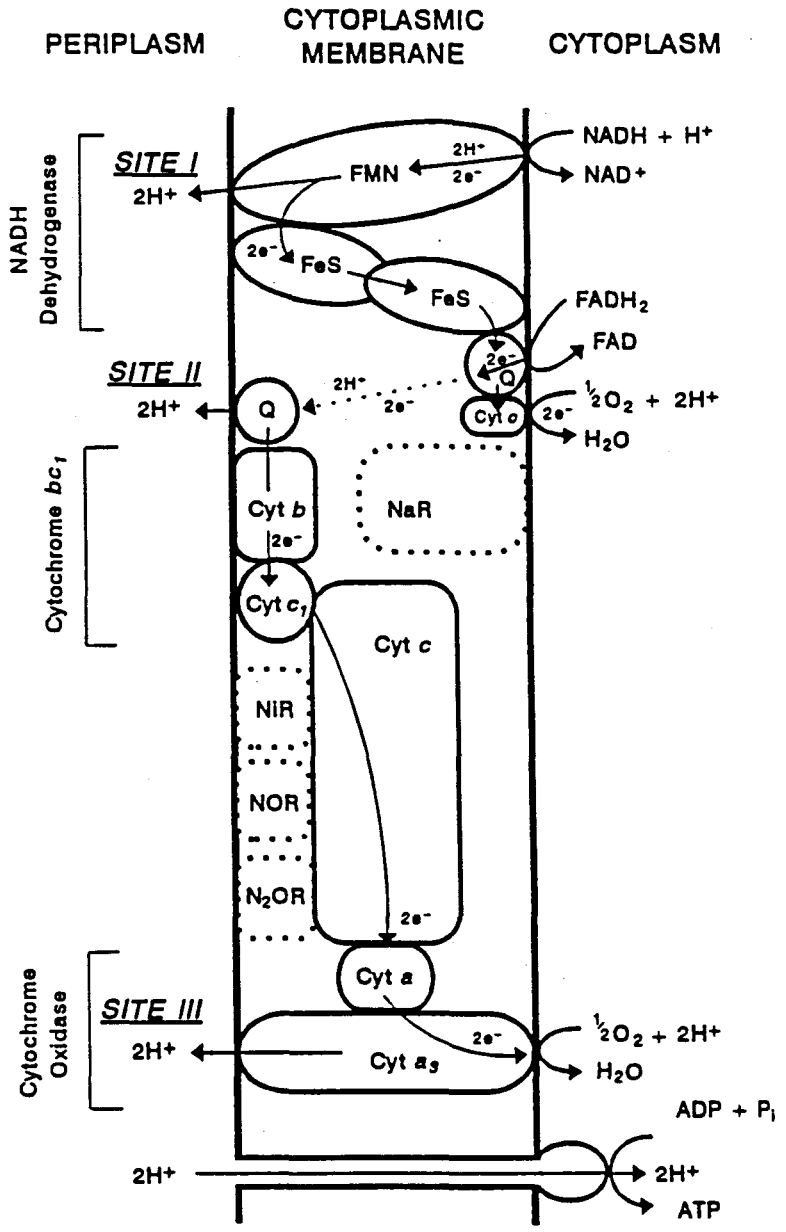

Fig 4.10: Electron transport pathway (ETP) for a typical facultative aerobic heterotrophic organism grown under aerobic conditions, indicating the presence of the oxidases, and synthesis at a low level of the nitrogen oxide reductases. [Abbreviations: NAD – nicotinamide adenine dinucleotide; FMN – flavomononucleotide; FeS – iron sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone, Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate].

mononucleotide (FMN) or *flavin* which acts as the electron and proton transferring site of the polypeptide.

The NADH dehydrogenase complex catalyzes the transfer of two protons and a pair of electrons from NADH to ubiquinone in three steps.

In the first step, the prosthetic group, FMN of the flavoprotein (Fp) (which spans, and is exposed on both sides of the membrane), gains two protons and a pair of electrons to give the reduced form $FMNH_2$. One proton and a pair of electrons are donated from NADH on the cytoplasmic side of the membrane and the other proton, (the one left free in the formation of NADH), is gained from the cytoplasmic medium. The overall transfer of protons and electrons from NADH to FMN is as follows:

$$NADH + H^+ + FMN \rightarrow FMNH_2 + NAD^+ \qquad (4.3)$$

In the second step, a pair of electrons are transferred from $FMNH_2$ to a series of iron–sulphur protein complexes (FeS) on the periplasmic side of the membrane at which point the two protons are released to the periplasm. The net result is the transfer of two protons from the cytoplasmic side of the membrane to the periplasmic side. The point at which protons are extruded through the membrane to the periplasm is conventionally regarded as the first of three proton–pumping or energy conserving positions along the pathway and is referred to as *Site I* (see Section 4.6 below for a more complete description of the mechanism of energy formation). In accepting a pair of electrons from $FMNH_2$, each of the iron atom(s) of the FeS complexes undergoes a valency change[5], from the oxidized ferric (+3) state to the reduced ferrous (+2) state as follows:

$$FMNH_2 + 2Fe^{3+} \rightarrow 2Fe^{2+} + FMN + 2H^+ \qquad (4.4)$$

In the third step, a pair of electrons are transferred between the FeS complexes, and each reduced FeS complex donates one electron to the next carrier in the ETC, ubiquinone.

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[5]Although the FeS complexes are catalytic in that they switch between the oxidized and reduced states, the reaction is not shown as a reversible reduction–oxidation reaction because Fe^{2+} reverts to the Fe^{3+} form only following transfer of electrons to the next electron transferring molecule, ubiquinone.

Ubiquinone complex

Ubiquinone is a mobile molecule which transports electrons and protons from the cytoplasmic to periplasmic side of the membrane. The mechanisms of electron and proton transfer to ubiquinone are different depending on whether electrons originate from NADH or $FADH_2$. For NADH as the initial electron source, the ubiquinone molecule accepts two electrons from the reduced FeS complex of NADH dehydrogenase on the cytoplasmic side of the membrane and takes up two protons from the cytoplasm, forming reduced ubiquinone called ubiquinol (QH_2) in the process of which the reduced iron (Fe^{2+}) atom of the FeS complex returns to the oxidized (Fe^{3+}) state as follows:

$$Q + 2Fe^{2+} + 2H^{+} \rightarrow QH_2 + 2Fe^{3+} \qquad (4.5)$$

For $FADH_2$ as the electron source, the ubiquinone molecule accepts two protons and a pair of electrons from $FADH_2$ forming reduced ubiquinone (QH_2), and oxidized FAD as follows:

$$Q + FADH_2 \rightarrow QH_2 + FAD \qquad (4.6)$$

Ubiquinone is named as a result of its ubiquitous occurrence amongst organisms. It is the only proton and electron carrier in the respiration chain which is not tightly bound or covalently attached to a protein, and being a small, non-polar, and lipid-soluble molecule, can move freely within the cytoplasmic membrane. As a result it serves as a highly mobile carrier of electrons between the flavoproteins and the subsequent electron carriers. In this manner, ubiquinone transports a pair of electrons and 2 protons to the periplasmic side of the membrane, where it extrudes two protons to the periplasm and transfers the pair of electrons to cytochrome *b* contained in the second of the main respiratory complexes, the cytochrome bc_1 complex, as follows:

$$QH_2 + 2 \text{ cyt } b_{ox} (Fe^{3+}) \rightarrow 2 \text{ cyt } b_{red} (Fe^{2+}) + 2H^{+} + Q \qquad (4.7)$$

The point at which two electrons are transferred to cytochrome *b* of the cytochrome bc_1 complex, and two protons are transferred to the periplasmic side of the membrane, is conventionally regarded as the second of the three energy conserving sites, i.e. *Site II.*

An important characteristic of cytochromes is that they are able to transfer electrons only, unlike ubiquinone, NADH, NADH dehydrogenase, $FADH_2$ and ubiquinone which transport both electrons and protons.

Cytochrome bc_1 complex

The cytochrome bc_1 complex contains two sequential electron transporting proteins, cytochrome *b* and cytochrome c_1. The role of the cytochromes of the cytochrome bc_1 complex is to act as an intermediate in the transfer of electrons from ubiquinol to cytochrome *c*, the next complex in the pathway. In the cytochrome bc_1 complex, a pair of electrons are transferred sequentially from ubiquinol to cytochrome *b*, then to cytochrome c_1, and then to the next electron transporting complex, cytochrome *c*. Cytochromes are electron transporting proteins which belong to a class of haem (iron-containing) proteins which also includes haemoglobin. All proteins in the group contain haem in a prosthetic (attached) group. Cytochrome *b*, cytochrome c_1 and cytochrome *c* all contain the same haem group, the difference between the three being the type of bond by which the group is attached to the protein. In cytochrome *b* the haem is not covalently bonded to the protein whereas in cytochrome *c* and cytochrome c_1 the haem is attached to the protein by covalent bonds. As with the iron atom of the FeS complexes, the iron atom in the haem group of the cytochromes alternates between an oxidized ferric (+3) state and a reduced ferrous (+2) state during electron transport. Regarding electron transport, it should be noted that the haem groups of the cytochromes are able to transfer only one electron at a time, unlike NADH, $FMNH_2$ and ubiquinone which transfer two electrons. Thus even though it is not shown as such in Fig 4.10, for each molecule of ubiquinol carrying two electrons and two protons, two molecules of cytochrome *b*, cytochrome c_1, and cytochrome *c* are required.

Cytochrome *c* complex

The cytochrome *c* complex receives electrons from cytochrome c_1 of the cytochrome bc_1 complex and transfers them to the terminal electron transferring complex for aerobic respiration, the cytochrome oxidase complex, cytochrome aa_3.

Cytochrome oxidase complexes

The terminal complex in the aerobic electron transport pathway, the cytochrome oxidase complex, transfers electrons to the terminal electron acceptor, oxygen. For obligate aerobic organisms, the aerobic cytochromes in the electron transport pathway to oxygen are contained in the cytochrome aa_3 complex. For

facultative denitrifying organisms, an additional cytochrome oxidase complex, cytochrome *o* is present, often termed alternative cytochrome oxidase. In facultative organisms undergoing aerobic respiration, both cytochrome aa_3 and cytochrome *o* are operative, although the greater fraction of electron transport to oxygen is via cytochrome aa_3. The roles of cytochrome *o* and cytochrome aa_3 in facultative organism respiration are described in the biochemical respiratory model for facultative organisms in Chapter 5. For electron transport mediated by cytochrome aa_3, under aerobic conditions, cytochrome *a* of the cytochrome aa_3 complex accepts electrons from cytochrome *c* and transfers them to cytochrome a_3. The cytochrome aa_3 complex contains four electron transferring metal centres; the iron (Fe) atoms contained in each of cytochrome *a* and cytochrome a_3 which alternate between a reduced ferrous (+2) state and an oxidized ferric (+3) state during electron transport, and two bound copper (Cu) atoms which also mediate electron transport and alternate between an oxidized (+2) form and a reduced (+1) form (Poole, 1982). Cytochrome a_3 lies in the membrane, the active site located on the cytoplasmic side. At this site, 4 protons from the cytoplasm combine with an oxygen molecule and 4 electrons from the four metal centres of the cytochrome aa_3 complex to form water as follows:[6]

$$\text{cyt } a\,(Fe^{2+},Cu^{+}) + \text{cyt } a_3\,(Fe^{2+},Cu^{+}) + O_2 + 4H^{+} \rightarrow 2H_2O + \text{cyt } a\,(Fe^{3+},Cu^{2+}) + \text{cyt } a_3\,(Fe^{3+},Cu^{2+}) \qquad (4.8)$$

The cytochrome aa_3 complex is conventionally regarded as the third of the three energy conserving sites, i.e. *Site III*, at which protons are pumped through the cytoplasmic membrane to the periplasm. The factor that distinguishes the cyt aa_3 complex (Site III) from the other two proton pumping sites, i.e. the NADH dehydrogenase complex (Site I) and the cytochrome bc_1 complex (Site II), is that at Sites I and II, two protons associated with the pair of electrons being transferred are ejected to the periplasm by the *proton transferring molecules*, $FMNH_2$ and ubiquinol respectively, whereas at Site III, proton transferring molecules are not present. The mechanism for removing protons from the cytoplasm to the periplasm at cytochrome aa_3 is thought to result from electron transport which induces a change in the cytochrome a_3 protein conformation causing part of the complex which spans the membrane to eject protons to the periplasm.

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[6]The mechanism of electron transport within the cyt aa_3 unit is very complex and it is not the intention here to discuss this; a detailed review of the subject is given by Babcock and Wikström (1992).

Under the environmental conditions in which cytochrome *o* acts as oxidase, electrons flow directly from ubiquinone to cytochrome *o*; cytochromes bc_1 and *c* are not involved. Accordingly, the electrons pass only 2 energy conserving sites, Sites I and II, for NADH as electron donor and one site, Site II, for $FADH_2$ as electron donor. For NADH as electron donor, electrons are transferred to NADH dehydrogenase, then to ubiquinone and for $FADH_2$ as electron donor, to ubiquinone directly. For cytochrome *o* as oxidase, 4 protons from the cytoplasm combine with an oxygen molecule and 2 pairs of electrons from ubiquinone to form water as follows:

$$2\text{cyt } o\ (Fe^{2+},Cu^{+}) + O_2 + 4H^{+} \rightarrow 2H_2O + 2\text{cyt } o\ (Fe^{3+},Cu^{2+}) \qquad (4.9)$$

The environmental conditions under which cytochrome *o* or cytochrome aa_3 act as terminal electron acceptors are described in Part II of this review.

A significant physical difference between cytochrome *o* and cytochrome aa_3 is in the electron transferring centres contained in each cytochrome. Cytochrome *o* contains two iron–histidine (Fe–hs) centres and one copper–histidine (Cu–hs) centre whereas cytochrome aa_3 contains an additional copper–histidine–cysteine (Cu–hs–cs) centre (Babcock and Wikström, 1992). The implications of this difference will become apparent in Part II of this review – in investigation of the mechanisms of regulation of respiration. The active site of cytochrome *o* has been assigned to the cytoplasmic side of the membrane since measurements of electron–transport–linked proton translocation indicate that protons required for reduction of O_2 are taken from the cytoplasm, (Boogerd *et al.*, 1981; Willison and Haddock, 1981). Van Verseveld *et al.* (1981) proposed also a cytoplasmic orientation for cytochrome aa_3.

4.5 ANOXIC RESPIRATION

The description above of aerobic respiration is for conditions where dissolved oxygen (DO) is in adequate supply. When DO becomes limiting, facultative heterotrophs are able to switch from oxygen to nitrate/nitrite as terminal electron acceptor and respire anoxically. For nitrate/nitrite as electron acceptor, the first three stages and the greater part of the fourth stage of the electron transport pathway continue functioning unchanged; as mentioned in passing above, a difference in electron transport between aerobic and anoxic respiration becomes apparent only after the ubiquinone complex, i.e. in the electron and proton transferring complexes of cytochrome steps in Stage IV of Fig 4.2. Under anoxic conditions not all of the

electron–transferring complexes shown in Fig 4.9 are synthesized. Figure 4.11 illustrates the respiratory complexes present in a facultative aerobic organism grown under anoxic conditions. Synthesis of the complexes shown in dotted outline is repressed under aerobic conditions, an aspect discussed in greater detail in Part II of this Chapter.

Definition of terms

Organisms can utilize nitrate through two processes, *assimilatory nitrogen removal* and *dissimilatory nitrogen removal.*

Assimilatory nitrogen removal occurs by the reduction of nitrate to ammonium (NH_4^+), the ammonium formed being used for synthesis of cellular material. This process is energy consuming and is *not* associated with the respiratory electron transport chain. The process can occur under both aerobic and anoxic conditions. Because this process is not associated with the respiratory electron transport chain, it is not considered further.

Dissimilatory nitrogen reduction is the reduction of nitrate to nitrite or nitrite to one of the more reduced gaseous nitrogen oxide compounds, nitric oxide (NO), nitrous oxide (N_2O) or dinitrogen (N_2). The process in which nitrate or nitrite act as terminal electron acceptors during the utilization of organic substrate is referred to as *nitrate* or *nitrite respiration.* The term *nitrate reduction* applies to the reduction of nitrate to nitrite only. The term *denitrification* is applied to the reduction of one of the ionic nitrogen oxides (nitrate or nitrite), to one of the gaseous nitrogen compounds, nitric oxide, nitrous oxide, or dinitrogen. The pathway for denitrification is composed of a number of sequential steps by which electrons are passed from the ETP to one of the nitrogen oxides, to produce a more reduced nitrogen oxide. Although opinions differ on the structure of the denitrification pathway (these differences are examined in Appendix D), according to Payne (1973), the denitrification pathway can be described as:

$$\text{Nitrate } (\overset{(+5)}{NO_3^-}{}_{aq}) \rightarrow \text{Nitrite } (\overset{(+3)}{NO_2^-}{}_{aq}) \rightarrow \text{Nitric Oxide } (\overset{(+2)}{NO}{}_{g}) \rightarrow$$
$$\rightarrow \text{Nitrous Oxide } (\overset{(+1)}{N_2O}{}_{g}) \rightarrow \text{Dinitrogen } (N_2{}_{g}).\ ^{7} \qquad (4.10)$$

[7] The values in brackets are the oxidation states of the nitrogen atom for each nitrogen oxide compound. The subscripts $_{aq}$ and $_{g}$ denote aqueous and gaseous species respectively.

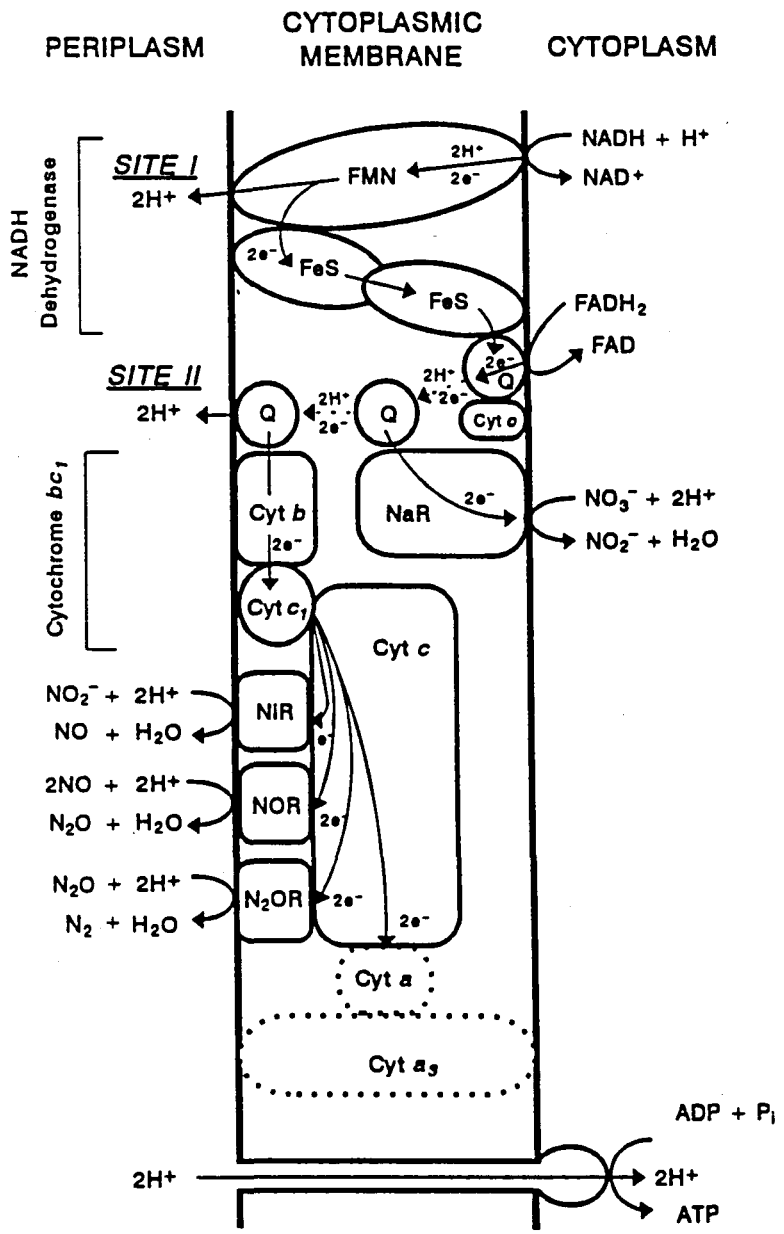

Fig 4.11: Electron transport pathway (ETP) for a typical facultative aerobic organism grown under anoxic conditions, indicating the presence of the reductases and cytochrome *o*, and the synthesis at a low level of the oxidase, cytochrome aa_3. [Abbreviations: NAD – nicotinamide adenine dinucleotide; FMN – flavomononucleotide; FeS – iron sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone, Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate].

The nitrogen oxides of the pathway, which comprise the more reduced species, i.e. nitrite, nitric oxide and nitrous oxide, are referred to as the *intermediates* of the denitrification pathway. Dinitrogen is referred to as the *endproduct* of denitrification. Reduction of one nitrogen oxide intermediate to another is accompanied by the transfer of electrons along the pathway to the specific nitrogen oxide. Reduction of each specific nitrogen oxide is catalyzed by a specific enzyme; these enzymes belong to a group which generally are referred to as the nitrogen oxide reductases. Specifically, these are the nitrate–, nitrite–, nitric oxide–, and nitrous oxide reductases. The electron transport pathways (ETPs) for aerobic and anoxic respiration have some common complexes, i.e. some complexes which are utilized in both aerobic and anoxic respiration; other complexes are specific to aerobic or anoxic respiration. Figure 4.9 illustrates the complexes present in both the aerobic and anoxic ETPs of a typical facultative organism. The pathways for electron flow under anoxic conditions differ depending on the nitrogen oxide available as terminal electron acceptor. With NO_3^- as terminal electron acceptor, electrons are transferred from NADH to NADH dehydrogenase to ubiquinone (Q) and then to nitrate reductase. With NO_2^-, NO and N_2O as electron acceptors, electrons pass from NADH to NADH dehydrogenase, to ubiquinone, to cytochrome bc_1 complex, to cytochrome c_1 and then to either nitrite–, nitric oxide–, or nitrous oxide reductase, for NO_2^-, NO, and N_2O respectively.

Not all organisms classified as denitrifiers can execute the entire pathway from nitrate to dinitrogen. Some organisms are capable of executing only part of the pathway, from one of the ionic nitrogen compounds (i.e. NO_3^- or NO_2^-) to one of the gaseous nitrogen compounds (i.e. NO or N_2O or N_2) and these organisms are referred to as *partial denitrifiers.* As mentioned above, some organisms reduce nitrate to nitrite only and these are referred to as *nitrate reducers.*

<u>Electron transferring complexes and energetic yield associated with anoxic respiration</u>

With oxygen as terminal electron acceptor, electrons pass from the cytochrome bc_1 complex, through cytochrome *c* to cytochrome aa_3, whereas with the nitrogen oxides as terminal electron acceptors, electrons pass directly from ubiquinone to nitrate reductase for nitrate as electron acceptor and from ubiquinone via the cytochrome bc_1 and cytochrome *c* complexes for the other nitrogen oxides as electron acceptor. These differences in electron transport have significant implications concerning energy conservation. For aerobic respiration, during electron transfer to

cytochrome aa_3, electrons pass all three proton–pumping sites whereas under anoxic respiration, during electron transfer to the nitrogen oxide reductases, electrons pass only two proton–pumping sites. The consequences of these differences in the aerobic and anoxic biochemical pathways for organisms switching between aerobic and anoxic respiration in activated sludge processes are discussed below.

In this section of the review much of the work is devoted to nitrate reductase, reflecting not so much the particular importance of that complex in anoxic respiration, but more so the greater volume of research devoted to it. This situation arose because nitrate is the first choice nitrogen oxide for reduction because it is the most oxidized of all the nitrogen oxides (+5), as indicated in Eq 4.10.

For the complete reduction of nitrate to dinitrogen, electrons are transferred through each of the nitrogen oxide reductases to the specific nitrogen oxides which become reduced. The complexes NADH dehydrogenase, ubiquinone, cytochrome bc_1, and cytochrome *c* are common to aerobic and anoxic ETPs. Their roles in mediating electron/proton transfer have been discussed in detail under aerobic respiration above. Accordingly, the electron transport role between these will not be discussed, only their role in electron transport to the nitrogen oxide reductases. To illustrate the mechanism by which each reductase accepts and transfers electrons, the *structure, function* and *location* with respect to the cytoplasmic membrane of each reductase is discussed in turn.

Nitrate reductase

The function of nitrate reductase is to reduce nitrate to nitrite. Nitrate reductase, is situated on the cytoplasmic side of the membrane (John 1977, Kristjansson *et al.*, 1978, Alefounder and Ferguson 1980). Nitrate reductase is composed of three polypeptide subunits, labelled α, β and γ (Craske and Ferguson, 1986). Each subunit has a function, either in electron transport or in attachment to the membrane. The catalytic site of electron transfer is the α–subunit, as a consequence of the electron transferring iron–sulphur and molybdenum centres it contains. The β–subunit has a role in attachment of nitrate reductase to the membrane without which electron transfer could not take place (Ingledew and Poole, 1984; Stouthamer, 1988; Stewart, 1988). The γ–subunit has a role in associating nitrate reductase with the electron donating complex ubiquinone (Enoch and Lester, 1974) and, along with the β–subunit, also is essential for attachment of nitrate reductase to the membrane (MacGregor, 1975b). In short, electron transfer to nitrate reductase is from

ubiquinone via the γ–subunit to the iron–sulphur and molybdenum centres of the α–subunit, none of which can occur without the association of the β and γ–subunits with the membrane as illustrated in Fig 4.12. From the catalytic site at the α–subunit, 2 electrons are passed to nitrate which in the process also takes up 2 protons from the periplasm to form nitrite and water as follows:

$$2H^+ + 2e^- + NO_3^- \rightarrow NO_2^- + H_2O \qquad (4.11)$$

In the process of transferring 2 electrons to nitrate reductase, ubiquinone ejects 2 protons to the periplasm and establishes the site of NO_3^- reduction as the second (Site II) of the energy conserving (proton–pumping) sites of the anoxic respiratory pathway as indicated in Fig 4.11. This site corresponds to Site II of aerobic respiration at which ubiquinone ejects 2 protons to the periplasm in the process of transferring electrons to the cytochrome bc_1 complex. Thus, for each molecule of nitrate reduced, 2 electrons originating from NADH pass 2 proton–pumping sites, Sites I and II, at each of which 2 protons are translocated to the periplasm. Electrons originating from $FADH_2$ and transferred to nitrate pass only one proton–pumping site, Site II.

Given the cytoplasmic placement of the active site of nitrate reductase, nitrate has to be translocated from outside to inside the cytoplasmic membrane. The literature is not clear on the precise mechanism for this translocation. John (1977) proposed a transmembrane ionic carrier specific to nitrate. Boogerd *et al.* (1983a) proposed two uptake systems for nitrate which operate in sequence. The first system is an H^+–NO_3^- symport mechanism which initiates nitrate uptake and is dependent on the proton motive force (pmf) established by the translocation of protons across the cytoplasmic membrane. Nitrate crosses the membrane together with two or more protons during production of ATP, and is reduced to nitrite at the α–subunit on the cytoplasmic side. The second nitrate uptake system is an NO_3^-/NO_2^- antiport, the function of which is to take over NO_3^- uptake from the first system. For each nitrate molecule crossing the membrane to the cytoplasm, one nitrite molecule passes back to the periplasm. The symport and antiport mechanisms are illustrated in Fig 4.13. However, because no physical evidence exists for the presence of such systems, Craske and Ferguson (1986) suggested that the nitrate reductase complex itself incorporates a nitrate–specific channel which provides access for nitrate to the active site of its reductase.

Nitrite Reductase

The function of nitrite reductase is to reduce nitrite originating from the bulk solution or from the reduction of nitrate to nitric oxide. Nitrite reductase, situated on the periplasmic side of the membrane, (Meijer *et al.*, 1979b; Alefounder and Ferguson, 1980; Boogerd *et al.*, 1981) accepts 1 electron from cytochrome *c* and at the catalytic site of the reductase the electron is passed to nitrite, which in the process also takes up 2 protons from the periplasm to form nitric oxide (NO) and water as follows:

$$2H^+ + e^- + NO_2^- \rightarrow NO + H_2O \qquad (4.12)$$

The electron from cytochrome *c* originated from NADH and passed through each of FMN, FeS, ubiquinone and cytochrome bc_1, in the process passing 2 energy conserving sites, Sites I and II. Thus, the same number of protons (4) are pumped across the membrane for each 2 electrons which pass to either nitrate or nitrite. Electrons originating from $FADH_2$ and transferred to nitrite through ubiquinone, cytochrome bc_1 and cytochrome *c* pass only one proton pumping site, Site II, the same as for electrons from $FADH_2$ transferred to nitrate. The reduction of NO_2^- on the periplasmic side of the membrane necessitates the transport of NO_2^- from the cytoplasm where it is formed, to the periplasm where it is reduced, this transport apparently occurring as part of the NO_3^-/NO_2^- antiport mechanism described for the movement of nitrate across the membrane.

Two different types of nitrite reductase have been identified; a cytochrome complex containing haems of the *c*- and *d*-type, referred to as cytochrome cd_1 and a copper containing cytochrome complex, both of which perform the same function but are present in different denitrifying organisms.

Some dispute has arisen over identification of the product of nitrite reduction because usually, nitrous oxide (N_2O), but not nitric oxide (NO) is identified in denitrifying cultures. This anomaly has been resolved with the finding that under normal denitrifying conditions, NO is maintained at constant low concentrations intracellularly and is absent extracellularly, but under adverse situations (e.g. high concentrations of NO_2^-, in the presence of toxins or in physiologically old cells), NO can be excreted extracellularly.

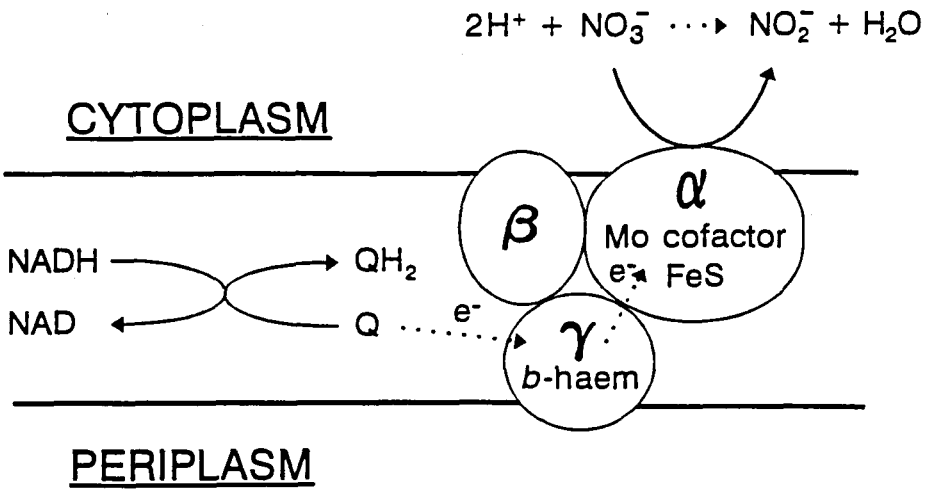

Fig 4.12: Subunits of nitrate reductase indicating the position of each with respect to the membrane and the role of each in electron transport to nitrate. [Abbreviations: NAD – nicotinamide adenine dinucleotide; Q – ubiquinone; Mo – molybdenum; FeS – iron-sulphur]. (redrawn from Stewart, 1988)

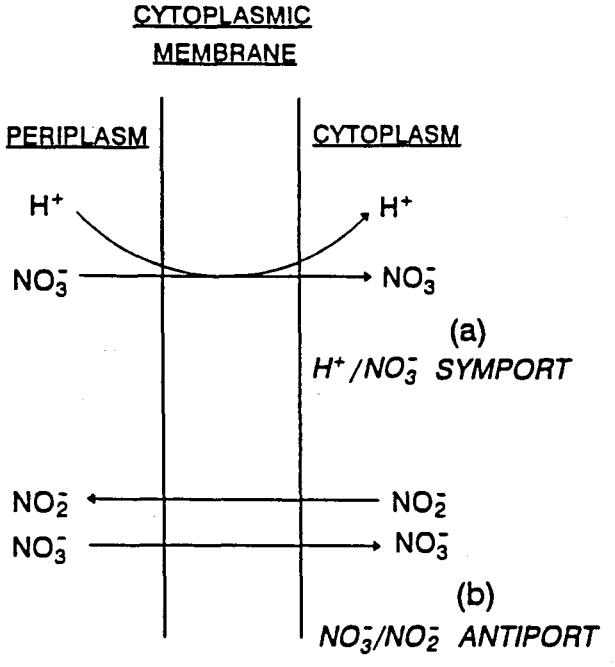

Fig 4.13: Illustration of the mechanism of (a) an H^+/NO_3^- symport and, (b) an NO_3^-/NO_2^- antiport nitrate transport systems (redrawn from Stouthamer, 1988).

Nitric Oxide Reductase

The function of nitric oxide reductase is to reduce nitric oxide to nitrous oxide. Considerable uncertainty exists concerning the position of the active site of nitric oxide reductase. It has been tentatively placed on the periplasmic side of the membrane on the basis of proton uptake experiments (Ferguson, 1988; Stouthamer, 1988; Zumft, 1993), but other opinions differ (Bell *et al.*, 1992). Nitric oxide reductase receives 2 electrons from cytochrome *c* and at the catalytic site of the reductase the electrons are passed to two molecules of nitric oxide which in the process take up 2 protons from the periplasm to form one molecule of nitrous oxide and water as follows:

$$2H^+ + 2e^- + 2NO \rightarrow N_2O + H_2O \qquad (4.13)$$

The two electrons gained by nitric oxide were transferred between the same complexes as electrons gained by nitrite, i.e. electrons passed the same two proton-pumping sites, Sites I and II, for electrons originating from NADH and Site II only for electrons originating from $FADH_2$. Thus the same number of protons (4) are pumped across the membrane per 2 electrons gained by nitric oxide as for nitrite, which equals the number of protons pumped across the membrane per 2 electrons gained by nitrate.

Transfer of electrons from nitric oxide reductase to nitric oxide occurs via the reactive centre of the reductase, a *bc*-type haem. The periplasmic location of the active site of nitric oxide reductase obviates any requirement for a transport mechanism for movement across the membrane of periplasmically produced nitric oxide. However, since nitric oxide is a small gaseous molecule it could move across the membrane by diffusion. An interesting aspect concerning nitric oxide reductase is that in the formation of nitrous oxide from nitric oxide a dinitrogen bond is formed, which is necessary for the ultimate production of dinitrogen (N_2).

Nitrous oxide reductase

The function of nitrous oxide reductase is to reduce nitrous oxide to dinitrogen. The active site of nitrous oxide reductase situated on the periplasmic side of the membrane (Boogerd *et al.*, 1981) receives 2 electrons from cytochrome *c* and at the catalytic site of the reductase the electrons are passed to nitrous oxide which in the process takes up 2 protons from the periplasm as follows:

$$2H^+ + 2e^- + N_2O \rightarrow N_2 + H_2O \qquad (4.14)$$

The endproduct N_2 is released from the cell. The two electrons gained by nitrous oxide are transferred between the same complexes as electrons gained by each of nitrite and nitric oxide, i.e. electrons pass the same proton pumping sites as for nitrite and nitric oxide; Sites I and II for electrons originating from NADH and Site II only for electrons originating from $FADH_2$. As mentioned above for NO_3^- and NO_2^- transport, although the existence of transport mechanisms has been hypothesized, no active or passive transport system for nitrous oxide has yet been found.

4.6 ENERGETIC ASPECTS OF RESPIRATION

The primary role of respiration, irrespective of whether it occurs under aerobic or anoxic conditions, is for the production of energy for the purposes of biosynthesis of the complex molecular components of the organism and for cell maintenance.

In the following section considerable attention is devoted to the mechanisms contributing to the production of energy from respiration and in particular the position of the proton-pumping sites in the ETP since it is imperative to compare the energy gained from aerobic and anoxic respiration, the two respiratory processes which occur in facultative aerobic organisms.

In the aerobic and anoxic respiratory pathways, the electrons and protons gained from organic substrate oxidation are transported to successively lower redox potentials during their passage between enzyme complexes. In this transport process, the free energy associated with the electrons and protons is released; much of this free energy is conserved by the organism.

In the foregoing discussion, mention was made of the three energy conserving sites in the ETP, alternatively referred to as proton-pumping sites. At these sites, the free energy released by the electrons is used to pump protons across the cytoplasmic membrane. In a subsequent mechanism, termed oxidation phosphorylation, the protons flow back across the membrane and energy is captured in the form of high energy phosphate bonds through the formation of the molecule, adenosine triphosphate (ATP) from adenosine diphosphate (ADP). Figure 4.9 illustrates the position of the proton-pumping sites and denotes them as Sites I, II, and III.

Site I is at the NADH dehydrogenase complex, where the flavoprotein (Fp) complex expels 2 protons to the periplasm for each pair of electrons transferred to the next electron transferring complex, the iron–sulphur (FeS) complex. Site II is at the point at which the ubiquinone (Q) molecule expels 2 protons to the periplasm during the transfer of a pair of electrons to the cytochrome bc_1 complex. Site III is at the oxidase complex cytochrome aa_3, at which point 2 protons are expelled to the periplasm and 2 electrons are transferred to oxygen during its reduction to water. This coupling of respiration and oxidative phosphorylation was proposed in 1961 by Mitchell and is known as the chemiosmotic theory.

The first step in oxidative phosphorylation involves the translocation of protons across the normally proton-impermeable cytoplasmic membrane during the passage of electrons through the electron transport pathway in the manner described above. The mechanism by which this movement of protons is coupled to energy production is illustrated in Fig 4.14 and can be described as follows: the translocation of protons from the cytoplasmic to the periplasmic side of the membrane establishes an electrochemical potential across the membrane referred to as proton motive force (pmf). Proton motive force ($\Delta\mu_H$) has both a pH and a charge ($\Delta\psi$) component which can be expressed as follows:

$$\Delta\mu_H = \Delta\psi + \frac{2{,}3RT}{F}\Delta pH \qquad (4.15)$$

where

R = Universal gas constant (1,987 cal/mol.K)

T = Temperature (K)

F = Faraday's constant (23062 cal/volt)

Proton motive force is a form of potential energy and as the name implies acts to drive the protons on the periplasmic side of the membrane back to the cytoplasmic side during which process, energy is converted from a potential form to a chemical form. The protons pass through the membrane from the periplasm to the cytoplasm via a channel created by a protein called adenosine triphosphatase (ATPase). This complex molecule spans the cytoplasmic membrane and is composed of three protein units denoted F_0, F_1 and I. The F_0 protein transfers protons across the lipid layer of the membrane; the F_1 protein catalyzes the phosphorylation of ADP to ATP; and the I protein is a regulatory protein that prevents premature hydrolysis of the newly formed ATP. The energy-rich ATP serves as an energy source for the

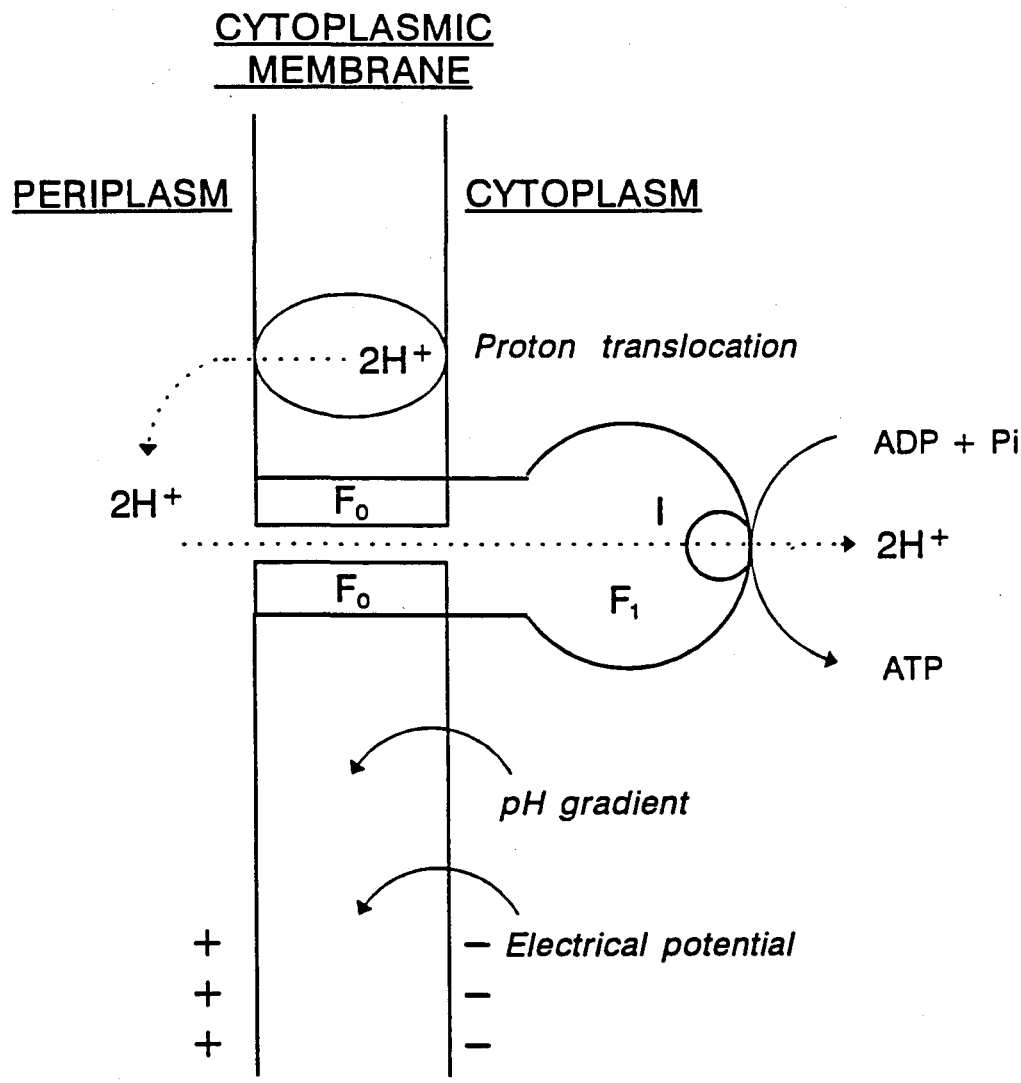

Fig 4.14: Chemiosmotic hypothesis of oxidative phosphorylation, indicating the roles of the combined electrical potential and pH gradient in establishing membrane potential, and the F_0, F_1 and I proteins responsible for phosphorylation (redrawn from Martin *et al.*, 1981)

organism. To generate one molecule of ATP, two protons must transverse the membrane via the ATPase enzyme. Accordingly, one ATP will be generated for each pair of protons pumped across the membrane at each of Sites I, II and III, or in other words, for aerobic respiration with a pair of electrons flowing past Sites I to III, 3 ATPs will be generated per electron pair passing from $NADH_2$ to O_2, and for anoxic respiration with a pair of electrons flowing past Sites I and II only, 2 ATPs will be generated per electron pair passing from $NADH_2$ to one of the nitrogen oxides.

Establishing the position of the energy sites in the electron transport pathway

The chemiosmotic theory hinges on the certain identification of the position and function of the three proton–pumping sites described above. These sites have been identified experimentally through three different approaches; (i) comparison of the ATP yield of different substrates, (ii) thermodynamic estimates of electron and proton flow between complexes, and (iii) inhibition of electron flow at specific sites on the electron transport pathway (Stryer, 1981).

In the first approach, artificial substrates are used which donate electrons and protons at different positions on the electron transport pathway producing different growth yields. For example, it is known that under aerobic conditions the electron and proton donors, NADH, succinate and ascorbate, donate electrons to NADH dehydrogenase, ubiquinone and cytochrome *c* respectively, as shown in Fig 4.15. Theoretically, electrons supplied by NADH pass Sites I, II, and III, yielding 3 moles of ATP per mole of electrons passed to oxygen; electrons supplied by succinate pass Sites II and III only, yielding 2 moles of ATP per mole of electrons passed to oxygen and; electrons supplied by ascorbate pass Site III only, yielding 1 mole of ATP per mole of electrons supplied to oxygen. Experiments conducted with NADH, succinate, and ascorbate as substrates produced organism growths in the approximate ratio, 3:2:1 respectively, providing support for the identification of the 3 sites at the complexes described above (Stryer, 1981).

In the second approach, from the known reduction–oxidation (redox) potentials for each cytochrome couple on the pathway, the free energy gained through transmission of electrons between cytochromes can be calculated and compared to the known free energy required to drive the synthesis of ATP. The relationship between the redox potentials and corresponding free energies for each cytochrome complex are shown diagrammatically in Fig 4.16. At each of Sites I, II and III the

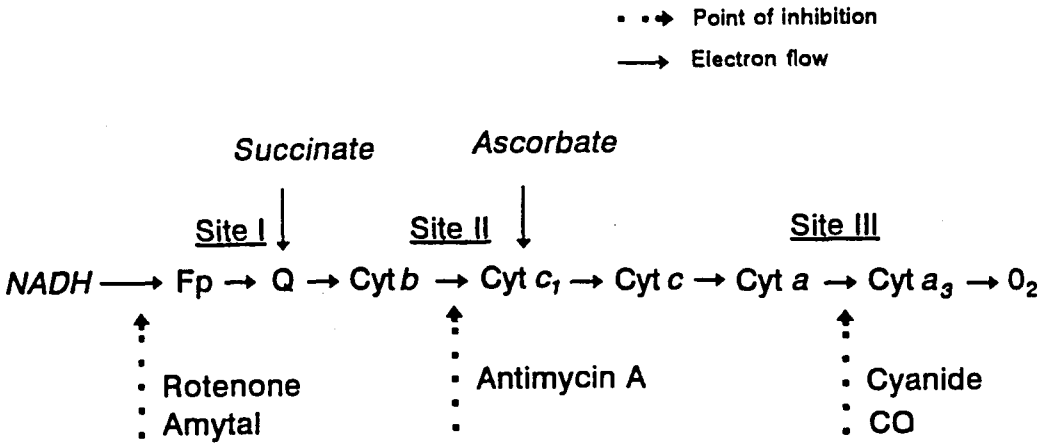

Fig 4.15: Proposed sites of electron donation by NADH, succinate, and ascorbate to the electron transport pathway of a typical facultative aerobic organism. [Abreviations: NADH – nicotinamide adenine dinucleotide; Cyt – Cytochrome; Fp – flavoprotein; Q – ubiquinone]. (redrawn from Martin *et al.*, 1981)

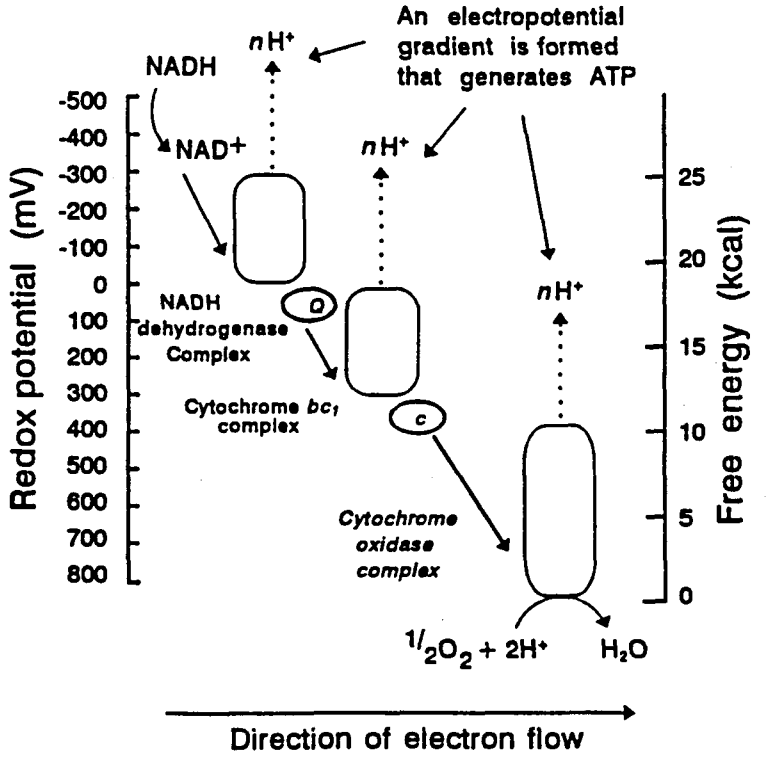

Fig 4.16: Relationship between redox potential (E_h) and the free energy associated with each complex of the electron transferring pathway (ETP) (redrawn from Alberts *et al.*, 1983).

change in redox potential is such that sufficient free energy is released for the synthesis of ATP. The standard free energy ($\Delta G_o'$) generated by the transfer of electrons between two complexes can be calculated from the standard reduction-oxidation (redox) potentials of the complexes:

$$\Delta G_o' = -n\ F\Delta E_h' \qquad (4.16)$$

where

n = number of electrons transferred in the reaction
F = Faraday constant (23 062 cal/volt)
E_h' = redox potential difference between complexes (mV)

The third approach involves the use of known electron transport inhibitors which block electron transport at specific points on the ETP. In this way, electrons pass 1, 2, or 3 proton-pumping sites depending on the inhibitor used. For example, the inhibitors rotenone and amytal prevent electron flow proceeding past the NADH dehydrogenase complex, antimycin A prevents electron flow proceeding past cytochrome *b*, and cyanide and carbon monoxide prevent electron flow to cytochrome a_3. Figure 4.15 indicates the points on the ETP at which each inhibitor and other inhibitors act. The observed growth yields with the use of these chemicals supported the proposed positions of the three proton-pumping sites. The details of these experiments are described in Appendices C and D.

These three approaches established the position of the three proton-pumping - energy conserving sites and are useful tools in determining the efficiency of growth under aerobic and anoxic conditions in which oxygen and nitrogen oxides respectively serve as terminal electron acceptors at different positions on the electron transport pathway. The necessity for establishing the positions on the aerobic electron transport pathway of the three proton-pumping sites will become evident in the following section which investigates the relationship between the electron transferring pathways involved in aerobic and anoxic respiration.

<u>Energetic yield associated with aerobic and anoxic growth</u>

Considering Fig 4.9 as typical of the cytochromes present and reactions associated with a facultative organism, it is possible to determine the comparative ATP yields associated with the use of oxygen and the nitrogen oxides as terminal electron acceptors.

For oxygen as electron acceptor, two cytochrome oxidases are available, cytochrome *o* and cytochrome aa_3. For cytochrome aa_3 as terminal oxidase and with NADH as electron donor, electrons pass all three of the proton-pumping sites with the concomitant energy conservation of 3 moles of ATP formed for the acceptance of 2 moles of electrons by 1 mole of oxygen atoms reduced. For the alternative oxidase cytochrome *o* as terminal oxidase, and NADH as electron donor, electrons pass Sites I and II only, and the yield associated with electron flow to oxygen mediated by this enzyme would be about 2/3rds of that associated with electron flow to oxygen via cytochrome aa_3 in which three sites are passed. An hypothesis for the development of the alternative oxidase cytochrome *o* under aerobic and anoxic conditions is given in Appendix C.

For the nitrogen oxides as terminal electron acceptors and with NADH as electron donor, the electrons pass only the first two of the three proton-pumping sites with the concomitant energy conservation of 2 moles of ATP formed for the acceptance of 2 moles of electrons by 1 mole of nitrogen oxide atoms reduced. Experimental evidence of a yield with nitrogen oxides under anoxic conditions of approximately 2/3rds of the yield with oxygen under aerobic conditions has been presented by Koike and Hattori (1975a), Justin and Kelly (1978), Van Verseveld *et al.* (1981), Parsonage and Ferguson (1983), and Stouthamer (1988).

Concerning the different nitrogen oxides, NO_3^-, NO_2^-, NO and N_2O, it has been established that each can serve as electron acceptor under anoxic conditions and that approximately the same amount of energy is gained in the use of each (Hadjipetrou and Stouthamer, 1965; Koike and Hattori, 1975b; Van Verseveld *et al.*, 1977; Kristjansson *et al.*, 1978; McCarty and Ferguson, 1981; Stouthamer *et al.*, 1982; Garber *et al.*, 1982; Shapleigh and Payne, 1985b).

<u>Comparison of energetic yield of nitrate reducers and denitrifiers</u>

As noted earlier, organisms which respire under anoxic conditions can be classified either as nitrate reducers or denitrifiers. Under conditions in which nitrate is not limiting, the yield of the two groups of organisms is the same. However, a difference in yield between the nitrate reducing and denitrifying groups of organisms results when the concentrations of nitrate and nitrite are present in quantities which are limiting. To illustrate this, consider two cases, the presence of one molecule of nitrate only, and the presence of one molecule of nitrite only under conditions of a non-limiting supply of electrons.

Considering the first case: For denitrifying organisms the reduction of one nitrate ion through each nitrogen oxide intermediate to a dinitrogen molecule requires four pairs of electrons. During transfer through the denitrification complexes to the nitrogen oxides, the electrons pass two energy conserving sites, Sites I and II (i.e. one pair of electrons is required for the reduction of each nitrogen oxide intermediate to the next intermediate), resulting in the transferal of 4 pairs of protons across the membrane and the concomitant generation of 4 ATP. In comparison, for nitrate reducing organisms, the reduction of a nitrate ion to a nitrite ion requires only one pair of electrons, which during transfer from NADH through the denitrification complexes passes the first two energy conserving sites, resulting in the generation of 2 ATP, a considerably lower energetic yield than that gained by the denitrifying organisms.

Considering the second case: For denitrifying organisms, the reduction of a nitrite ion through each nitrogen oxide intermediate to a dinitrogen molecule requires three pairs of electrons, which during transfer from NADH through the denitrification complexes to one of the nitrogen oxides, pass Sites I and II, resulting in the generation of 3 ATP. In comparison, nitrate reducers cannot utilize nitrite and gain no energy from its presence under anoxic conditions.

PART II: MECHANISMS OF REGULATION OF AEROBIC AND ANOXIC RESPIRATION IN FACULTATIVE ORGANISMS

4.7 INTRODUCTION

In Part I the biochemical pathways and enzyme complexes present in facultative organisms under steady–state aerobic and steady–state anoxic conditions were described. Also, it was established that the enzyme complexes (the oxidases and nitrogen oxide reductases) which transfer electrons to the terminal electron acceptors are adaptive, i.e. are synthesized when aerobic or anoxic conditions respectively are imposed. Facultative organisms have the propensity to respire under anoxic or aerobic conditions. In nitrogen and nutrient removal activated sludge systems, these organisms are constantly cycled between anoxic and aerobic conditions. This raises the question as to how the organisms regulate the synthesis and activity of the required enzyme complexes when changing from anoxic to aerobic conditions and *vice versa*.

In this section – Part II – it is the intention to examine the factors associated with initiation, regulation, and termination of aerobic and anoxic respiration. Each of

these three phenomena involve changes in both synthesis and activity of specific enzyme complexes. To investigate the synthesis and activity of the denitrifying complexes of facultative organisms in pure culture, research workers conducted experiments with two sets of growth conditions:

(i) Aerobically grown organisms (in which the denitrifying enzymes are absent and aerobic enzymes are present) were exposed to anoxic conditions to examine how denitrifying enzymes are synthesized and aerobic enzymes are inactivated and degraded.

(ii) Anoxically grown organisms (in which the denitrifying enzymes are present and aerobic enzymes are absent) were exposed to aerobic conditions to examine how aerobic conditions affect the activity of the synthesized denitrifying enzymes, and synthesis of the aerobic enzymes.

(This approach is fortuitous in that the two sets of laboratory experimental conditions are the same as the conditions encountered in aerobic–anoxic activated sludge systems (i.e. changes in conditions from aerobic to anoxic, and from anoxic to aerobic), and because of this similarity the laboratory experimental behaviour is applicable to behaviour of facultative organisms in activated sludge systems.)

In the discussion that follows, the electron transport pathway (ETP) used for illustrative purposes is the one concluded in Appendix C [8] to be the most current, complete, and applicable to organisms subjected to changes between anoxic and aerobic conditions, and is illustrated in Fig 4.9. The ETP is shown with both aerobic and anoxic respiratory complexes present.

4.8 AEROBIC CONDITIONS CHANGED TO ANOXIC CONDITIONS

Aerobic and denitrifying enzyme synthesis under aerobic conditions

Under *aerobic* conditions the status of synthesis of the aerobic and anoxic reductases is as illustrated in Fig 4.10. Enzymes shown in dotted outline are not synthesized or are synthesized at a low level only.

Sapshead and Wimpenny (1972) designed experiments to measure the effect of oxygen on the development of the aerobic enzymes. *Pa. denitrificans* was grown at different concentrations of oxygen, and the levels of the oxidases, cytochrome aa_3 and cytochrome *o* were measured. The level of cytochrome aa_3 of organisms grown at low aeration was intermediate between the high level developed under aerobic

[8] A review of research into electron transport pathways of facultative heterotrophic organisms.

conditions and total absence when grown under anoxic conditions, and although cytochrome *o* was synthesized under both aerobic and anoxic/anaerobic conditions, under aerobic conditions it developed to only 70% of its level under anoxic conditions.

Under oxygen saturation conditions, synthesis of nitrate reductase of the nitrate reducing organism *E. coli* is reduced to its basal level (Showe and de Moss, 1968) and similarly for the denitrifying organism *Pa. denitrificans* nitrate and nitrite reductase were measured at a low level (Lam and Nicholas, 1969a).

Requirements for induction of denitrifying enzyme synthesis

When aerobic conditions are changed to anoxic conditions, the potential exists for induction of synthesis of the denitrifying enzymes and concomitant denitrification. (Although nitrate reductase and the other nitrogen oxide reductases are present under aerobic conditions at low levels, with the onset of anoxic conditions, the level of enzyme synthesis is too low to allow an immediate measurable rate of denitrification). Considerable variability exists between organisms in their requirements for synthesis of the denitrifying enzymes. Most organisms, e.g. *Pa. denitrificans, Klebsiella aerogenes* and *Alcaligenes faecalis* require the absence of oxygen for derepression of nitrate and nitrite reductase and the presence of nitrate and nitrite for induction of nitrate and nitrite reductase synthesis respectively (Lam and Nicholas, 1969a; Pichinoty and d'Ornano, 1961c; Kakutani *et al.*, 1981). Other organisms such as *Bacillus licheniformis, Proteus mirabilis and Ps. stutzeri* require only the absence of oxygen for nitrate and nitrite reductase synthesis (Schulp and Stouthamer, 1970; de Groot and Stouthamer, 1970b; Kodama, 1970), but even in these organisms nitrate and nitrite reductase is synthesized at only about 40% of the level to which it is synthesized in the presence of nitrate and nitrite. Under aerobic conditions, synthesis of the reductases is *repressed*, and nitrous oxide more so than the others; in aerobically grown *Ps. perfectomarinus* switched to anoxic conditions in the absence of nitrate and nitrite, synthesis of nitrous oxide reductase began first (Payne, 1973). For aerobically grown *Pa. denitrificans* switched to anoxic conditions, synthesis of all the denitrifying enzymes began simultaneously (Payne, 1973).

To examine the relative importance of oxygen and nitrate concentration on the synthesis of nitrate reductase, *Pa. denitrificans* was grown at different concentrations of oxygen with nitrate present (Sapshead and Wimpenny, 1972). At

high concentrations of oxygen, synthesis of nitrate reductase was repressed, irrespective of the presence or absence of nitrate. However, as the concentration of oxygen decreased, the relative importance of nitrate in the synthesis of nitrate reductase increased, such that at a very low concentration of oxygen or under anoxic conditions, the presence or absence of nitrate was of primary importance.

Little definitive information is available regarding the effect of oxygen and the nitrogen oxides on the induction of the other denitrifying enzymes, i.e. nitric oxide and nitrous oxide reductase. What is known is that aerobically grown *Ps. perfectomarinus* in which synthesis of all the denitrifying enzymes was repressed, synthesized all the denitrification enzymes in the *absence* of oxygen and nitrogen oxides (Payne *et al.*, 1971). Additionally, in a *Flavobacterium* sp. grown with nitrate, the denitrification enzymes were synthesized sequentially in the same order as the denitrification sequence, i.e. from nitrate reductase through to nitrous oxide reductase (Firestone and Tiedje, 1979), implying that the reductases are synthesized in response to the sequentially produced nitrogen oxides.

In summary:

- The minimum requirements for most facultative organisms for induction of synthesis of the nitrogen oxide reductases are low levels or the absence of oxygen.

- For some facultative organisms an additional requirement for synthesis is the presence of the specific nitrogen oxide which is the reactant for the reductase, e.g. nitrate, for nitrate reductase, etc.

- For virtually all facultative organisms, maximum levels of the reductases are synthesized only in the presence of the specific nitrogen oxide which is the reactant for the reductase.

For reductases synthesized to their maximum level (i.e. under anoxic conditions) the factors which regulate synthesis and activity (electron transferring capability) are described in the following section.

<u>Regulation of synthesis and activity of the denitrifying enzymes under anoxic conditions</u>

Under anoxic conditions denitrification is regulated both by control of enzyme synthesis and control of enzyme activity.

Control of the *synthesis* of the reductases is exerted at the level of expression of the specific gene for synthesis of the reductases. The details of this mechanism are given in Appendix D.

Control of the *activity* of the denitrifying enzymes under anoxic conditions involves complex interactions between the nitrogen oxides and the nitrogen oxide reductases. Each of the nitrogen oxides interacts with its own reductase and other reductases, the nett effect of which is a mechanism which ensures that none of the nitrogen oxide intermediates, (NO_2^-, NO, N_2O) increases to high concentrations. A summary of the results of experimental work conducted on the interactions between the nitrogen oxides and the nitrogen oxide reductases is given in Table 4.1. The interactions in which each intermediate inactivates each nitrogen oxide reductase other than its own appears to be virtually general, except for interactions of nitric oxide with nitrate reductase and nitrate with nitrous oxide reductase. In the literature search, no report appears to have yet been published on these interactions. Additionally the interaction of nitrate with nitrite reductase appears to be anomalous in that it is not repressive, but is an activating interaction. This effect has been confirmed in four out of five investigations. An explanation is that the end-product of nitrate reduction, i.e. nitrite, activates nitrite reductase.

Effect of aerobic growth conditions on the synthesis of the denitrifying enzymes of organisms isolated from activated sludge

In the foregoing section, the effects of aerobic and anoxic conditions on the aerobic and denitrifying enzymes of organisms in pure culture have been examined. Because of the applied nature of this investigation it would be advantageous to establish whether similar behaviour occurs in facultative organisms from activated sludge. In this section the effects of similar conditions on the denitrifying enzymes of facultative organisms from activated sludge are reviewed.

In 24 facultative bacteria isolated from denitrifying and aerobic sludges, the synthesis of nitrate reductase after growth under aerobic conditions varied from total repression in some bacteria to near non-repression in others (Krul and Veeningen, 1977).

In a similar investigation (Simpkin and Boyle, 1988), the level of synthesis of nitrate and nitrite reductase was analysed for a mixed culture activated sludge subjected to various aerobic/anoxic conditions. The synthesis of these reductases was repressed

Table 4.1: Summary of experimental findings with regard to the effect of the nitrogen oxides on the activity of the nitrogen oxide reductases under anoxic conditions (+ indicates an activating effect, and – indicates an inactivating effect).

References to research concerning interactions between nitrogen oxides and nitrogen oxide reductase				
	NITRATE REDUCTASE	**NITRITE REDUCTASE**	**NITRIC OXIDE REDUCTASE**	**NITROUS OXIDE REDUCTASE**
NITRATE (NO_3^-)	• *Paracoccus denitrificans* (Fewson & Nicholas, 1961a +) (Lam & Nicholas, 1969a +)	• *Achromobacter cycloclastes* (Iwasaki & Matsubara, 1972 +) • *Alcaligenes faecalis* (Kakutani *et al.*, 1981 +) • *Paracoccus denitrificans* (Newton, 1969 +) • *Pseudomonas perfectomarinus* (Payne *et al.*, 1971 +) • *Pseudomonas stutzeri* (Kodama, 1969; 1970 -)	• *Paracoccus denitrificans* (Payne & Riley, 1969 -) (Frunzke & Zumft, 1986 +) • *Pseudomonas perfectomarinus* (Miyata, 1971 -)	
NITRITE (NO_2^-)	• *Escherichia coli* (Showe & De Moss, 1968 -) • *Paracoccus denitrificans* (Kučera *et al.*, 1983a -) (Alefounder *et al.*, 1983 +) • *Pseudomonas mirabilis* (De Groot & Stouthamer, 1970a -) • *Pseudomonas stutzeri* (Kodama *et al.*, 1969 -) • *Thiobacillus denitrificans* (Baalsrud & Baalsrud, 1954 -)	• *Alcaligenes faecalis* (Kakutani *et al.*, 1981 +) • *Paracoccus denitrificans* (Lam & Nicholas, 1969a +) • *Pseudomonas stutzeri* (Kodama, 1970 +) • *Thiobacillus denitrificans* (Justin & Kelly, 1978 +) (Baalsrud & Baalsrud, 1954 -)	• *Pseudomonas aeruginosa* (Swain *et al.*, 1978 +) (Carlson & Ingraham, 1983 -) • *Pseudomonas perfectomarinus* (Payne *et al.*, 1971 -)	• *Pseudomonas aeruginosa* (Lan, 1973 -) (Carlson & Ingraham, 1983 -)
NITRIC OXIDE (NO)		• *Achromobacter cycloclastes* • *Paracoccus denitrificans* • *Pseudomonas aeruginosa* • *Pseudomonas perfectomarinus* (Shapleigh *et al.*, 1987 -)	• *Bacillus sp.* (Pichinoty *et al.*, 1978 +) • *Pseudomonas perfectomarinus* (Payne & Riley, 1969 +)	• *Pseudomonas perfectomarinus* (Frunzke & Zumft, 1986 -) (Payne *et al.*, 1971 -)
NITROUS OXIDE (N_2O)	• *Paracoccus denitrificans* (Alefounder *et al.*, 1982 -) (Alefounder *et al.*, 1983 -) (Kučera *et al.*, 1983a -)	• *Paracoccus denitrificans* (Alefounder *et al.*, 1983 -)	• *Pseudomonas perfectomarinus* (Frunzke & Zumft, 1986 -)	• *Achromobacter cycloclastes* (Matsubara, 1975 +) • *Alcaligenes* (Matsubara, 1975 +) • *Paracoccus denitrificans* (Carlson & Ingraham, 1983 +) • *Pseudomonas perfectomarinus* (Payne *et al.*, 1971 +) • *Pseudomonas stutzeri* (Carlson & Ingraham, (1983 +)

by less than 50% on exposure to aerobic conditions in comparison to almost complete repression for the pure culture organisms described above. The authors concluded that less than 50% change in synthesis of nitrate and nitrite reductase between aerobic and anoxic growth could not account for the very large difference in nitrate and nitrite reduction between aerobic and anoxic conditions and attributed the difference to the effect of oxygen on the *activity* and not the *synthesis* of the reductases.

4.9 ANOXIC CONDITIONS CHANGED TO AEROBIC CONDITIONS

Inhibition of activity and repression of synthesis of the denitrifying enzymes

It is generally accepted that oxygen has an immediate inhibitory effect on denitrification. It can be assumed that upon exposure to oxygen the immediate reduction in denitrification rate does not result from changes in the level of synthesized enzymes, but from a change in the activity of the denitrification enzymes.

To investigate the effect of oxygen on denitrification generally, facultative organisms were subjected to slowly increasing concentrations of dissolved oxygen. From work conducted with *Hyphomicrobium* X (Meiberg *et al.,* 1980) and *Thiobacillus denitrificans* (Justin and Kelly, 1978) it was established that low concentrations of oxygen inhibit the activity of the nitrogen oxide reductases and higher concentrations repress synthesis of the enzymes. Hochstein *et al.* (1984) demonstrated that for the activities of the reductases of *Pa. halodenitrificans* the order of decreasing sensitivity to oxygen is; nitrous oxide–, nitrite–, and nitrate reductase.[9] Thus, under conditions of increasing concentration of dissolved oxygen, but at oxygen concentrations at which enzyme synthesis is not repressed, the products and intermediates of denitrification appeared in the order; dinitrogen, nitrous oxide, and then nitrite. Tiedje (1985) illustrates the effect of oxygen at different concentrations on the activity and synthesis of the denitrifying reductases of *Hyphomicrobium* in the manner of Fig 4.17. Because the author did not accept nitric oxide as an obligatory intermediate in the denitrification pathway, it was not included in the original work, but is included here between nitrite reductase and

[9]Because of its very rapid instability in aerobic environments, nitric oxide does not appear as an external intermediate during inhibition of nitrogen oxide activity by oxygen. In these experiments nitric oxide was not measured and consequently the workers did not consider nitric oxide as an obligatory compound in the denitrification pathway and thus did not consider the effect of oxygen on nitric oxide reductase.

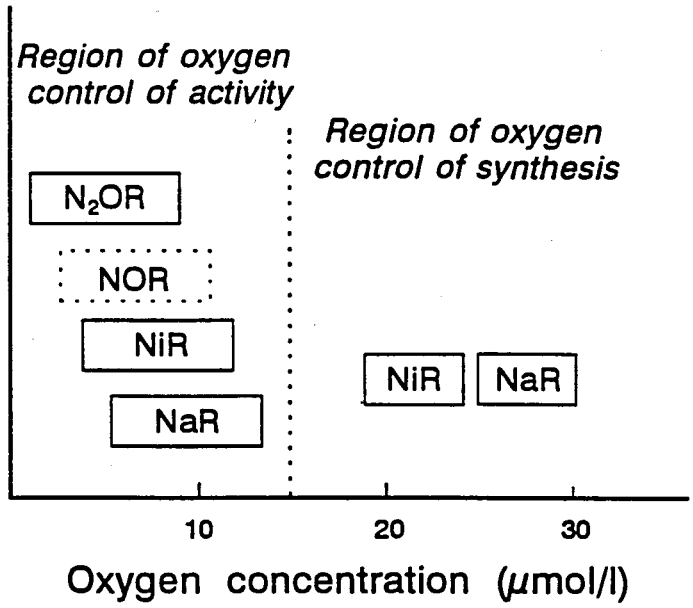

Fig 4.17: Effect of oxygen at different concentrations on the activity and synthesis of the nitrogen oxide reductases. [N_2OR – nitrous oxide reductase; NOR – nitric oxide reductase; NiR –nitrite reductase; NaR – nitrate reductase] (adapted from Tiedje, 1985).

nitrous oxide reductase.

In order to examine more specifically the effect of oxygen on the activity of denitrifying enzymes, workers grew organisms under anoxic conditions such that the denitrifying enzymes were fully synthesized and then exposed the organisms to aerobic conditions which caused that denitrification was reduced or terminated and closely monitored the electron transferring capacity of the enzymes. From these investigations, the inactivation of the denitrifying enzymes (and nitrate reductase in particular), under aerobic conditions was hypothesized to occur by one or a combination of the following four mechanisms:

- Oxygen interferes directly with the enzymes thereby preventing transfer of electrons from the reductases to the appropriate electron acceptors.
- Extracellular oxygen affects the intracellular redox potential of the organism, thereby affecting the flow of electrons between electron transport complexes.
- Oxygen prevents insertion of the subunits of nitrate reductase into the cytoplasmic membrane, thereby preventing transport of electrons from electron transferring complexes to nitrate reductase.
- Oxygen affects the permeability of the cytoplasmic membrane, thereby regulating the movement of nitrate to nitrate reductase, and preventing electron transport from nitrate reductase to nitrate.

Each of these proposed mechanisms is discussed in turn.

<u>Inactivation of nitrogen oxide reductases through direct interference by oxygen</u>

A mechanism by which oxygen could inactivate the nitrogen oxide reductases is through direct attachment to, or reaction with the reductases, in a manner which prevents the transfer of electrons to the nitrogen oxide electron acceptors. However, in research into this effect no such mechanism has been found; as proof that oxygen does not act in this manner, workers have considered it sufficient to demonstrate that oxygen inhibits the reduction of the nitrogen oxidases in a manner other than through direct interference with the reductases, such as by the mechanisms listed above and described below.

<u>Inhibition of electron flow to nitrogen oxide reductases due to intracellular redox changes</u>

Payne (1973) suggested that inactivation of nitrogen oxide reductase results from

oxygen acting as an electron acceptor at cytochrome oxidase, increasing the redox potential at the oxidase and thereby reducing electron flow to other parts of the ETP, in particular to nitrate reductase. Later work allocated the point of distribution of electron flow between nitrate reductase and cytochrome oxidase variously to cytochromes *c* and c_1 (Alefounder *et al.* 1981), cytochrome *b* (John and Whatley, 1975), cytochrome *cd* of nitrite reductase (Kučera *et al.* 1981), and also ubiquinone (Alefounder *et al.* 1981).

Alefounder *et al.*, 1981 proposed a mechanism whereby the redox state of ubiquinone controls electron flow, shown diagrammatically in Fig 4.18. The concentration of dissolved oxygen coupled with the K_m of the oxidase affects the rate of electron transfer to oxygen thereby affecting the extent of reduction of ubiquinone. A high extent of reduction of ubiquinone resulting from a low rate of electron flow to cytochrome oxidase (cytochrome *o* and cytochrome aa_3) would operate a switch allowing electron flow to nitrate reductase (Alefounder *et al.*, 1981).

Alefounder *et al.* (1983) reviewed the work concerned with the point of electron distribution. From their own experiments and accepting the ETP of Ferguson (1982) (in which ubiquinone is the last common electron transferring complex to nitrate reductase and the cytochrome oxidases aa_3 and *o*) they concluded that their proposed mechanism of 1981 was still valid. Stouthamer (1988) concluded that the work of Alefounder *et al.* (1981) and Kučera *et al.* (1981) indicates that the inhibitory action of oxygen on nitrate reductase results from the inhibition of electron flow to that reductase, as a consequence of changes in the reduction–oxidation state of one or more of the complexes of the ETP due to the presence of oxygen. However, due to the difficulty in measuring the extent of reduction of ubiquinone, little experimental evidence has been produced to support this proposal.

<u>Prevention by oxygen of insertion of nitrate reductase subunits into the membrane</u>

During nitrate reduction, the three nitrate reductase subunits (α, β, and γ) play a role in electron transfer to nitrate. Stewart (1988) suggested that the incorporation of nitrate reductase subunits α and β into the membrane requires electron flow, and because oxygen prevents electron flow to nitrate reductase, the subunits cannot be incorporated in the presence of oxygen. This proposal was based on the function of the cytochrome *b* (γ) subunit of nitrate reductase, to associate with the membrane, and the finding of Hackett and MacGregor (1981) with *E.coli* that the synthesis of

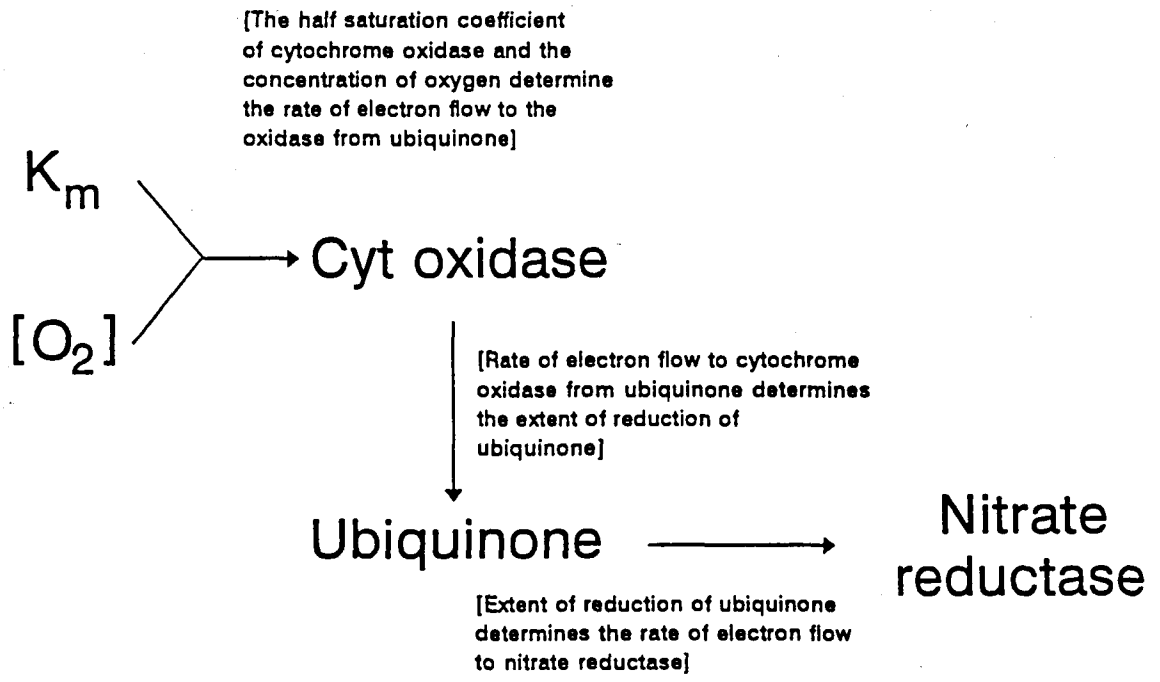

Fig 4.18: Proposed mechanism for the control of electron flow via the half saturation coefficient of the oxidase (k_m) and the redox state of ubiquinone (from Alefounder *et al.*, 1981).

the γ–subunit occurs only in the absence of oxygen and presence of nitrate and that the incorporation of the nitrate reductase subunits α and β into the membrane is blocked following a shift from anoxic to aerobic conditions.

Regulation of nitrate movement through changes in the permeability of the cytoplasmic membrane effected by oxygen

The two mechanisms above describe the manner by which oxygen exerts control on the transfer of electrons to and from nitrate reductase. Hypothetically, nitrate reduction could also be regulated by controlling the movement of nitrate across the cytoplasm to the active site of nitrate reductase on the cytoplasmic side of the membrane (Alefounder and Ferguson, 1980). Attention has been directed at the role of the cytoplasmic membrane in restricting nitrate movement to nitrate reductase under aerobic conditions. Five experiments, described below, contributed to the promotion of this mechanism as a primary means of restricting nitrate reduction under aerobic conditions. In the first experiment, it was shown that restriction of electron flow is not a significant mechanism for decreasing reduction of *nitrate* under aerobic conditions (but not necessarily so for *nitrite* reduction under aerobic conditions). The other four experiments demonstrate that oxygen reduces the permeability of the membrane to nitrate movement thereby preventing nitrate reduction under aerobic conditions.

- A non–limiting supply of electrons to nitrate reductase in the form of the chemical dihydroquinone was fed to *Pa. denitrificans* under aerobic conditions – no nitrate reduction was observed (Alefounder *et al.*, 1983).

- Intact cells of denitrifying *Pa. denitrificans* did not reduce nitrate under aerobic conditions. However, inside–out membrane vesicles (in which access of nitrate to nitrate reductase was not prevented by the cytoplasmic membrane) reduced nitrate under aerobic conditions (John ,1977).

- The cytoplasmic membrane of cells of *Pa. denitrificans* was made permeable to nitrate by the addition of the chemical Triton X–100, to allow passage of nitrate to its reductase – a simultaneous reduction of oxygen and nitrate was observed under aerobic conditions. In the absence of Triton X–100, nitrate was not reduced under aerobic conditions (Alefounder and Ferguson, 1980; Kučera *et al.*, 1983b).

- The addition of transmembrane nitrate carriers such as benzyl or heptyl viologen radicals caused nitrate reduction under aerobic conditions with *E. coli.* Without such carriers, nitrate reduction did not occur under aerobic conditions (Noji and Taniguchi, 1987).

- Nitrate reduction did not occur in intact cells of *Ps. aeruginosa* at low concentrations of oxygen, but in cell extracts in which the cytoplasmic membrane was destroyed, nitrate reduction was observed (Hernandez and Rowe, 1987, 1988).

These experiments provide strong evidence that under aerobic conditions the membrane becomes impermeable to nitrate (i.e. restricts the movement of nitrate across the membrane) thereby limiting nitrate reduction.

An additional finding in the third experiment was that when the membrane was made permeable with Triton X–100, nitrate was reduced to nitrite intracellularly and when a certain nitrite concentration was attained, electron flow to cytochrome oxidase was reduced and electron flow to the nitrogen oxide reductases increased. This finding has major implications with regard to aerobic respiration and is the subject of further scrutiny in Section 4.10 below which examines mechanisms of regulation of aerobic respiration.

An additional finding in the fifth experiment was that, in intact cells in which nitrate reduction was prevented at low concentrations of oxygen, nitrite reduction was not inhibited, and at higher concentrations of oxygen, nitrite reduction was only partially inhibited. This finding is due to differences in position between the active sites of nitrate and nitrite reductase; the active site of nitrate reductase is situated on the cytoplasmic side of the membrane and the active site of nitrite reductase on the periplasmic side. The impermeability of the membrane to nitrate caused by oxygen, does not apply to nitrite because it is not necessary for extracellular nitrite to cross the membrane to the active site of its reductase. If the only source of nitrite is intracellular (i.e., from cytoplasmically reduced nitrate) then regulation of nitrate movement across the membrane by oxygen would represent a primary mechanism by which the whole denitrification pathway could be controlled. However, if an extracellular source of nitrite is available (e.g. from oxidation of ammonium by *Nitrosomonas* in a mixed culture such as activated sludge), then control of membrane permeability by oxygen will not regulate the reduction of

nitrite or the nitrogen oxides which result from the reduction of nitrite (i.e. nitric oxide and nitrous oxide). (This assumes that under aerobic conditions the gaseous intermediates nitric oxide and nitrous oxide are not restricted in their movement across the cytoplasmic membrane).

In summary:

- Nitrate respiration under aerobic conditions is controlled by (i) regulation of electron movement to the reductases by the intracellular redox potential established by oxygen, and by preventing insertion into the membrane of nitrate reductase subunits, and (ii) restricting nitrate movement through changes in the permeability of the cytoplasmic membrane.

- Although nitrate reduction under aerobic conditions is completely inhibited, nitrite reduction under aerobic conditions is only partially inhibited, as a consequence of the periplasmic location of the active site of nitrite reductase which obviates the need for extracellular nitrite to cross the cytoplasmic membrane to be reduced.

- Under aerobic conditions, the attainment of a certain intracellular nitrite concentration inhibits electron flow to cytochrome oxidase and increases electron flow to the nitrogen oxide reductases (except nitrate reductase).

4.10 MECHANISMS OF REGULATION OF AEROBIC RESPIRATION

It was noted earlier that in passing from aerobic to anoxic conditions, the oxidases are fully developed while the reductases are absent and require synthesis. In passing from anoxic to aerobic conditions, the reductases are fully developed and primary oxidase cytochrome aa_3 is absent and synthesis is initiated. The significant difference between the synthesis of the oxidases and the reductases in a change from anoxic to aerobic conditions is that all the reductases are inducible but of the two oxidases, cytochrome *o* is constitutive (synthesized under all conditions) and cytochrome aa_3 is inducible (synthesized only when oxygen is present). It has been shown that in passing from anoxic to aerobic conditions, oxygen inhibits nitrogen oxide reduction. In an anomalous finding, in artificially created situations in which nitrate and oxygen reduction occurred simultaneously, one of the reduced products of nitrate reduction inhibits oxidase activity. This finding is of crucial importance to development of the biochemical model for aerobic facultative organism respiration

in Chapter 5. Accordingly, in the section below, research into this aspect will be reviewed. Because the inhibition of oxidase activity was a consequence of an increase in nitrite concentration, the interaction of nitrite with cytochrome oxidase will be investigated as a first step.

Mechanisms of inhibition of aerobic respiration by nitrite

The inhibition of oxidase activity by nitrite has been investigated by a number of workers, using different techniques. Using *Pa. denitrificans*, Kučera and Dadak (1983) examined electron flow under aerobic conditions to cytochrome oxidase and nitrite reductase. An uncoupler was added to reduce the transmembrane potential of the cytoplasmic membrane, allowing access of nitrite to cytochrome oxidase. After addition of the uncoupler, electron flow to cytochrome oxidase decreased and electron flow to nitrite reductase increased, indicating that the mechanism of inhibition requires the access of nitrite to the cytoplasmically placed cytochrome oxidases.

However, without any changes to the membrane, Yang (1985) measured an inhibition of oxidase activity in *Ps. aeruginosa* following the addition of nitrite but no inhibition following the addition of nitrate. The result of non-inhibition with nitrate can be explained by the necessity for nitrate to cross the membrane but given that the inhibitory nitrite was not produced intracellularly but was added extracellularly, a means of traversing the membrane would also be required for nitrite to reach and inhibit cytochrome oxidase. Such a result would tend to indicate that oxidase inhibition is due to an effect of nitrite, but not due to nitrite *per se*. Further evidence for this view was provided in the following experiment.

Kučera *et al.* (1986b) investigated the effect of increasing electron flow to cytochrome oxidase and nitrite reductase in *Pa. denitrificans* through the addition of the artificial electron donor, ascorbate and a chemical TMPD (referred to as a mediator for its role in selectively increasing the flow of electrons to cytochrome oxidase and nitrite reductase to ensure the availability of electrons to nitrite reductase under aerobic conditions). In the absence of nitrite, oxidase activity increased with increased concentration of TMPD, but with nitrite present, oxidase activity initially increased with increased concentration of TMPD but decreased above a certain concentration of TMPD. The authors suggested that this decrease in oxidase activity was caused by the production of an inhibitory but unidentified intermediate resulting from the reduction of nitrite, possibly one of the

denitrification intermediates, nitric oxide or nitrous oxide. To determine if either of these intermediates could inhibit oxidase activity, nitric oxide and nitrous oxide were added separately to membrane vesicles of *Pa. denitrificans*; *nitric oxide severely inhibited oxidase activity but nitrous oxide had no effect.* As a further indication that the inhibitory agent is a product of nitrite reduction, a culture of aerobically grown *Pa. denitrificans* (i.e. lacking nitrite reductase and therefore also not producing nitric oxide or nitrous oxide) demonstrated uninhibited oxidase activity in the presence of nitrite.

Mechanisms of inhibition of aerobic respiration by nitric oxide

Krul (1976) examined the effect of nitrate, nitrite and nitric oxide on the oxygen utilization rate of an anoxically grown (with nitrate) *Alcaligenes* sp. isolated from denitrifying activated sludge. Nitrate inhibited the rate of oxygen utilization only when the dissolved oxygen concentration was less than 0,5 mgO/ℓ, nitrite inhibited the rate only when the dissolved oxygen concentration was less than 2,0 mgO/ℓ, and nitric oxide inhibited the rate when the dissolved oxygen concentration was as high as 4,0 mgO/ℓ. The rate of nitrate and nitrite reduction which resulted from inhibition of oxidase activity due to the addition of nitric oxide was equal to the rate of reduction of nitrate and nitrite under anoxic conditions.

From these results two important conclusions can be drawn concerning nitrate and nitrite reduction under aerobic conditions. Firstly, inhibition of oxidase activity results from the interaction of nitric oxide with cytochrome oxidase and not from nitrite or nitrous oxide; inhibition of oxidase activity resulting from the addition of nitrate or nitrite is due to nitric oxide formation from the reduction of nitrate and nitrite, not nitrate and nitrite *per se.* Secondly, when nitric oxide inhibits the flow of electrons to cytochrome oxidase, a large proportion of electrons passing to the oxidase are diverted to the denitrification ETP, and nitrate and nitrite are reduced.

Other research groups conducted experiments to determine the identity of the inhibitory species. Kučera *et al.*, (1987b) conducted experiments to determine whether nitrite is reduced aerobically and to determine the identity of the product. (Considerable resistance was still to be found to acceptance of nitric oxide as an obligatory intermediate of denitrification). The addition of ferricytochrome *c* to an aerobic suspension of anoxically grown (with nitrite) *Pa. denitrificans* resulted in the formation of a complex of ferricytochrome *c* and nitric oxide (nitric oxide reacts with the Fe^{3+} centre of ferricytochrome *c*) under aerobic conditions. Although

giving no indication of the inhibitory nature of nitric oxide (being bound to ferricytochrome *c* it was unable to interact with cytochrome oxidase), the experiment demonstrates that the product of the reduction of nitrite under aerobic conditions *is* nitric oxide.

From a hypothetical viewpoint, a concern with the proposal for nitric oxide as an inhibitory species was that its extremely reactive nature (with oxygen in particular) would not allow it to be present in sufficient quantity intracellularly to account for the degree of inhibition measured in laboratory studies (Parsonage *et al.*, 1985). In this regard Carr and Ferguson (1990b) demonstrated that under anoxic conditions the nitric oxide reductase of *Pa. denitrificans* maintained the steady–state concentration of dissolved nitric oxide sufficiently low that reaction with oxygen was insignificant, but sufficiently high that reaction with the active site of cytochrome oxidase would inhibit oxygen reduction.

Concerning the biochemical mechanism of inhibition of oxidase activity by nitric oxide; although the experimental work described above demonstrated that nitric oxide is the inhibitory species, the work did not describe the mechanism of inhibition. Four possible mechanisms are outlined and discussed below:

(1) Under aerobic conditions, electrons are directed away from cytochrome oxidase to the nitrogen oxide reductases by an intracellular redox effect due to the presence of nitric oxide. Kučera *et al.* (1983a) proposed that under anoxic conditions electron distribution between reductases is effected by redox control exerted by the concentration of the denitrification intermediates, and proposed that control of nitrate reduction by oxygen is effected by the same mechanism, thereby making it feasible that inhibition of oxidase activity by nitric oxide also occurs by the same mechanism.

(2) Nitric oxide accepts electrons directly from cytochrome oxidase, thereby reducing the transfer of electrons from cytochrome oxidase to oxygen; However, enzymes have a conformation specific to their oxidizing molecule (Robertis and Robertis, 1980) and it is unlikely that nitric oxide would have the correct conformation to effect electron transfer from cytochrome oxide.

(3) Nitric oxide reacts with dissolved intracellular molecular oxygen, thereby preventing oxygen attaining its site of reduction at cytochrome oxidase.

However, it has been suggested that this reaction occurs for nitric oxide at high concentrations but slows to a negligible rate when the concentration of nitric oxide has fallen to a low level through reduction by nitric oxide reductase (Parsonage *et al.* 1985; Carr and Ferguson, 1990b).

(4) Nitric oxide interacts with cytochrome oxidase to form a complex which cannot then transfer electrons to oxygen.

Considering the four mechanisms of inhibition: for (2) and (3) there is sufficient contradictory evidence in their listing to discard them; for (1) and (4), there is sufficient supporting evidence to accept them. With regard to (1), evidence for its acceptance is presented in its listing. With regard to (4), its acceptance is based on the finding that the reactive centres of cytochrome oxidase comprise iron (Fe) and copper (Cu) complexes (Babcock and Wikström, 1992). The Fe–containing complexes have a propensity to react with nitric oxide to form iron–nitric oxide (Fe–NO) complexes (Wood *et al.*, 1981; Reddy *et al.*, 1983) which are incapable of transferring electrons to oxygen. Thus, a mechanism integrating (1) and (4) will be considered the means by which nitric oxide inhibits aerobic respiration (see Chapter 5).

In concluding that inhibition of oxidase activity results from interaction of nitric oxide with cytochrome oxidase, it is not clear which of the two oxidases, cytochrome *o* or cytochrome aa_3 is inhibited. A number of findings indicate that nitric oxidase inhibits cytochrome *o* but may not necessarily inhibit cytochrome aa_3:

- Cytochrome *o*, but not cytochrome aa_3, is synthesized under anoxic conditions such that under subsequent aerobic conditions, in which oxidase activity is inhibited (Krul, 1976) cytochrome *o* is the only oxidase available for inhibition (Lam and Nicholas, 1969a; Yang, 1982; Kučera *et al.*, 1983b; Casella *et al.*, 1986).

- Although cytochromes *o* and aa_3 both contain iron (Fe) and copper (Cu) in their electron–transferring centres, the centres are different; in cytochrome aa_3 the iron and copper centres are associated with each other, in cytochrome *o* one iron–containing centre is unassociated (Babcock and Wikström, 1992).

Thus, it is probable that nitric oxide interacts with the unassociated Fe–containing centre of cytochrome *o* but not with the Fe–Cu complexes of cytochrome aa_3.

4.11 AEROBIC DENITRIFICATION

One of the principles arising from the work on inhibition of aerobic respiration is that whereas nitrate respiration cannot occur under aerobic conditions, nitrite reduction can. This is because nitrate reductase is situated on the cytoplasmic side of the membrane and oxygen makes the membrane impermeable to nitrate movement, whereas nitrite reductase is on the periplasmic side of the membrane and nitrite is not required to cross the membrane. Accordingly, nitrite can be reduced under aerobic conditions. Although aerobic denitrification has been an area of some debate the literature reports its occurrence among a great diversity of facultative organisms (Skerman *et al.*, 1951; Mechsner and Wuhrmann, 1963; Hernandez and Rowe, 1987; Robertson and Kuenen, 1983, 1984a,b, 1990). However the majority of literature is concerned with aerobic denitrification of nitrate, not nitrite, and it appears that denitrification of nitrate in those cases is attributable to organism tolerance of reduced oxygen levels, not redirection of electrons to nitrite reductase due to inhibition of cytochrome oxidase by nitric oxide. In contrast Krul (1976) in examining the effect of nitrate, nitrite, and nitric oxide on the oxygen uptake of an unidentified organism from activated sludge noted aerobic denitrification of nitrate and nitrite to nitric oxide but found the phenomenon difficult to explain. The aspect of aerobic denitrification forms an integral part of the biochemical model postulated in Chapter 5 for facultative organism respiration.

4.12 SUMMARY

In this Chapter a review of literature concerned with the respiration of facultative organisms was conducted in two parts: Part I reviewed the biochemical mechanisms involved in utilization of substrate for aerobic and anoxic respiratory processes, and the effect of the presence of oxygen, the absence of oxygen, and the presence of nitrogen oxides, on synthesis of the aerobic and anoxic electron transferring complexes of the ETP. Part II reviewed the mechanisms which initiate, regulate, and terminate aerobic and anoxic respiration.

To summarize briefly, the major conclusions of the review are:

From Part I;

- An electron transport pathway (ETP) can be described which can serve as a general pathway for facultative aerobic organisms, representing the major electron transferring complexes and sequences of these organisms.

- For facultative organisms respiring under aerobic conditions, two oxidases are synthesized and denitrifying electron transport complexes (reductases) are not synthesized or are synthesized at a low level. For facultative organisms respiring under anoxic conditions, the reductases are synthesized and of the oxidases, one is not synthesized and the other is synthesized at a higher level than under aerobic conditions.

- From the general ETP for facultative organisms, the mechanisms resulting in lower yields under anoxic than aerobic conditions are identifiable; a consequence of electrons passing two, and not all three available proton-pumping (energy conserving sites).

From Part II;

- For facultative organisms respiring under aerobic conditions, then transferred to anoxic conditions, the oxidases are inactivated, and synthesis of the reductases is initiated.

- The nitrogen oxides regulate synthesis and activity of the reductases under anoxic conditions; the nitrogen oxides promote synthesis of their own reductases and inhibit synthesis of other reductases.

- For facultative organisms respiring under anoxic conditions, then transferred to aerobic conditions, three mechanisms have an effect on, or result in inactivation of the reductases; (i) the presence of oxygen affects the intracellular potential of the organism, increasing electron flow to the oxidases, and reducing electron flow to the reductases, (ii) oxygen prevents insertion of nitrate reductase subunits into the cytoplasmic membrane preventing electron transport to nitrate, (iii) oxygen reduces the permeability of the cytoplasmic membrane to nitrate, thereby reducing the accessibility of nitrate to its reductase.

- An additional finding for organisms subjected to the conditions described above is that intracellular nitric oxide accumulated under anoxic conditions inhibits oxidase activity (aerobic respiration) under the following aerobic conditions.

CHAPTER 5

METABOLIC BEHAVIOUR OF FACULTATIVE AEROBIC ORGANISMS UNDER AERATED/UNAERATED CONDITIONS

ABSTRACT

A model is outlined for the major biochemical respiratory mechanisms of a conceptualized heterotrophic facultative organism (representing the heterotrophic facultative mass of activated sludge) subjected to four different sets of conditions; steady–state aerobic, steady–state anoxic, steady–state aerobic changed to anoxic, and steady–state anoxic/anaerobic changed to aerobic. Proposals and implications of the model regarding the effect of these conditions and changes between conditions on respiration under aerobic and anoxic conditions are tested experimentally and verified with special aerobic batch tests on sludges from IAND, 2RND, MUCT, continuous aerobic, and continuous anoxic systems fed municipal sewage.

PART I: A CONCEPTUAL BIOCHEMICAL MODEL

5.1 INTRODUCTION

In Chapter 4 a review was presented of the biochemical respiratory pathways and mechanisms operative in facultative heterotrophic organisms under a variety of environmental conditions. The review established that research has been conducted almost entirely with pure cultures of a number of organisms selected as representative of the main classes of heterotrophs, i.e. obligate aerobes, facultative aerobes which reduce nitrate, and facultative aerobes which denitrify. However, in nature, organisms are virtually never found in pure culture – they always exist in mixed cultures of unknown microbial constitution. An example of such a mixed culture is the microbial mass that develops in the activated sludge system. In the nutrient removal activated sludge sewage treatment process the microbial population is subjected to cyclic aerated and unaerated conditions. As a consequence of exposure to the sequences of aerated/unaerated conditions in these systems, it can be assumed that the microbial population will include a significant fraction of facultative heterotrophic organisms which have a capacity for substrate utilization under both aerated and unaerated conditions. With this assumption as a basis, and from the knowledge gained from the review of research on pure cultures, the overall objective of this investigation is to develop a microbiological/biochemical model that will allow the macroscopic biological responses observed in activated

sludge (in particular the proliferation of filamentous organisms) to be predicted with reasonable surety. In the first steps towards the development of such a model, a conceptual biochemical model is presented that describes the response of the facultative heterotrophic biomass in the activated sludge system to a variety of environmental conditions.

From the review of the behaviour of heterotrophic organisms in pure culture under a variety of conditions (Chapter 4 and Appendices C and D), a general similarity in biochemical pathway organization was noted between different heterotrophic species. In mixed cultures these individual species are likely to give rise to a conjoined response which retains features commonly present in the pure cultures, but which may not be entirely representative of the response of any single species. Consequently, in developing a biochemical model of the behaviour of a mixed culture of facultative heterotrophic organisms, one needs to "synthesize" a conceptualized facultative organism, the behaviour of which is representative of the behaviour of the mixed culture. This approach in utilizing a representative conceptualized organism is implicit in the procedure used by Dold *et al.*, (1980) in modelling activated sludge kinetics.

In this Chapter a similar approach will be followed – it is the intention to develop general biochemical mechanisms and include these in a biochemical model for a conceptualized organism which represents the heterotrophs in the mixed cultures of activated sludge systems. That is, more specifically, to describe the biochemical mechanisms operative in a facultative heterotrophic organism subjected to separate aerobic and anoxic conditions and to changes from anoxic to aerobic and from aerobic to anoxic conditions.

5.2 RESPIRATION IN FACULTATIVE AEROBIC ORGANISMS

For the purposes of this model, the complexes which mediate proton and electron transport in the electron transport pathway (ETP) and their arrangement about the cytoplasmic membrane can be conceptualized as illustrated in Fig 5.1. This ETP is a modification of that proposed by Ferguson (1982) for the facultative organism *Paracoccus denitrificans* (*Pa. denitrificans*), incorporating the reduction of nitric oxide at nitric oxide reductase as an obligatory step. The association of the complexes of the ETP with the cytoplasmic membrane is illustrated in Fig 5.2. This ETP is proposed as the operative one for the conceptualized facultative organism. It should be noted that Figs 5.1 and 5.2 indicate all the enzyme

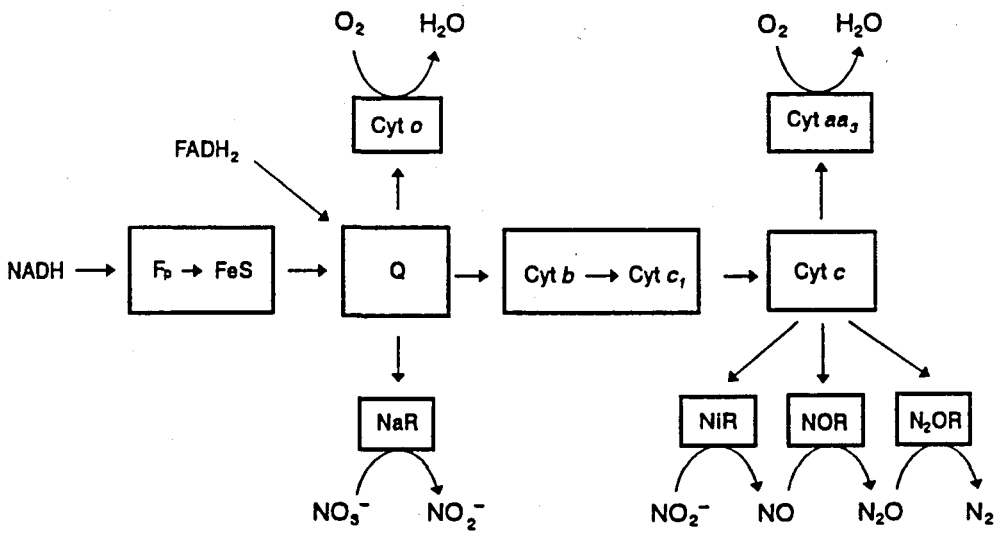

Fig 5.1: The electron transport pathway (ETP) for a conceptualized facultative heterotrophic organism as representative of the facultative heterotrophic organism mass in activated sludge [modification of pathway proposed by Ferguson (1982) for *Pa. denitrificans*].

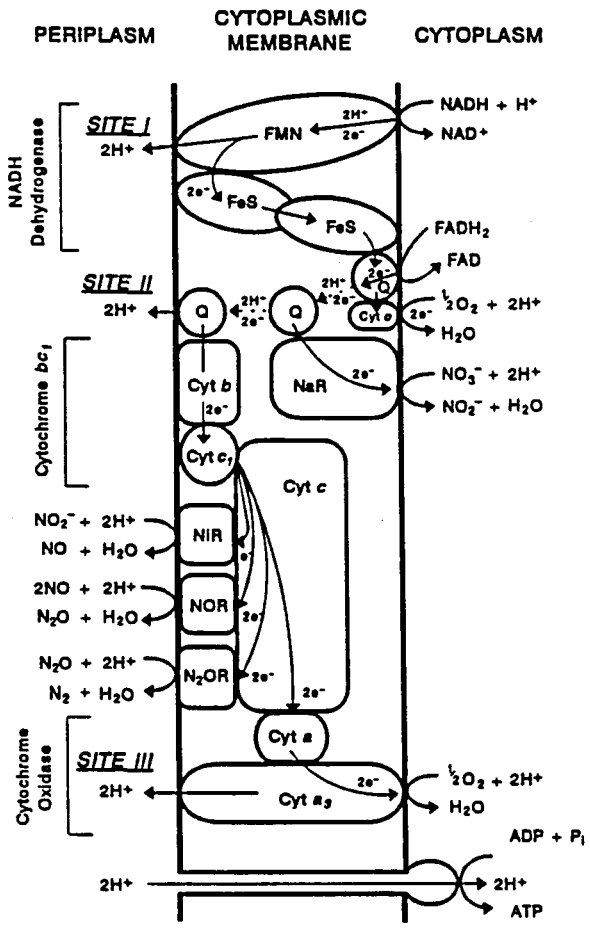

Fig 5.2: The electron transport pathway (ETP) for a conceptualized facultative organism, in which all of the electron transferring complexes are present and illustrated with respect to the cytoplasmic membrane.

complexes synthesized by facultative heterotrophic organisms, irrespective of whether the complexes are synthesized only under aerobic or anoxic conditions (adaptive) or under both aerobic and anoxic conditions (constitutive). The pathway establishes the structure within which the various biochemical processes can be identified under anoxic and aerobic conditions, and during changes from anoxic to aerobic and from aerobic to anoxic conditions. Description of the biochemical processes under these conditions is set out in six parts.

- Part 1 examines the biochemical processes which are common to both the anoxic and aerobic ETPs, that is, which mediate the transport of electrons and protons (associated with NADH produced in the tricarboxylic acid – TCA cycle) through the first three complexes of the ETP (NADH dehydrogenase, the FeS complexes, and ubiquinone).

- Parts 2 and 3 examine the biochemical processes associated with respiration under aerobic and anoxic conditions respectively.

- Part 4 examines the biochemical processes associated with a change from aerobic to anoxic conditions.

- Part 5 examines the biochemical processes associated with a change from anoxic to anaerobic conditions (i.e. a situation in which nitrate (NO_3^-) and nitrite (NO_2^-) decrease from concentrations sufficient for denitrification, to conditions which, as a consequence of the process of denitrification, are absent in nitrate and nitrite.

- Part 6 examines the biochemical processes associated with a change from anoxic to aerobic conditions. In this part, two anoxic conditions are investigated. The first is one in which nitrate or nitrite is present for all of the period (anoxic conditions) before the change to aerobic conditions and the second is one in which nitrate or nitrite is present only for some of the period and is absent (anaerobic conditions) immediately prior to the change to aerobic conditions. These two sets of conditions are of particular significance to the biochemical model since they significantly influence respiration under aerobic conditions which follow anoxic conditions. The biochemical model in turn has implications regarding the microbiological/biochemical model for filament and floc–former substrate competition (bulking model) described in the following Chapter.

Electron and proton production and transport preceding the processes associated with the cytoplasmic membrane ETP

Each of the six parts of the model describe the transport of electrons, protons, and electron acceptors in processes associated with the ETP under aerobic or anoxic conditions. Other processes which precede these, such as the biochemical processes which result in the production of electrons and protons (captured by NADH) are common to both aerobic and anoxic respiration; these processes have been described fully in Part I of Chapter 4, and the descriptions are not repeated here. The role of NADH in the respiratory process is to serve as the link between electron/proton production and the ETP. NADH transports protons and electrons generated in the catabolic processes associated with the tricarboxylic acid (TCA) cycle to ETP complexes. These complexes extrude the protons to the periplasm, indirectly resulting in the production of energy, and transfer the electrons to ETP complexes, which in turn transfer them to one of the terminal electron acceptors, oxygen under aerobic conditions and nitrate or nitrite under anoxic conditions. The rate of electron transfer to the terminal electron acceptors is limited by the rate of production of NADH in the TCA cycle (Lehninger, 1975)[1].

Each of the six parts of the biochemical model is discussed in turn below.

Part 1 – Electron and proton transport from NADH to FMN, the FeS complexes and ubiquinone under both aerobic and anoxic conditions

The ETP complexes, NADH dehydrogenase, the FeS complexes, and ubiquinone are common to both aerobic and anoxic conditions. Under both aerobic and anoxic conditions, NADH transfers a pair of electrons and two protons (one originating from the cytoplasm) to the NADH dehydrogenase complex which comprises flavomononucleotide and FeS complexes. In this transfer two protons are removed from the cytoplasm and translocated to the periplasm at the first proton-pumping (energy conserving) site, Site I. Ubiquinone accepts a pair of electrons from the NADH dehydrogenase complex and two protons from the cytoplasm, mediates the transfer of electrons to the complexes involved in aerobic or anoxic respiration, and transports the protons to the periplasm at the second proton-pumping site, Site II. Thus, the first two sites (Sites I and II) are common to aerobic and anoxic respiration; a difference in energy production between aerobic and anoxic

[1] As indicated in Chapter 4, electrons supplied to the ETP originate from both NADH and $FADH_2$. However, for simplification, where mention is made of the source of electrons, it is assumed to be NADH.

respiration is introduced only in subsequent electron transfer steps and this difference is highlighted in the relevant sections below.

Part 2 – Aerobic conditions

For conditions in which oxygen only, or oxygen and nitrate/nitrite are present, aerobic respiration is the preferred mechanism. For the experimental work conducted in this investigation and for the purposes of the biochemical model, conditions in which oxygen is present at a concentration equal to or exceeding 0,2 mgO/ℓ are defined as aerobic. At DO concentrations < 0,5 mgO/ℓ, some of the biochemical mechanisms of the aerobic ETP are adversely affected and in the following model the role of oxygen concentration in respiration is examined by description of the biochemical mechanisms mediated under two ranges of dissolved oxygen concentration; $0,2 < DO < 0,5$ and $DO \geq 0,5$ mgO/ℓ.

DO ≥ 0,5 mgO/ℓ

For $DO \geq 0,5$ mgO/ℓ, the status of the complexes of the ETP present is illustrated in Fig 5.3. Both of the oxidases cytochrome *o* and cytochrome aa_3 are present; cytochrome aa_3 is synthesized to a greater extent than cytochrome *o*, and at these concentrations of DO, cytochrome *o* is synthesized to a level 30% of that under anoxic conditions (Sapshead and Wimpenny, 1972). For both oxidases, the active site (the site at which oxygen is reduced) is situated on the cytoplasmic side of the membrane. This implies that oxygen crosses the membrane to the cytoplasmic side for reduction. The nitrogen oxide reductases are present at a low (basal) level (Lam and Nicholas, 1969a).

Oxygen enters the organism through the cell wall, and crosses the cytoplasmic membrane to the active sites of cytochrome *o* and cytochrome aa_3 situated on the cytoplasmic side. Electrons associated with NADH are passed via FMN to the FeS complexes, and then to ubiquinone as described above. From ubiquinone, a small proportion of the electrons are transferred to cytochrome *o* (≈ 30%) and the greater proportion (≈ 70%) are transferred to the cytochrome aa_3 complex via the cytochrome bc_1 complex and cytochrome *c*. At each of the active sites of the two oxidase cytochromes, oxygen accepts two electrons as follows:

$$2H^+ + 2e^- + \frac{1}{2} O_2 \rightarrow H_2O \qquad (5.1)$$

At this point it should be stated parenthetically that the two oxidases have different

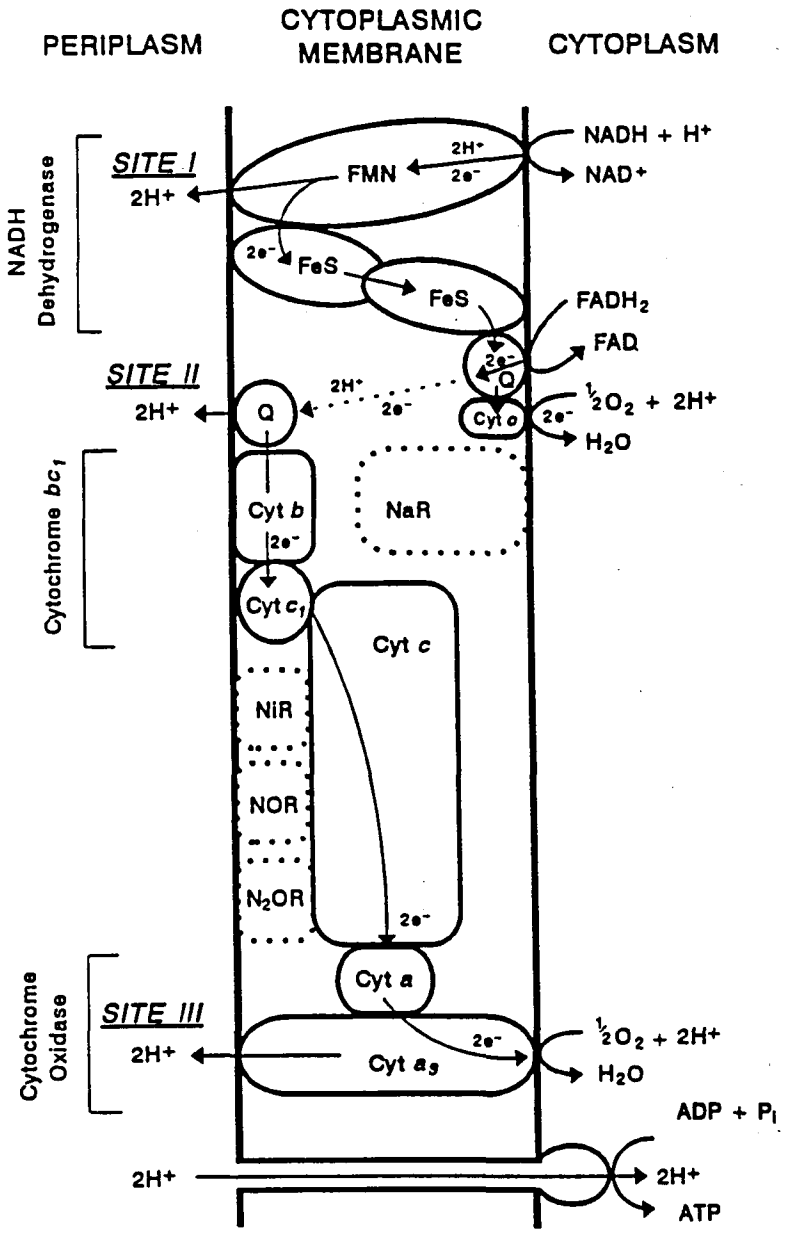

Fig 5.3: The ETP of the conceptualized facultative organism under aerobic conditions with dissolved oxygen (DO) $\geq$ 0,5 mgO/ℓ.

reactive centres as described in Section 4.10. To recap, the reactive centre of cytochrome aa_3 comprises two iron–histidine (Fe–hs) complexes, one copper–histidine (Cu–hs) complex and a copper–histidine–cysteine (Cu–hs–cs) complex whereas the reactive centre of cytochrome *o* comprises two Fe–hs complexes and one Cu–hs complex (Babcock and Wikström, 1992). The difference in composition between the reactive centres of cytochrome *o* and aa_3 has considerable implications with regard to inhibition of aerobic respiration through interaction of nitric oxide with cytochrome oxidase following a change from anoxic to aerobic conditions and these implications are highlighted in Part 6.

Cytochrome aa_3 is referred to as the principal oxidase and cytochrome *o* as the alternative oxidase since under aerobic conditions the greater proportion of oxygen is reduced at cytochrome aa_3. The cytochrome aa_3 complex is the third of the three proton–pumping (energy conserving) sites (Site III) at which two protons are translocated from the cytoplasm across the membrane to the periplasm for each pair of electrons transferred to oxygen; no such energy conserving site exists at cytochrome *o*. Thus, the energetic yield associated with the reduction of oxygen at cytochrome *o* is approximately two–thirds that of the yield associated with the reduction of oxygen at cytochrome aa_3 because reduction of oxygen at cytochrome *o* requires that electrons pass two proton–pumping sites and reduction of oxygen at the cytochrome aa_3 complex requires that electrons pass three proton–pumping sites; the pumping of 2 protons at any site results in the formation of 1 ATP, through the mechanisms of oxidative phosphorylation described in Section 4.6.

0,2 < DO < 0,5 mgO/ℓ

For conditions in which the DO is between 0,2 and 0,5 mgO/ℓ, the status of the complexes of the ETP is as illustrated in Fig 5.2. In this "twilight" DO range, it is considered that the oxidases (cytochrome *o* and cytochrome aa_3) and all the nitrogen oxide reductases are present. Cytochrome aa_3 is synthesized to a lower level and cytochrome *o* is synthesized to a higher level than under the higher DO conditions and each of the reductases are synthesized to a lower level than under conditions of DO < 0,2 mgO/ℓ, and to a higher level than under conditions of DO > 0,5mgO/ℓ. It is implicit to the analysis that at the lower concentrations of DO within this range, the proportion of cytochrome aa_3 decreases and cytochrome *o* increases.

Electrons associated with NADH are passed via FMN to the FeS complexes and

then to ubiquinone, but a smaller fraction of the electrons pass to the oxidases and a larger fraction pass to the reductases than at DO > 0,5 mgO/ℓ, and of the electrons which do pass to the oxidases, a greater proportion pass to cytochrome *o* than to cytochrome aa_3. The electrons which do not pass to the oxidases pass to the denitrifying enzymes, the nitrogen oxide reductases. Because of their low level of synthesis the reductases have only a very small electron transferring capacity. Within the range of low DO examined here, a priority in the order of reductase synthesis is established. At the higher concentrations of DO in the low DO range, only nitrate reductase is synthesized, but as the DO concentration decreases to 0,2 mgO/ℓ, each of the successive reductases of the denitrifying pathway is synthesized, i.e. nitrite–, nitric oxide–, and nitrous oxide reductase.

Part 3 – Anoxic conditions

The anoxic condition is defined as one in which oxygen is absent and nitrate or nitrite, or both, are present. Under anoxic conditions in the presence of the electron acceptors nitrate and nitrite, the status of the complexes of the ETP present in a facultative organism are shown in Fig 5.4. All of the nitrogen oxide reductases are present (Payne 1973), and also the oxidase cytochrome *o*, but the oxidase cytochrome aa_3 either is not present (Gray *et al.*, 1966; Lam and Nicholas, 1969c; Sapshead and Wimpenny, 1972), or is present at a low level (Alefounder *et al.*, 1981). The reduction of the nitrogen oxides can be best modelled by describing the sequential steps involved in the formation of one molecule of dinitrogen (N_2) from nitrate. Nitrate enters the organism through the cell wall and crosses the cytoplasmic membrane via one of two mechanisms; (i) with a proton (H^+), in a symport system which initiates nitrate reduction (Boogerd *et al.*, 1983a), or (ii) in exchange for an outgoing nitrite molecule in a nitrate/nitrite antiport system which functions once the process of nitrate reduction has been established by the first mechanism.

With nitrate present at nitrate reductase, electrons associated with NADH produced in the TCA cycle are passed via the NADH dehydrogenase complex to ubiquinone and then to nitrate reductase, where nitrate serves as electron acceptor and is reduced to nitrite on the cytoplasmic side of the membrane (John, 1977; Alefounder and Ferguson, 1980) at the catalytic site of nitrate reductase (the α subunit) (Chaudhry and MacGregor, 1983b). Reduction of nitrate (NO_3^-) to nitrite (NO_2^-) is as follows:

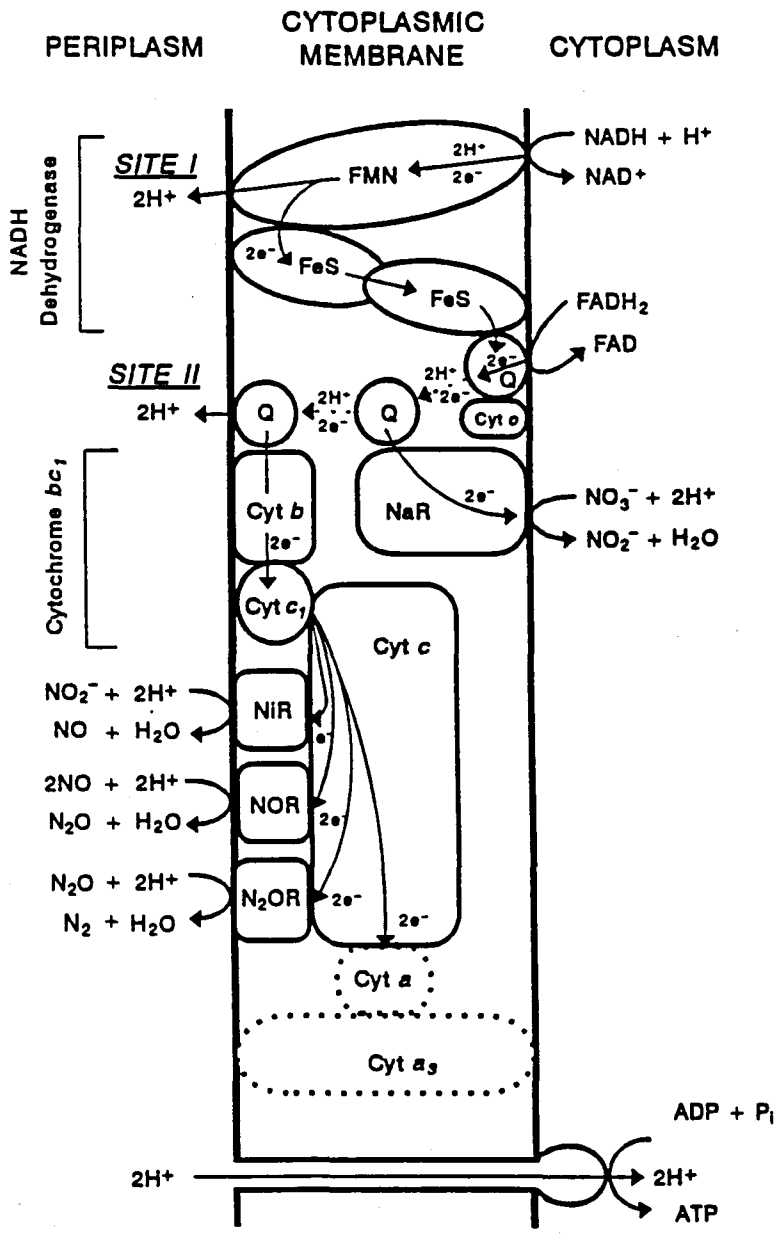

Fig 5.4: The ETP of the conceptualized facultative organism under steady-state anoxic conditions with nitrate and nitrite present.

$$2H^+ + 2e^- + NO_3^- \rightarrow NO_2^- + H_2O \qquad (5.2)$$

From the cytoplasmic side, the nitrite molecule crosses the membrane via the NO_3^-/NO_2^- antiport system to the nitrite reductase complex at the periplasm (Wood, 1978; Alefounder and Ferguson, 1980). With nitrite present at nitrite reductase, electrons associated with NADH produced in the TCA cycle are passed via the NADH dehydrogenase complex to ubiquinone, to the cytochrome bc_1 complex, to cytochrome *c* and then to nitrite reductase, where nitrite produced from the reduction of nitrate and any other extracellularly produced nitrite are reduced to nitric oxide (NO) at the nitrite reductase complex as follows:

$$2H^+ + e^- + NO_2^- \rightarrow NO + H_2O \qquad (5.3)$$

On the periplasmic side of the membrane, the product nitric oxide, passes to the nitric oxide reductase complex, also situated on the periplasmic side of the membrane (Zumft, 1993). With nitric oxide present at the active site of nitric oxide reductase, electrons associated with NADH produced in the TCA cycle are passed through the same complexes as for nitrite reduction to cytochrome *c* and then to nitric oxide reductase where nitric oxide produced from the reduction of nitrite is reduced to nitrous oxide (N_2O) as follows:

$$2H^+ + 2e^- + 2NO \rightarrow N_2O + H_2O \qquad (5.4)$$

On the periplasmic side, the nitrous oxide molecule then passes to the active site of the nitrous oxide reductase complex also situated on the periplasmic side of the membrane (Boogerd *et al.*, 1981). With nitrous oxide present at nitrous oxide reductase, electrons associated with NADH produced in the TCA cycle are passed through the same complexes as for nitrite reduction and nitric oxide reduction, and then to nitrous oxide reductase where nitrous oxide is reduced to dinitrogen (N_2) as follows:

$$2H^+ + 2e^- + N_2O \rightarrow N_2 + H_2O \qquad (5.5)$$

The endproduct of the reaction, dinitrogen, is released through the cell wall to the external medium.

The denitrification processes as described above are somewhat simplistic in that the

processes are described as sequential stepwise mechanisms in which nitrate is reduced through each of the denitrification intermediates to dinitrogen. However, during steady–state denitrification, all of the denitrification intermediates are present and are denitrified simultaneously. Electron supply from NADH is continuous, and distribution of electrons between the nitrogen oxide reductases is dependent on the redox potentials established by the nitrogen oxides at the reductases. The redox potential at each reductase increases as the concentration of the nitrogen oxide associated with that reductase increases, thereby attracting electrons to the reductase and stimulating the activity of electron transfer from the reductase to the associated nitrogen oxide. The rate of electron supply to the nitrogen oxide reductases affects the intracellular accumulation of the nitrogen oxides as a consequence of their rates of reduction and formation. Figures 5.5a and 5.5b illustrate the proposed steady–state concentrations of the nitrogen oxide intermediates with low and high rates of supply of electrons respectively. At low rates of electron supply (slowly biodegradable substrate), the intracellular level of the more reduced nitrogen oxides is high due to a high proportion of electrons passing to nitrate reductase and an insufficient supply of electrons to the other reductases to maintain low–level steady–state concentrations of the nitrogen oxides as illustrated in Fig 5.5a. At high rates of electron supply (readily biodegradable substrate), the electron supply is sufficient to maintain low–level steady–state concentrations of the nitrogen oxides and they do not accumulate as illustrated in Fig 5.5b. Under steady–state anoxic conditions, the total flow of electrons to the reductases is limited by the rate of production of NADH in the TCA cycle. As a consequence of the limited electron supply, and under conditions in which nitrite is initially at a low concentration, but is produced from the reduction of nitrate, electron flow to nitrate reductase decreases as a consequence of electron flow to nitrite reductase. Thus, nitrite has an apparently inhibitory effect on the activity of nitrate reductase. The other denitrification intermediates, nitric oxide and nitrous oxide, produced sequentially from the reduction of nitrite and nitric oxide respectively, also have an inhibitory effect on electron transfer, not only on nitrate reductase, but also on all other reductases other than their own. As an example to illustrate the mechanisms involved in the stimulation and inhibition of the activity of the reductases, consider nitrite reductase; nitrite itself stimulates electron flow to and from the reductase and nitrate, nitric oxide, and nitrous oxide indirectly inhibit electron flow to the reductase as a consequence of stimulating electron flow to their own reductases. Similar mechanisms occur for each of the reductases, and these mechanisms in combination act to control the extracellular and intracellular levels

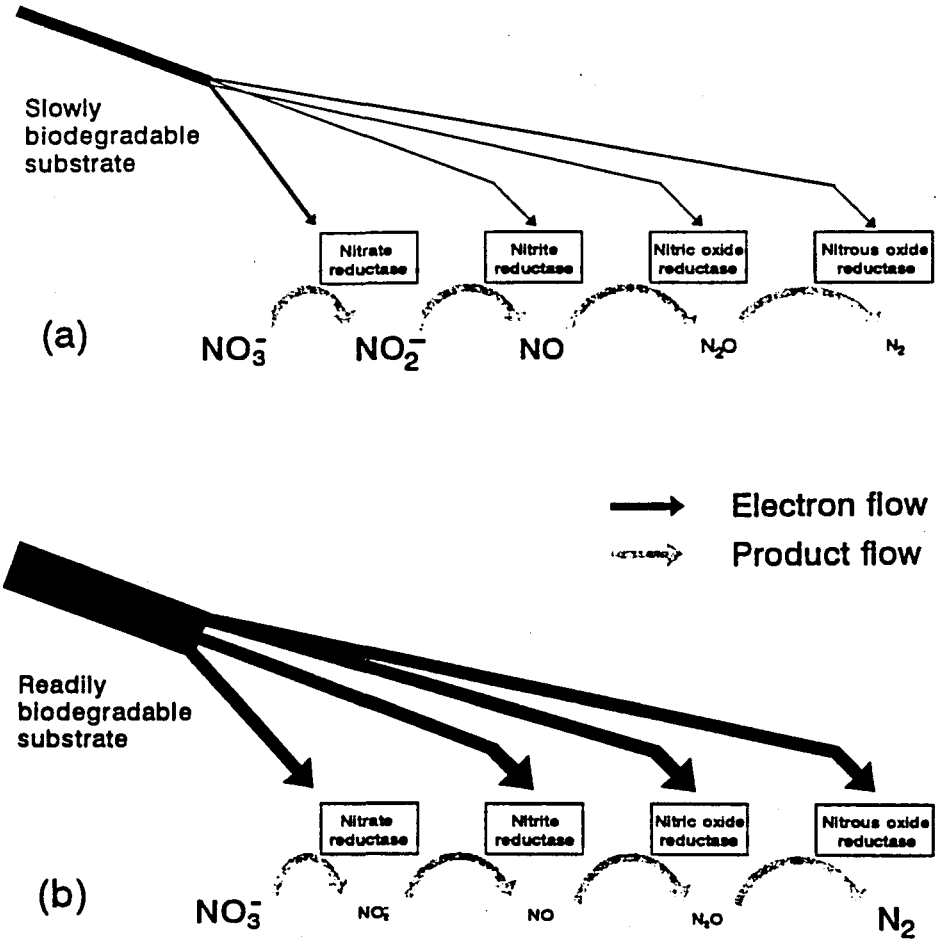

Fig 5.5: Diagrammatic representation of the kinetics of the denitrification pathway indicating rates of electron flow and steady-state intracellular levels of the nitrogen oxides under anoxic conditions with adequate nitrate present and the ETP supplied with; (a) a low rate of electron supply (from slowly biodegradable substrate), and (b) a high rate of electron supply (from readily biodegradable substrate).

of the nitrogen oxides. A summary of the interactions between all of the reductases and nitrogen oxides is illustrated in Fig 5.6.

The periplasmic location of the reductases for nitrite, nitric and nitrous oxide means that the products of each reductive step do not need to pass across the membrane in order to reach the active site of their specific reductase.

Under certain environmental conditions, the permeability of the membrane changes, thereby reducing the accessibility of the nitrogen oxides to their specific reductases. For example, it is well established that the presence of oxygen reduces the membrane permeability to nitrate, reducing the rate of nitrate reduction through the reduced accessibility of nitrate to its reductase. This mechanism becomes a dominant feature when conditions change from aerobic to anoxic, as discussed below, and when conditions change from anoxic to aerobic, as discussed in Part 6.

Part 4 – Steady–state aerobic conditions changed to anoxic conditions

For organisms respiring under steady–state aerobic conditions and then exposed to anoxic conditions in which nitrate and nitrite are present, changes occur in the synthesis of intracellular complexes and in the reactions associated with the ETP.

The status of the ETP under steady–state aerobic conditions (DO > 0,5 mgO/ℓ) is described by Fig 5.3 and the status of the ETP under steady–state anoxic conditions is described by Fig 5.4. Thus, for organisms respiring under steady–state aerobic conditions, exposure to anoxic conditions for a period sufficient to achieve steady–state levels of the enzymes, causes the ETP to change from that described by Fig 5.3 to that described by Fig 5.4, i.e. all the nitrogen oxides are synthesized and cytochrome aa_3 is degraded. The changes are illustrated in Fig 5.7 and occur in the following manner: In the absence of oxygen the effects of membrane impermeability to nitrate, and inhibition of nitrogen oxide reductase synthesis are removed. Additionally, the presence of nitrate induces synthesis of nitrate reductase, resulting in an increase in level of the end–product nitrite, which in turn induces the synthesis of nitrite reductase. The same mechanism occurs sequentially for each of the nitrogen oxide reductases in that each nitrogen oxide induces the synthesis of its own reductase, in the process of which the next nitrogen oxide in the denitrification sequence is formed. This results in the sequential formation of the nitrogen oxides, nitrate, nitrite, nitric oxide, nitrous oxide and then dinitrogen with time (or equivalently, with decrease in oxygen concentration).

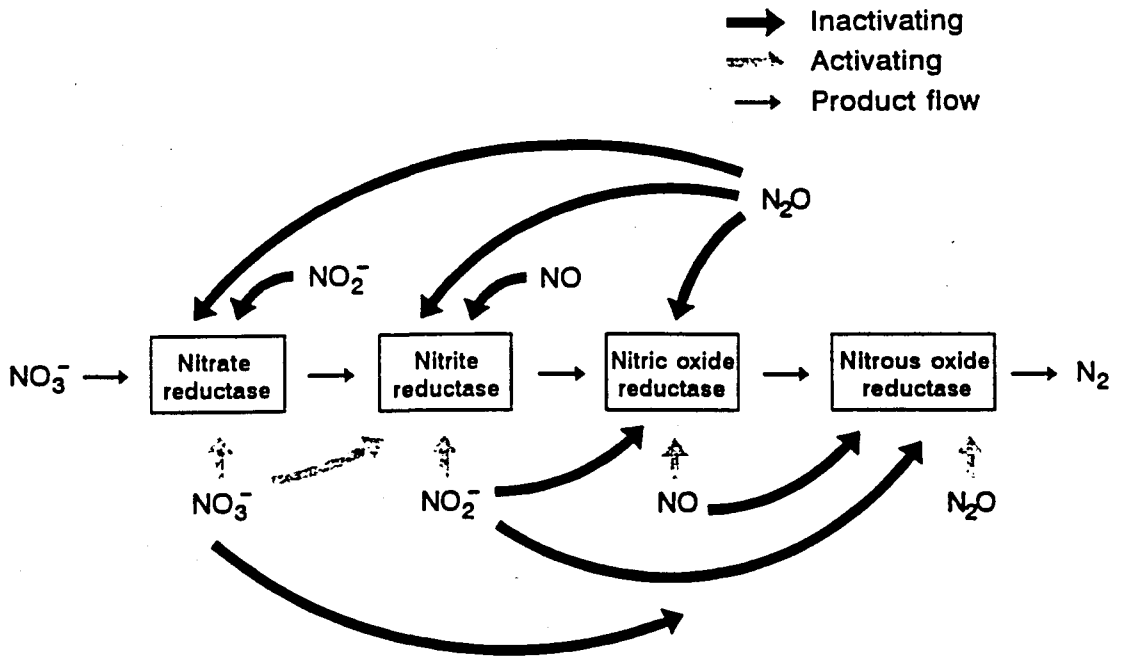

Fig 5.6: Summary of the effect of the nitrogen oxides on the activities of the nitrogen oxide reductases of the denitrifying electron transport pathway (ETP) and the direction of product (nitrogen oxide) flow.

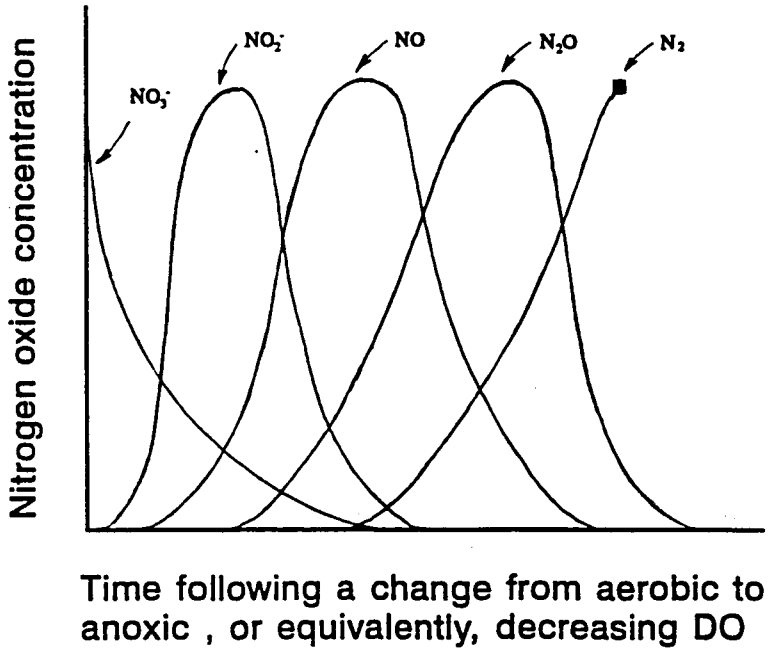

Fig 5.7: Sequential formation, with time (or equivalently dissolved oxygen concentration) of the nitrogen oxides, nitrate, nitrite, nitric oxide, and nitrous oxide for a facultative organism under steady-state aerobic conditions transferred to anoxic conditions with nitrate present (redrawn from Tiedje, 1985).

Under anoxic conditions, cytochrome aa_3 is not synthesized, or is synthesized at a basal level, and the level of cytochrome *o* increases from the level established under fully aerobic conditions, to an increased level under anoxic conditions. The implication of an increase in cytochrome *o* under anoxic conditions will become apparent in the discussion of the changes in the ETP during a change from steady–state anoxic conditions to aerobic conditions (Part 6).

From the literature, it is unclear as to the period of time required for complete transformation of the ETP from that synthesized under steady–state aerobic conditions, to that synthesized under steady–state anoxic conditions. However, from experiments conducted in this investigation (described at a later stage) it appears that a period of 7–10 days is necessary. During this period, in which the system approaches steady–state, oxygen is absent and nitrate and nitrite are present in sufficient quantity so as not to limit the rate of electron transfer. Electrons do not flow to the oxidases, but to the reductases, to be utilized in two processes; (i) for the synthesis of new enzymatic material, and (ii) for the reduction of the nitrogen oxides as described by Eqs 5.2 to 5.5. Electron transfer to nitrite is initially limited by the level of the nitrogen oxide, and immediately following a change to anoxic conditions with nitrate and nitrite present, from steady–state aerobic conditions, this limitation on electron transfer to the nitrogen oxides is considerable since nitrate – and nitrite reductase and the other nitrogen oxide reductases are not synthesized under aerobic conditions. The implication of this scenario is an initial reduced growth rate of the organism under anoxic conditions. As the reductases are synthesized with time, electron transfer to the nitrogen oxides from the reductases increases, and the growth rate of the organism increases until steady–state anoxic conditions are achieved and the transfer of electrons is limited by the rate of electrons supplied by NADH.

<u>Part 5 – Anoxic conditions changed to anaerobic conditions</u>

The major difference between anoxic and anaerobic conditions is the absence of nitrate and nitrite under the latter. Exposure of organisms to anaerobic conditions results in a decrease in the synthesis of the nitrogen oxide reductases which require the presence of the nitrogen oxides for maximum synthesis. Additionally, anaerobic conditions with substrate present ensures the removal of any intracellularly accumulated nitrogen oxides. The relevance of this aspect to aerobic respiration will be appreciated in the following section.

Part 6 – Anoxic conditions changed to aerobic conditions

In Parts 2 and 3 it was ascertained that different complexes are synthesized under steady–state anoxic conditions (Fig 5.4) and steady–state aerobic conditions (Fig 5.3), and that the reactions mediated by these complexes are also different. This implies that in undergoing a change from steady–state anoxic with one or both of the ionic nitrogen oxides nitrate or nitrite present, to aerobic with oxygen present, the status of the ETP changes with time from that described by Fig 5.4 to that described by Fig 5.3. At the onset of aerobic conditions four major changes occur in the complexes and transport processes associated with the ETP. Briefly, these are:

- reduction in nitrogen oxide reductase activities,
- reduction in permeability of the cytoplasmic membrane to nitrate,
- cessation of synthesis of nitrogen oxide reductases, reduction in the synthesis of cytochrome oxidase *o*, and initiation of synthesis of cytochrome aa_3,
- interaction of nitric oxide with the cytochrome oxidases.

In combination, the four changes contribute to or are implicated in a mechanism which results in the inhibition of aerobic respiration following a transition from anoxic to aerobic conditions, and the four changes are dealt with in the order in which they affect aerobic respiration after the transition. Each change is dealt with in turn.

Changes in nitrogen oxide reductase activities

Oxygen inhibits the activities of the nitrogen oxide reductases in the order corresponding to the reductases for the increasingly less reduced nitrogen oxides, i.e. the nitrous oxide –, nitric oxide –, nitrite –, and nitrate reductases (Hochstein *et al.*, 1984), either with time (e.g. nitrous oxide reductase activity is inhibited prior to nitric oxide reductase at any constant oxygen concentration) or with increase in oxygen concentration (e.g. nitrous oxide reductase activity is inhibited at a lower oxygen concentration than is nitric oxide reductase activity). This is illustrated in Fig 5.8 which describes the effect of oxygen on the activities (and synthesis) of the reductases. The result of exposure to aerobic conditions is the accumulation of the nitrogen oxides in the order, nitrous oxide, nitric oxide, nitrite, and nitrate. The mechanism by which oxygen affects nitrogen oxide reductase activity is through redirection of electrons, from the reductases to the oxidases as a consequence of the greater redox potential for electron flow created by the presence of oxygen at the oxidases than by the presence of the nitrogen oxides at the reductases (Alefounder

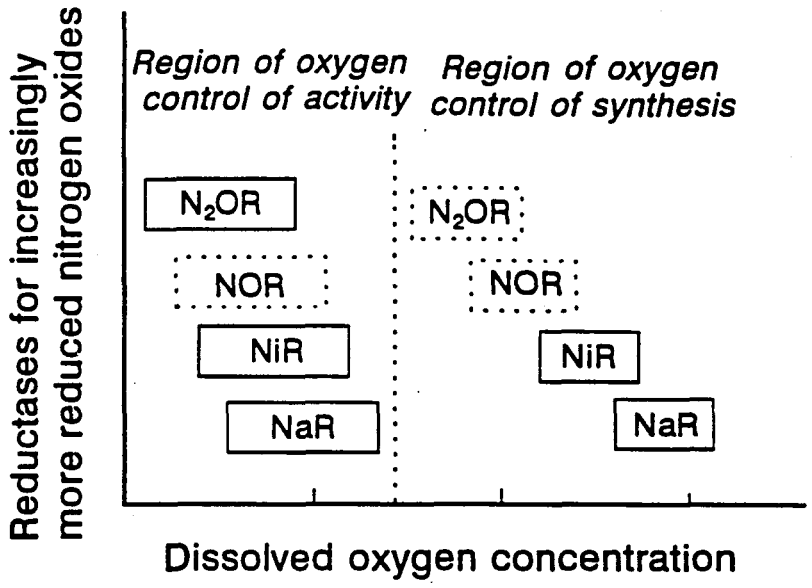

Fig 5.8: Effect of oxygen on the synthesis and activity of the nitrogen oxide reductases (redrawn from Tiedje, 1985 – the proposed positions of the reductases shown in dotted outline are not included in the original work). [Abbreviations: NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase].

et al., 1981).

Change in the permeability of the cytoplasmic membrane

Oxygen induces a change in the permeability of the cytoplasmic membrane such that the movement of nitrate to its reductase is prevented (John, 1977; Alefounder and Ferguson, 1980; Kučera *et al.*, 1983b; Noji and Taniguchi, 1987). This effect is apparently specific to the movement of nitrate. Assuming that under steady–state anoxic conditions nitrate movement across the membrane occurs via an NO_3^-/NO_2^- antiport mechanism, it is assumed that impermeability of the membrane to nitrate movement refers to the effect of oxygen in preventing operation of the antiport mechanism.

Change in the synthesis of the anoxic and aerobic enzyme complexes

Oxygen prevents the synthesis of the nitrogen oxide reductases but induces the synthesis of the principal oxidase cytochrome aa_3. The synthesis of the alternative oxidase cytochrome *o* continues under aerobic conditions (Lam and Nicholas, 1967; Newton, 1967; Lam and Nicholas, 1969c) but is synthesized at only 30% of the level that it is synthesized under anoxic conditions. It is considered that synthesis of cytochrome aa_3 occurs at a rate commensurate with the rate of degradation of cytochrome *o*,[2] such that the nett level of available cytochrome oxidase (cytochrome *o* + cytochrome aa_3) is at the least, *maintained* under aerobic conditions. Under aerobic conditions, electron flow to the oxidases is utilized in two processes; (i) for the reduction of oxygen and, (ii) for the synthesis of cytochrome aa_3. It is unclear from the literature as to the period of time required for the synthesis of cytochrome aa_3 to steady–state level, but from experimental work conducted in this investigation, it would appear that given a high rate of supply of electrons, a period of 6–8 hours is necessary and given a low rate of supply of electrons, a number of days may be required. The mechanism by which oxygen represses synthesis of nitrate reductase is through oxygen binding to a protein (which regulates nitrate reductase synthesis) causing a conformational change, enabling the protein to bind to a segment of DNA specific to synthesis of nitrate reductase, thereby preventing its synthesis (Stouthamer 1988).

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[2] Modelling the heterotrophic facultative sludge mass as a surrogate heterotrophic facultative organism necessitates the degradation with time of cytochrome *o*. In individual organisms in the sludge mass, it is likely that the level of cytochrome *o* under anoxic conditions does not degrade, but that "new" organisms have a significantly lower level of cytochrome *o*, yielding a lower nett level for the sludge mass with time, modelled by a decreasing level of cytochrome *o* in the surrogate organism.

Interaction of nitric oxide with cytochrome oxidase

Interaction of intracellular nitric oxide with cytochrome *o* is possible only if nitric oxide is present (from the reduction of nitrate or nitrite) at the time of introduction of oxygen, i.e. if the conditions just prior to aerobic conditions are anoxic, not anaerobic. Under anoxic conditions, nitrate is reduced on the cytoplasmic side of the membrane, and simultaneously, nitrite, nitric oxide and nitrous oxide undergo reduction on the periplasmic side. Of the two oxidases, cytochrome *o* is the only one immediately available for reduction of oxygen, because cytochrome aa_3 is not synthesized under the prior anoxic conditions. As described in Part 2, cytochrome *o* is different to cytochrome aa_3 in that the reactive centre of cytochrome *o* contains two iron atoms and one copper atom whereas the reactive centre of cytochrome aa_3 comprises two iron atoms and two copper atoms. For this model it is assumed that nitric oxide interacts only with Fe–containing complexes which have not formed electron transferring couples with Cu–containing complexes[3]. Thus, nitric oxide will not interact with cytochrome aa_3 (in which both of the Fe and Cu containing complexes are coupled), but will interact with cytochrome *o* which contains one uncoupled Fe–containing complex. Figures 5.9(a) and (b) illustrate electron transfer to oxygen by the oxidases in the absence and presence of nitrite respectively.

Immediately following a change in conditions from anoxic to aerobic, intracellular nitric oxide[4] *interacts with the Fe–containing complexes of cytochrome o, creating iron–nitric oxide (Fe–NO) complexes which are incapable of transferring electrons. For conditions in which cytochrome* aa_3 *is present at the commencement of aerobic conditions (i.e. for an organism exposed to short alternating anoxic–aerobic conditions), nitric oxide does not inhibit electron transport to oxygen by that oxidase but does inhibit electron transport by cytochrome o for the reasons stated above.* Electrons, unable to be passed to oxygen via cytochrome *o* are diverted to the nitrogen oxide reductases. From redox potential considerations of the reactions for

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[3] From the literature, the exact conformation and electron–transferring mechanisms of cytochrome aa_3 and cytochrome *o* are unclear. It appears that for cytochrome aa_3, each of the Fe–containing complexes forms a couple with each of the Cu–containing complexes such that each of cytochromes *a* and a_3 contain one Fe–Cu couple. For cytochrome *o*, it is assumed that one of the Fe–containing complexes forms a couple with the Cu–containing complex, leaving the other Fe–containing complex uncoupled. In a couple the physical conformation of the Fe–containing complex is such that its close proximity to the Cu–containing complex (< 5Å – Holm *et al.*, 1987) prohibits interaction with nitric oxide.

[4] In the biochemical model, mention of nitric oxide refers specifically to *intracellular* nitric oxide. Extracellular nitric oxide is unstable, particularly under aerobic conditions where it reacts rapidly with oxygen.

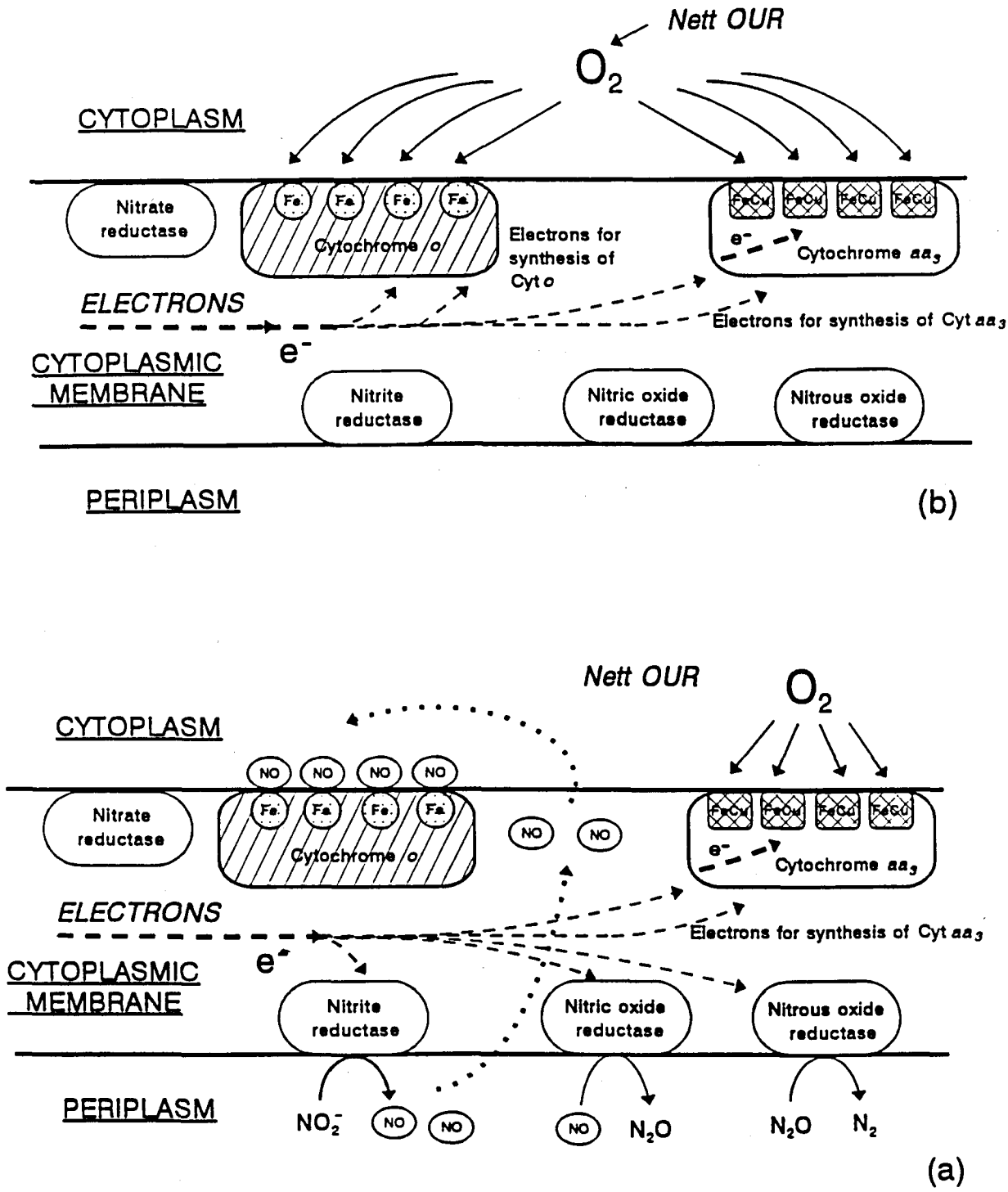

Fig 5.9: Model of interactions about the cytoplasmic membrane of a conceptualized facultative organism grown under alternating anoxic–aerobic conditions, illustrating electron transfer to oxygen under aerobic conditions in the (a) absence of nitrite and (b) presence of nitrite, illustrating the mechanisms resulting in inhibition of aerobic respiration.

reduction of the nitrogen oxides, the preferred pathway for electron flow is to nitrate reductase (Payne, 1973). But, because of the effect of oxygen in changing the permeability of the membrane and preventing the movement of nitrate to its reductase (preventing nitrate acting as electron acceptor), the major fraction of the electrons are passed to the second choice pathway, i.e. to nitrite reductase. At nitrite reductase, nitrite is reduced to nitric oxide at the periplasmic side of the membrane and nitric oxide traverses the membrane to meet one of two fates on the cytoplasmic side; (i) reduction by nitric oxide reductase to form nitrous oxide, or (ii) reaction with any uninhibited cytochrome *o* resulting in continued inhibition of electron transfer to oxygen (Carr and Ferguson, 1990b) and redirection of electrons to nitrite reductase.

Consideration of organism respiration when conditions change from anoxic to aerobic in the presence and absence of readily biodegradable substrate and nitrite

Two factors are of particular importance in the analysis of the mechanisms of respiration: (i) the presence or absence of nitrite during the transition from anoxic to aerobic conditions, and (ii) the availability or not of a large source of electrons as a consequence of the availability or not of readily biodegradable substrate.[5] Four scenarios are described below detailing the effect of nitrite and readily biodegradable substrate on organism respiration during a change from anoxic to aerobic conditions. For each scenario the role in respiration of the four changes associated with the cytoplasmic membrane, viz. reductase activities, membrane permeability, enzyme synthesis, and nitric oxide–cytochrome oxidase interactions is described. It should be emphasized that each of the different scenarios is examined for an organism subjected to alternating anoxic–aerobic conditions such that the oxidases (cytochrome *o* and aa_3) and the nitrogen oxide reductases are present, and both are synthesized at some level intermediate between the levels established under steady–state aerobic and anoxic conditions. The ETP for such an organism is illustrated in Fig 5.2 in which all the complexes of the ETP are included. For each scenario, an additional situation is examined in which the aerobic period of the alternating anoxic–aerobic conditions is not changed back to anoxic conditions but maintained aerobic, as a means of examining the effect of extended aerobic conditions (in the presence of readily or slowly biodegradable substrate) on inhibition of aerobic respiration.

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[5] In this analysis an assumption is that slowly biodegradable substrate is always present irrespective of the presence or absence of readily biodegradable substrate. Thus, when it is stated that electrons are supplied by slowly biodegradable substrate, it can be assumed that readily biodegradable substrate is absent.

(i) *Readily biodegradable substrate absent and nitrite present under sequential anoxic and aerobic conditions:*

Figures 5.10a to 5.10c illustrate the interactions involving electrons, nitrite reductase, the oxidases (cytochrome *o* and cytochrome aa_3), nitric oxide and oxygen, which occur about the cytoplasmic membrane with time with a slowly biodegradable substrate as electron source and with nitrite *present* at the start of aerobic conditions following steady–state anoxic conditions with readily biodegradable substrate absent and nitrite present.

Under the preceding anoxic conditions in which slowly biodegradable substrate supplies electrons to reduce nitrite, nitric oxide accumulates (Fig 5.10a). As shown in Fig 5.5, the kinetics of the denitrification pathway indicate that with slowly biodegradable substrate the rate of formation of nitric oxide is greater than its rate of reduction and as a result it accumulates. Under the subsequent aerobic conditions with slowly biodegradable substrate and nitrite present, nitric oxide interacts with the Fe–containing centres of the cytochrome *o* complex, preventing the transfer of electrons to oxygen. Electrons are then directed to the electron–transferring site at which the next highest redox potential is established – nitrite reductase. (Since the cytoplasmic membrane is impermeable to nitrate movement, nitrate cannot pass to nitrate reductase and only a low redox potential is established at that reductase). At nitrite reductase, nitrite is reduced to nitric oxide, which passes across the membrane and interacts with the Fe–containing centres of any uninhibited cytochrome *o* (Fig 5.10b). Thus, under conditions in which cytochrome oxidase is inhibited, nitrite is reduced to nitric oxide under aerobic conditions (i.e. aerobic denitrification). With extended time under aerobic conditions, inhibition is relieved through two processes: (1) new cytochrome aa_3 is synthesized (the rate of synthesis is low due to a low availability of electrons from slowly biodegradable substrate), and since nitric oxide does not interact with the cytochrome aa_3 complex, this complex transfers electrons to the terminal electron acceptor oxygen, at a higher rate than the inhibited cytochrome *o*, (2) the nitrogen oxide reductases are degraded (see footnote 2), and less nitric oxide is produced. These changes are illustrated in Fig 5.10c, the consequence of which is an increased rate of aerobic respiration. *Rapid* relief of inhibition, through rapid synthesis of cytochrome aa_3 requires a high rate of electron supply, which can be

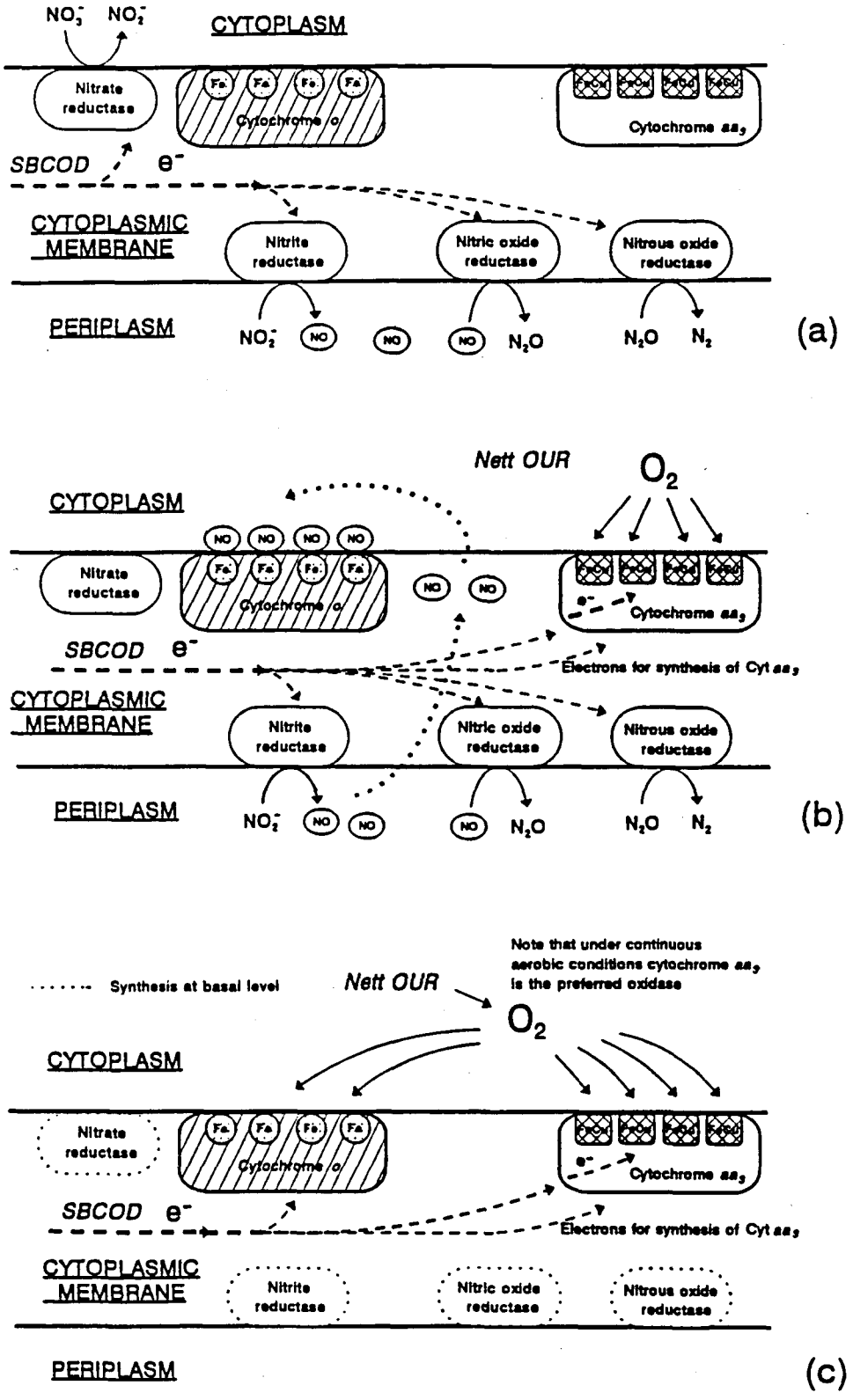

Fig 5.10: Model of interactions about the cytoplasmic membrane of a conceptualized facultative organism grown under alternating anoxic–aerobic conditions, subjected to (a) anoxic conditions followed by (b) aerobic conditions and (c) continuous aerobic conditions with nitrite present and slowly biodegradable substrate (SBCOD) as electron source under all conditions.

achieved only with readily biodegradable substrate.

(ii) *Readily biodegradable substrate and nitrite absent under sequential anoxic (anaerobic) and aerobic conditions:*

Figures 5.11a to 5.11c illustrate the interactions involving nitrite reductase, the oxidases (cytochrome *o* and cytochrome aa_3), nitric oxide, and oxygen which occur about the cytoplasmic membrane under aerobic conditions with time, with slowly biodegradable substrate as electron source, following anoxic/anaerobic conditions with slowly biodegradable substrate present and nitrite absent.

Under the preceding anoxic/anaerobic conditions, denitrification of nitrite is essentially complete and only a residual amount of nitric oxide remains (Fig 5.11a). Under subsequent aerobic conditions, aerobic respiration is essentially uninhibited (Fig 5.11b). Inhibition can be induced in such organisms through the imposition of a high external concentration of nitrite which leads to a high intracellular level of nitrite on the periplasmic side of the membrane. Since the active site of nitrite reductase is situated on the periplasmic side of the membrane a high redox potential is established at nitrite reductase such that electrons are attracted to the reductase and nitrite is reduced to nitric oxide. Nitric oxide then interacts with the Fe–containing complex of cytochrome *o* and aerobic respiration is inhibited. Inhibition does not continue indefinitely with time; degradation of the nitrogen oxide reductases under continuous aerobic conditions results in reduced levels of nitric oxide and eventually its absence, irrespective of the concentration of nitrite. Under continuous aerobic conditions (Fig 5.11c), cytochrome aa_3 is the dominant oxidase and will transfer a greater proportion of electrons than under alternating anoxic–aerobic conditions.

Inhibition *cannot* be induced through the imposition of a high external concentration of *nitrate*, since the active site of nitrate reductase is situated on the cytoplasmic side of the cytoplasmic membrane, and oxygen changes the permeability of the membrane, preventing access of nitrate to its reductase.

(iii) *Readily biodegradable substrate absent and nitrite present under anoxic conditions; readily biodegradable substrate and nitrite present under subsequent aerobic conditions:*

It was noted in (i) above that inhibition can be relieved by the further

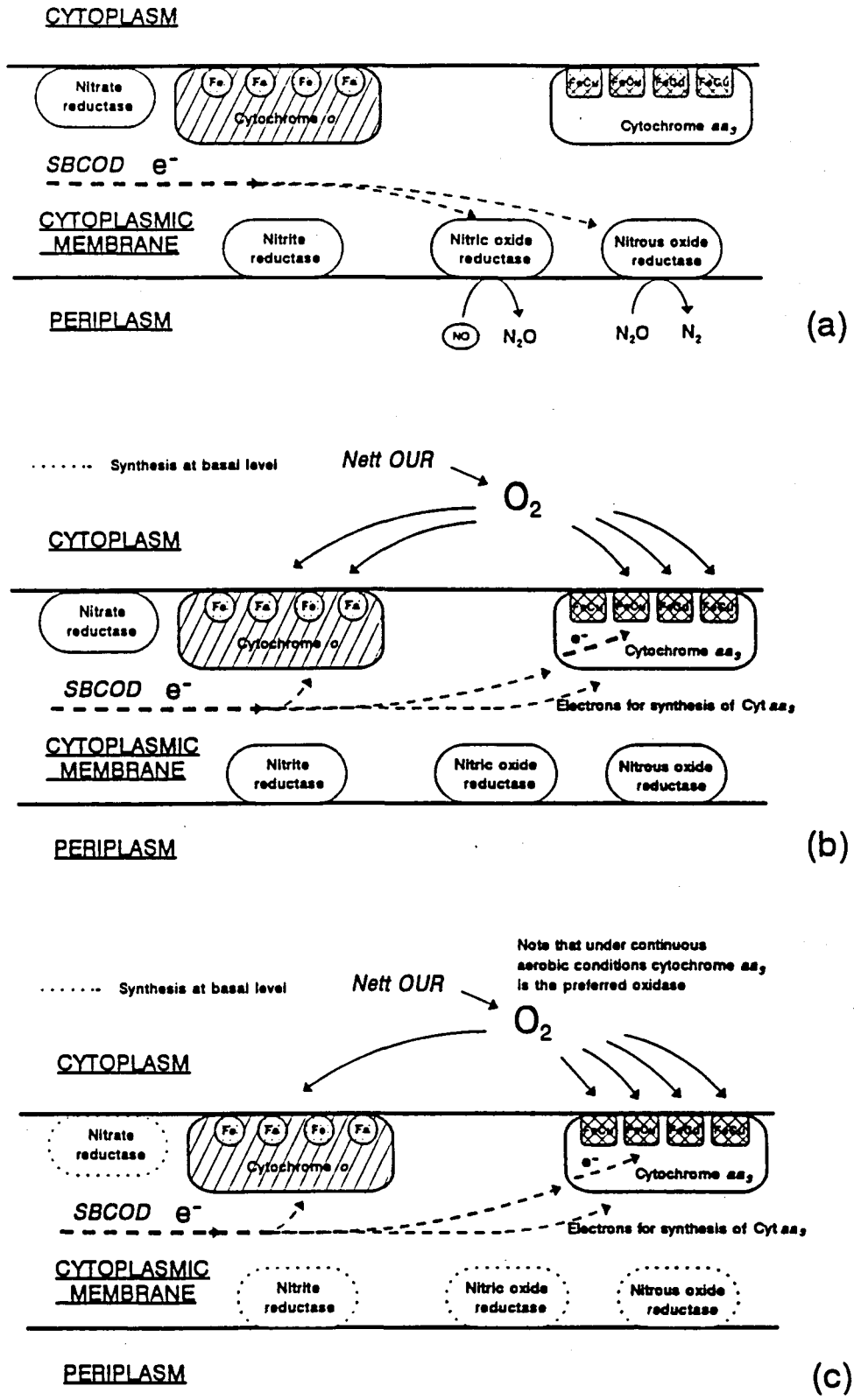

Fig 5.11: Model of interactions about the cytoplasmic membrane of a conceptualized facultative organism grown under alternating anoxic–aerobic conditions subjected to (a) anoxic conditions followed by (b) aerobic conditions and (c) continuous aerobic conditions, with nitrite absent and slowly biodegradable substrate (SBCOD) as electron source under all conditions.

synthesis of cytochrome aa_3, the Fe–Cu electron transferring couples of which do not interact with nitric oxide. A high rate of synthesis requires a higher rate of supply of electrons than can be achieved with slowly biodegradable substrate. Figures 5.12a to 5.12d describe the interactions involving nitrite reductase, the oxidases (cytochrome *o* and cytochrome aa_3), nitric oxide, and oxygen, which occur about the cytoplasmic membrane under aerobic conditions with electrons supplied by readily biodegradable substrate.

Under the preceding anoxic conditions with readily biodegradable substrate absent and nitrate and nitrite present, nitric oxide accumulates to a high level as illustrated in Fig 5.12a (see also Fig 5.5). Under subsequent aerobic conditions with both readily biodegradable substrate and nitrite present, cytochrome oxidase is initially inhibited by the nitric oxide and electrons are redirected to nitrite reductase where nitrite is reduced to nitric oxide (Fig 5.12b). The high rate of electron supply from readily biodegradable substrate serves three purposes, (a) to supply electrons for synthesis of cytochrome aa_3, (b) to supply electrons for transfer to oxygen, and (b) to supply electrons for reduction of nitric oxide and other nitrogen oxides resulting in an increasing rate of aerobic respiration (Fig 5.12c). With time under aerobic conditions, the high rate rate of electron supply allows a high rate of cytochrome aa_3 synthesis, promoting an increased flow of electrons to that oxidase, the nitrogen oxide reductases are degraded, less nitric oxide is produced and inhibition is relieved (Fig 5.12d).

The nett result of these mechanisms is that inhibition induced by a high concentration of nitrite and slowly biodegradable substrate under anoxic conditions can be relieved under subsequent aerobic conditions by an adequate supply of electrons from readily biodegradable substrate.

(iv) *Readily biodegradable substrate and nitrite present under sequential anoxic and aerobic conditions:*

Sections (i) to (iii) above have dealt exclusively with the differences between respiration with readily and slowly biodegradable substrate under *aerobic conditions* following anoxic conditions with nitrite present or absent. The mechanisms associated with the use of readily biodegradable substrate under anoxic conditions have not been examined. Figures 5.13a and 5.13b describe the interactions involving electrons, nitrite reductase and nitrogen oxides

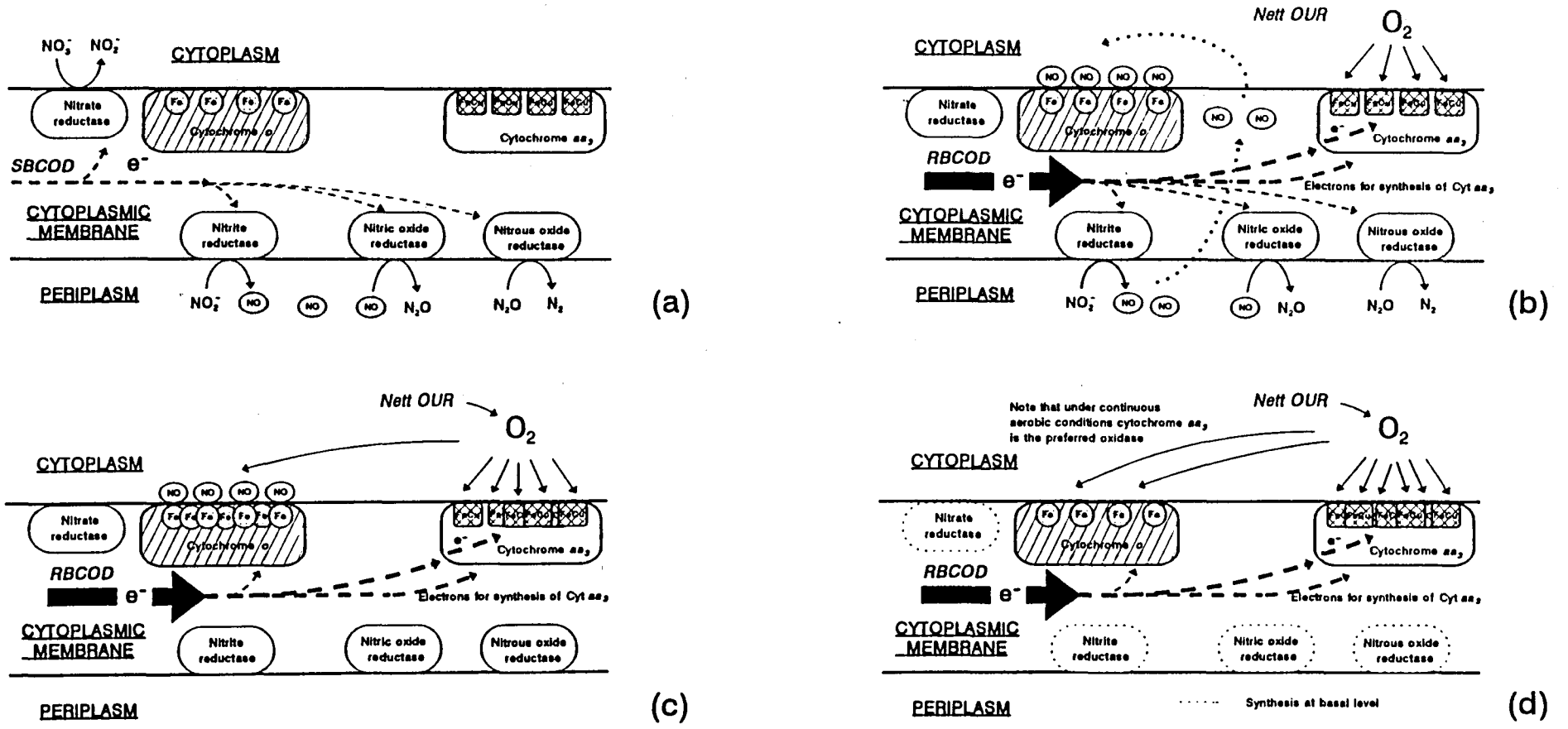

Fig 5.12: Model of interactions about the cytoplasmic membrane of a conceptualized facultative organism grown under alternating anoxic–aerobic conditions, subjected to (a) anoxic conditions with nitrite present and slowly biodegradable substrate (SBCOD) as electron source followed by (b,c) aerobic conditions and (d) continuous aerobic conditions with nitrite present and readily biodegradable substrate (RBCOD) as electron source.

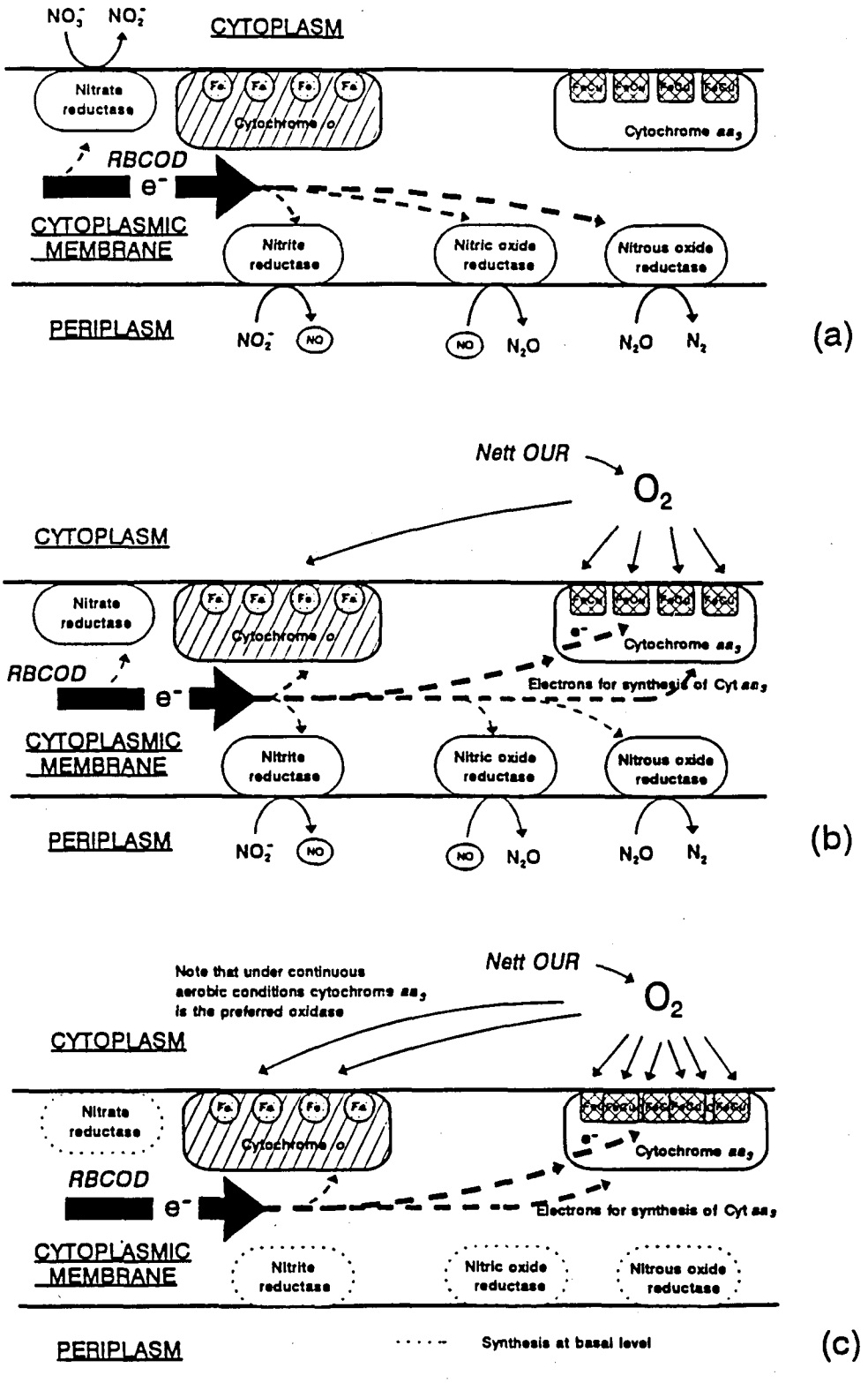

Fig 5.13: Model of interactions about the cytoplasmic membrane of a conceptualized facultative organism grown under alternating anoxic–aerobic conditions subjected to (a) anoxic conditions followed by (b) aerobic conditions and (c) continuous aerobic conditions with nitrite present and readily biodegradable substrate (RBCOD) as electron source under all conditions.

which occur about the cytoplasmic membrane under *anoxic conditions* with a readily biodegradable substrate.

As indicated in Fig 5.13a, under anoxic conditions with readily biodegradable substrate, the rate of reduction of nitric oxide is as fast as its rate of formation and it does not accumulate. This occurs for two reasons, (i) the level of nitric oxide reductase is at a sufficiently high level to provide high rates of reduction of nitric oxide, and (ii) the rate of supply of electrons is high (see Fig 5.5). Thus, under the subsequent aerobic conditions, cytochrome oxidase is not inhibited by nitric oxide (Fig 5.13b). Under continuous aerobic conditions the nitrogen oxide reductases degrade with time and the organism remains uninhibited (Fig 5.13c).

In conclusion; it is evident that a major consideration as to whether aerobic respiration will, or will not be inhibited following a transition from anoxic to aerobic conditions is whether nitrite and/or readily biodegradable substrate is present prior to the transition. When nitrite is present and readily biodegradable substrate absent, inhibition of aerobic respiration is induced, but irrespective of whether nitrite is present or absent, if readily biodegradable substrate is present in sufficient quantity, no inhibition of aerobic respiration is induced.

5.3 ENERGETIC YIELD ASSOCIATED WITH INHIBITION

In Chapter 4 it was established that during aerobic respiration, for each pair of electrons reaching oxygen at cytochrome aa_3, three proton–pumping sites were passed, at which a total of 6 protons were transferred across the membrane resulting in the production of 3 ATP; for each pair of electrons reaching oxygen at cytochrome *o*, two proton–pumping sites were passed, at which a total of 4 protons were transferred across the membrane resulting in the production of 2 ATP. It was also established that during anoxic respiration, for each pair of electrons reaching one of the nitrogen oxide reductases, two proton–pumping sites were passed, at which a total of 4 protons were transferred across the membrane resulting in the production of 2 ATP.

From this analysis it can be implied that a lower energetic yield results from inhibited than from uninhibited aerobic respiration. In the inhibited case a proportion of electrons is redirected to the nitrogen oxide reductases which results in two–thirds of the energy had the electrons passed to uninhibited cytochrome aa_3.

5.4 PROPOSALS AND IMPLICATIONS OF THE BIOCHEMICAL MODEL

(1) Effect of nitrite concentration under anoxic conditions on induction of inhibition of respiration under subsequent aerobic conditions

- During denitrification of nitrite under anoxic conditions (i.e. $NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) with slowly biodegradable substrate, nitric oxide accumulates which inhibits utilization of oxygen under subsequent aerobic conditions.

- With nitrite absent under anoxic/anaerobic conditions, no accumulation of nitric oxide occurs and aerobic respiration will not be inhibited under subsequent aerobic conditions.

- An increase in the concentration of nitrite being denitrified with slowly biodegradable substrate under anoxic conditions gives rise to an increased oxygen utilization inhibition under subsequent aerobic conditions.

(2) Effect of nitrite and nitrate under aerobic conditions on induction of inhibition of aerobic respiration

- With nitrite added or present under aerobic conditions with only slowly biodegradable substrate present, inhibition of respiration will be induced.

- The extent of inhibition of aerobic respiration, induced by denitrification of nitrite with slowly biodegradable COD, will be proportional to the length of time nitrite is present due to low rates of electron flow to nitrite reductase.

- With nitrate added or present under aerobic conditions with only slowly biodegradable substrate present, inhibition of respiration will not be induced due to impermeability of the membrane to nitrate movement.

(3) Effect of substrate biodegradability under aerobic conditions on induction and relief of inhibition of aerobic respiration

- With only slowly biodegradable substrate present under aerobic conditions, nitrite added or present is denitrified and inhibition of respiration will be induced.

- Readily biodegradable substrate under aerobic conditions relieves inhibition of aerobic respiration induced by denitrification of nitrite with slowly

biodegradable substrate under preceding anoxic conditions.

- With readily biodegradable substrate present under aerobic conditions, nitrite added or present will *not* result in accumulation of nitric oxide and inhibition of respiration will *not* be induced.

- Inhibition relieved with readily biodegradable substrate will be reinduced when the readily biodegradable substrate has been exhausted with nitrite present.

(4) Inhibition of aerobic respiration is a general mechanism

- For sludge subjected to alternating anoxic–aerobic conditions with nitrite present and readily biodegradable substrate absent, inhibition will be induced irrespective of the system configuration (i.e. intermittently aerated, 2 reactor anoxic–aerobic or multi–reactor unaerated–aerated systems).

(5) Synthesis of aerobic and anoxic pathway enzymes under aerobic and anoxic growth conditions

- For anoxically grown sludge subjected to aerobic conditions, inhibition of aerobic respiration will decrease with continued exposure to aerobic conditions through decreased synthesis of the nitrogen oxide reductases and increased synthesis of the oxidase cytochrome aa_3.

- For aerobically grown sludge subjected to anoxic conditions, inhibition of aerobic respiration will increase with continued exposure to anoxic conditions through increased synthesis of the nitrogen oxide reductases and decreased synthesis of the oxidase cytochrome aa_3.

- Exposure of anoxically grown sludge to continuous aerobic conditions will reduce the denitrification potential of the sludge through decreased synthesis of the nitrogen oxide reductases.

PART II: EXPERIMENTAL TESTING OF THE CONCEPTUAL BIOCHEMICAL MODEL

5.5 INTRODUCTION

In Part I a conceptual model was formulated for the biochemical mechanisms of aerobic–facultative heterotrophic organisms operative under various environmental conditions. The model was formulated from an amalgamation of (i) a literature review which highlighted the significant biochemical mechanisms of facultative aerobic organisms, especially with regard to interaction between the aerobic and anoxic respiration pathways of those organisms, (ii) hypotheses regarding the kinetics of denitrification under aerobic and anoxic conditions with readily biodegradable substrate present and absent, and (iii) results from experiments conducted with defined artificial substrate and municipal sewage.

A difficulty in testing the model is in the examination of organism behaviour on a level as fundamental as biochemical pathways. This problem was exacerbated by the limited facilities available in a wastewater treatment laboratory designed to examine extracellular macroscopic microbiological phenomena such as nitrification, aerobic substrate utilization rates, denitrification rates, phosphorus removal, and biomass production. Using such facilities, changes in intracellular biochemical processes such as enzyme synthesis and activity can be measured only indirectly, and as such, the experimental programme which follows can be considered as an attempt to accumulate an overwhelming body of circumstantial evidence for or against the hypothesized biochemical model. Experimental work to test the model was oriented at examining the proposals and implications, listed above.

It is important to note that just as the implications listed under categories (1), (2), and (3) are for organisms subjected to frequent exposure to anoxic–aerobic alternating conditions (such that both aerobic and anoxic respiratory enzymes are synthesized), so the experimental work to test the implications was conducted on sludge subjected to frequent anoxic–aerobic alternating conditions, i.e. a 2RND (MLE) system (System 9) with 3:1 a–recycle and 1:1 s–recycle, unless otherwise stated. For the implications listed under category (4), experimental work to test these was conducted on sludge subjected to steady–state aerobic and anoxic periods.

5.6 INHIBITION OF AEROBIC RESPIRATION IN ACTIVATED SLUDGE

A large number of experiments with aerobic batch tests fed municipal sewage were

conducted in examining the implications of the model. Each of the experiments is described below with an explanation of the results and the relevance of the results to the hypothesis.

(1) Respiration under aerobic conditions following anoxic conditions with nitrite present at different concentrations

The objective of these experiments was two-fold; firstly to determine if the presence of nitrite under anoxic conditions with slowly biodegradable substrate could induce inhibition of aerobic respiration under subsequent aerobic conditions, and secondly, if inhibition is induced, to determine if the degree of inhibition is related to the concentration of extracellular nitrite under anoxic conditions and at the start of aerobic conditions. Four aerobic batch tests (BT2, BT4, BT5, BT11) were conducted on sludges held anoxic for a 2 hour period and then aerated. Different concentrations of nitrite were added to each batch during the anoxic period 1 hour before aeration commenced, and sewage was added to the start of the aeration period. The first test (BT2) had a very high concentration of nitrite (25,0 $mgNO_2^-$-N/ℓ) at the onset of aerobic conditions, the other three tests (BT4, BT5, and BT11) had successively lower concentrations of nitrite (5,5; 0,6 and 0,1 $mgNO_2^-$-N/ℓ respectively).[6]

Figure 5.14 indicates that as the concentration of nitrite present at the onset of aerobic conditions decreases, the degree of inhibition[7] of oxygen utilization also decreases. The percentage inhibitions were 68%, 36%, 30% and 8% for the nitrite concentrations 25,0; 5,5; 0,6 and 0,1 $mgNO_2$-N/ℓ respectively. From these results, which are in agreement with the model, the following conclusions can be made:

(i) Nitrite present with slowly biodegradable substrate under anoxic conditions, and at the start of subsequent aerobic conditions results in inhibition of aerobic respiration.

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[6]The experimental procedure for conducting such tests and the reasons for the procedure are described in Appendix B.

[7]The measure of the degree of inhibition is obtained by calculating the fraction the initial specific oxygen utilization rate ($\hat{\mu}_i$) is of the maximum specific oxygen utilization rate ($\hat{\mu}_m$). A problem encountered with this method is that in cases in which inhibition is very large, the RBCOD fraction of the sewage is utilized completely (indicated by a precipitous drop in OUR) before the $\hat{\mu}_m$ is reached.

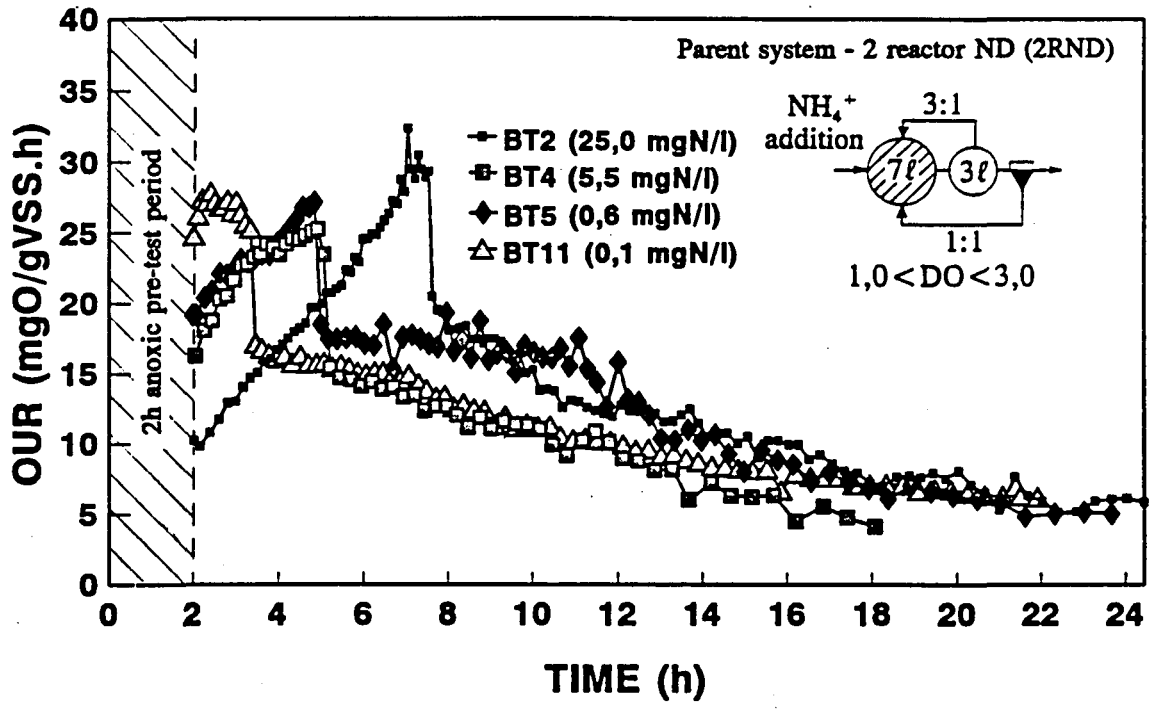

Fig 5.14: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Tests 2, 4, 5 and 11 with different concentrations of nitrite present at the onset of aerobic conditions.

(ii) With nitrite absent both under anoxic conditions and at the start of subsequent aerobic conditions, aerobic respiration is not inhibited.

(iii) Increases in the concentration of nitrite under anoxic conditions and at the start of subsequent aerobic conditions result in increased inhibition of aerobic respiration.

(2) <u>The effect of the presence of nitrite and nitrate under aerobic conditions on aerobic respiration</u>

- ***Effect of different periods of time for denitrification of nitrite under aerobic conditions***

During development of the model an uncertainty arose concerning the capability of facultative organisms to denitrify nitrite to nitric oxide under *aerobic* conditions. The periplasmic orientation of nitrite would suggest that nitrite has access to its reductase under aerobic conditions, but it was uncertain whether aerobic denitrification could occur at a rate sufficient to produce nitric oxide in quantities which would inhibit oxidase activity. The series of experiments described below investigated two aspects of aerobic denitrification of nitrite. The first aspect was the mechanism of aerobic denitrification as a means of producing intracellular, oxidase inhibitory, nitric oxide. Given that such a mechanism can occur, the second aspect was to determine the time period required for aerobic denitrification of nitrite such that inhibition is induced. In Fig 5.15, four tests (BT13, BT14, BT15, and BT16) are shown in which nitrite was added to sludge under aerobic conditions at different time periods before the addition of substrate (i.e. 60, 30, 15, and 5 minutes respectively). Nitrite was added with the objective of ensuring that at the addition of substrate feed, each of the tests had similar concentrations of nitrite. In the same order for the tests as above, the concentrations of nitrite measured immediately after the introduction of feed were 11,9; 15,5; 16,5 and 12,0 $mgNO_2^-$-N/ℓ respectively.

From these results, in agreement with the model, the following conclusions can be made:

(i) Denitrification of nitrite occurs under aerobic conditions and causes inhibition of aerobic respiration.

(ii) For a given concentration of nitrite, the longer the period for aerobic

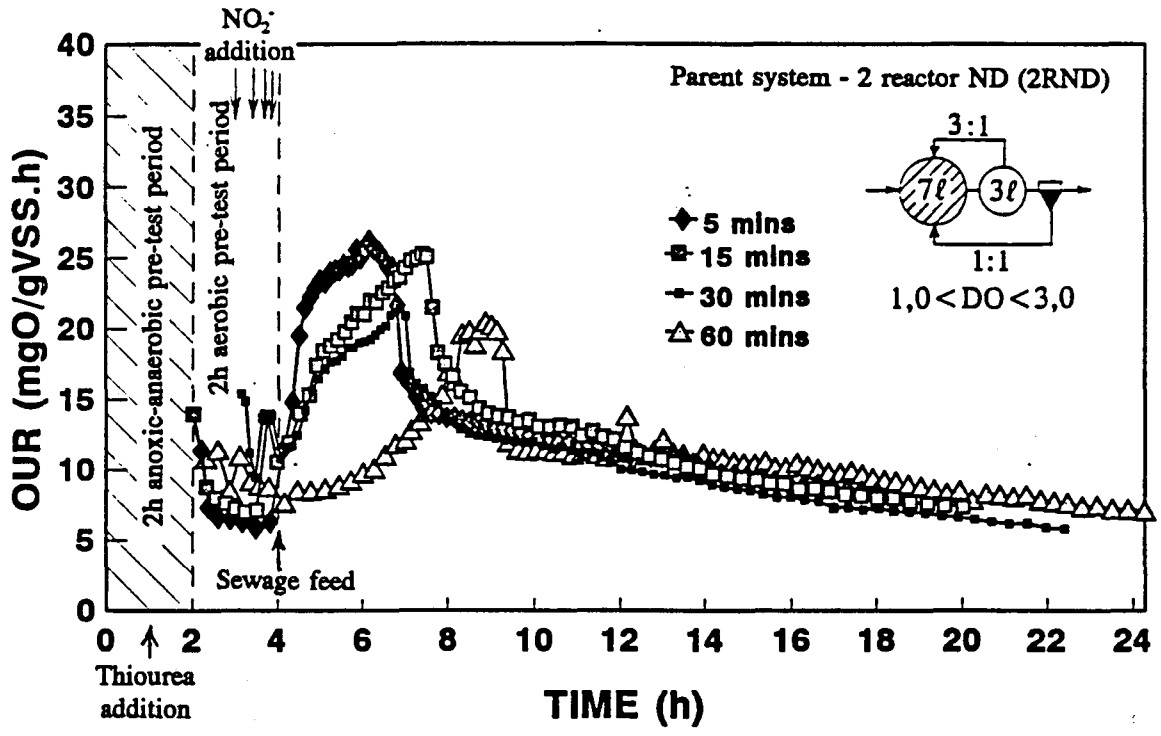

Fig 5.15: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Tests 13, 14, 15 and 16 with nitrite addition under aerobic conditions at different time periods before the addition of substrate.

denitrification of nitrite, the greater the degree of inhibition induced. This conclusion was based on the length of time required to overcome the inhibition effect, i.e. following the addition of feed the time required to raise the OUR from the initial OUR to the maximum OUR. For nitrite added 5, 15, 30 and 60 minutes before the addition of feed, the periods required for relief of inhibition were approximately 2, $3^1/_4$, 3, and $4^1/_2$ hours respectively, a generally increasing trend following the increase in time allowed for aerobic denitrification of nitrite. (The time required for relief of inhibition is not a good measure of the inhibition induced since relief of inhibition is also a function of the amount of readily biodegradable substrate available – complete relief will not be achieved with a small amount of readily biodegradable substrate).

- ***Denitrification of nitrate under aerobic conditions***

The concept of aerobic denitrification of nitrite developed as a consequence of the periplasmic orientation of nitrite reductase such that nitrite has unrestricted access to it. With regard to aerobic denitrification of nitrate, the biochemical model proposes that nitrate cannot be reduced under aerobic conditions since nitrate reductase is situated on the cytoplasmic side of the membrane and oxygen reduces the permeability of the membrane restricting the movement of nitrate. These proposals were tested experimentally. In Batch Test 13 discussed in the previous section and shown again in Fig 5.16, nitrite was added under aerobic conditions in the absence of nitrate an hour before the addition of substrate, and inhibition was induced. To test experimentally aerobic denitrification of nitrate, the same experimental conditions were applied, except nitrate instead of nitrite was added under aerobic conditions an hour before the addition of substrate. Nitrate was added such that the concentration of nitrate on addition of substrate (i.e. 14,9 mgNO_3^-–N/ℓ) was similar to the concentration of nitrite (i.e. 11,9 mgNO_2^-–N/ℓ) at the time of substrate addition in BT13 described above. As indicated in Fig 5.17 for Batch Test 12, addition of nitrate under aerobic conditions did not result in inhibition. From this result it can be concluded that, in agreement with the model, nitrate is not denitrified under aerobic conditions.

However, in a result that appears to be contrary to the proposals of the model concerning aerobic denitrification (nitrite but not nitrate is reduced under aerobic conditions), Batch Test 13 described by Fig 5.16 indicates that after the addition of substrate, the concentration of nitrate decreased by 1 mgNO_3^-–N/ℓ in the first hour

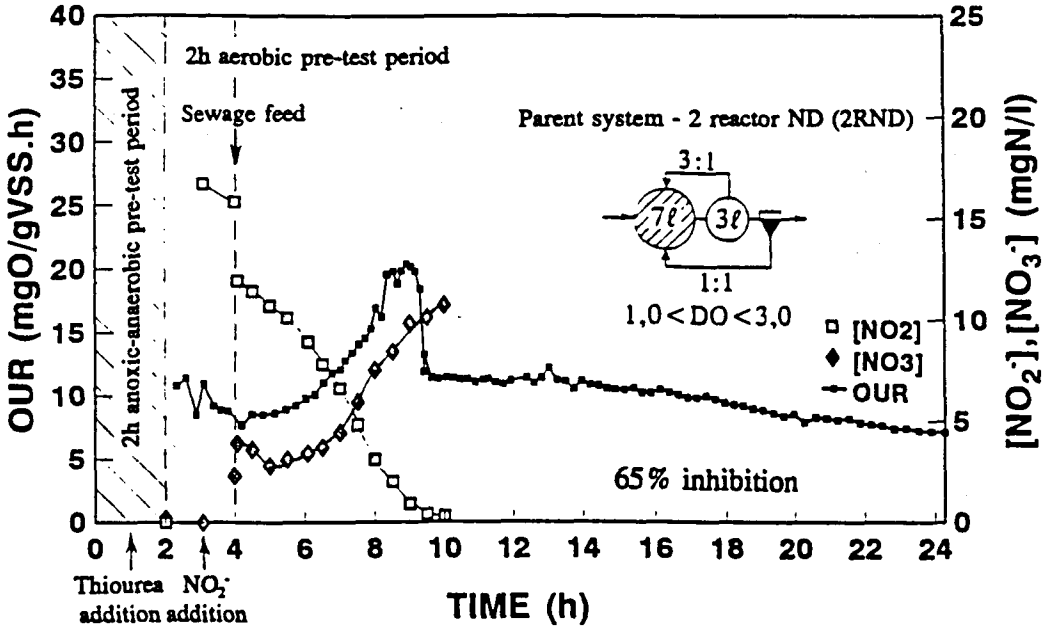

Fig 5.16: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 13 with nitrite added under aerobic conditions in the absence of readily biodegradable substrate.

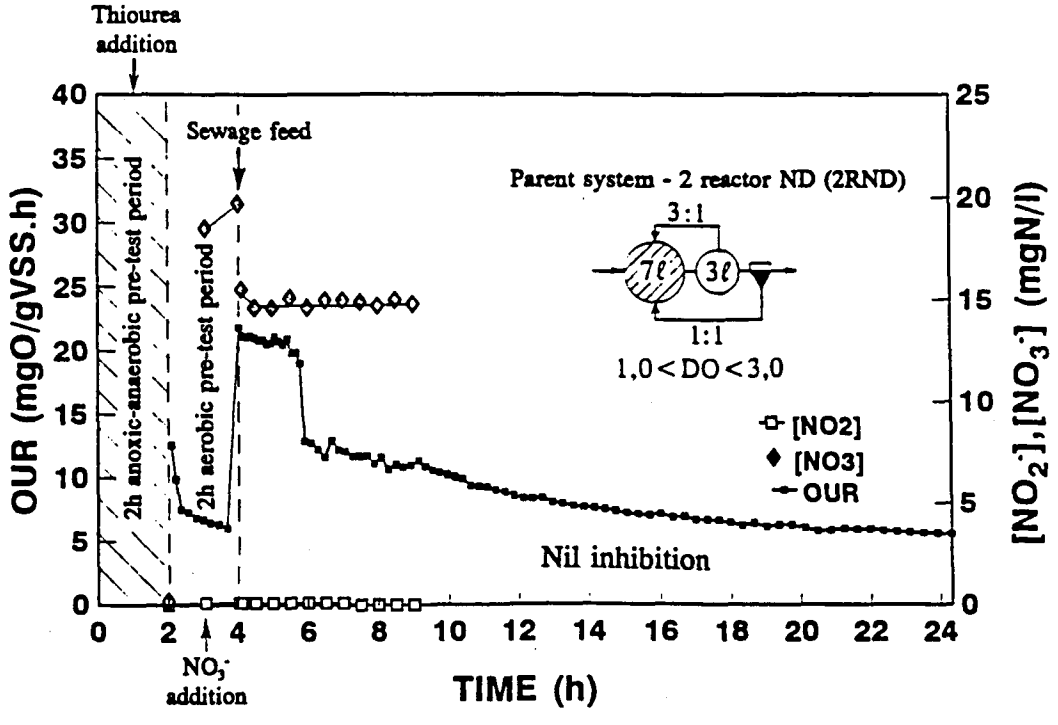

Fig 5.17: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 12 with nitrate added under aerobic conditions in the absence of readily biodegradable substrate demonstrating no induction of inhibition.

due to aerobic denitrification. (The subsequent increase in nitrate concentration results from nitrification of nitrite, see Appendix E). The result can be explained by examining the conditions under which nitrate was reduced in Batch Test 13 but not reduced in Batch Test 12. In Batch Test 12 in which nitrate was added, but not reduced, nitrite was not present, but in Batch Test 13 in which nitrate was reduced, nitrite was present. *Thus, nitrate was reduced under aerobic conditions only when nitrite was present.* It was not clear as to the mechanisms which contribute to this phenomenon but it is suggested that the answer lies in a difference in the extent to which oxygen is inhibitory of the H^+/NO_3^- symport and NO_2^-/NO_3^- antiport mechanisms. However since these nitrogen oxide transport mechanisms are hypothetical, any suggested differences between the operation of the two under aerobic conditions are also hypothetical.

(3) Effect of substrate biodegradability under aerobic conditions on induction and relief of inhibition of aerobic respiration

- ***With slowly biodegradable substrate, aerobic denitrification of nitrite will induce inhibition of respiration***

In (2) above it was demonstrated in Batch Tests 13 to 16 that for nitrite added under aerobic conditions, inhibition of aerobic respiration is induced. In these tests, nitrite was added under aerobic conditions in which only slowly biodegradable substrate was present. Thus it can be concluded that with slowly biodegradable substrate under aerobic conditions, aerobic denitrification of nitrite induces inhibition of aerobic respiration.

- ***Readily biodegradable substrate under aerobic conditions will relieve inhibition of respiration induced by denitrification of nitrite with slowly biodegradable substrate under preceding anoxic conditions***

In (1) above it was demonstrated in Batch Tests 2, 4, 5, and 11, that nitrite addition under anoxic conditions will induce inhibition of aerobic respiration under subsequent aerobic conditions. In these tests, nitrite was added under anoxic conditions in which only slowly biodegradable substrate was present. Under subsequent aerobic conditions with readily biodegradable substrate present, inhibition of aerobic respiration was relieved, reflected in an increasing OUR with time. Thus it can be concluded that readily biodegradable substrate under aerobic conditions relieves inhibition of respiration induced by denitrification of nitrite under preceding anoxic conditions.

- ***With readily biodegradable substrate present under aerobic conditions, nitrite added will not be denitrified and inhibition will not be induced***

The biochemical model proposes that after a change from anoxic to aerobic conditions, when NADH supplies electrons in excess to the ETP, the intracellular concentration of nitric oxide is maintained low enough by nitric oxide reductase to prevent inhibition of aerobic respiration. When the supply of electrons from NADH is restricted, the intracellular concentration of nitric oxide increases because electrons flow preferentially to nitrite reductase to produce nitric oxide, than to nitric oxide reductase to reduce it; the increased nitric oxide concentration inhibits aerobic respiration.

From the foregoing batch tests it is apparent that addition of RBCOD relieves the inhibition effect, reflected in a steadily increasing OUR following the addition of substrate.

To test this aspect more directly, three batch tests were conducted; Batch Test 17 in which nitrite was added in the presence of readily biodegradable substrate and inhibition was not induced (Fig 5.18), Batch Test 1 in which no nitrite was added to the batch test and inhibition was not induced (Fig 5.19), and Batch Test 13 in which nitrite was added in the absence of readily biodegradable substrate and inhibition was induced, as described previously (Fig 5.16) and shown again to facilitate comparison of the tests. In Batch Test 17, nitrite was added about an hour after the addition of substrate and the OUR increased due to nitrification of nitrite. An alternative conclusion is that inhibition was induced, but that the decrease in OUR due to inhibition was offset by a larger increase in OUR due to nitrification. However, this would appear not to be the case, given that for BT13 (Fig 5.16) the addition of nitrite in the absence of readily biodegradable substrate had an oxygen requirement (for nitrification) of 3,0 mgO/gVSS.h, a requirement similar to that for the addition of nitrite in the presence of readily biodegradable substrate in BT17 (3,6 mgO/gVSS.h). These results would indicate that the increase in OUR following the addition of nitrite was due to nitrification only, not the nett result of inhibition and nitrification.

- ***Inhibition effect relieved on a sludge with readily biodegradable substrate will be re-induced in the absence of readily biodegradable substrate***

From the model, in the presence of an adequate supply of electrons, inhibition is relieved, and in the absence of an adequate supply of electrons, inhibition is induced

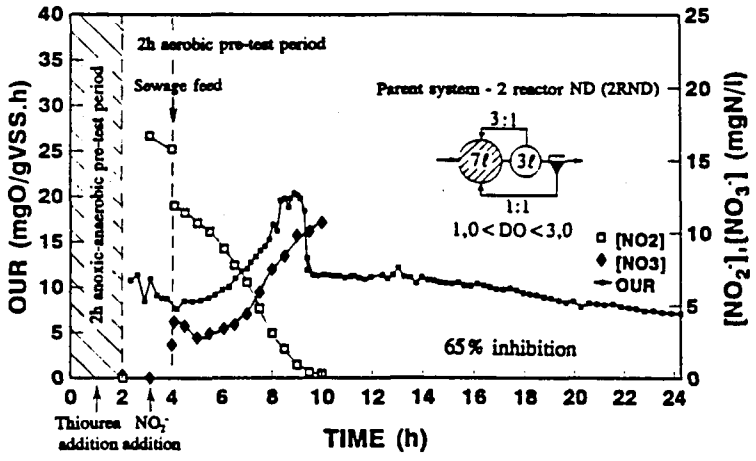

Fig 5.16: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 13 with nitrite added under aerobic conditions in the absence of readily biodegradable substrate.

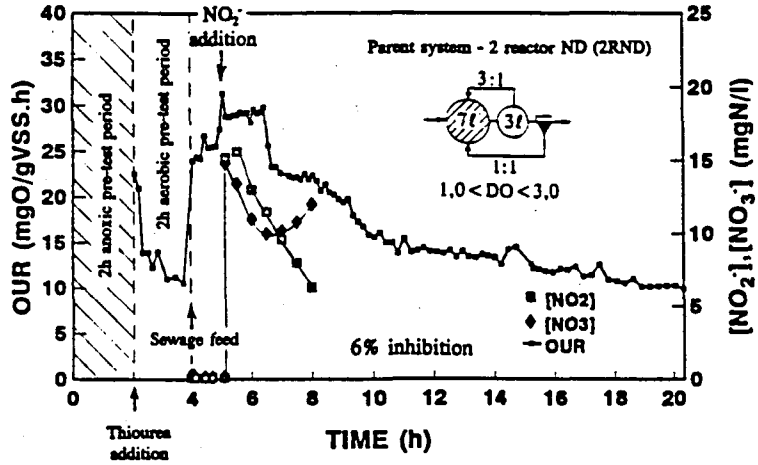

Fig 5.18: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 17 with nitrite added under aerobic conditions in the presence of readily biodegradable substrate demonstrating no induction of inhibition.

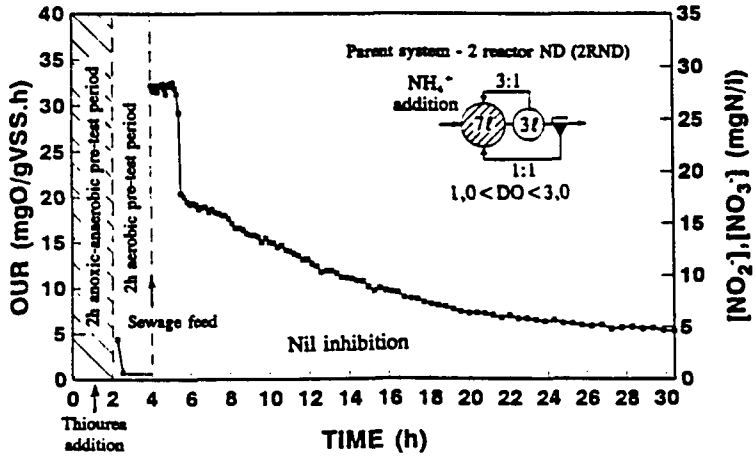

Fig 5.19: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 1 with nitrate and nitrite absent before addition of substrate demonstrating no induction of inhibition.

in the presence of nitrite. In the foregoing batch tests in which inhibition is relieved with readily biodegradable substrate, it is unclear as to whether relief is permanent or temporary. To examine this aspect, Batch Tests 7 and 8 were conducted, in which substrate was added to sludge in which nitrite and nitrate had been removed, as illustrated in Fig 5.20. The OUR profile indicates that the RBCOD was completely utilized after 2 hours and that the sludge was inhibition-free. About 20 hours after substrate addition, nitrite was added at a concentration of 6,0 $mgNO_2^-$-N/ℓ and 5 minutes later substrate added. The presence of nitrite, slowly biodegradable substrate, but not readily biodegradable substrate in the 5 minute period prior to substrate addition induced inhibition, which took $4^1/_2$ hours to overcome. From the results of the experiments, it can be concluded that neither induction nor relief of inhibition is permanent, but is a function of the presence or absence of readily biodegradable substrate and nitrite. The results of the experiments are in agreement with the proposals of the model; relief and absence of inhibition occurs in the presence of an adequate supply of electrons (readily biodegradable substrate) and inhibition occurs or is induced in the presence of an inadequate supply of electrons (slowly biodegradable substrate) with nitrite present, irrespective of whether the sludge has previously been relieved of or was free of inhibition.

(4) <u>Inhibition of respiration is a general mechanism applicable to all sludges subjected to alternating anoxic-aerobic conditions irrespective of system configuration</u>

The inhibition tests described above were all conducted on sludges developed in a 2RND post denitrification (MLE) configuration with a 3:1 a-recycle and a 1:1 s-recycle. To ensure that inhibition of respiration is not specific to MLE configurations, sludges from a multi-reactor MUCT configuration and an intermittently aerated nitrification-denitrification (IAND) system were each subjected to the same conditions which induced inhibition in a sludge from an MLE system.

For the MUCT system, Batch Tests 22 and 24, illustrated in Figs 5.21 and 5.22 respectively, indicate that similar results are measured for inhibition of aerobic respiration for sludge from MUCT systems as for 2RND systems.

For the IAND system, Fig 5.23 describes the results of Batch Tests 20 and 21 in which nitrite was present and absent respectively during a 2 hour anoxic period prior to aeration and addition of substrate. The tests indicate that similar results

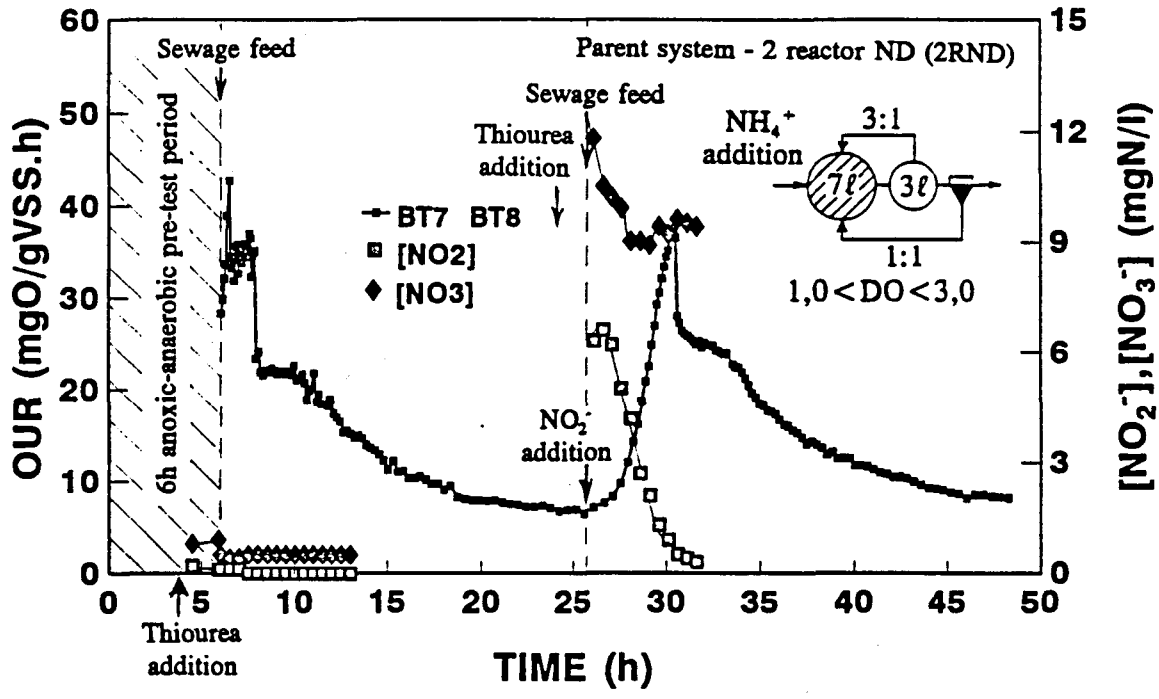

Fig 5.20: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Tests 7 and 8 with inhibition relieved and then induced in the same sludge.

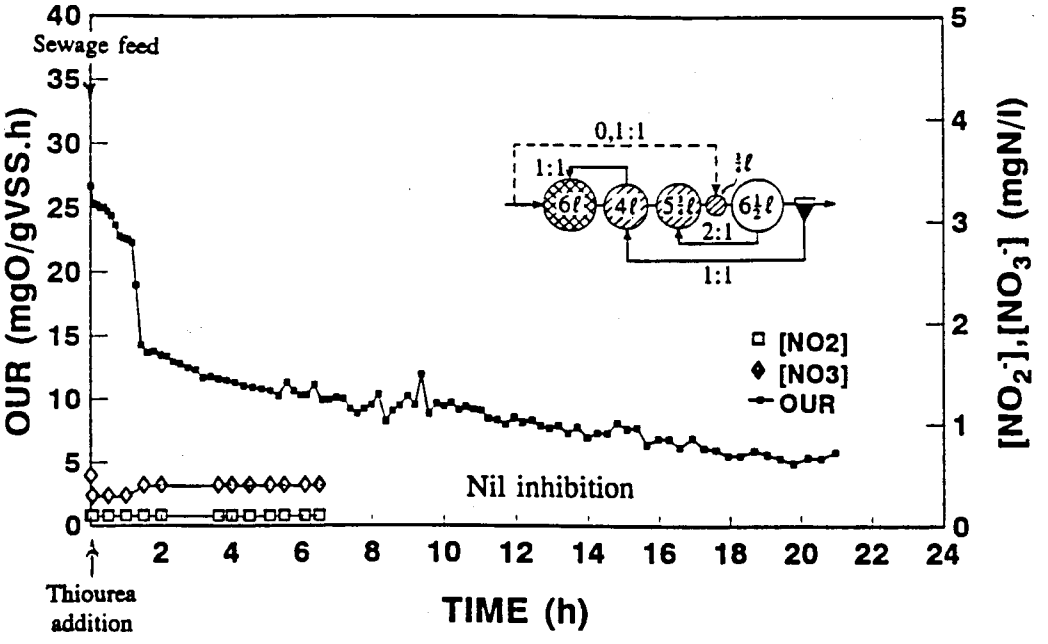

Fig 5.21: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 22 for sludge from a multireactor nitrification–denitrification biological excess phosphorus removal (NDBEPR) system with nitrite present under prior anoxic conditions.

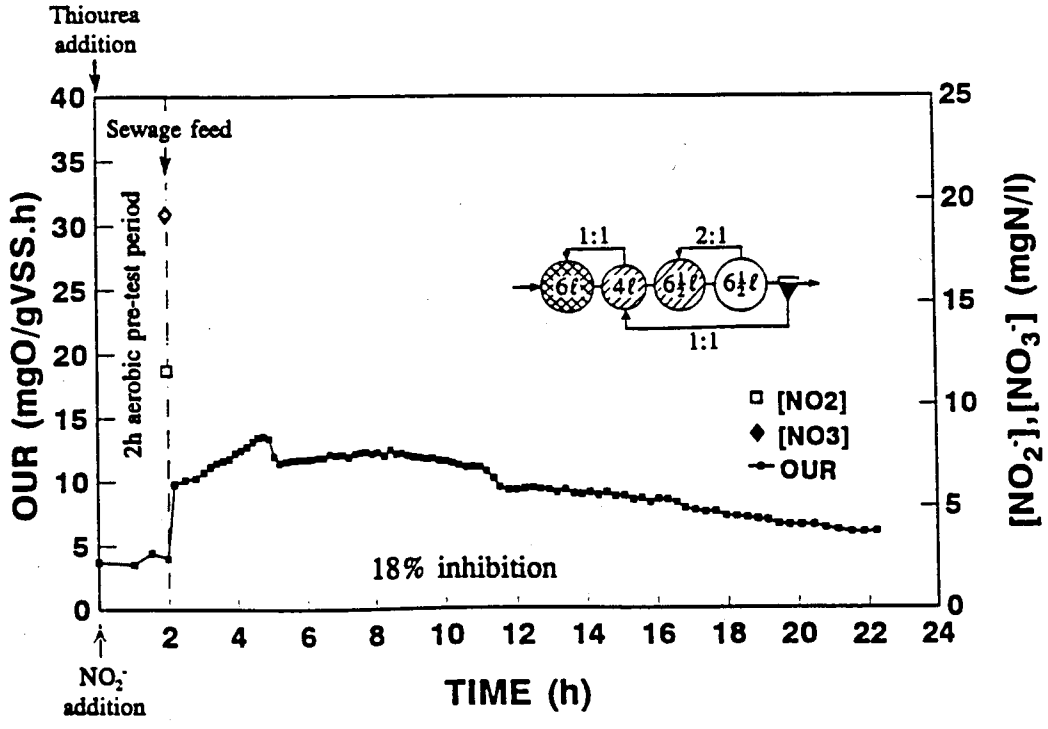

Fig 5.22: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 24 for sludge from a multireactor nitrification–denitrification biological excess phosphorus removal (NDBEPR) system with nitrite absent under prior anoxic conditions.

are measured for inhibition of aerobic respiration for sludge from IAND systems as for 2RND systems.

Apart from the general trend in OUR measurements in the inhibited case, other similarities exist between tests conducted on the IAND, MUCT and 2RND sludges. The first similarity is the initial rapid decrease in nitrate concentration due to denitrification and then the increase in nitrate concentration due to nitrification. The second similarity is the nett loss in nitrogen unaccounted for by the nitrification of nitrite to nitrate. For Batch test 20, conducted on IAND System 5 on Day 300, in the first 7 hours after feeding, 2,6 mgNO_2^-–N/ℓ disappeared and only 1,1 mgNO_3^-–N/ℓ appeared, indicating a loss of 1,5 mgN/ℓ through denitrification of nitrate and nitrite. In Fig 5.24, the initial rapid decrease in nitrate and the initial rapid increase in nitrite are extrapolated to the time at which all of the RBCOD is utilized. Over the $4^1/_4$ hours in which readily biodegradable substrate was present, ≈ 1,4 mgNO_3^-–N/ℓ disappeared and ≈ 0,9 mgNO_2^-–N/ℓ appeared, suggesting that 0,5 mgNO_3^-–N/ℓ was denitrified. Since 1,5 mgN/ℓ in total was denitrified, 1,0 mgNO_2^-–N/ℓ was denitrified. It appears that under aerobic conditions for sludge with inhibited aerobic respiration, twice as much nitrite as nitrate is denitrified and denitrification is initially rapid but decreases with time as inhibition is overcome. This mechanism is in agreement with the concepts of the biochemical model. With regard to the general concept of aerobic denitrification, electrons are diverted to the nitrogen oxide reductases as a consequence of inhibition of electron flow to the oxidases, but as inhibition is relieved, electron flow to the oxidases increases and electron flow to the reductases decreases such that aerobic denitrification also decreases. With regard to more specific details of the model; the experimental results indicate that nitrate was denitrified along with nitrite under aerobic conditions and that the amount of nitrate denitrified (0,5 mgNO_3^-–N/ℓ) was less than the amount of nitrite denitrified (1,0 mgNO_2^-–N/ℓ) As noted in (2), nitrate was denitrified only when nitrite was present. However, a principle of the model is that due to the periplasmic and cytoplasmic placements of nitrite and nitrate reductase respectively, under *aerobic* conditions nitrite (but not nitrate) has access to its reductase and with a high concentration of nitrite, a high redox potential develops at nitrite reductase and a large flow of electrons passes to it. From the results it appears that the membrane is not completely impermeable to nitrate; some nitrate can pass across the membrane to nitrate reductase.

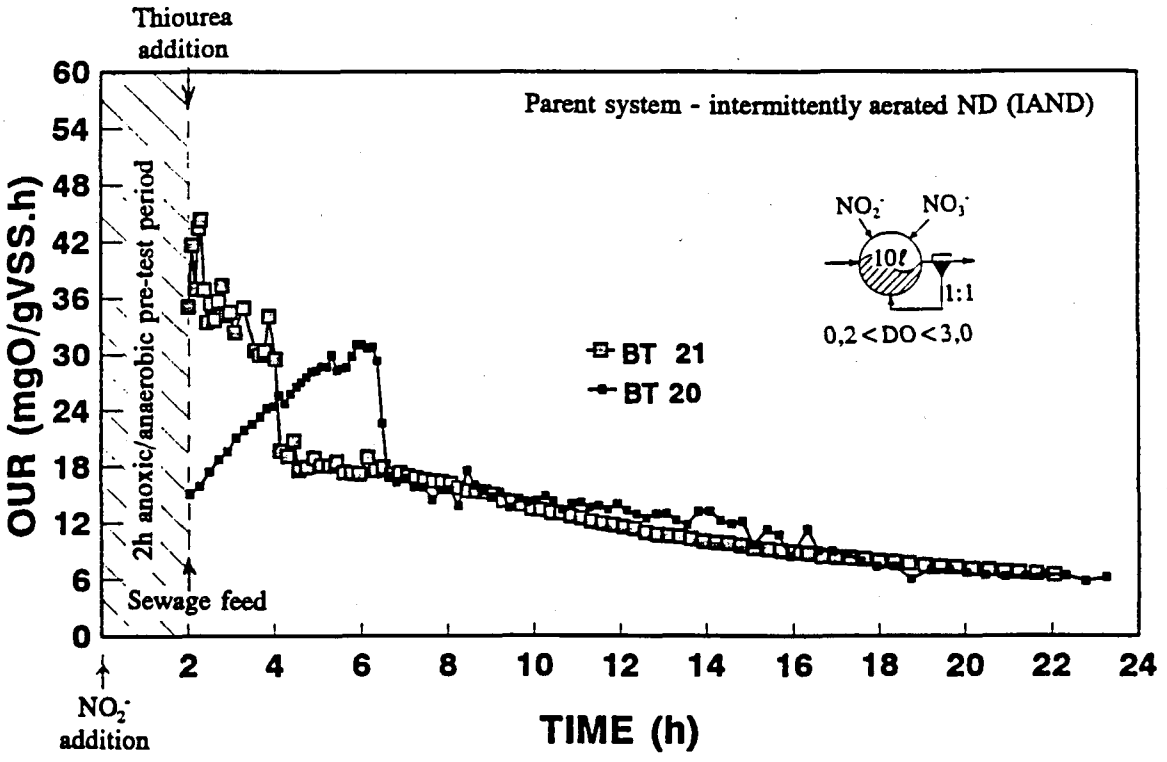

Fig 5.23: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Tests 20 and 21 for sludge from an intermittently aerated nitrification–denitrification (IAND) system with nitrite present (BT20) and absent (BT21) under prior anoxic conditions.

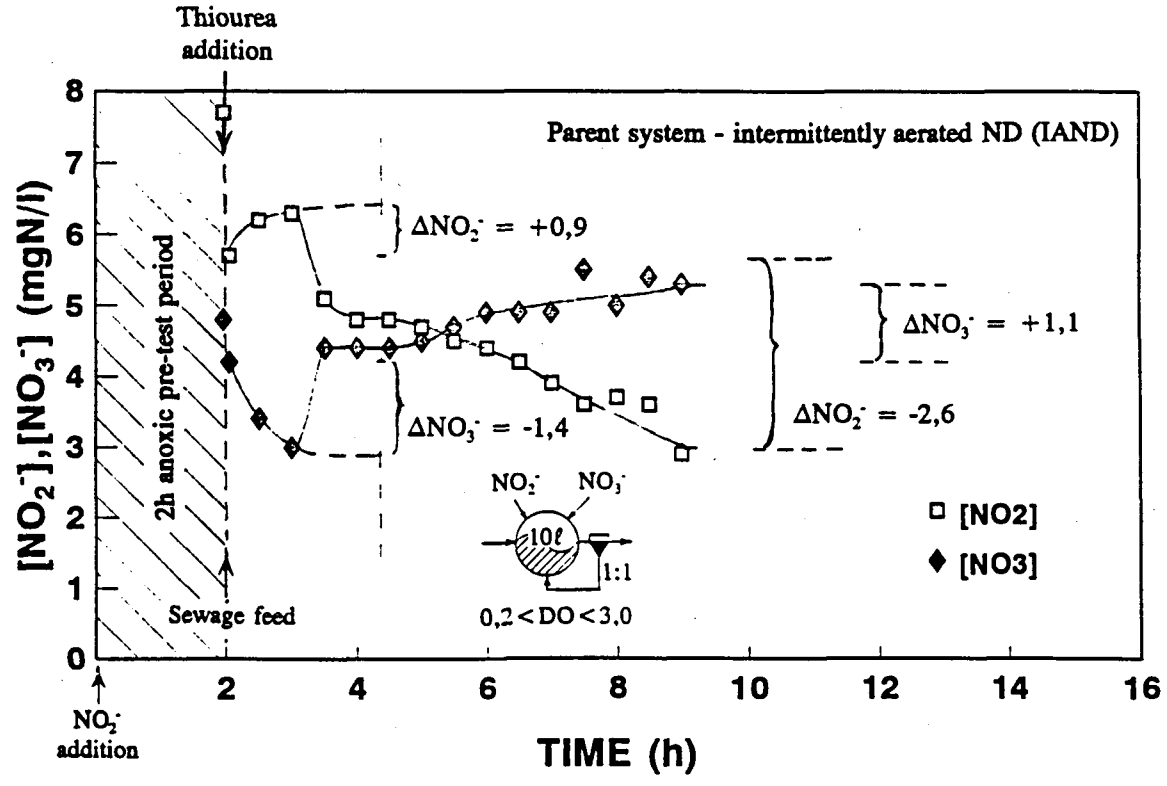

Fig 5.24: Changes in nitrate ($mgNO_3^-$–N/ℓ) and nitrite ($mgNO_2^-$–N/ℓ) concentration with time after feeding for Batch Test 20.

(5) Synthesis of aerobic and anoxic pathway enzymes under aerobic and anoxic conditions

A principal aspect of the conceptual model concerns the effect of aerobic and anoxic conditions on the synthesis of the enzymes of the aerobic and anoxic electron transport pathways. The model proposes that (i) facultative organisms grown under aerobic conditions, when placed under anoxic conditions are unable to denitrify immediately, because under aerobic conditions synthesis of the denitrifying enzymes is repressed, and (ii) facultative organisms grown under anoxic conditions can immediately utilize substrate under aerobic conditions as a consequence of the presence of the denitrifying enzymes under anoxic conditions.

To examine the effect of aerobic and anoxic conditions on the synthesis of aerobic and denitrifying enzymes, sludge in a single reactor (System 7) was maintained under sequential continuous anoxic conditions and continuous aerobic conditions for varying periods of time. The uptake of substrate under aerobic inhibition batch test conditions was examined periodically during the anoxic and aerobic conditions. Since inhibition is a function of the level of the nitrogen oxide reductases (in particular nitrite–, and nitric oxide reductase) and cytochrome oxidase (in particular cytochrome aa_3), measurement of the level of inhibition provided information concerning the levels of these enzymes.

To examine the effect of aerobic and anoxic conditions on synthesis of the denitrifying enzymes, anoxic denitrification batch tests were conducted before and after a change from aerobic to anoxic conditions.

Figure 5.25 illustrates the degree of inhibition measured in aerobic inhibition batch tests A to R conducted on System 7 from Days 222 to 372. The tests were conducted during continuous anoxic periods (Days 262 to 305, and Days 352 to 372) and continuous aerobic periods (Days 255 to 261, and Days 306 to 351). The anoxic period, from Days 222 to 254 is included to provide information regarding conditions to which the sludge was subjected prior to the first aerobic period.

The objective of the analyses that follow is to test the proposals of the model regarding the major changes that occur in the synthesis and activity of the enzymes of the aerobic and anoxic pathway during changes between aerobic and anoxic conditions.

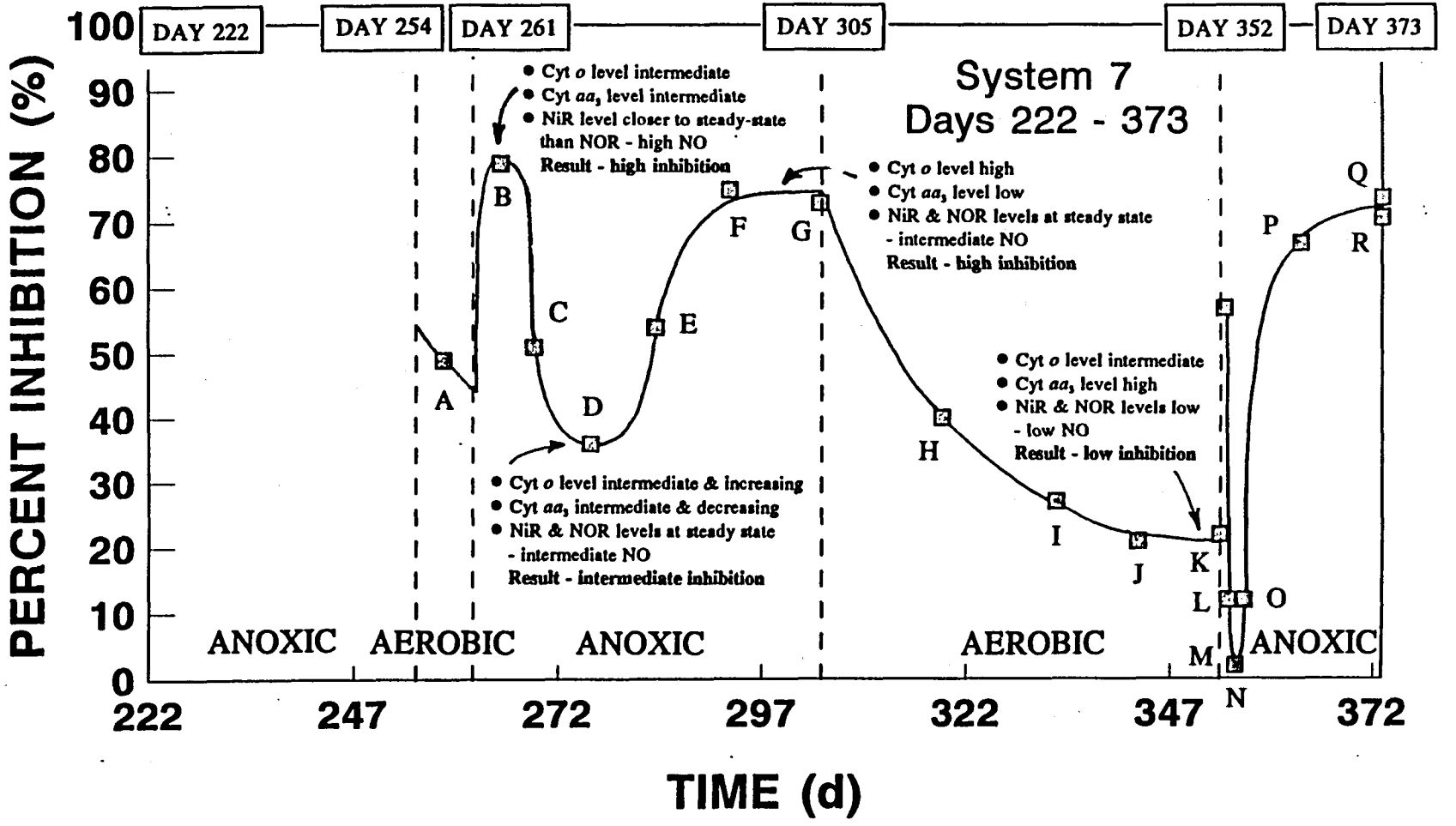

Fig 5.25: Percentage aerobic inhibition (%) with time for sludge from System 7, resulting from changes between aerobic and anoxic conditions.

With reference to Fig 5.25, two periods are of primary interest, the change from steady–state anoxic to aerobic conditions on Day 306, and the change from steady–state aerobic to anoxic conditions on Day 352. At these times, the long anoxic period resulted in steady–state conditions and expected maximum synthesis of the denitrifying enzymes at Day 306, and the long aerobic period resulted in steady–state conditions and expected maximum synthesis of the aerobic enzymes at Day 352.

- ***Inhibition of aerobic respiration will decrease with continuous exposure of anoxically grown sludge to aerobic conditions***

At the end of the second anoxic period (Days 262 to 305), inhibition was about 76%. The high level of inhibition results from interaction of cytochrome *o* (synthesized to its maximum level under anoxic conditions) with high levels of nitric oxide produced through denitrification of nitrite with slowly biodegradable substrate. During the subsequent aerobic period (Days 306 to 352), the maximum inhibition decreased asymptotically to a value of 21% by Day 352. The reduction in maximum inhibition results from a combination of two changes: (i) with continuous exposure to aerobic conditions, cytochrome aa_3 (which is not inhibited by nitric oxide) is synthesized, cytochrome *o* (which is strongly inhibited by nitric oxide) degrades, and the greater flow of electrons passes to cytochrome aa_3. (ii) during the aerobic period the denitrifying reductases degrade with time, so that very much reduced concentrations of nitric oxide are produced. In combination, a low concentration of nitric oxide, a low level of cytochrome *o* and a high concentration of cytochrome aa_3 result in a low level of inhibition of aerobic respiration.

- ***Inhibition of aerobic respiration will increase with continuous exposure of aerobically grown sludge to anoxic conditions***

Similar trends in maximum inhibition were measured for the two anoxic periods illustrated in Fig 5.25. Both trends initially increased, then decreased, and then increased to a similar high steady–state value ($\approx$ 72%). It appears that the difference in rates of increase and decrease in level of inhibition between the two anoxic periods is a consequence of the effect on the denitrifying reductases of the preceding aerobic period. The aerobic period preceding the anoxic period from Day 261 to Day 305 was only 7 days; in comparison the aerobic period preceding the anoxic period from Day 352 to Day 373 was 47 days. However it is not clear as to how this difference would affect the rate of change of inhibition.

From the principles of the model, the nett increase in maximum inhibition with time results from an increase in nitrite reductase which produces nitric oxide, an increase in cytochrome *o*, and a reduction in cytochrome aa_3 synthesis, the interaction between the nitric oxide and the cytochrome *o* resulting in inhibition.

The initial rapid increase, then decrease in inhibition can be explained by examining the *rates* of synthesis of nitrite reductase and nitric oxide reductase. In modelling synthesis of the nitrogen oxide reductases after an aerobic period during which the levels of the reductases decrease, each of the reductases is synthesized in response to the presence of its reactant and at a rate proportional to the concentration of the reactant. Thus, nitrite reductase is synthesized in response to nitrite, and nitric oxide reductase is synthesized in response to nitric oxide. A change from aerobic to anoxic conditions with nitrite present, results in an initial synthesis of nitrite reductase; reduction of nitrite causes a high concentration of nitric oxide prior to synthesis of nitric oxide reductase. The period between nitrite reductase synthesis and nitric oxide reductase synthesis will result in a high nitric oxide concentration and an initial high level of inhibition due to interaction of nitric oxide with cytochrome *o*. Synthesis of nitric oxide reductase results in a reduction in nitric oxide concentration and a decrease in inhibition with time. Between Day 276 and Day 305 the level of cytochrome o increases and the level of cytochrome aa_3 decreases, during which time the level of intracellular nitric oxide is stable and inhibition increases with time.

Although the results of the experiments described above are only an indirect measure of the level of synthesis of the enzymes of the aerobic and anoxic ETP's, several aspects provide credibility for the aspects of the conceptual model describing changes in the synthesis of aerobic and anoxic respiratory ETP enzymes; the very definite trends associated with inhibition of aerobic respiration following aerobic and anoxic growth conditions, and the reproducibility of the trend in changes in inhibition associated with changes from aerobic to anoxic conditions.

- ***Exposure of aerobically grown sludge to anoxic conditions will reduce the denitrification potential of the sludge***

Anoxic denitrification batch tests (DBT) were conducted on Day 350 (DBT1) and Day 352 (DBT2) on sludge from System 7, operated under aerobic conditions , and on Day 355 (DBT3) and Day 364 (DBT4) on sludge from System 7 operated under anoxic conditions. The results of the batch tests are illustrated in Fig 5.26. For

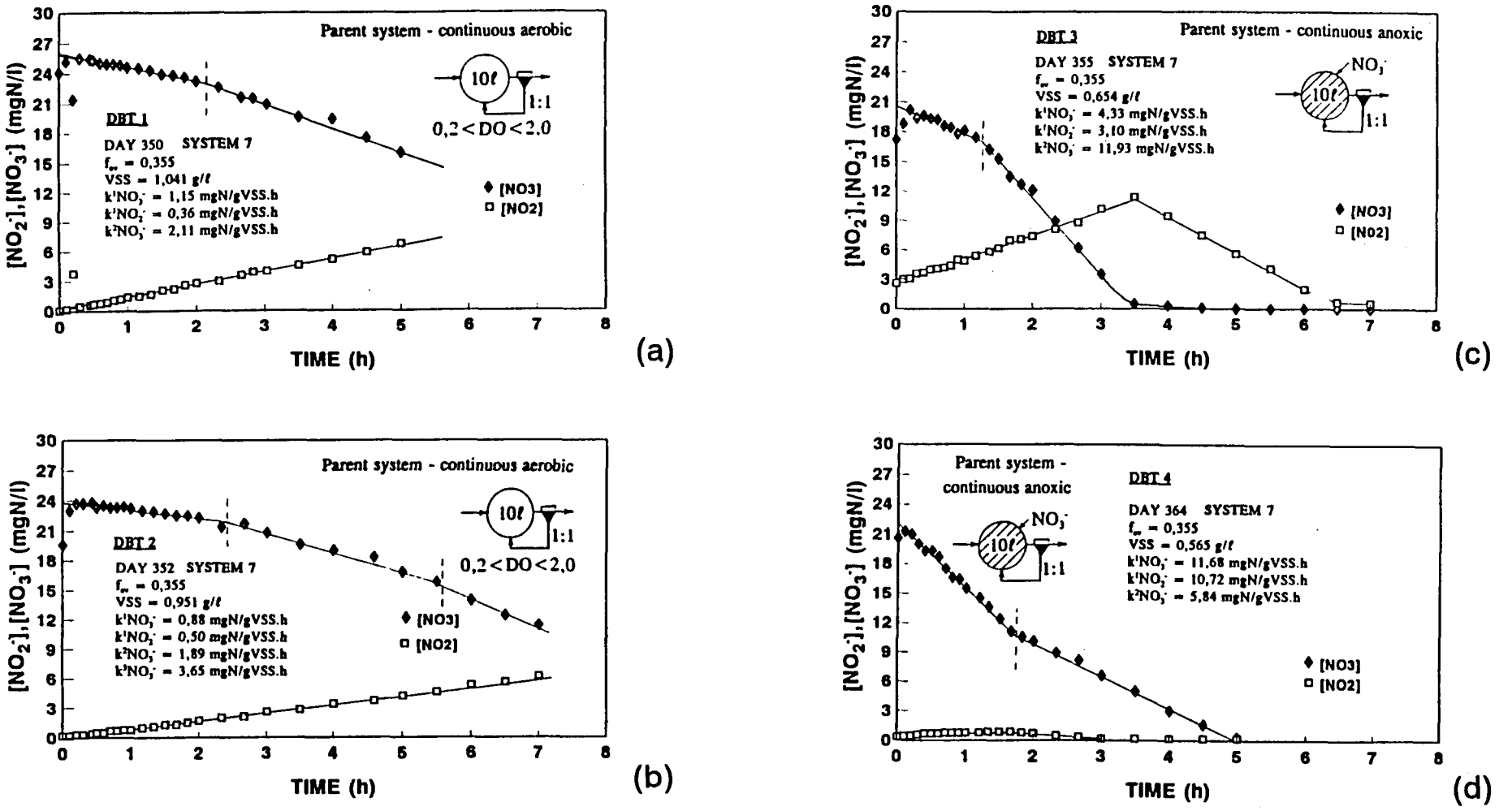

Fig 5.26: Change in denitrification rate of nitrate and nitrite with time for sludge from System 7, resulting from changes in operating conditions, from steady-state aerobic to anoxic.

analysis purposes, the change in nitrate concentration is divided into approximately linear sections and the progressively increasing rates of nitrate reduction in any one test are labelled $k^1_{NO_3^-}$, $k^2_{NO_3^-}$, and $k^3_{NO_3^-}$ and the rate of nitrite reduction is labelled $k^1_{NO_2^-}$. The increase in nitrate concentration during the first 20 minutes of each test is attributed to nitrate mixing problems after the addition of nitrate at the same time as addition of substrate. The variations in $k^1_{NO_3^-}$ and $k^1_{NO_2^-}$ from DBT1 to DBT4 are plotted in Fig 5.27 and are discussed separately below.

Nitrate reduction: For sludge developed under aerobic conditions, the initial rates of nitrate reduction ($k^1_{NO_3^-}$) were very low for DBT1 (1,15 mgN/gVSS.h) and DBT2 (0.88 mgN/gVSS.h).[8] The initial rate of nitrate reduction for DBT2 for the sludge subjected to aerobic conditions for a slightly longer time (2 days) than DBT1, is slightly lower than that for DBT1. This resulted from a slightly greater degree of degradation of nitrate reductase with continued exposure to aerobic conditions. On Day 352 System 7 was made anoxic, and after 3 days of anoxic conditions with nitrate present, the initial rate of nitrate reduction ($k^1_{NO_3^-}$ = 4,33 mgN/gVSS.h) was considerably higher than the value under aerobic conditions, and after a further 12 days of anoxic conditions, the initial rate of nitrate reduction ($k^1_{NO_3^-}$ = 11,68 mgN/gVSS.h) increased further. This resulted from increased synthesis of nitrate reductase with exposure to anoxic conditions, in agreement with the principles of the model.

Nitrite reduction: Figure 5.27 illustrates that similar results to those described for nitrate reduction were measured also for nitrite reduction under aerobic and anoxic conditions. The true rate of nitrite reduction ($k^1_{NO_2^-}$) was calculated by subtracting the average of the first two rates of nitrate reduction (which equals the rate of formation of NO_2^-), from the measured rate of nitrite formation during the same period. Under continuous aerobic conditions, initial rates of nitrite reduction were very low for DBT1 ($k^1_{NO_2^-}$ = 0,36 mgN/gVSS.h) and DBT2 ($k^1_{NO_2^-}$ = 0,50 mgN/gVSS.h). This resulted from decreased synthesis of nitrite reductase with time under aerobic conditions in agreement with the principles of the model. Under anoxic conditions, the initial nitrite denitrification rate increased from DBT3 ($k^1_{NO_2^-}$ = 3,10 mgN/gVSS.h) to DBT4 (10,72 mgN/gVSS.h). A noteworthy feature of the

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[8] The notation $k^1_{NO_3^-}$, $k^2_{NO_3^-}$ and $k^3_{NO_3^-}$ to indicate the initial (first), second and third rates of nitrate reduction are not to be confused with the first and second rates of nitrate reduction normally employed in wastewater research to denote nitrate reduction with RBCOD and SBCOD and endogenous matter respectively.

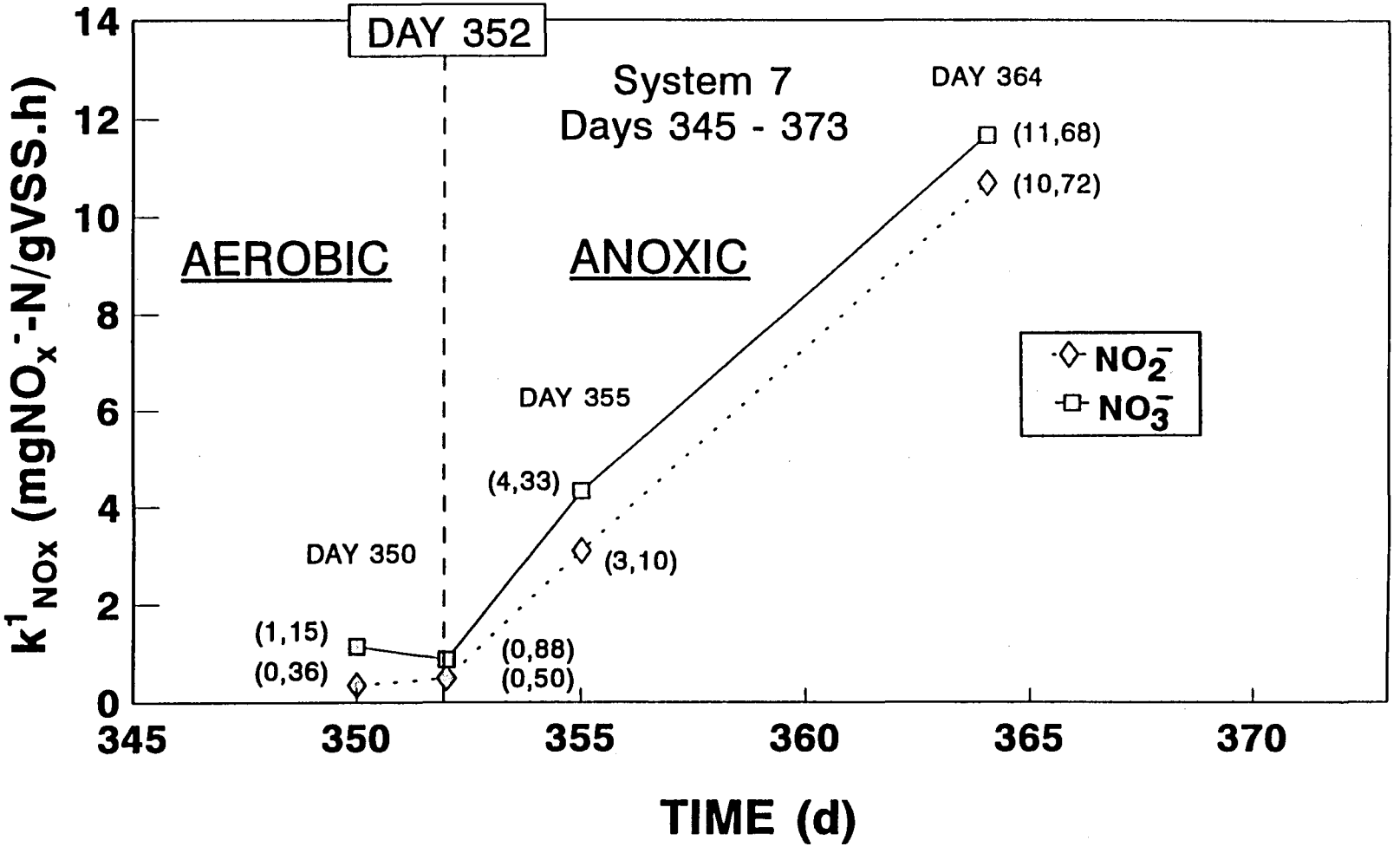

Fig 5.27: Rates of denitrification of nitrate ($k'_{NO_3^-}$) and nitrite ($k'_{NO_2^-}$) with time for sludge from System 7, for a change from aerobic to anoxic operating conditions.

results is that for sludge grown under anoxic conditions and subjected to anoxic batch tests (DBT3 and DBT4), nitrite accumulated to a greater degree in DBT3 (5,0 mgNO_2^--N/gVSS.h) than in DBT4 (1,2 mgNO_2^--N/gVSS.h), indicating that after changing System 7 from aerobic to anoxic conditions, nitrate reductase synthesis was initially considerably more rapid than nitrite reductase synthesis. These results are in agreement with the principles of the model in which sequential formation of the nitrogen oxide reductases (in the order, nitrate–, nitrite–, nitric oxide– and nitrous oxide reductase) under anoxic conditions following steady–state aerobic conditions, results in sequential formation of the nitrogen oxides in the order, nitrate, nitrite, nitric oxide, and nitrous oxide (see Fig 5.7).

5.7 CLOSURE

In Part I of this Chapter a conceptual model was outlined for the major biochemical respiratory mechanisms involved when a heterotrophic facultative organism (representing the heterotrophic facultative mass of activated sludge) is subjected to steady–state aerobic, steady–state anoxic and changes from steady–state aerobic to anoxic and steady–state anoxic to aerobic conditions. In Part II the major proposals and implications of the model were tested with aerobic inhibition batch test procedures which examined the effect on aerobic respiration of anoxic and anaerobic pre–test conditions, nitrite and nitrate concentrations under anoxic conditions, readily and slowly biodegradable substrate, and synthesis of aerobic and denitrifying enzymes under anoxic and aerobic conditions. Anoxic batch tests examined the effect of aerobic and anoxic conditions on the denitrifying enzymes, nitrate– and nitrite reductase. The experimental results provide substantive evidence that supports the conceptual biochemical model for facultative organism respiration.

CHAPTER 6

PROLIFERATION OF AA FILAMENTOUS ORGANISMS: A BIOCHEMICAL/MICROBIOLOGICAL MODEL FOR BULKING

ABSTRACT

A model is presented that describes the competitive growth behaviour of floc-forming and AA filamentous[1] organisms in long sludge age nitrogen (N) and nitrogen and phosphorus (N & P) removal activated sludge systems. The model, referred to as the bulking model, establishes the potential for filamentous organism proliferation under various aeration and substrate feeding régimes. Based on the principles of the model, system configuration and operational procedures are proposed for the amelioration of bulking and are tested experimentally. To examine the general applicability of the bulking model it is tested through application to the results of the experiments described in Chapter 3.

6.1 INTRODUCTION

The objective of the conceptual biochemical model for aerobic facultative heterotrophic organism respiration developed in Chapter 5, was to establish a basis by which the mechanisms of respiration of the facultative organism mass in activated sludge could be understood. In this Chapter the biochemical model is applied to filamentous and floc-forming organisms, to develop a microbiological model for substrate competition by these organisms as a means of explaining the proliferation of AA filaments in N and N & P removal systems. Implicit to the formulation of the bulking model is the assumption required by the biochemical model, that both filamentous and floc-forming organisms are aerobic facultative heterotrophs.

6.2 STATEMENT OF HYPOTHESIS

In activated sludge systems, floc-formers and filaments compete for mutually growth-limiting substrate. Under completely aerobic or completely anoxic conditions, the floc-formers outcompete the filaments for substrate due to higher substrate utilization rates and filament growth is restricted. In nitrification-denitrification

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[1] Throughout description of the bulking model, the terms *filamentous* and *filaments* refer specifically to *AA filáments*, (formerly designated low F/M filaments) which are associated with poorly settling sludges in long sludge age N and N & P removal systems.

(ND) and nitrification–denitrification biological excess phosphorus removal (NDBEPR) activated sludge systems, competition between filamentous and floc–forming organisms for mutually growth–limiting substrate is influenced by inhibition of the floc–formers substrate utilization under aerobic conditions. Under anoxic conditions, in utilization of substrate the floc–formers execute the denitrification of nitrate through each of the denitrification intermediates to dinitrogen as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

nitrate → nitrite → nitric oxide → nitrous oxide → dinitrogen

In the absence of, or at low concentrations of readily biodegradable substrate, the intermediate nitric oxide is accumulated intracellularly in the floc–formers. In the subsequent aerobic zone, intracellular nitric oxide inhibits the utilization of oxygen by floc–forming organisms as a result of the interaction of nitric oxide with the enzymes specific to aerobic respiration, the cytochrome oxidases, cytochrome o and cytochrome aa_3. Under these conditions, floc–forming organisms are inhibited in aerobic respiration. The inhibition of aerobic respiration causes electrons to be redirected to the nitrite, nitric oxide and nitrous oxide reductases for the reduction of nitrogen oxide (aerobic denitrification) and this continues as long as nitrite remains available. Thus, under aerobic conditions a low concentration of nitrite maintains the intracellular accumulation of nitric oxide and thereby the inhibitory effect, higher concentrations of nitrite exacerbate the inhibitory effect. The inhibition of aerobic respiration and the phenomenon of aerobic denitrification in floc–formers results in lower substrate utilization rates, and lower nett energy yields.

In contrast to floc–formers, the filamentous organisms are nitrate reducers and execute only part of the denitrification pathway, i.e. the reduction of nitrate to nitrite as follows:

$$NO_3^- \rightarrow NO_2^-$$

They do not accumulate nitric oxide and hence are not inhibited in aerobic respiration in the subsequent aerobic zone.

Thus, in systems in which sludge is exposed to alternating anoxic–aerobic conditions with nitrite present in the anoxic zone, floc–formers are placed at a disadvantage in

the competition for substrate under subsequent aerobic conditions and filaments gain an advantage. Given sufficient advantage, the filaments proliferate with time.

6.3 EXAMINATION OF HYPOTHESES

Steady–state anoxic conditions

Under anoxic conditions in which one or both of the ionic nitrogen oxides nitrate or nitrite are available and oxygen is absent, a major difference between the filaments and floc–formers is in their anoxic respiratory pathways, as described above.

For the floc–formers; of the oxidases, only cytochrome *o* is synthesized and cytochrome aa_3 either is absent, or present at a basal level; of the reductases, all are synthesized to their maximum level as illustrated in Fig 6.1a.

For the filaments; of the oxidases, only cytochrome *o* is synthesized, and cytochrome aa_3 either is absent or present at a basal level; of the reductases, only nitrate reductase is synthesized, as illustrated in Fig 6.1b.

Under conditions in which nitrate and nitrite are present in sufficient quantities so as not to be limiting, for the two groups of organisms the energetic yield of their respective pathways is equal. Electrons, which are transferred to nitrate–, nitrite–, nitric oxide–, or nitrous oxide reductase for the floc–formers, or to nitrate reductase for the filaments, pass two proton–pumping (energy conserving) sites, Sites I and II. Therefore, under conditions in which nitrate is not limiting, the ability to execute the denitrification pathway through each of the intermediates, does not endow the floc–formers with an energetic benefit in comparison with the filaments. Under conditions in which nitrate is limiting and substrate is in excess, the ability of floc–formers to utilize each of the sequentially produced denitrification intermediates as an electron acceptor endows them with a greater energetic benefit than the filaments (for which only nitrate can be utilized as electron acceptor). A more complete discussion of this aspect is given in Chapter 4.

In conclusion, under steady–state anoxic conditions with sufficient nitrate present, theoretically, floc–formers gain no energetic advantage over the filaments for each nitrate molecule reduced, and the low proportion of filaments which develop under steady–state anoxic conditions (see Chapter 3) is a consequence of a lower specific substrate utilization/growth rate for filaments than floc–formers.

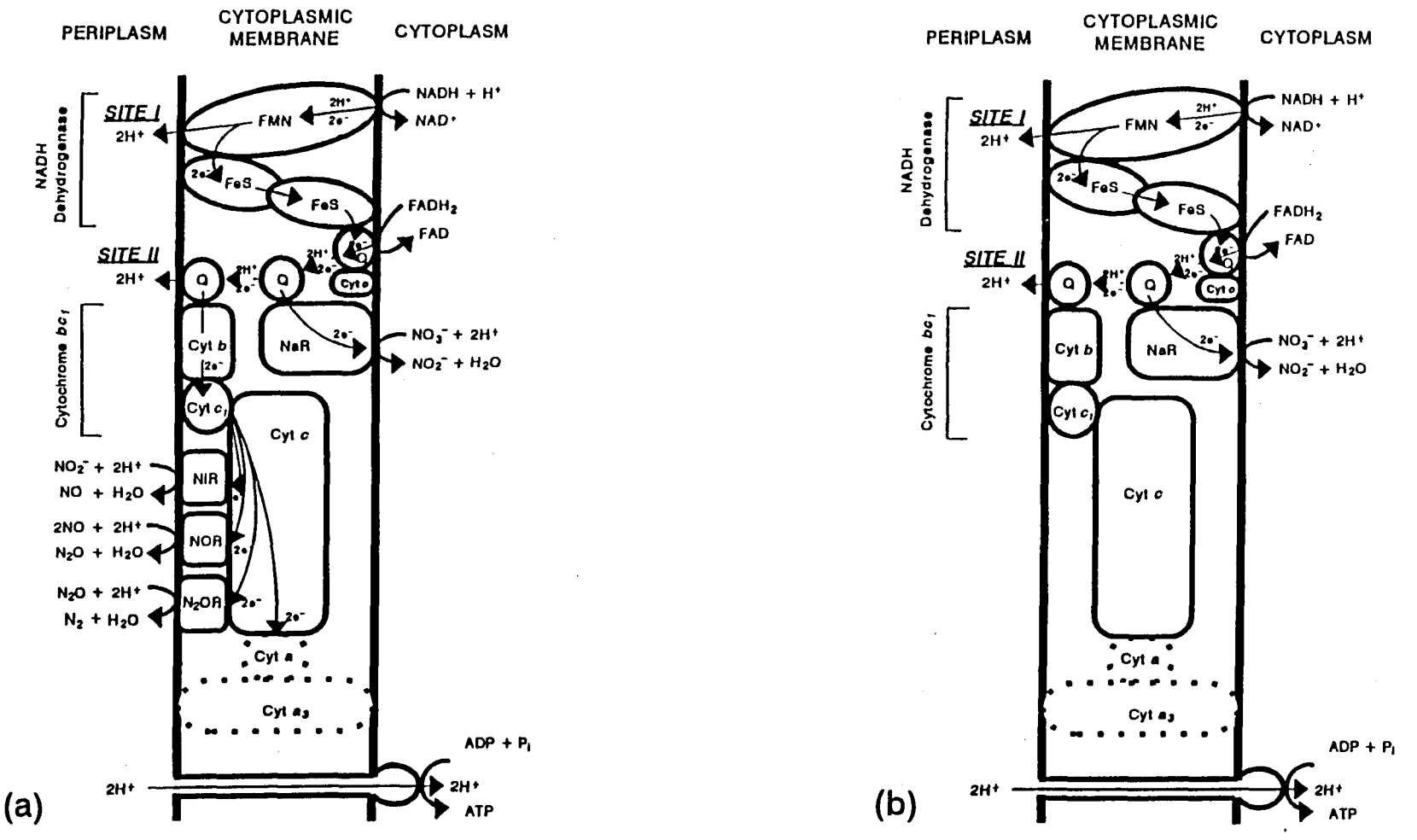

Fig 6.1: Models of the electron transport pathway (ETP) for (a) floc-forming and (b) filamentous organisms under steady state anoxic conditions. [Abbreviations: NAD – nicotinamide adenine dinucleotide; FMN – flavomononucleotide; FeS – iron-sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone, Cyt – Cytochrome; NaR – Nitrate reductase; NiR – Nitrite reductase; NOR – Nitric oxide reductase; N_2OR – Nitrous oxide reductase; ADP – adenosine diphosphate, ATP – adenosine triphosphate.]

Steady-state aerobic conditions

Under aerobic conditions with a concentration of DO greater than 2 mgO/ℓ, the filaments and floc-formers utilize oxygen as electron acceptor.[2] For floc-formers and filaments, both of the oxidases cytochrome *o* and cytochrome aa_3 are synthesized, cytochrome aa_3 to its maximum level and cytochrome *o* to a considerably reduced level ($\approx$ 30% of the level under anoxic conditions), and the reductases are synthesized at a basal level as indicated in Figs 6.2a and 6.2b for floc-formers and filaments respectively. For both floc-formers and filaments, the mechanisms of electron transfer to the complexes and finally to oxygen during respiration are the same as described in the biochemical model for facultative heterotrophs under aerobic conditions. In their transferal to oxygen the greater proportion of electrons pass to cytochrome aa_3, in the process of which three proton-pumping (energy conserving) sites, Sites I, II and III are passed. Under steady-state aerobic conditions, theoretically, floc-formers gain no energetic advantage over the filaments for each oxygen molecule reduced, and it is concluded that the low proportion of filaments which develop under steady-state aerobic conditions (see Chapter 3) results from a lower specific substrate utilization/growth rate for filaments than floc-formers.

Frequent alternation between anoxic and aerobic conditions

Level of synthesis of enzymes of the ETP

In the preceding two sections, the status of the ETP under steady-state aerobic and steady-state anoxic conditions is described. As demonstrated in Chapter 5, maximum synthesis of aerobic enzymes may take up to three sludge ages to be effected when conditions change between anoxic and aerobic conditions. However, in ND and NDBEPR systems the residence times in the anoxic and aerobic zones are in the order of hours, and with respect to the electron transferring complexes of the ETP, neither the oxidases nor the reductases will be synthesized at their maximum levels. Consequently, the ETPs of both the filaments and floc-formers are conceptualized as having all the complexes present but at *lower* levels than under steady state anoxic and aerobic conditions, irrespective of the immediate conditions to which they are exposed. The ETPs of the floc-formers and filaments

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[2]For the purposes of the model, it is assumed that the filaments and floc-formers switch from nitrate/nitrite as electron (acceptor) to oxygen as electron acceptor at about the same concentration of DO. However, equal justification could be given to the argument that as a consequence of the protrusion of the filaments from the floc into the bulk liquid, they react to a change in DO more rapidly than the floc-formers (oxygen at low concentrations may not penetrate into the floc).

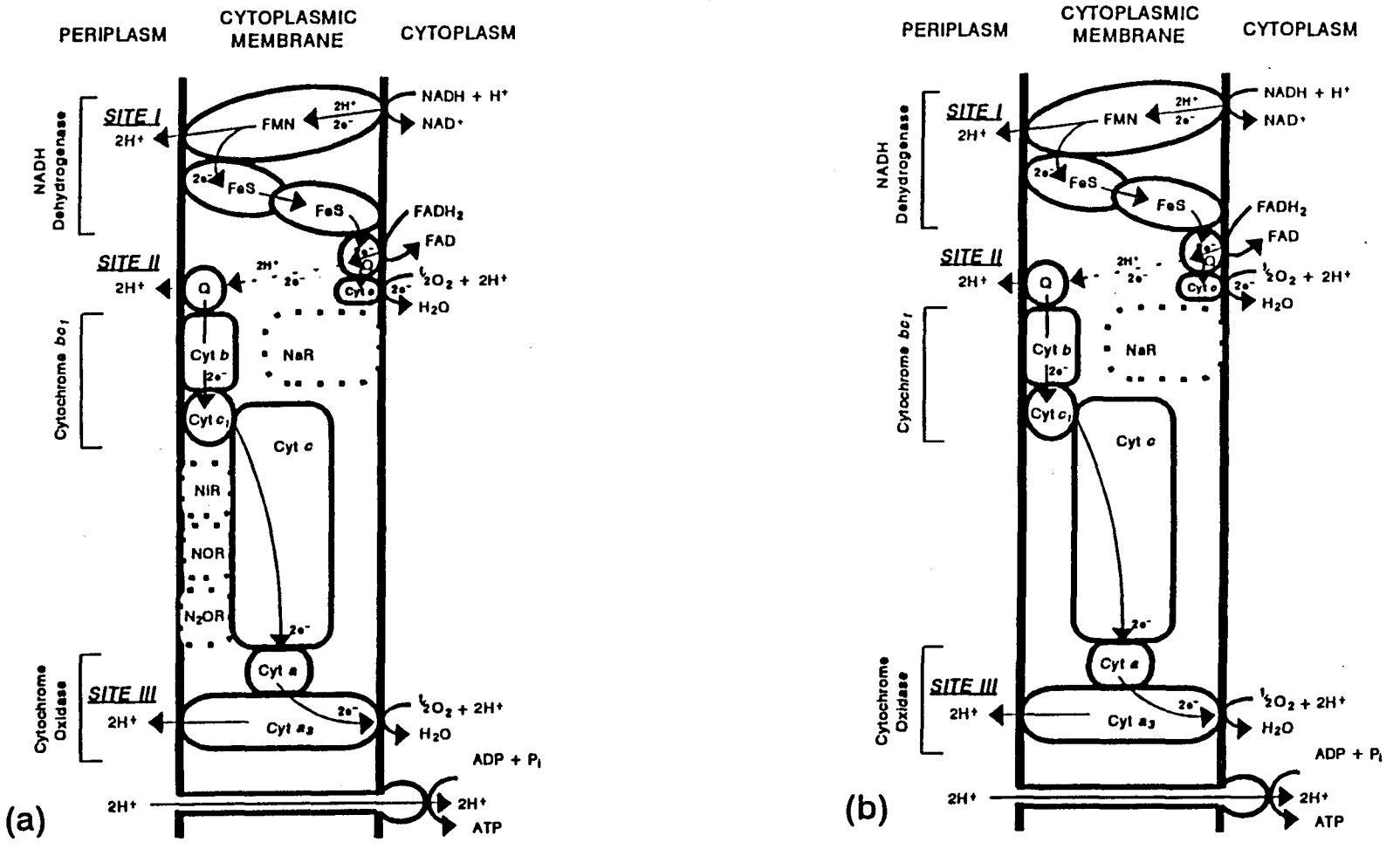


Fig 6.2: Models of the electron transport pathway (ETP) for (a) floc-forming and (b) filamentous organisms under steady-state aerobic conditions. [Abbreviations: NAD – nicotinamide adenine dinucleotide; FMN – flavomononucleotide; FeS – iron-sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone; Cyt – Cytochrome; NaR – Nitrate reductase; NiR – Nitrite reductase; NOR – Nitric oxide reductase; N_2OR – Nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate.]

are illustrated in Figs 6.3a and 6.3b respectively for conditions under which filaments proliferate, i.e. 30 to 40 percent aerobic. The complexes are present at a level intermediate between the levels under steady–state aerobic and steady–state anoxic conditions, depending on the proportion of exposure of the organisms to aerobic and anoxic conditions. For the denitrifying enzymes, the level of each of the nitrogen oxide reductases increases with time under anoxic conditions and decreases with time under aerobic conditions. For the oxidases under anoxic conditions, cytochrome aa_3 decreases and cytochrome *o* increases, and under aerobic conditions, cytochrome aa_3 increases and cytochrome *o* decreases.

Activity of enzymes of the ETP

Given that the level of the enzymes does not vary considerably during exposure to relatively short (hours) alternating anoxic–aerobic conditions, low or negligible rates of nitrogen oxide reduction under aerobic conditions are a consequence of inactivation of the reductases by oxygen. Activation of the reductases occurs in the absence of, or at low concentrations of oxygen. This applies to both filaments and floc–formers.

Biodegradability of substrate available under anoxic and aerobic conditions

For long sludge age ND and NDBEPR systems fed normal municipal substrate, by far the majority of substrate available under aerobic and anoxic conditions is slowly biodegradable COD (SBCOD). As noted in Chapter 5, this has implications with respect to intracellular accumulation of nitric oxide generated by denitrification of nitrite under anoxic and aerobic conditions. Nitric oxide does not accumulate through denitrification with readily biodegradable substrate under anoxic or aerobic conditions but does accumulate through denitrification with slowly biodegradable substrate under both conditions.

Inhibition of floc–former substrate utilization under aerobic conditions

Of particular interest to this model is a mechanism described at length in Chapter 5; when facultative organisms are subjected to a change from anoxic to aerobic conditions with nitrite present, they are inhibited in their utilization of substrate by accumulated intracellular nitric oxide. It is not the intention to again describe in detail the biochemical mechanisms which contribute to inhibition, but rather to indicate the applicability of the mechanisms of inhibition of substrate utilization under aerobic conditions to filamentous and floc–forming organisms.

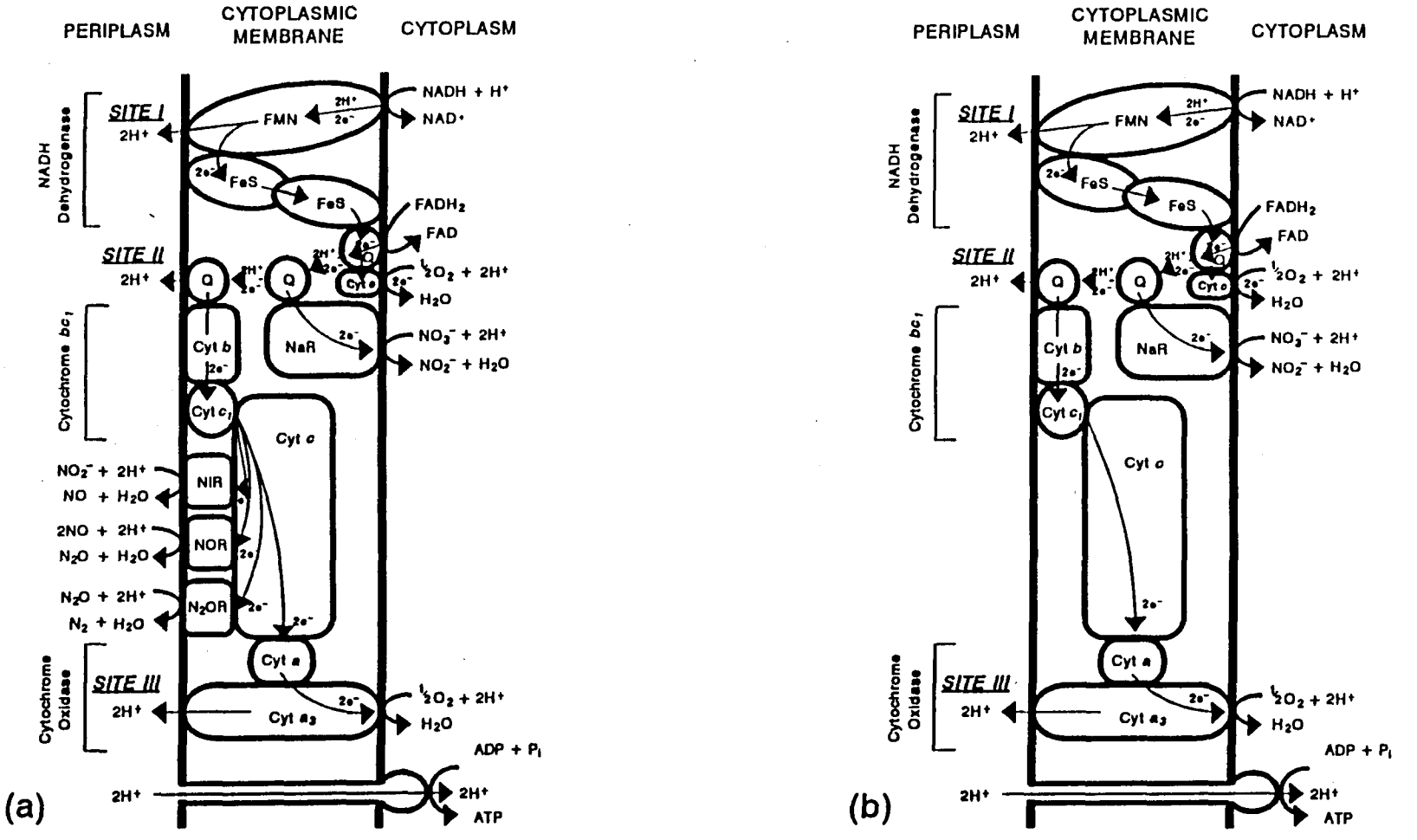


Fig 6.3: Models of the electron transport pathway (ETP) for (a) floc-forming and (b) filamentous organisms under conditions of alternation between anoxic (60–70%) and aerobic (30–40%) conditions. [Abbreviations: NAD – nicotinamide adenine dinucleotide; FMN – flavomononucleotide; FeS – iron–sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone; Cyt – Cytochrome; NaR – Nitrate reductase; NiR – Nitrite reductase; NOR – Nitric oxide reductase; N_2OR – Nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate.]

The mechanism for inhibition of substrate utilization under aerobic conditions by nitric oxide is applicable to floc–formers, but not to filaments. This is a consequence of the postulate that under anoxic conditions which precede aerobic conditions, floc–formers denitrify nitrate to the end–product dinitrogen through each of the intracellular denitrification intermediates, nitrite, nitric oxide, and nitrous oxide, with nitric oxide accumulating with utilization of slowly biodegradable substrate. Under subsequent aerobic conditions, intracellular nitric oxide inhibits aerobic respiration. In the biochemical model, facultative organisms inhibited in aerobic respiration transfer electrons to the denitrification pathway and denitrify nitrite under aerobic conditions. (As indicated in the biochemical model, nitrate is not denitrified under aerobic conditions due to impermeability of the cytoplasmic membrane to nitrate, preventing access to its reductase). Similarly, floc–formers inhibited in aerobic respiration denitrify nitrite via the same mechanisms, with an accompanying reduction in substrate utilization rate and energetic yield with nitrite as electron acceptor to that with oxygen as electron acceptor. In contrast to floc–formers, under the same conditions, filaments reduce nitrate to the end–product nitrite, and do not produce intracellular nitric oxide. Consequently, under subsequent aerobic conditions, aerobic respiration in filaments is not inhibited providing them with an advantage in competition with the floc–formers for substrate under aerobic conditions.

As described in the biochemical model for facultative respiration, a requirement for inhibition of aerobic respiration is the presence of intracellular nitric oxide during a change from anoxic to aerobic conditions. *However intracellular nitric oxide cannot be directly or easily monitored; since nitrite is the precursor to nitric oxide in the denitrification pathway, in the bulking model, nitrite is used as an indicator of the presence of nitric oxide.* In the absence of nitrite, during a change from anoxic to aerobic conditions, floc–formers are not inhibited and compete for substrate, such that filaments gain no advantage under aerobic conditions. The biochemical interactions which take place during a change to aerobic conditions from anoxic conditions in which nitrite is present or absent are described extensively in the biochemical model for facultative organism respiration and apply directly to floc–formers. A requirement to maintain inhibition under aerobic conditions is the presence of nitrite in the aerobic zone and the conditions which cause nitrite to be present at concentrations sufficient to induce inhibition of respiration and to maintain inhibition are described below.

Cause of high nitrite concentrations

A condition necessary for filament proliferation is a concentration of extracellular nitrite under anoxic conditions adequate to establish an intracellular concentration of nitrite and through reduction, of nitric oxide in floc-formers under anoxic conditions, such that during a change in conditions from anoxic to aerobic the floc-former's cytochrome oxidase activity is inhibited. To maintain inhibition under the aerobic conditions, a sufficient concentration of extracellular nitrite (> 1,0 mgNO_2-N/ℓ) is required to maintain the intracellular concentration of nitric oxide (from aerobic denitrification) at a level at which inhibition of oxidase activity is maintained.

The objective of this section is to postulate causes for the high nitrite concentrations (> 1 mgNO_2-N/ℓ) measured in the aerobic zones of ND and NDBEPR systems.

In anoxic-aerobic activated sludge systems there are two sources of nitrite. In the anoxic zone, nitrite is the product of the reduction of nitrate by nitrate reducing (filamentous) and denitrifying (floc-forming) organisms. In the aerobic zone, nitrite is the product of nitrification of ammonium by the group of nitrifying organisms, *Nitrosomonas.*

Concerning the source of nitrite under anoxic conditions, a common perception is that under steady-state anoxic conditions the rate of nitrite reduction is equal to the rate of nitrite production (such that nitrite does not accumulate), a perception based on the results of denitrification batch tests with high concentrations of nitrate (> 15 mg NO_3-N/ℓ) in which nitrite remains at relatively low concentrations (< 2 mg NO_2-N/ℓ). It was established in Chapter 4 that for denitrifying organisms under anoxic conditions subjected to aerobic conditions, oxygen affects the synthesis of the reductases in the order, nitrous oxide -, nitric oxide -, nitrite -, and then nitrate reductase. Thus, for denitrifying organisms subjected to frequent changes between anoxic and aerobic conditions, the nitrogen oxide reductases are not synthesized to the levels achieved under steady-state anoxic conditions; this effect is least pronounced for nitrate reductase, but is increasingly more pronounced for each successive reductase from nitrate reductase to nitrous oxide reductase. Thus, under the anoxic conditions of alternating anoxic-aerobic systems, nitrate reductase is present at a level closer to its anoxic steady-state level than is nitrite reductase resulting in an increase in nitrite concentration during reduction of nitrate under each successive anoxic condition.

Concerning the source of nitrite under aerobic conditions, it is well established that under steady–state aerobic conditions in which ammonium is not limiting, the rate of nitrification of nitrite to nitrate by *Nitrobacter* is somewhat faster than the rate of nitrification of ammonium to nitrite by *Nitrosomonas* and nitrite does not accumulate. However, a finding of the review of nitrification in Appendix E is that unaerated (anoxic or anaerobic) conditions inactivate the two groups of nitrifying organisms *Nitrosomonas* and *Nitrobacter* such that their rates of oxidation are reduced under subsequent aerobic conditions, and of the two groups, *Nitrobacter* are more sensitive to low concentrations of DO or the absence of DO than *Nitrosomonas*. As a consequence, nitrite is not nitrified to nitrate at the same rate as it is produced and it accumulates under each successive aerobic condition of the alternating anoxic–aerobic conditions.

Conceivably, both production of nitrite under aerobic conditions, and production of nitrite under anoxic conditions can contribute to the pool of extracellular nitrite in systems with alternating anoxic–aerobic conditions, and it is unclear as to which source is the cause of high concentrations of nitrite frequently observed in these systems. However given the significant intolerance of the nitrifying organisms to changes in other environmental conditions such as temperature, pH, and toxins, it would seem likely that the nitrifying organisms, more so than the denitrifying organisms are responsible for high nitrite concentrations.

6.4 MODEL EVALUATION AND EXPERIMENTAL TESTING

In evaluating the model, two avenues of investigation are employed; (1) the implications of the model are experimentally tested to determine the general validity of the principles of the model, (2) the model is tested against experimental observations made during the exploratory phase of the experimental programme to determine its general applicability.

(1) Examination of model implications

A difference in the relative extents of reduction of nitrate by floc–formers and filaments

If floc–formers denitrify nitrate through each of the denitrification intermediates to the end–product dinitrogen, and filaments reduce nitrate to the end–product nitrite, then under anoxic conditions a sludge containing a high proportion of filaments (high DSVI) should accumulate nitrite, but a sludge containing a high proportion of floc–formers (low DSVI) should produce greater quantities of dinitrogen.

Sludge samples from a fully anoxic system (System 7, Day 255) with a low DSVI and a 2RND system (70% unaerated mass fraction) (System 9, Day 228) with a high DSVI, both fed municipal sewage, were subjected to a nitrate reduction test, a test which determines the generation of NO_2^- and/or N_2 from reduction and denitrification respectively of NO_3^-. The sample with the high DSVI (many filaments) accumulated NO_2^-, but no N_2 was detected in 8 out of 10 tests. The sample with the low DSVI (few filaments) accumulated N_2 but no NO_2^- in 8 out of 10 tests. The results are shown in Table 6.1. The results were interpreted as supportive of the hypothesis that the denitrification pathway is mediated to different extents by filaments ($NO_3^- \rightarrow NO_2^-$) and floc-formers ($NO_3^- \rightarrow N_2$). However, the results *could* be interpreted as indicative of the parent system conditions, in that sludges developed in systems with high unaerated mass fractions (i.e. fully anoxic systems) would develop a greater proportion of denitrifying organisms than systems with lower unaerated mass fractions (i.e. the anoxic-aerobic 2RND system), such that the extent of formation of N_2 from NO_3^- would be increased both in the parent system and in the nitrate reduction test. However, in neither of the two parent systems was the generated NO_2^- mass greater than 10% of the NO_3^- mass denitrified to N_2, indicating that the unaerated mass fraction has little effect on whether NO_2^- accumulates through incomplete denitrification of NO_3^-.

Inhibition of floc-former respiration and filament proliferation results in reduced cell yield

Inhibition of floc-former aerobic respiration results in aerobic denitrification of nitrite. Substrate utilization through denitrification produces two-thirds the energetic yield of aerobic substrate utilization. This should result in a lower cell yield for floc-formers and a lower nett sludge mass production.

Experiments were not conducted expressly to investigate this aspect. However in examining the results of systems in which a high DSVI developed (floc-formers inhibited) (Systems 12 and 13), the relationship between DSVI and VSS was investigated. This relationship is examined in (2) below.

Effect of presence and absence of nitrite on proliferation of filaments

Under alternating anoxic-aerobic conditions, the presence of nitrite during a change from the anoxic to the aerobic zone should result in the proliferation of filaments, whereas the absence of nitrite under the same conditions should result in reduced filament proliferation.

Table 6.1: Results of nitrate reduction tests conducted on a sludge sample from a continuous anoxic system (System 7, Day 255) with a low DSVI, and an intermittent aeration system (System 9, Day 228) with high DSVI.

NITRATE REDUCTION TESTS					
System 7 Continuous anoxic Day 255	NO_2^-	$N_2(g)$	System 9 MLE 70% unaerated Day 228	NO_2^-	$N_2(g)$
1	-	+	1	+	-
2	+(weak)	+(weak)	2	+	-
3	-	+	3	+	-
4	-	+	4	+	-
5	-	+	5	+	-
6	-	+	6	+	-
7	-	+	7	-	+
8	-	+	8	+	-
9	+	-	9	+	-
10	+	-	10	-	+

To study the effect of nitrite on filament proliferation, two identical MUCT configurations were set up; System 14 was operated as a control, with a high TKN/COD ratio ≈ 0,13 mgN/mgCOD (NH_4^+ added to the influent), System 15 was operated with a low TKN/COD ratio ≈ 0,09 mgN/mgCOD (no NH_4^+ added to the influent). In System 14, the nitrate concentration in the 2nd anoxic reactor ranged between 0,5 and 1,5 mgNO_2-N/ℓ/ℓ and the DSVI between 200 and 250 mℓ/g. For System 15 with the low TKN/COD ratio, the nitrate concentration in the 2nd anoxic reactor ranged between zero and 0,4 mgN/ℓ and the DSVI between 100 and 150 mℓ/g (see Fig 6.4). After 62 days operation, nitrite was added by continuous drip feeding to the 2nd anoxic reactor of System 15. The nitrite concentration in the 2nd anoxic reactor increased to a high but variable value (1–10 mgN/ℓ) and the DSVI increased rapidly to between 200 and 230 mℓ/g. After 35 days, nitrite addition was terminated; the nitrite concentration in the 2nd anoxic reactor reduced to ≈ 0,3 mgNO_2-N/ℓ and the DSVI declined within 3 days to 170 mℓ/g and within 25 days to 150 mℓ/g. The dominant filament identified in the systems was type 0092. Similar experiments into the role in bulking of the ionic nitrogen oxides, nitrate and nitrite were conducted by Musvoto *et al.* (1992). These experiments are described in Chapter 3 in examination of the relative roles of nitrite and nitrate on filament proliferation, but because of their applicability at this point, the results are again described briefly. Nitrate addition to the 2nd anoxic reactor resulted in an increase in DSVI, and removal of nitrate addition resulted in a decrease in DSVI. Nitrite addition to the 2nd anoxic reactor resulted in an increase in DSVI at a rate considerably faster than with nitrate addition. In the experiments conducted by Musvoto *et al.* (1992) and in the experiments described above, the increase in DSVI was a consequence of the proliferation of filament type 0092. In comparing the results of the experiments described above with those of Musvoto *et al.* (1992) with nitrite addition, an interesting finding was made. In the experiments described above the unaerated mass fraction (66%) was the optimum for filament proliferation, the rate of increase in DSVI was rapid, and the maximum DSVI was relatively high (230 mℓ/g). For the experiments of Musvoto, the unaerated mass fraction was very large (80%), the rate of increase in DSVI with nitrite added was relatively slow and the maximum DSVI attained (170 mℓ/g) was considerably less than for the experiments described earlier. In terms of the model, the smaller aerobic mass fraction in the experiments of Musvoto *et al.* (1992) restricted the advantage gained by the filaments at the expense of inhibited floc-formers under aerobic conditions due to the smaller aerobic residence time.

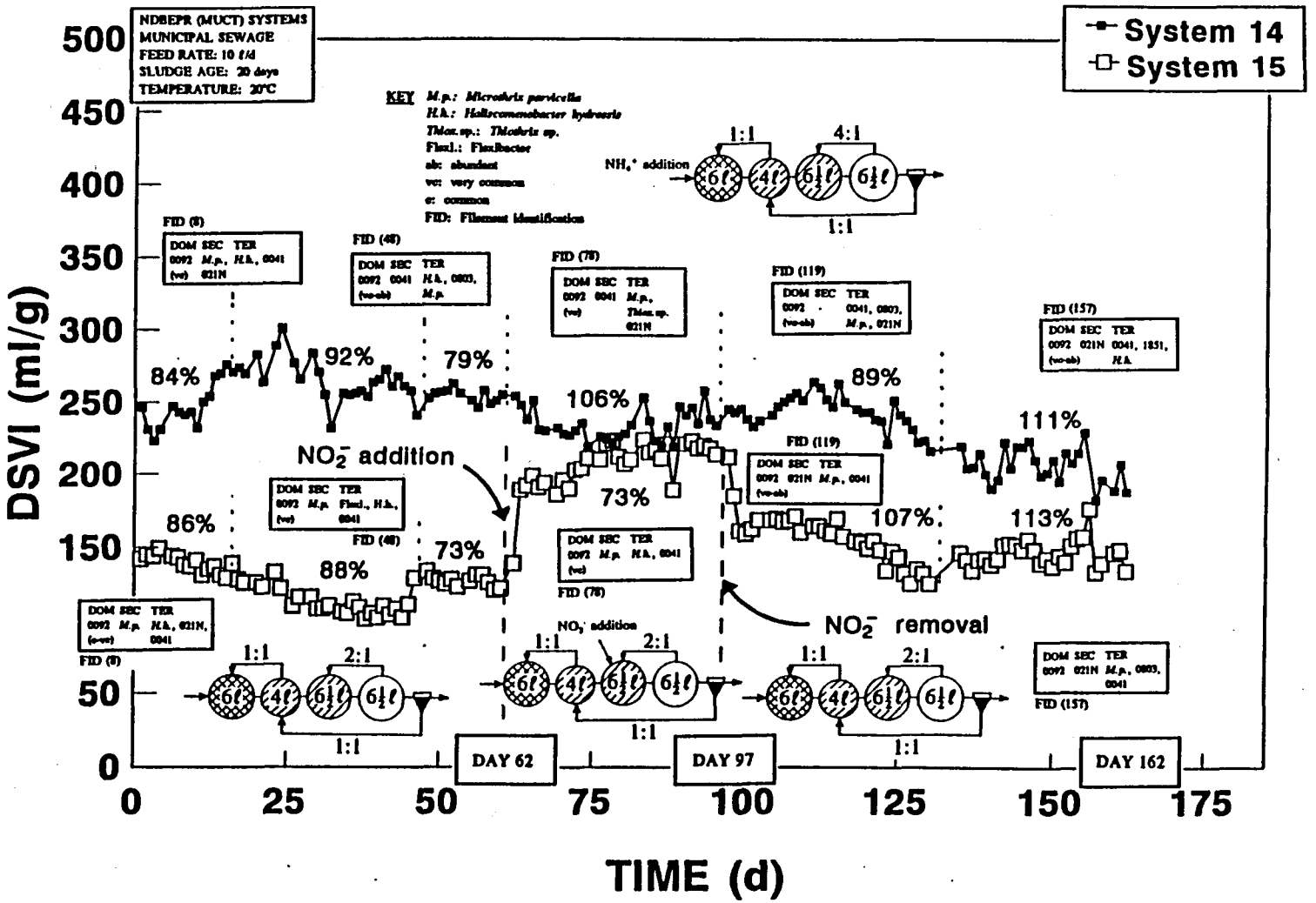


Fig 6.4: Sludge settleability as dilute sludge volume index (DSVI in mℓ/g) with time (h) for System 14 (NH_4^+ addition to influent) and System 15 (NO_2^- addition to 2nd anoxic reactor).

(2) Application of the model to experimental results

In Chapter 3 a wide-ranging exploratory experimental programme was undertaken in which a diversity of experimental configurations, aeration patterns and substrates were examined. In order to promote confidence in the general applicability of the model, it is necessary to demonstrate that the results of the experimental programme conform to the expectations of the model. The results of the experimental programme against which the model is tested, and the results of application of the model to the experimental results are described below.

Aeration pattern and anoxic/aerobic mass fractions

(i) *Continuous aerobic and continuous anoxic conditions resulted in low DSVI (< 150 mℓ/g) values and systems incorporating 30 to 40 percent aerobic mass fraction developed high DSVI (> 150 mℓ/g) values (System 2):* In the exploratory experimental programme of Chapter 3, a relationship was established for System 2 between DSVI and aerobic mass fraction during steady-state operation. The relationship is illustrated in Fig 6.5. For aerobic mass fractions decreasing from 30 percent, the DSVI decreases. At aerobic mass fractions between 30 and 40 percent the DSVI attains maximum values, and at aerobic mass fractions increasingly greater than 40 percent the DSVI decreases.

At low aerobic mass fractions (low DSVI), the large anoxic mass fraction (> 70 percent) has three effects. Firstly, it provides a denitrification potential in excess of that required for the nitrate and nitrite produced in the system (for an influent TKN/COD ratio < 1,0 mgN/mgCOD), and nitrate and nitrite are not present at the end of the anoxic zone of intermittently aerated systems, or in the anoxic reactor of 2-reactor, or multi-reactor systems. Thus, intracellular nitric oxide is not present and the floc-formers are not inhibited under subsequent aerobic conditions. Secondly, if nitrite is present under anoxic conditions, or if the floc-formers do contain intracellular nitric oxide and are inhibited when subjected to aerobic conditions, the small aerobic residence time (< 30 percent of the total residence time) does not allow the filaments sufficient time to gain an advantage. Thirdly, at high anoxic mass fractions (> 70 percent), the reductases are synthesized to a greater extent than at lower anoxic mass fractions and the difference between the levels of each of the reductases and their steady-state anoxic levels is small. Consequently, little nitric oxide or nitrite accumulate intracellularly,

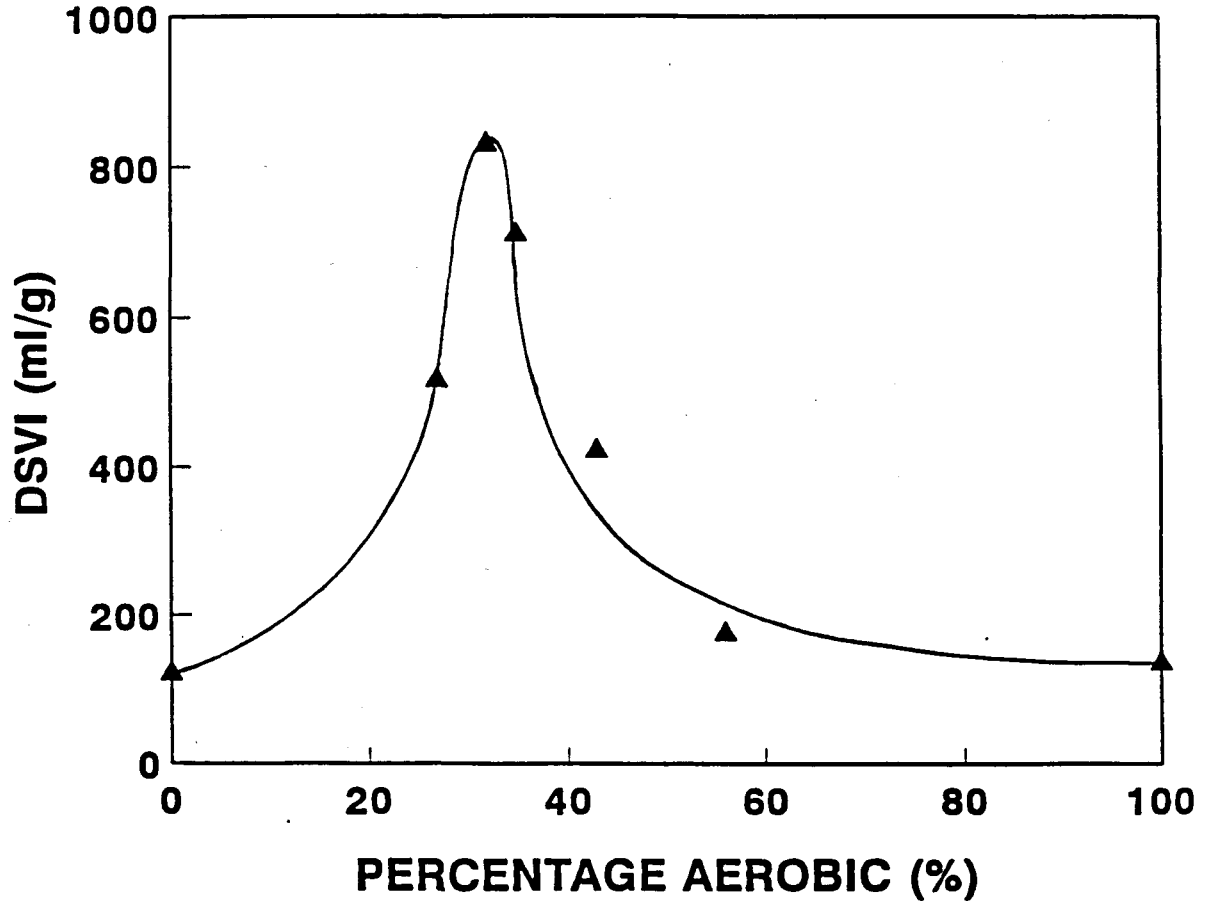


Fig 6.5: Sludge settleability as dilute sludge volume index (DSVI in mℓ/g) with percentage aerobic mass fraction for steady–state periods of System 2.

and little inhibition is induced.

At high aerobic mass fractions (low DSVI) the denitrifying enzymes are synthesized at a low level, little nitric oxide is produced and there is little interaction between nitric oxide and the oxidases.

At 30 to 40 percent aerobic mass fraction, the denitrification potential of the system is exceeded by the nitrate and nitrite load, the denitrifying reductases are synthesized at sufficiently high levels such that intracellular nitric oxide accumulates, and the oxidases (especially cytochrome *o*) are synthesized at sufficiently high levels such that sufficient interaction occurs between nitric oxide and the oxidases to inhibit floc–former aerobic respiration, allowing filament proliferation.

(ii) *With continuous aerobic low DO (0,2 < DO < 0,5) conditions, low DSVI (<100 mℓ/g) values developed (System 5, Days 62 to 94; System 6, Days 95 to 152):* Under conditions of low continuous DO, both of the oxidases are synthesized at an intermediate level, as are the reductases. For the low DO conditions, it is assumed that both filaments and floc–formers respire aerobically (i.e. with oxygen as electron acceptor). Interaction between nitric oxide and the oxidases inhibits floc–former aerobic respiration causing redirection of electrons to the denitrification pathway; under normal high DO conditions, anoxic respiration would result in a reduced yield. However the low concentrations of oxygen (in which oxygen is limiting) do not provide an aerobic yield to filaments much greater than is provided by anoxic respiration to floc–formers. Thus floc–formers are not out competed and low DSVIs result.

(iii) *With intermittent aeration conditions, the higher the DO the higher the DSVI (System 5, Days 95 to 149, Days 150 to 180, and Days 1 to 61):* For intermittent aeration with low DO (0,2 < DO < 0,5 mgO/ℓ) in the aerobic period, a low DSVI results for the same reasons as for continuous low DO systems described above; i.e. the low DO concentration (in which oxygen is limiting) does not give the aerobically respiring filaments a large advantage over the floc–formers which as a consequence of aerobic inhibition respire with nitrite as electron acceptor. For intermittent aeration systems with increasingly higher DO, floc–formers continue to be inhibited, but filaments

gain an increasing advantage in respiring under aerobic conditions because oxygen is not limiting. This allows filaments to proliferate to a greater degree at increasingly higher DO concentrations in the aerobic zone of intermittently aerated systems.

Substrate biodegradability

(i) *RBCOD fed throughout the intermittent aeration sequence resulted in high DSVI values (System 7, Days 63 to 94; System 8, Days 95 to 127):* This result was unexpected since the rate of supply of electrons from RBCOD should be sufficient to remove any accumulated intracellular nitric oxide. When RBCOD was fed to System 8 the DSVI initially decreased rapidly as the growth of the AA filament *M. parvicella* was reduced, but the DSVI then increased due to *H. hydrossis*, a filament categorized as low DO, a category of filaments to which the model does not apply.

(ii) *SBCOD fed throughout the intermittent aeration sequence resulted in high DSVI values (System 7, Days 95 to 127, System 8, Days 64 to 94):* Under conditions of high nitrite concentration entering the aerobic zone, a low rate of supply of electrons from SBCOD would be insufficient to remove the intracellular accumulation and inhibitory effect of nitric oxide.

(iii) *RBCOD fed during the anoxic zone of the intermittent aeration sequence resulted in high DSVI values (System 7, Days 128 to 162), but RBCOD fed during the aerobic zone of the intermittent aeration sequence resulted in low DSVI values (System 8, Days 163 to 192):* For RBCOD fed during the anoxic zone only, in the aerobic zone, where the concentration of nitrite was > 1,0 mgN/ℓ, SBCOD was the only available substrate, and although the floc–formers were not inhibited upon entering the aerobic zone, the low rate of supply of electrons from SBCOD under aerobic conditions with nitrite present induced inhibition and a high DSVI resulted. For RBCOD fed during the aerobic zone only, with the high nitrite concentration (> 1,0 mgN/ℓ) in the anoxic zone with only SBCOD available, the floc–formers are inhibited. Upon entering the aerobic zone with RBCOD present, irrespective of the concentration of nitrite present, floc–former inhibition is relieved and they out–compete the filaments for substrate.

Frequency of alternation between aerobic and anoxic conditions

Ketley *et al.* (1991) demonstrated that in an anoxic–aerobic system, irrespective of the frequency of alternation of sludge between the zones (from 72 cycles/d to 1 cycle/3 days), the DSVI is high.

With nitrite and SBCOD present under aerobic conditions, the floc–formers are inhibited. Floc–former inhibition is maintained with time under aerobic conditions until the denitrifying enzymes producing and reducing nitric oxide, degrade, and the oxidase (cytochrome aa_3) which is only partly inhibited is fully synthesized, i.e. 1 to 3 sludge ages. Thus, under conditions in which the sludge is subjected to alternating anoxic–aerobic conditions, unless the aerobic period is longer than the time required to synthesize cytochrome aa_3, inhibition of floc–formers is maintained and bulking results.

Relationship between sludge settleability (in DSVI), nitrite concentration in the 2nd anoxic reactor, volatile suspended solids (VSS) and nitrogen mass balances

In experiments conducted on Systems 12 and 13 to examine the effect on DSVI of ammonium addition to the influent, increases and decreases in nitrite concentration in the 2nd anoxic reactor were accompanied by increases and decreases respectively in DSVI (see Chapter 3). An unexpected result in these systems was that when the 2nd anoxic nitrite concentration and DSVI increased, the VSS decreased, and when the 2nd anoxic nitrite concentration and DSVI decreased, the VSS increased. For Systems 14 and 15 described above which examined directly the effect of nitrite concentration in the 2nd anoxic reactor, similar results were noted and are illustrated in Fig 6.6 and Fig 6.7 respectively. In addition, for each of the System pairs, 10 and 11, 12 and 13, and 14 and 15, during periods of low or decreasing DSVI, the nitrogen mass balance was generally higher than during periods of high or increasing DSVI (see Fig 3.13, Fig 3.16, and Fig 6.4 respectively). This relationship is particularly noticeable for System 15 during the period of nitrite addition (Days 63 to 97), when the DSVI was at its highest ($\approx$ 225 mℓ/g) and the nitrogen balance was at its lowest (73%); in the corresponding period in System 14, the DSVI was at its lowest to that time ($\approx$ 220 mℓ/g), and the nitrogen balance was at its highest (106%). Because COD mass balances were not measured for Systems 12 to 15, no relationship between COD balance, sludge settleability, and the nitrogen mass balance could be determined. However, for Systems 5 to 8, although nitrogen mass balances were not conducted, a relationship was established between the COD mass balance and sludge settleability. For systems with high DSVIs (produced through

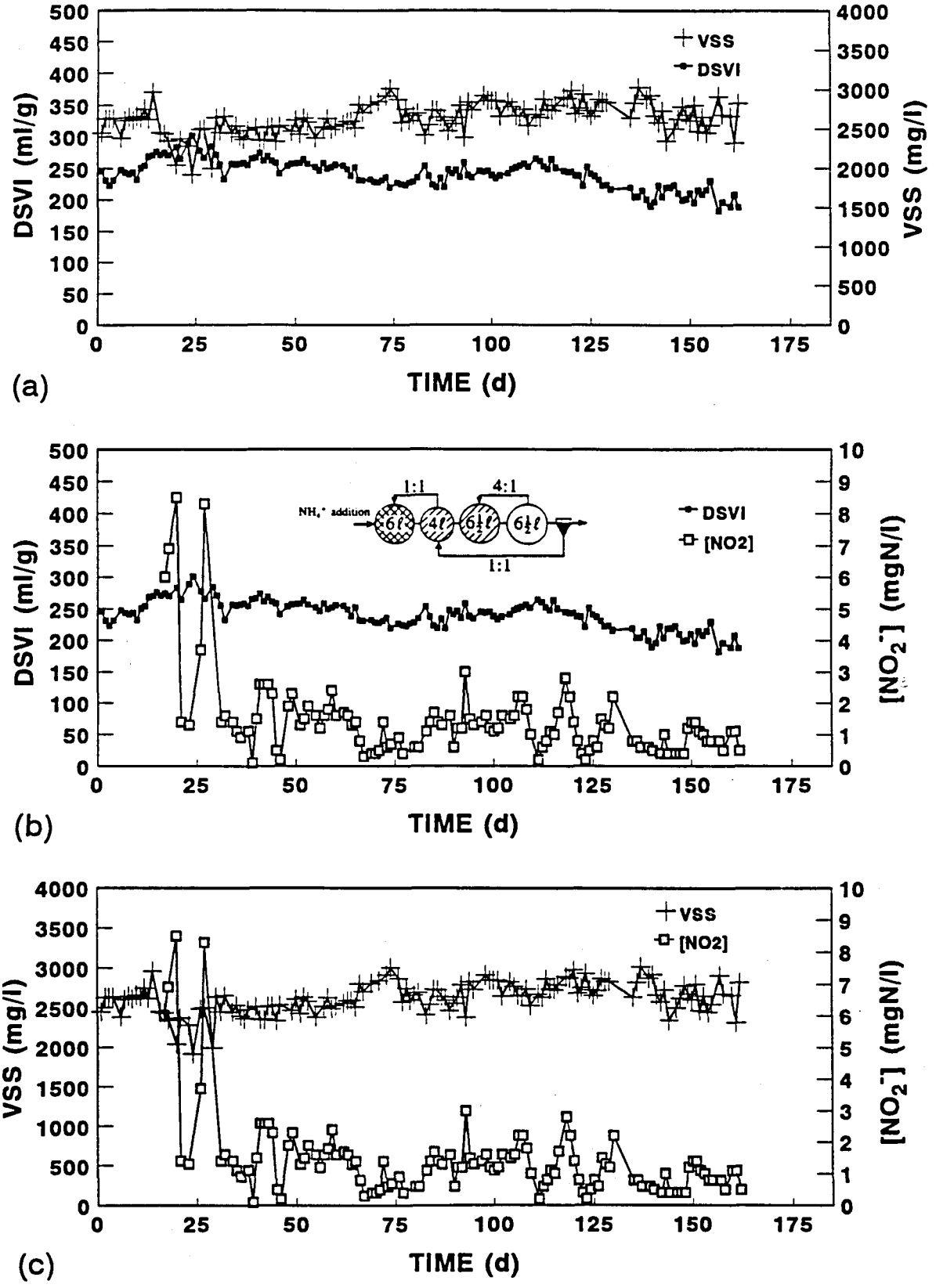


Fig 6.6: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor NO_2^- concentration with time, and (c) VSS and 2nd anoxic reactor NO_2^- concentration with time for System 14.

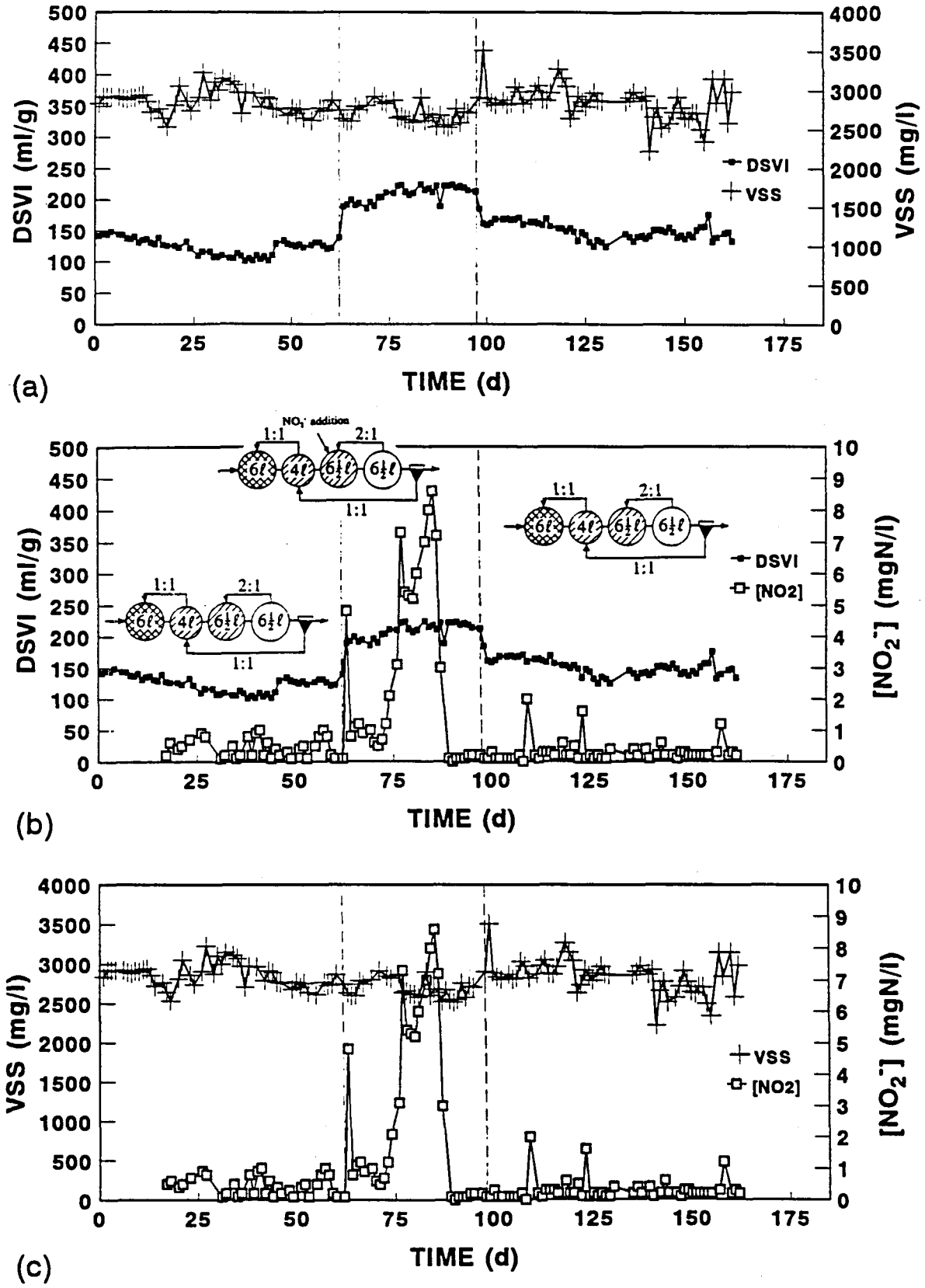


Fig 6.7: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor NO_2^- concentration with time, and (c) VSS and 2nd anoxic reactor NO_2^- concentration with time for System 15.

intermittent aeration) the COD mass balances were low, and for systems with low DSVIs (produced through continuous aeration) the COD mass balances were high. In summary, combining the results of multi-reactor systems (Systems 12 to 15) and single reactor systems (Systems 5 to 8) it can be concluded that: In systems in which high DSVI values develop (many filaments) (i.e. systems in which sludge is exposed to alternating anoxic-aerobic conditions), both the COD and nitrogen mass balances are low, and in systems in which low DSVI values develop (few filaments) (i.e. continuous aerobic systems), both the COD and nitrogen mass balances are high.

Concerning the relationship between VSS and nitrite concentration, interaction of nitric oxide with cytochrome oxidase results in redirection of electrons to nitrite reductase, and the resultant aerobic denitrification yields only two-thirds the energy of uninhibited aerobic respiration. The consequence is that production of sludge mass is reduced. Concerning the relationship between the high DSVIs produced by high nitrite concentrations, and the low COD and nitrogen mass balances which accompany high DSVIs, a consequence of aerobic denitrification of nitrite by floc-formers is that nitrogen and COD removed under aerobic conditions will be unaccounted for in the nitrogen and COD mass balances. Thus during periods of high or increasing DSVI, low nitrogen and COD mass balances are measured.

In summary, for sludge subjected to alternating anoxic-aerobic conditions with nitrite present, floc-former aerobic respiration is inhibited by interaction of nitric oxide with cytochrome oxidase, filaments proliferate, and the DSVI increases. Electrons (originating from COD) are then directed to nitrite reductase and under aerobic conditions nitrite is reduced. Because of the lower yield of respiration with nitrite, compared to respiration with oxygen, the VSS of the sludge is less than under conditions in which inhibition of floc-former aerobic respiration is not induced (i.e. under continuous aerobic conditions, or under alternating anoxic-aerobic conditions in which nitrite is absent). Under conditions in which floc-formers are not inhibited, electrons are not redirected to nitrite reductase and nitrite is not utilized under aerobic conditions, and the nitrogen and COD balances are high.

6.5 CONTROL PROCEDURES FOR AA FILAMENT BULKING

In summary, experimental examination of the implications of the model and application of the model to a range of experimental results provide support for the validity of the model. To assist in development of procedures for control of filament

proliferation, the major aspects of the model can be devolved to four main principles, which in combination result in filament proliferation:

(1) Alternating anoxic–aerobic conditions result in development of both the respiratory oxidases and the reductases in the facultative organisms, the floc–formers, and the filaments.

(2) The absence of high concentrations of readily biodegradable substrate under anoxic conditions results in the accumulation of intracellular nitric oxide in floc–formers during denitrification.

(3) Under aerobic conditions, accumulated nitric oxide results in inhibition of floc–formers, and high concentrations of nitrite under aerobic conditions maintain floc–former inhibition.

(4) Filamentous organisms do not produce nitric oxide and are not inhibited under aerobic conditions.

The ultimate objective in the investigation was to formulate a procedure by which AA filament bulking in ND and NDBEPR systems could be ameliorated. To this end, methods have been developed for avoiding, preventing or removing one or more of the conditions listed above, which in combination result in filamentous organism bulking. With reference to the principal aspects of the model listed above, attention is directed at points (2) and (3) and not (1) and (4), since regarding (1), a necessity for nitrogen removal is that sludge be exposed to a combination of aerobic (for nitrification) and anoxic (for denitrification) conditions and regarding (4), the physiology of the filaments (or floc–formers) cannot be altered. With reference to points (2) and (3) the bulking model proposes that if the nitrite concentration is reduced to near zero in the anoxic zone, or if intracellular nitric oxide is significantly reduced under aerobic or anoxic conditions, then the floc–formers will not be inhibited under aerobic conditions and significant bulking is unlikely to occur.

In a design situation, estimation of NO_2^- concentration is still uncertain as the denitrification kinetics of this parameter have not been well established for the activated sludge process. To prevent high concentrations of NO_2^- prior to the aerobic zone in ND and NDBEPR systems, the most appropriate solution at present

is to design the anoxic mass fraction/a- (aerobic–anoxic) recycle of the system such that the denitrification potential of the system is greater than the mass of NO_3^- and NO_2^- recycled to it, i.e. complete denitrification in the anoxic zones is achieved. This can be accomplished by; (i) increasing the unaerated mass fraction, or, (ii) reducing the a–recycle.

In the event that inflexible system configurations do not allow for reduction of the a–recycle, or unusual influent wastewater characteristics do not practically allow for removal of all NO_3^- and NO_2^- recycled to the anoxic reactor or zone, attention is directed not at the extracellular NO_2^-, but at intracellular NO, and provisions are made for its reduction under both aerobic and anoxic conditions. This can be accomplished by:

(i) Inclusion of a small aerobic reactor immediately upstream of the main aerobic reactor to which a fraction of the influent sewage is fed. From the biochemical model, intracellular NO is reduced rapidly under aerobic conditions with RBCOD and inhibition is not induced. Experiments with aerobic batch tests (see Chapter 5) in which inhibition is relieved in the presence of sufficient quantities of RBCOD offer support for this proposal which is currently under investigation but which is not described here.

(ii) Inclusion of a small anoxic reactor immediately upstream of the main aerobic reactor to which a fraction of the influent sewage is fed – from the biochemical model, intracellular NO is reduced rapidly under anoxic conditions with RBCOD and inhibition is not induced under subsequent aerobic conditions. This proposal has been investigated and the experiments are described below.

A denitrifying anoxic (DENOX) reactor comprising 4 percent of the total sludge mass of MUCT System 17 was added between the 2nd anoxic reactor and the aerobic reactor, and 10 percent of the influent feed was introduced into the DENOX reactor. The MUCT + DENOX configuration and changes in DSVI with time are illustrated in Fig 6.8. Between days 50 and 76, the DSVI decreased from > 150 mℓ/g to < 100 mℓ/g. During the same period, the DSVI of a control system, System 16, with ammonium added to the influent, increased from 160 mℓ/g to > 220 mℓ/g. Following removal of the DENOX reactor from System 17 and addition of the DENOX to System 16, the DSVIs of the two systems initially acted in

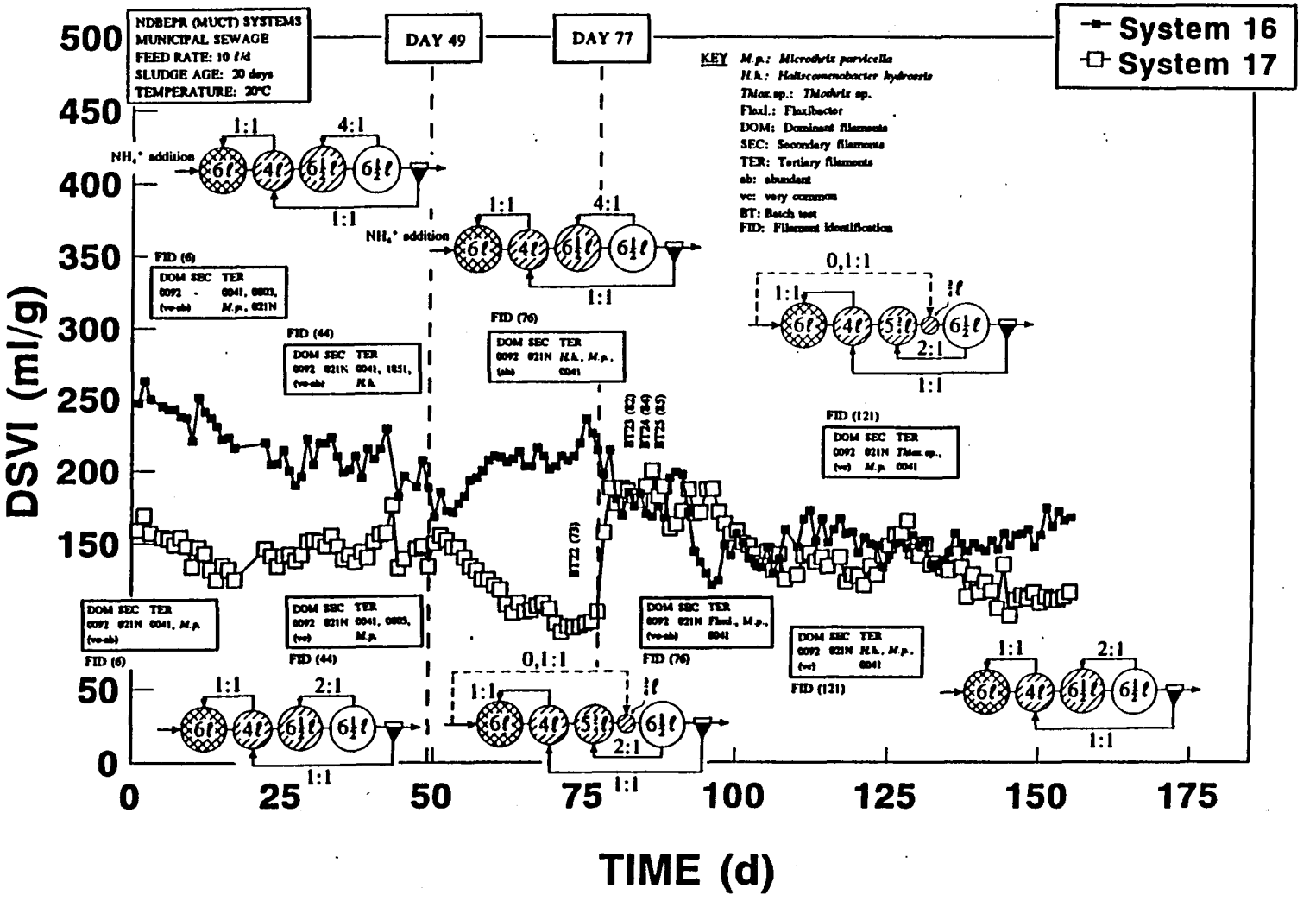


Fig 6.8: Dilute sludge volume index (DSVI in mℓ/g) for Systems 16 and 17 to which a small denitrifying anoxic (DENOX) reactor was added and removed.

accordance with expectation; the DSVI of System 17, now without the DENOX reactor, increased from < 100 mℓ/g to ≈ 170 mℓ/g in 20 days (up to day 97) and the DSVI of the System 16, incorporating the DENOX reactor, decreased from > 220 mℓ/g to ≈ 110 mℓ/g in 20 days. On Day 97 the DSVIs of the systems began to deviate from the expected behaviour. The DSVI of the DENOX system (System 16) increased to between 150 and 170 mℓ/g and the DSVI of the system without the DENOX reactor (System 17) decreased to ≈ 100 mℓ/g. At present, the causes of this deviant behaviour are unclear. However, from the experimental results obtained thus far, introduction of a small unaerated reactor between the anoxic and aerobic zones and addition of substrate containing an adequate supply of RBCOD to the small reactor appears to hold promise as a strategy for control of AA filaments.

As an aside: it should be noted that during testing of the DENOX reactor control procedure, relationships between the parameter DSVI, VSS, and nitrite concentration in the second anoxic reactor were the same as were found throughout the experimental programme; generally DSVI increased and VSS decreased with increase in nitrite concentration and DSVI decreased and VSS increased with decrease in nitrite concentration as illustrated in Fig 6.9 and Fig 6.10 for Systems 16 and 17 respectively.

6.6 CLOSURE

A wide–ranging experimental program (Chapter 3) investigating at laboratory–scale the problem of filamentous organism bulking (formerly low F/M bulking – now renamed AA bulking), resulted in the development of a biochemical model describing the fundamental biochemical mechanisms of facultative heterotrophic organisms (Chapter 5). Application of the biochemical model for facultative organism respiration to filamentous and floc–forming facultative organisms provided the means to develop a conceptual model that explains AA filamentous organism proliferation or non–proliferation. The model was evaluated against experimental data and shown to provide an explanation for observations relating to AA filament bulking. From the conceptual model, proposals for measures by which the bulking problem can be reduced or eliminated were identified and examined. Initial testing of the proposals produced encouraging results.

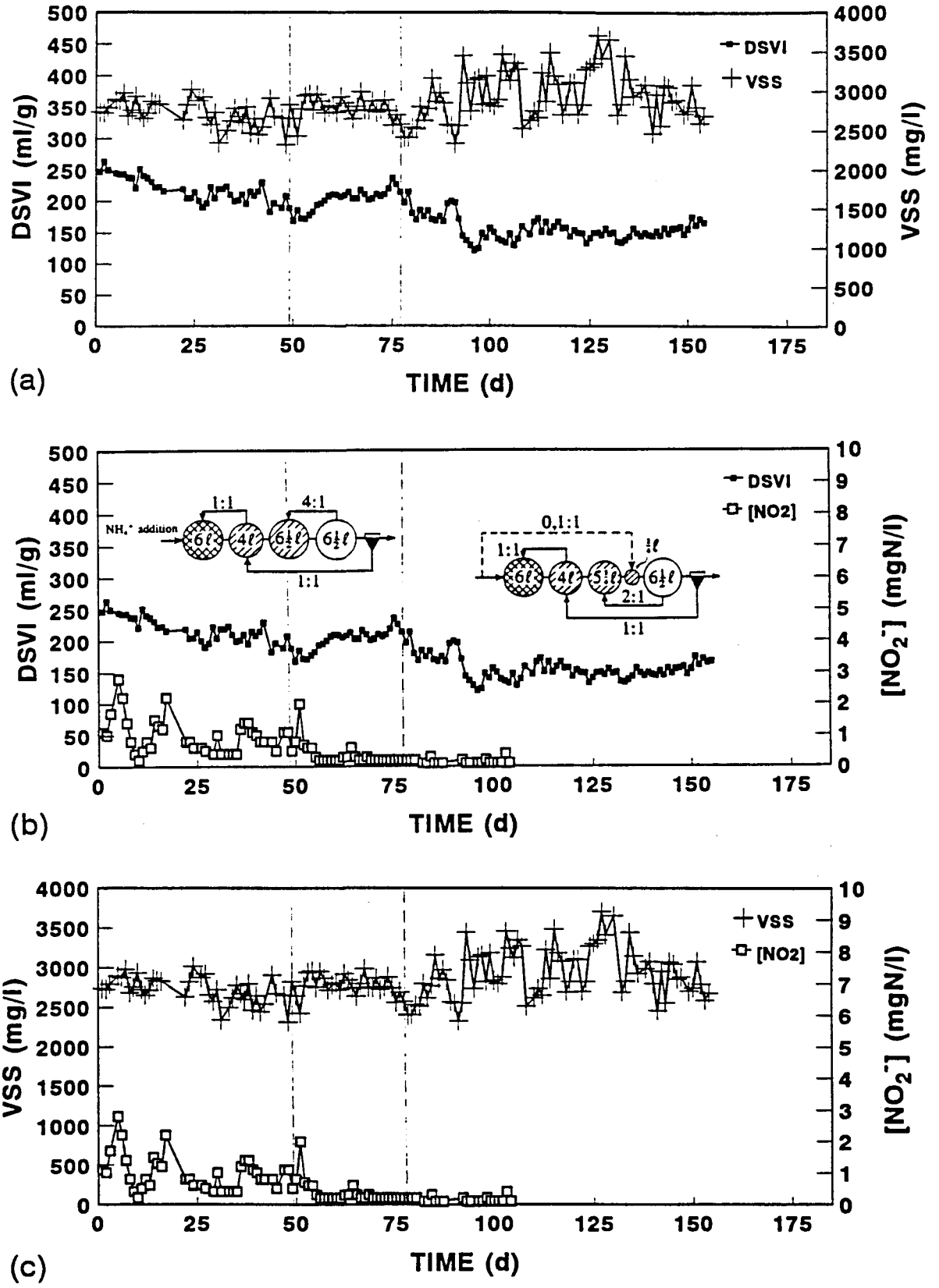


Fig 6.9: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor NO_2^- concentration with time, and (c) VSS and 2nd anoxic reactor NO_2^- concentration with time for System 16.

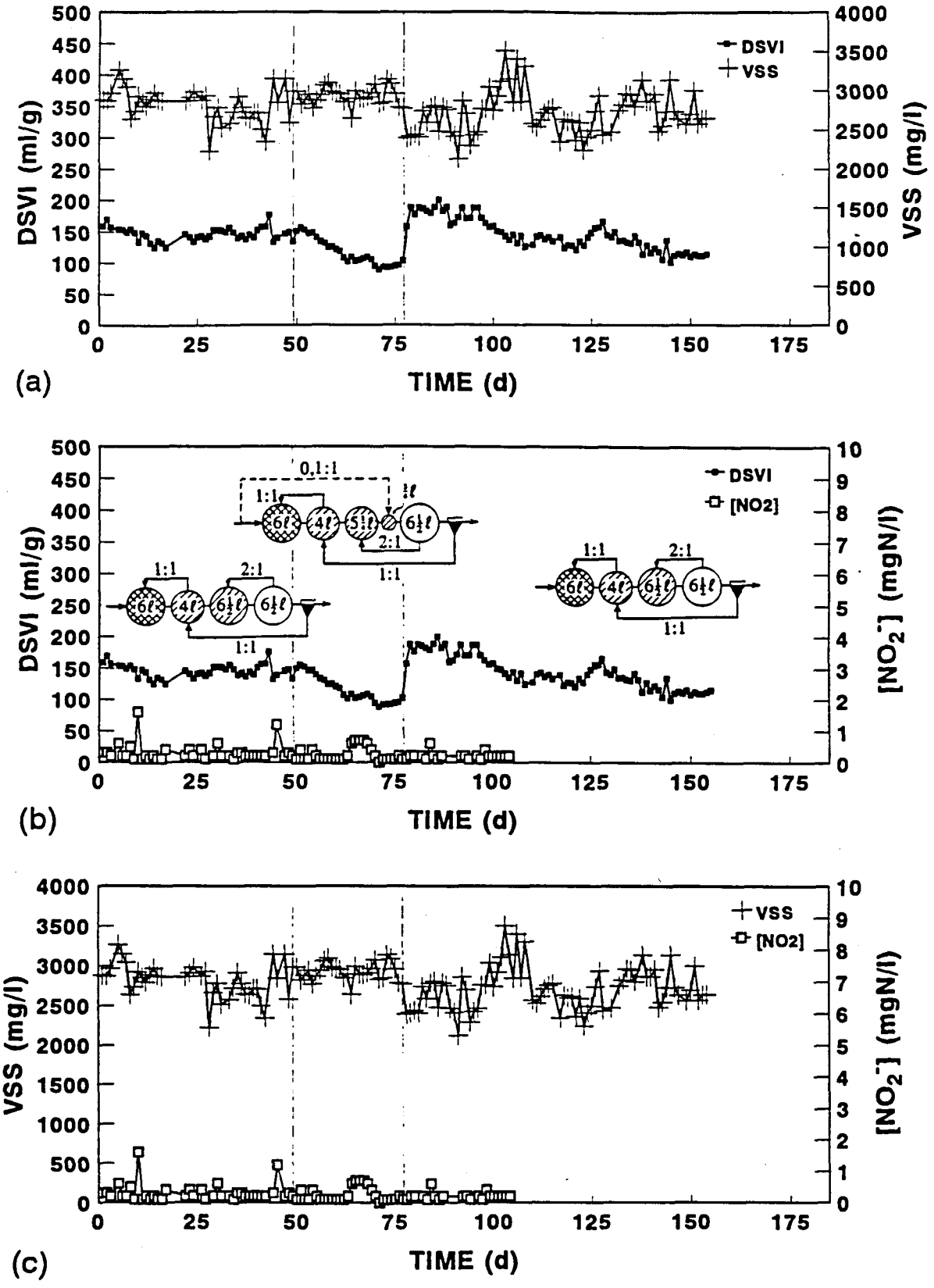


Fig 6.10: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor NO_2^- concentration with time, and (c) VSS and 2nd anoxic reactor NO_2^- concentration with time for System 17.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

The objective of the investigation was to determine the fundamental causes for the proliferation of low F/M filamentous organisms in long sludge age, nitrogen (N) and nutrient (N & P) removal systems.

In meeting this objective the investigation evolved in 4 sequential stages; viz., (1) a review of bulking problems and practices world-wide, (2) an exploratory experimental investigation to examine the effect of numerous factors on filament proliferation, (3) formulation and experimental testing of a conceptual biochemical model for facultative heterotrophic organism respiration, and (4) formulation and experimental testing of a conceptual microbiological model for proliferation of filamentous organisms.

(1) <u>Review of bulking</u>

- Procedures for specific control of filamentous organism bulking have suffered from lack of a single broadly applicable approach. Where procedures have been successful in specific cases, for example, with incorporation of selector reactors, application of the same procedures to other situations has often proven unsuccessful.

- The filamentous organism classification procedure of Jenkins *et al.*, (1984a) in identifying conditions giving rise to proliferation of specific organisms, has brought a measure of order to the field. However, the procedure has also proven somewhat inadequate in that filaments often proliferate under conditions not assigned to their classification. For example, in long sludge age systems, which support low F/M conditions, filaments not classified as low F/M often proliferate. Additionally, the procedure does not describe the fundamental biochemical and microbiological mechanisms which allow filaments to find a niche in activated sludge.

(2) <u>Exploratory experimental investigation</u>

- As a starting point to the experimental investigation, the use of a defined

artificial substrate provided a useful tool by which the different fractions of sewage could be manipulated and their effect on filament proliferation observed. The results indicate that defined substrates can be used to determine general responses of filaments and floc–formers to certain conditions and the requirements of filaments for proliferation (i.e. alternating anoxic–aerobic conditions in the correct proportions, with nitrite present). However, specific responses, such as the type of filaments which proliferate with municipal sewage and the extent of proliferation are not necessarily reproducible with defined substrate.

- Three factors were found to have a major influence on filament proliferation; (i) exposure to alternating anoxic–aerobic alteration, (ii) proportion of the mass fraction which is aerobic and anoxic, and (iii) concentration of nitrite and nitrate at the end of the anoxic zone, or in the anoxic reactor. As a consequence of the finding that filamentous organism types which normally proliferate in long sludge age (low F/M) plants (referred to as low F/M filaments), require exposure to alternating anoxic–aerobic conditions, these filaments were re–named anoxic–aerobic (AA) filaments.

- Laboratory–scale activated sludge systems fed municipal sewage serve as an excellent experimental means of examining filament proliferation. With due care given to regular cleaning of these systems to prevent the incursion of *Sphaerotilus natans*, type 021N, and *Thiothrix*, the same filament types develop in laboratory–scale systems as develop in similar full–scale systems (i.e., *M. parvicella* in ND systems, and type 0092 in NDBEPR systems), and the extent of proliferation is similar.

(3) <u>Biochemical model</u>

- A conceptual biochemical model was formulated to describe the respiratory mechanisms involved in heterotrophic facultative organism respiration. The biochemical model can explain experimentally determined changes in rates of aerobic and anoxic respiration when activated sludge is subjected to changes between aerobic and anoxic conditions.

- Although information concerning nitrate reductase and nitrite reductase is reasonably well documented in the literature, the available information regarding nitrous oxide reductase, nitric oxide reductase, cytochrome aa_3 and

cytochrome *o* is sparse, and more biochemical research is necessary to establish, (i) rates of synthesis and degradation of the reductases and oxidases under anoxic and aerobic conditions; (ii) placement of the reductases and oxidases with respect to the cytoplasmic membrane; (iii) mechanisms of nitrogen oxide transport to and from the reductases; (iv) mechanisms of nitrogen oxide reduction, and (v) mechanisms of interaction between nitric oxide and cytochromes *o* and aa_3.

- A method was described and tested by which indirect measurement could be made of the levels of synthesis of both the reductases and oxidases (aerobic inhibition batch tests) and the reductases only (anoxic denitrification batch tests) of facultative organisms in activated sludge subjected to changes between aerobic and anoxic conditions. The examination of this aspect was conducted on a cursory level only, and a more rigorous investigation is required, focusing in particular on the sequential production and removal of the denitrification intermediates produced by the reductases during changes between aerobic and anoxic conditions.

- The aerobic inhibition batch test method is a useful tool for determining the *potential* for filamentous organism growth via the *potential* for inhibition of floc-forming organisms through indication of the levels of their oxidases and reductases. However, filamentous organisms will proliferate in the parent system only in the presence of certain other conditions, (e.g. nitrite present prior to aerobic conditions and readily biodegradable substrate absent or at very low concentrations under aerobic conditions).

- A rapid analytical procedure is required to determine the level of aerobic and denitrifying enzymes in the mixed culture organisms of activated sludge (as opposed to procedures applicable to pure culture organisms) so as to establish the potential for inhibition of aerobic respiration and concomitant filament growth which results in bulking.

(4) <u>Microbiological/Biochemical (Bulking) model</u>

- The bulking model can explain the proliferation or not of AA filamentous organisms under essentially all the conditions to which sludge is subjected in nitrogen and nutrient removal activated sludge systems.

- The proposal of the bulking model, that floc–formers denitrify from nitrate to dinitrogen and filaments reduce nitrate to nitrite is based mainly on theoretical considerations. Microbiological studies to develop pure cultures of filaments are required to determine the relative extents of denitrification/nitrate reduction of the organisms.

- The bulking model provides a useful framework for evaluation of the effect on AA filament proliferation, of changes in (i) configuration modification, (ii) changes in wastewater composition, and (iii) changes in aeration pattern, particularly where the changes are introduced as a strategy for control of filaments.

- At present, the most promising strategy for control of AA filaments is to ensure that the denitrification potential of the anoxic reactor immediately upstream of the main aeration reactor is not exceeded (i.e. that nitrate and nitrite are not present in the mixed liquor for sludge passing from anoxic to aerobic conditions).

- Further experimental work is required to determine the efficacy of a control strategy in which a small denitrifying anoxic (DENOX) reactor fed a fraction of the influent is situated immediately upstream of the main aeration reactor to ensure complete denitrification of extracellular nitrate and nitrite, and intracellular nitrite and nitric oxide in floc–formers, prior to aerobic conditions.

- It is recommended that for systems with high DSVIs in which sludge is subjected to alternating anoxic–aerobic conditions with high nitrite concentrations, a control strategy be examined in which influent is fed to a small aerobic reactor situated immediately upstream of the main aeration reactor, as a means of aerobically denitrifying intracellular nitrite and nitric oxide, thereby preventing inhibition of aerobic respiration in floc–formers and resultant proliferation of filaments.

- The conceptual biochemical and bulking models are powerful tools in the hands of those acquainted with their development and application. Because of the complexity of the biochemical mechanisms upon which the models are based, a more useful tool would be a mathematical model for bulking by AA

filamentous organisms, which can be used in the design and operation of full-scale wastewater treatment facilities.

(5) General

- Historically, the philosophy governing attempts to control filament growth has been to *increase the growth rate of floc-formers* above that of filaments for the substrate concentrations found in activated sludge, through induction of the selector effect. In contrast the results of this investigation indicate that a more appropriate philosophy is to *remove the disadvantage to floc-former growth* which results from inhibition of aerobic respiration.

APPENDIX A

DEVELOPMENT OF AND EXPERIMENTS WITH A DEFINED ARTIFICIAL SUBSTRATE

Appendix A describes the motivation behind the development and use of a defined artificial substrate. The defined substrate was used in Part I of the experimental investigation described in Chapter 3. The results of all the experimental work including daily experimental data, aerobic batch tests, COD and Nitrogen mass balances, substrate testing, and filament identifications are described herein.

A.1 BACKGROUND TO SUBSTRATE DEVELOPMENT

(1) Literature survey of sewage compositions

(2) Establishing readily and slowly biodegradable substrate/sewage fractions

(3) Bacterial population development

A.2 PREPARATION AND STORAGE OF SUBSTRATE

A.3 NDBEPR (MUCT) SYSTEM FED DEFINED SUBSTRATE

A.4 IAND SYSTEMS FED DEFINED SUBSTRATE

Operation of IAND systems

Daily measured data, mass balances, and system operating changes

Substrate composition

Relationship between Sludge Settleability (as DSVI) and Percentage Aerobic Period

Effect of system configuration, aeration pattern, nitrate concentration, and substrate composition on the proliferation of filamentous organism types

A physical effect of the "Fats & Oils" suspension of the defined substrate on the settleability of sludge during the DSVI test

A.5 INVESTIGATION OF THE "SELECTOR EFFECT"

Effect of proportion of RBCOD in the influent on induction of the selector effect

Induction of the selector effect by aerobic selector reactor configuration

Selector effect removed with time under non-inductive conditions

A.6 DETERMINATION OF THE INFLUENT RBCOD FRACTION OF THE DEFINED SUBSTRATE

Introduction

Background

Results

- *Biological determination of the influent readily biodegradable COD*
- *Determination of the theoretical COD of the RBCOD suspension of the defined substrate*
- *Physical separation of the soluble (<0,45μm) fraction of defined substrate*
- *Determination of the biodegradability of the Complex Carbohydrates suspension of the defined substrate*
- *Determination of the contribution to RBCOD from the Organic Nitrogen suspension*

A.1 BACKGROUND TO SUBSTRATE DEVELOPMENT

In the initial investigations conducted by Gabb (1988) into filamentous organism bulking in laboratory–scale nitrogen and nutrient removal systems fed municipal sewage, a problem encountered was that for each batch of sewage obtained from Mitchell's Plain full–scale sewage treatment plant (Cape Town) (approximately every 2 weeks), the initial response of the laboratory system differed.

In order to eliminate the uncertainty associated with changes in filamentous organism growth resulting from variations in municipal sewage composition, Gabb (1988) motivated the development of a defined artificial substrate as a substitute for municipal sewage as influent to laboratory systems. Additionally, the flexibility of the defined artificial substrate composition is advantageous for work concerned with the utilization of specific fractions of sewage by organisms, in which the effect of various components of the substrate on filamentous organism proliferation can be examined through their addition to and removal from the substrate.

In development of the substrate, three key objectives were set; that the artificial substrate would have; (i) a similar chemical composition to municipal sewage, (ii) a similar kinetic response, and (iii) similar filamentous organism types and bulking behaviour as the laboratory–scale units being fed municipal sewage.

A similar chemical composition refers to the classification of compounds as either soluble or particulate, as well as the correct proportions of essential nutrients such as carbon, nitrogen and phosphorus and other nutrients such as potassium, magnesium, zinc and manganese. The kinetic response of the sludge to the substrate is dependent on having the correct proportions of readily biodegradable and slowly biodegradable material as well as the correct biodegradability of both fractions, and can be determined by the oxygen utilization rate (OUR) of the sludge under specific aerobic batch test conditions. The filamentous organism types which developed in the systems fed defined substrate were identified microscopically and compared to known filament types which develop at full–scale and laboratory–scale on municipal sewage. Methods for filamentous organism identification followed the procedure of Eikelboom (1975, 1977) and Jenkins *et al.* (1984a).

The investigation was conducted in three steps:

(1) Literature survey of sewage compositions

Gabb (1988) conducted a literature survey of the nutrient requirements of most of the bacteria found in activated sludge systems. The chemical composition of Mitchell's Plain raw sewage was established and compared with the composition of other sewages reported in the literature. It was important that the characteristics of Mitchell's Plain raw sewage be identified, as this was the municipal sewage used in the laboratory-scale systems to which the systems fed a defined substrate were compared. From this survey, a sewage composition was established which formed a guideline for the defined substrate recipe.

(2) Establishing readily and slowly biodegradable substrate/sewage fractions

Gabb (1988) compared the proportions of readily biodegradable COD (RBCOD) and slowly biodegradable COD (SBCOD) contained in defined substrate and municipal sewage by operating 2 batch fed single reactor, fully aerobic activated sludge units (sludge age 10 days); one fed defined substrate, the other fed Mitchell's Plain raw sewage. After 2-3 sludge ages, aerobic batch tests were conducted on the sludges developed in each system to compare the response of the activated sludge organisms to the RBCOD fraction contained in the defined substrate with the response to the RBCOD fraction in municipal sewage. The procedure for operation of aerobic batch tests is described by Ekama *et al.* (1986). The sludge harvested from the aerobic system fed municipal sewage was fed municipal sewage in the batch test and the sludge harvested from the aerobic system fed defined substrate was fed defined substrate in the batch test. The kinetic response of the sludges to municipal sewage and defined substrate was monitored by measuring changes in the maximum specific OUR. In order to match the maximum specific OUR measured for municipal sewage, modifications were made to the defined substrate by adding or removing amounts of the readily biodegradable fraction of the substrate to increase or decrease respectively the maximum specific OUR. The 'new' defined substrate was then fed to the system for a period of 2-3 sludge ages whereupon the same batch test procedure was performed on the sludge, until similar OUR profiles were measured for the sludges from the systems fed municipal sewage and artificial substrate.

A criticism of determining the proportion of RBCOD in a substrate by this

procedure is that the maximum specific OUR is not a good parameter for assessment of the proportions of readily and slowly biodegradable COD present in the substrate because other factors affect this parameter to a considerable degree; the method of feeding (whether intermittent or continuous) (Still *et al.*, 1986) and the system configuration (Chudoba *et al.*, 1973b). In the procedure described by Ekama *et al.* (1986) for aerobic batch tests, the proportions of RBCOD and SBCOD in a substrate are determined by comparing the areas under specific parts of the OUR profile. Thus, although the two systems tested by Gabb (1988) were operated under the same conditions (i.e. same influent COD system volume, feed pattern, temperature, etc.) the method in which the response of organisms to RBCOD was determined by comparing the maximum specific OUR rather than the area under the OUR curve is not a good means of comparing the values of RBCOD contained in municipal and defined substrate. A better method for determination of readily and slowly biodegradable fractions in substrates is the "flow through activated sludge system" method (Ekama *et al.*, 1986). The results of the application of this method to the defined substrate are described in Section A.4.

(3) <u>Bacterial population development</u>

In order to apply at full scale, the results of laboratory research, it is important that the filamentous organisms developed at laboratory-scale on either municipal sewage or artificial substrate are similar to those found in full-scale systems. Gabb (1988) operated 2 single reactor batch fed long sludge age systems with a 24 hr aeration pattern of 6 hrs unaerated, 16 hrs aerated and 2 hrs for sludge settling and liquor decanting. In previous studies (Gabb et al., 1989a) laboratory-scale systems fed municipal sewage and operated with this aeration pattern developed similar filamentous organism types to those found in full-scale systems. The two laboratory-scale systems developed sludges with similar filament types and with DSVI values greater than 150 mℓ/g for longer than 3 sludge ages.

Gabb (1988) concluded that these results indicated that the competence of the defined substrate to substitute as a laboratory wastewater had been adequately demonstrated, the substrate having satisfied the three criteria outlined above, and work with the defined substrate was continued by that worker. The defined substrate composition fed to the intermittently aerated

systems 1 to 4 in this investigation (see Chapter 3) is the same as the final substrate composition developed by Gabb (1988).

A.2 PREPARATION AND STORAGE OF SUBSTRATE

The defined substrate comprised a mixture of 8 suspensions and after preparation suspensions were retained for the following periods:

1. RBCOD	(2–3 weeks)
2. Complex carbohydrates	(2–3 weeks)
3. Fats & Oils	(2 weeks)
4. Organic nitrogen	(2 weeks)
5. Vitamins	(1 month)
6. Macro inorganic nutrients	(2–3 weeks)
7. Micro inorganic nutrients	(1 month)
8. Additional micronutrients	(3 weeks)

The length of the period for preparation of each suspension was determined by the degradability of the constituents. The suspension of Fats & Oils was prepared with boiling water to enable the dissolution of sodium oleate, stearic acid and palmitic acid which are immiscible in water at 20°C. Due to the degradability of some of the constituents of suspensions 1, 4 and 5, these were stored under refrigeration at 4°C. Fats & Oils were not refrigerated because of solidification problems at low temperature. The final substrate for the activated sludge systems was prepared daily by mixing specific quantities of each of the suspensions and diluting to 10 ℓ with tap water. Because the experimental programme required changes in the substrate composition at different times, the quantities of each suspension in the final substrate mixture varied with time for any one system and also varied between systems. The individual constituents in each of the 8 suspensions are detailed in Table A.1.

A.3 NDBEPR (MUCT) SYSTEM FED DEFINED SUBSTRATE

The bulking behaviour of full–scale nutrient removal systems has been investigated at laboratory–scale by the UCT Water Research Group with the Modified UCT (MUCT) configuration. Invariably these laboratory–scale systems develop bulking sludges when fed Mitchell's Plain raw municipal sewage. Given the apparent similarity in composition, kinetic response and bacterial population development between the defined substrate and Mitchell's Plain raw sewage, the first step of this

Table A.1: Constituents of the suspensions of the defined artificial substrate.

CONSTITUENTS OF THE SUSPENSIONS OF DEFINED SUBSTRATE					
RBCOD	**Suspension concentration (g/5l)**	**Vitamins**	**Suspension concentration (g/5l)**	**Fats & Oils**	**Suspension concentration (g/10l)**
Lactose	3,3	Pantothenic Acid	1,400	Sodium Oleate	19,5
Sodium acetate	13,8	Nicotinic Acid	1,400	Stearic acid	4,5
Sodium succinate	8,7	Biotin (D-7)	0,07	Palmitic acid	11,38
Tri-sodium citrate	26,4	Cyanocobalamin (Vit.B-12)	0,07	Tween 20	1,88
D-Glucose	3,3	Folic Acid	0,07	Tween 40	6,0
Maltose	3,3	Pyridoxine (alhydrochloride)	1,400	Tween 60	3,75
Glycerol	5,4	Cocarboxylate	1,400	Tween 80	12,0
	(*ml/5l*)	p-Aminobenzoic acid	1,400	Mineral Oil	3,75
Lactic Acid	20,0	Inositol-(meso)	1,400		
Ethanol	9,0	Thiaminium dichloride	1,400		
Butanol	4,5	Riboflavin	1,400		
		Choline Chloride	1,400		
Micro-inorganic nutrients 2	**Suspension concentration (g/5l)**	**Macro-inorganic nutrients 1**	**Suspension concentration (g/15l)**	**Micro-inorganic nutrients 1**	**Suspension concentration (g/5l)**
$NiCl_2.6H_2O$	0,250	NH_4Cl	162,0	$FeSO_4.7H_2O$	8,62
$AlCl_3.6H_2O$	0,100	K_2HPO_4	57,0	$ZnSO_4.7H_2O$	2,46
NH_4VO_3	0,025	KH_2PO_4	3,0	$MnSO_4.4H_2O$	2,46
Na_2SeO_3	0,010	$MgCl_2.6H_2O$	198,0	$CuSO_4.5H_2O$	0,50
TiO_2	0,040	$CaCl_2.2H_2O$	49,5	$CoCl_2.6H_2O$	0,50
$Na_2WO_4.2H_2O$	0,015			$Na_2MoO_4.2H_2O$	0,25
				H_3BO_3	0,50
				KI	0,12
Macro-inorganic nutrients 2	**Suspension concentration (g/15l)**	**Complex Carbohydrates**	**Suspension concentration (g/15l)**	**Organic Nitrogen**	**Suspension concentration (g/8l)**
K_2HPO_4	57,0	Starch	50,55	Casein	10,80
KH_2PO_4	3,0	Cellulose	39,60	Peptone	20,20
$MgCl_2.6H_2O$	198,0	Agar	7,95	Yeast Extract	20,20
$CaCl_2.2H_2O$	49,5	Dextrin	69,15	Gelatin	15,80

investigation was to feed defined substrate to a laboratory–scale MUCT system (MUCTA) and examine the system response in COD and nutrient removal and bacterial population (filamentous organism) development.

MUCTA was fed defined substrate and operated for 135 days during which time the system was monitored by testing for COD, phosphorus and nitrogen removal, and sludge settleability. During the investigation it became clear that the bulking behaviour in response to defined substrate was very different to that exhibited by a similar configuration fed municipal sewage. The system demonstrated a steady decline in sludge settleability as measured by the Dilute Sludge Volume Index (DSVI) test even though considerable opportunity was given the system to re–establish filamentous organism growth through seeding with bulking sludges. It was concluded that as a starting point for the investigation with defined substrate, the MUCT configuration was too complex to elucidate the mechanisms inducing bulking and other configurations were sought for further experimental investigations. Figure A.1 presents the daily measured data for the 135 days of operation. The daily measured data for MUCTA is contained in a spreadsheet configured in QUATTRO and stored in the file MUCTA.WQ1.

A.4 IAND SYSTEMS FED DEFINED SUBSTRATE

The results described above highlighted the need for a relatively simple laboratory configuration capable of developing bulking sludges. Analysis of full–scale plants indicated that filamentous organism bulking occurs frequently in Orbal systems in which the mixed liquor is subjected to intermittent anoxic–aerobic periods. These configurations are generally referred to as intermittently aerated nitrification–denitrification (IAND) configurations. IAND configurations have been examined with municipal sewage as feed and considerable success has been gained in developing sludges containing high proportions of filamentous organisms (Gabb *et al.*, 1988).

Operation of IAND systems

In operation of a typical IAND system, the dissolved oxygen (DO) is raised in 1 minute to about 2 mgO/ℓ at which point the air is switched off. For a sludge operated at about 15 days sludge age, the OUR is 30–40 mgO/ℓ.h, and the DO falls to zero after 3–4 minutes, this period being aerobic. The following 6–7 minutes before the oxygen switches on is the anoxic period and the cycle is repeated continuously. The percentage aerobic period is calculated as the percentage of the

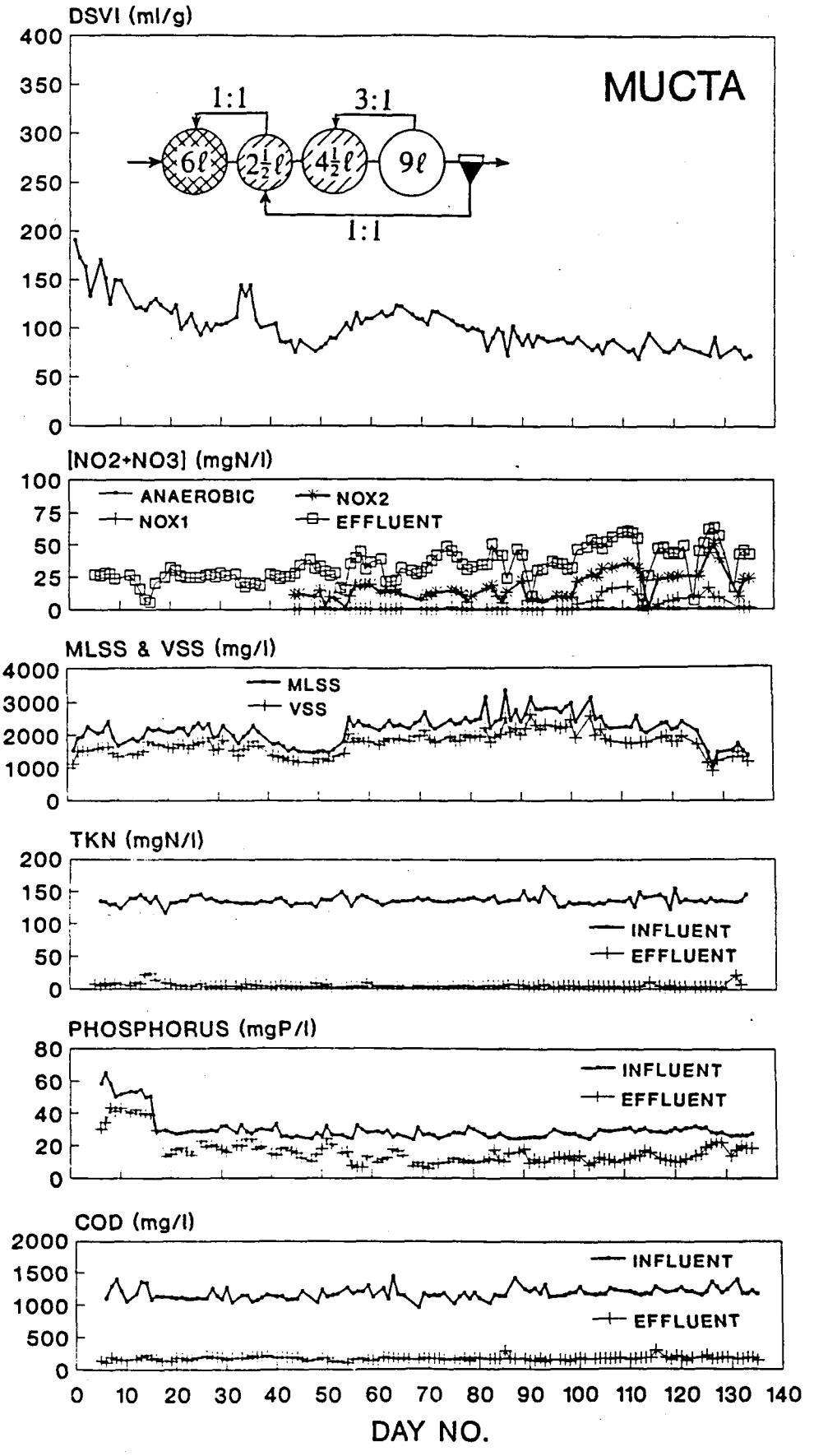


Fig A.1: Daily measured data for System MUCTA.

total cycle at which the DO is greater than 0,2 mgO/ℓ. The percentage aerobic period is maintained at 30–40%. Depending on the OUR it may be necessary to lengthen or shorten the total cycle time to maintain the aerobic period at 30–40% of the total period.

Daily measured data, mass balances and system operating changes

Four IAND systems (Systems 1–4) were operated at a sludge age of 15 days and 20° C. The daily measured data is presented in Figs A.2 to A.5 and mass balances for COD and TKN are presented in Tables A.2 to A.5 for Systems 1–4 respectively. The method for calculation of the mass balances is described in Appendix B.1 in discussion of the results of IAND systems fed municipal sewage. The changes in operating parameters for Systems 1 to 4 are given in Tables A.6 to A.9 respectively, along with a discussion of changes in system response.

The daily measured data for Systems 1–4 are contained in spreadsheets configured in QUATTRO and stored in files SYS1.WQ1 to SYS4.WQ1 respectively.

Substrate composition

During operation of the systems, changes were made to the composition of the substrate, i.e. the quantities and types of suspensions included in the final substrate mixture. The different substrate compositions are labelled A, B, C, D, and E and the volumes of suspensions included in each composition are given in Table A.10. The periods during which the different substrate types were fed to Systems 1–4 are given in Table A.11. The contribution to COD of each suspension for each of substrate types A, B, C, D, and E is shown in Table A.12.

Relationship between Sludge Settleability (as DSVI) and Percentage Aerobic Period

The finding that continuous aerobic conditions (Gabb *et al.*, 1989a) and continuous anoxic conditions (Ketley *et al.*, 1991) suppress filamentous organism growth and that the aerobic sludge mass fraction influences the proliferation of filamentous organisms was examined with System 2. The experimental programme of System 2 was not designed with the objective of determining the relationship between sludge settleability and percentage aerobic period, but there was sufficient variation in percentage aerobic period throughout the testing period to enable such an analysis.

The objective was to determine the average steady state DSVI values which result from a system operated for a long period (1 sludge age) at any given percentage

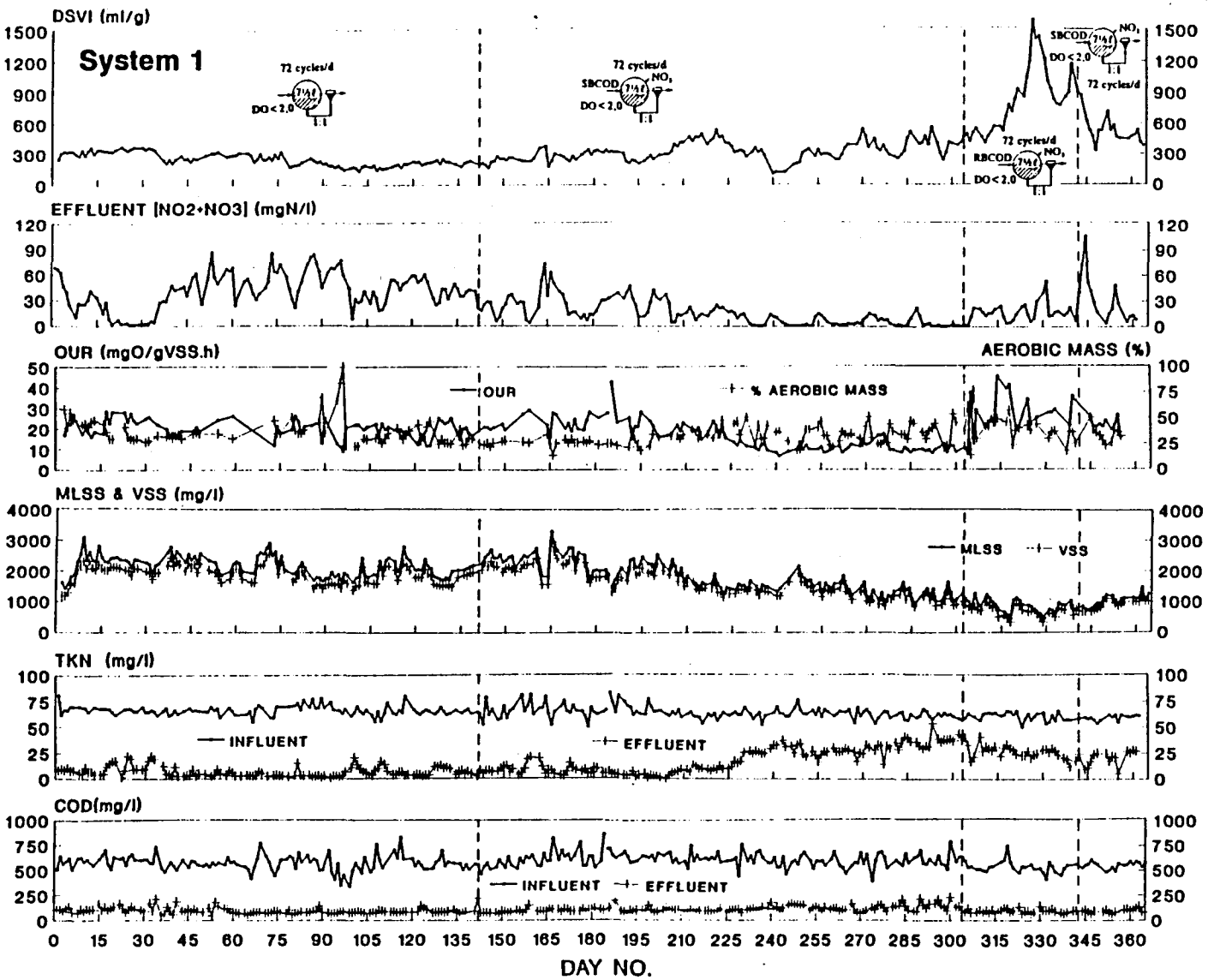


Fig A.2: Daily measured data for System 1.

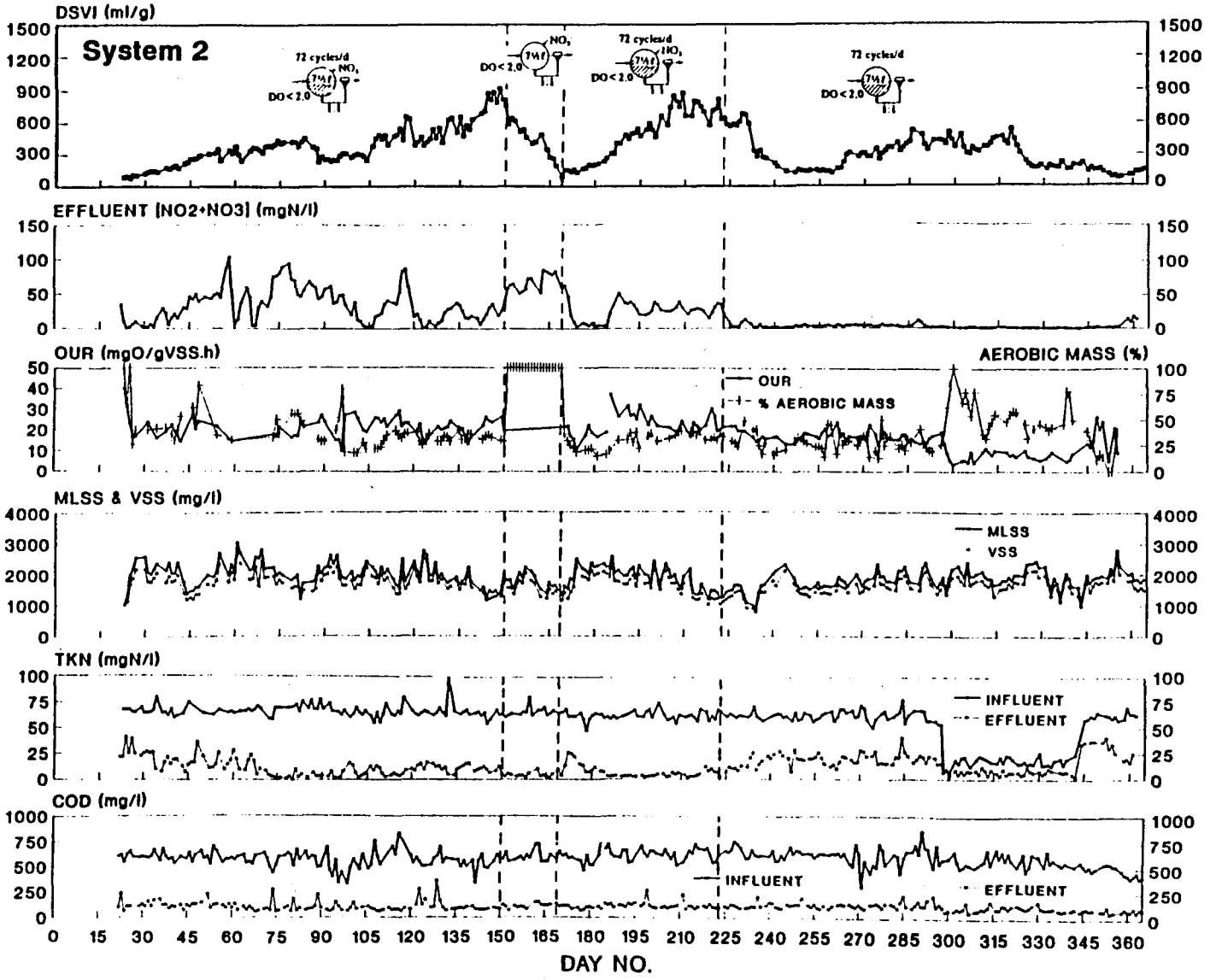


Fig A.3: Daily measured data for System 2.

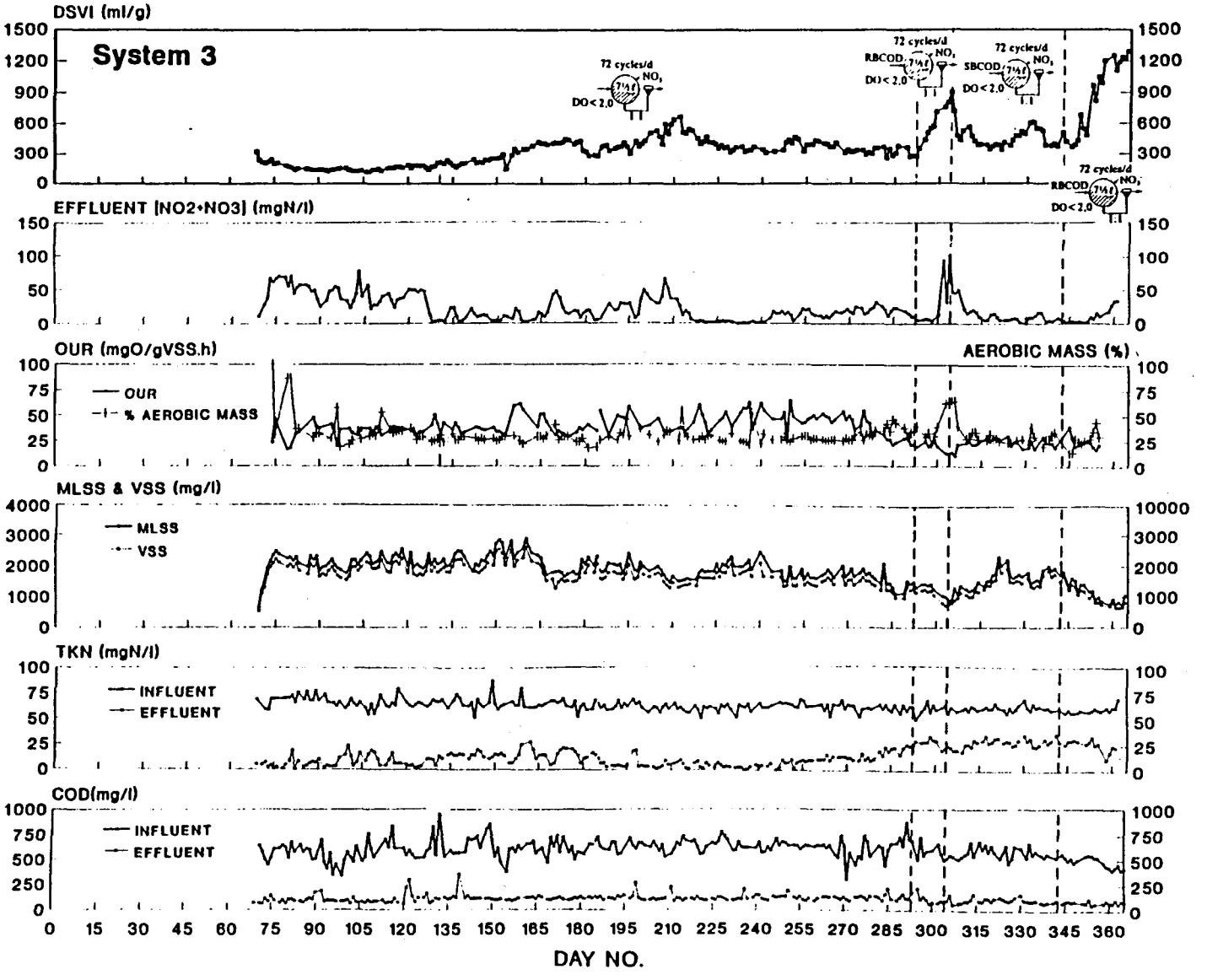


Fig A.4: Daily measured data for System 3.

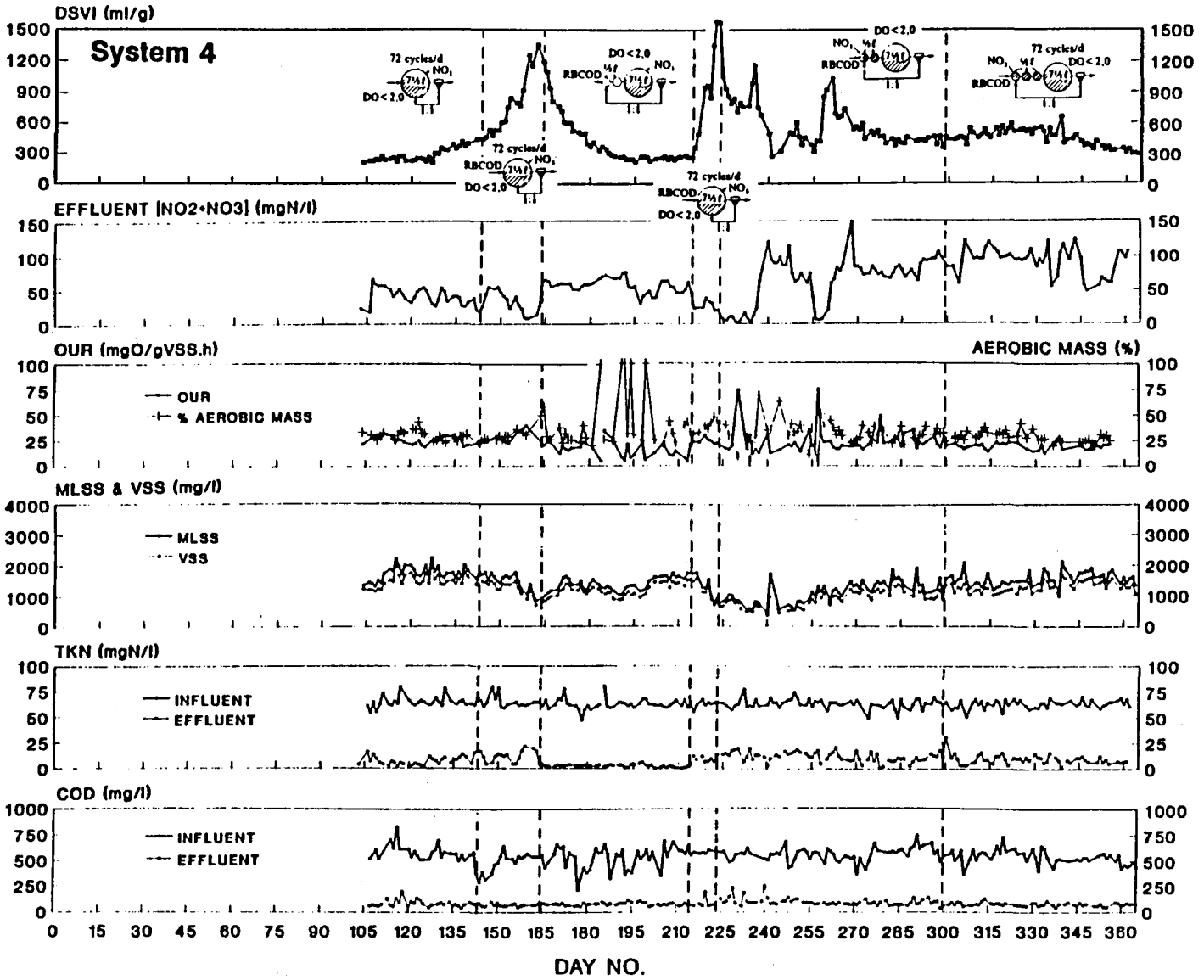


Fig A.5: Daily measured data for System 4.

Table A.2: Mass balances of COD and Nitrogen for System 1.

COD & NITROGEN Balance System 1

Q =	10	l/d
Vp =	7.5	l
Rs =	15	d
w =	500	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day		MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1	31	5968	1086	1759	1302	2839	88	1
2	32	84	5675	914	2034	1505	2356	84	2
3	85	141	5586	833	1747	1293	1188	59	3
4	142	216	6144	989	1981	1466	1862	70	4
5	217	259	5986	1160	1393	1031	1191	57	5
6	260	303	5790	1283	1131	837	1127	56	6
7	304	341	5348	905	701	519	1402	53	7
8	342	365	5489	905	912	675	735	42	8

Steady State Period	Day-Day		MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1	31	675	98	88	211	229	472	489	100	1
2	32	84	660	53	102	485	452	538	505	100	2
3	85	141	655	60	87	269	464	312	507	100	3
4	142	216	647	70	99	247	246	479	478	100	4
5	217	259	630	223	70	261	92	507	338	100	5
6	260	303	618	323	57	258	50	447	239	100	6
7	304	341	604	261	35	254	164	398	308	100	7
8	342	365	590	204	46	266	295	312	340	100	8

Steady State Period	Day-Day		OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1	31	43.7	47	3725	2235	1490	1349	1
2	32	84	40.7	43	3127	2309	818	1538	2
3	85	141	32.7	44	2614	2319	295	893	3
4	142	216	44.5	33	2675	2184	491	1371	4
5	217	259	17.1	42	1288	1546	-258	1449	5
6	260	303	12.5	42	941	1092	-151	1278	6
7	304	341	19.4	48	1669	1406	263	1140	7
8	342	365	19.1	41	1399	1556	-157	892	8

Table A.3: Mass balances of COD and Nitrogen for System 2.

COD & NITROGEN Balance System 2

Q =	10	l/d
Vp =	7.5	l
Rs =	15	d
w =	500	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day		MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1	66	6011	1218	1783	1319	3074	93	1
2	67	103	5425	978	1783	1320	1333	67	2
3	104	149	5117	1025	1713	1268	1725	79	3
4	150	169	5962	1096	1541	1140	4133	107	4
5	170	222	6009	917	1631	1207	1565	61	5
6	223	259	5958	1003	1359	1006	444	41	6
7	260	297	5690	954	1650	1221	626	49	7
8	298	342	4774	655	1696	1255	1421	70	8
9	343	365	5244	1036	1717	1271	658	57	9

Steady State Period	Day-Day		MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1	66	662	198	89	250	280	345	375	100	1
2	67	103	680	53	89	208	486	260	538	100	2
3	104	149	648	97	86	263	250	478	465	100	3
4	150	169	650	45	77	256	660	123	528	100	4
5	170	222	618	68	82	249	249	469	469	100	5
6	223	259	617	175	68	0	38	336	374	100	6
7	260	297	605	201	83	0	34	288	322	100	7
8	298	342	173	67	85	0	1	20	22	100	8
9	343	365	618	303	86	0	46	183	229	100	9

Steady State Period	Day-Day		OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1	66	34.4	61	3800	1714	2086	988	1
2	67	103	35.1	48	3048	2460	588	745	2
3	104	149	36.1	38	2482	2125	358	1368	3
4	150	169	34.4	100	6192	2412	3780	353	4
5	170	222	36.5	36	2365	2141	223	1342	5
6	223	259	21.5	31	1191	1708	-518	962	6
7	260	297	24.2	29	1272	1471	-198	824	7
8	298	342	12.2	67	1462	99	1363	58	8
9	343	365	28.5	23	1182	1048	135	524	9

Table A.4: Mass balances of COD and Nitrogen for System 3.

COD & NITROGEN Balance System 3

Q =	10	l/d
Vp =	7.5	l
Rs =	15	d
w =	500	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxm: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day	MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1 66	5621	988	1838	1360	1409	67	1
2	67 103	6287	1143	1907	1411	1954	72	2
3	104 149	6230	1232	1681	1244	1860	70	3
4	150 169	6463	1148	1670	1236	2752	79	4
5	170 222	6404	1248	1448	1072	2141	70	5
6	223 259	6079	1264	1413	1045	2173	74	6
7	260 297	6061	1139	1051	778	1114	50	7
8	298 342	5746	1034	1401	1037	1420	61	8
9	343 365	4883	833	1012	749	1291	59	9

Steady State Period	Day-Day	MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxm	Nitrogen Mass Balance	Steady State Period
1	1 66	665	73	92	224	421	303	500	100	1
2	67 103	654	145	95	237	125	525	413	100	2
3	104 149	606	69	84	250	273	430	453	100	3
4	150 169	609	40	83	247	40	692	486	100	4
5	170 222	652	72	72	239	145	601	507	100	5
6	223 259	622	151	71	258	173	486	401	100	6
7	260 297	600	266	53	248	276	254	282	100	7
8	298 342	621	279	70	242	129	386	272	100	8
9	343 365	605	262	51	246	105	433	293	100	9

Steady State Period	Day-Day	OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1 129	34.9	45	2826	2283	543	867	1
2	130 178	39.1	33	2340	1888	453	1502	2
3	179 212	42.4	35	2698	2069	629	1231	3
4	213 244	47.1	35	2991	2219	772	1980	4
5	245 259	46.8	33	2740	2317	423	1718	5
6	260 291	38.6	38	2615	1832	783	1391	6
7	292 304	19.8	47	1674	1287	387	727	7
8	305 341	22.9	38	1562	1245	317	1103	8
9	342 365	24.0	32	1391	1337	54	1238	9

Table A.5: Mass balances of COD and Nitrogen for System 4.

COD & NITROGEN Balance System 4

Q =	10	l/d
Vp =	7.5	l
Rs =	15	d
w =	500	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day	MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1 141	5807	838	1507	1115	1149	53	1
2	142 163	4876	714	1312	971	1213	59	2
3	164 214	5283	799	1172	867	-148	29	3
4	215 224	5820	858	1047	775	1486	54	4
5	225 257	5601	1159	660	488	608	40	5
6	258 299	5375	884	1074	795	309	37	6
7	300 365	5299	786	1284	950	-421	25	7

Steady State Period	Day-Day	MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1 141	647	67	75	255	420	339	505	100	1
2	142 163	654	127	66	244	310	395	461	100	2
3	164 214	635	26	59	244	568	227	550	100	3
4	215 224	635	94	52	302	266	525	489	100	4
5	225 257	648	137	33	556	467	567	478	100	5
6	258 299	630	98	54	573	801	250	478	100	6
7	300 365	624	91	64	525	924	70	469	100	7

Steady State Period	Day-Day	OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1 141	38.2	36	2487	2308	180	970	1
2	142 163	35.8	34	2191	2107	84	1129	2
3	164 214	18.7	51	1718	2514	-796	648	3
4	215 224	26.2	47	2218	2233	-16	1502	4
5	225 257	17.7	37	1172	2185	-1013	1621	5
6	258 299	25.6	39	1779	2186	-407	715	6
7	300 365	24.9	34	1525	2145	-620	199	7

Table A.6: Changes in operating conditions and their effect on system response for System 1.

SYSTEM 1 :	CHANGES IN OPERATING CONDITIONS	
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 32	Intermittent aeration (0,2 < DO < 2,0) Average substrate Continuous NO_3^- addition	To develop a sludge with high DSVI containing *M.parvicella*: DSVI remained between 300 and 350 ml/g. Dominant filaments were type 0092, 0914 and *H.hydrossis*. *M.parvicella* secondary at start then reduced to tertiary.
33 - 141	Intermittent aeration (0,2 < DO < 2,0) Average substrate without fats and oils Continuous NO_3^- addition	To determine effect of lack of fats and oils on *M.parvicella*: DSVI decreased from ≈ 350 to ≈ 200 ml/g; *H.hydrossis* was dominant, type 0092 the secondary filament.
142 - 303	Intermittent aeration (0,2 < DO < 2,0) SBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with SBCOD and as a complement to System 3 (Days 292-303): DSVI increased from ≈ 200 to ≈ 450 ml/g and *H.hydrossis* and type 1851 were dominant. Decrease in DSVI on Day 238 due to solids loss resulting in high percent aerobic fraction (> 40%). Note the low COD balance as the DSVI increased (see Fig. 3.1), most likely due to solids lose in effluent.
304 - 341	Intermittent aeration (0,2 < DO < 2,0) RBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with RBCOD and as a complement to System 3 (Days 304-341): DSVI increased from ≈ 450 to ≈ 1200 ml/g and *H.hydrossis* was dominant. Note the very low COD mass balance (53%) due to high DSVI values and loss of solids in effluent.
342 - 365	Intermittent aeration (0,2 < DO < 2,0) SBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with SBCOD and as a complement to System 3 (Days 342-365): DSVI decreased from ≈ 1200 to ≈ 400 ml/g; the dominant filament was *H.hydrossis*.

Summary:

- *M.parvicella* did not proliferate with fats and oils in the substrate
- With average substrate composition, *H.hydrossis* and type 1851 were dominant
- With RBCOD-substrate, DSVI increased and *H.hydrossis* and type 1851 were dominant
- With SBCOD-substrate, DSVI decreased and filaments *H.hydrossis* and type 1851 were dominant
- In changing between substrate types (RBCOD to SBCOD), filament type 1701 increased in abundance

Table A.7: Changes in operating conditions and their effect on system response for System 2.

SYSTEM 2 :	CHANGES IN OPERATING CONDITIONS	
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
22 - 66	Intermittent aeration (0,2 < DO < 2,0) Average substrate Continuous NO_3^- addition	To develop a sludge with high DSVI, containing *M.parvicella*: DSVI increased from < 100 to ≈ 350 ml/g; dominant filaments were *H.hydrossis* and type 1851, *M.parvicella* was a tertiary filament.
67 - 149	Intermittent aeration (0,2 < DO < 2,0) Average substrate without fats and oils Continuous NO_3^- addition	To determine effect of lack of Fats & Oils on *M.parvicella*: DSVI increased from ≈ 350 to ≈ 900 ml/g and *H.hydrossis* and type 1851 were dominant.
150 - 169	Continuous aeration (0,2 < DO < 2,0) average substrate - without fats and oils Continuous NO_3^- addition	To reduce filament proliferation: DSVI decreased from ≈ 900 to ≈ 100 ml/g. Note that the COD mass balance (107%) (see Table A.3) is higher than for the intermittent aeration periods (≈ 80%)
170 - 222	Intermittent aeration (0,2 < DO < 2,0) Average substrate - without fats and oils Continuous NO_3^- addition	To develop a sludge with high DSVI: DSVI increased from ≈ 100 to ≈ 700 ml/g; dominant filaments were *H.hydrossis* and type 1851. Note the low COD mass balance (61%) with intermittent aeration.
223 - 297	Intermittent aeration (0,2 < DO < 2,0) Average substrate - without fats and oils Continuous NO_3^- addition	To determine if presence of NO_3^- affects filament proliferation: DSVI decreased from ≈ 700 to ≈ 130 ml/g by Day 259 (type 1701 was dominant) and then increased to ≈ 430 ml/g (filament type 1851 was dominant). Effluent $NO_{x,t}^-$ decreased from ≈ 25 to < 5 mgN/ℓ.
298 - 342	Intermittent aeration (0,2 < DO < 2,0) Average substrate - without fats and oils Low TKN	To reduce influent NH_4^+ to decrease NO_3^-: DSVI decreased from ≈ 430 to ≈ 150 ml/g; filament type 1851 was dominant. Effluent $NO_{x,t}^-$ decreased from < 5 to ≈ 0,1 mgN/ℓ.
343 - 365	Intermittent aeration (0,2 < DO < 2,0) Average substrate - without fats and oils Normal TKN	To increase influent NH_4^+ to increase NO_3^-: DSVI initially decreased, then increased to 150 ml/g; dominant filament was type 1851.

Summary:

- *M.parvicella* did not proliferate with Fats & Oils in the substrate
- Under intermittent aeration with average substrate, the DSVI increased, *H.hydrossis* was dominant and type 1851 secondary
- Under continuous aeration the DSVI decreased and filament type 0041 was dominant
- With NO_3^- removal, the DSVI decreased and filament type 1701 was dominant

Table A.8: Changes in operating conditions and their effect on system response for System 3.

SYSTEM 3 :	CHANGES IN OPERATING CONDITIONS	
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
69 - 129	Intermittent aeration (0,2 < DO < 2,0) average substrate - without Fats & Oils Continuous NO_3^- addition	To develop a sludge with high DSVI containing *M.parvicella*: DSVI remained 150-200 ml/g; dominant filaments were *H.hydrossis* and type 1851. *M.parvicella* was not identified.
130 - 291	Intermittent aeration (0,2 < DO < 2,0) Average substrate - with Fats & Oils Continuous NO_3^- addition	To determine if addition of Fats & Oils induces *M.parvicella* growth: DSVI fluctuated between 200 and 650 ml/g and *H.hydrossis* and type 1851 were dominant filaments. Increases and decreases in DSVI are related to increases and decreases in effluent $NO_{3,n}^-$ concentrations (see Fig 3.5) in particular between Days 212 and 365. Note that the COD mass balance decreased when the DSVI was decreasing (Days 213-244, 79%) and was less than when DSVI was increasing (Days 179-212, 70%) (see Table A.4).
292 - 303	Intermittent aeration (0,2 < DO < 2,0) RBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with RBCOD and as a complement to System 1 (Days 142-303): DSVI increased from ≈ 280 to ≈ 900 ml/g; type 1851 was dominant and *H.hydrossis* secondary. Note the very low COD balance (50%).
304 - 341	Intermittent aeration (0,2 < DO < 2,0) SBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with SBCOD and as a complement to System 1 (Days 304-341): DSVI decreased from ≈ 900 to ≈ 380 ml/g; *H.hydrossis* was the dominant filament.
342 - 365	Intermittent aeration (0,2 < DO < 2,0) RBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with RBCOD and as a complement to System 1 (Days 342-365): DSVI increased from ≈ 380 to ≈ 1300 ml/g; *H.hydrossis* and type 1851 were dominant filaments.

Summary:

- *M.parvicella* did not proliferate with fats and oils
- With average substrate composition, *H.hydrossis* and type 1851 were dominant
- With RBCOD-rich substrate, DSVI increased and *H.hydrossis* and type 1851 were dominant
- With SBCOD-rich substrate, DSVI decreased

Table A.9: Changes in operating conditions and their effect on system response for System 4.

SYSTEM 4 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
104 - 141	Intermittent aeration (0,2 < DO < 2,0), Average substrate without fats and oils Continuous NO_3^- addition	To develop sludge with high DSVI: DSVI increased from ≈ 210 to ≈ 400 ml/g; dominant filaments were *H.hydrossis* and type 1851.
142 - 163	Intermittent aeration (0,2 < DO < 2,0), RBCOD-rich substrate Continuous NO_3^- addition	To develop a bulking sludge with RBCOD-rich substrate for work with selector reactors: DSVI increased from ≈ 400 to ≈ 1300 ml/g; *H.hydrossis* was the dominant filament.
164 - 214	Intermittent aeration (0,2 < DO < 2,0), RBCOD-rich substrate Continuous NO_3^- addition 2 aerobic selectors added	To determine the efficacy of aerobic selector reactors with RBCOD as substrate: DSVI decreased from ≈ 1300 to ≈ 250 ml/g; *H.hydrossis* was the dominant filament. The reason for the very low COD mass balance (29%) (see Table A.5), is that the OUR was measured in the main aeration chamber, not in the selector where the COD was utilized. To test that no RBCOD was passing through the selectos, COD profiles were conducted through the system on Days 181, 188, 194 and 209; virtually all the RBCOD was used in the selectors as shown in Table A.10. Batch tests to determine the extent of induction of a selector effect were conducted on Days 194, 198 and 207, and the results are described in Section A.5.
215 - 224	Intermittent aeration (0,2 < DO < 2,0), RBCOD-rich substrate Continuous NO_3^- addition	To determine if removing the aerobic selectors promotes filament proliferation: DSVI increased from ≈ 250 to ≈ 1600 ml/g; *H.hydrossis* was the dominant filament and types 1701 and 1851 the accondary.
225 - 257	Intermittent aeration (0,2 < DO < 2,0), RBCOD-rich substrate Continuous NO_3^- addition to 2 anoxic selectors	To determine the efficacy of anoxic selectors with RBCOD as substrate: DSVI decreased from ≈ 1600 to ≈ 450 ml/g; filament type 1701 was dominant. Aerobic batch tests to determine the extent of induction of a selector effect were conducted on Days 222 and 286 and the results are described in Section A.5. COD profiles were conducted on Days 283, 292 and 286 and the results are shown in Table A.11. Two perturbations occurred in the System, on Days 230 and 257 which resulted in rapid increases in DSVI. On Day 230 the sludge (s) recycle from the settler to the selectors was blocked, the VSS in the selectors decreased and RBCOD passed through to the intermittently aerated main reactor and filaments proliferated. On Day 257, the NO_3^- supply to the selectors was stopped, the RBCOD passed to the intermittently aerated main reactor and filaments proliferated.
258 - 299	Intermittent aeration (0,2 < DO < 2,0), RBCOD-rich substrate 3 anoxic selectors added	To determine if adding a 3rd anoxic selector would reduce the DSVI to a value similar to that established by aerobic selectors (≈ 250 ml/g) by removing more COD: DSVI increased from ≈ 450 to ≈ 550 ml/g by Day 330 and then decreased to ≈ 300 ml/g. The cause of the low COD balance (25%) is unclear.

Summary:

- With average substrate under intermittent aeration, *H.hydrossis* and type 1851 proliferated
- With RBCOD-rich substrate under intermittent aeration, *H.hydrossis* proliferated
- With 2 aerobic selectors prior to intermittent aeration, the DSVI decreased
- With 2 & 3 anoxic selectors prior to intermittent aeration, the DSVI decreased

Table A.10: Volumes of suspensions for inclusion in substrate feed types A, B, C, D, and E.

VOLUMES OF SUSPENSIONS FOR DIFFERENT SUBSTRATE FEED TYPES					
SUBSTRATE SUSPENSIONS	Substrate feed types and volumes (ml)				
	A	B	C	D	E
RBCOD	20	20	-	140	20
Complex carbohydrates	170	300	360	-	300
Fats and oils	140	-	-	-	-
Organic nitrogen	140	140	140	140	140
Vitamins	30	30	30	30	30
Macro-inorganic nutrients 1	170	170	170	170	–
Macro-inorganic nutrients 2	–	–	–	–	170
Micro-inorganic nutrients 1	30	30	30	30	30
Micro-inorganic nutrients 2	50	50	50	50	50

Table A.11: Periods for different substrate types fed to Systems 1 to 4.

PERIODS DURING WHICH DIFFERENT SUBSTRATE TYPES FED TO SYSTEMS 1-4			
SYSTEM 1	SYSTEM 2	SYSTEM 3	SYSTEM 4
1 - 31 (A)	1 - 66 (A)	1 - 129 (B)	1 - 141 (B)
32 - 141 (B)	67 - 297 (B)	130 - 291 (A)	142 - 365 (D)
142 - 303 (C)	298 - 342 (E)	292 - 303 (D)	
304 - 341 (D)	343 - 380 (B)	304 - 341 (C)	
342 - 365 (C)		342 - 365 (D)	

Table A.12: Contribution to COD of each substrate suspension for each of substrate types A, B, C, D, and E.

CONTRIBUTION TO TOTAL SUBSTRATE COD OF EACH SUSPENSION FOR DIFFERENT SUBSTRATE TYPES					
SUSPENSION	SUBSTRATE TYPE & COD CONTRIBUTION				
	A	B	C	D	E
	mgCOD (%)	mgCOD (%)	mgCOD (%)	mgCOD (%)	mgCOD (%)
RBCOD	60 (10)	60 (10)	-	435 (73)	60 (10)
Complex Carbohydrates	180 (30)	375 (63)	435 (73)	_	320 (53)
Fats & Oils	195 (33)	-	-	-	-
Organic Nitrogen	85 (14)	85 (14)	85 (14)	85 (14)	85 (14)
Others	80 (13)	80 (13)	80 (13)	80 (13)	80 (13)
TOTAL	600	600	600	600	600

aerobic period. For this purpose, time periods of 20–60 days were chosen during which the reactor configuration and aeration pattern did not change. The average percentage aerobic period was calculated over the chosen time period but the corresponding average DSVI was calculated over a much shorter time at the end of this period when the DSVI had reached a steady–state value. As can be seen in Fig A.3, for System 2, the DSVI changes quite considerably over the chosen time periods and for this reason the average DSVI value is calculated only over a time period at the end of the steady state period over which the value of the average percentage aerobic period is calculated. Figure A 6 illustrates the relationship between DSVI and percentage aerobic period for System 2. Table A.13 indicates the time periods over which the percentage aerobic periods and DSVIs were determined. The investigation was conducted on System 2 because of its long steady–state periods; Systems 1, 3 and 4 experienced regular changes in configuration, aeration pattern and/or substrate composition which excluded a similar analysis being conducted on them.

Effect of system configuration, aeration pattern, nitrate concentration and substrate composition on the proliferation of filamentous organism types

Filament identifications with time for Systems 1 to 4 are given in Tables A.14 to A.17 respectively. The effects of system configuration, aeration pattern, nitrate concentration and substrate composition on the type of filamentous organisms which develop and extent of their proliferation in systems fed defined substrate are summarized in Table A.18. The effects of these changes on filamentous organisms not identified at the Dominant or Secondary abundance levels is difficult to ascertain and are not reported on. It should be noted that the filaments which proliferate on a particular defined artificial substrate composition under specific conditions and may not necessarily proliferate in systems fed municipal sewage with similar configurations and aeration patterns.

A physical effect of the "Fats & Oils" suspension of the defined substrate on the settleability of sludge during the DSVI test

As a result of the finding in Systems 1, 2 and 3 that the substrate suspension Fats & Oils does not affect the proliferation of *Microthrix parvicella*, this suspension was excluded from subsequent substrate compositions. However, although Fats & Oils did not appear to affect the type of filament which proliferated in the systems, small changes in settleability of the sludge (DSVI) were noticed after removal of the Fats & Oils suspension from the substrate (System 1, Day 32) and after addition of the

Table A.13: Time periods over which average values of percentage aerobic period and DSVI were calculated for System 2.

SYSTEM 2			
PERCENTAGE AEROBIC		DSVI (ml/g)	
Time period (Day No.)	Value	Time period (Day No.)	Value
26 - 83	43	74 - 83	425
104 - 149	32	143 - 149	833
150 - 169	100	167 - 169	140
170 - 200	27	192 - 200	519
201 - 222	35	210 - 222	713
298 - 342	56	327 - 342	179
Data of Ketley (1992)	Nil	-	125

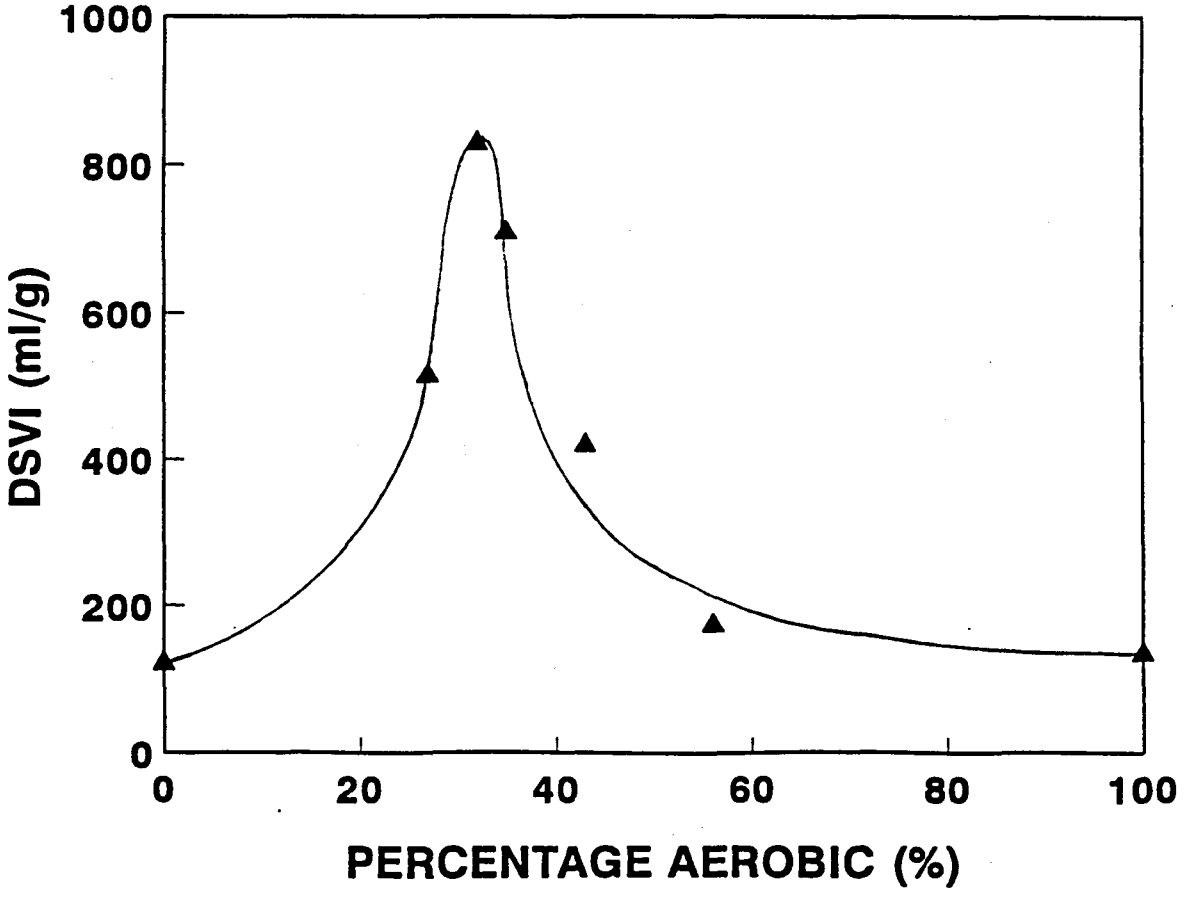


Fig A.6: Relationship between sludge settleability (DSVI in mℓ/g) and percentage aerobic period (%) for System 2.

Table A.14: Filament identifications with time for System 1.

Day No. System 1	Filament abundance	Dominant	Secondary	Tertiary	Remarks
9		0092	*M.parvicella*	0041, 0675, Flexibacter, *N.limicola II*	
25		0092	0914	1851, *M.parvicella*, 0041	
58		*H.hydrossis*	0092	0803, 0041, *M.parvicella*	
94		*H.hydrossis*	0092	0675, 0041, Flexibacter	•strings of beads
107	common-very common	*H.hydrossis*	0092	1851, 0675, 0041	
128	common-very common	*H.hydrossis*	0092	0041, 1851	
144	very common	*H.hydrossis*	0092	0041, 1851, 0914	•strings of beads
170	very common	*H.hydrossis*	1851	0914, 0041, 0092	•strings of beads
192	common-very common	1851	*H.hydrossis*	0092, *Thiothrix I*, 0914, 0041	•strings of beads
213	common-very common	1851, *H.hydrossis*	-	0092, 0041, *Beggiatoa*, 0914, *N.limicola II*	
263	common	*H.hydrossis*	1851	021N, 1701, 0041, Flexibacter, *Beggiatoa*	
304	common-very common	*H.hydrossis*	1851	1701, 0041	
334	very common-abundant	*H.hydrossis*	1701	021N, 0092, 1851	
365	abundant	*H.hydrossis*	-	Flexibacter, 0041, 1851, 1701	•some *algae* sp. present

Table A.15: Filament identifications with time for System 2.

Day No. System 2	Filament abundance	Dominant	Secondary	Tertiary	Remarks
25		0914	0092	*H.hydrossis*, 0041	
58		*H.hydrossis*	1851	0803, 0041, 0092, *M.parvicella*	
74		0092	*H.hydrossis*	1851, 0041, *N.limicola I*	
107	very common	*H.hydrossis*	1851	*N.limicola* I, 0041, 0961, 0675, 0092	
128	very common-abundant	*H.hydrossis*	1851	0041, 0092	
144	very common-abundant	*H.hydrossis*	1851	0041, 0092, 1701, *Nocardia* sp.	
170	few	0041	*M.parvicella*	*H.hydrossis*, 0092, *N.limicola III*, Flexibacter, strings of beads	
192	common	*H.hydrossis*	1851	0041, 0092, strings of beads, *N.limicola II*	
213	very common-abundant	*H.hydrossis*	1851	0041, 0092, *N.limicola II*	
222	very common-abundant	*H.hydrossis*	1851	0041, 0092, *N.limicola II*	
263	common-very common	1701	1851	*H.hydrossis*, 0041, 0092	• Large unidentifiable clusters of amorphous zooglea
304	very common-abundant	1851	1701	*M.parvicella*, 0041, strings of beads, 0092	
334	abundant	1851	1701	*H.hydrossis*, 0041, *Beggiatoa*, *M.parvicella*,	• Fungus spp. present
365	very common	1851	1701	0041, *H.hydrossis*	

Table A.16: Filament identifications with time for System 3

Day No. System 3	Filament abundance	Dominant	Secondary	Tertiary	Remarks
74		*H.hydrossis*, 0092	-	0675, 0041, Flexibacter, 1851, *N.limicola I*	
107	common	*H.hydrossis*	1851	0092, 0041, *M.parvicella*	
128	common	*H.hydrossis*	1851	0041	•Rotifers present
144	very common	*H.hydrossis*	1851	0041	
170	very common-abundant	*H.hydrossis*	1851	*Beggiatoa*, 1863, 0041, 0092, *N.limicola II*	
192	very common-abundant	*H.hydrossis, 1851*	-	1863, 0041, strings of beads, 0092	
213	common-very common	*H.hydrossis*	1851	0041, 0092	
263	very common-abundant	1851, *H.hydrossis*	-	0092, 0041, 1863, *N.limicola II, Beggiatoa*	•unidentified
304	very common-abundant	*1851*	*H.hydrossis*	0092, 0041, *N.limicola III*	filaments •chains of large
334	abundant	*H.hydrossis*	1851	0041, *1701, 0092*	ovoid cells
363	abundant	*H.hydrossis*	1851	0041, *Thiothrix* sp., 0803	

Table A.17: Filament identifications with time for System 4.

Day No. System 4	Filament abundance	Dominant	Secondary	Tertiary	Remarks
107	common	*H.hydrossis*	1851	0041, 0092, *M.parvicella* 0675, *Thiothrix* sp.,	• Many rotifers
128	common	*H.hydrossis*	1851	0092, 0041, strings of beads, *N.limicola II*	
144	very common	*H.hydrossis*, 1851	-	1701, 0092, 0041, Flexibacter	
170	common-very common	1863	*H.hydrossis*	*M.parvicella*, 0041, 0092	
192	common	*H.hydrossis*	1863	0041, 0092, strings of beads	
213	some	*H.hydrossis*	1863	0041, 0092	
222	abundant	*H.hydrossis*	1701, 1851	0041, 0092, *N.limicola II*	
263	common-very common	1701	1851	*M.parvicella*, 0092, *N.limicola II*, 0041, *H.hydrossis*, many finger zooglea	
304	common	1701	0803	*H.hydrossis*, 0092	
334	very common-abundant	1701	0803, *H.hydrossis*	0041, 0675, 0092, coccoid zooglea cells	• Many finger-like zooglea
365	common	1701	1851	*H.hydrossis*, 0041, 0803, strings of beads.	

Table A.18: Effect of system configuration, aeration pattern, and substrate composition on the type of filamentous organisms which develop and their extent of proliferation in Systems 1–4 fed defined artificial substrate.

THE EFFECT OF VARIOUS CONDITIONS ON FILAMENTOUS ORGANISMS DEVELOPED ON DEFINED ARTIFICIAL SUBSTRATE			
ORGANISM TYPE	**Conditions which promote or sustain proliferation**	**Conditions which reduce proliferation**	**Conditions which have little or no effect on proliferation**
Haliscomenobacter hydrossis	• Intermittent aeration + RBCOD-rich substrate or SBCOD-rich substrate	• Fully aerobic • Low [NO] (<5,0 mgN/ℓ) • Aerobic selectors + RBCOD-rich substrate • Anoxic selectors + RBCOD-rich substrate	• FATS & OILS present or absent
1851	• Intermittent aeration • RBCOD-rich substrate • SBCOD-rich substrate	• Fully aerobic • FATS & OILS absent • Aerobic selectors + RBCOD-rich substrate	• FATS & OILS present or absent
0092		• SBCOD-rich substrate	• FATS & OILS absent
1701	• RBCOD-rich substrate • Aerobic selectors + RBCOD-rich substrate • Anoxic selectors + RBCOD-rich substrate • Low [NO] (<5,0 mgN/ℓ)	• SBCOD-rich substrate	• Fully aerobic
0041			• Fully aerobic
Microthrix parvicella		• Intermittent aeration	• Fully aerobic
0914		• Intermittent aeration	
0803	• Anoxic selectors + RBCOD-rich substrate		

same suspension to the substrate (System 3, Day 130), but not after its removal from substrate fed to System 2 on Day 67. Because of the slowly biodegradable nature of the constituents of the Fats & Oils suspension which are long chain molecules, these substances are enmeshed in the sludge in accordance with the assumptions of the bisubstrate model (Dold *et al.*, 1980). It was considered that the presence of such substances in substrate mixtures fed to Systems 1, 2 and 3, possibly could affect the settleability of sludge by a physical mechanism. It can be hypothesized that the constituents of the mixture increased the viscosity of the mixed liquor and bridging of the interstices between the flocs, thereby altering the physical nature of the floc, and influencing the DSVI. This aspect was examined by conducting six settleability tests on sludge from System 4, (a system fed defined substrate in which Fats & Oils were not included) to which different volumes of the Fats & Oils suspension (0, 20, 40, 80, 160 and 300 mℓ) were added to the sludge of the settling test and diluted to 1 litre. The DSVI values, and the volumes of the Fats & Oils mixture added, are plotted in Fig A.7. In the substrate mixtures A to E, 140 mℓ of the Fats & Oils suspension are included. Since the volume of sludge selected for settling comprises only 5,7% of the total reactor volume, the maximum amount of Fats & Oils that could be enmeshed in one day is 3,8 mℓ, assuming that all of the Fats & Oils suspension is enmeshed, none is utilized and none passes out in the effluent. Figure A.7 indicates that this volume would have little if any effect on sludge settleability.

A.5 INVESTIGATION OF THE "SELECTOR EFFECT"

Studies concerned with the comparison of the biodegradability of defined substrate with municipal sewage were undertaken by Gabb (1988). In that work aerobic batch tests conducted on sludge from systems fed either municipal sewage or defined substrate were fed municipal sewage and defined substrate respectively and exhibited similar OUR responses, indicating similar proportions of RBCOD and SBCOD in municipal sewage and defined substrate.

In this investigation, aerobic batch tests were conducted on Systems 1–4 to determine the extent of imposition of the selector effect (high maximum specific substrate utilization rate) as a consequence of (i) the inclusion of aerobic and anoxic selector reactors, and (ii) feeding RBCOD–rich substrate. The procedure for operation of the batch tests follows that described by Ekama *et al.* (1986) for determination of the maximum specific growth rate of heterotrophs. Tables A.19 to A.29 indicate the operating conditions for Defined Substrate Batch Test 1 (DSBT1)

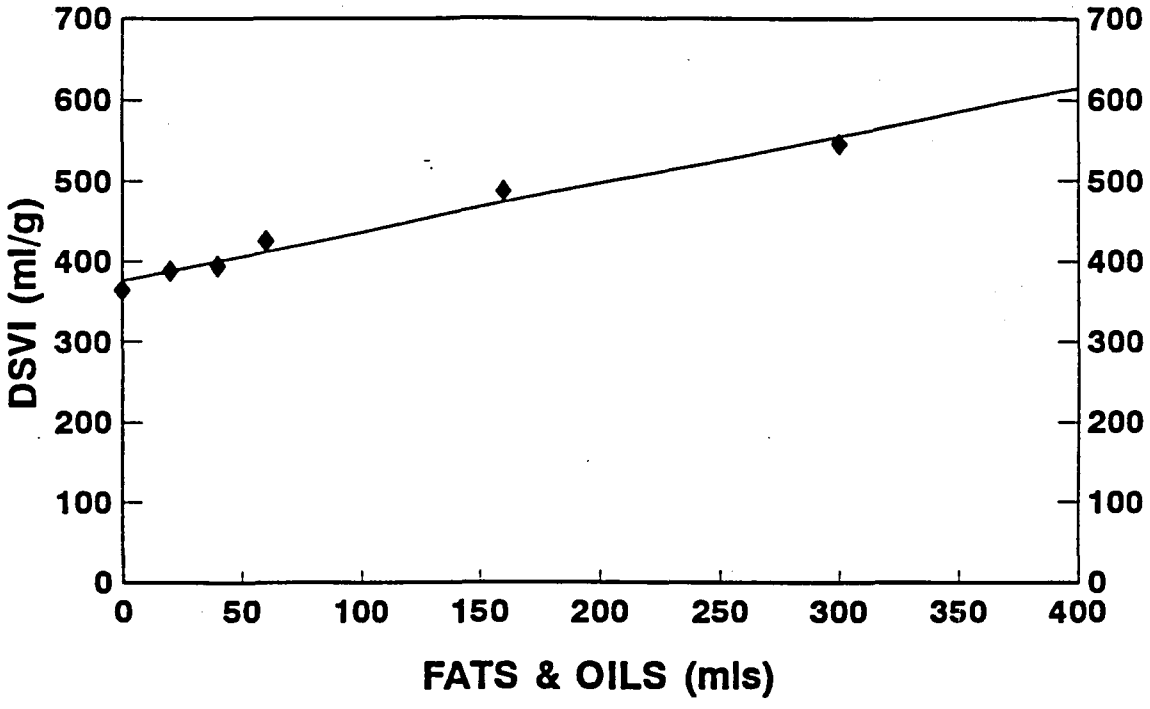


Fig A.7: The physical effect of different volumes of Fats & Oils (mℓ) on sludge settleability (DSVI in mℓ/g).

to DSBT11 respectively and Figs A.8 to A.18 describe the results of batch tests DSBT1 to DSBT11. The measured values for Batch test 1 to Batch test 11 are contained in a spreadsheet configured in QUATTRO and stored in the file DSBT.WQ1. The batch tests can be categorized in the following manner:

Effect of proportion of RBCOD in the influent on induction of the selector effect

The results of three batch tests can be compared to determine the effect of RBCOD in the influent on induction of a selector effect; DSBT5 (System 2, Day 205, fed particulate substrate – no selector effect measured); DSBT3 (System 3, Day 199, fed average substrate – no selector effect measured); DSBT10 (System 1, Day 328, fed RBCOD–rich substrate – selector effect measured).

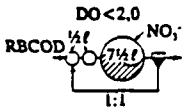
The results indicate that for systems fed substrate with low proportions of RBCOD (i.e. particulate or average substrate) no selector effect is induced, but for systems fed substrate with high proportions of RBCOD (i.e. RBCOD–rich substrate) a selector effect is induced and can be measured under aerobic batch test conditions. The differences between systems in the induction or not of a selector effect could not be attributed to system configuration since all systems were operated intermittently aerated and System 1 did not incorporate selector reactors.

Induction of the selector effect by aerobic selector reactor configuration

Three aerobic batch tests were conducted on System 4 during the period it incorporated 2 aerobic selector reactors and was fed RBCOD–substrate: DSBT1 (Day 194 – selector effect measured); DSBT2 (Day 198 – selector effect measured); and DSBT6 (Day 207 – no selector effect measured). It is unclear from DSBT1 and DSBT2 whether the selector effect results from the selector reactor configuration since at the time of the batch tests, System 4 was fed RBCOD–rich substrate which as shown earlier can induce a selector effect in the absence of selector reactors. No selector effect was measured for DSBT6 since the batch test was fed particulate substrate, in which RBCOD was not present.

To determine the efficacy of the aerobic selectors in removing RBCOD, four COD profiles were conducted when System 4 incorporated 2 aerobic selectors. The results of the profiles are given in Table A.30 and indicate that 87% of the COD (as a percentage of the influent) was removed in the first selector.

Table A.19: Operating conditions for Defined Substrate Batch Test 1 (DSBT1).

BATCH TEST DSBT1 OPERATING CONDITIONS		
Objective: To examine extent of induction of selector effect in system with 2 aerobic selectors fed RBCOD.		
PARENT SYSTEM CONDITIONS		
System 4; Day 194 System configuration:IAND & 2 aerobic selectors Substrate type: RBCOD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	443
VSS	(mg/ℓ)	333
F/M	(mgCOD/mgVSS	0,85
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	284

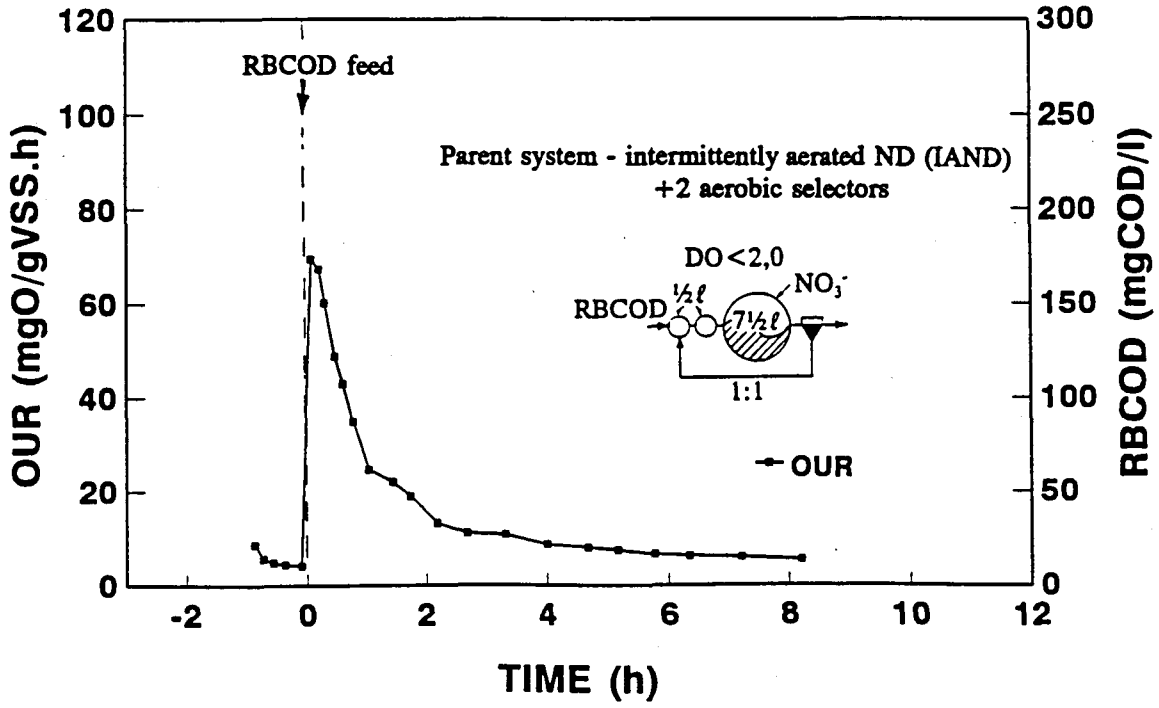
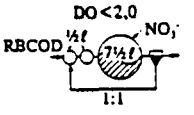


Fig A.8: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 1 (DSBT1) conducted on Day 194 on sludge from System 4.

Table A.20: Operating conditions for Defined Substrate Batch Test 2 (DSBT2).

BATCH TEST DSBT2 OPERATING CONDITIONS		
Objective: To examine extent of induction of selector effect in system with 2 aerobic selectors fed RBCOD.		
PARENT SYSTEM CONDITIONS		
System 4; Day 198 System configuration:IAND & 2 aerobic selectors Substrate type: RBCOD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	452
VSS	(mg/ℓ)	389
F/M	(mgCOD/mgVSS	1,04
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	404

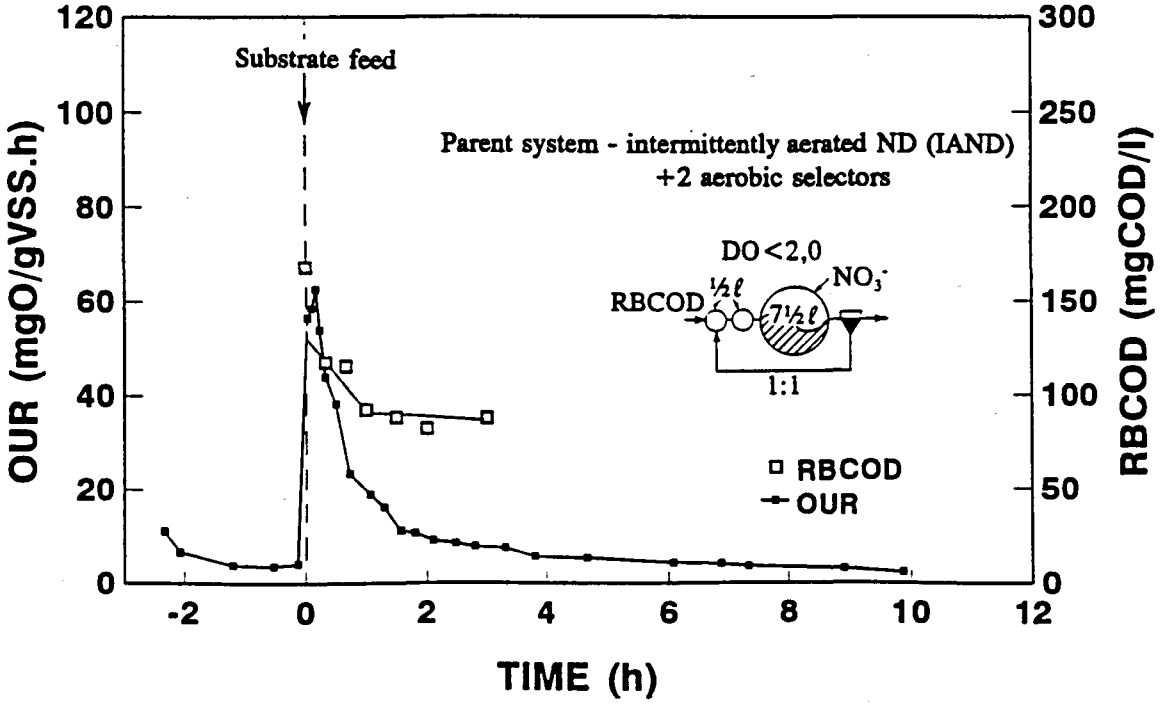
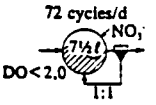


Fig A.9: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 2 (DSBT2) conducted on Day 198 on sludge from System 4.

Table A.21: Operating conditions for Defined Substrate Batch Test 3 (DSBT3).

BATCH TEST DSBT3 OPERATING CONDITIONS		
Objective: To examine extent of induction of selector effect in system fed average substrate.		
PARENT SYSTEM CONDITIONS		
System 3; Day 199 System configuration: IAND Substrate type: Average COD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	663
VSS	(mg/ℓ)	580
F/M	(mgCOD/mgVSS	0,70
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	404

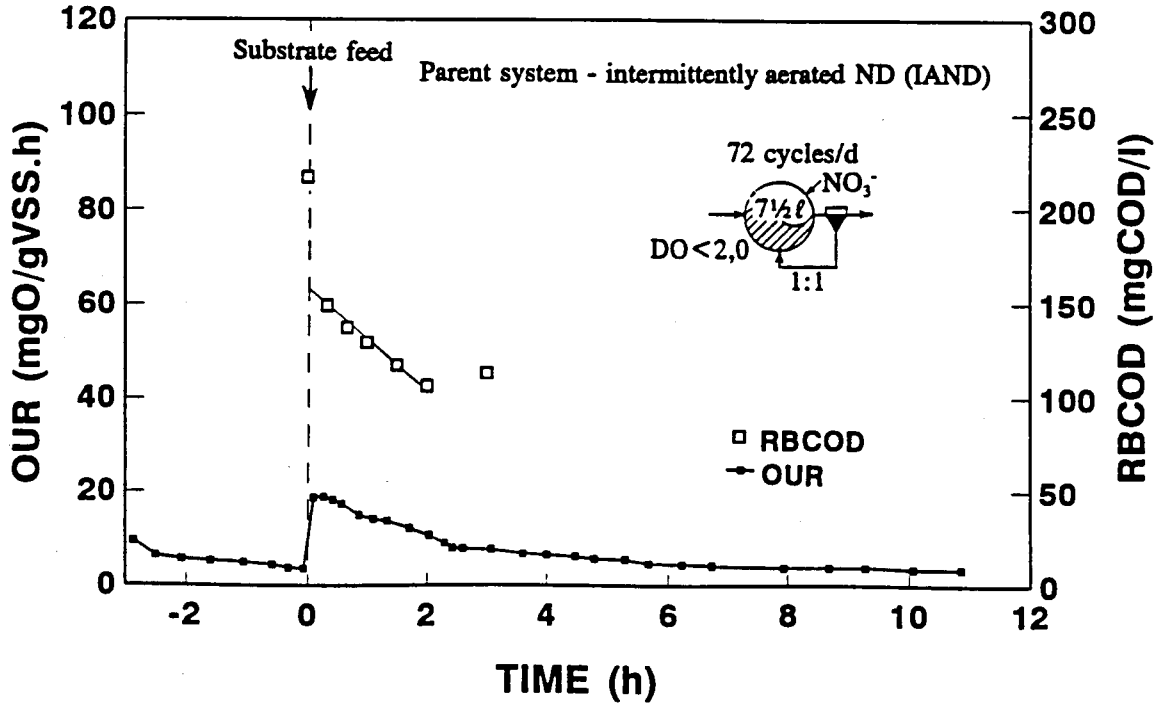


Fig A.10: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 3 (DSBT3) conducted on Day 199 on sludge from System 3.

Table A.22: Operating conditions for Defined Substrate Batch Test 4 (DSBT4).

BATCH TEST DSBT4 OPERATING CONDITIONS		
Objective: To examine extent of induction of selector effect in system fed SBCOD.		
PARENT SYSTEM CONDITIONS		
System 2; Day 202 **System configuration: IAND** **Substrate type: Average COD**		72 cycles/d, NO_3^-, 7½ℓ, DO<2,0, 1:1
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**555**
VSS	**(mg/ℓ)**	**474**
F/M	**(mgCOD/mgVSS**	**0,62**
DO	**(mgO/ℓ)**	**2 - 4**
Temperature	**(°C)**	**20**
SUBSTRATE TO BATCH TEST		
RBCOD	**(mgCOD/ℓ-final batch vol)**	**295**

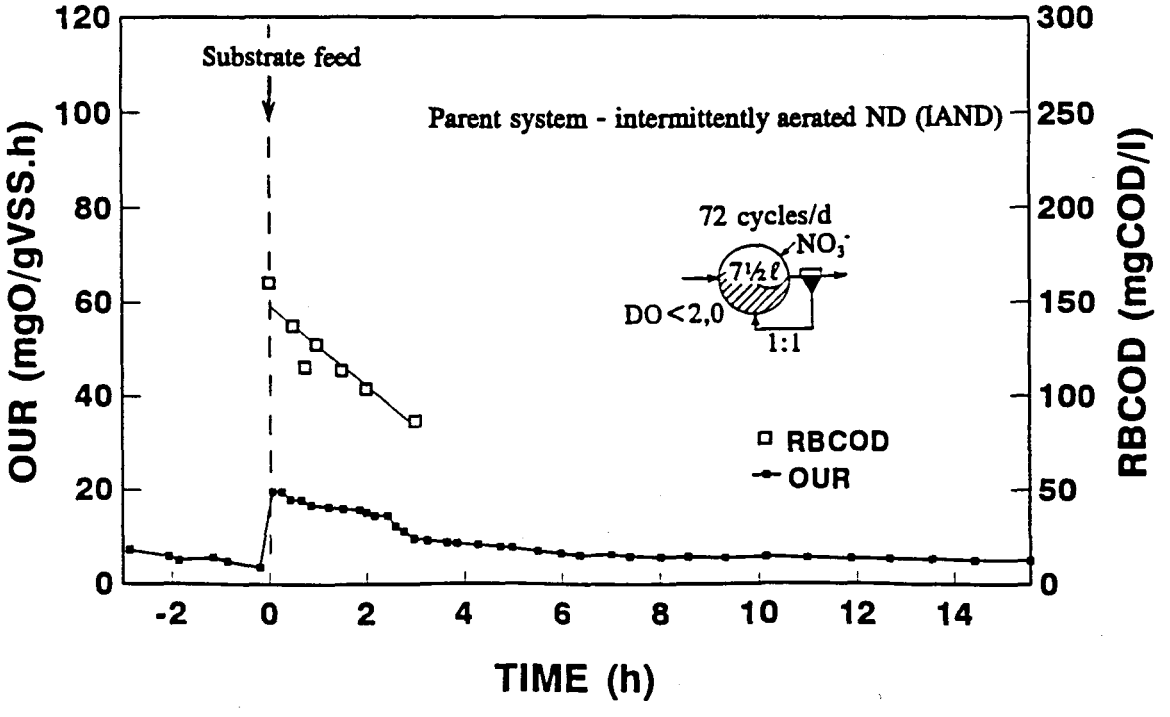


Fig A.11: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 4 (DSBT4) conducted on Day 202 on sludge from System 2.

Table A.23: Operating conditions for Defined Substrate Batch Test 5 (DSBT5).

BATCH TEST DSBT5 OPERATING CONDITIONS		
Objective: To examine extent of induction of selector effect in system fed SBCOD.		
PARENT SYSTEM CONDITIONS		
System 2; Day 205 **System configuration: IAND** **Substrate type: Average COD** **Sludge age**	**(d)**	**15**
BATCH TEST CONDITIONS		
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**749**
VSS	**(mg/ℓ)**	**676**
F/M	**(mgCOD/mgVSS**	**0,48**
DO	**(mgO/ℓ)**	**2 - 4**
Temperature	**(°C)**	**20**
SUBSTRATE TO BATCH TEST		
RBCOD	**(mgCOD/ℓ-final batch vol)**	**324**

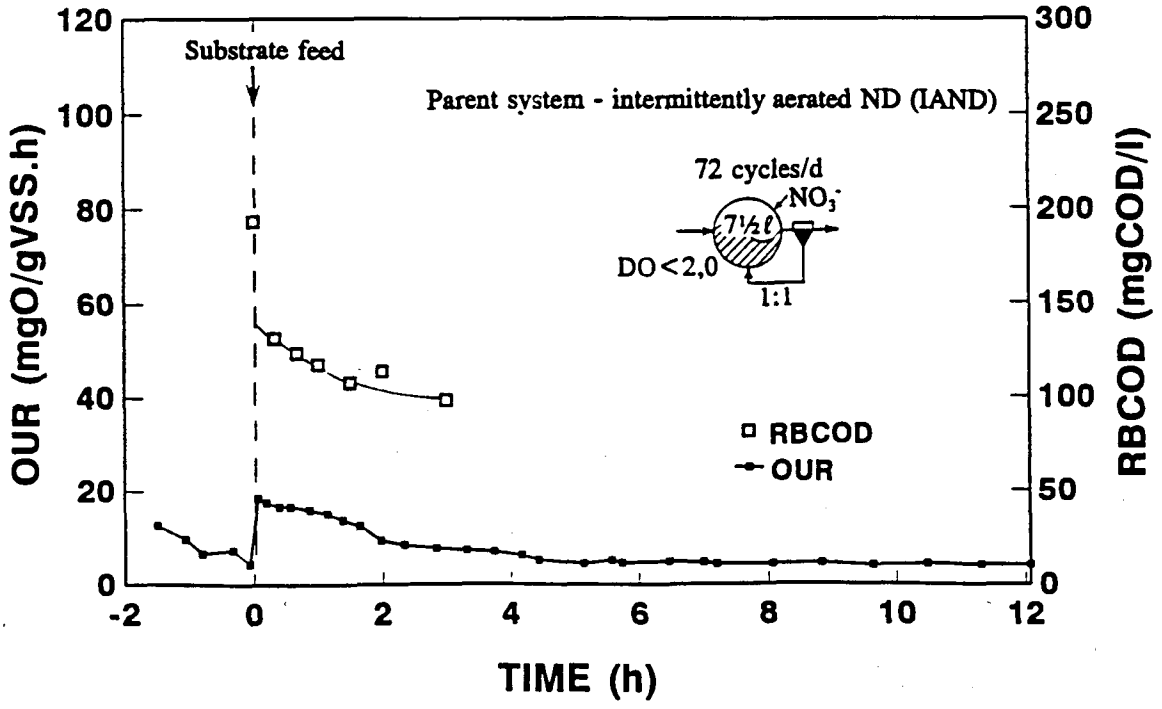
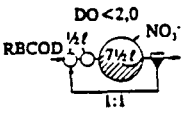


Fig A.12: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 5 (DSBT5) conducted on Day 205 on sludge from System 2.

Table A.24: Operating conditions for Defined Substrate Batch Test 6 (DSBT6).

BATCH TEST DSBT6 OPERATING CONDITIONS		
Objective: To demonstrate that the SBCOD fraction does not contain sufficient RBCOD to measure a selector effect.		
PARENT SYSTEM CONDITIONS		
System 4; Day 207 System configuration:IAND & 2 aerobic selectors Substrate type: RBCOD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	621
VSS	(mg/ℓ)	536
F/M	(mgCOD/mgVSS	0,62
DO	(mgO/ℓ)	2.- 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	330

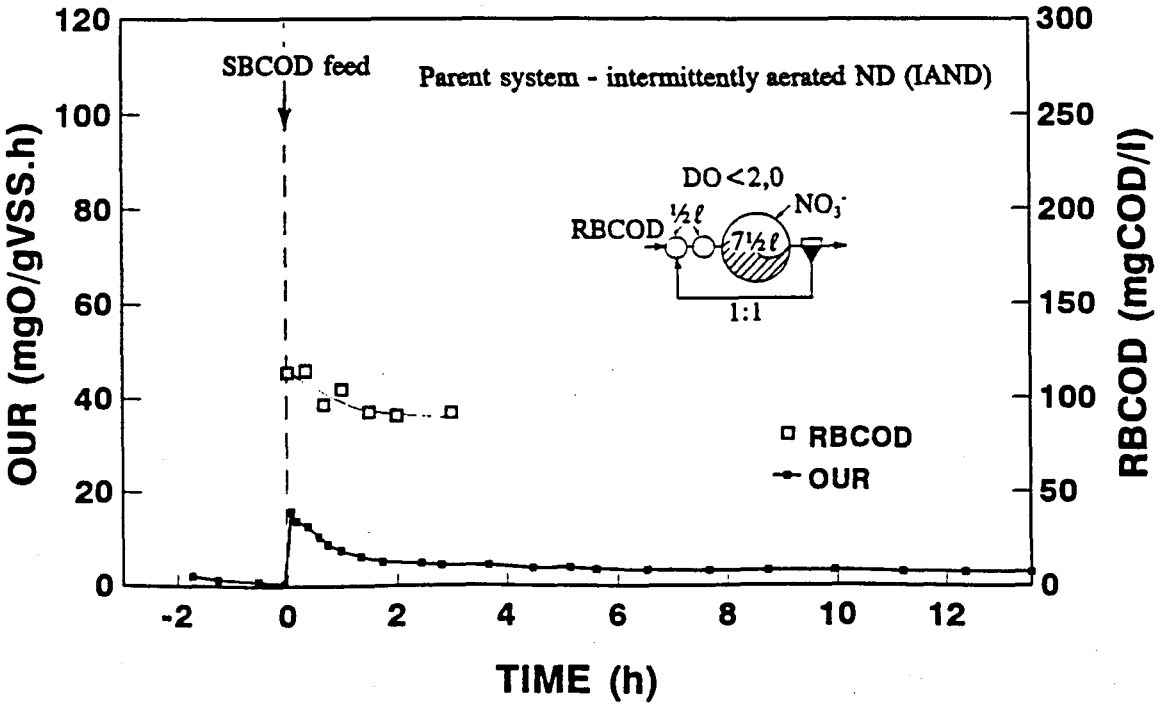


Fig A.13: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 6 (DSBT6) conducted on Day 207 on sludge from System 4.

Table A.25: Operating conditions for Defined Substrate Batch Test 7 (DSBT7).

BATCH TEST DSBT7 OPERATING CONDITIONS		
Objective: To examine the role of RBCOD in inducing a selector effect.		
PARENT SYSTEM CONDITIONS		
System 4; Day 222 System configuration: IAND Substrate type: RBCOD Sludge age	(d)	72 cycles/d RBCOD, NO_3^-, 7½ℓ DO<2,0 1:1 15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	363
VSS	(mg/ℓ)	322
F/M	(mgCOD/mgVSS	0,66
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	211

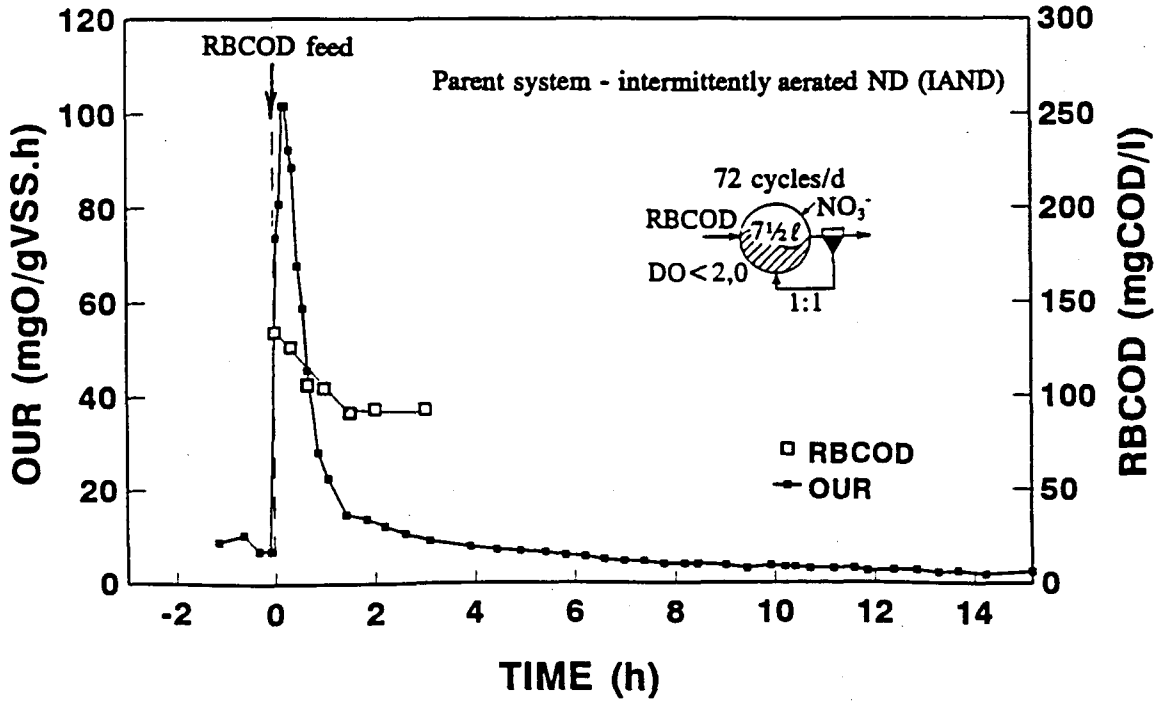
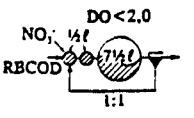


Fig A.14: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 7 (DSBT7) conducted on Day 222 on sludge from System 4.

Table A.26: Operating conditions for Defined Substrate Batch Test 8 (DSBT8).

BATCH TEST DSBT8 OPERATING CONDITIONS		
Objective: To examine the extent of induction of selector effect in system with 2 anoxic selectors fed RBCOD.		
PARENT SYSTEM CONDITIONS		
System 4; Day 286 System configuration:IAND & 2 anoxic selectors Substrate type: RBCOD Sludge age	(d)	 15
BATCH TEST CONDITIONS		
Total volume MLSS VSS F/M DO Temperature	(ℓ) (mg/ℓ) (mg/ℓ) (mgCOD/mgVSS (mgO/ℓ) (°C)	3,0 642 519 0,67 2 - 4 20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	346

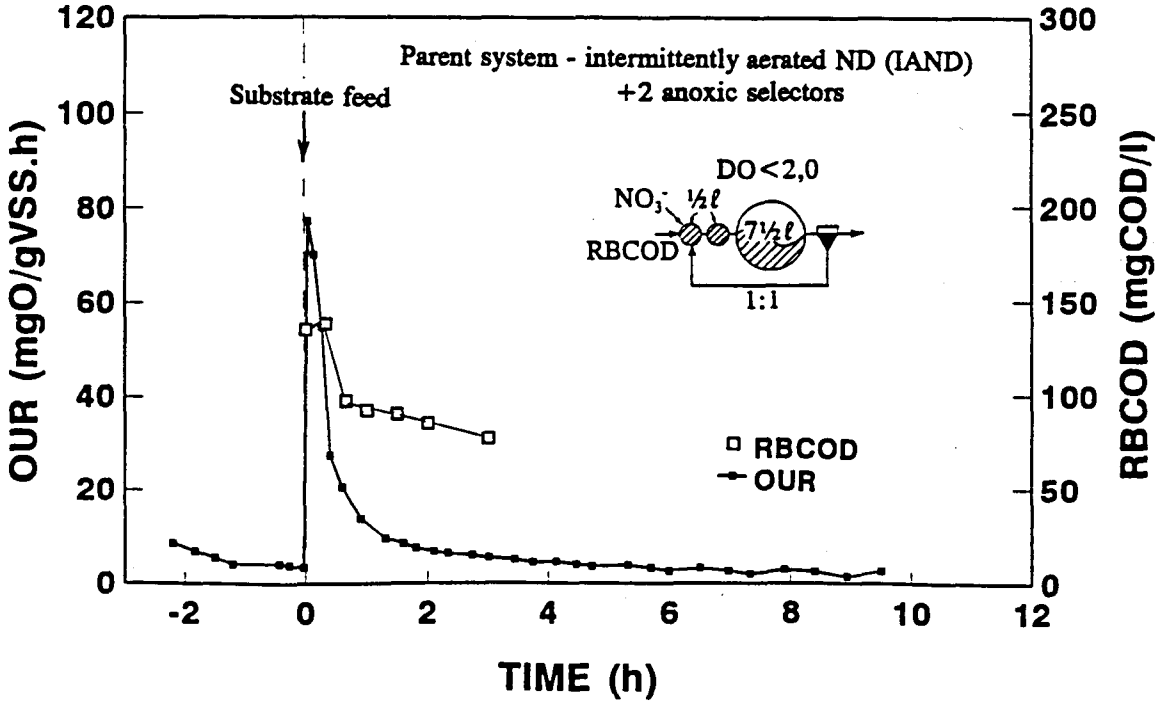
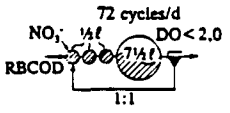


Fig A.15: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 8 (DSBT8) conducted on Day 286 on sludge from System 4.

Table A.27: Operating conditions for Defined Substrate Batch Test 9 (DSBT9).

BATCH TEST DSBT9 OPERATING CONDITIONS		
Objective: To examine the extent of induction of selector effect in system with 3 anoxic selectors.		
PARENT SYSTEM CONDITIONS		
System 4; Day 326 System configuration:IAND & 3 anoxic selectors Substrate type: RBCOD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	526
VSS	(mg/ℓ)	392
F/M	(mgCOD/mgVSS	0,88
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	346

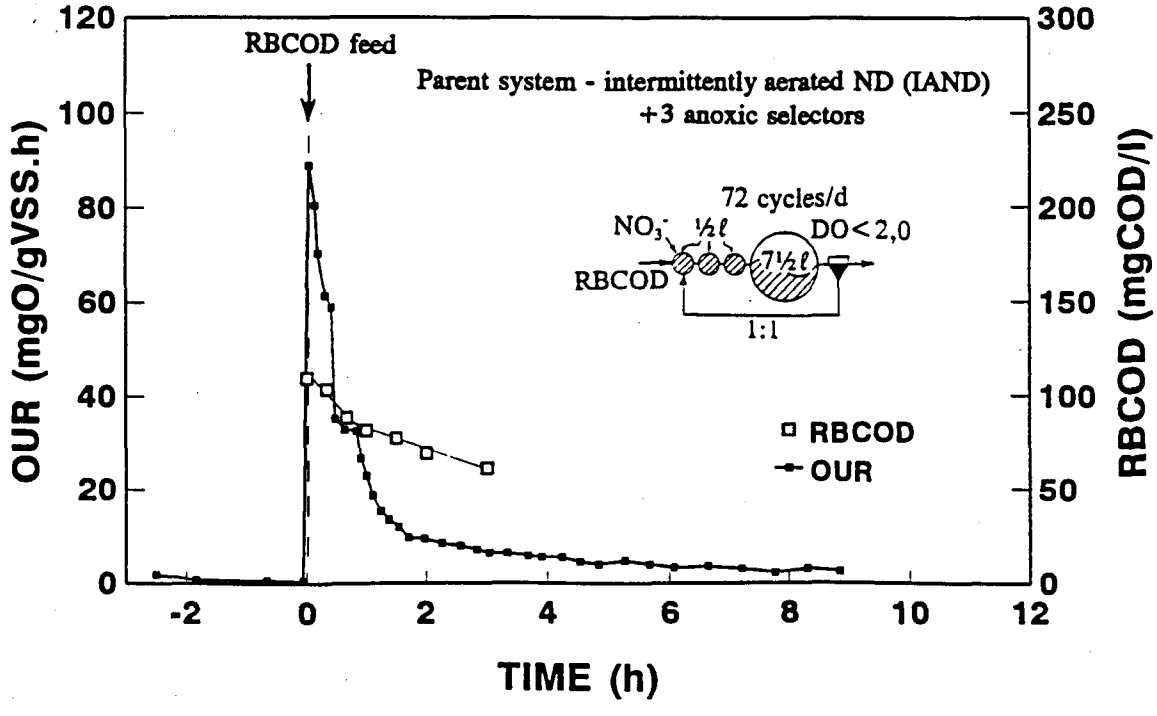
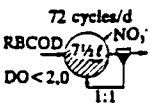


Fig A.16: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 9 (DSBT9) conducted on Day 326 on sludge from System 4.

Table A.28: Operating conditions for Defined Substrate Batch Test 10 (DSBT10).

BATCH TEST DSBT10 OPERATING CONDITIONS		
Objective: To examine the extent of induction of selector effect in IAND system fed RBCOD.		
PARENT SYSTEM CONDITIONS		
System 1; Day 328 System configuration: IAND Substrate type: RBCOD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3;0
MLSS	(mg/ℓ)	307
VSS	(mg/ℓ)	255
F/M	(mgCOD/mgVSS	-
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	-

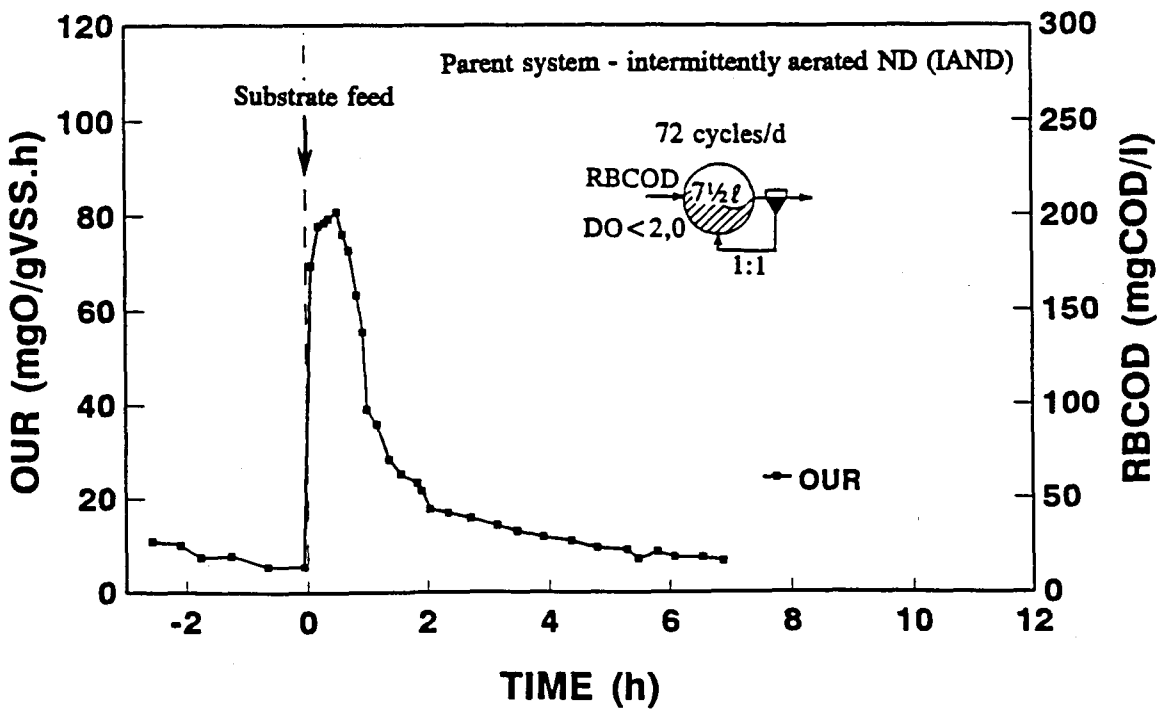


Fig A.17: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 10 (DSBT10) conducted on Day 328 on sludge from System 1.

Table A.29: Operating conditions for Defined Substrate Batch Test 11 (DSBT11).

BATCH TEST DSBT11 OPERATING CONDITIONS		
Objective: To examine the extent of induction of selector effect in IAND system fed SBCOD.		
PARENT SYSTEM CONDITIONS		
System 3; Day 334 System configuration: IAND Substrate type: SBCOD		72 cycles/d SBCOD, NO_3^-, 7½ℓ, DO<2,0, 1:1
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	562
VSS	(mg/ℓ)	481
F/M	(mgCOD/mgVSS	0,72
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	346

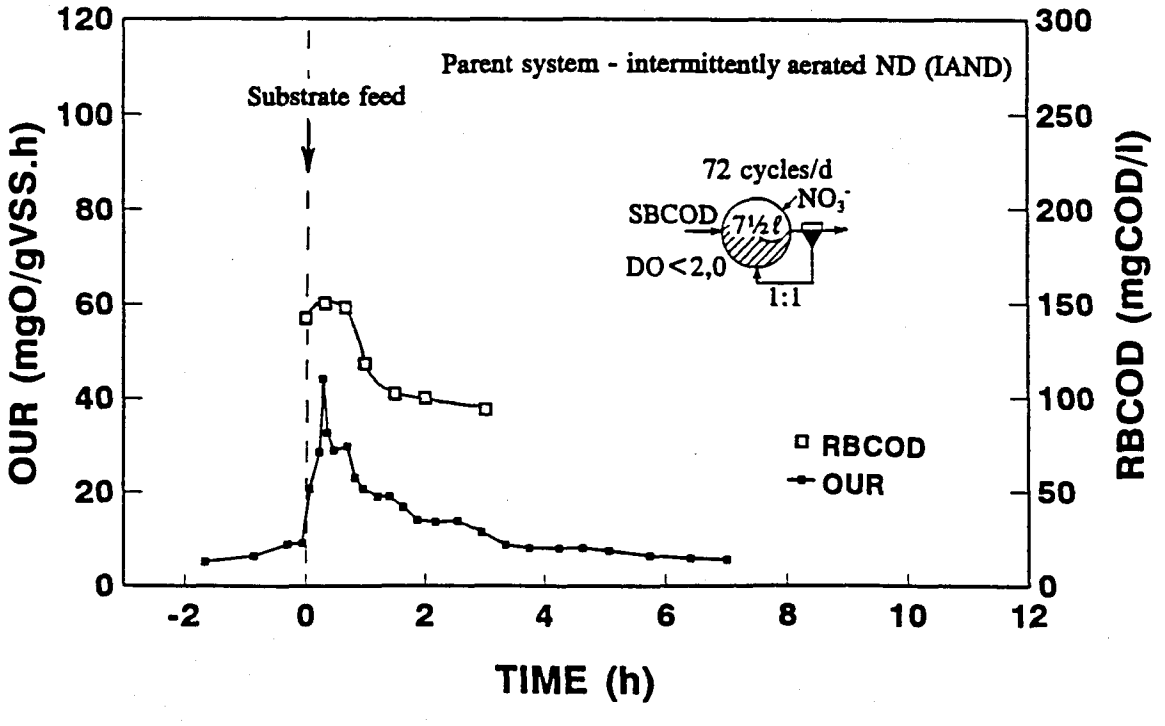
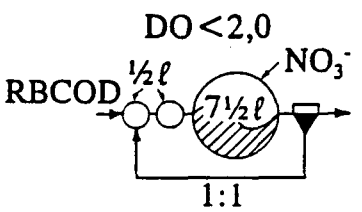


Fig A.18: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 11 (DSBT11) conducted on Day 334 on sludge from System 3.

Table A.30: COD profiles through System 4 incorporating 2 aerobic selectors.

COD PROFILES FOR 2 AEROBIC SELECTORS FOR SYSTEM 4						
Profile No. Day No.	1 181	2 188	3 194	4 209	Average	COD removed as percentage of influent (%)
Influent	410	341	417	387	389	-
Selector 1	102	93	102	151	112	87
Selector 2	57	89	114	104	91	5
Reactor	65	72	118	88	86	1
Effluent	26	57	77	83	61	-



	COD removal as % entering reactor	COD removal as % of influent
Selector 1	$\frac{389+61-112}{389+61} = 75$	$\frac{389+61-112}{389} = 87$
Selector 2	$\frac{112-91}{112} = 19$	$\frac{112-91}{389} = 5$
Reactor 3	$\frac{91-86}{91} = 5$	$\frac{91-86}{389} = 1$

Induction of the selector effect by anoxic selector reactor configuration

Three aerobic batch tests were conducted on System 4 during the period it incorporated 2 or 3 anoxic selector reactors: DSBT7 (Day 286 – selector effect measured); DSBT8 (Day 324 – selector effect measured); and DSBT9 (Day 324 – selector effect measured). As for the conclusions regarding induction of the selector effect by aerobic selectors, it is unclear as to whether the selector effect is a consequence of the anoxic selector reactor configuration, or the RBCOD–rich substrate fed to the system. However it is interesting to note that although the RBCOD–rich substrate was utilized under *anoxic* conditions in the 2 and 3 anoxic selector–reactor configurations, high substrate utilization rates were measured under *aerobic* batch test conditions.

To determine the efficacy of the anoxic selector reactors in removing RBCOD, three COD profiles were conducted when System 4 incorporated 2 anoxic selectors and eight COD profiles when System 4 incorporated 3 anoxic selectors. The results of the profiles for System 4, with 2 and 3 anoxic selectors are given in Table A.31 and Table A.32 respectively and indicate that 90% and 92% of the COD was utilized in the first selector reactor of the 2 and 3 anoxic selector reactor configurations respectively.

Selector effect removed with time under non–inductive conditions

An apparently anomalous result was measured for DSBT11 (System 3, Day 334) in which System 3 at the time of the batch test was being fed *particulate* substrate and a partial selector effect was measured. This can be attributed to the system being fed RBCOD–rich substrate for a period of a sludge age, 2 sludge ages previously. This conforms with the findings of Still *et al.* (1986) in which a period of 3 sludge ages was required for complete removal of the selector effect.

A.6 DETERMINATION OF THE INFLUENT RBCOD FRACTION OF THE DEFINED SUBSTRATE

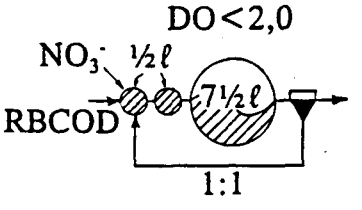
Introduction

After operation of IAND Systems 1–4 for more than 3 sludge ages it was recognized that filament proliferation with artificial substrate (DSVI ≈ 500 mℓ/g) was far greater than filament proliferation in configurations with the same conditions but fed municipal sewage (DSVI ≈ 250 mℓ/g).

It was concluded that certain characteristics of the artificial substrate were

Table A.31: COD profiles through System 4 incorporating 2 anoxic selectors.

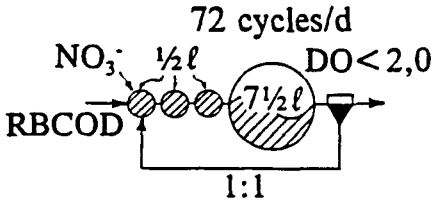
COD PROFILES FOR 2 ANOXIC SELECTORS FOR SYSTEM 4						
Profile No. Day No.	5 286	6 292	7 296	Average	COD Removal	COD removed as percentage of influent (%)
Influent	567	642	496	568	-	-
Selector 1	123	138	122	128	514	90
Selector 2	91	122	102	105	23	4
Reactor	83	100	81	88	17	3
Effluent	63	98	61	74	-	-



	COD removal as % entering reactor	COD removal as % of influent
Selector 1	$\frac{568+74-128}{568+74} = 80$	$\frac{568+74-128}{568} = 90$
Selector 2	$\frac{128-105}{128} = 18$	$\frac{128-105}{568} = 4$
Reactor	$\frac{105-88}{105} = 16$	$\frac{105-88}{568} = 3$

Table A.32: COD profiles through System 4 incorporating 3 anoxic selectors.

COD PROFILES FOR 3 ANOXIC SELECTORS FOR SYSTEM 4										
Profile No. Day No.	8 312	9 314	10 317	11 319	12 321	13 325	14 333	15 340	Average	COD removed as percentage of influent (%)
Influent	503	575	478	506	522	511	525	585	526	-
Selector 1	130	134	64	111	124	113	94	118	111	92
Selector 2	91	75	60	75	132	96	101	114	931	3
Selector 3	87	-	54	75	116	98	82	83	85	2
Reactor	77	75	58	79	132	88	57	77	80	1
Effluent	60	63	60	87	100	75	49	75	71	-



	COD removal as % entering reactor	COD removal as % of influent
Selector 1	$\frac{526-111+71}{526+71} = 81$	$\frac{526-111+71}{526} = 92$
Selector 2	$\frac{111-93}{111} = 16$	$\frac{111-93}{526} = 3$
Selector 3	$\frac{93-85}{93} = 9$	$\frac{93-85}{526} = 2$
Reactor	$\frac{85-80}{85} = 6$	$\frac{85-80}{526} = 1$

responsible for the greater proliferation of the filamentous organisms than with municipal sewage. According to the proposal of Chudoba (1985) the concentration of RBCOD in a system is of particular importance in the proliferation of filamentous organisms and attention was directed at determining the proportions of RBCOD and SBCOD in the artificial substrate to ascertain if a significant difference could be determined between the RBCOD fractions of the defined substrate and municipal sewage.

Background

The COD of municipal wastewaters has been categorized into two main fractions; biodegradable and unbiodegradable. Each fraction has 2 subfractions; for the biodegradable COD, these are soluble readily biodegradable and particulate slowly biodegradable, and for the unbiodegradable COD, soluble unbiodegradable and particulate unbiodegradable. The subdivision of biodegradable COD into soluble readily biodegradable COD (RBCOD) and particulate slowly biodegradable COD (SBCOD) is made on the basis of biological response to the aerobic batch test, as described in Section A.1.

Determination of the RBCOD fraction of artificial substrate as influent to the activated sludge process is essential for the proper operation of activated sludge systems for two reasons; (i) the concentration of RBCOD in the reactor has been hypothesized to play a role in filamentous organism proliferation (Chudoba, 1985) and therefore by implication so also the fraction of RBCOD in the influent, and (ii) research at the University of Cape Town has indicated that knowledge of the proportions of RBCOD and SBCOD in a wastewater or substrate is essential for accurate modelling of the behaviour of the activated sludge process to which the wastewater or substrate is fed (Dold *et al.*, 1980). However, the objective of this section is to determine the proportion of RBCOD in the wastewater with regard to its implication in bulking; modelling of systems fed defined substrate was not an objective of this investigation.

From the work of Gabb (1988) in characterizing the defined substrate it was concluded that the defined substrate had proportions of RBCOD and SBCOD similar to municipal wastewater. As noted in Section A.1, the method of assessment of the results enabling this conclusion to be drawn was somewhat unsound and given the finding that filament proliferation is very different between systems fed defined substrate and those fed municipal sewage, the proportion of RBCOD in the defined

substrate was again investigated.

Results

Biological determination of the influent readily biodegradable COD

In the determination of the influent readily biodegradable COD by the biological "square wave" method, the oxygen utilization rate (OUR) was monitored with time for a single reactor completely mixed aerobic activated sludge unit with sludge recycle, fed defined substrate with composition A. The system was operated at a sludge age of about 2 to 3 days (to avoid nitrification) under daily cyclic square wave loading conditions (i.e. 12 hours with feed, 12 hours without feed). Figure A.19 describes the change in OUR of a sludge developed on composition A under such conditions along with the OUR response predicted by the UCT Activated Sludge Model (Dold *et al.*, 1991). During feeding, the OUR remains high, and on feed termination there is a precipitous decrease in OUR, after which the OUR remains nearly constant for a period of 1 to 2 hours before decreasing to the rate associated with endogenous respiration. In terms of the general activated sludge model (Dold and Marais, 1985) the difference between the plateau values before and a short period ($\approx$ 15 mins) after feed termination gives a measure of the soluble readily biodegradable fraction of the feed (Ekama *et al.*, 1986). Taking the average of the results of 6 such tests, the soluble readily biodegradable fraction was calculated as 30% of the total influent COD. In comparison, application of the same procedure to municipal sewage yields an average value for the RBCOD fraction of 20–25% of the total COD. *Thus, it would appear that the artificial substrate comprises* 5–10% *more RBCOD than an average municipal sewage.* Attention was then directed at determining the source of the large RBCOD fraction by examining the contribution to RBCOD of the RBCOD suspension which is assumed to be the only source of RBCOD in the substrate.

Determination of the theoretical COD of the RBCOD suspension of the defined substrate

To analyse the contribution to COD of the RBCOD suspension, the theoretical COD was calculated for each of the constituents. The measured COD of the unfiltered RBCOD suspension was 10% of the total COD of the defined substrate mixture. The theoretical COD of each of the constituents was determined assuming complete oxidation to CO_2 and H_2O. As an example, the COD of acetic acid (HAc) is calculated as follows:

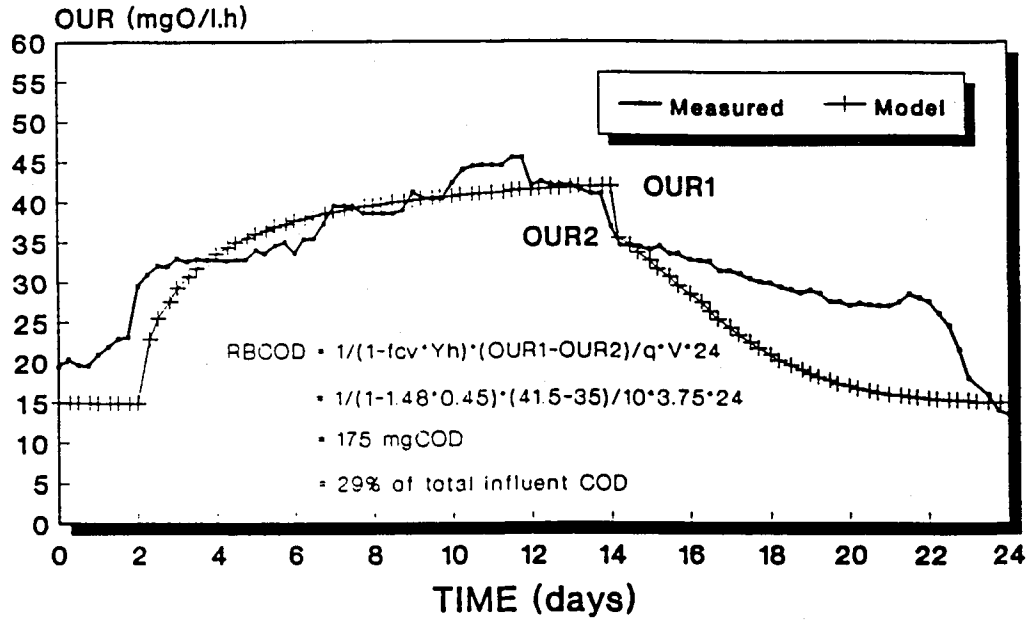


Fig A.19: Oxygen utilization rate (OUR in mgO/g.VSS.h) with time (h) for long sludge age (15 d) single reactor, cyclically loaded (12 h feed on, 12 h feed off) system fed defined substrate composition A.

Acetic acid: (mol.wt = 60 g/mol)

$CH_3\ COOH + 2\ H_2O \rightarrow 2\ CO_2 + 8\ H^+ + 8\ e^-$

$8\ H^+ + 8\ e^- + 2\ O_2 \rightarrow 4\ H_2O$

1 mole acetic acid requires 2 moles oxygen for complete combustion

i.e. 60 mg HAc requires 64 mg O_2, or, 60 mgHAc is equivalent to 64 mgCOD.

The 5ℓ stock solution of the RBCOD suspension includes 13,8 g of HAc and 20 mℓ of this stock solution is included in composition A. The contribution to total COD of HAc in the final composition can be calculated as follows:

$$\text{HAc fed daily} = \frac{20}{5\text{x}10^3} \cdot 13{,}8 = 55{,}2 \text{ mg}$$

$$\text{i.e. } [55{,}2 \text{ mgHAc}] \cdot \left[\frac{64 \text{ mgCOD}}{60 \text{ mgHAc}}\right] = 58{,}9 \text{ mg COD}$$

The contribution to COD of each of the constituents of the RBCOD suspension was calculated in the manner above and the values for each constituent are shown in Table A.33. The total theoretical COD of the RBCOD suspension is the sum of the theoretically calculated COD of each constituent, i.e., 488 mgCOD. Given that the experimentally determined total COD of the substrate is ≈ 6 000 mg, and assuming that the RBCOD suspension contributes essentially all of the RBCOD in the substrate mixture, the theoretically calculated RBCOD of the substrate mixture is 8,1% of the total COD. This value is considerably less than the value of 30% determined by the biological square wave method and alternative procedures were employed to examine this aspect. The results for each of the procedures are given in Table A.34 and the methods for the procedures are described below.

Physical separation of the soluble (< 0,45μm) fraction of defined artificial substrate

Because the biological square wave method requires at least 3 sludge ages (i.e. 6–9 days) for the system to become acclimatized to the loading conditions and a considerable amount of effort to obtain satisfactory results, Dold *et al.* (1986) directed attention at developing alternative rapid physical methods for determining the RBCOD fraction, Ultrafiltration of sewage through membranes of 100 000 molecular weight gave values for the readily biodegradable COD which closely correlated with values determined by the square–wave biological method. However, ultrafiltration requires special facilities and the authors suggested that in the absence of such facilities, filtrates from a vacuum filtered 0,45μm filter can give an approximate measure of the RBCOD; values of filtered COD being 25% higher than

Table A.33: Theoretically calculated contribution of each of the constituents of the RBCOD suspension.

COD OF CONSTITUENTS OF RBCOD SUSPENSION (From oxidation calculations)	
CONSTITUENTS	COD (mg/d)
Lactose	28
Acetate	59
Succinate	33
Citrate	44
D-Glucose	14
Maltose	148
Glycerol	13
Lactic Acid	52
Ethanol	60
Butanol	37
SUSPENSION	488

Table A.34: Summary of contribution to RBCOD of substrate suspensions as determined by microfiltration, biological square wave, and theoretical means.

RBCOD FRACTION OF SUSPENSIONS OF SUBSTRATE MIXTURE "A" (PERCENTAGE OF TOTAL SUBSTRATE MIXTURE COD)				
METHOD	MICROFILTRATION		BIOLOGICAL SQUARE WAVE	THEORETICAL
SUBSTRATE SUSPENSIONS	Measured (COD <0.45μm)	Calculated (4/5*COD <0.45μm)	Procedure of Ekama *et al.* (1986)	Oxidation calculations
RBCOD	10	7.5	-	8.1
Complex carbohydrates	4	3	-	-
Organic nitrogen	12.5	10	13	-
SUBSTRATE	-	-	30	-

those measured by ultrafiltration.

Accordingly, as a further measure of the RBCOD of the defined substrate (assumed to originate entirely from the RBCOD suspension), the RBCOD suspension was passed through a vacuum filtered 0,45μm filter. The COD of the filtrate was 10% of the total COD of the composition. Given that this procedure overestimates the true value of RBCOD by 25%, the RBCOD of the artificial substrate by this method is 7,5% of the total COD. This value is comparable with that determined by theoretical calculations (8,1%) for the RBCOD suspension as described above. Thus, it would appear that the RBCOD fraction measured by the biological square wave method (30%) is a measure not only of the RBCOD in the RBCOD suspension of the substrate (7,5%), but also includes the RBCOD of another component which is classified as "particulate slowly biodegradable COD" (SBCOD). The results of the biological square wave method indicate that this "particulate" COD fraction is utilized by the activated sludge organisms as RBCOD.

Attention was directed at the complex carbohydrates suspension as a source of RBCOD, this suspension having been assumed to comprise the SBCOD fraction of the substrate.

Determination of the biodegradability of the Complex Carbohydrate suspension of the defined substrate

Due to the large size of the molecules of the constituents of the Complex Carbohydrate suspension, it is unlikely that they would be utilized as RBCOD by the microorganisms. It was hypothesized that following preparation of the suspension in which the constituents are dissolved in water, one or some of the constituents degrades (solubilizes) to lower molecular weight compounds with time and are utilized as RBCOD. Experiments were designed to determine whether any of the Complex Carbohydrate group of substances solubilize with time and contribute to the RBCOD fraction, when

(a) stored in their concentrated (stock) form at 4° C for 1 month, or

(b) diluted with the other suspensions of the defined substrate mixture and stored in the stirred feed bucket at 4° C during the 24 hr feeding period.

Experimental set–up

Solutions of each of the individual constituents of the Complex Carbohydrates suspension, i.e. starch, dextrin and agar (but not cellulose) were made up to the same concentration as they are in the stock solutions and stored in 2ℓ closed glass flasks at 4°C. A fourth solution comprising a mixture of these solutions in the proportions found in the final substrate feed was stored under the same conditions. Cellulose was not included in the testing programme because of its unbiodegradable nature. The concentrated solutions were tested on a daily basis for a period of 29 days for the fraction of COD passing a 0,45μm filter.

Twice weekly, each of the concentrated solutions (agar, dextrin, starch and mixture) was diluted to the same concentration as is in the final substrate feed mixture, placed in an open 50 mℓ glass flask and fixed to a mechanical shaker for 24 hours to simulate the action of the stirrer in the feed bucket. The 50 mℓ flasks were open to the atmosphere as were the feed buckets in the laboratory investigation. The experiments were terminated after 14 days, this being the longest period for which the concentrated Complex Carbohydrate suspension was stored.

Results

During the 30 day storage experiment, the total COD of each of the samples remained essentially constant; any solubilization of constituents was a transformation of COD > 0,45μm to COD < 0,45μm.

Figure A.20 illustrates the change in COD < 0,45μm (due to solubilization) with time for the complex carbohydrate suspension and the 3 particulate slowly biodegradable constituents, agar, dextrin and starch each stored in concentrated form, as a percentage of the total COD of the complex carbohydrate suspension. The COD fraction < 0,45μm was about 10% of the total COD of the Complex Carbohydrates suspension. Because the Complex Carbohydrates suspension accounts for about 30% of the total COD of the defined substrate, solubilization of Complex Carbohydrates under storage in the concentrated (stock) form is about 3% of the total influent COD of the defined substrate mixture. Note that essentially all solubilization occurs in the first 3 days.

For the diluted, stirred solutions of the Complex Carbohydrate suspension, the changes in COD < 0,45μm with time are shown in Fig A.21. On average, about 14% of the COD of the mixture solubilizes under these conditions, equal to about

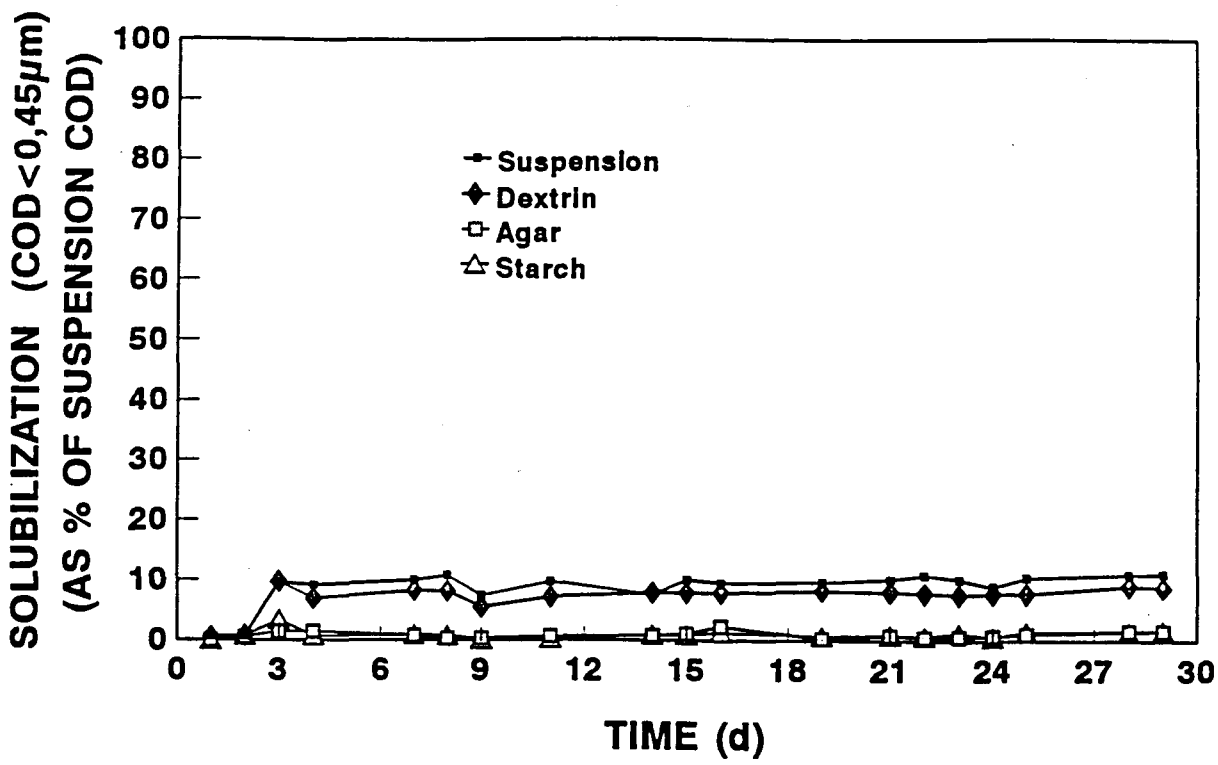


Fig A.20: Solubilization (COD < 0,45µm), with time for the complex carbohydrates suspension and its 3 constituents, agar, dextrin, and starch, as a percentage of the total COD of the suspension while stored in their concentrated form at 5° C.

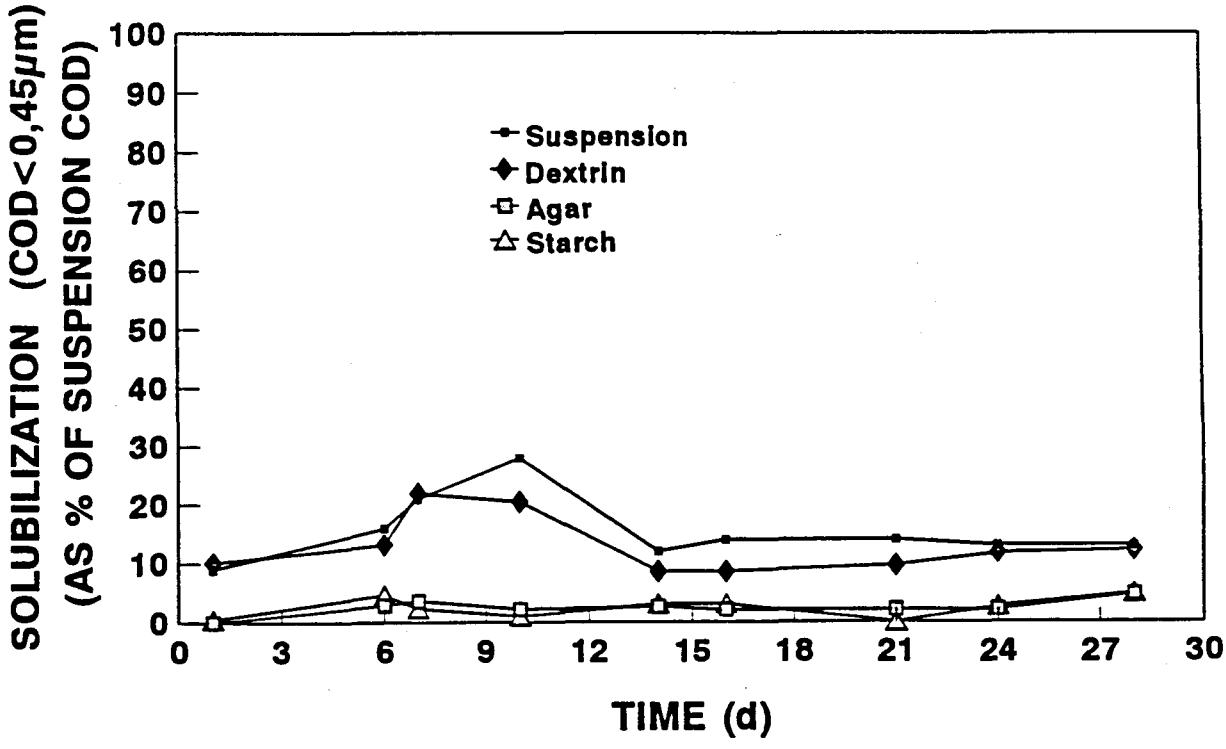


Fig A.21: Solubilization (COD < 0,45µm), with time, for the complex carbohydrates suspension and its 3 constituents, agar, dextrin, and starch, as a percentage of the total COD of the suspension, while stored in their diluted, stirred form at 5° C.

4% of the total influent COD of the defined substrate mixture, an additional solubilization of about 1% on the unstirred concentrated suspension as a percentage of the total COD of the substrate.

From the above it can be accepted that the compounds comprising the Complex Carbohydrate suspension did represent a particulate (> 0,45μm) fraction; solubilization of these constituents did not contribute greatly to the RBCOD of the suspension under storage conditions either in the concentrated form (for 1 month), or in the dilute stirred condition (for 24 hrs).

Solubilization of the particulate complex carbohydrate material (> 0,45μm) to soluble (< 0,45μm) material accounted for 4% of the total COD of the defined substrate mixture. Because the < 0,45μm filtration method overestimates the RBCOD content by 25%, the value of COD < 0,45μm (4%) corresponds to a RBCOD content of 3% of the total COD. Adding this amount of < 0,45μm COD (3%) to the < 0,45μm COD measured for the RBCOD suspension (7,5%) (i.e. 10,5% total), leaves 19,5% of the total influent RBCOD measured by the biological method (30%) unaccounted for.

The possibility that some other constituents of the defined substrate, regarded as particulate SBCOD could behave as soluble RBCOD was investigated. Attention was focussed on the Organic Nitrogen suspension.

Determination of the contribution to RBCOD from the Organic Nitrogen suspension
Two methods were used for determination of the RBCOD fraction of the Organic Nitrogen suspension of the defined substrate.

The first method was the biological square wave feeding procedure described earlier. The system was operated with defined substrate which did not include the RBCOD suspension (i.e. substrate feed type C); the only sources of COD were from the Organic Nitrogen and Complex Carbohydrate suspensions. In the defined substrate fed to the square wave system in these tests, the amount of Complex Carbohydrates was increased to accommodate the loss of COD through the omission of the RBCOD suspension (10% of total COD). This was an increase in Complex Carbohydrates from substrate feed type B (see Table A.10). Assuming 14% solubilization of the Complex Carbohydrates suspension, the contribution to COD < 0,45μm is 10% of the total COD of the SBCOD-rich substrate mixture. Given that filtration

$< 0{,}45\mu m$ yields a value of COD 25% less than the true value of RBCOD, this value corresponds to an RBCOD fraction of 8% of the total COD.

From the average of the results of 4 biological square wave tests, the RBCOD contribution from the Complex Carbohydrate and Organic Nitrogen mixtures was 21%. Accepting the value of 8% determined above for the contribution to RBCOD from the Complex Carbohydrates, the contribution to RBCOD from the Organic Nitrogen suspension is 13%.

The second method for the determination of the RBCOD contribution from the Organic Nitrogen suspension was physical separation by $0{,}45\mu m$ filtration after testing for solubilization with time in a procedure similar to that described earlier for the Complex Carbohydrates suspension. Solutions of each of the individual components of the Organic Nitrogen suspension, i.e. casein, peptone, yeast, and gelatin were made up to the same concentration as they were present in the concentrated solution and stored in 2ℓ closed glass flasks at 4° C for 2 weeks. Each day during this period the solutions were measured for COD $< 0{,}45\mu m$. Figure A.22 illustrates the change in $< 0{,}45\mu m$ COD for each constituent and the Organic Nitrogen suspension with time for the 2 week period. Little change in COD$< 0{,}45\mu m$ with time is evident. The COD $< 0{,}45\mu m$ of the Organic Nitrogen suspension measured immediately after making up each solution constitutes 90 percent of the total COD of the suspension[1]. The Organic Nitrogen suspension contributes 14% to the total COD of the substrate (Table A.12). Thus, the Organic Nitrogen suspension COD $< 0{,}45\mu m$ contributes 12,5% to the total COD of the defined substrate which corresponds to a value of 10% as RBCOD given that filtration $< 0{,}45\mu m$ overestimates the RBCOD by 25%. This value compares favourably with the value of 13% calculated from biological measurements.

The total RBCOD of the average defined substrate is the addition of the RBCOD fractions measured (with $0{,}45\mu m$ filtration) for the RBCOD suspension (7,5%), for the complex carbohydrates (3%) and the organic nitrogen (10%), that is, 20,5% in total. This does not compare well with the value of 30% determined by the biological square wave test, and it would appear that the assumption that the fraction passing $0{,}45\mu m$ overestimates the true RBCOD by 25% is applicable to raw sewage (on which the procedure was developed), but not necessarily to the defined

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[1]This value was calculated by taking the average value of the COD $< 0{,}45\mu m$ as a percentage of the unfiltered COD of the suspension.

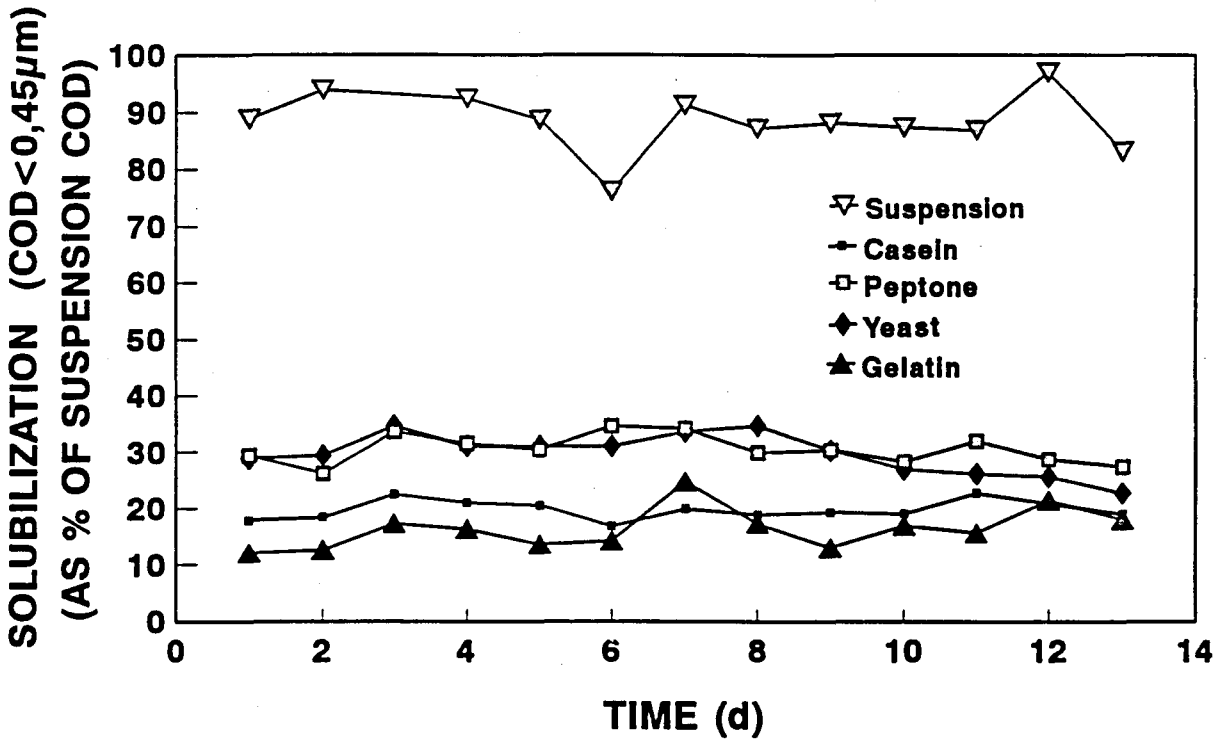

Fig A.22: Solubilization (COD < 0,45μm), with time, for the Organic Nitrogen suspension and its 4 constituents, casein, peptone, yeast, and gelatin, as a percentage of the total COD of the suspension while stored in their concentrated form at 5° C.

substrate used in this investigation. If this assumption is not made and it is considered that the fractions passing 0,45μm are a true estimate of RBCOD, the total RBCOD is 26,5%, i.e. RBCOD suspension (10%), complex carbohydrates (4%) and organic nitrogen (12,5%). The contribution to RBCOD of the suspensions, calculated by the methods described above are shown in Table A.34.

APPENDIX B

EXPERIMENTS WITH MUNICIPAL SEWAGE

In the experimental investigation using municipal sewage as influent, three types of system configuration were employed;

(i) Single reactor; either intermittently aerated nitrification–denitrification (IAND), continuous aerobic, or continuous anoxic – Systems 5 to 8;

(ii) Two–reactor nitrification–denitrification (2RND) – System 9;

(iii) Multi–reactor nitrification–denitrification biological excess phosphorus removal (NDBEPR) Modified UCT (MUCT) – Systems 10 to 17.

APPENDIX B.1

SINGLE REACTOR; INTERMITTENTLY AERATED NITRIFICATION-DENITRIFICATION (IAND) CONTINUOUS AEROBIC, AND CONTINUOUS ANOXIC SYSTEMS

Single reactors were operated with many different aeration and substrate feeding patterns to investigate the effect of these on filamentous organisms

Daily measured data

Figures B.1, B.2, B.3 and B.4 present daily measured data for Systems 5, 6, 7 and 8 respectively (DSVI, Effluent NO_2/NO_3^-, OUR, MLSS & VSS, TKN, COD). The values of the daily measured data for Systems 5–8 are contained in spreadsheets configured in QUATTRO and stored in files SYS5.WKQ to SYS8.WKQ respectively.

Changes in operating conditions

Tables B.1, B.2, B.3 and B.4 present changes in operating parameters for Systems 5, 6, 7 and 8 respectively.

Filament identifications

Tables B.5, B.6, B.7 and B.8 present changes in filament types and abundances for Systems 5, 6, 7 and 8 respectively. Table B.9 summarizes the conditions which, from the experimental work with Systems 5 to 8 appear to promote, reduce, or sustain growth of filamentous organisms.

COD and Nitrogen (N) mass balances

Tables B.10, B.11, B.12 and B.13 present the COD and Nitrogen mass balances for Systems 5, 6, 7 and 8 respectively. The procedures for calculation of the COD and Nitrogen mass balances are described and a sample calculation is illustrated for Steady State Period 1 of System 5.

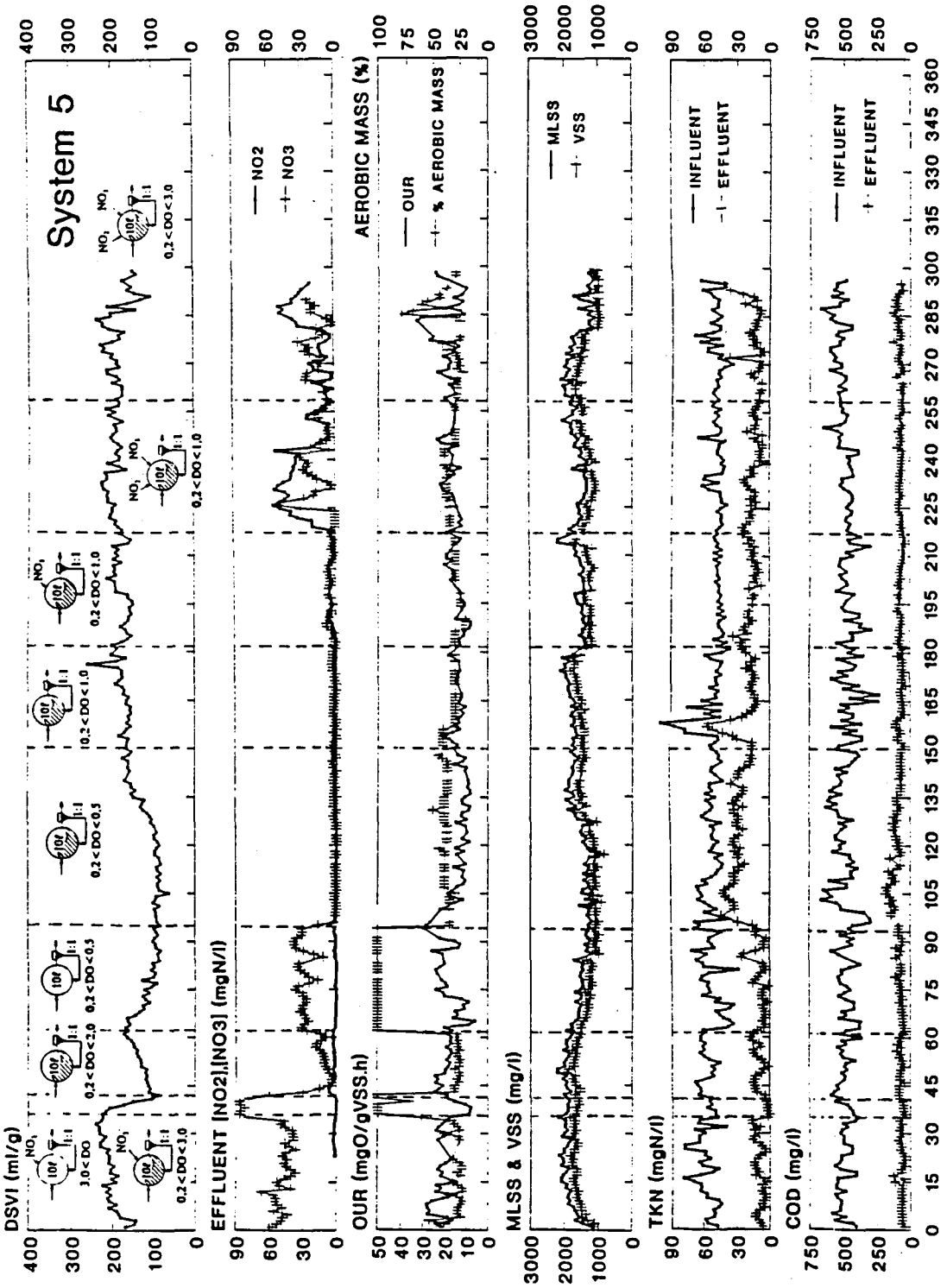
System 5
DSVI (ml/g)
EFFLUENT [NO2],[NO3] (mgN/l)
NO2
NO3
OUR (mgO/gVSS.h)
AEROBIC MASS (%)
OUR
% AEROBIC MASS
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
DAY NO.

Fig B.1

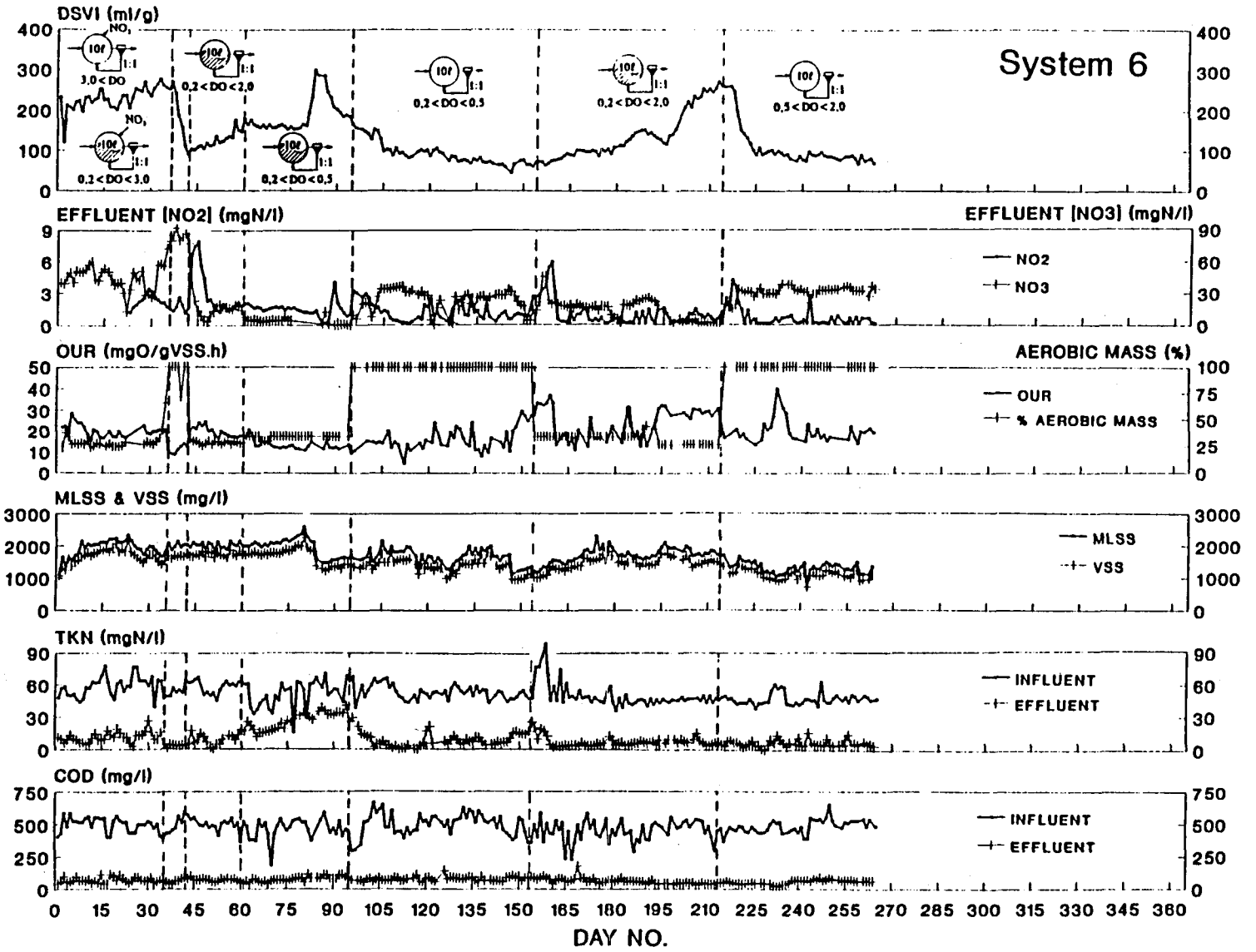
DSVI (ml/g)
System 6
3,0<DO
0,2<DO<2,0
0,2<DO<0,5
0,2<DO<2,0
0,5<DO<2,0
0,2<DO<3,0
0,2<DO<0,5
EFFLUENT [NO2] (mgN/l)
EFFLUENT [NO3] (mgN/l)
NO2
NO3
OUR (mgO/gVSS.h)
AEROBIC MASS (%)
OUR
% AEROBIC MASS
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360
DAY NO.

Fig B.2

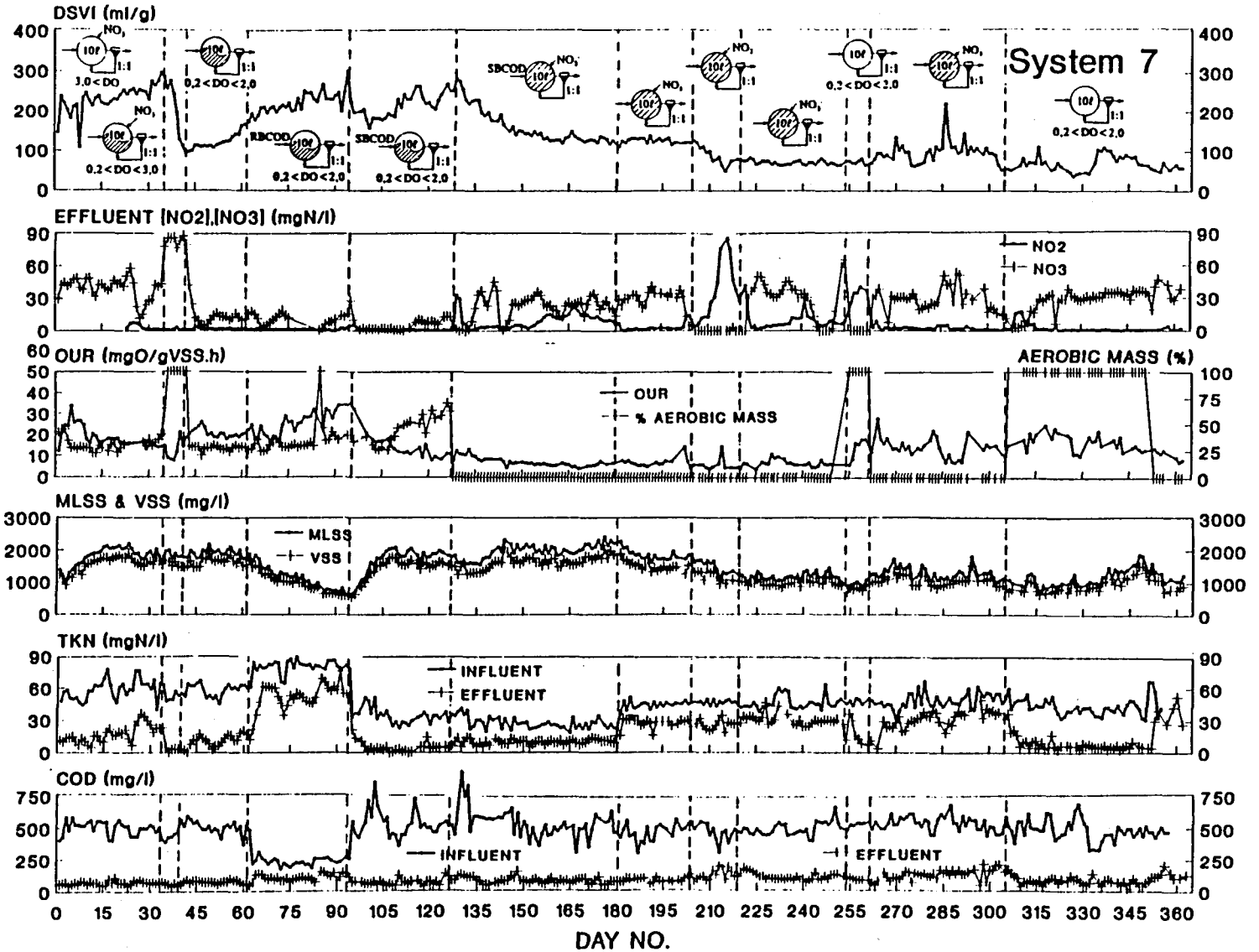
DSVI (ml/g)
System 7
EFFLUENT [NO2],[NO3] (mgN/l)
NO2
NO3
OUR (mgO/gVSS.h)
AEROBIC MASS (%)
OUR
% AEROBIC MASS
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
DAY NO.

Fig B.3

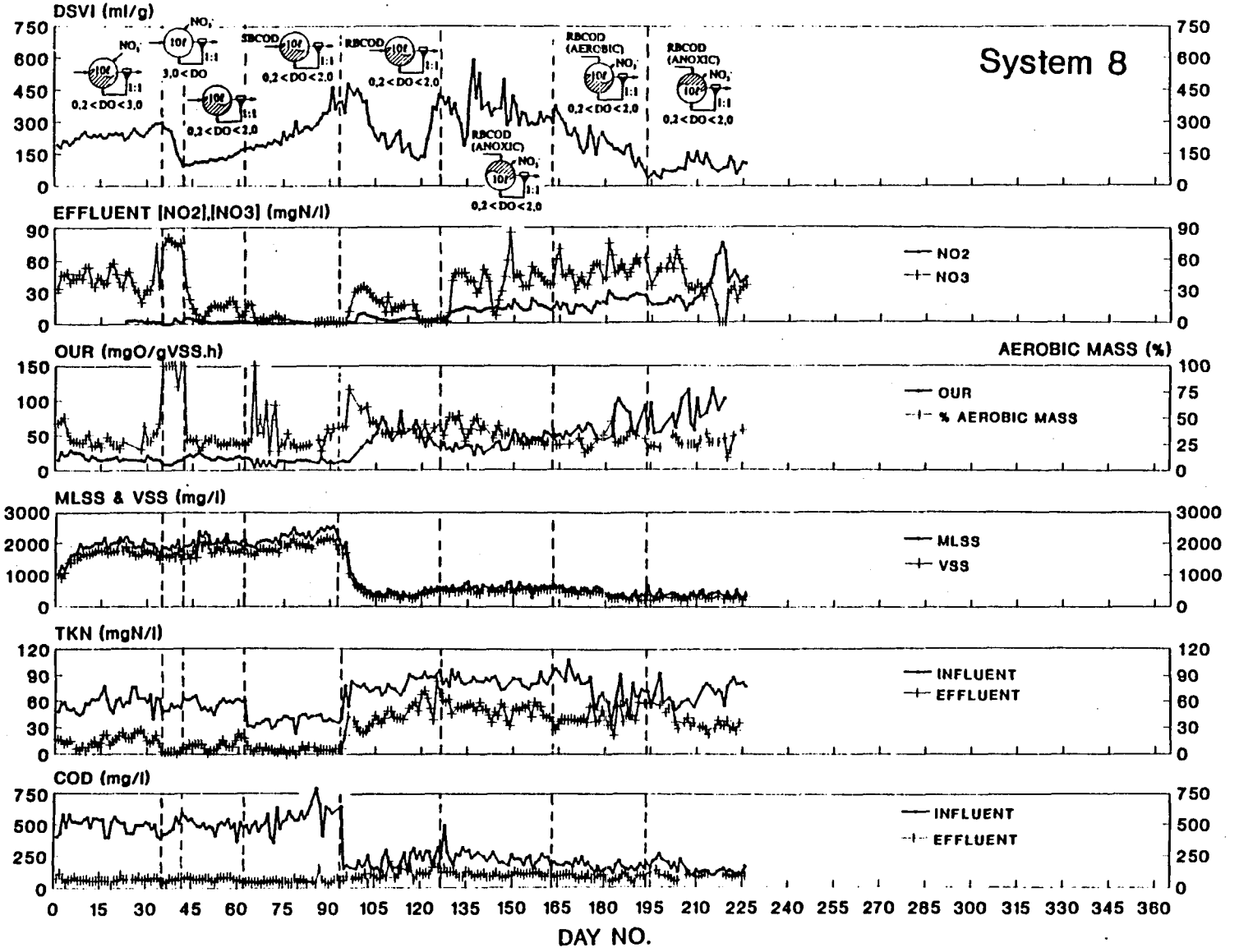
DSVI (ml/g)
System 8
EFFLUENT [NO2],[NO3] (mgN/l)
NO2
NO3
OUR (mgO/gVSS.h)
AEROBIC MASS (%)
OUR
% AEROBIC MASS
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
DAY NO.

Fig B.4

Table B.1

SYSTEM 5 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 35	Intermittent aeration - very high DO (0,2 < DO < 3,0 mgO/ℓ) 30-35% aerobic	To determine if intermittent aeration induces AA filament proliferation: The DSVI increased rapidly, from ≈ 150 to ≈ 225 ml/g; a rate similar to the increase in DSVI during the same period in Systems 6 - 8 and *M. parvicella* was the dominant filament.
36 - 42	Continuous aeration (DO > 3,0 mgO/ℓ)	To determine if continuous aeration reduces AA filament proliferation: The DSVI decreased rapidly from ≈ 225 to < 100 ml/g; a rate similar to the decrease in DSVI during the same period in Systems 6 - 8.
43 - 61	Intermittent aeration - high DO (0,2 < DO < 2,0 mgO/ℓ) 30-35% aerobic	To increase the DSVI to above 150 ml/g: The DSVI increased from < 100 to ≈ 165 ml/g and *M. parvicella* was dominant.
62 - 94	Continuous aeration - low DO (0,2 < DO < 0,5 mgO/ℓ)	To determine if AA filaments proliferate in the low DO period (0,2 < DO < 0,5) of IAND systems: The DSVI decreased from ≈ 265 ml/g to ≈ 100 ml/g. Note that the concentration of NO_3^- in the effluent (< 1,0 mgN/ℓ) is considerably less than the concentration of NO_3^- (≈ 30 mgN/ℓ)
95 - 150	Intermittent aeration - low DO (0,2 < DO < 0,5 mgO/ℓ) 30-35% aerobic	To determine if AA filaments can proliferate under intermittent aeration conditions if the aerobic period of the low DO period is DO (0,2 < DO < 0,5): The DSVI did not increase until Day 120, then increased to a constant value ≈ 160 ml/g by Day 150. The dominant filament was *H. hydrossis*. Note that in contrast to the continuous low DO conditions, the concentrations of NO_2^- (1,5 mgN/ℓ) and NO_3^- (1,5 mgN/ℓ) are similar. This aspect is discussed in Appendix E in dealing with nitrification.
151 -181	Intermittent aeration - intermediate DO (0,2 < DO < 1,0 mgO/ℓ) 30-35% aerobic	To determine if filaments proliferate more when the DO in the aerobic period is increased: The DSVI increased from ≈ 160 ml/g to ≈ 200 ml/g and type 0092 was dominant.
182 - 217	Intermittent aeration - intermediate DO (0,2 < DO < 1,0 mgO/ℓ) 30-35% aerobic Continuous NO_3^- addition	To determine if high concentrations of NO_3^- increase filament proliferation: The DSVI was not constant but remained between 150 and 200 ml/g and filament types 0092 and 0041 were both dominant for some of the time. Note that although NO_3^- was being added, the proportion of NO_2^- (≈ 4 mgN/ℓ) to NO_3^- (≈ 4 mgN/ℓ) did not change.
218 - 258	Intermittent aeration - intermediate DO (0,2 < DO < 1,0 mgO/ℓ) 30-35% aerobic Continuous NO_3^- and NO_2^- addition	To determine the effect of high concentrations of NO_2^- (> 10 mgN/ℓ): The DSVI fluctuated between 180 and 220 ml/g. The dominant filament was *M. parvicella*.
259 - 300	Intermittent aeration - high DO (2,0 < DO < 3,0 mgO/ℓ) 30-35% aerobic Continuous NO_3^- and NO_2^- addition	To determine if increasing the DO in the aerobic period (2,0 < DO < 3,0) under intermittent aeration conditions results in filament proliferation: The DSVI increased to ≈ 225 ml/g by Day 276 and then decreased to ≈ 150 ml/g by Day 300. The increase in DSVI resulted from an increase in percent aerobic fraction caused a loss of sludge in the settler. This aspect is discussed in greater detail in chapter 3.

Table B.2

SYSTEM 6 :	CHANGES IN OPERATING CONDITIONS	
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 35	Intermittent aeration - very high DO. (0,2 < DO < 3,0 mgO/ℓ) 30-35% aerobic	To determine if intermittent aeration induces filament proliferation: The DSVI increased steadily from ≈ 200 to ≈ 250 mℓ/g (compare Systems 5, 7 and 8). Filament type 021N was dominant. Effluent NO_{3+2}^- (≈ 47 mg N/ℓ).
36 - 42	Continuous aeration - very high DO. (DO > 3,0 mgO/ℓ)	To determine if continuous aeration reduces filament proliferation: The DSVI decreased rapidly from ≈ 250 to < 100 mℓ/g (compare Systems 5, 7 and 8).
43 - 60	Intermittent aeration - high DO. (0,2 < DO < 2,0 mgO/ℓ) 30-35% aerobic	Note that the increase in effluent NO_{3+2}^- (≈ 85 mgN/ℓ) compared to intermittent aeration. Note that the COD mass tolerance (93%) is better than the previous intermittent aeration period (see Table B.11 & Fig. 3.10). To increase the DSVI to above 150 mℓ/g: The DSVI increased from < 100 to ≈ 165 mℓ/g (compare with Systems 5, 7 and 8). Filament type 021N was dominant.
61 - 94	Intermittent aeration - low DO (0,2 < DO < 0,5) 30-35% aerobic	To determine if filaments proliferate under intermittent aeration conditions when the aerobic period of the cycle is low DO (0,2 < DO < 0,5): The DSVI did not increase until Day 80, then increased rapidly to Day 86, then decreased to ≈ 160 mℓ/g by Day 95. The dominant filament was type 021N. Note that the effluent NO_2^- (≈ 1,5 mgN/ℓ) and NO_3^- (1-4,5 mgN/ℓ) concentrations are similar and low, due to incomplete nitrification of NH_4^+. Compare with Days 94-150 of System 5.
95 - 153	Continuous aeration - low DO (0,2 < DO < 0,5 mgO/ℓ)	To determine if filaments proliferate in the low DO period (0,2 < DO < 0,5) of IAND systems: The DSVI decreased from ≈ 160 to ≈ 65 mℓ/g. Initially filament type 0041 was dominant and then type 0092 became dominant. Note that the proportions of effluent NO_2^- (≈ 1,5 mgN/ℓ) and NO_3^- (≈ 20 mgN/ℓ) are very different to intermittent aeration due to more complete nitrification of NH_4^+. Compare change in DSVI with Days 62-94 of System 5 in which DSVI decreased and type 0041 became secondary.
154 - 213	Intermittent aeration - high DO (0,2 < DO < 2,0 mgO/ℓ) 30-35% aerobic	To determine if filaments proliferate more when the DO in the aerobic period is high: The DSVI increased steadily to ≈ 270 mℓ/g and *H.hydrossis* was dominant. Note that the average COD mass balance (94%) is generally better than periods of intermittent aeration (see Fig. 3.10 & Table B.11).
214 - 265	Continuous aeration - high DO (0,2 < DO < 2,0)	To compare the ameliorating affect of high DO with continuous low DO (Days 95-153) and continuous very high DO (Days 36-42): The DSVI decreased initially rapidly to < 100 mℓ/g and then slowly to ≈ 70 mℓ/g. The dominant filament was type 0041. The rate of decrease in DSVI was proportional to the concentration of DO. Note that the proportion of effluent NO_2^- (< 1 mgN/ℓ) to NO_3^- (25-30 mgN/ℓ) are different to those at high DO (see Days 95 - 153), due to increased nitrification of NO_2^-. Note that as with Days 36-42 and Days 95-153, the average COD mass balance (94%) is better than periods of intermittent aeration (see Fig. 3.10 & Table B.11).

Table B.3

SYSTEM 7 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 35	Intermittent aeration - very high DO (0,2 < DO < 3,0) 30-35% aerobic	To determine if intermittent aeration induces filament proliferation: The DSVI increased steadily from ≈ 200 to ≈ 290 ml/g; (compare Systems 5, 6 and 8). Filament type 021N was dominant. Effluent $NO_{x,e}^-$ (≈ 45 mgN/ℓ).
36 - 42	Continuous aeration - very high DO (DO > 3,0 mgO/ℓ)	To determine if continuous aeration reduces filament proliferation: The DSVI decreased rapidly from ≈ 290 ml/g to < 100 ml/g; a rate similar to the decrease in DSVI during the same period in Systems 5, 6 and 8. Note the increase in effluent $NO_{x,e}^-$ (≈ 85 mgN/ℓ) due to more complete nitrification of NH_4^+. Note that COD mass balance (100%) is better than the previous intermittent aeration period (95%) (see Fig. 3.11 & Table B.12).
43 - 62	Intermittent aeration - very high DO (0,2 < DO < 2,0) 30-35% aerobic	Note that the increase in effluent $NO_{x,e}^-$ (≈ 85 mgN/ℓ) compared to intermittent aeration. Note that the COD mass balance (93%) is better than the previous intermittent aeration period (see Fig. 3.11 & Table B.12) To increase the DSVI to above 150 ml/g: The DSVI increased from < 100 to ≈ 165 ml/g (compare with Systems 5, 7 and 8). Filament type 021N was dominant.
63 - 94	Intermittent aeration - high DO (0,2 < DO < 2,0) 30-35% aerobic RBCOD substrate	To determine filaments proliferate with RBCOD: The DSVI increased to 250 ml/g (compare with System 8, Days 95-127). The dominant filament was type 021N. The high influent NH_4^+ (72 mgN/ℓ) was a result of NH_4^+ in the soluble fraction of sewage, effluent NO_3^- and NO_2^- were ≈ 1 and ≈ 10 mgN/ℓ respectively.
95 - 127	Intermittent aeration - high DO (0,2 < DO < 2,0) 30-35% aerobic SBCOD substrate	To determine if filaments proliferate with SBCOD: The DSVI decreased initially, then increased to ≈ 250 ml/g (compare with System 8, Days 63-94). The dominant filament was type 021N. The effluent NO_3^- (≈ 0,7 mgN/ℓ) and NO_2^- (≈ 6 mgN/ℓ) were not very different even through the influent NH_4^+ (17 mgN/ℓ) was less than for RBCOD as substrate.
128 - 180	Continuous anoxic Continuous NO_3^- addition SBCOD substrate	To determine if filaments proliferate under anoxic conditions: The DSVI decreased to 130 ml/g. The dominant filament was initially *H. hydrossis*, and then type 0092. The effluent NO_3^- concentration increased from 1 to ≈ 10 mgN/ℓ as the DSVI decreased.
181 - 202	Continuous anoxic Continuous NO_3^- addition	Change from SBCOD to non-fractionated average sewage: The DSVI remained constant at ≈ 130 ml/g.
203 - 221	Continuous anoxic Continuous NO_2^- addition	To determine if filaments can proliferate with NO_2^- as electron acceptor under anoxic conditions: The DSVI decreased to ≈ 75 ml/g.
222 - 254	Continuous anoxic Continuous NO_3^- addition	To determine if changing electron acceptor to NO_3^- results in increase in DSVI: The DSVI remained constant at ≈ 75 ml/g.
255 - 261 262 - 305 306 - 352 353 - 363	Continuous aerobic - Continuous anoxic - Continuous aerobic - Continuous anoxic -	Experiments to determine the extent of synthesis of aerobic and denitrifying enzymes under aerobic and anoxic conditions (see chapter 6): DSVI remained < 100 ml/g. Note that the COD mass balance for the aerobic period, Days 335-352 (90%) is better than for periods of intermittent aeration (see Table B.12).

Table B.4

SYSTEM 8 :	CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 35	Intermittent aeration - very high DO (0,2 < DO,3,0) 30-35% aerobic	To determine if intermittent aeration induces filament proliferation: The DSVI increased steadily from ≈ 200 to ≈ 300 ml/g (compare with Systems 5, 6 and 7). Effluent NO_{x+3}^- ≈ 48 mgN/ℓ. Filament *M.parvicella* was dominant.
36 - 42	Continuous aeration - very high DO (DO > 3mgO/ℓ)	To determine if continuous aeration reduces filament proliferation: The DSVI decreased rapidly from ≈ 300 ml/g to < 100 ml/g (compare with Systems 5, 6 and 7). The effluent NO_{x+3}^- (≈ 79 mgN/ℓ) was higher than for intermittent aeration (≈ 48 mgN/ℓ). Note that COD mass balance (99%) for continuous aerobic is better than all intermittent aeration periods (see Fig. 3.12 & Table B.13).
43 - 62	Intermittent aeration - high DO (0,2 < DO < 2,0)	To increase the DSVI to above 150 ml/g: The DSVI increased from < 100 to ≈ 165 ml/g (compare System 7). *M.parvicella* was dominant. Effluent NO_2^- (≈ 2 mgN/ℓ) and NO_3^- (≈ 15 mgN/ℓ).
63 - 94	Intermittent aeration - high DO (0,2 < DO < 2,0) SBCOD substrate	To determine if filaments proliferate with SBCOD: The DSVI increased to ≈ 400 ml/g (compare System 7, Days 95-127). *M.parvicella* was dominant. The effluent NO_2^- (<0,5 mgN/ℓ) and NO_3^- (≈ 4 mgN/ℓ) were less than for complete sewage (Days 43-62).
95 - 127	Intermittent aeration - high DO (0,2 < DO < 2,0) 30-35% aerobic RBCOD substrate	To determine if filaments proliferate with RBCOD: the DSVI initially decreased to ≈ 130 ml/g by Day 119 but then increased to ≈ 420 ml/g the increase due to *H.hydrosis* (compare with System 7, Days 63-94). High influent NH_4^+ (≈ 75 mgN/ℓ) resulted from NH_{4+} in soluble fraction of sewage, effluent NO_2^- (≈ 3 mgN/ℓ) and NO_3^- (<2 mgN/ℓ).
128 - 162	Intermittent aeration - high DO (0,2 < DO < 2,0) RBCOD substrate fed to anoxic zone Continuous NO_3^- addition	To determine if filaments proliferate with RBCOD under anoxic conditions: The DSVI initially decreased to ≈ 200 ml/g by Day 135, then increased to ≈ 300 ml/g. Effluent NO_2^- (≈ 15 mgN/ℓ) and NO_3^- (≈ 40 mgN/ℓ).
163 - 193	Intermittent aeration - high DO (0,2 < DO < 2,0) RBCOD substrate fed to aerobic zone Continuous NO_3^- addition	To determine if filaments proliferate with RBCOD under aerobic conditions: The DSVI decreased to ≈ 50 ml/g and filament type 0041 was dominant.
194 - 226	Intermittent aeration (0,2 < DO < 2,0) RBCOD substrate fed to anoxic zone Continuous NO_3^- addition	To repeat experiments conducted on Days 128-162: The same trend occurred, the DSVI increased to ≈ 100 ml/g.

Table B.5

Day No. System 5	Filament abundance	Dominant	Secondary	Tertiary	Remarks
7	abundant	021N	*M.parvicella*, 0092	0041, 1851, 0961	
38	some-common	*M.parvicella*	0092	*Thiothrix* sp.	•chains of large cocci
69	common-very common	*M.parvicella*	0092	*H.hydrossis*, 0961, 0041, 021N	
96	few-some	*H.hydrossis*	0041	*M.parvicella*, 0961	•*Fungus* sp. present
129	common-very common	0092	021N	*M.parvicella*, *Beggiatoa* sp., 0841, *H.hydrossis*	
152	common-very common	0092	*H.hydrossis*	*Beggiatoa* sp., 0961, *M.parvicella*, 0041	
187	very common	0041	0092	0961, *M.parvicella*, *Beggiatoa* sp., *H.hydrossis*, *N.limicola I*	
227	common-very common	0041	0092	021N, *H.hydrossis*, *M.parvicella*	•chains of cocci
257	very common-abundant	*M.parvicella*	0092	0041, *H.hydrossis*, 021N	•chains of cocci
298	very common-abundant	*M.parvicella*	0092	*Beggiatoa* sp., *Flexibacter*, 0041, 1851, 021N	•attached ciliates common

Table B.6

Day No. System 6	Filament abundance	Dominant	Secondary	Tertiary	Remarks
7	abundant	021N	*M.parvicella*, 0092	0041, 1851, 0961	
38	very common	021N	*M.parvicella*	*0092, 0041, Thiothrix* sp.	
69	very common	021N	0041	Flexibacter sp., *M.parvicella*, 0961, 0092	•flagellates (*Monosiga* sp.) common •finger-like zooglea
96	few-some	021N	*H.hydrossis*	*M.parvicella*, 0092, 0961, 0041	
129	common-very common	0041	*H.hydrossis*	1701, 0092	•*Aeolosoma* sp. present •chains of cocci
152	some-common	0092	0041	0961, 0803	
187	very common	*H.hydrossis*	0041	021N, 0961, 0092	•a few finger-like zooglea
227	very common	0041	1851	*M.parvicella*, 021N, 0092	•fungus sp. •chains of cocci
257	common-very common	0041	*M.parvicella*	*H.hydrossis*, 0092, 0961	

Table B.7

Day No. System 7	Filament abundance	Dominant	Secondary	Tertiary	Remarks
7	abundant	021N	*M.parvicella*, 0092	0041, 1851, 0961	
38	common-very common	021N	*M.parvicella*	0092, 0041	
69	abundant	021N	*M.parvicella*	*H.hydrossis*, 0041, 0092	•Attached cilates common
96	some-common	021N	0041	*H.hydrossis*, *M.parvicella*, 0092	•Fungus sp. present
129	very common-abundant	021N	*H.hydrossis*, 0092	0041, *M.parvicella*	
152	very common	*H.hydrossis*	0092	*M.parvicella*, 0041, 021N	
187	very common	0092	021N	*H. hydrossis*, 0041	
227	few-some	0092	-	*H.hydrossis*, 0041, 1851	•Amoeba fairly common
257	some	021N	-	*S.natans*, 0092, 0041, 0803, *H.hydrossis*	
298	some-common	0092	*M.parvicella* (broken)	*Beggiatoa* sp., *H.hydrossis*	•Free-swimming ciliates common
336	some-common	*H.hydrossis*	-	0041, 0092, 021N, 0803	•Finger-like zooglea

Table B.8

Day No. System 8	Filament abundance	Dominant	Secondary	Tertiary	Remarks
7	abundant	021N	*M.parvicella*, 0092	0041, 1851, 0961	
38	very common-abundant	*M.parvicella*	021N	1851, 0041, 0092	•chains of large cocci present
69	abundant	*M.parvicella*	021N	*H.hydrossis*, 0041, 0092	
96	very common	*M.parvicella*	021N	0041, 0961, 0092	
129	abundant-excessive	*H.hydrossis*	1701	0092, 0041, *M.parvicella*	•chains of cocci •finger-like zooglea
152	excessive	*H.hydrossis*	0041	021N, 0092	
187	common	0041	0092	*H.hydrossis*, 021N	•chains of large cocci
227	none identified	none identified	none identified	none identified	

Table B.9

THE EFFECT OF VARIOUS CONDITIONS ON FILAMENTOUS ORGANISMS DEVELOPED ON MUNICIPAL SEWAGE			
ORGANISM TYPE	**Conditions which promote growth**	**Conditions which reduce growth**	**Conditions which sustain growth**
Microthrix parvicella	• Intermittent aeration - high DO (DO > 2,0 mgO/ℓ) + NO_2 concentration > 1 mgN/ℓ + SBCOD as substrate	• Intermittent aeration - low DO (DO < 2,0 mgO/ℓ) + NO_2 concentration < 1 mgN/ℓ • Intermittent aeration - high DO (DO > 3,0 mgO/ℓ) + RBCOD substrate • Continuous aeration - high DO (DO > 3,0 mgO/ℓ) • Continuous aeration - low DO (02 < DO < 0,5 mgO/ℓ)	
0092	• Intermittent aeration - low DO (0,2 < DO < 0,5) • Intermittent aeration - high DO (DO > 2,0 mgO/ℓ) + anaerobic zone in system (not immediate to aerobic reactor)	• Continuous aeration - high DO (DO > 2,0 mgO/ℓ)	• Intermittent aeration - high DO + low NO_3 concentrations • Continuous aeration - low DO (0,2 < DO < 0,5)
Haliscomenobacter Hydrossis	• Intermittent aeration - high DO + RBCOD (DO > 2,0 mgO/ℓ)	• Continuous aeration - high DO (DO > 2,0 mgO/ℓ) • Continuous anoxic	
0041	• Change in aeration pattern: intermittent (high DO) - continuous (low DO) intermittent (low DO) - intermittent (intermediate DO)		• Continuous aeration - high D (0,5 < DO < 2,0) • RBCOD under aerobic conditions
021N	• Septic sewage under: intermittent high DO (DO > 2,0 mgO/ℓ)	• Continuous high DO (DO > 3,0 mgO/ℓ) + removal of septic sewage	

Table B.10

COD & NITROGEN Balance: SYSTEM 5

Q =	10	l/d
Vp =	10	l
Rs =	15	d
w =	667	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day		MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1	35	4940	639	1617	1595	2034	86	1
2	36	42	4884	573	1638	1616	2852	103	2
3	43	61	4941	704	1687	1665	1231	73	3
4	62	77	4609	610	1380	1362	2486	97	4
5	78	94	4820	798	1167	1152	3091	105	5
6	95	120	4849	1261	1112	1097	1175	73	6
7	121	150	5156	849	1436	1417	1089	65	7
8	151	181	4421	643	1576	1555	1498	84	8
9	182	217	4547	664	1310	1293	1735	81	9
10	218	233	4687	716	1261	1245	2007	85	10
11	234	258	4998	674	1377	1359	2012	81	11
12	259	276	5429	890	1554	1534	2180	85	12
13	277	300	5401	899	1061	1047	2602	84	13

Steady State Period	Day-Day		MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1	35	583	88	108	541	479	448	387	100	1
2	36	42	542	24	109	426	812	22	408	100	2
3	43	61	581	110	112	0	112	246	358	100	3
4	62	77	470	61	92	0	293	0	317	95	4
5	78	94	565	103	78	0	296	88	384	100	5
6	95	120	548	324	74	0	15	134	149	100	6
7	121	150	519	265	96	0	14	144	158	100	7
8	151	181	550	229	105	0	19	197	215	100	8
9	182	217	457	173	87	286	40	443	197	100	9
10	218	233	457	173	84	300	131	368	199	100	10
11	234	258	472	119	92	288	149	400	261	100	11
12	259	276	455	111	104	300	162	378	240	100	12
13	277	300	504	170	71	300	146	417	264	100	13

Steady State Period	Day-Day		OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1	35	34.1	31	2520	1768	752	1281	1
2	36	42	20.2	96	4654	1864	2790	62	2
3	43	61	32.7	28	2163	1637	526	704	3
4	62	77	16.4	100	3934	1448	2486	0	4
5	78	94	19.1	100	4592	1753	2839	252	5
6	95	120	13.5	46	1473	683	790	385	6
7	121	150	12.4	47	1400	722	677	411	7
8	151	181	20.4	39	1921	985	936	563	8
9	182	217	17.2	33	1368	900	468	1267	9
10	218	233	17.8	44	1866	910	955	1052	10
11	234	258	21.1	41	2059	1192	867	1145	11
12	259	276	26.4	35	2196	1098	1098	1082	12
13	277	300	21.2	51	2614	1206	1408	1194	13

Table B.11

COD & NITROGEN Balance: SYSTEM 6

Q =	10	l/d
Vp =	10	l
Rs =	15	d
w =	667	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day		MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1	35	4940	695	1685	1662	2141	91	1
2	36	42	4884	607	1699	1676	2503	98	2
3	43	60	4928	710	1756	1732	1311	76	3
4	61	80	4596	662	1852	1827	1289	82	4
5	81	85	4477	870	1771	1747	1547	93	5
6	86	94	4784	964	1353	1335	1095	71	6
7	95	120	4849	748	1468	1449	2355	94	7
8	121	153	5087	814	1312	1295	3123	103	8
9	154	188	4392	734	1412	1393	1493	82	9
10	189	196	4500	496	1520	1499	1943	88	10
11	197	213	4767	421	1532	1512	1920	81	11
12	214	225	4545	470	1271	1254	3041	105	12
13	226	265	4953	593	1038	1024	2454	82	13

Steady State Period	Day-Day		MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1	35	583	110	112	577	443	494	360	100	1
2	36	42	542	31	113	414	835	-23	397	100	2
3	43	60	580	89	117	0	149	225	374	100	3
4	61	80	475	214	123	0	43	94	138	100	4
5	81	85	539	311	118	0	11	99	110	100	5
6	86	94	566	348	90	0	18	110	128	100	6
7	95	120	548	76	98	0	253	121	374	100	7
8	121	153	518	91	87	0	196	143	339	100	8
9	154	188	532	67	94	0	178	193	371	100	9
10	189	196	446	67	101	0	202	76	278	100	10
11	197	213	465	73	102	0	22	268	290	100	11
12	214	225	456	55	85	0	242	74	316	100	12
13	226	265	475	60	69	0	314	31	345	100	13

Steady State Period	Day-Day		OUR mgO/Lh	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1	35	33.4	30	2376	1647	729	1412	1
2	36	42	19.0	96	4384	1814	2570	-67	2
3	43	60	34.3	29	2376	1708	668	643	3
4	61	80	20.0	34	1648	629	1019	270	4
5	81	85	21.0	35	1767	505	1263	284	5
6	86	94	13.5	42	1365	584	781	314	6
7	95	120	15.5	100	3718	1709	2009	346	7
8	121	153	17.8	100	4264	1548	2715	408	8
9	154	188	26.1	42	2635	1694	941	552	9
10	189	196	29.7	42	2995	1270	1726	217	10
11	197	213	31.9	32	2480	1325	1155	765	11
12	214	225	17.8	100	4272	1443	2829	213	12
13	226	265	16.4	100	3943	1579	2364	90	13

Table B.12

COD & NITROGEN Balance: SYSTEM 7

Q = 10 l/d (daily flow rate)
Vp = 10 l (proces volume)
Rs = 15 d (sludge age)
w = 667 ml (daily waste vol)
fn = 0.1 mgN/mgVSS
fcv = 1.48 mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added

MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: beterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day	MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1 35	4940	646	1562	1542	2489	95	1
2	36 42	4884	573	1531	1511	2300	100	2
3	43 62	4933	700	1658	1636	1615	80	3
4	63 94	4582	2072	957	944	871	85	4
5	95 127	5210	643	1444	1425	2087	80	5
6	128 158	5451	794	1566	1545	1566	72	6
7	159 180	4872	670	1701	1679	1807	85	7
8	181 202	4474	752	1494	1474	1486	83	8
9	203 221	4345	1229	1197	1181	984	78	9
10	222 254	4849	999	969	956	1450	70	10
11	255 261	5257	845	833	822	2242	74	11
12	262 305	5307	1259	1055	1041	1667	75	12
13	306 334	5035	824	824	813	2333	79	13
14	335 352	4542	659	1185	1169	2731	100	14
15	353 362	4690	1305	921	909	1403	77	15

Steady State Period	Day-Day	MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1 35	583	165	104	566	402	477	313	100	1
2	36 42	542	20	102	429	835	13	420	100	2
3	43 62	582	117	111	0	137	218	355	100	3
4	63 94	1614	1057	64	0	206	287	493	100	4
5	95 127	336	46	96	0	57	137	193	100	5
6	128 158	282	91	104	746	199	548	0	92	6
7	159 180	247	100	113	860	229	632	0	97	7
8	181 202	444	276	100	831	312	520	0	95	8
9	203 221	464	268	80	891	315	575	0	91	9
10	222 254	469	299	65	795	289	507	0	92	10
11	255 261	485	185	56	0	45	199	244	100	11
12	262 305	491	312	70	867	284	583	0	92	12
13	306 334	439	93	55	0	218	73	291	100	13
14	335 352	438	52	79	0	336	-29	307	100	14
15	353 362	442	428	61	840	349	491	0	104	15

Steady State Period	Day-Day	OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1 35	28.0	38	2557	1432	1124	1365	1
2	36 42	19.5	100	4680	1918	2762	37	2
3	43 62	34.0	32	2613	1622	991	624	3
4	63 94	23.6	41	2304	2252	52	819	4
5	95 127	20.3	53	2580	884	1695	392	5
6	128 158	12.1	0	0	0	0	1566	6
7	159 180	9.5	0	0	0	0	1807	7
8	181 202	10.5	0	0	0	0	1486	8
9	203 221	7.0	0	0	0	0	984	9
10	222 254	6.6	0	0	0	0	1450	10
11	255 261	11.6	100	2787	1115	1672	570	11
12	262 305	15.0	0	0	0	0	1667	12
13	306 334	14.4	100	3456	1331	2125	208	13
14	335 352	17.6	100	4218	1404	2814	-84	14
15	353 362	10.1	0	0	0	0	1403	15

Table B.13

COD & NITROGEN Balance: SYSTEM 8

Q = 10 l/d
Vp = 10l (1-97); 15l (96-226)
Rs = 15 d
w = 667ml (10l); 1000ml (15l)
fn = 0.1 mgN/mgVSS
fcv = 1.48 mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxm: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day	MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1 35	4940	677	1612	1590	2207	91	1
2	36 42	4884	650	1593	1572	2633	99	2
3	43 62	4933	712	1795	1771	1525	81	3
4	63 94	5465	564	1880	1855	1864	78	4
5	95 119	3852	1979	445	658	1248	101	5
6	120 127	5065	2633	444	657	2033	105	6
7	128 135	5643	2288	492	728	1650	83	7
8	136 162	4489	1976	499	738	1250	88	8
9	163 193	3291	1605	388	575	848	92	9
10	194 226	2904	1763	291	431	1967	143	10

Steady State Period	Day-Day	MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxm	Nitrogen Mass Balance	Steady State Period
1	1 35	583	150	107	440	429	336	325	100	1
2	36 42	542	29	106	374	754	26	406	100	2
3	43 62	582	106	120	0	149	208	357	100	3
4	63 94	381	49	125	0	43	164	207	100	4
5	95 119	1524	792	30	0	391	312	703	100	5
6	120 127	1779	1276	30	0	36	437	473	100	6
7	128 135	1718	1089	33	510	701	405	596	100	7
8	136 162	1659	981	33	544	797	392	645	100	8
9	163 193	1451	829	26	532	999	129	596	100	9
10	194 226	1452	734	19	565	676	587	698	100	10

Steady State Period	Day-Day	OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1 35	28.6	40	2732	1485	1247	960	1
2	36 42	18.4	100	4413	1856	2557	76	2
3	43 62	33.7	32	2561	1630	931	594	3
4	63 94	22.5	41	2223	827	1396	468	4
5	95 119	17.2	51	3167	2811	355	893	5
6	120 127	16.3	46	2676	1893	783	1250	6
7	128 135	15.6	51	2876	2385	491	1159	7
8	136 162	19.6	38	2709	2579	129	1121	8
9	163 193	23.3	34	2862	2384	478	370	9
10	194 226	27.9	31	3082	2793	289	1679	10

Nitrogen mass balance [1]

Assume: (1) All ammonium (NH_4^+) is nitrified to NO_3^-

(2) NO_3^- is denitrified to N_2 (i.e. any NO_2^- formed from NO_3^- reduction is denitrified to N_2 and the gaseous denitrification intermediates NO, and N_2O formed are also denitrified to N_2).

Nitrogen in:

(i) TKN in influent: MN_{ti}

$M(N_{ti}) = Q \cdot N_{ti}$

where

Q = daily flow rate (ℓ/d)

N_{ti} = influent TKN concentration (mgN/ℓ)

(ii) $(NO_2^- + NO_3^-)$ added: MN_{oxi}

where

MN_{oxi} = average daily mass of $(NO_2^- + NO_3^-)$ added (mgN/d)

Nitrogen out:

(i) TKN in effluent: MN_{te}

$MN_{te} = Q \cdot N_{te}$

where

N_{te} = average daily effluent TKN concentration (mgN/ℓ)

(ii) $(NO_2^- + NO_3^-)$ in effluent: MN_{oxe}

$MN_{oxe} = Q \cdot N_{oxe}$

where

N_{oxe} = average daily effluent $(NO_2^- + NO_3^-)$ concentrations (mgN/ℓ)

(iii) TKN in waste sludge: MN_w

$MN_w = f_n \cdot M\Delta X_v$

where

---

[1]For the IAND system, in order to calculate the mass of $(NO_3^- + NO_2^-)$ denitrified, the concentrations of $(NO_3^- + NO_2^-)$ at the start and end of the anoxic period of each cycle would need to have been measured. These were not measured and consequently it was necessary to assume a 100% nitrogen mass balance, and on this basis the mass of $(NO_3^- + NO_2^-)$ denitrified, $M(N_{oxd})$ was calculated.

f_n = nitrogen fraction of VSS
= 0,10 mgN/mgVSS
= $M\Delta X_v$ = mass of sludge wasted/d (mgVSS/d)

(iv) $(NO_2^- + NO_3^-)$ denitrified: MN_{oxd}

$MN_{oxd} = MN_{oxi} + (MN_{ti} - MN_{te} - MN_w) - MN_{oxe}$

$\%N \text{ balance} = 100 \cdot (MN_{te} + MN_{oxe} + MN_w + MN_{oxd})/MN_{ti} + MN_{oxi}$

Example calculation

$MN_{ti} = 10\ \ell/d \cdot 58{,}3\ mgN/\ell = 583\ mgN/d$

$MN_{oxi} = 541\ mgN/d$

$MN_{te} = 10\ \ell/d \cdot 8{,}8\ mgN/\ell = 88\ mgN/d$

$MN_{oxe} = 10\ \ell/d \cdot 47{,}9\ mgN/\ell = 479\ mgN/d$

for MN_w : $M\Delta X_v = 1617\ mgVSS/\ell \cdot 0{,}667\ \ell/d = 1079\ mgVSS/d$

$MN_w = 0{,}10\ mgN/mgVSS \cdot 1079\ mgVSS/d = 108\ mgVSS/d$

$MN_{oxd} = 541 + \{583 - 88 - 108\} - 479 = 449\ mgN/d$

$\%\ N \text{ balance} = 100 \cdot (88 + 479 + 108 + 449)/(583 + 541) = 100$

COD mass balance

COD in:

(i) COD in influent: MS_{ti}

$MS_{ti} = Q \cdot S_{ti}$

where

S_{ti} = average of daily effluent COD concentration

COD out:

(i) COD utilized under aerobic conditions (in O_2 units): $M(O_h)$

$MO_h = MO_t - MO_n$

where

MO_t = mass of oxygen utilized in aerobic period

MO_n = mass of oxygen for nitrification

$MO_t = (OUR \cdot V_t \cdot 24 \cdot p_a)$

where

OUR = oxygen utilization rate
V_t = total volume of process
p_a = fraction aerobic period

$MO_n = 4{,}57 \cdot MN_{non}$
where
4,57 = mass of oxygen required for nitrification of 1 $mgNH_4^+$ to 1 $mgNO_3^-$ ($mgO/mgNO_3^-N$)

$MN_{non} = MN_{ti} - MN_w = M_{nte}$
where
MN_{oxn} = mass of NO_3^- from nitrification of NH_4^+

(ii) COD itilized under anoxic conditions (in O_2 units): MO_d

$MO_d = 2{,}86 \cdot MN_{oxd}$
where
2,86 = oxygen equivalent of NO_3^- ($mgO/mgNO_3^-N$)

(iii) COD in waste sludge: MZ_w

$MZ_w = f_{cv} \cdot M\Delta X_v$
where
f_{cv} = COD fraction of VSS
= 1,48 mgCOD/VSS

(iv) COD in effluent: MS_{te}

$MS_{te} = Q \cdot S_{te}$
where
S_{te} = average of daily effluent COD concentrations

$$\% \text{ COD balance} = 100\ (MO_h + MO_d + MZ_w + MS_{te})/MS_{ti}$$

Example calculation:

MS_{ti} = 10 ℓ/d · 494 mgCOD/ℓ = 4940 mgCOD/d
for MO_n : MN_{oxn} = (583–108–88) mgN/d = 387 mgN/d
MO_n = (4,57 mgO/mgN · 387 mgN/d = 1769 mgO/d
MO_t = 34,1 mgO/ℓ · h · 10ℓ · 24h:0,31 = 2537 mgO/d [2]

[2]The small differences in values between those calculated here and those in Table B.1

MO_h = (2537–1769) mgO/d = 768 mgO/d

MO_d = 2,86 mgO/mgN · 449 mgN/d = 1284

MZ_w = 1,48 mgCOD/mgVSS · 1079 mgVSS/d = 1597 mgCOD/d

MS_{te} = 10 ℓ/d · 63,9 mgCOD/ℓ = 639 mgCOD/d

% COD balance = 100 (768 + 1284 + 1597 + 639)/4940 = 87

(e.g. MO_t; 2537 and 2520 mgO/d) are a consequence of working with values rounded off to the nearest unit as opposed to the 4 decimal places in the computer programme used for calculation of the tabulated data.

APPENDIX B.2

TWO-REACTOR NITRIFICATION-DENITRIFICATION (2RND) SYSTEMS

System 9 was operated at the start of the experimental period as a 2RND system. During the experimental period changes were made to the system to determine differences in filament growth between 2RND and IAND systems.

Daily measured data

Figure B.5 presents daily measured data for System 9.

Changes in operating conditions

Table B.14 presents changes in operating parameters for System 9.

Filament identifications

Table B.15 describes changes in filament types and abundances for System 9.

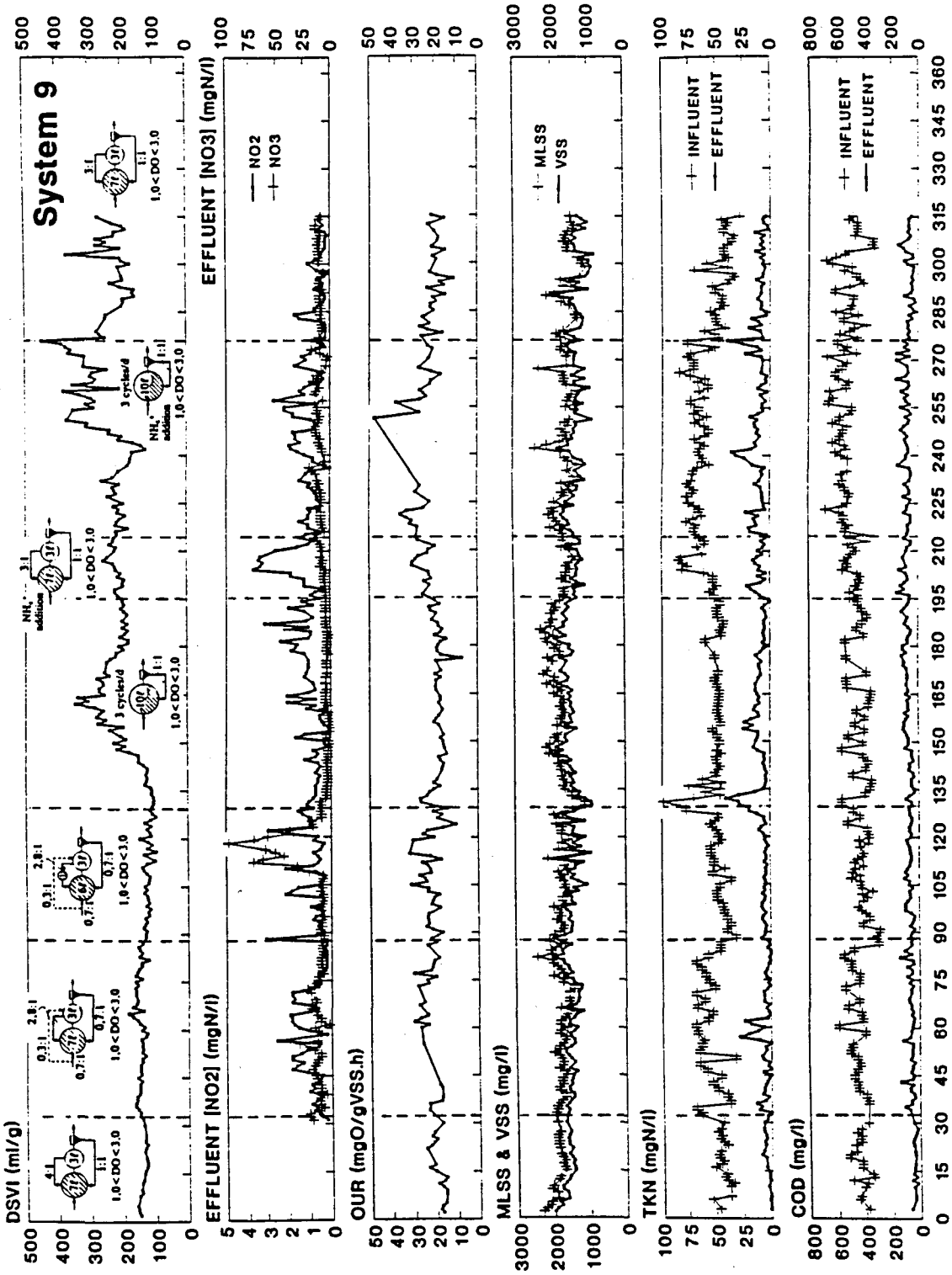
System 9
DSVI (ml/g)
EFFLUENT [NO2] (mgN/l)
EFFLUENT [NO3] (mgN/l)
NO2
NO3
OUR (mgO/gVSS.h)
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
DAY NO.

Fig B.5

Table B.14

SYSTEM 9 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 32	Pre-denitrification 2-reactor modified Ludzack-Ettinger (MLE) system	To attempt to develop a bulking sludge: DSVI constant 130-160 ml/g; dominant filament type 021N.
34 - 88	MLE - feed split between anoxic & aerobic reactors in proportion to mass fractions	To approximate feeding conditions similar to IAND in which the influent feeds during both aerobic and anoxic parts of cycle: DSVI constant at ≈ 150 ml/g; dominant filament type 021N. Note that seeding with *M.parvicella* (Day 61) did not result in a significant increase
89 - 130	MLE - feed split between anoxic & aerobic reactors and small unaerated reactor in (a) aerobic-anoxic recycle	To approximate feeding conditions in an IAND system in which sludge is exposed to a period of low and decreasing DO at the end of the aerobic period: DSVI decreased from ≈ 130 to ≈ 110 ml/g; filament type 0041 dominant
131 - 195	Intermittently aerated single reactor (IAND) system with three, 8h cycles/d	To determine if changing from a 2-reactor to a single reactor system with apparently similar conditions (i.e. frequency of exposure of sludge to anoxic-aerobic conditions), affects filament proliferation: DSVI initially increased from ≈ 110 to ≈ 350 ml/g by Day 160 but then decreased to ≈ 200 ml/g. On Day 189, measurements were made of NO_2^-, NO_3^- & ϵ_a throughout an 8h anoxic-aerobic cycle (see Fig. 3.20)
196 - 214	Intermittently aerated single reactor (IAND) system with ammonium (NH_4^+) addition to the influent	To increase the concentration of NO_2^- in the system: DSVI increased from ≈ 200 to ≈ 240 ml/g; dominant filament type 0041. Generally, when the concentration of NO_2^- increased, the DSVI increased. On Day 209 measurements were made of NO_2^-, NO_3^- & ϵ_a throughout an 8h anoxic-aerobic cycle (see Fig. 3.21)
215 - 277	MLE configuration with NH_4^+ addition to the influent	To determine if a high concentration of NO_2^- results in a high DSVI: DSVI remained high ≈ 220 ml/g until Day 232 when low DO in the aerobic zone resulted in reduced nitrification and reduced concentration of effluent NO_2^- and DSVI decreased to ≈ 120 ml/g. From Day 242 the DSVI increased to 490 ml/g. The dominant filament type was 0041
278 - 316	MLE configuration	To determine if a reduced concentration of NO_2^- results in reduced filament proliferation: DSVI decreased to ≈ 230 ml/g; dominant filament type 0803. The concentration of NO_2^- in the effluent was ≈ 0,5 mgN/ℓ

Summary:

- A 2RND configuration with similar conditions to an IAND system in terms of frequency of exposure of sludge to anoxic-aerobic conditions, feeding pattern and periods of low DO did not develop a sludge with a similarly high DSVI (<150 ml/g)
- Addition of NH_4^+ to the influent of an IAND system resulted in an increased DSVI
- Addition of NH_4^+ to the influent of a 2RND system resulted in an increased DSVI and removal of NH_4^+ resulted in a decreasing DSVI

Table B.15

Day No. System 9	Filament abundance	Dominant	Secondary	Tertiary	Remarks
11	very common	021N	0092	*H.hydrossis*, *M.parvicella*, *Nocardia*, 0041, *Aeolosoma* sp.	• Chains of cocci
42	very common-abundant	021N	0092	*M.parvicella*, 0041, 0092	
69	common	021N	*M.parvicella*	*Beggiatoa* sp., 0041, 0092	
102	abundant	0041	*M.parvicella*	*H.hydrossis*, 0092, 021N, Flexibacter	• Finger-like zooglea
125	very common	0041	0092	*M.parvicella*, *H.hydrossis*, 1851	• Diatoms common • Free-swimming ciliates common • Finger-like zooglea
160	very common-abundant	*H.hydrossis*	0041, 0092	*Beggiatoa*, 021N, *M.parvicella*	
200	very common	0041	-	Flexibacter, 0092, *H.hydrossis*, *M.parvicella*, 0803	• Diatoms common
230	some-common	0041	-	*M.parvicella*, *S.natans*, 1851, 0092	
271	common-very common	0041	*M.parvicella*	021N, *S.natans*, 0092	
309	very common	0803	0041	*H. hydrossis*, 0092	• Spirochaetes abundant

APPENDIX B.3

MULTI-REACTOR NITRIFICATION-DENITRIFICATION BIOLOGICAL EXCESS PHOSPHORUS REMOVAL (NDBEPR) MODIFIED UCT (MUCT) SYSTEMS

Eight NDBEPR configurations (Systems 10 to 17) were operated in the MUCT mode during four experimental periods in which experiments were conducted to examine the effects of the following parameters on filament proliferation.

- aerated/unaerated mass fractions (Systems 10 and 11)
- high and low influent TKN concentrations (Systems 12 and 13)
- high and low NO_2^- concentrations in the 2nd anoxic reactor (Systems 14 and 15)
- a small unaerated reactor between the 2nd anoxic and aerobic reactor for removal of extracellular NO_3^- and NO_2^- and intracellular NO (Systems 16 and 17).

Daily measured data

Figures B.6 to B.13 present daily measured data for MUCT Systems 10 to 17 respectively.

Changes in operating conditions

Tables B.16 to B.19 present changes in operating parameters for MUCT Systems 10 to 17 respectively.

Filament identifications

Tables B.20 to B.27 describe changes in filament types and abundances for MUCT Systems 10 to 17 respectively.

Nitrogen mass balances

Tables B.28 to B 35 present the nitrogen mass balances for Systems 10 to 17 respectively. Because OURs were not measured, COD mass balances were not determined.

The procedure for calculation of the nitrogen mass balance about the unaerated (anaerobic and anoxic) reactors is described. It should be noted that for Systems 10

to 13, combined NO_2^- and NO_3^- concentrations were measured, and for Systems 14 to 17 individual NO_2^- and NO_3^- concentrations were measured. The nitrogen mass balance example is for System 14, steady state period 2.

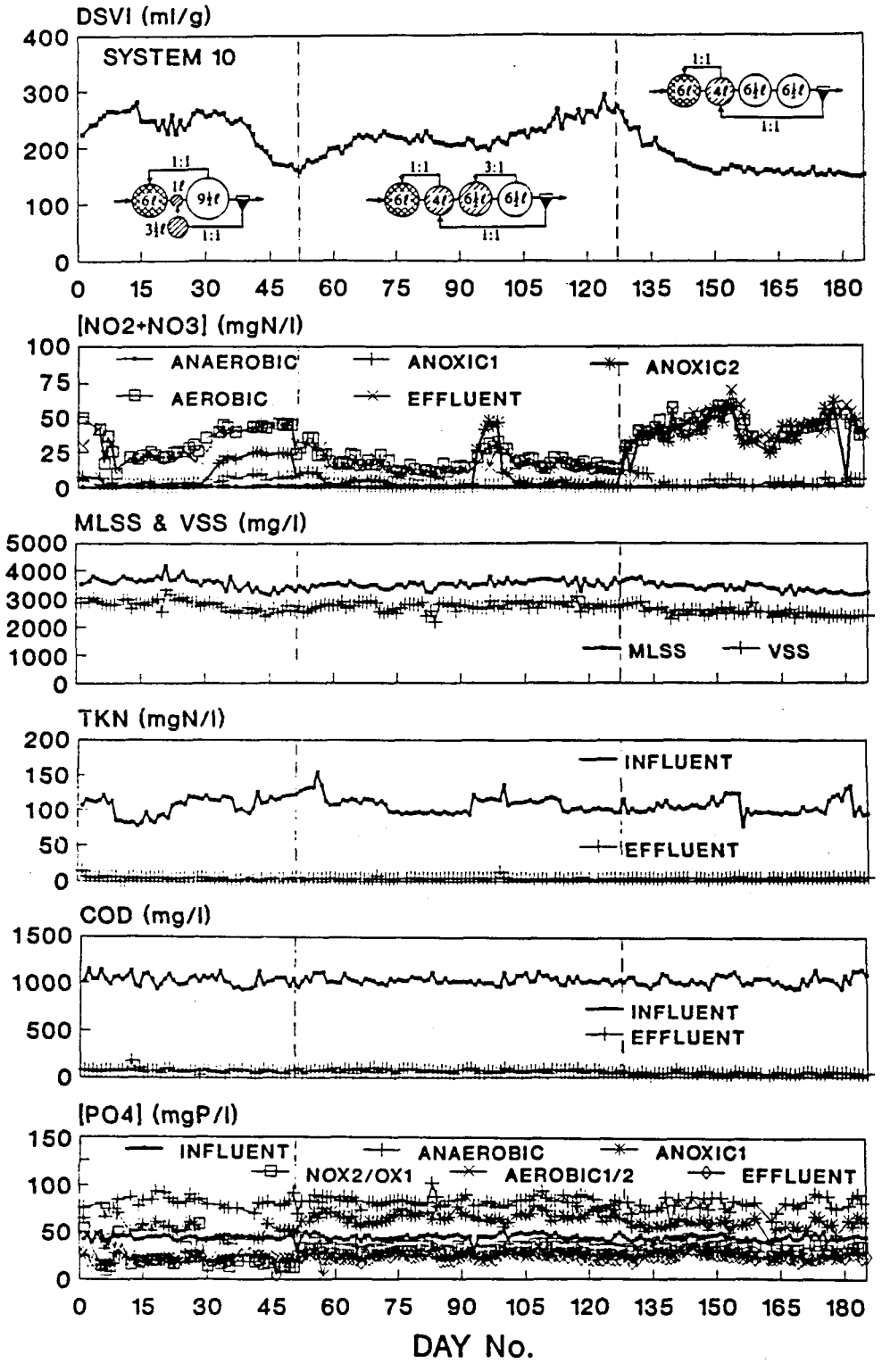
DSVI (ml/g)
SYSTEM 10
[NO2+NO3] (mgN/l)
ANAEROBIC
ANOXIC1
ANOXIC2
AEROBIC
EFFLUENT
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
[PO4] (mgP/l)
INFLUENT
ANAEROBIC
ANOXIC1
NOX2/OX1
AEROBIC1/2
EFFLUENT
DAY No.

Fig B.6

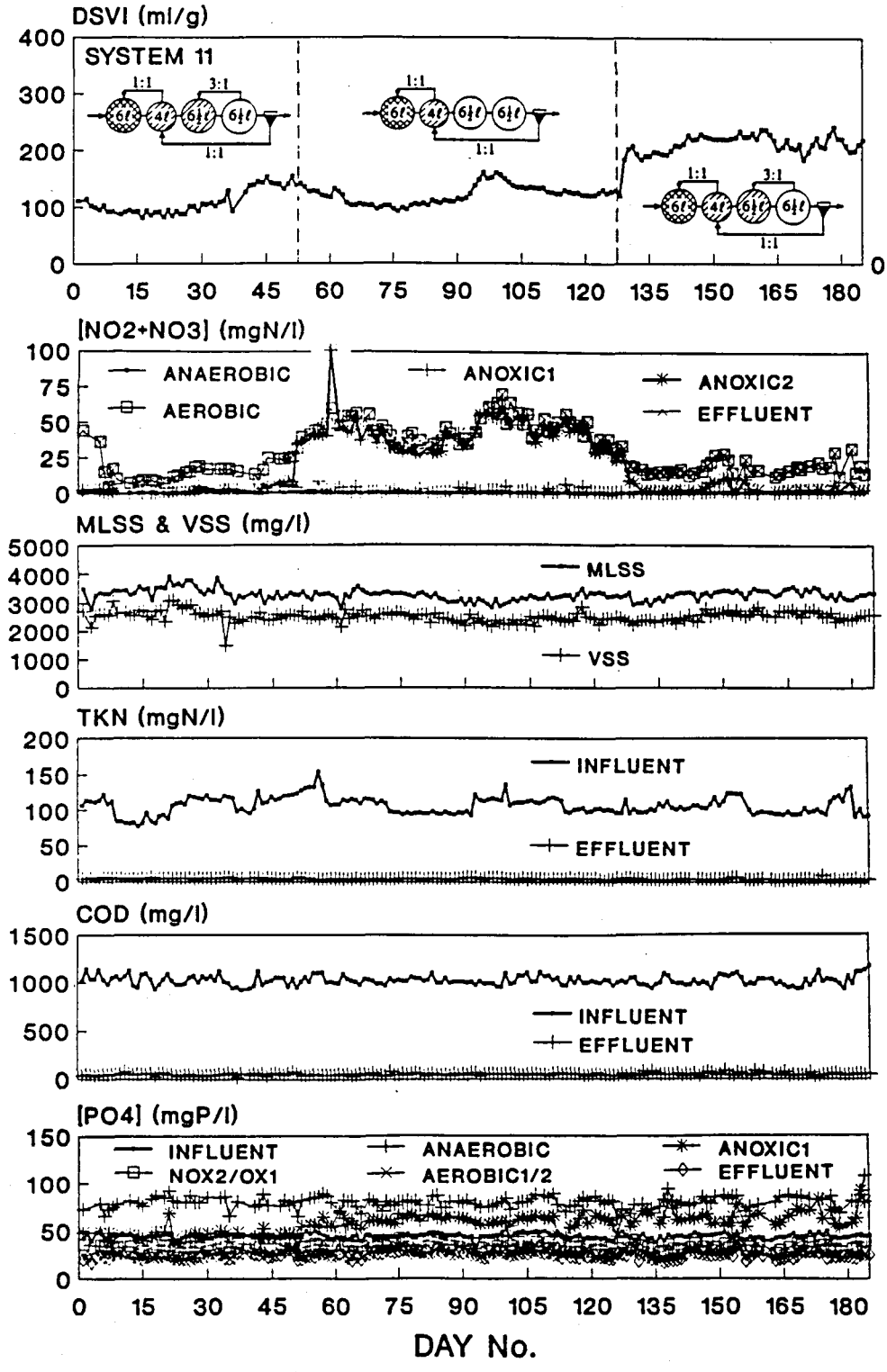
DSVI (ml/g)
SYSTEM 11
[NO2+NO3] (mgN/l)
ANAEROBIC
AEROBIC
ANOXIC1
ANOXIC2
EFFLUENT
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
[PO4] (mgP/l)
INFLUENT
NOX2/OX1
ANAEROBIC
AEROBIC1/2
ANOXIC1
EFFLUENT
DAY No.

Fig B.7

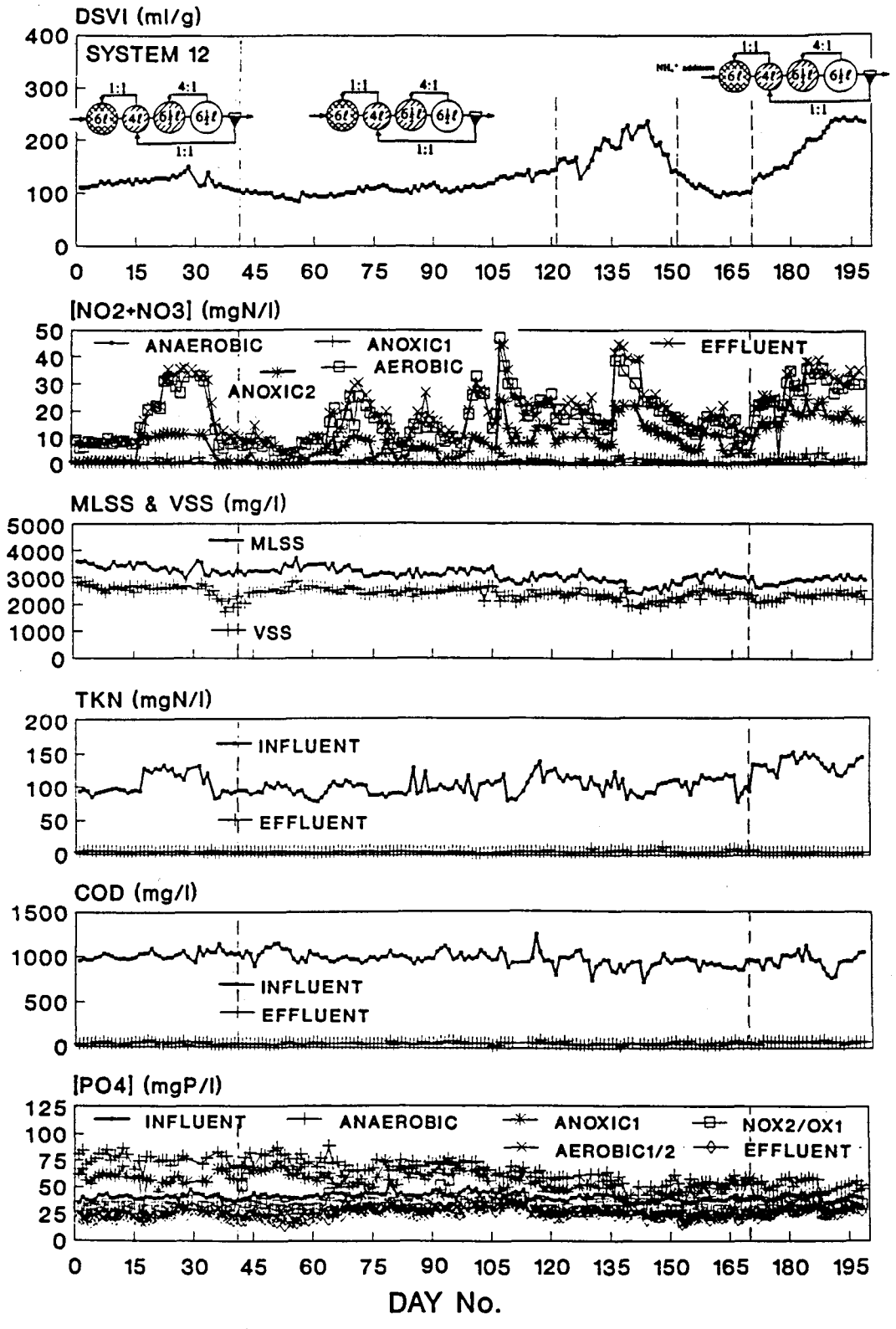
DSVI (ml/g)
SYSTEM 12
[NO2+NO3] (mgN/l)
ANAEROBIC
ANOXIC1
AEROBIC
ANOXIC2
EFFLUENT
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
[PO4] (mgP/l)
INFLUENT
ANAEROBIC
ANOXIC1
NOX2/OX1
AEROBIC1/2
EFFLUENT
DAY No.

Fig B.8

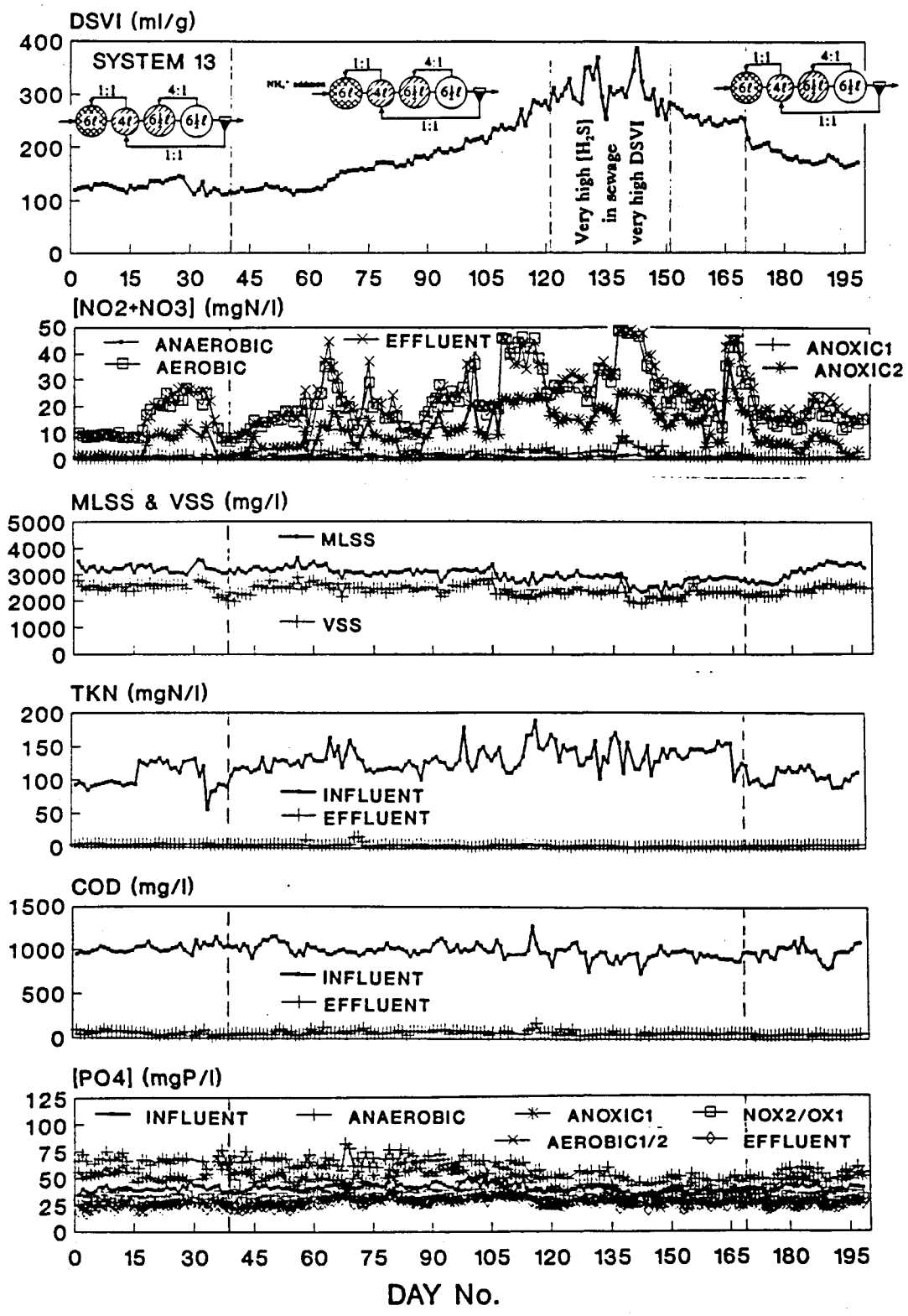
DSVI (ml/g)
SYSTEM 13
Very high [H2S] in sewage very high DSVI
[NO2+NO3] (mgN/l)
ANAEROBIC
AEROBIC
EFFLUENT
ANOXIC1
ANOXIC2
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
[PO4] (mgP/l)
INFLUENT
ANAEROBIC
ANOXIC1
NOX2/OX1
AEROBIC1/2
EFFLUENT
DAY No.

Fig B.9

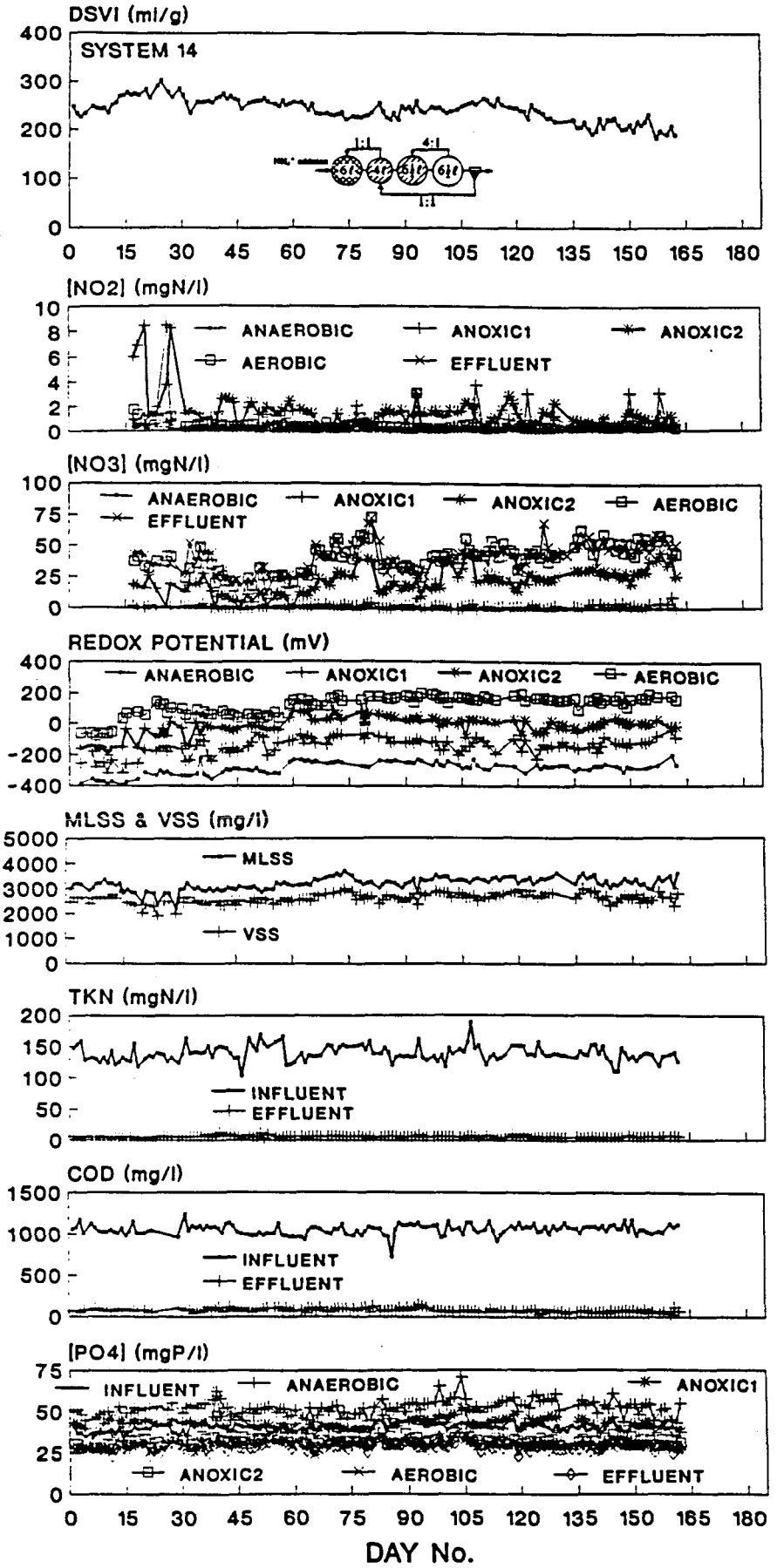
DSVI (ml/g)
SYSTEM 14
[NO2] (mgN/l)
ANAEROBIC
ANOXIC1
ANOXIC2
AEROBIC
EFFLUENT
[NO3] (mgN/l)
REDOX POTENTIAL (mV)
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
[PO4] (mgP/l)
DAY No.

Fig B.10

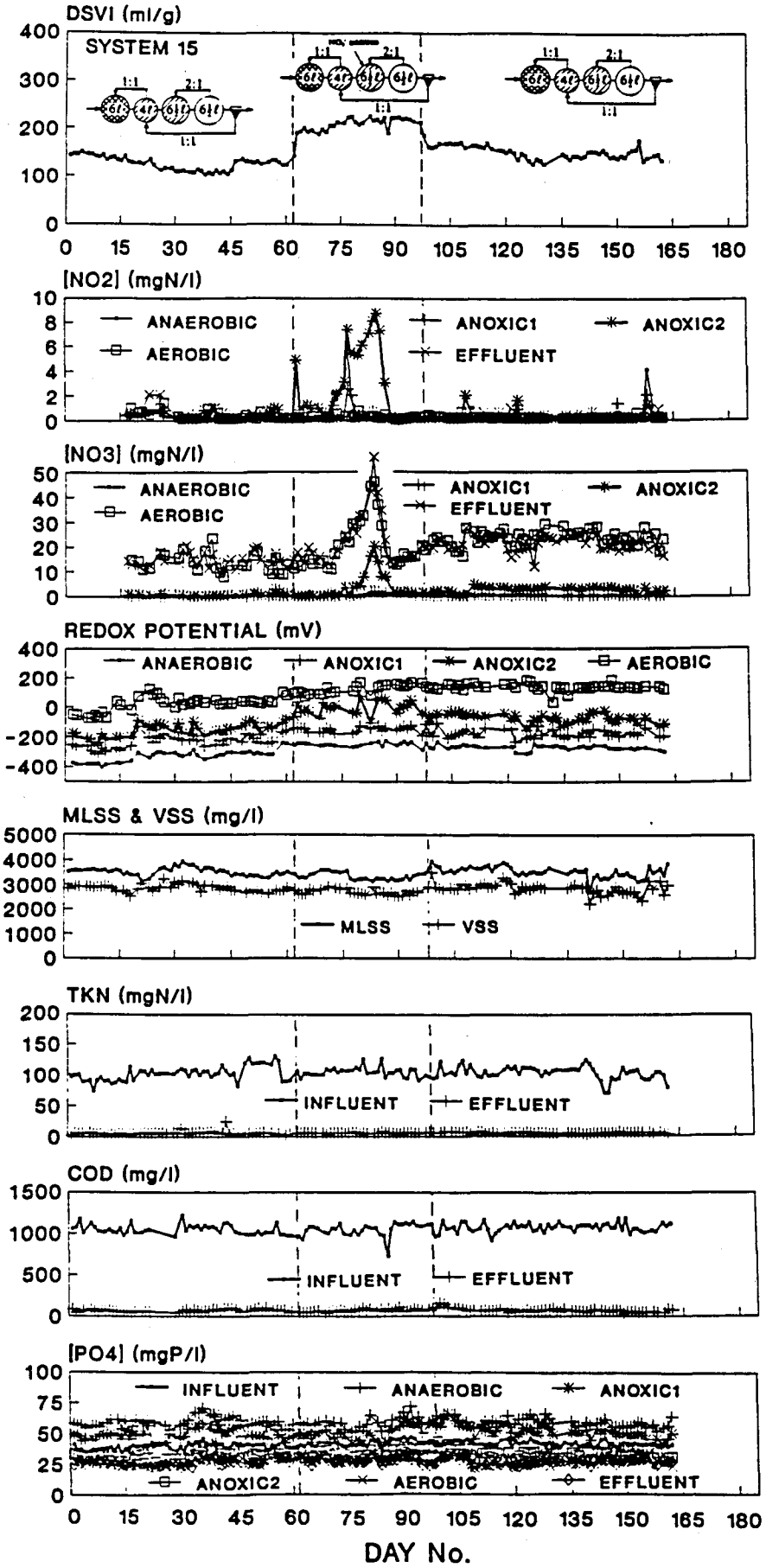
DSVI (ml/g)
SYSTEM 15
[NO2] (mgN/l)
ANAEROBIC
AEROBIC
ANOXIC1
EFFLUENT
ANOXIC2
[NO3] (mgN/l)
REDOX POTENTIAL (mV)
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
[PO4] (mgP/l)
DAY No.

Fig B.11

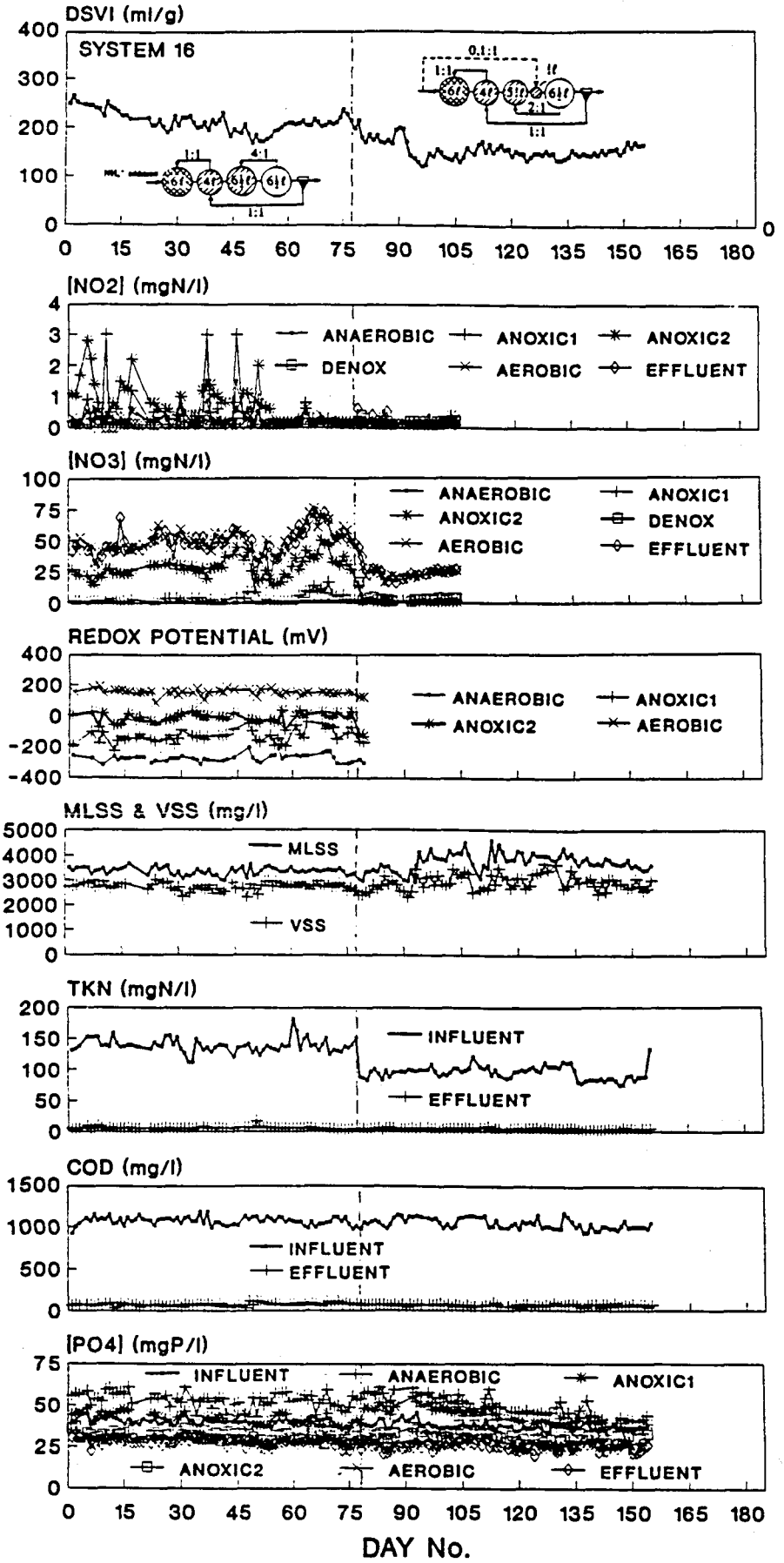
DSVI (ml/g)
SYSTEM 16
[NO2] (mgN/l)
ANAEROBIC
ANOXIC1
ANOXIC2
DENOX
AEROBIC
EFFLUENT
[NO3] (mgN/l)
REDOX POTENTIAL (mV)
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
COD (mg/l)
[PO4] (mgP/l)
DAY No.

Fig B.12

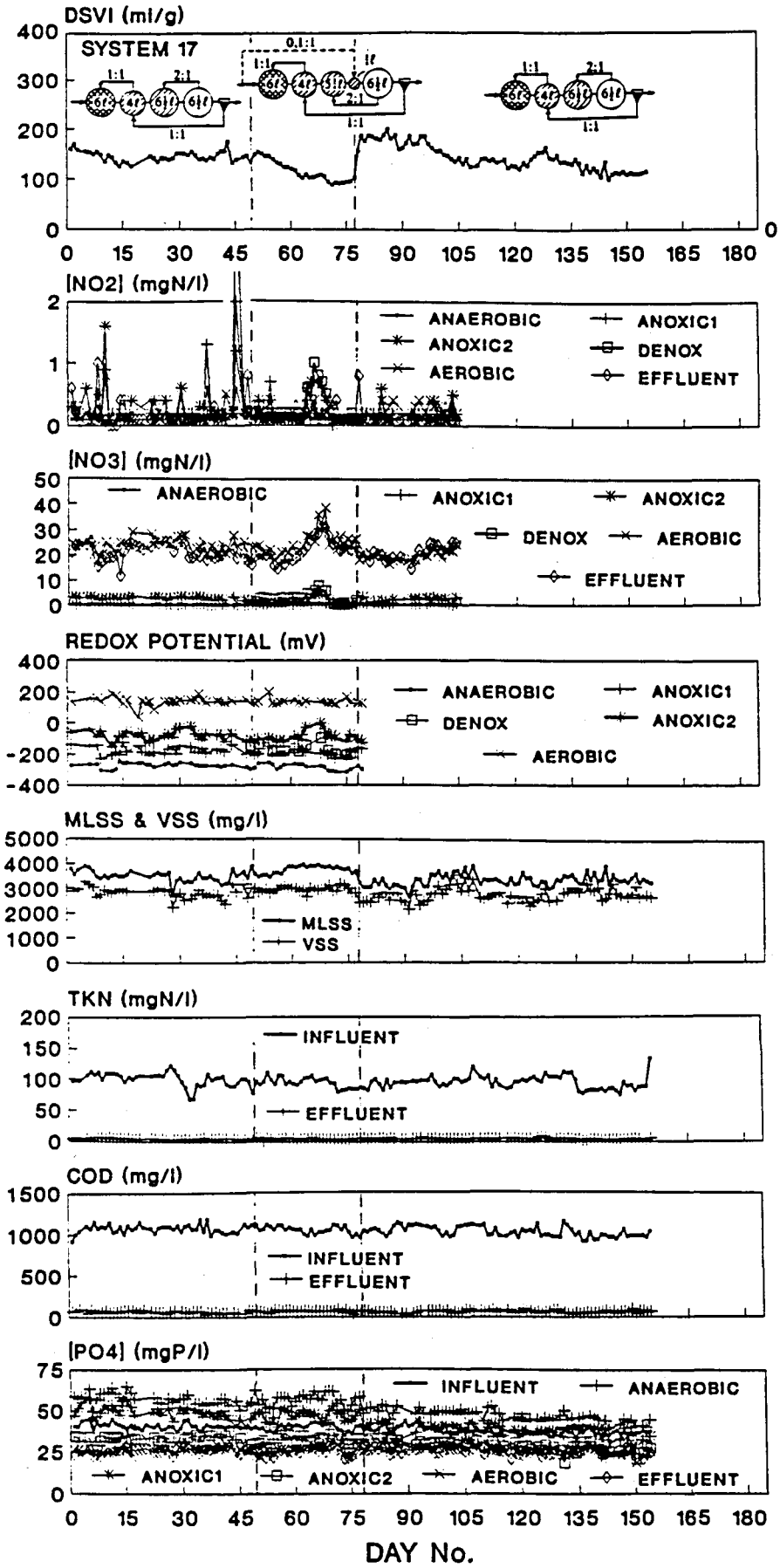
DSVI (ml/g)
SYSTEM 17
[NO2] (mgN/l)
ANAEROBIC
ANOXIC1
ANOXIC2
DENOX
AEROBIC
EFFLUENT
[NO3] (mgN/l)
REDOX POTENTIAL (mV)
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
COD (mg/l)
[PO4] (mgP/l)
DAY No.

Fig B.13

Table B.16

Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
	SYSTEM 10 :	**CHANGES IN OPERATING CONDITIONS**
1 - 52	Modified Johannesburg configuration (53% aerated)	This was the configuration "inherited" from a prior set of activated sludge experiments. The DSVI remained constant at ≈ 250 ml/g to Day 28 and then decreased from ≈ 225 to ≈ 160 ml/g as the dominant filament changed from *M.parvicella* to type 0092. Note that for Days 1-28 with DSVI increasing the N balance was lower (71%) than Days 29-52 with DSVI decreasing (81%) (see Fig. 3.13 & Table B.28).
53 - 127	MUCT configuration (33% aerated)	To ensure the aerated mass fraction was in the range (30-35%), for optimum filament proliferation (see Fig. 3.8): DSVI increased from ≈ 160 to ≈ 270 ml/g. Filament identification indicated type 0092 dominant and *M.parvicella* secondary. Note that for the rapid increase in DSVI (Days 98-127), the N mass balance was lower (66%) than all other periods.
128 - 185	MUCT configurations 65% aerated	To determine effect of large aerated mass fractions on filament proliferation: DSVI decreased from ≈ 270 to ≈ 150 ml/g and type 0092 was dominant. From Days 128-150, N balance increased (98%) and from Day 151-185, N balance increased (107%) (compare with System 11).
	SYSTEM 11	
1 - 52	MUCT configuration (33% aerated)	To determine if the percent aerobic mass fraction determined for Systems 1-8 (30-35%) applies to multi-reactor systems: DSVI increased from ≈ 100 to ≈ 130 ml/g. Filament type 0092 was dominant. From Days 1-28, when DSVI was < 100 ml/g, N balance was high (97%), but when DSVI increased from Days 28-52, N balance decreased (84%) (see Fig. 3.13 & Table B.29).
53 - 127	MUCT configuration (65% aerated)	To determine effect of large aerated mass fraction on DSVI: DSVI remained between 100-150 ml/g and type 0092 was dominant.
128 - 185	MUCT configuration (33% aerated)	To determine effect of small aerated mass fractions on DSVI: DSVI increased from ≈ 125 to ≈ 720 ml/g and type 0092 was dominant. Between Days 128-150, N balance was low (83%), and between Days 151-185, when DSVI decreased slightly, N balance increased slightly (91%)

Summary

- For small aerated mass fractions (33%) the DSVI is generally high (>150 ml/g) and the N balance is generally low
- For large aerated mass fractions (65%) the DSVI is generally low (<150 ml/g) and the N balance is generally high
- Type 0092 was the dominant filament irrespective of whether the percent aerobic period was high or low

Table B.17

SYSTEM 12 :	CHANGES IN OPERATING CONDITIONS	
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 40	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10)	This configuration used to establish a sludge containing filament type 0092 and as a complement to System 13 (Days 1-40): DSVI constant at 100-150 ml/g; dominant filament is type 0092. Note the increase in DSVI (Days 21-28) is paralleled by an increase in NO_{x+3}^- concentration in the 2nd anoxic and aerobic reactors and effluent (see Fig. 3.17).
41 - 121	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10)	Configuration same as for Days 1-40 as a complement to System 13 (Days 41-121): DSVI remained between 100 and 150 ml/g; dominant filament is type 0092 and type 021N secondary. Note the gradual decrease in VSS with increase in DSVI (see Fig. 3.17).
122 - 150	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10)	No change in configuration: DSVI increased rapidly as a consequence of proliferation of 021N due to the high concentration of H_2S in the sewage. System cleaned - tubing disinfected.
151 - 170	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10)	No change in configuration: DSVI decreased from ≈ 140 to < 100 ml/g; dominant filament is type 0092. Note the decrease in concentration of NO_{x+3}^- in the second anoxic reactor.
171 - 198	MUCT configuration 33% aerated High TKN/COD (0,12-0,14) (from NH_4^+ addition to influent)	To determine effect of NO_{x+3}^- produced from nitrification of NH_4^+ on filament proliferation: DSVI increased from < 100 to ≈ 240 ml/g; dominant filament is type 0092. Note the decrease in VSS with increase in DSVI (see Fig. 3.17).
	SYSTEM 13	
1 - 40	MUCT configuration 33% aerated	This configuration used to establish a sludge containing filament type 0092 and as a complement to System 12 (Days 1-40): DSVI constant at 100-150 ml/g; dominant filament type 0092.
41 - 121	MUCT configuration 33% aerated High TKN/COD (0,12-0,14 mgN/mgCOD) (from NH_4^+ addition to influent)	To determine effect of NO_{x+3}^- produced from nitrification of NH4$^+$ on filament proliferation: DSVI increased from ≈ 120 to ≈ 280 ml/g. The dominant filament was type 0092 initially and then type 021N dominated and type 0092 was secondary.
122 - 150	MUCT configuration 33% aerated High TKN/COD (0,12-0,14 mgN/mgCOD) (from NH_4^+ addition to influent)	No change in configuration: DSVI increased rapidly as a consequence of proliferation of 021N due to the high concentration of H_2S in the sewage. Note the decrease in VSS (see Fig. 3.18). System cleaned - tubing disinfected.
151 - 170	MUCT configuration 33% aerated High TKN/COD (0,12-0,14 mgN/mgCOD) (from NH_4^+ addition to influent)	No change in configuration: DSVI decreased slightly from ≈ 280 to ≈ 250 ml/g; dominant filament type 0092, secondary 021N.
171 - 198	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10 mgN/mgCOD)	To determine effect of low NO_{x+3}^- concentration on filament proliferation and as a complement to System 12 (Days 171-198): DSVI decreased from ≈ 250 to ≈ 165 ml/g; dominant filament type 021N, type 0092 secondary.

Summary

- Low influent TKN/COD ratios and associated 2 anoxic reactor low NO_{x+3}^- concentrations, result in lower DSV values
- High influent TKN/COD ratios and associated 2 anoxic reactor high NO_{x+3}^- concentrations, result in higher DSVI values
- An increase in DSVI resulted in a decrease in VSS and a decrease in DSVI resulted in an increase in VSS
- Filament type 021N proliferated with septic sewage
- Filament type 0092 proliferated with high concentrations of NO_{x+3}^- in 2nd anoxic reactor

Table B.18

SYSTEM 14 :	CHANGES IN OPERATING CONDITIONS	
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 62	MUCT configuration 33% aerated high TKN/COD (0,12-0,14 mgN/mgCOD) from NH_4^+ addition to influent	This configuration used to establish a sludge with a high DSVI containing *M.parvicella*, as a complement to System 15 (Days 1-62): DSVI fluctuated 250-280 ml/g; dominant filament type 0092, *M.parvicella* secondary. Note the high concentration of NO_3^- in the 2nd anoxic reactor (≈ 2 mgN/ℓ) (see Fig. 6.6). Note the low N mass balance (84%) when the DSVI increased and the higher N mass balance (92%) when the DSVI decreased (see Fig. 6.4 & Table B.32).
63 - 97	MUCT configuration 33% aerated, high TKN/COD (0,12-0,14 mgN/mgCOD) from NH_4^+ addition to influent	No change in configuration: DSVI decreased slightly from ≈ 250 to ≈ 240 ml/g; filament type 0092 was dominant. Note that between Days 30 and 75 when the DSVI decreased, the VSS increased, and from Day 75 to 100 when the DSVI decreased, VSS increased.
98 - 162	MUCT configuration 33% aerated, high TKN/COD (0,12-0,14 mgN/mgCOD) from NH_4^+ addition to influent	No change in configuration: DSVI increased to ≈ 260 ml/g by Day 115, then decreased to ≈ 200 ml/g by Day 162. No apparent change in VSS. Note the highest N mass balance (111%) when the DSVI was decreasing between Days 131-162. Note the low N mass balance as the DSVI increased between Days 63-97 (73%).
	SYSTEM 15	
1 - 62	MUCT configuration 33% aerated	This configuration is used to establish a sludge with low DSVI, as a complement to System 14 (Days 1-62): DSVI was 100 to 150 ml/g, dominant filament type 0092, *M.parvicella* secondary. Note the low N mass balance between Days 48-62 (73%) as the DSVI begins to increase (see Fig. 6.4 & Table B.33).
63 - 97	MUCT configuration 33% aerated Continuous NO_3^- addition to 2nd anoxic reactor	To determine effect of increased concentration of NO_3^- in 2nd anoxic reactor on filament proliferation: DSVI increased rapidly from ≈ 130 to ≈ 220 ml/g; dominant filament is type 0092, *M.parvicella* secondary. Note that the increase in DSVI is associated with a decrease in VSS, and an increase in concentration of NO_3^- (1-10 mgN/ℓ) in the 2nd anoxic reactor (see Fig. 6.7).
98 - 162	MUCT configuration 33% aerated	To determine effect of decreased concentration of NO_3^- in 2nd anoxic reactor on filament proliferation: DSVI decreased rapidly from ≈ 220 to ≈ 160 ml/g by Day 100 and to ≈ 130 ml/g by Day 127. Thereafter, the DSVI remained 130-150 ml/g. Note that the decrease in DSVI is associated with an increase in VSS and a decrease in concentration of NO_3^- (< 1,0 mgN/ℓ) in the 2nd anoxic reactor. Note that the highest N mass balances (107 & 113%) occurred during this period of decreasing or low DSVI.

Summary

- For concentrations of NO_3^- in the 2nd anoxic reactor < 0,5 mgN/ℓ the DSVI generally was low or decreasing. For concentrations of NO_3^- in the 2nd anoxic reactor > 1,0 mgN/ℓ, the DSVI was generally high or increasing
- Increases in DSVI were associated with decreases in VSS and decreases in DSVI were associated with increases in VSS
- During periods of low or decreasing DSVI the N mass balance was generally higher than during periods of high or increasing DSVI
- Filament type 0092 dominated when the DSVI was either low, or high and filament types 0041 and *M.parvicella* were secondary

Table B.19

SYSTEM 16 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 49	MUCT configuration 33% aerated, high TKN/COD (0,12-0,14 mgN/mgCOD) *from NH_4^+ addition to* influent	To develop a sludge with high DSVI containing filament type 0092: DSVI decreased from ≈ 250 to ≈ 200 ml/g; dominant filament type 0092.
50 - 77	MUCT configuration 33% aerated, high TKN/COD (0,12-0,14 mgN/mgCOD) from NH_4^+ addition to influent	The NH_4^+ addition was stopped to provide a system with conditions complementary to System 17: DSVI increased from ≈ 180 to ≈ 225 ml/g as a consequence of filament type 021N secondary
78 - 153	MUCT configuration 33% aerated, sewage addition to small unaerated DENOX reactor	To denitrify the floc-former intracellular denitrification intermediates NO and NO_2^-, to prevent inhibitions and filament proliferation: DSVI decreased from ≈ 150 to ≈ 100 ml/g, filament type 0092 was dominant. Note the significant increase in MLSS with decrease in DSVI (see Fig 6.9).
	SYSTEM 17	
1-49	MUCT configuration 33% aerated	To develop a sludge with filament type 0092: DSVI constant at ≈ 150 ml/g; filament type 0092 dominant.
50 - 77	MUCT configuration 33% aerated, sewage addition to small DENOX reactor	To denitrify the floc-former intracellular denitrification intermediate NO & NO2 to prevent inhibition and filament proliferation: DSVI decreased from ≈ 150 to ≈ 100 ml/g, filament type 0092 was dominant. Note the significant increase in VSS with decrease in DSVI (see Fig 6.10). The reason for the high N balances is not apparent.
78 - 155	MUCT configuration 33% aerated	DENOX reactor removed to determine if filaments proliferate in its absence: DSVI increased initially from ≈ 100 to ≈ 200 ml/g by Day 86 but inexplicably decreased to ≈ 115 ml/g by Day 155; dominant filament type 0092. It is interesting that Systems 16 and 17 were decreasing and increasing after the change in configuration on Day 77, but the DSVIs of the systems then began increasing and decreasing respectively; most likely a consequence of changes in sewage composition.

Summary

- Incorporation of a small (4% of total process volume) unaerated reactor between the 2nd anoxic and aerobic reactors with 10% of the daily sewage addition initially demonstrated promising results in the control of filament type 0092.

Table B.20

Day No. System 10	Filament abundance	Dominant	Secondary	Tertiary	Remarks
1	abundant	*M.parvicella*	0092	0041, *H.hydrossis*	• Chains of coccoid cells
35	very common	0092	*M.parvicella*	0041, 021N	• Strings of beads
68	very common-abundant	0092	*M.parvicella*	021N, 0041, *Thiothrix* sp.	• Chains of large cocci present
98	abundant	0092	*M.parvicella*	*H.hydrossis*	• Finger-like zooglea • Chains of large coccoid cells present
127	very common-abundant	0092	021N	*M.parvicella*, 0041, 0675	• Algae quite common • Chains of large bead-like cells
185	very common-abundant	0092	021N	0041, *M.parvicella*	• Spirochaetes common

Table B.21

Day No. System 11	Filament abundance	Dominant	Secondary	Tertiary	Remarks
1	very common	0092	*M.parvicella*	0041, 1851, 1701	• Fungus spp. • Chains of coccoid cells
35	very common	0092	021N	0041, 0803, beads, *M.parvicella*, *Thiothrix sp.*	• Gliding algae
68	common-very common	0092	*M.parvicella*	0041, 021N, 1863, *H.hydrossis*	• Chains of large cocci
98	very common-abundant	0092	-	*M.parvicella*, 0041, *H.hydrossis*	• Chains of large coccoid cells
127	common-very common	0092	021N	*M.parvicella*, 0041	
185	abundant	0092	021N	*Nocardia* sp., 0041	• Motile algae

Table B.22

Day No. System 12	Filament abundance	Dominant	Secondary	Tertiary	Remarks
1	very common-abundant	0092	021N	0041	• Chains of large Gram negative and Gram positive cocci
29	common-very common	0092	-	0041, 021N, *H.hydrossis*, *Aeolosoma* sp.	• Chains of cocci
67	common-very common	0092	021N	0041, *H.hydrossis*	
110	very common-abundant	0092	021N	*H.hydrossis*, *M.parvicella*, 0041	• Spirochaetes common
141	abundant	0092	021N	*M.parvicella*, *H.hydrossis*	
172	abundant	0092	021N	*Nocardia* sp., 0041, *H.hydrossis*, *M.parvicella*	• Chains of large cocci present
198	abundant	0092	021N	*M.parvicella*, *H.hydrossis*, 0041	• Algae • Chains of large cocci

Table B.23

Day No. System 13	Filament abundance	Dominant	Secondary	Tertiary	Remarks
1	common-very common	0092	021N	0041, *M.parvicella*	• Chains of small Gram +ve cocci present
29	very common-abundant	0092	021N	0041, 1851, *H.hydrossis*	• Motile *algae* sp. common • *Aelosoma* sp., *Fungus* sp.
67	common-very common	0092	021N	0041, *H.hydrossis*	
110	very common	021N	0092	*M.parvicella*, 0041	• Extended finger-like zooglea • Spirochaetes common
141	abundant	021N	0041, 0092	*M.parvicella, H.hydrossis*	
172	abundant	0092	021N	0041, *H.hydrossis, M.parvicella*	• Gram positive cocci
198	abundant	021N	0092	*M.parvicella*, 0041	• Finger-like zooglea • Chains of large cocci • Algae

Table B.24

Day No. System 14	Filament abundance	Dominant	Secondary	Tertiary	Remarks
8	very common	0092	*M.parvicella* 021N	*H.hydrossis*, 0041	• Chains of cocci
48	very common-abundant	0092	0041	*H.hydrossis*, 0803, *M.parvicella*	
78	very common	0092	0041	*M.parvicella*, *Thiothrix* sp., 021N	• Chains of cocci
119	very common-abundant	0092	-	0041, 0803, *M.parvicella*, 021N	• Unicells common
157	very common-abundant	0092	021N	0041, 1851, *H.hydrossis*	

Table B.25

Day No. System 15	Filament abundance	Dominant	Secondary	Tertiary	Remarks
8	common-very common	0092	*M.parvicella*	*H.hydrossis*, 021N, 0041	• Chains of large cocci
48	very common	0092	*M.parvicella*	Flexibacter, *H.hydrossis*, 0041	• Long chains of cocci
78	very common	0092	*M.parvicella*	*H.hydrossis*, 0041	• Chains of cocci
119	very common-abundant	0092	021N	*M.parvicella*, 0041	• Algae
157		0092	021N	*M.parvicella*, 0803, 0041	• Chains of cocci

Table B.26

Day No. System 16	Filament abundance	Dominant	Secondary	Tertiary	Remarks
6	very common-abundant	0092	-	0041, 0803, *M.parvicella*, 021N	• Unicells common
44	very common-abundant	0092	021N	0041, 1851, *H.hydrossis*	
76	abundant	0092	021N	*H.hydrossis*, *M.parvicella*, 0041	
121	very common	0092	021N, *M.parvicella*	*Thiothrix* sp., 0041	• Chains of cocci

Table B.27

Day No. System 17	Filament abundance	Dominant	Secondary	Tertiary	Remarks
6	very common-abundant	0092	021N	0041, *M.parvicella*	• Chains of cocci
44	very common	0092	021N	0041, 0803, *M.parvicella*	• Algae • Chains of cocci
76	very common-abundant	0092	021N	Flexibacter, *M.parvicella*, 0041	• Spirochaetes very common
121	very common	0092	021N	*H.hydrossis*, *M.parvicella*, 0041	• Chains of cocci

Table B.28

NITROGEN Balance: SYSTEM 10

Q= 10 l/d
Vp= 17 l (days1-52), 20 l (days53-185)
Rs= 15 d
w = 0.85 l/d (days1-52), 1 l/d (days53-185)
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: nitrogen in waste sludge
MNoxe: eff nitrate + nitrite
MNo2e: effluent nitrite
MNoxd: nitrate+nitrite denit
MNo2d: nitrite denitrif
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
Noxox: 2nd anoxic nitrate + nitrite
No2ox: 2nd anoxic nitrite
Noxan: anaerobic nitrate + nitrite
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	MNw mgN/d	MNoxe mgN/d	MNo2e mgN/d	MNoxd mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	0- 28	1006	51	244	231		208		73
2	29- 52	1149	37	220	399		278		81
3	53- 72	1182	39	270	192		552		89
4	73- 97	974	40	264	104		395		83
5	98-127	1085	38	275	157		244		66
6	128-150	1045	35	257	396		339		98
7	151-185	1042	40	243	446		388		107

Steady State Period	Day-Day	Xv mgVSS/l	a	s	r	Noxox mgN/l	No2ox mgN/l	Noxan mgN/l	No2an mgN/l	Noxe mgN/l
1	0- 28	2868	0	1	1	25.9		1.1		23.1
2	29- 52	2592	0	1	1	39.9		6.1		39.9
3	53- 72	2703	3	1	1	20.9		5.4		19.2
4	73- 93	2644	3	1	1	12.5		1.7		10.4
5	94-127	2746	3	1	1	19.1		9.7		15.7
6	128-150	2569	0	1	1	42.2		2.9		39.6
7	151-185	2433	0	1	1	44.0		2.9		44.6

Table B.29

NITROGEN Balance: SYSTEM 11

Q= 10 l/d
Vp= 17 l (days1-52), 20 l (days53-185)
Rs= 15 d
w = 0.85 l/d (days1-52), 1 l/d (days53-185)
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: nitrogen in waste sludge
MNoxe: eff nitrate + nitrite
MNo2e: effluent nitrite
MNoxd: nitrate+nitrite denit
MNo2d: nitrite denitrif
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
Noxox: 2nd anoxic nitrate + nitrite
No2ox: 2nd anoxic nitrite
Noxan: anaerobic nitrate + nitrite
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti (mgN/d)	MNte (mgN/d)	MNw (mgN/d)	MNoxe (mgN/d)	MNo2e (mgN/d)	MNoxd (mgN/d)	MNo2d (mgN/d)	NITROGEN Mass Balance
1	0- 28	1006	45	269	151		514		97
2	29- 52	1149	44	247	231		445		84
3	53- 72	1183	34	251	507		467		106
4	73- 97	974	43	246	367		357		106
5	98-127	1085	41	239	492		458		113
6	128-150	1045	37	244	136		449		83
7	151-185	1052	40	258	151		508		91

Steady State Period	Day-Day	Xv (mgVSS/l)	a	s	r	Noxox mgN/l	No2ox mgN/l	Noxan mgN/l	No2an mgN/l	Noxe mgN/l
1	0- 28	2688	3	1	1	14.2		1.3		15.1
2	29- 52	2470	3	1	1	21.0		8.3		23.1
3	53- 72	2513	0	1	1	48.7		2.0		50.7
4	73- 97	2459	0	1	1	36.8		1.5		38.7
5	98-127	2369	0	1	1	47.2		1.7		49.2
6	128-150	2435	3	1	1	16.2		3.5		13.6
7	151-185	2583	3	1	1	17.8		3.5		15.1

Table B.30

NITROGEN BALANCE: SYSTEM 12

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: nitrogen in waste sludge
MNoxe: eff nitrate + nitrite
MNo2e: effluent nitrite
MNoxd: nitrate+nitrite denit
MNo2d: nitrite denitrif
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
Noxox: 2nd anoxic nitrate + nitrite
No2ox: 2nd anoxic nitrite
Noxan: anaerobic nitrate + nitrite
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	MNw mgN/d	MNoxe mgN/d	MNo2e mgN/d	MNoxd mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1-40	1039.0	39.0	254.5	182.0		481.2		92
2	41-63	919.4	35.5	251.1	82.7		269.4		69
3	64-99	973.9	40.0	246.1	159.9		373.2		84
4	100-121	1082.4	40.8	237.6	253.1		599.7		105
5	151-170	1084.6	53.0	239.2	155.5		273.2		66
6	171-198	1382.0	45.1	232.8	291.7		336.4		66

Steady State Period	Day-Day	Xv mgVSS/l	a	s	r	Noxox mgN/l	No2ox mgN/l	Noxan mgN/l	No2an mgN/l	Noxe mgN/l
1	1-40	2545	4	1	1	14.9		5.0		18.2
2	41-63	2511	4	1	1	6.8		1.4		8.3
3	64-99	2461	4	1	1	13.7		5.6		16.0
4	100-121	2376	4	1	1	25.9		11.5		25.3
5	151-170	2392	4	1	1	14.1		7.5		15.6
6	171-198	2328	4	1	1	27.7		17.7		29.2

Table B.31

NITROGEN BALANCE: SYSTEM 13

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: nitrogen in waste sludge
MNoxe: eff nitrate + nitrite
MNo2e: effluent nitrite
MNoxd: nitrate+nitrite denit
MNo2d: nitrite denitrif
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
Noxox: 2nd anoxic nitrate + nitrite
No2ox: 2nd anoxic nitrite
Noxan: anaerobic nitrate + nitrite
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	MNw mgN/d	MNoxe mgN/d	MNo2e mgN/d	MNoxd mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1-40	1042.8	43.3	250.4	149.5		431.6		84
2	41-63	1243.4	46.4	253.7	170.0		477.0		76
3	64-99	1292.3	53.0	246.0	214.2		436.7		74
4	100-121	1414.2	42.0	240.7	318.7		493.7		77
5	151-170	1420.7	36.0	229.4	282.3		365.7		64
6	171-198	1074.7	46.9	241.3	177.2		458.4		86

Steady State Period	Day-Day	Xv mgVSS/l	a	s	r	Noxox mgN/l	No2ox mgN/l	Noxan mgN/l	No2an mgN/l	Noxe mgN/l
1	1-40	2504	4	1	1	13.5		4.3		14.9
2	41-63	2537	4	1	1	16.2		5.7		17.0
3	64-99	2460	4	1	1	19.4		9.2		21.4
4	100-121	2407	4	1	1	33.3		19.3		31.9
5	151-170	2294	4	1	1	26.5		16.3		28.2
6	171-198	2413	4	1	1	15.5		5.6		17.7

Table B.32

NITROGEN BALANCE: SYSTEM 14

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNo3e: effluent nitrate
MNo2e: effluent nitrite
MNo3d: nitrate denitrified
MNo2d: nitrite denitrified
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
No3ox: 2nd anoxic nitrate
No2ox: 2nd anoxic nitrite
No3an: anaerobic nitrate
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	Xv mgVSS/l	MNw mgN/d	MNo3e mgN/d	MNo2e mgN/d	MNo3d mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1- 16	1337	44	2617	262	361	-	452	-	84
2	17- 47	1375	59	2391	239	339	3	754	-132	92
3	48- 62	1476	59	2529	253	260	1	682	-88	79
4	63- 97	1411	58	2677	268	411	1	795	-45	106
5	98-130	1425	56	2775	278	427	1	573	-64	89
6	131-162	1354	53	2668	267	492	1	722	-32	111

Steady State Period	Day-Day	a	s	r	No3ox mgN/l	No2ox mgN/l	No3an mgN/l	No2an mgN/l	No3e mgN/l	No2e mgN/l
1	1- 16	4	1	1	34.4	-	21.4	-	36.1	-
2	17- 47	4	1	1	30.7	0.6	13.5	2.7	33.9	0.3
3	48- 62	4	1	1	22.9	0.4	8.3	1.7	26.0	0.1
4	63- 97	4	1	1	41.1	0.4	21.0	1.1	41.1	0.1
5	98-130	4	1	1	43.6	0.3	26.6	1.3	42.7	0.1
6	131-162	4	1	1	51.1	0.3	30.2	0.8	49.2	0.1

Table B.33

NITROGEN Balance: SYSTEM 15

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNo3e: effluent nitrate
MNo2e: effluent nitrite
MNo3d: nitrate denitrified
MNo2d: nitrite denitrified
Xv: volatile suspended solids
MNo2a: daily nitrite addition

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
No3ox: 2nd anoxic nitrate
No2ox: 2nd anoxic nitrite
No3an: anaerobic nitrate
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	Xv mgVSS/l	MNw mgN/d	MNo3e mgN/d	MNo2e mgN/d	MNo3d mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1- 16	901	45	2879	288	124	-	315	-	86
2	17- 47	1024	65	2905	290	141	4	405	-6	88
3	48- 62	1118	45	2731	273	148	3	357	-9	73
4	63- 97	1033	54	2684	268	216	1	496	-103	73
5	98-130	1037	53	2927	293	208	2	555	-6	107
6	131-162	980	37	2748	275	214	2	591	-7	113

Steady State Period	Day-Day	a	s	r	No3ox mgN/l	No2ox mgN/l	No3an mgN/l	No2an mgN/l	No3e mgN/l	No2e mgN/l	MNo2a mgN/d
1	1- 16	2	1	1	10.8	-	0.6	-	12.4	-	0.00
2	17- 47	2	1	1	14.2	0.4	0.5	0.5	14.1	0.4	0.00
3	48- 62	2	1	1	12.3	0.2	0.9	0.4	14.8	0.3	0.00
4	63- 97	2	1	1	20.8	0.2	3.4	2.7	21.6	0.1	250.00
5	98-130	2	1	1	22.1	0.2	2.3	0.3	20.8	0.2	0.00
6	131-162	2	1	1	23.8	0.2	2.5	0.3	21.4	0.2	0.00

Table B.34

NITROGEN BALANCE: SYSTEM 16

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNo3e: effluent nitrate
MNo2e: effluent nitrite
MNo3d: nitrate denitrified
MNo2d: nitrite denitrified
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
No3ox: 2nd anoxic nitrate
No2ox: 2nd anoxic nitrite
No3an: anaerobic nitrate
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	Xv mgVSS/l	MNw mgN/d	MNo3e mgN/d	MNo2e mgN/d	MNo3d mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1- 17	1421	57	2802	280	435	1	804	-60	107
2	18- 49	1354	53	2668	267	492	1	722	-32	111
3	50- 77	1383	51	2772	277	517	1	793	-11	118
4	78- 97	941	44	2774	277	234	2	579	-1	121
5	98-124	973	46	2996	300	252	1	633	-2	126
6	125-153	911	40	2990	299	162	-	383	-	97

Steady State Period	Day-Day	a	s	r	No3ox mgN/l	No2ox mgN/l	No3an mgN/l	No2an mgN/l	No3e mgN/l	No2e mgN/l
1	1- 17	4	1	1	43.1	0.3	22.6	1.2	43.5	0.1
2	18- 49	4	1	1	51.1	0.3	30.2	0.8	49.2	0.1
3	50- 77	4	1	1	50.6	0.2	29.1	0.4	51.7	0.1
4	78- 97	2	1	1	23.7	0.1	3.2	0.1	23.4	0.2
5	98-124	2	1	1	25.3	0.2	3.1	0.2	25.2	0.1
6	125-153	2	1	1	14.0	-	1.5	-	16.2	-

Table B.35

NITROGEN BALANCE: SYSTEM 17

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNo3e: effluent nitrate
MNo2e: effluent nitrite
MNo3d: nitrate denitrified
MNo2d: nitrite denitrified
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
No3ox: 2nd anoxic nitrate
No2ox: 2nd anoxic nitrite
No3an: anaerobic nitrate
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	Xv mgVSS/l	MNw mgN/d	MNo3e mgN/d	MNo2e mgN/d	MNo3d mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1- 17	1040	49	2913	291	205	2	565	-7	106
2	18- 49	980	37	2748	275	214	2	591	-7	113
3	50- 77	950	41	2922	292	220	2	605	-5	122
4	78- 97	939	50	2555	255	192	1	498	-1	106
5	98-124	972	47	2773	277	232	1	570	-2	116
6	125-153	918	41	2749	275	123	-	310	-	82

Steady State Period	Day-Day	a	s	r	No3ox mgN/l	No2ox mgN/l	No3an mgN/l	No2an mgN/l	No3e mgN/l	No2e mgN/l
1	1- 17	2	1	1	23.8	0.2	2.9	0.3	20.5	0.2
2	18- 49	2	1	1	23.8	0.2	2.5	0.3	21.4	0.2
3	50- 77	2	1	1	24.3	0.2	2.6	0.2	22.0	0.2
4	78- 97	2	1	1	18.7	0.2	1.7	0.2	19.2	0.1
5	98-124	2	1	1	21.2	0.3	2.2	0.2	23.2	0.1
6	125-153	2	1	1	11.8	-	1.3	-	12.3	-

Nitrogen in:

(i) TKN in influent: MN_{ti}

$MN_{ti} = Q \cdot N_{ti}$

where

Q = daily flow rate (ℓ/d)

N_{ti} = influent TKN concentrations (mgN/ℓ)

(ii) Mass of nitrate and/or nitrite added: MN_{oxi}

Nitrogen out:

(i) Mass of nitrogen denitrified[3]

NO_2^- denitrified: MN_{o2d}

$MN_{o2d} = (a \cdot N_{o2ox} + s \cdot N_{o2e}) \cdot Q - (1+s+a) \cdot Q \cdot N_{o2an}$

where

N_{o2ox} = concentration of NO_2^- in aerobic zone (mgNO_2^-–N/ℓ)

N_{o2an2} = concentration of NO_2^- in 2nd anoxic zone (mgNO_2^-–N/ℓ)

s = sludge recycle ratio from settler to 1st anoxic reactor

a = mixed liquor recycle ratio from aerobic to anoxic reactor

NO_3^- denitrified: MN_{o3d}

$MN_{o3d} = (a \cdot N_{o3ox} + s \cdot N_{o3e}) \cdot Q \cdot N_{o3an}$

where

N_{o3ox} = concentration of NO_3^- in aerobic zone (mgNO_3–N/ℓ)

N_{o3an2} = concentration of NO_3^- in 2nd anoxic zone (mgNO_3^-–N/ℓ)

(iii) Mass of TKN in effluent: MN_e

$MN_{te} = N_{te} \cdot Q$

where

N_{te} = concentration of effluent TKN (mgN/ℓ)

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[3]For MUCT systems in which the concentration of NO_2^- is not separately measured but the combined concentration (NO_2^- + NO_3^-) is measured, the mass of nitrogen denitrified is calculated on the combined concentration. If a COD mass balance were conducted, the combined measurement of NO_2^- and NO_3^- would have implications regarding the oxygen requirements for nitrification of NO_2^- and NO_3^- and the equivalent oxygen for denitrification of NO_2^- and NO_3^-, but since a COD balance is not conducted here, separate calculations of NO_2^- and NO_3 denitrified do not influence the nitrogen balance and are conducted for the purpose of illustration.

(iv) Mass of nitrate and nitrite in effluent: MN_{o3e}, MN_{o2e}

$MN_{o3e} = N_{o3e} \cdot Q$

where

N_{o3e} = concentration of NO_3^- in effluent (mgN/ℓ)

$MN_{o2e} = N_{o2e} \cdot Q$

where

N_{o2e} = concentration of NO_2^- in effluent (mgN/ℓ)

(v) Mass of nitrogen in waste sludge: MN_w

$MN_w = f_n \, M\Delta X_v$

where

f_n = nitrogen fraction of VSS (mgN/mgVSS)
= 0,1 mgN/mgVSS

$M\Delta X_v$ = VSS in waste sludge (mgVSS)
$= 1/R_s \cdot V \cdot X_v$

where

V = volume of system (ℓ)

R_s = sludge age (d)

X_v = volatile suspended solids (mg/ℓ)

$$\% \text{ N balance} = 100 \, (MN_{o2d}+MN_{o3d}+MN_{te}+MN_{o2e}+MN_{o3e})/(MN_{ti}+MN)$$

$MN_{ti} = 10 \text{ ℓ/d} \cdot 137{,}5 \text{ mgN/ℓ} = 1375 \text{ mgN/d}$

$MN_{o2d} = (4 \cdot 0{,}6 + 1 \cdot 0{,}3) \cdot 10 - (1 + 1 + 4) \cdot 10 \cdot 2{,}7 = -135$

$MN_{o3d} = (4 \cdot 30{,}7 + 1 \cdot 33{,}9) \cdot 10 - (1 + 1 + 4) \cdot 10 \cdot 13{,}5 = 757$

$MN_e = (5{,}9 + 0{,}3 + 33{,}9) \text{ mgN/ℓ} \cdot 10 \text{ ℓ/d} = 401 \text{ mgN/d}$

$MN_w = 0{,}1 \cdot 1\text{ℓ} \cdot 2391 \text{ mgVSS/ℓ} = 239$

$$\% \text{ N balance} = 100 \, (-135+757+401+239)/1375 = 92$$

APPENDIX B.4

AEROBIC AND ANOXIC BATCH TESTS

Aerobic batch tests were conducted on sludge from a 2RND system (System 7) in order to examine the induction of inhibition of aerobic respiration. Aerobic batch tests were also conducted on sludge from an IAND system (System 5) and an MUCT system (System 17) to determine if a similar inhibitory effect could be measured for sludge from these systems as for sludge from a 2RND system. Additionally, aerobic batch tests were conducted on sludge from a system which was either continuous aerobic or continuous anoxic (System 7) to determine the effect of aerobic and anoxic conditions on synthesis of the denitrification and aerobic enzymes. In analysis of the results, it is assumed that the degree of inhibition reflects the interaction between nitric oxide and cytochrome oxidase which indicates the proportions of NO_2^- reductase, NO reductase, and cytochrome oxidase present.

Anoxic batch tests were conducted on sludge from a system which was either continuous aerobic, or continuous anoxic (System 7) to determine the effect of aerobic and anoxic conditions on the proportions of NO_3^-– and NO_2^-– reductase present. It is assumed that rapid rates of NO_3^- and NO_2^- removal indicate high levels of NO_3^- and NO_2^- reductase and conversely, low rates of NO_3^- and NO_2^- removal indicate low levels of NO_3^-– and NO_2^-– reductase.

Aerobic batch tests

Procedure

The procedure for the aerobic batch tests to determine the level of inhibition of OUR induced in a sludge follows closely the procedures described by Ekama *et al.* (1986) for determining influent COD fractions and the maximum specific growth rate of heterotrophs. Variations to the procedure are in the aerobic and anoxic pre–test conditions prior to the addition of substrate under aerobic conditions. The reasons for the variation in procedure are described below.

Anoxic–anaerobic pre–test conditions: The sludge was exposed to unaerated periods of 1 to 24 h as a means of eliminating residual NO_2^- and NO_3^- by

denitrification. In some batch tests (2, 3, 4, 11, 12, 13, 14, 15), denitrification was assisted by the addition of a small volume (0,25 ℓ) of sewage at the start of the unaerated period. In later batch tests, this procedure was discontinued and high concentrations of NO_2^- and NO_3^- in the sludge were removed by "washing" the sludge, i.e. diluting the sludge to 1 ℓ, settling for $^1/_2$ h, decanting, then repeating the procedure twice. *Thiourea* (10 mg/ℓ – final batch volume) was usually added during this unaerated period and for tests operated without an unaerated period, it was added during the aerated pre–test period.

Aerobic pre–test conditions: The sludge was exposed to an aerated period of 2 h during which time NO_2^- was added so as to induce inhibition in the sludge through aerobic denitrification of NO_2^-. The concentration of DO was maintained between 2 and 5 mgO/ℓ during this period and also during the period following substrate addition.

Origin of sludge

For anoxic–aerobic systems irrespective of the configuration, sludge was taken from an unaerated zone, i.e. during the anoxic period of an IAND system, from the anoxic reactor of a 2RND system, and from the 2nd anoxic reactor of an MUCT system. Sludge was taken from the unaerated as opposed to the aerated zone in these systems to avoid high concentrations of NO_2^- and NO_3^-.

Calculation of inhibition

The degree of inhibition induced in a sludge is determined by calculating the fraction of the maximum OUR which is inhibited immediately after feeding. For example, for Batch test A conducted on sludge from System 7 on Day 258, the OUR increases from 9,5 to 18,8 mgO/gVSS.h over a period of $5^1/_2$ h and the inhibition is calculated as follows:

$$100 \cdot (18{,}8 - 9{,}5)/18{,}8 = 49\%$$

Results

2RND configuration (System 9): Tables B.36 to B.54 indicate the operating conditions for Batch tests 1 to 19 respectively, and Figs B.14 to B.32 illustrate the results of Batch tests 1 to 19 respectively.

IAND configuration (System 5): Tables B.55 and B.56 indicate the operating

conditions for batch tests 20 and 21 respectively, and Figs B.33 and B.34 illustrate the results of batch tests 20 and 21 respectively.

MUCT configuration (System 17): Tables B.57 to B.60 indicate the operating conditions for batch tests 22 to 25 respectively, and Figs B.35 to B.38 illustrate the results of batch tests 22 to 25 respectively.

Continuous aerobic and continuous anoxic configuration (System 7): Different aerobic and anoxic pre–conditions were applied to the batch tests depending on whether System 7 was operated continuous anoxic or continuous aerobic at the time of the test. For batch tests conducted during periods when the system was continuous anoxic, sludge was subjected to an anoxic pre–test period of 1 h with NO_2^- added at the start of anoxic conditions in order to ensure the maximum degree of inhibition possible from intracellular accumulation of NO by floc–formers during denitrification of NO_2^-. For batch tests conducted during periods when the system was continuous aerobic, sludge was subjected to a pre–aerobic period of 2 h with NO_2^- added after $^1/_2$ h in order to ensure maximum intracellular accumulation of NO by floc–formers during denitrification of NO_2^-. Tables B.61 to B.78 indicate the operating conditions for Batch tests A to R, and Figs B.39 to B.56 describe the results of batch tests A to R respectively.

Discussion of results

The aerobic batch tests examined the effect on inhibition of OUR of many different factors. Many of these factors, such as, (i) effect of concentration of NO_2^- in the anoxic zone preceding aerobic conditions, (ii) effect of RBCOD and SBCOD on relief and induction of inhibition, (iii) aerobic denitrification of NO_3^- and NO_2^-, and (iv) time required for aerobic denitrification of NO_2, are directly implicated in the conceptual biochemical model for facultative organism respiration and are discussed in Chapter 5.

Other factors, such as (i) effect of the presence and absence of aerobic pretreatment of sludge, and (ii) differences between sludges from different configurations, do not impact directly on the biochemical model and are not described in discussion of the conceptual model for facultative organism respiration. However all the batch tests add to understanding of the inhibition effect and can be categorized are discussed according to their category.

(1) *The effect of different concentrations of NO_2^- at the end of the anoxic period, just before substrate addition under aerobic conditions*: Four tests were conducted, with different concentrations of NO_2^- present at the time of substrate addition, different percent inhibitions were induced; BT 2 (25,0 mgN/ℓ, 68%), BT 4 (5,5 mgN/ℓ, 36%), BT 5 (0,5 mgN/ℓ, 30%) and BT 11 (0,1 mgN/ℓ, 8%).

- Inhibition of OUR was proportional to the concentration of NO_2^-; as the concentration of NO_2^- decreased from 25,0 to 0,1 mgN/ℓ, inhibition decreased from 68 to 8%.

(2) *Comparison of inhibition induced from denitrification of NO_3^- and NO_2^- under aerobic conditions*: Three tests can be compared to determine the extent of aerobic denitrification of NO_3^- and NO_2^-; Batch test 12 (NO_3^- added under aerobic conditions, no inhibition), Batch test 13 (NO_2^- added under aerobic conditions, 65% inhibition), and Batch test 1 (no NO_2^- or NO_3^- added under aerobic conditions, no inhibition).

- NO_3^- is not, but NO_2^- is denitrified under aerobic conditions, resulting in inhibition.

(3) *Effect of RBCOD and SBCOD on induction of inhibition through aerobic denitrification of NO_2^-*: Effect of RBCOD and SBCOD on induction of inhibition through aerobic denitrification of NO_2^-. Two tests can be compared to determine the effect of RBCOD and SBCOD on aerobic denitrification of NO_2^-; Batch test 13 (NO_2^- added under aerobic conditions with SBCOD only present, 65% inhibition) and Batch test 17 (NO_2^- added under aerobic conditions with RBCOD only present, 6% inhibition).

- Inhibition is induced with NO_2^- and SBCOD, but is not induced with NO_2^- and RBCOD present under aerobic conditions. The relief of inhibition with time in Batch test 13 after feeding indicates that RBCOD not only prevents inhibition but also relieves it.

(4) *Time required for aerobic denitrification of NO_2^- for induction of inhibition*: Three tests can be compared to determine the effect of time for NO_2^- denitrification under aerobic conditions; Batch test 14 (NO_2^- present for 30

mins, 50% inhibition), Batch test 15 (NO_2^- present for 15 mins, 55% inhibition), and Batch test 16 (NO_2^- present for 5 mins, 41% inhibition). Although there appears to be little difference in % inhibition between the 3 tests, a very distinct feature in each OUR profile is the 2 rates of relief of inhibition. A difference is apparent between the first rates of relief of inhibition in the 3 batch tests. For Batch tests 14, 15 and 16, the time periods for the first rate were 127, 71, and 44 mins. respectively, indicating that as the time for aerobic denitrification of NO_2^- decreased (from 30 to 5 mins.), the time for relief of the first inhibition also decreased (from 127 to 44 mins.).

- The cause of the 2 rates of relief of inhibition are not immediately apparent, but may be explained from an aspect of the biochemical respiratory model, in which inhibition is overcome (i) through a reduction in NO accumulated and (ii) by an increase in cytochrome aa_3 synthesized. The 2 rates of relief of inhibition may result from these 2 aspects.

(5) *Re-induction of inhibition after removal with RBCOD*: In addition to the experiments described in (3) above, two batch tests can be compared to examine the roles of RBCOD and SBCOD in relief and induction respectively of inhibition. In Batch test 7, inhibition was relieved and the maximum OUR was attained. Approximately 2 h after feeding, the RBCOD was completely utilized and the OUR decreased to a constant value until all the SBCOD was utilized, whereafter the OUR decreased to the endogenous rate. Batch test 8 describes the OUR from the end of Batch test 7 when NO_2^- was added, 5 mins. before the addition of substrate. The OUR increased slowly with time to a minimum value, indicating that inhibition had been re-induced in the absence of RBCOD (presence of SBCOD only) after it had been relieved by the presence of RBCOD in Batch test 7.

- As with the conclusion in (3) above, inhibition is induced with NO_2^- present in the absence of RBCOD.

(6) *Effect of continuous aerobic and continuous anoxic conditions on inhibition*: A series of 18 aerobic batch tests, A to R, were conducted on sludge from System 7, during periods when the system was either continuous aerobic or continuous anoxic. In changing from aerobic to anoxic conditions, the level of

inhibition initially increased rapidly (1–4 days) to ≈ 80%, then decreased (2–8 days) to ≈ 40% before increasing (15–30 days) to ≈ 70%. In changing from anoxic to aerobic conditions, the level of inhibition decreased (40 days) from ≈ 70% to ≈ 20%.

- Changes between anoxic and aerobic conditions result in changes in synthesis of anoxic and aerobic respiratory enzymes. These results are discussed at length in Chapter 5.

(7) *Inhibition of sludge from IAND and MUCT systems*: Two tests conducted on sludges from an IAND system, one with NO_2^- present under anoxic pre–test conditions (Batch test 20, 51% inhibited), the other with NO_2^- present under anoxic pre–test conditions (Batch test 21, no inhibition), demonstrated similar results to sludges from 2RND systems. Two tests conducted on sludges from the 2nd anoxic reactor of an MUCT system, one with NO_2^- present under aerobic pre–conditions (Batch test 25, 29% inhibition), the other without NO_2^- present in the sludge (Batch test 22, no inhibition) demonstrated similar results to sludges from IAND and 2RND systems.

- Inhibition can be induced in sludge from ND and NDBEPR systems irrespective of the configuration.

(8) *Inhibition induced in sludge from either aerobic or anoxic reactors*: Two tests conducted on sludges from the aerobic reactor of an MUCT system, one with NO_2^- present under aerobic pre–conditions (Batch test 24, 18% inhibition), the other without NO_2^- present in the sludge (Batch test 23, no inhibition) demonstrated similar results to sludges from the aerobic reactor of MUCT systems (Batch tests 25 and 22).

- Inhibition can be induced in sludges from either the aerobic or anoxic reactors of an NDBEPR system.

Aspects (1) to (8) above have considerable implications with regard to the biochemical respiratory model for facultative organisms. Aspects (9) to (12) below describe experimental factors which although not directly implicated in the biochemical respiratory model, were examined to determine their effect on the results of aerobic inhibition batch tests.

(9) *Effect of anoxic pre–test conditions on inhibition (i)*: Two batch tests can be compared to determine the effect on aerobic substrate utilization of a long unaerated pre–condition; Batch test 9 (11 h unaerated period; NO_2^- present during a 2 h aerobic pre–period 62% inhibition) and Batch test 10 (12 h unaerated period; $NO_2^- \approx 0{,}1$ mgN/ℓ during a 2 h aerobic pre–period, 14% inhibition).

- It is unclear as to whether the small degree of inhibition in the system with a very low concentration of NO_2^- resulted from the NO_2^- or was a consequence of the long unaerated period.

(10) *Effect of anoxic pre–test conditions on inhibition (ii)*: Two other batch tests can be compared to examine the effect on inhibition of anoxic pre–test conditions, inconclusively tested in (9) above; Batch test 3 (6 h unaerated, no inhibition) and Batch test 11 (2 h unaerated, 8% inhibition).

- Unaerated periods < 6 h do not, and unaerated periods > 6 h *may* have an effect on induction of inhibition in sludge.

(11) *Effect of aerobic pre–conditions on inhibition*: Two batch tests can be compared to examine the effect on inhibition of aerobic pre–test conditions; Batch test 18 (no aerobic pre–test condition – sludge taken directly from the parent system, no inhibition) and Batch test 19 (2 h aerobic pre–test condition, no inhibition).

- The aerobic pre–test condition has no effect on induction of inhibition in sludge.

(12) *Determination of whether increase in OUR is a result of increase in nitrification*: It was considered that the increase in OUR in some batch tests immediately after feeding could result from an increase in nitrification by *Nitrosomonas* and *Nitrobacter*, only partially inhibited by the thiourea (10 mg/ℓ – final batch volume) added during the preceding anoxic and aerobic periods. Doubling the concentration of thiourea (20 mg/ℓ – final batch volume) added to Batch test 6 (86% inhibition did not prevent an increase in OUR. From analysis of NO_2^- and NO_3^- concentration in many tests, it is apparent that the thiourea inhibits only the first nitrification step, $NH_4^+ \rightarrow$

NO_2^- (*Nitrosomonas*) and not the second nitrification step, $NO_2^- \rightarrow NO_3^-$ *(Nitrobacter)*.

Conceivably the increase in OUR after substrate addition could result from an increase in *Nitrobacter*. However it should be noted that in Batch test 17 in which NO_2^- was added (after 5 h) when the maximum OUR had been reached, the OUR increased immediately by 3,6 mgO/gVSS, an increase attributable to nitrification of NO_2^-. Examination of the oxygen requirements for nitrification of $NO_2^- \rightarrow NO_3^-$ (1,14 mgO/mgNO_2^-) calculated from the change in NO_3^- concentration after the addition of NO_2^- for Batch tests 13, 9, 6, 4, and 2, yielded oxygen consumption values of 3,0; 1,0; 1,3; 4,7; and 6,1 mgO/gVSS.h respectively. These values are insignificant compared to many of the increases in OUR.

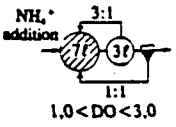
- Increases > 3,5 mgO/gVSS.h in OUR in batch tests are not a consequence of nitrification of NO_2^-.

Anoxic batch tests

Procedures and results

Anoxic, denitrification batch tests, conducted to determine the extent of synthesis of denitrification enzymes, follow the procedures described by Ekama *et al.* (1986) for determination of nitrate reduction rates. These tests were conducted on System 7, during periods when the system was continuous anoxic and continuous aerobic. The conditions of operation of Batch tests DBT1 to DBT4 are shown in Tables B.79 to B.82, and the results of the batch tests are illustrated in Figs B.57 to B.60.

Table B.36

BATCH TEST 1 OPERATING CONDITIONS		
Objective: To determine OUR response following absence of NO_2^-/NO_3^- at end of 2 h anoxic/anaerobic pre-test conditions followed by 2 h aerobic period.		
PARENT SYSTEM CONDITIONS		
System 9; Day 225 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,4
Sewage volume	(ℓ)	0,8
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	468
VSS	(mg/ℓ)	441
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	309
TKN	(mgN/ℓ-final batch volume)	26,9

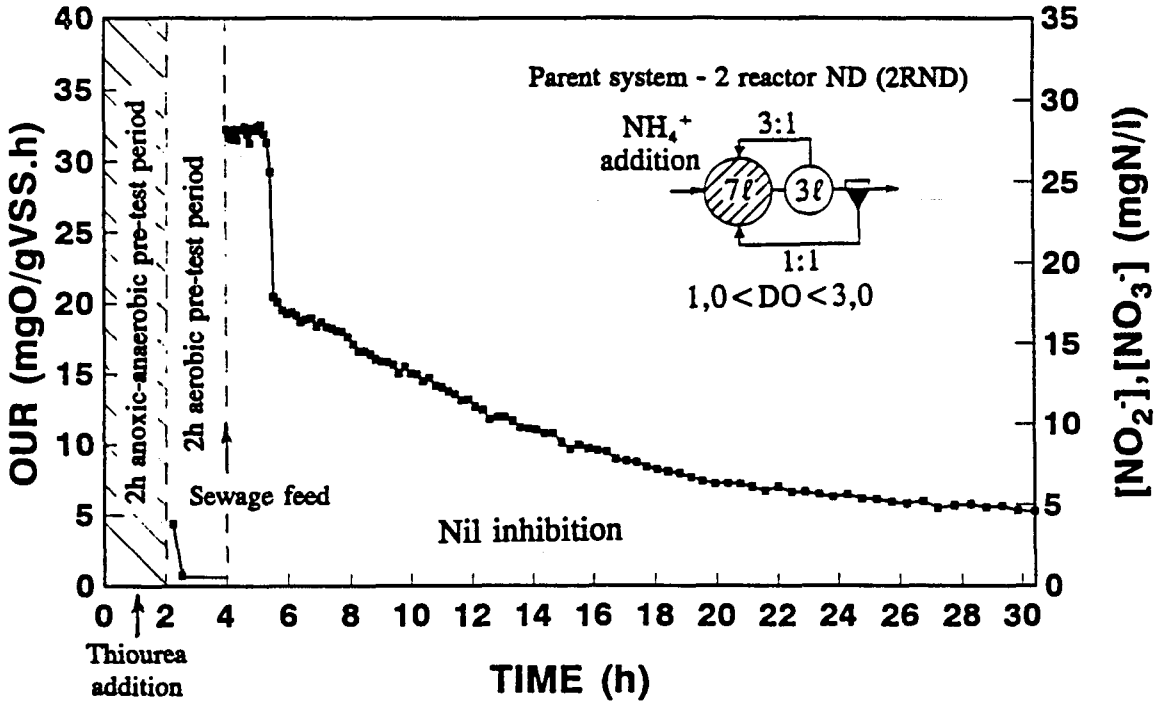
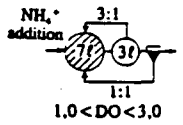


Fig B.14

Table B.37

BATCH TEST 2 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- addition under anoxic pre-test conditions to give NO_2^- (25,0 mgN/ℓ) at start of aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 227 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1;0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,4 @ t=0; 0,8 @ t=2
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	626
VSS	(mg/ℓ)	510
F/M	(mgCOD/mgVSS)	0,6
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	307
TKN	(mgN/ℓ-final batch volume)	28,3

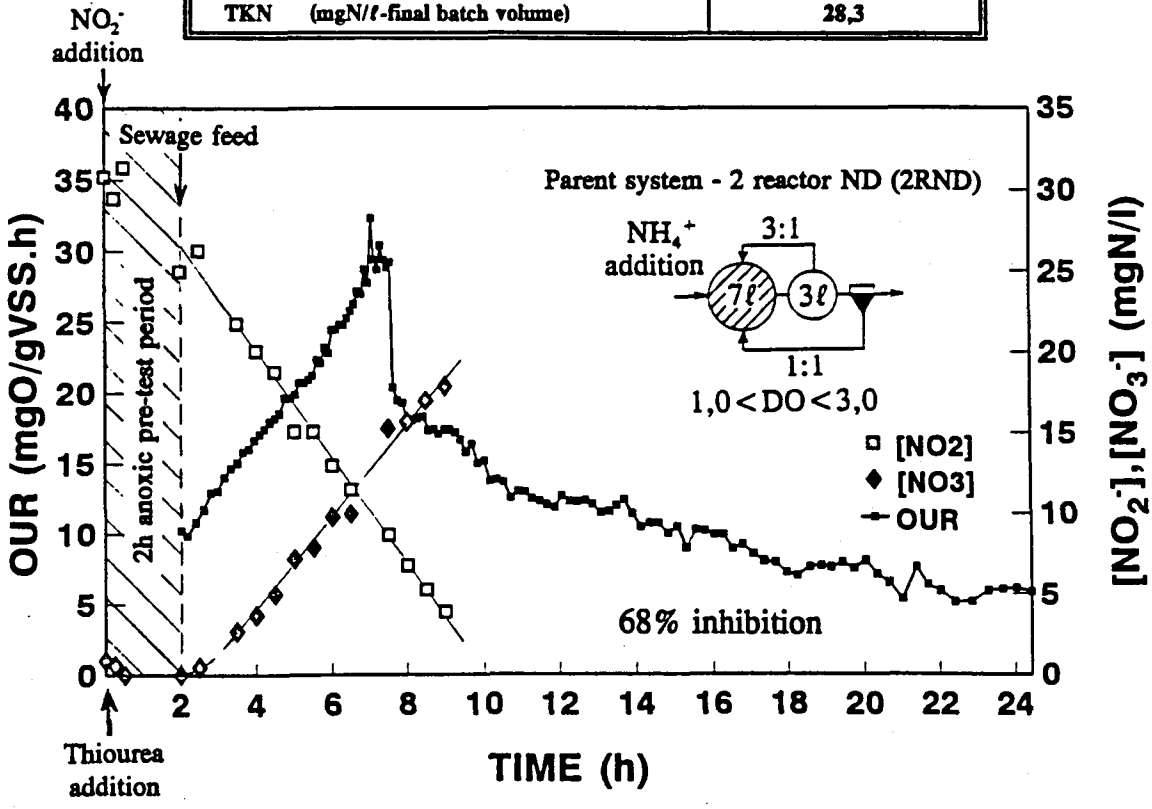
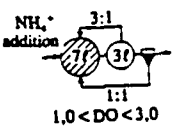


Fig B.15

Table B.38

BATCH TEST 3 OPERATING CONDITIONS		
Objective: To determine OUR response following 6 h anoxic/anaerobic conditions with NO_3^- absent at the end.		
PARENT SYSTEM CONDITIONS		
System 9; Day 229 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,2
Sewage volume	(ℓ)	0,3 @ t=0; 0,7 @ t=6
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	515
VSS	(mg/ℓ)	457
F/M	(mgCOD/mgVSS)	0,6
Anoxic pre-test period	(h)	6,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	370
TKN	(mgN/ℓ-final batch volume)	28,3

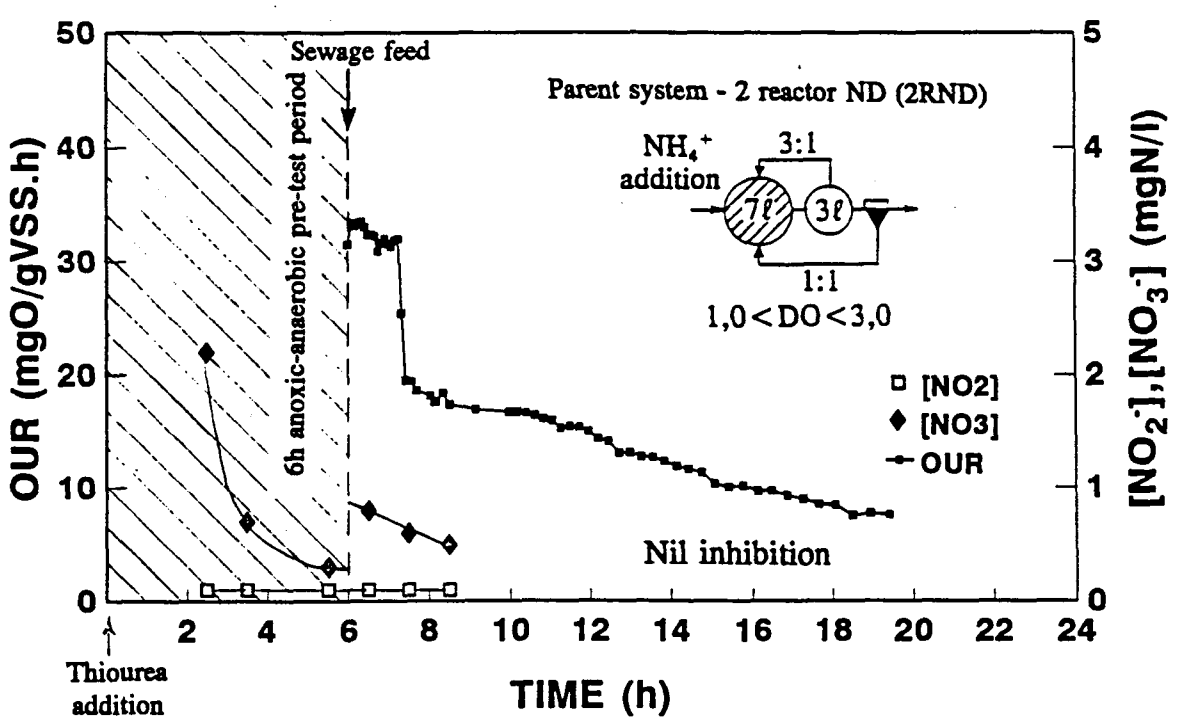
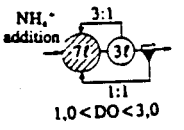


Fig B.16

Table B.39

BATCH TEST 4 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- addition under anoxic pre-test conditions to give NO_2^- (5,5 mgN/ℓ) at start of aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 233 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,2
Sewage volume	(ℓ)	0,25 @ t=0;0,75 @ t=2
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	490
VSS	(mg/ℓ)	451
F/M	(mgCOD/mgVSS)	0,6
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	288
TKN	(mgN/ℓ-final batch volume)	21,3

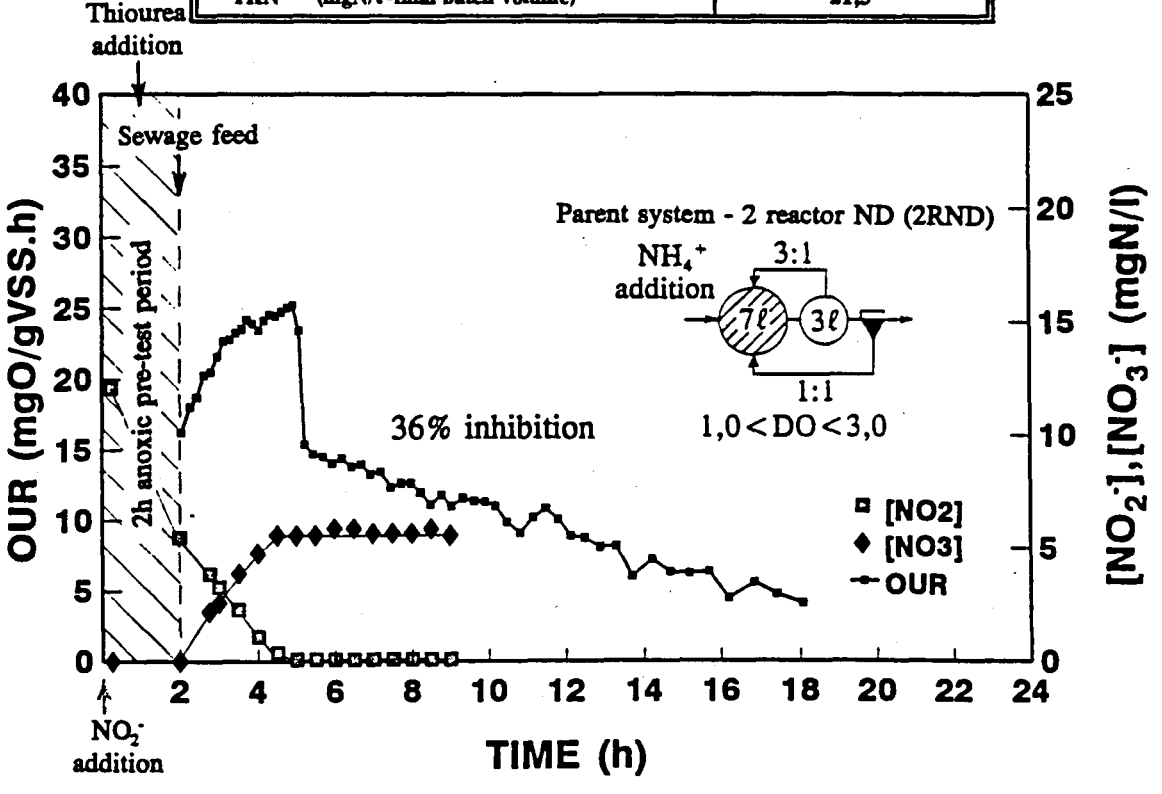
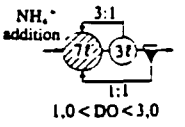


Fig B.17

Table B.40

BATCH TEST 5 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- addition under anoxic pre-test conditions to give NO_2^- (0,6 mgN/ℓ) at start of aerobic feed conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 240 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,2
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	577
VSS	(mg/ℓ)	465
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	383
TKN	(mgN/ℓ-final batch volume)	28,3

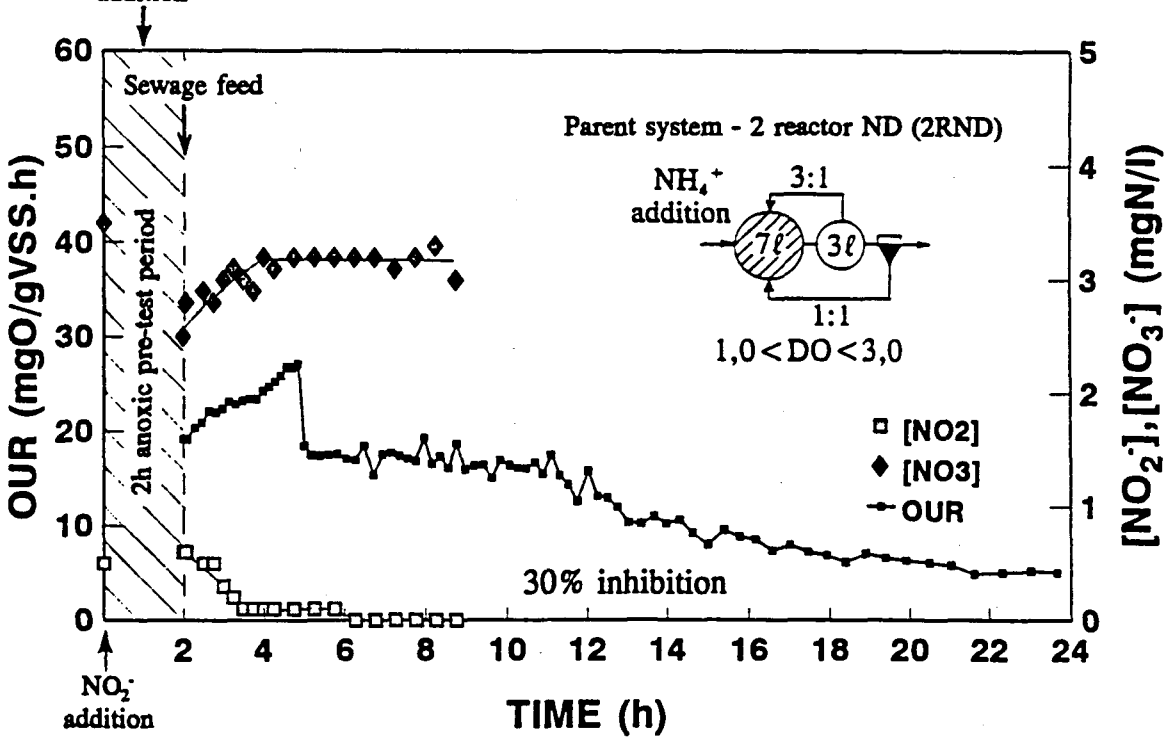
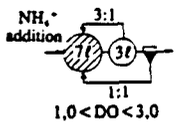


Fig B.18

Table B.41

BATCH TEST 6 OPERATING CONDITIONS		
Objective: To determine the effect of doubling the concentration of thiourea on OUR response following NO_2^- addition under aerobic pre-test period.		
PARENT SYSTEM CONDITIONS		
System 9; Day 241 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,2
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	631
VSS	(mg/ℓ)	567
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	1,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	376
TKN	(mgN/ℓ-final batch volume)	31,0

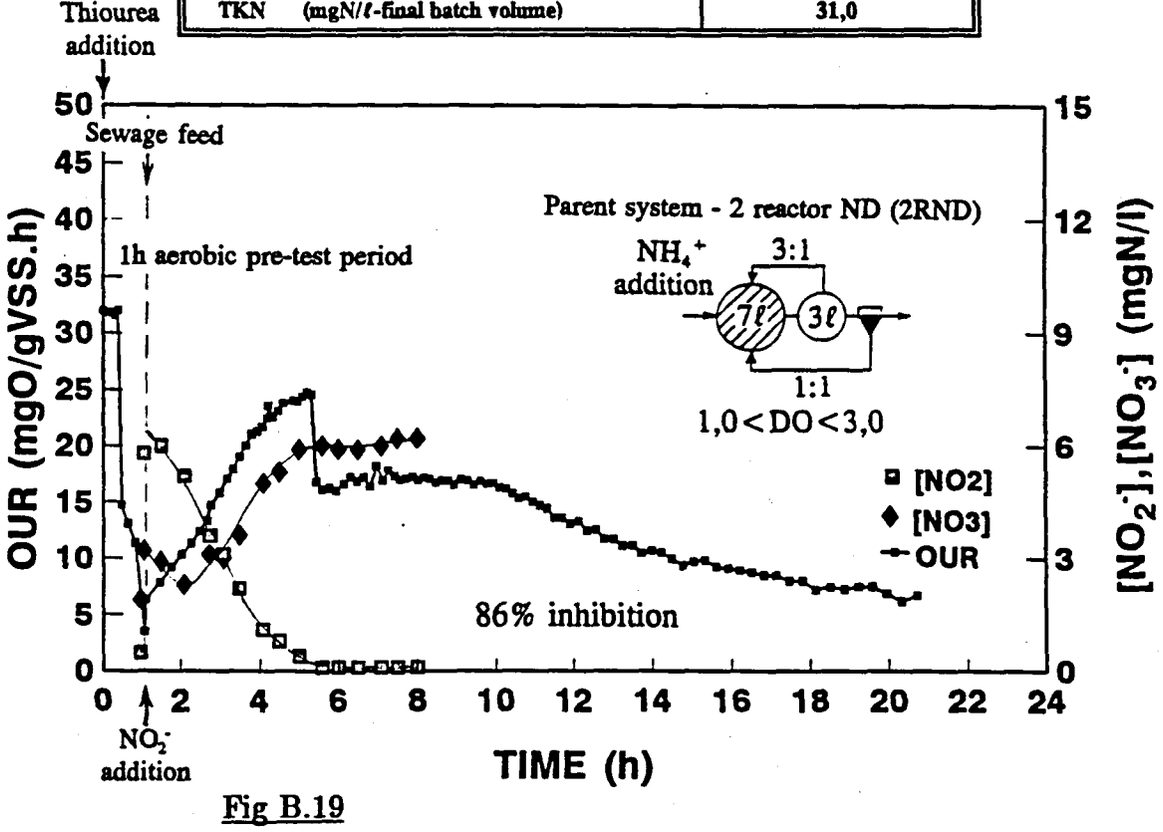
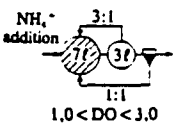


Fig B.19

Table B.42

BATCH TEST 7 OPERATING CONDITIONS		
Objective: To determine role of length of the anoxic/anaerobic pre-test conditions on OUR response.		
PARENT SYSTEM CONDITIONS		
System 9; Day 252 **System configuration: 2RND (MLE)**		
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**0,8**
Water volume	**(ℓ)**	**1,4**
Sewage volume	**(ℓ)**	**0,8**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**518**
VSS	**(mg/ℓ)**	**462**
F/M	**(mgCOD/mgVSS)**	**0,8**
Anoxic pre-test period	**(h)**	**6,0**
Aerobic pre-test period	**(h)**	**-**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**392**
TKN	**(mgN/ℓ-final batch volume)**	**33,4**

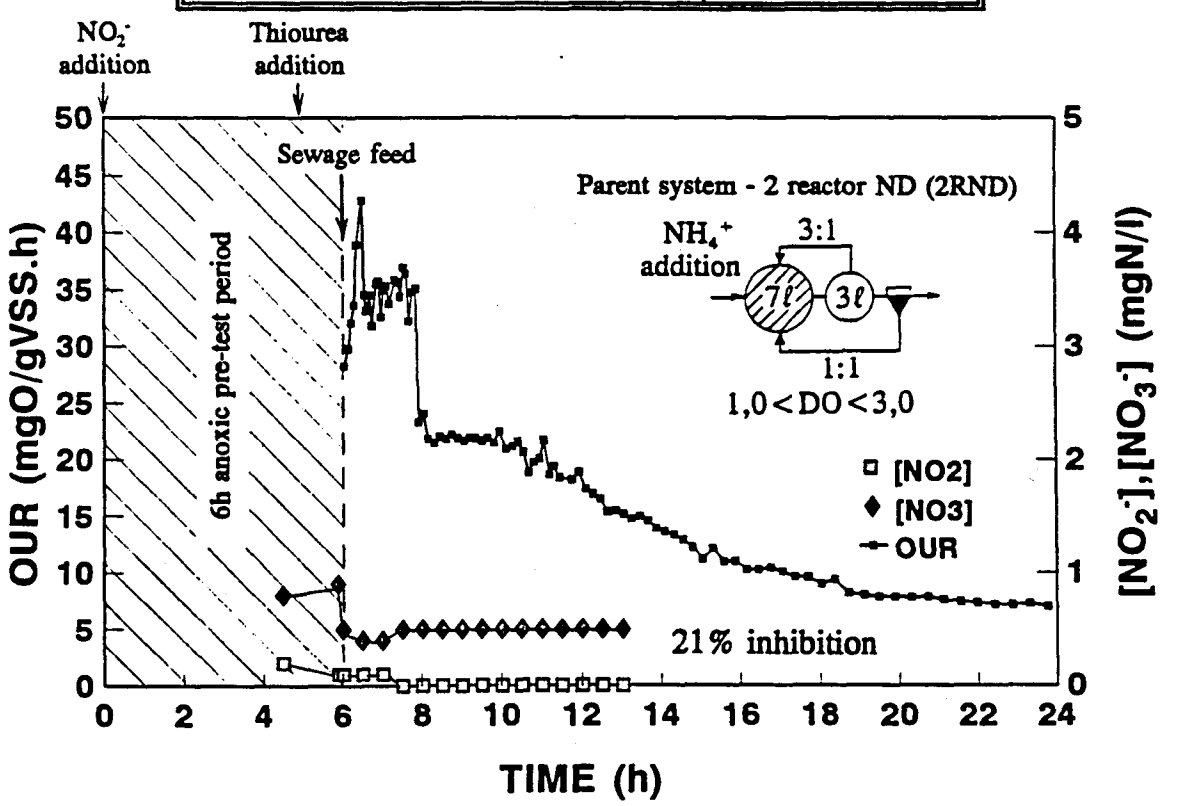
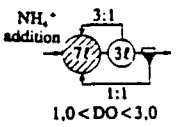


Fig B.20

Table B.43

BATCH TEST 8 OPERATING CONDITIONS		
Objective: To determine if inhibition can be re-induced in a sludge in which inhibition has been relieved.		
PARENT SYSTEM CONDITIONS		
System 9; Day 253 **System configuration: 2RND (MLE)**		
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**From Batch Test 13**
Water volume	**(ℓ)**	**1,0**
Sewage volume	**(ℓ)**	**1,0**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**459**
VSS	**(mg/ℓ)**	**409**
F/M	**(mgCOD/mgVSS)**	**0,3**
Anoxic pre-test period	**(h)**	**See end of Batch Test 20**
Aerobic pre-test period	**(h)**	**See end of Batch Test 20**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**392**
TKN	**(mgN/ℓ-final batch volume)**	**33,4**

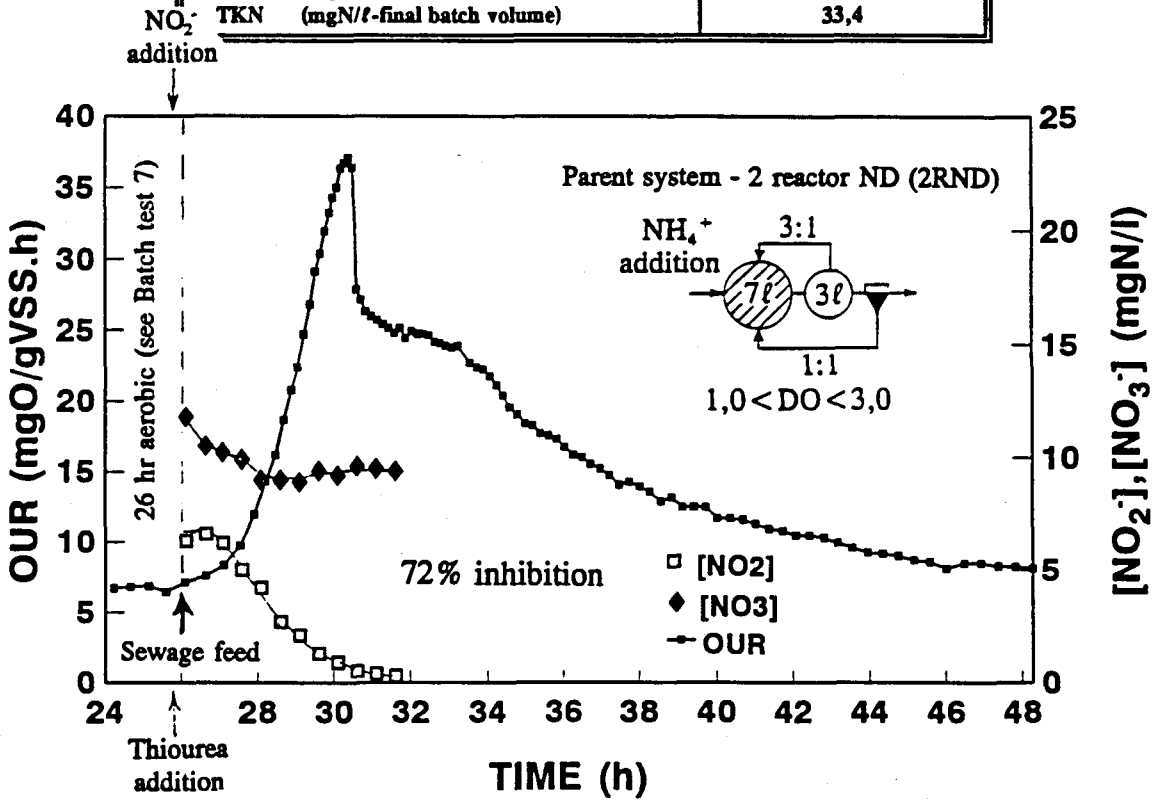
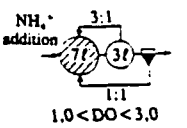


Fig B.21

Table B.44

BATCH TEST 9 OPERATING CONDITIONS		
Objective: To determine the role of the length of the anoxic/anaerobic pre-test conditions on OUR response to NO_3^- addition under aerobic pre-test conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 261 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	499
VSS	(mg/ℓ)	457
F/M	(mgCOD/mgVSS)	0,9
Anoxic pre-test period	(h)	12,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	392
TKN	(mgN/ℓ-final batch volume)	33,4

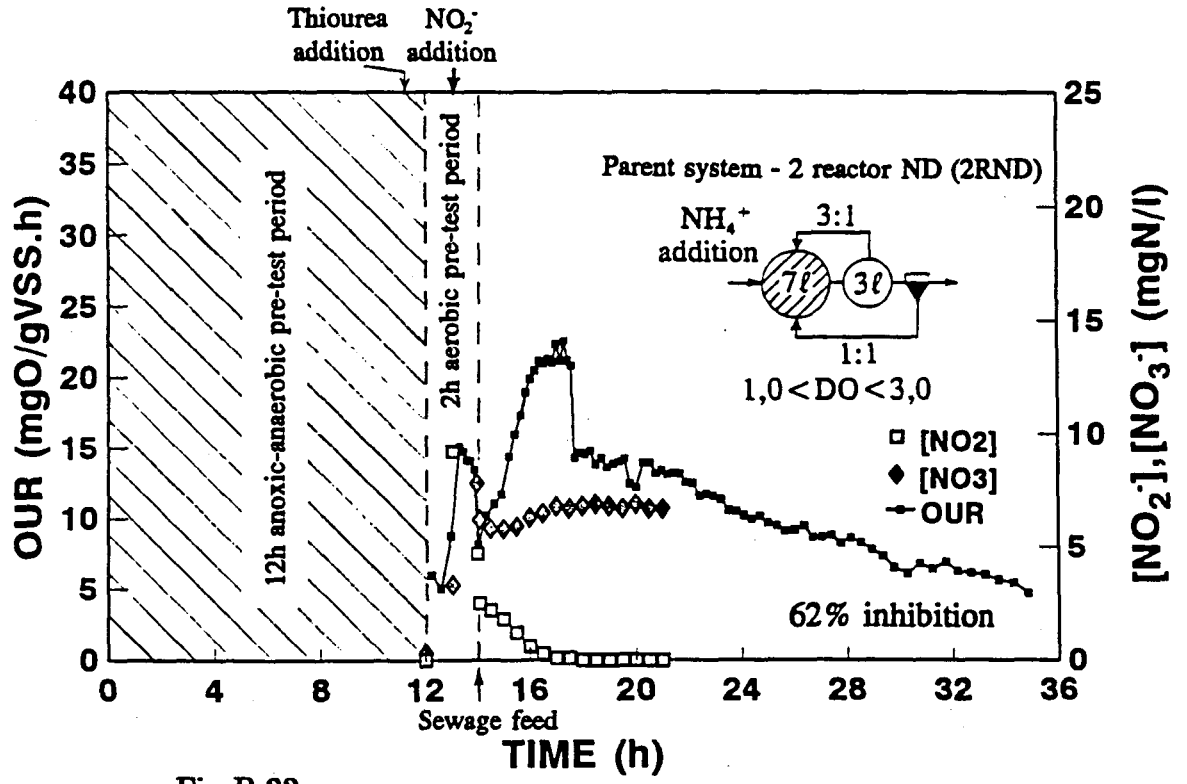
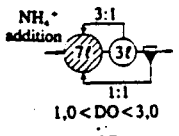


Fig B.22

Table B.45

BATCH TEST 10 OPERATING CONDITIONS		
Objective: To determine OUR response to absence of NO_2^-/NO_3^- in aerobic pre-test period.		
PARENT SYSTEM CONDITIONS		
System 9; Day 262 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,3
Sewage volume	(ℓ)	0,9
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	595
VSS	(mg/ℓ)	511
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	12,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	353
TKN	(mgN/ℓ-final batch volume)	30,1

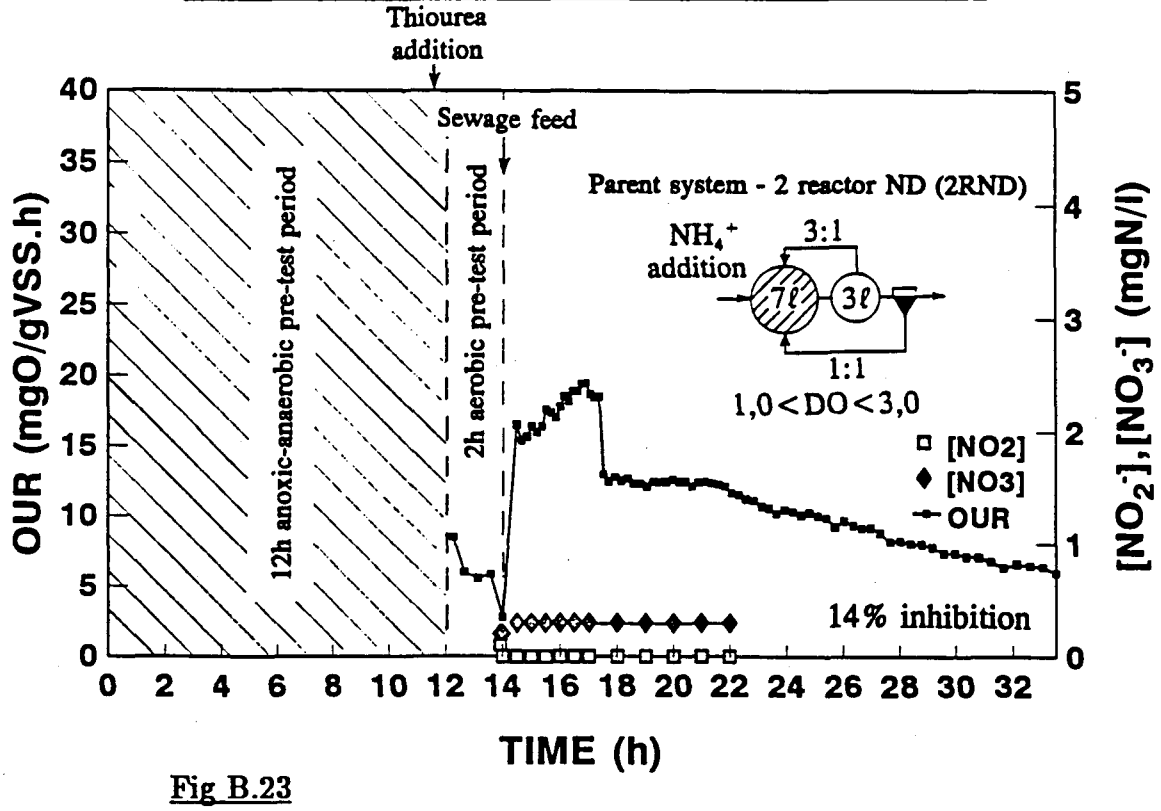
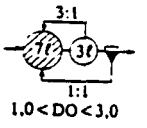


Fig B.23

Table B.46

BATCH TEST 11 OPERATING CONDITIONS		
Objective: To determine OUR response to absence of NO_3^- < 0,2 mgN/ℓ at end of 2 h anoxic/anaerobic pre-test conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 280 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,25 @ t=0;0,751 @ t=2
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	652
VSS	(mg/ℓ)	628
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	322
TKN	(mgN/ℓ-final batch volume)	30,8

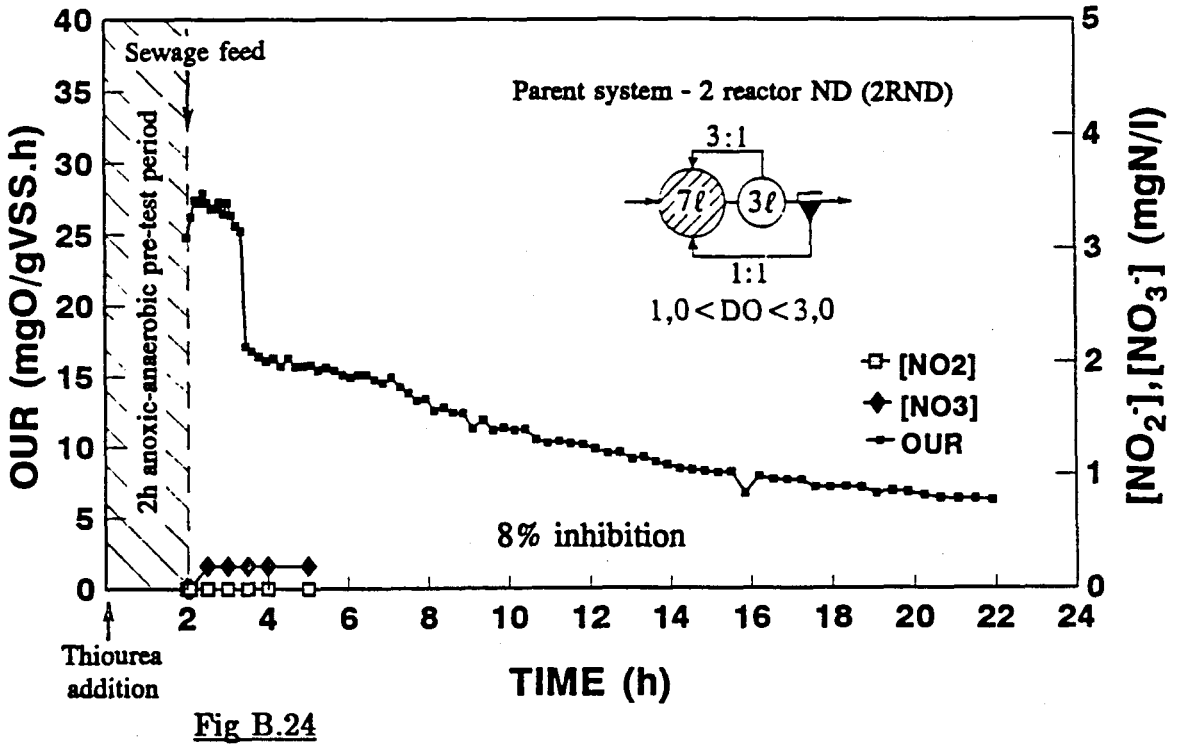
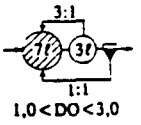


Fig B.24

Table B.47

BATCH TEST 12 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_3^- (≈ 20 mgN/ℓ) addition under aerobic pre-test conditions 60 minutes before substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 284 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,25 @ t=0;0,75 @ t=4
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	641
VSS	(mg/ℓ)	594
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	272
TKN	(mgN/ℓ-final batch volume)	22,5

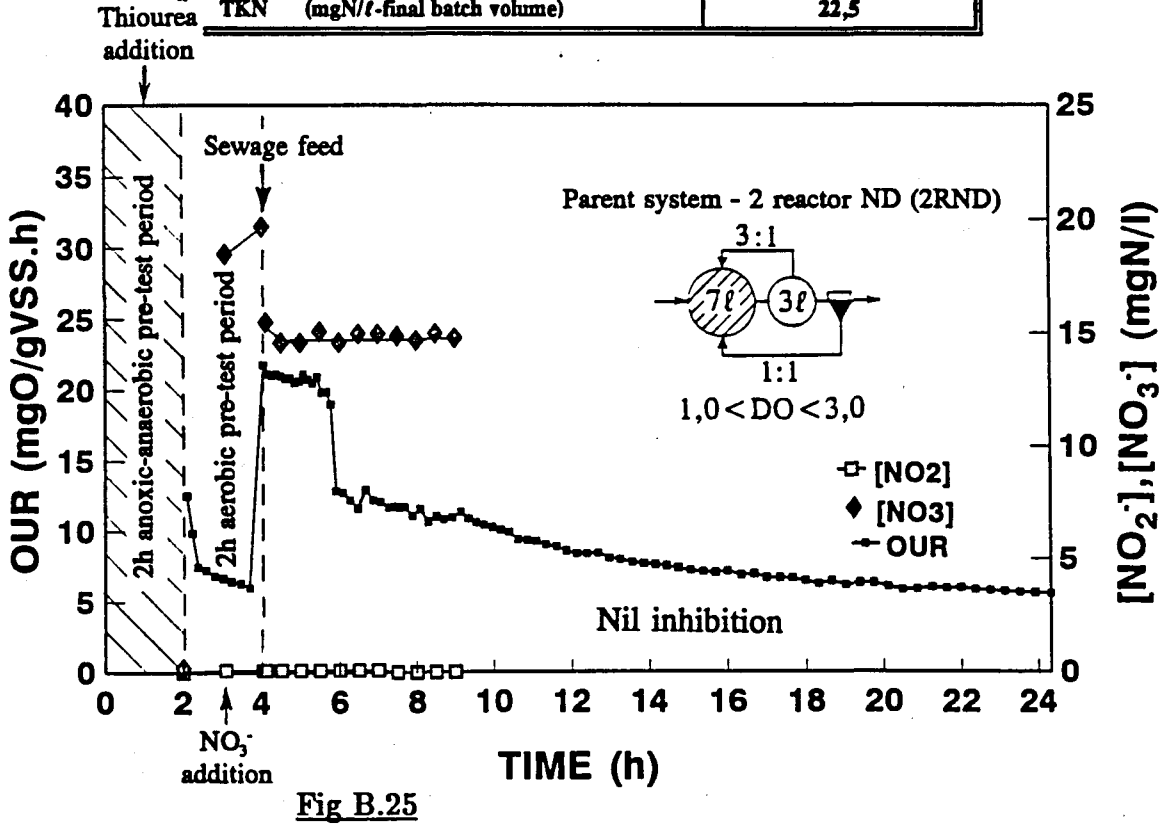
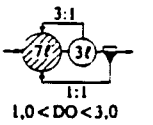


Fig B.25

Table B.48

BATCH TEST 13 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- (> 10 mgN/ℓ) addition under aerobic pre-test conditions 60 minutes before substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 285 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,25 @ t=0;0,75 @ t=4
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	628
VSS	(mg/ℓ)	512
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	272
TKN	(mgN/ℓ-final batch volume)	22,5

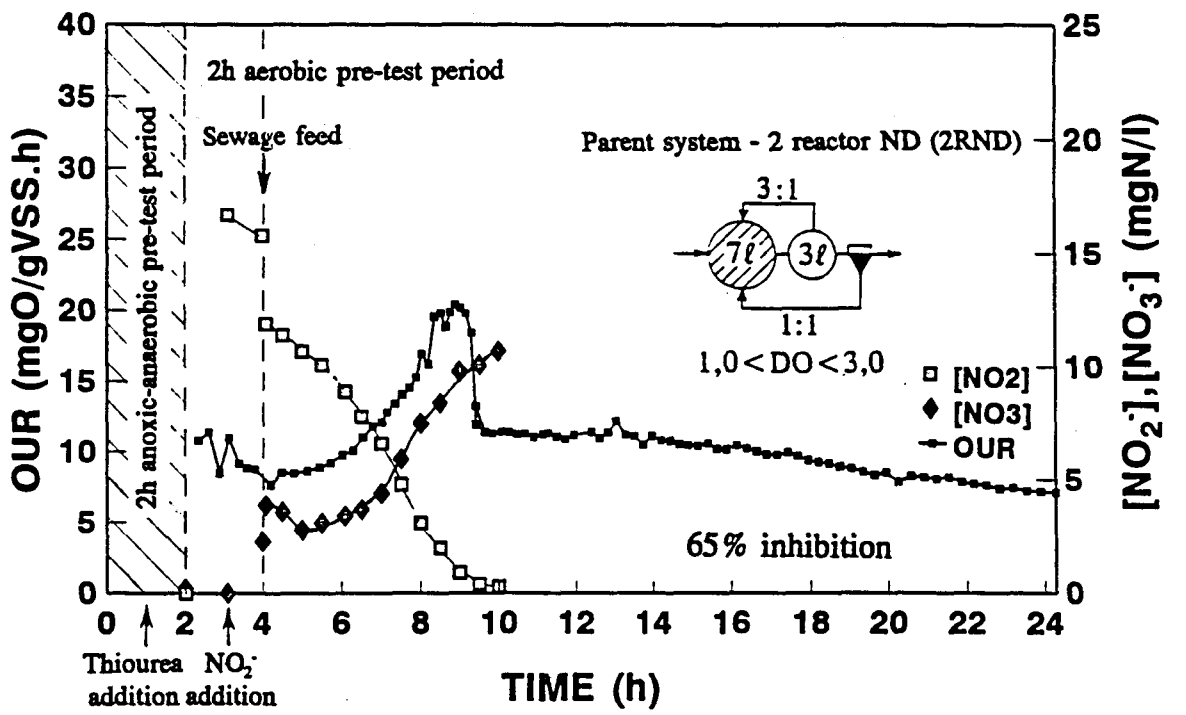
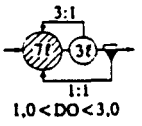


Fig B.26

Table B.49

BATCH TEST 14 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_3^- (> 10 mgN/ℓ) addition under aerobic pre-test conditions 30 minutes before substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 330 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,25 @ t=0;0,75 @ t=4
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	754
VSS	(mg/ℓ)	648
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	303
TKN	(mgN/ℓ-final batch volume)	30,0

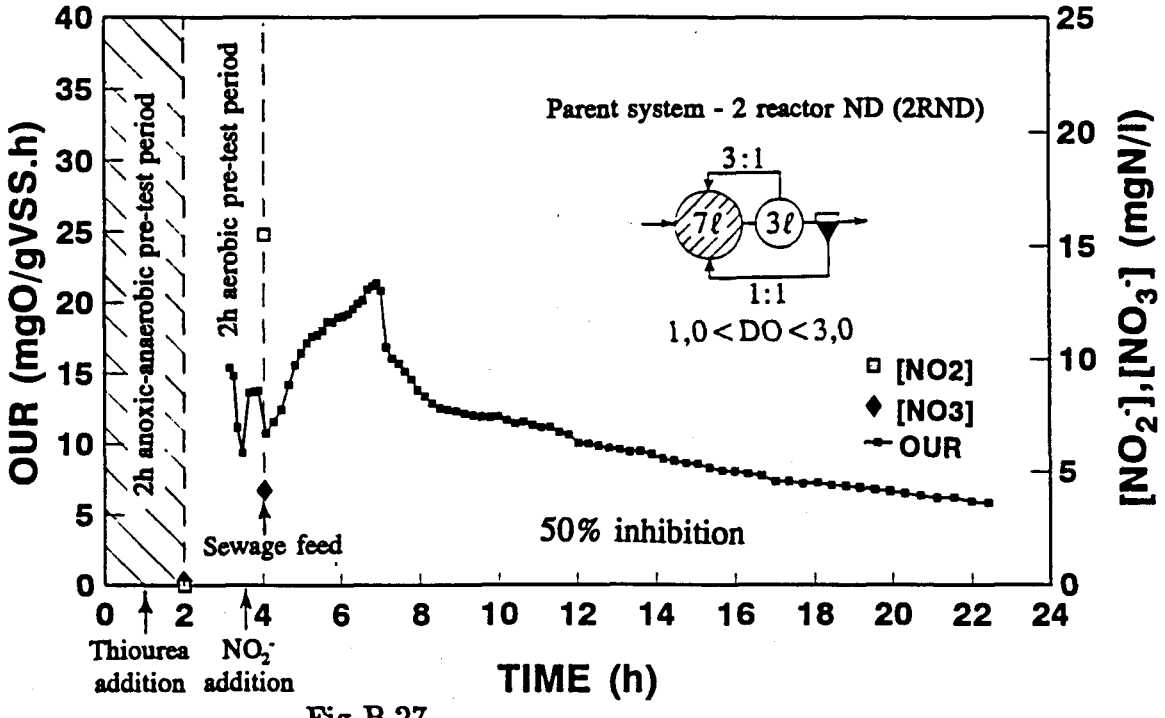
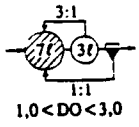


Fig B.27

Table B.50

BATCH TEST 15 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- (> 10 mgN/ℓ) addition under aerobic pre-test conditions 15 minutes before substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 333 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,25 @ t=0;0,75 @ t=4
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	587
VSS	(mg/ℓ)	509
F/M	(mgCOD/mgVSS)	0,6
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	303
TKN	(mgN/ℓ-final batch volume)	30,0

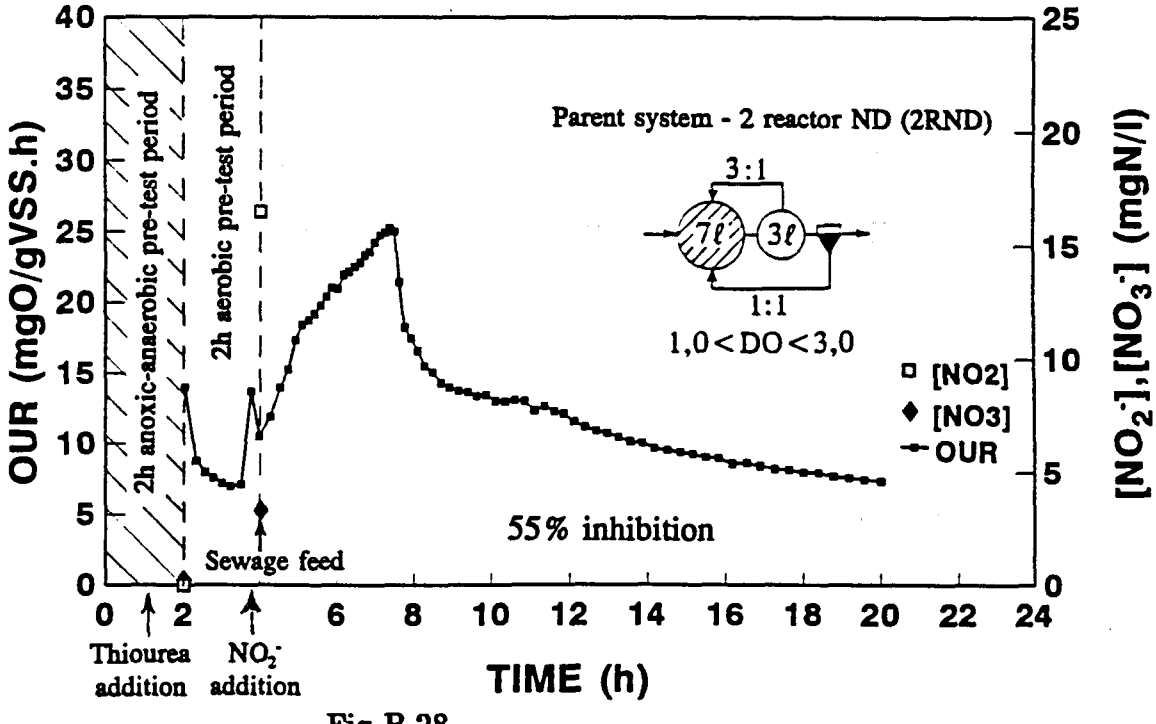
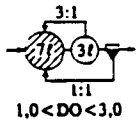


Fig B.28

Table B.51

BATCH TEST 16 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- addition (>10 mgN/ℓ) under aerobic pre-test conditions 5 minutes before substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 334 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	606
VSS	(mg/ℓ)	546
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	403
TKN	(mgN/ℓ-final batch volume)	40,0

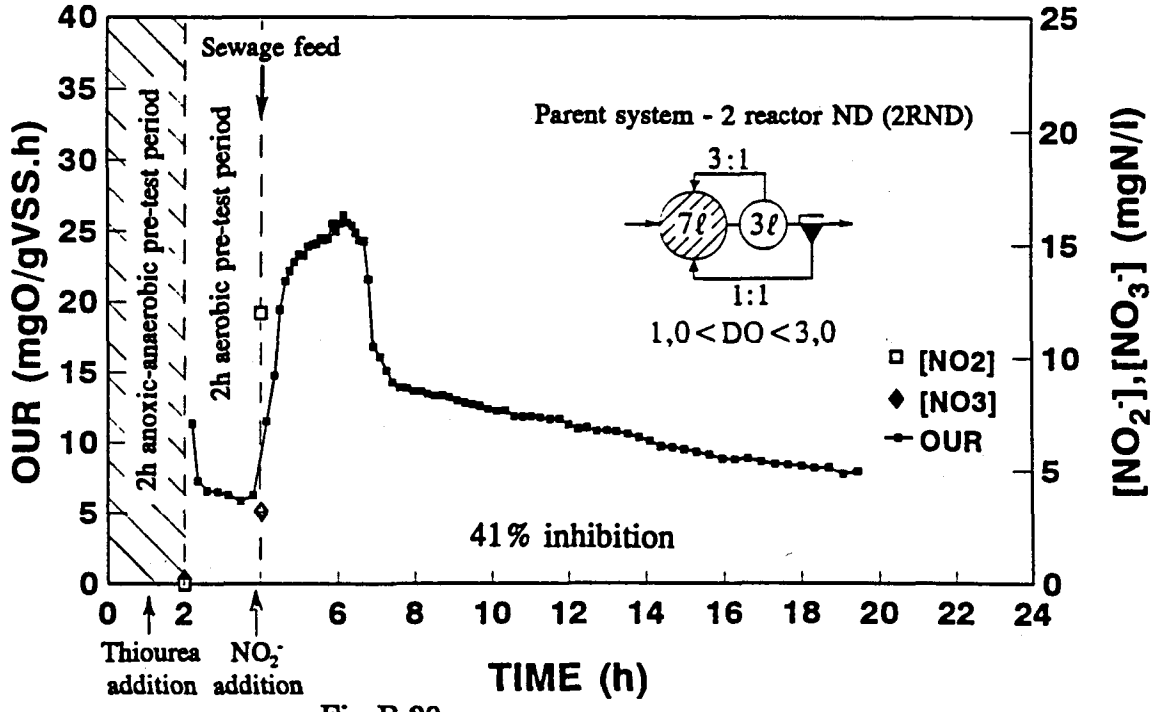
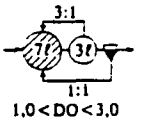


Fig B.29

Table B.52

BATCH TEST 17 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- (>10 mgN/ℓ) addition 60 minutes after substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 335 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	535
VSS	(mg/ℓ)	470
F/M	(mgCOD/mgVSS)	0,9
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	403
TKN	(mgN/ℓ-final batch volume)	39,9

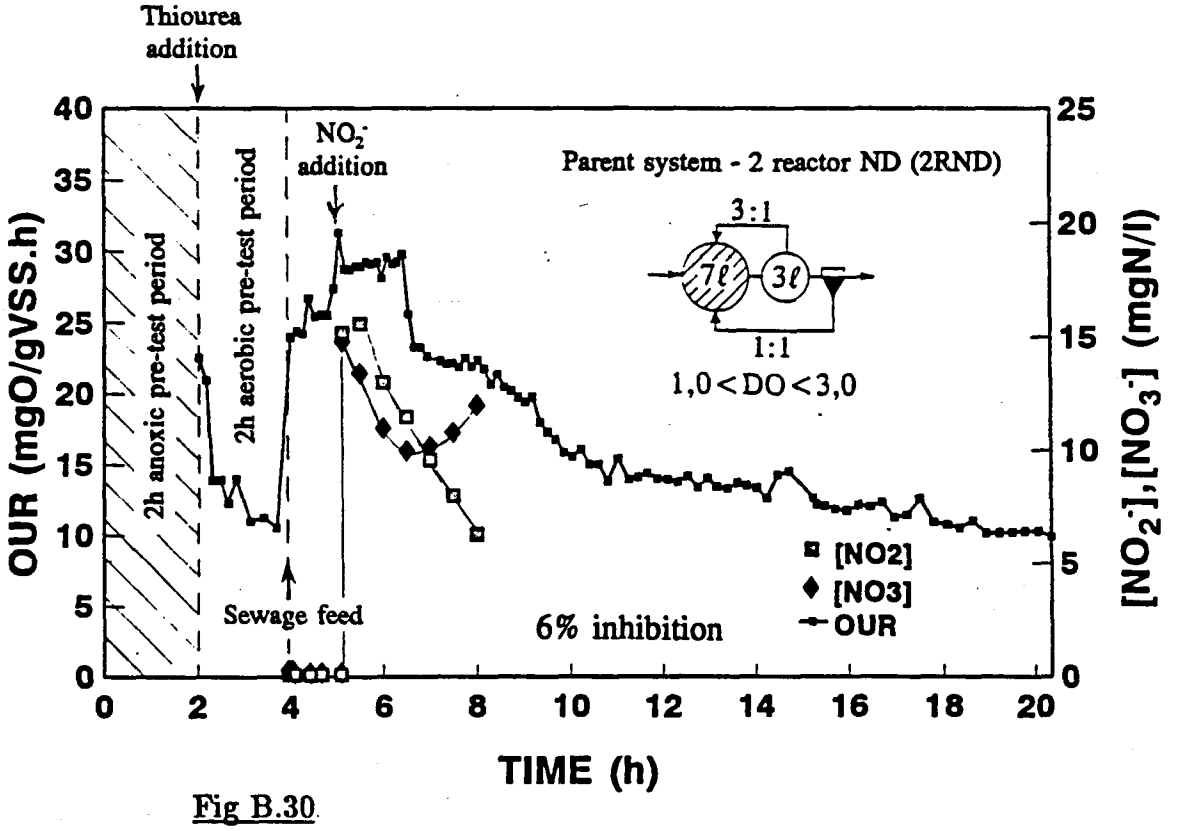
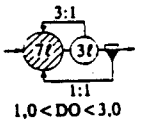


Fig B.30

Table B.53

BATCH TEST 18 OPERATING CONDITIONS		
Objective: To determine OUR response following transfer of sludge directly from parent system, i.e. no anoxic or aerobic pre-test conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 343 System configuration: 2RND (MLE) Sludge age	(d)	
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,2
Sewage volume	(ℓ)	0,8
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	705
VSS	(mg/ℓ)	627
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	339
TKN	(mgN/ℓ-final batch volume)	27,0

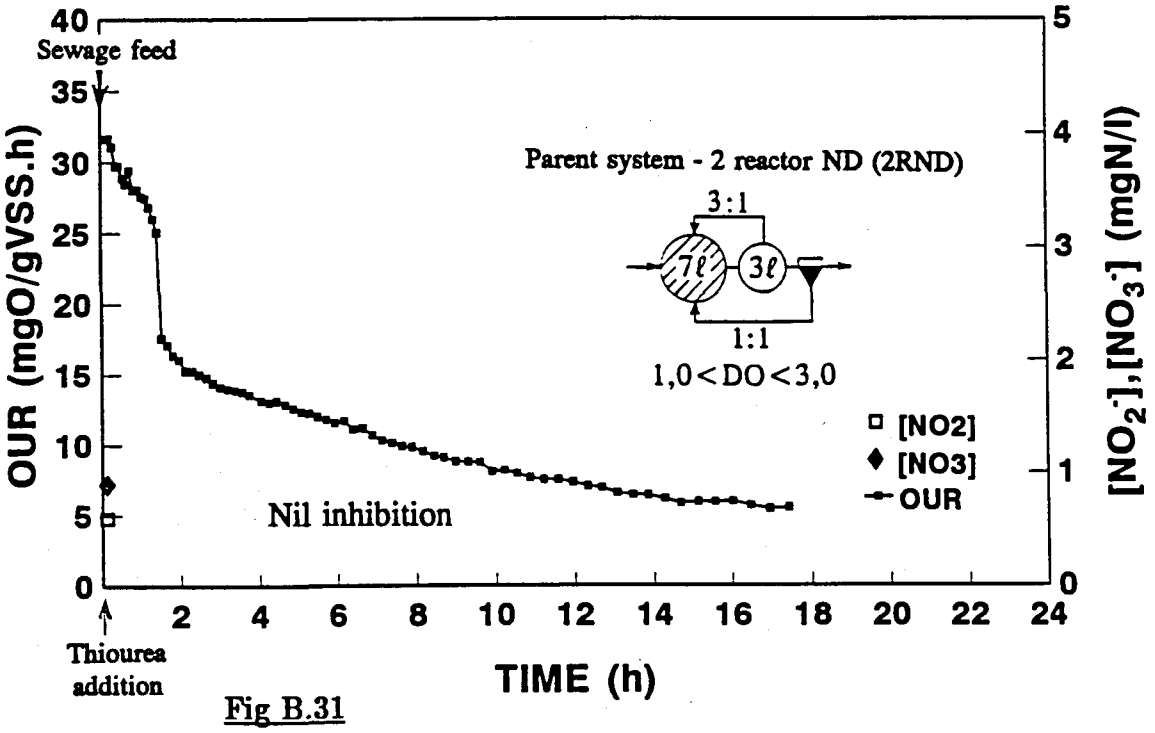
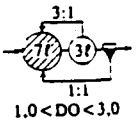


Fig B.31

Table B.54

BATCH TEST 19 OPERATING CONDITIONS		
Objective: To determine OUR response following aerobic pre-test conditions without NO_3^- addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 344 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,2
Sewage volume	(ℓ)	0,8
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	773
VSS	(mg/ℓ)	625
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	339
TKN	(mgN/ℓ-final batch volume)	27,0

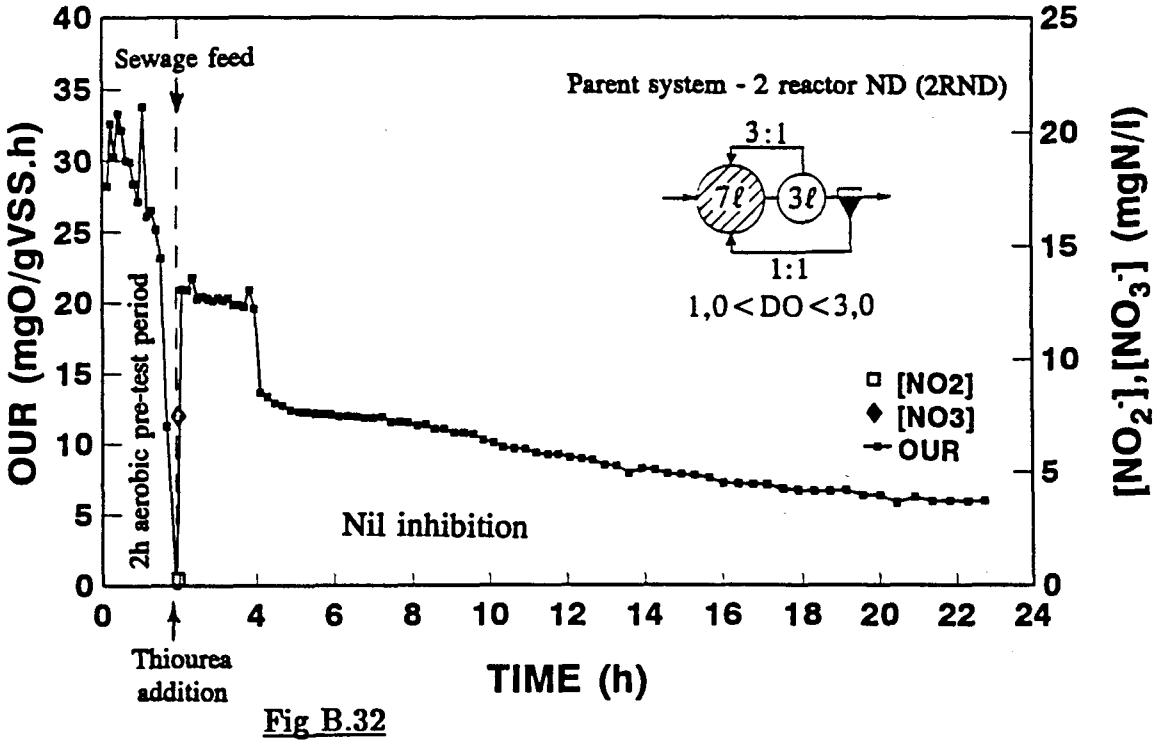
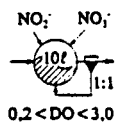


Fig B.32

Table B.55

BATCH TEST 20 OPERATING CONDITIONS		
Objective: To determine OUR response in sludge from IAND configuation following anoxic pre-test period with NO_2^- present.		
PARENT SYSTEM CONDITIONS		
System 5; Day 300 System configuration IAND		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	503
VSS	(mg/ℓ)	435
F/M	(mgCOD/mgVSS)	1,0
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	729
TKN	(mgN/ℓ-final batch volume)	41,1

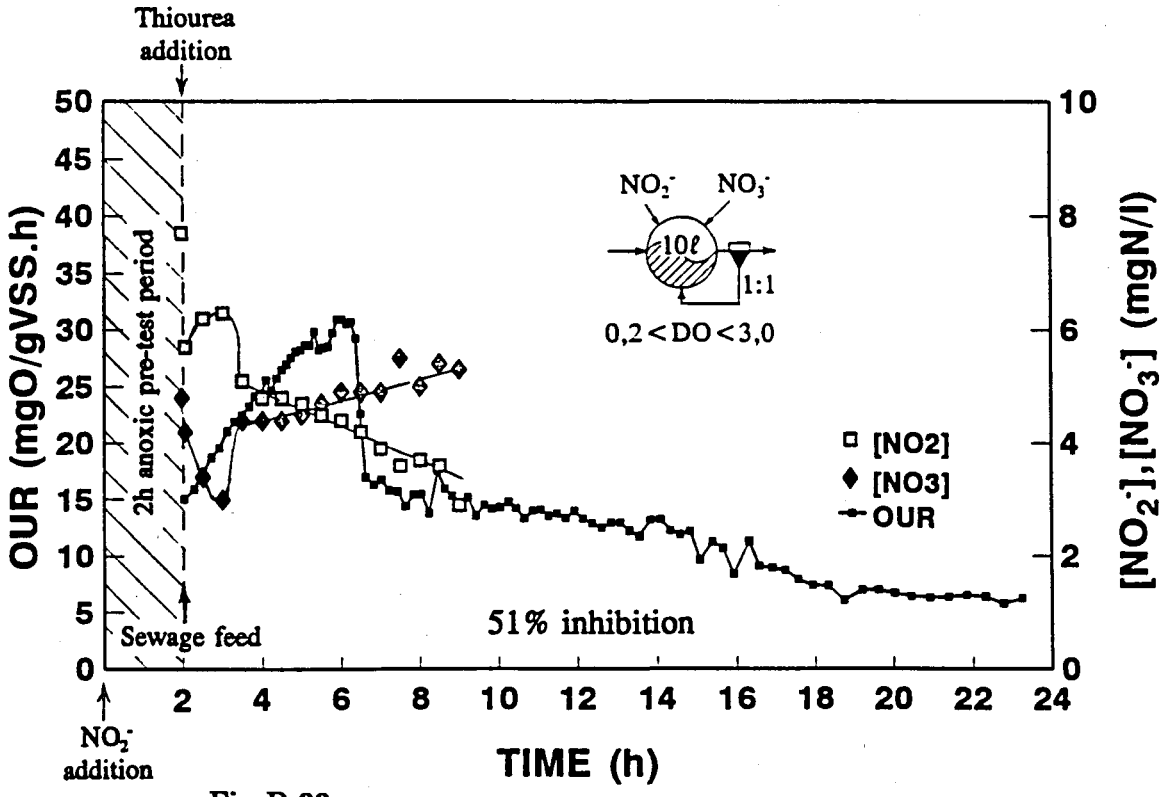
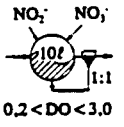


Fig B.33

Table B.56

BATCH TEST 21 OPERATING CONDITIONS		
Objective: To determine OUR response with NO_3^- absent at the end of anoxic-aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 5; Day 306 System configuration: IAND		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,25 @ t=0 ; 0,75@t=2
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	638
VSS	(mg/ℓ)	622
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	322
TKN	(mgN/ℓ-final batch volume)	30,8

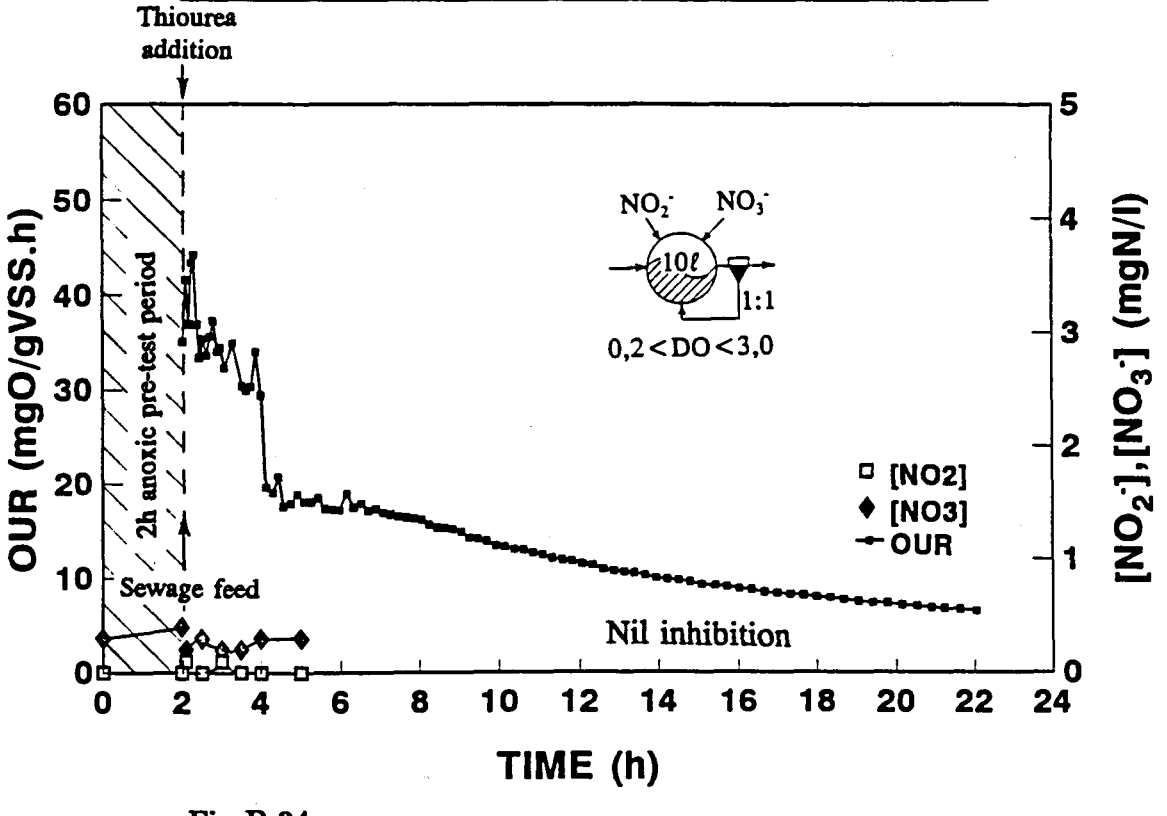
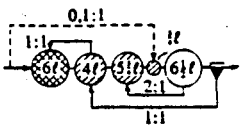


Fig B.34

Table B.57

BATCH TEST 22 OPERATING CONDITIONS		
Objective: To determine OUR response following direct transfer of sludge from 2nd anoxic reactor of parent system to batch test; i.e. anoxic or aerobic pre-test conditions.		
PARENT SYSTEM CONDITIONS		
System 17; Day 73 System configuration: NDBEPR (MUCT)		
Sludge age	(d)	20
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,6
Water volume	(ℓ)	1,4
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	740
VSS	(mg/ℓ)	627
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2,0
Temperature	(°C)	2 - 5 20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	423
TKN	(mgN/ℓ-final batch volume)	33,8

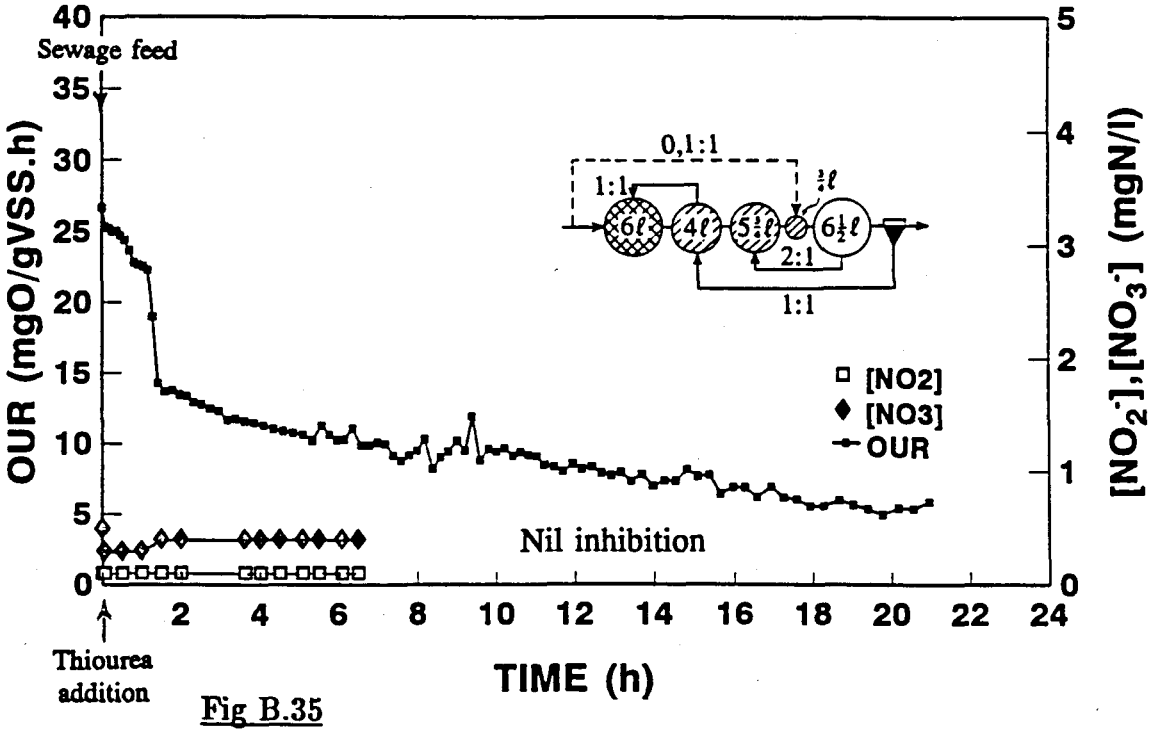
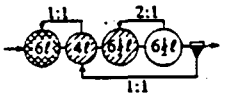


Fig B.35

Table B.58

BATCH TEST 23 OPERATING CONDITIONS		
Objective: To determine OUR response following direct transfer of sludge from aerobic reactor of parent system to batch test; i.e. no anoxic or aerobic pre-test conditions.		
PARENT SYSTEM CONDITIONS		
System 17; Day 82 System configuration: NDBEPR (MUCT)		
Sludge age	(d)	20
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,5
Water volume	(ℓ)	1,5
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	651
VSS	(mg/ℓ)	524
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	423
TKN	(mgN/ℓ-final batch volume)	33,8

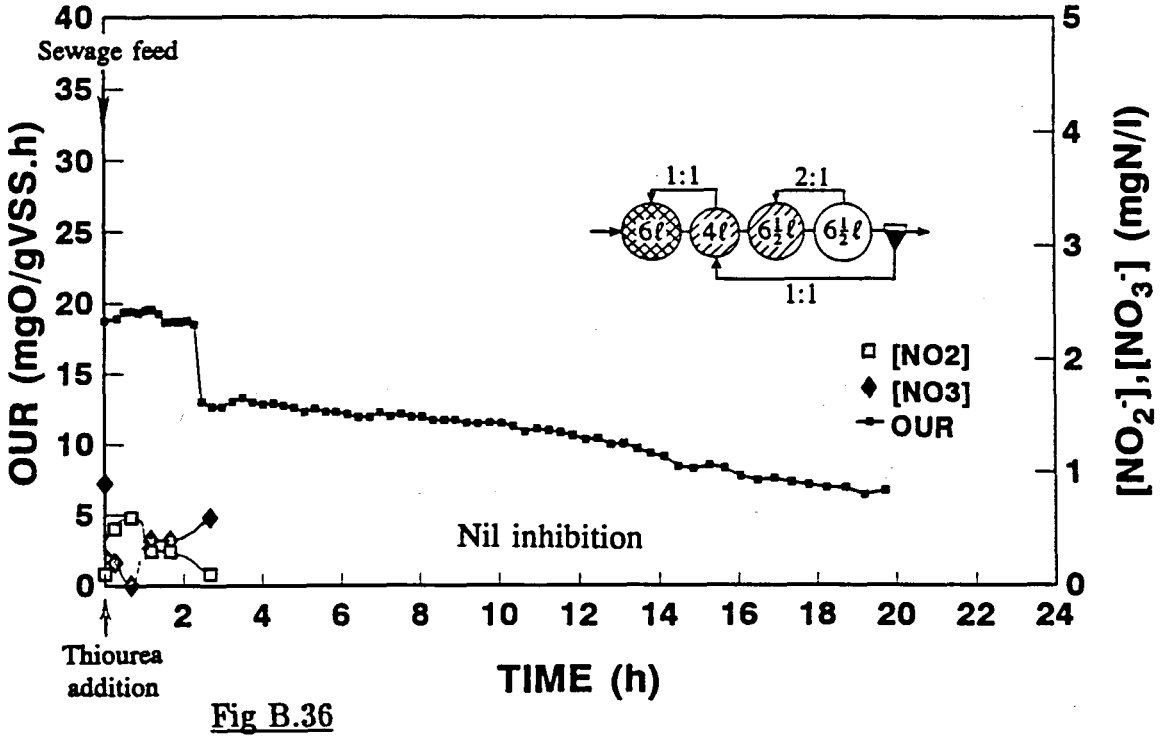
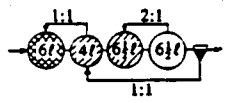


Fig B.36

Table B.59

BATCH TEST 24 OPERATING CONDITIONS		
Objective: To determine OUR response on sludge from the aerobic reactor following aerobic pre-test conditions with NO_2^- present.		
PARENT SYSTEM CONDITIONS		
System 17; Day 84 System configuration: NDBEPR (MUCT)		
Sludge age	(d)	20
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,5
Water volume	(ℓ)	1,5
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	707
VSS	(mg/ℓ)	655
F/M	(mgCOD/mgVSS)	0,6
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	423
TKN	(mgN/ℓ-final batch volume)	33,8

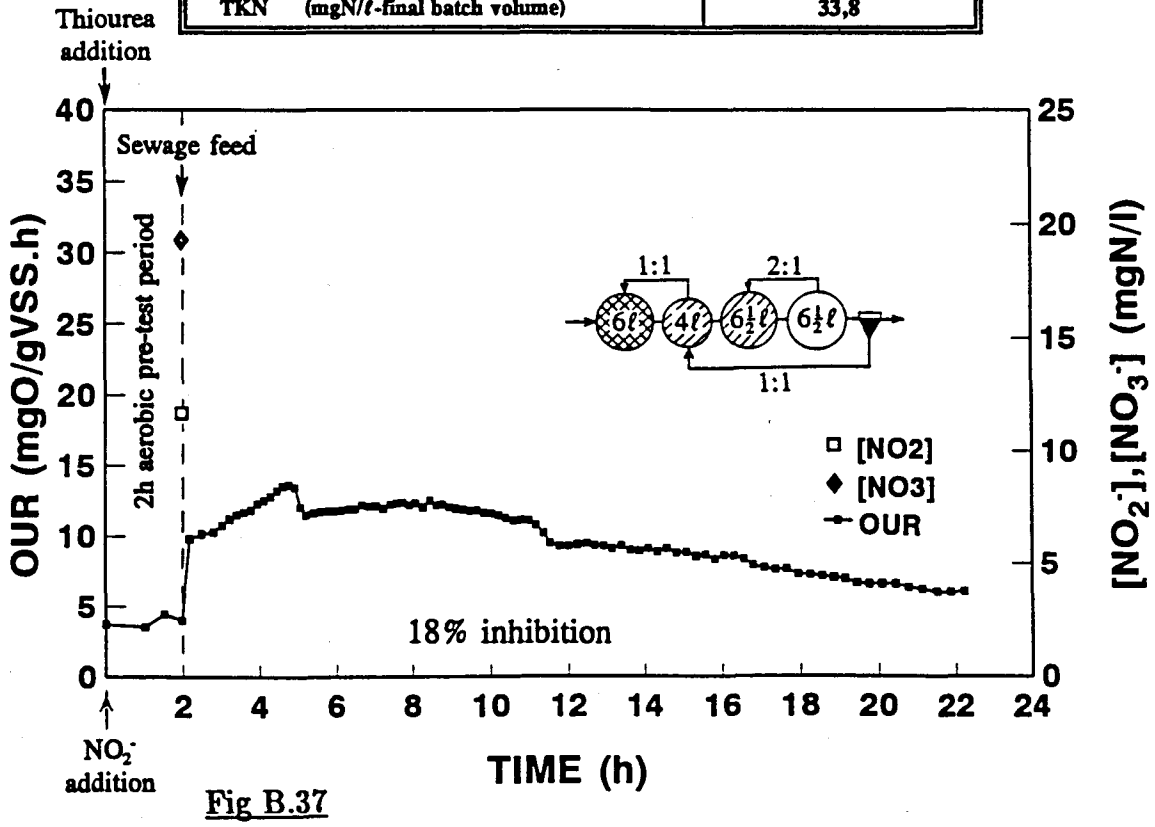
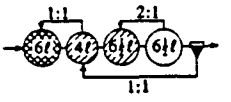


Fig B.37

Table B.60

BATCH TEST 25 OPERATING CONDITIONS		
Objective: To determine OUR response or sludge from the 2nd anoxic reactor following aerobic pre-test period with NO_3^- present		
PARENT SYSTEM CONDITIONS		
System 17; Day 85 System configuration: NDBEPR (MUCT)		
Sludge age	(d)	20
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,5
Water volume	(ℓ)	1,5
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	657
VSS	(mg/ℓ)	570
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	463
TKN	(mgN/ℓ-final batch volume)	40,9

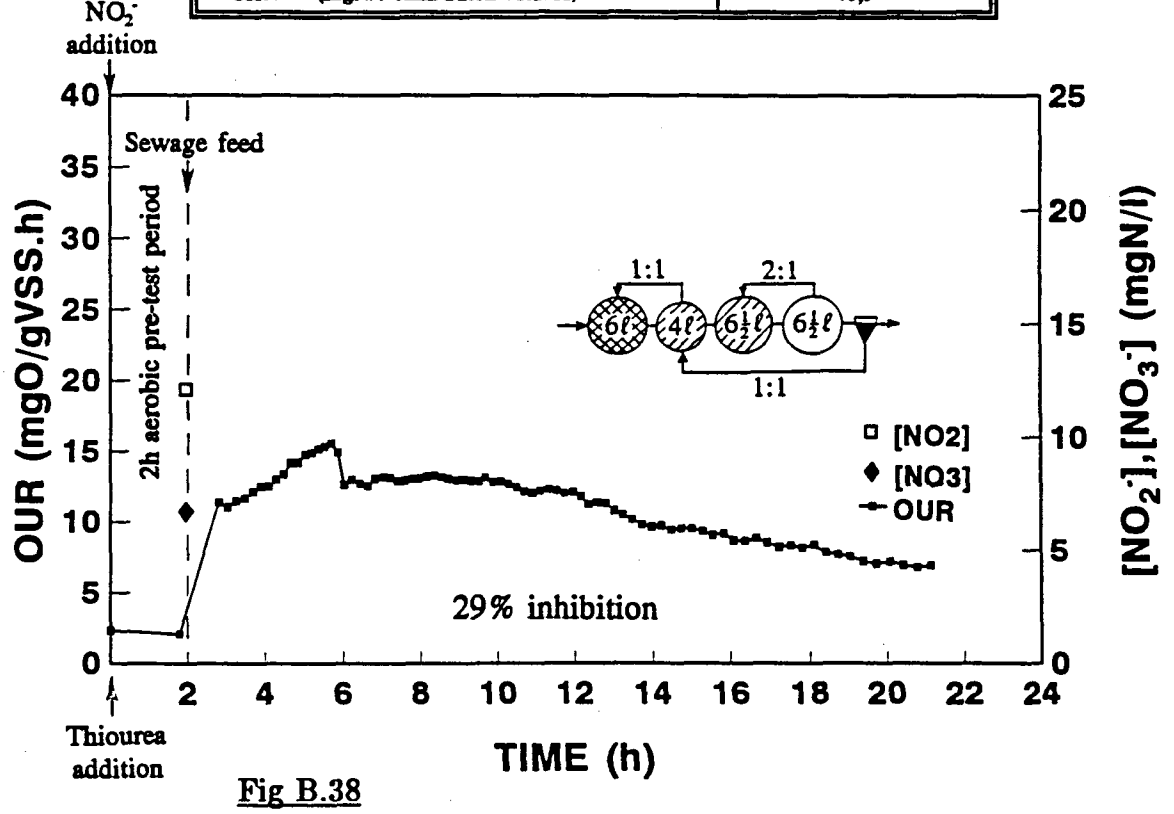


Fig B.38

Table B.61

BATCH TEST A OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 258 System configuration: continuous aerobic		10ℓ 1:1 0,2 < DO < 2,0
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,4
Sewage volume	(ℓ)	0,8
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	632
VSS	(mg/ℓ)	576
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	385
TKN	(mgN/ℓ-final batch volume)	39,3

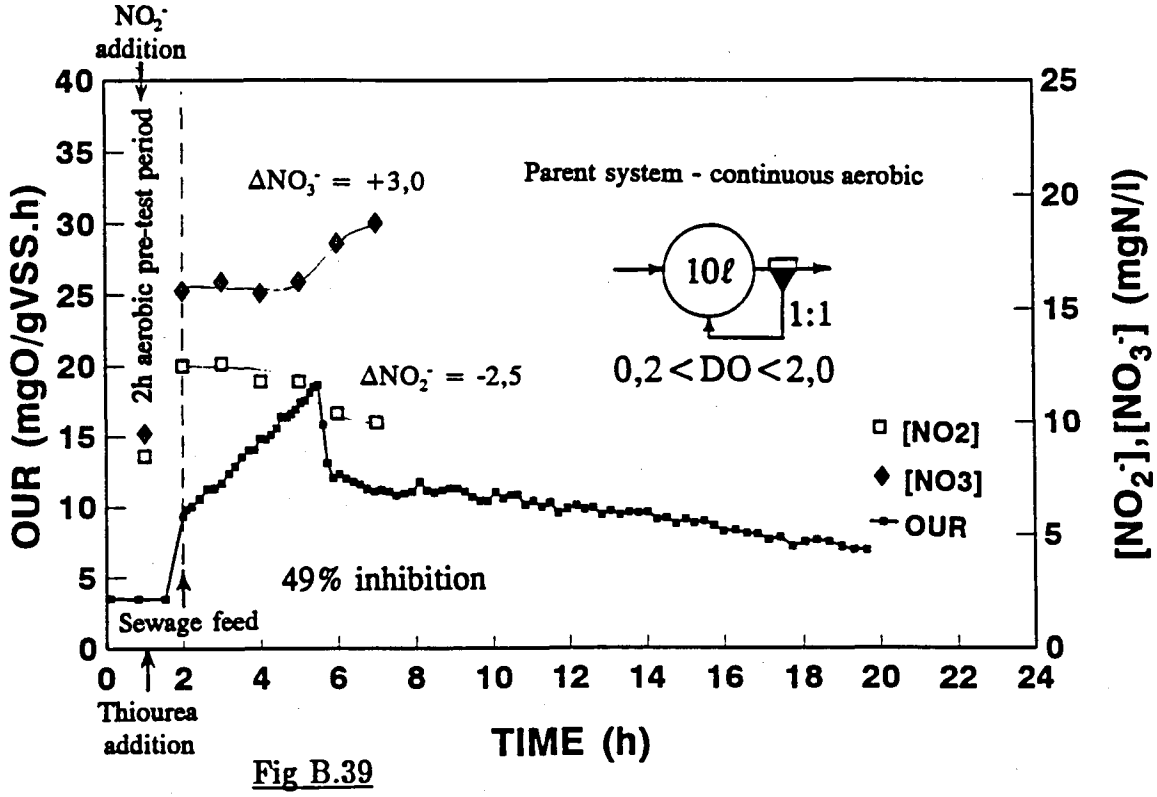


Fig B.39

Table B.62

BATCH TEST B OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 265 System configuration: continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	419
VSS	(mg/ℓ)	318
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	0,5
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	243
TKN	(mgN/ℓ-final batch volume)	31,1

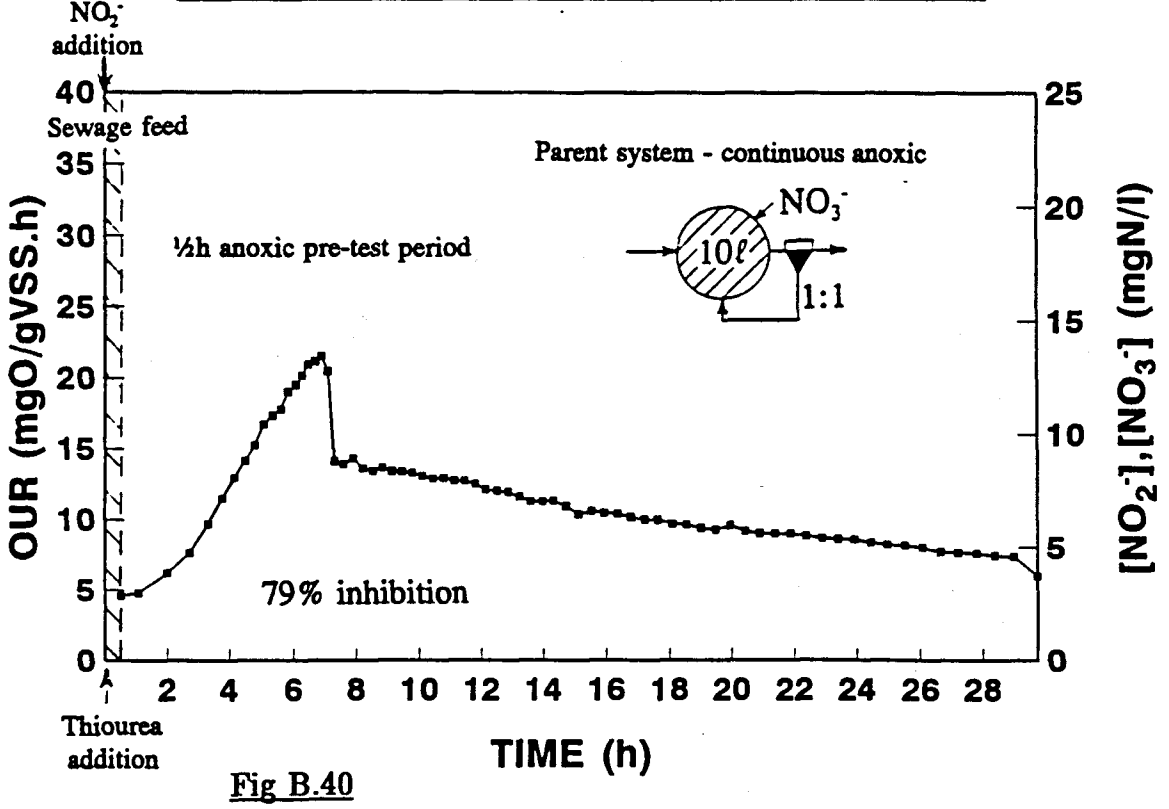
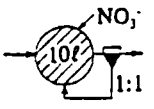


Fig B.40

Table B.63

BATCH TEST C OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 269 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,4
Sewage volume	(ℓ)	0,8
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	426
VSS	(mg/ℓ)	369
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	301
TKN	(mgN/ℓ-final batch volume)	27,9

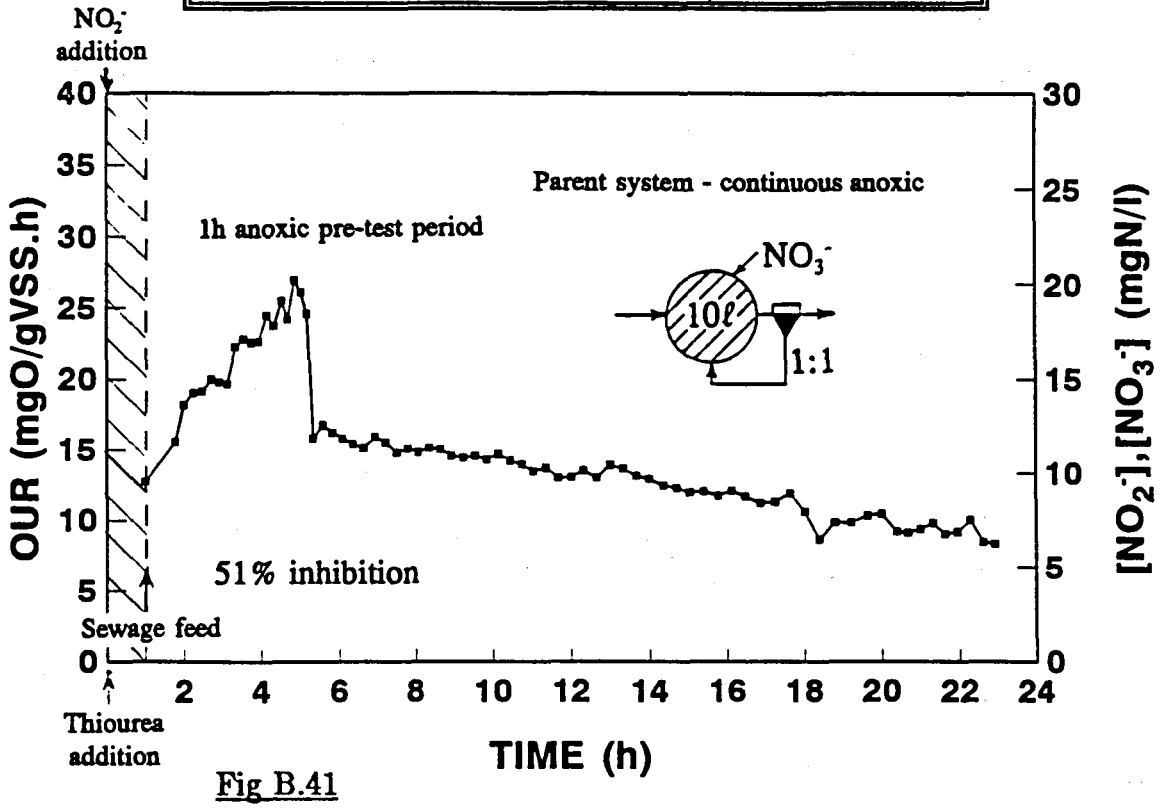
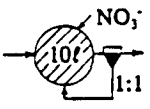


Fig B.41

Table B.64

BATCH TEST D OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 276 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,1
Water volume	(ℓ)	1,1
Sewage volume	(ℓ)	0,8
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	449
VSS	(mg/ℓ)	369
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	301
TKN	(mgN/ℓ-final batch volume)	27,9

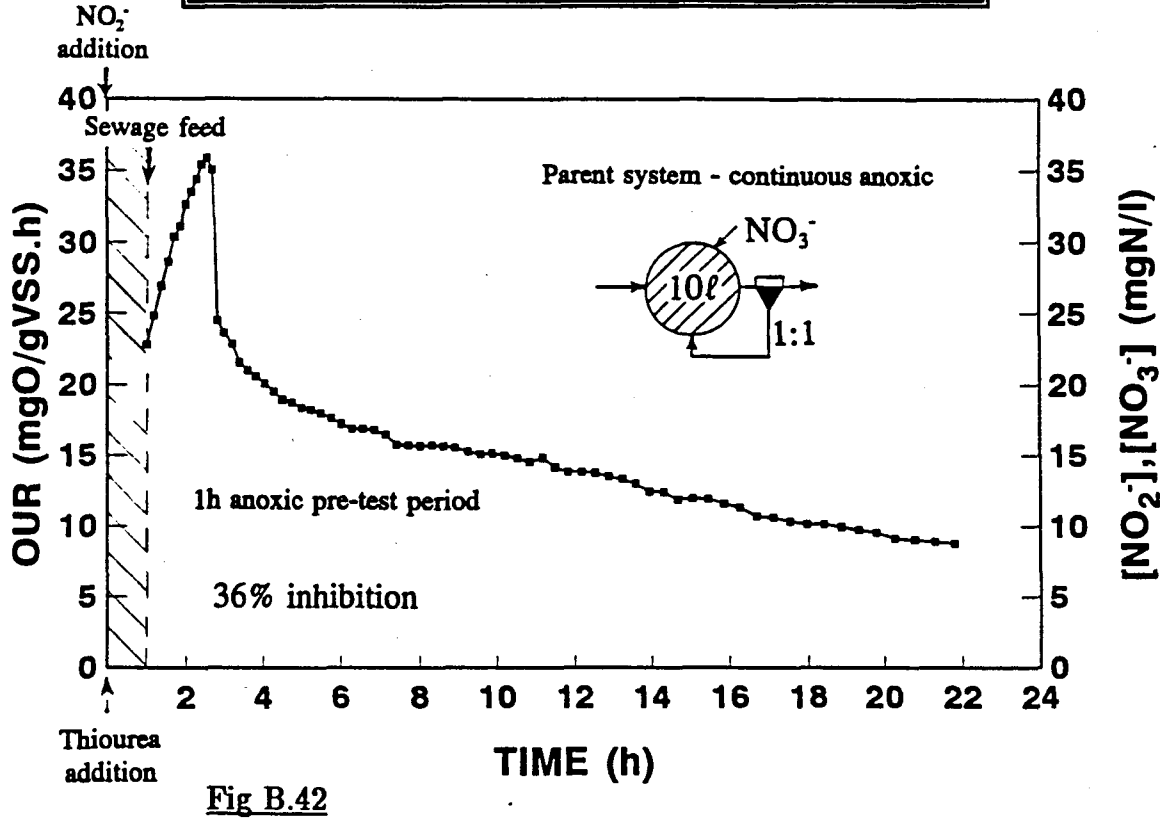


Fig B.42

Table B.65

BATCH TEST E OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 284 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,1
Sewage volume	(ℓ)	0,9
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	565
VSS	(mg/ℓ)	444
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	353
TKN	(mgN/ℓ-final batch volume)	30,1

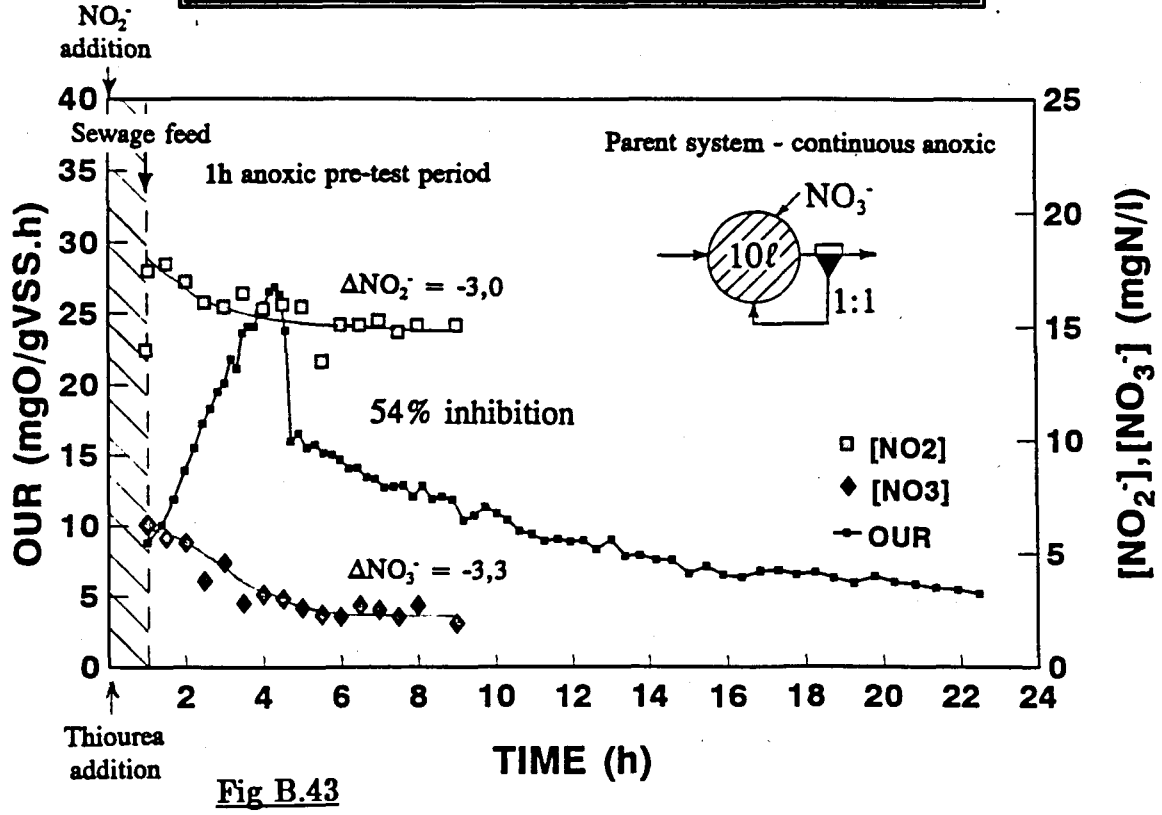
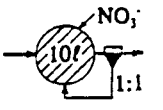


Fig B.43

Table B.66

BATCH TEST F OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 293 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	564
VSS	(mg/ℓ)	474
F/M	(mgCOD/mgVSS)	0,9
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	429
TKN	(mgN/ℓ-final batch volume)	41,1

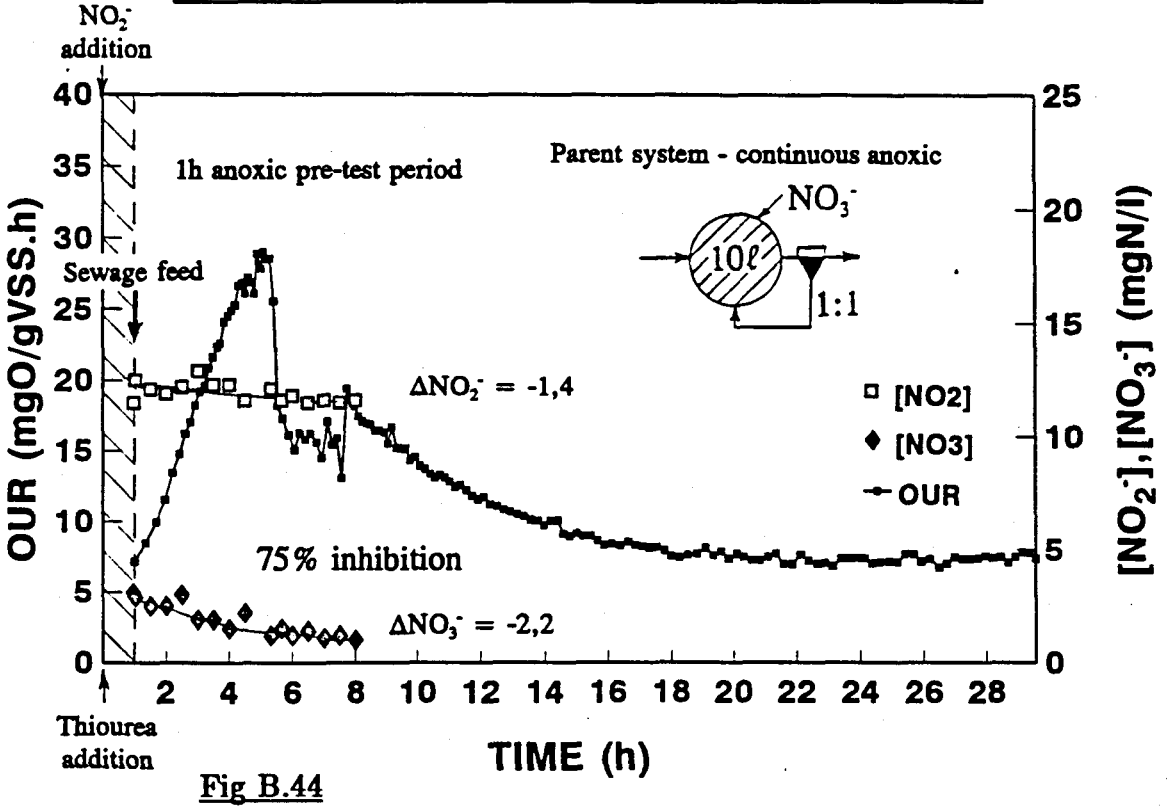
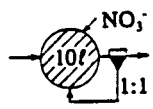


Fig B.44

Table B.67

BATCH TEST G OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 304 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	366
VSS	(mg/ℓ)	349
F/M	(mgCOD/mgVSS)	1,2
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	409
TKN	(mgN/ℓ-final batch volume)	39,1

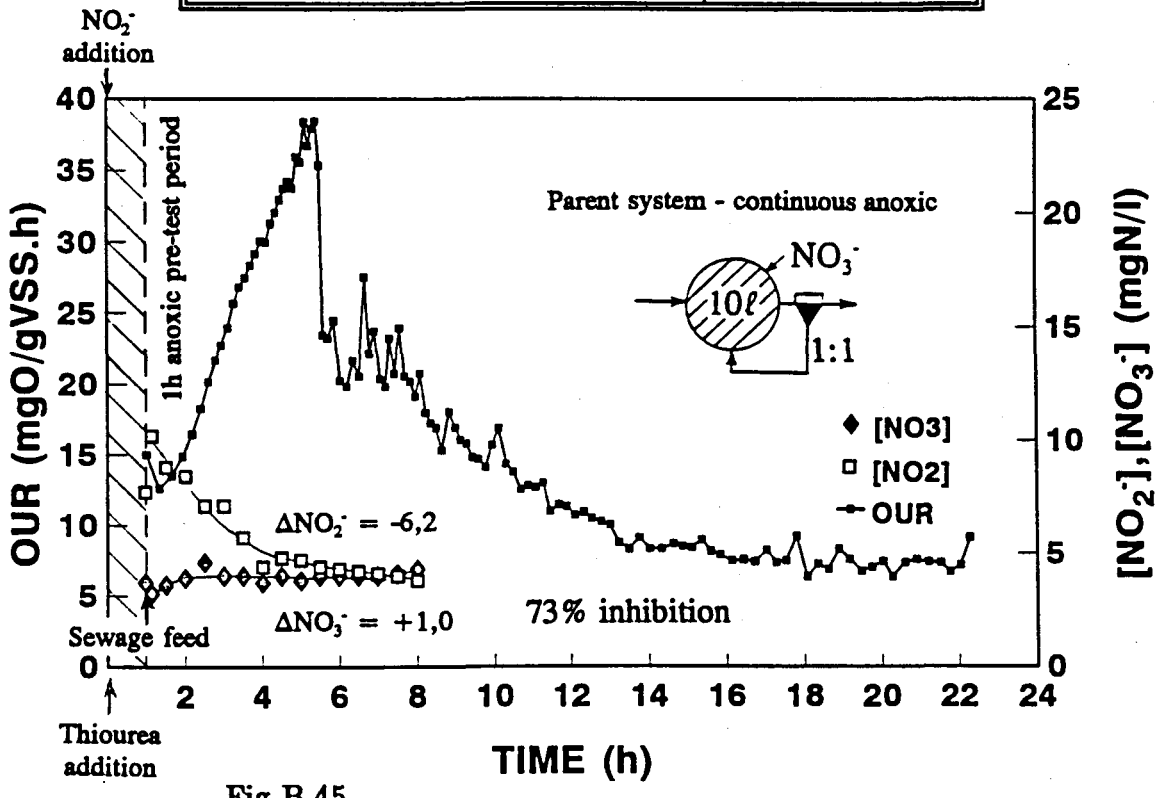


Fig B.45

Table B.68

BATCH TEST H OPERATING CONDITIONS		
Objective: To examine the effect of *aerobic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 319 System configuration: Continuous aerobic		10ℓ 1:1 0,2 < DO < 2.0
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	512
VSS	(mg/ℓ)	446
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	363
TKN	(mgN/ℓ-final batch volume)	29,2

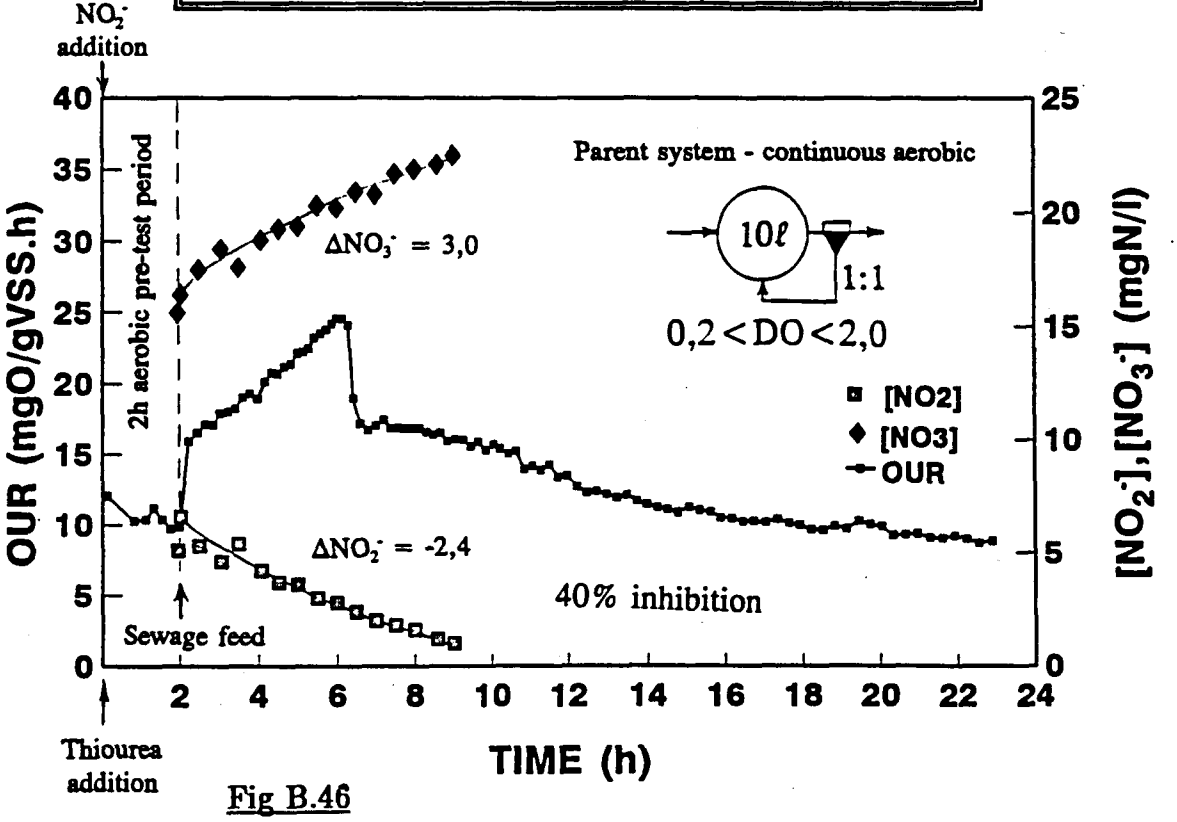


Fig B.46

Table B.69

BATCH TEST I OPERATING CONDITIONS		
Objective: To examine the effect of *aerobic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 333 System configuration: Continuous aerobic		10ℓ 1:1 0.2 < DO < 2.0
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,6
Water volume	(ℓ)	0,4
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	596
VSS	(mg/ℓ)	447
F/M	(mgCOD/mgVSS)	1,0
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	436
TKN	(mgN/ℓ-final batch volume)	31,2

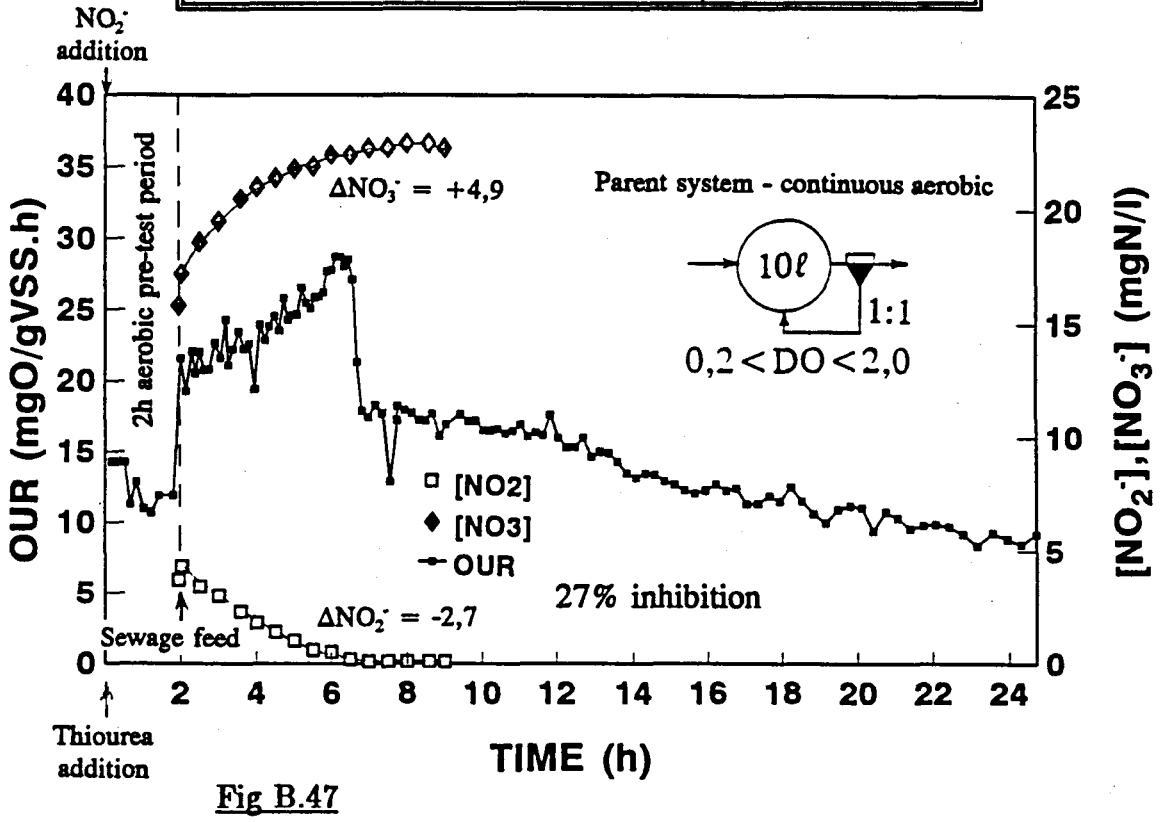
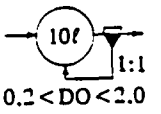


Fig B.47

Table B.70

BATCH TEST J OPERATING CONDITIONS		
Objective: To examine the effect of *aerobic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 343 System configuration: Continuous aerobic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,2
Water volume	(ℓ)	0,8
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	650
VSS	(mg/ℓ)	466
F/M	(mgCOD/mgVSS)	1,0
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	473
TKN	(mgN/ℓ-final batch volume)	36,8

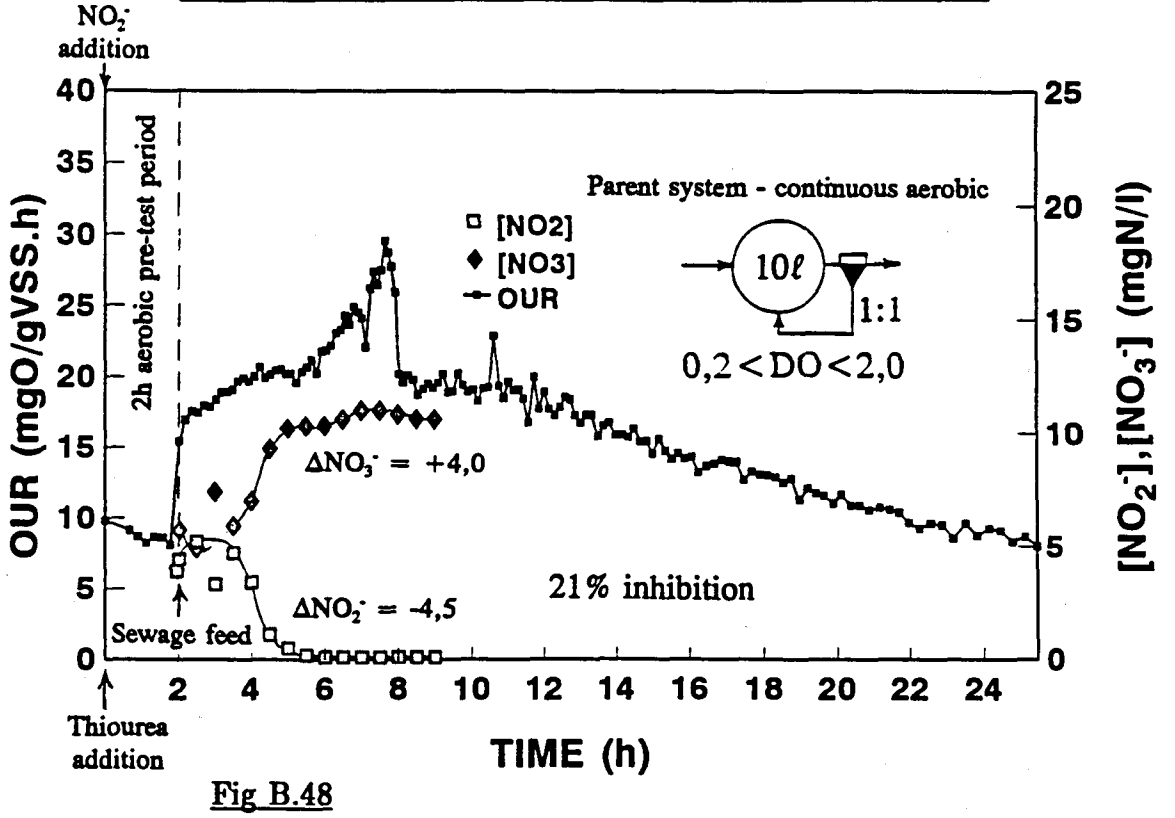


Fig B.48

Table B.71

BATCH TEST K OPERATING CONDITIONS		
Objective: To examine the effect of *aerobic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 353 System configuration: Continuous aerobic		10ℓ 1:1 0,2 < DO < 2,0
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	449
VSS	(mg/ℓ)	393
F/M	(mgCOD/mgVSS)	1,2
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	473
TKN	(mgN/ℓ-final batch volume)	36,8

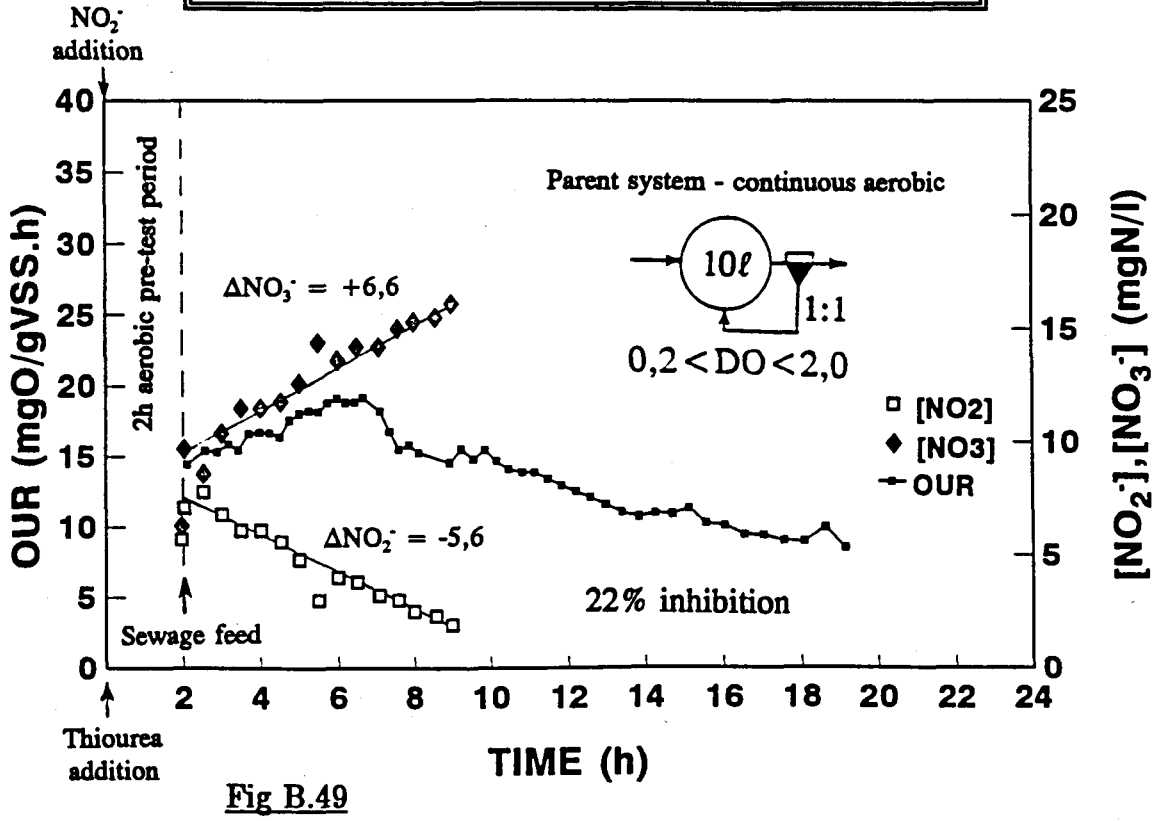
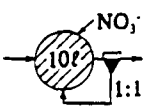


Fig B.49

Table B.72

BATCH TEST L OPERATING CONDITIONS		
Objective: To examine the effect of *aerobic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 353,5 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,7
Water volume	(ℓ)	1,3
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	449
VSS	(mg/ℓ)	382
F/M	(mgCOD/mgVSS)	1,2
Anoxic pre-test period	(h)	8,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	473
TKN	(mgN/ℓ-final batch volume)	36,8

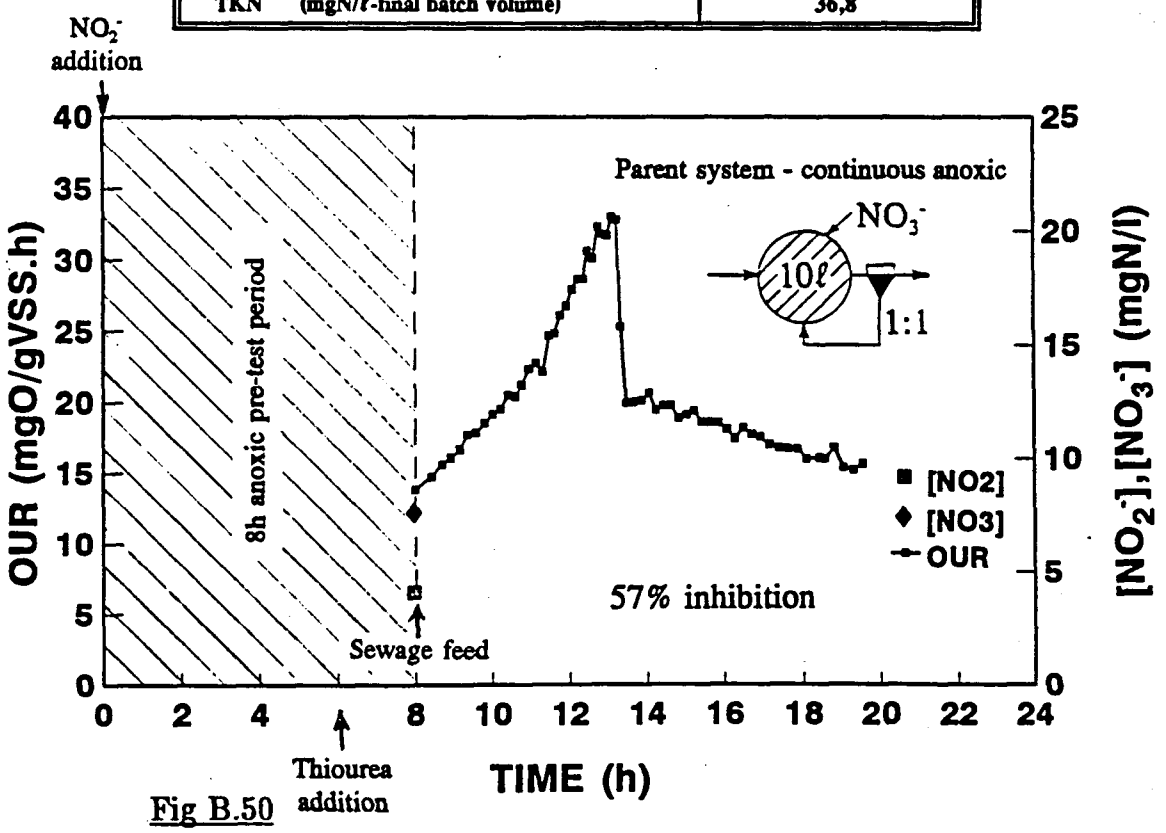


Fig B.50

Table B.73

BATCH TEST M OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 354 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	496
VSS	(mg/ℓ)	444
F/M	(mgCOD/mgVSS)	1,1
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	473
TKN	(mgN/ℓ-final batch volume)	36,8

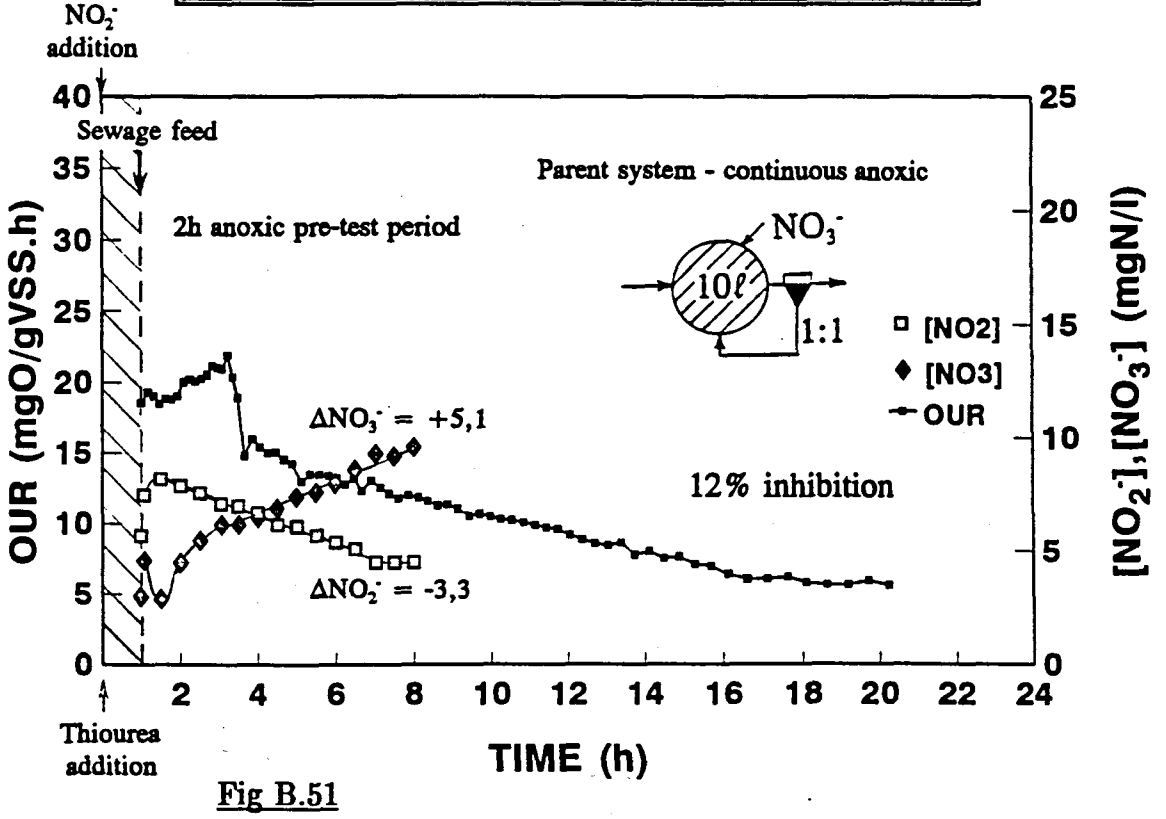


Fig B.51

Table B.74

BATCH TEST N OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 355 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	649
VSS	(mg/ℓ)	488
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	8,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	403
TKN	(mgN/ℓ-final batch volume)	39,9

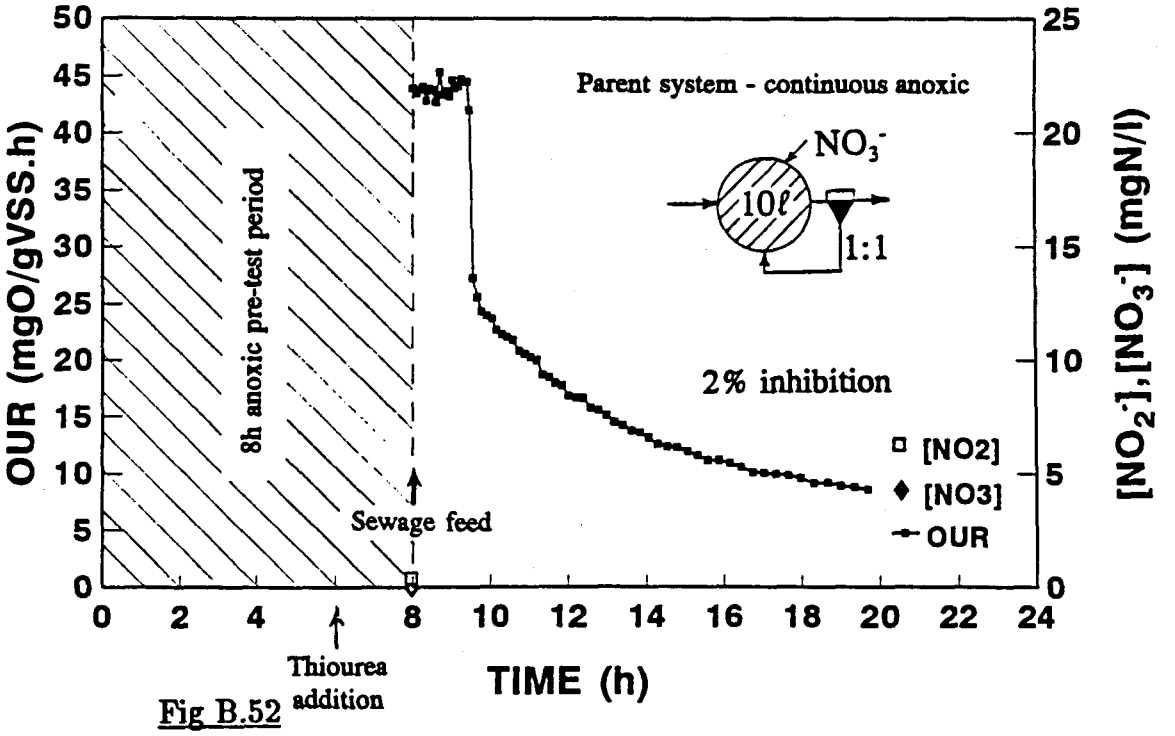
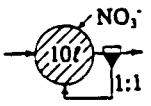


Fig B.52

Table B.75

BATCH TEST O OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 356 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,2
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,8
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	427
VSS	(mg/ℓ)	388
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	1,0
DO	(mgO/ℓ)	-
Temperature	(°C)	2 - 5 20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	323
TKN	(mgN/ℓ-final batch volume)	31,9

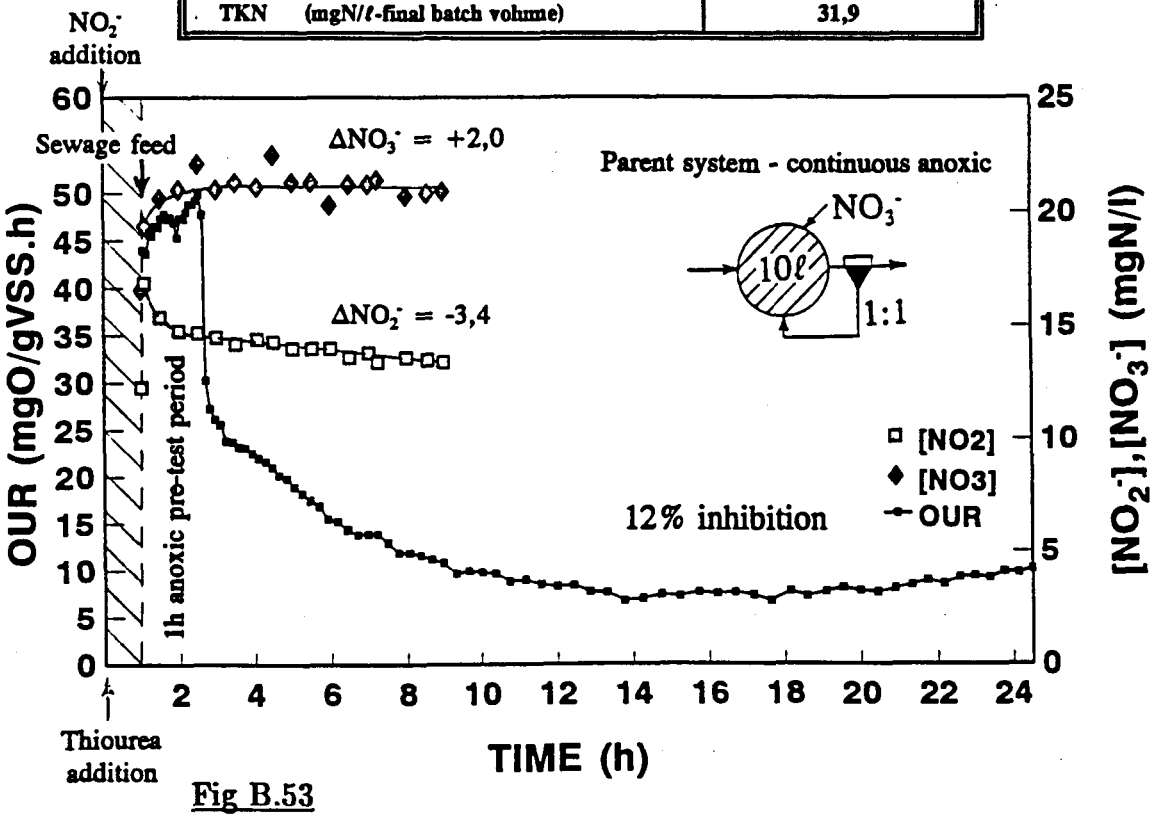
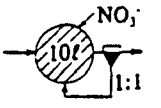


Fig B.53

Table B.76

BATCH TEST P OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 363 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,2
Water volume	(ℓ)	0,8
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	579
VSS	(mg/ℓ)	478
F/M	(mgCOD/mgVSS)	0,9
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	423
TKN	(mgN/ℓ-final batch volume)	33,8

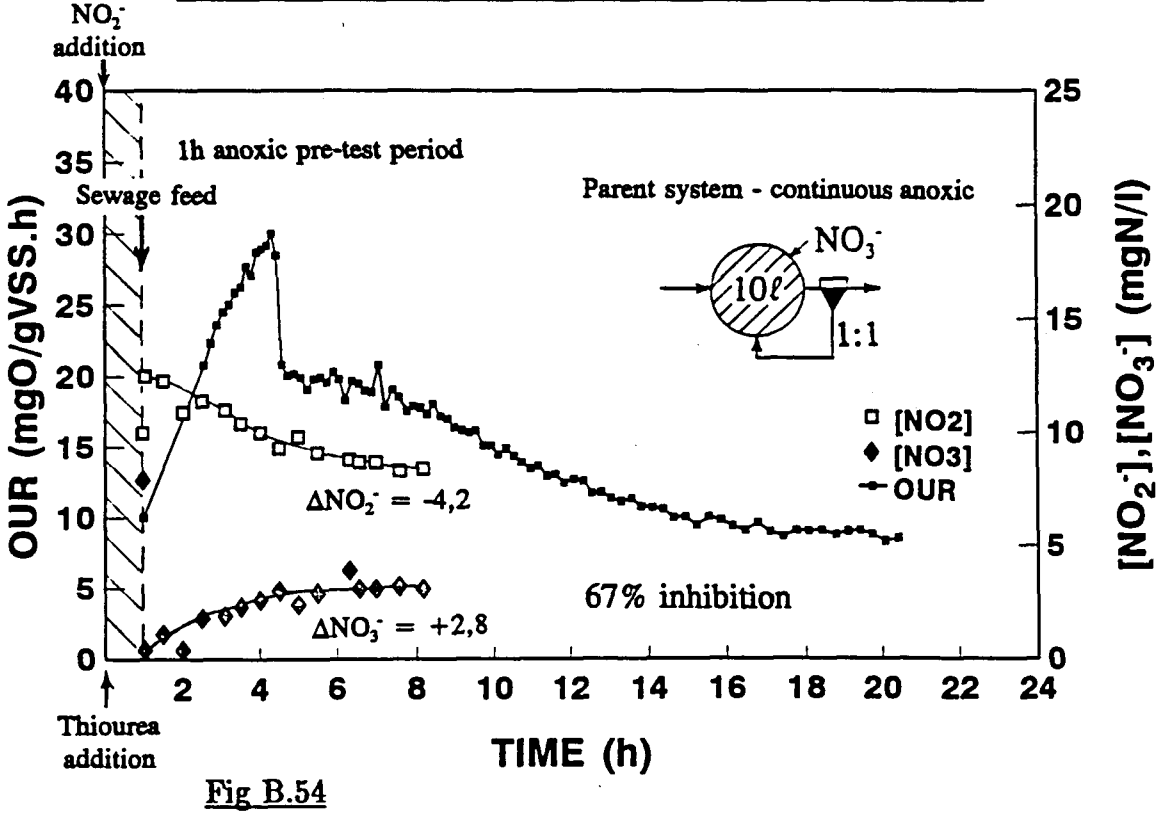
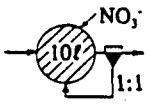


Fig B.54

Table B.77

BATCH TEST Q OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 373 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,5
Water volume	(ℓ)	0,5
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	696
VSS	(mg/ℓ)	581
F/M	(mgCOD/mgVSS)	0,9
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	423
TKN	(mgN/ℓ-final batch volume)	33,8

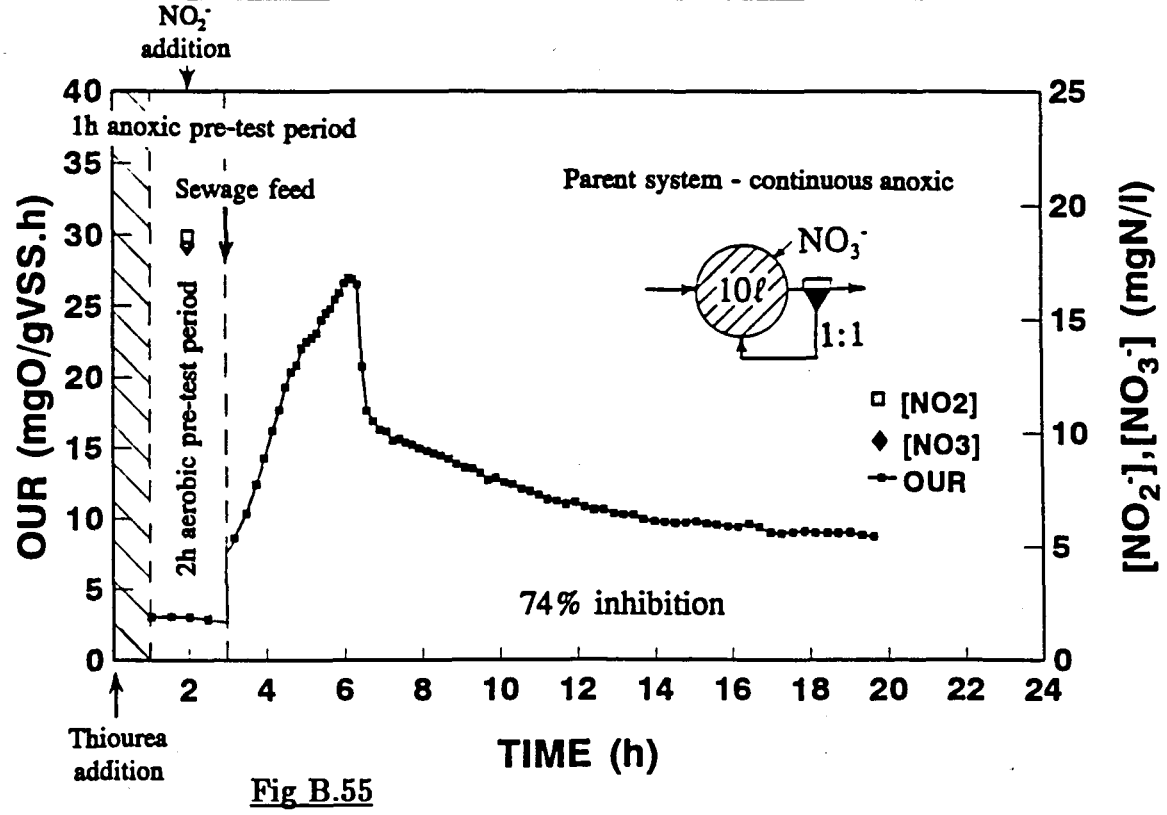


Fig B.55

Table B.78

BATCH TEST R OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 373 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,5
Water volume	(ℓ)	0,5
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	731
VSS	(mg/ℓ)	541
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	423
TKN	(mgN/ℓ-final batch volume)	33,8

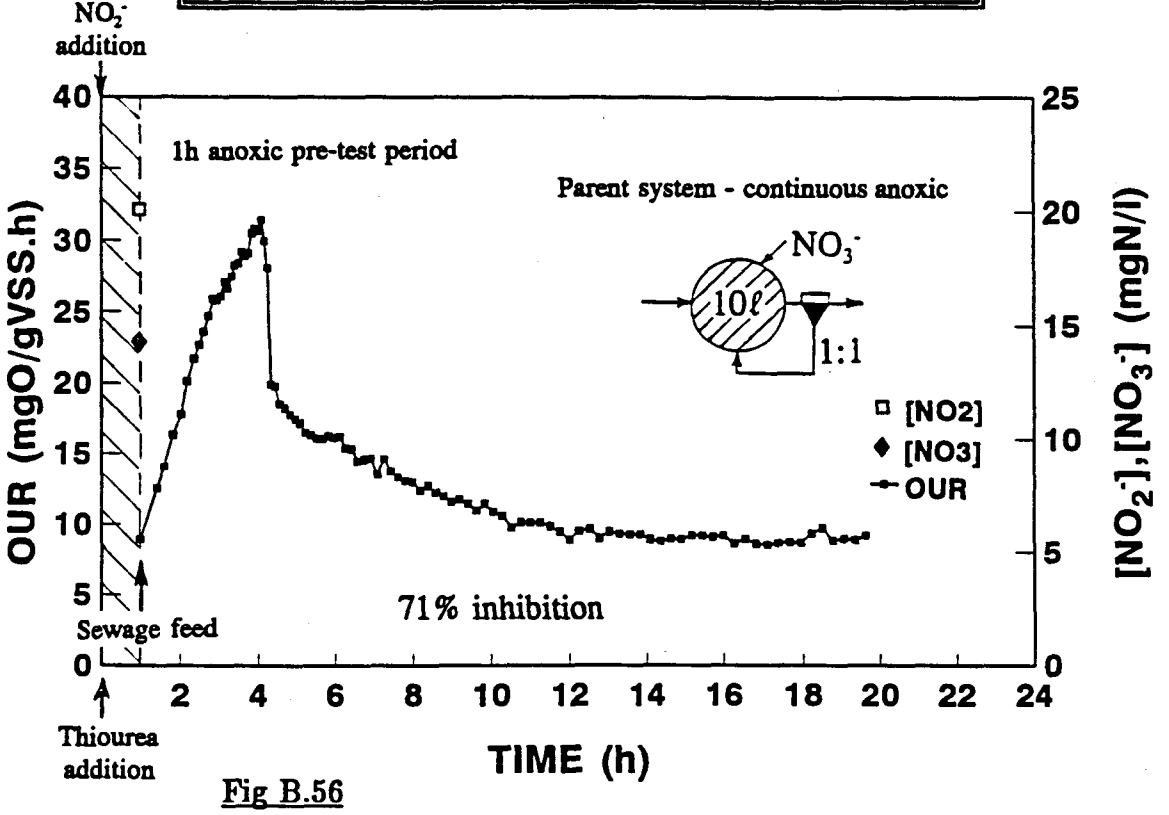
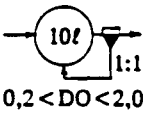


Fig B.56

Table B.79

DENITRIFICATION BATCH TEST 1 OPERATING CONDITIONS		
Objective: To determine the effect on the denitrification rate of a long period of exposure to aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 7 ; Day 350 System configuration: Continuous aerobic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	2,3
Water volume	(ℓ)	0,3
Sewage volume	(ℓ)	0,7
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	1237
VSS	(mg/ℓ)	1041
F/M	(mgCOD/mgVSS	0,3
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	327

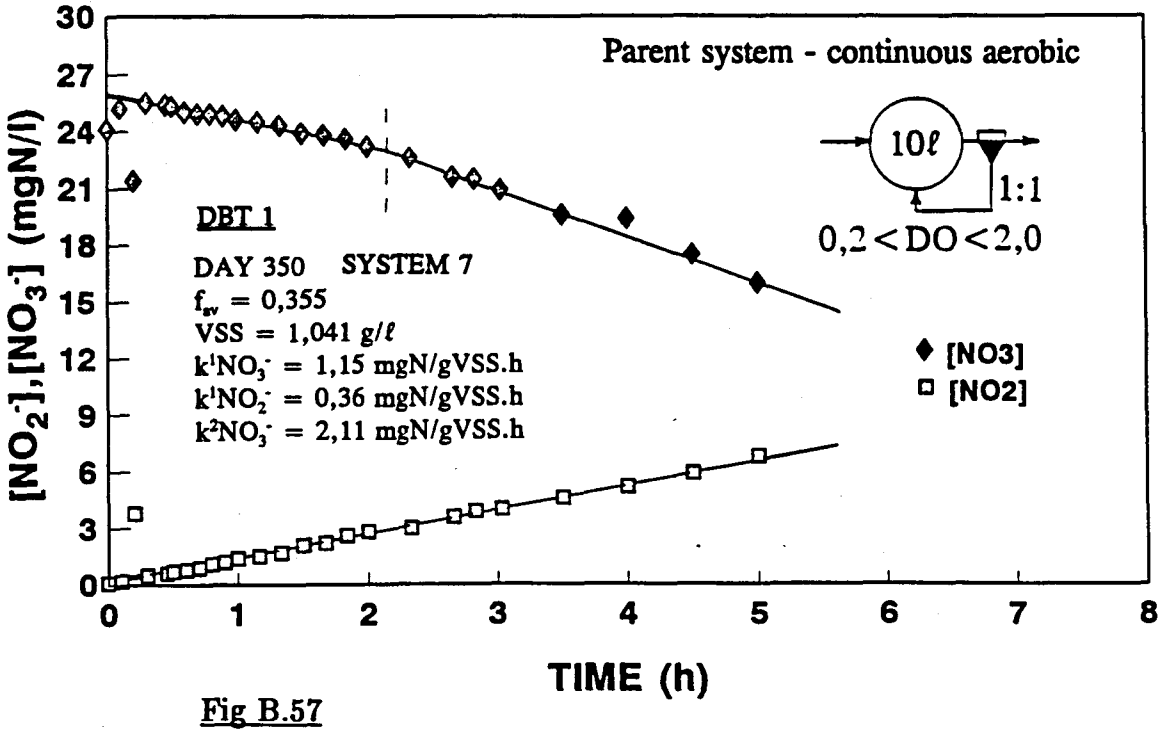


Fig B.57

Table B.80

DENITRIFICATION BATCH TEST 2 OPERATING CONDITIONS		
Objective: To determine the effect on the denitrification rate of an increase in exposure to aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 7 ; Day 352 System configuration: Continuous aerobic		 0,2 < DO < 2,0
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	2,0
Water volume	(ℓ)	0,3
Sewage volume	(ℓ)	0,7
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	449
VSS	(mg/ℓ)	382
F/M	(mgCOD/mgVSS	0,8
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	315

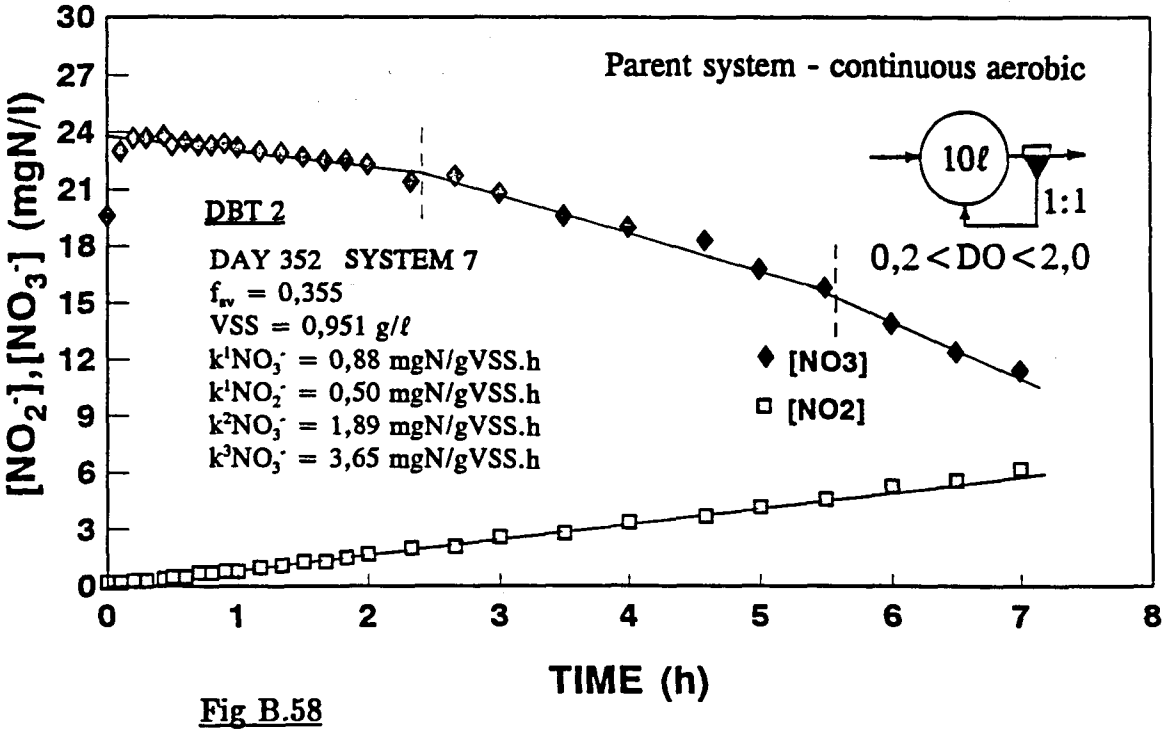
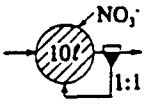


Fig B.58

Table B.81

DENITRIFICATION BATCH TEST 3 OPERATING CONDITIONS		
Objective: To determine the effect on the denitrification rate of a change from steady-state aerobic conditions to anoxic conditions.		
PARENT SYSTEM CONDITIONS		
System 7 ; Day 355 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	2,0
Water volume	(ℓ)	0,3
Sewage volume	(ℓ)	0,7
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	857
VSS	(mg/ℓ)	654
F/M	(mgCOD/mgVSS	0,5
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	321

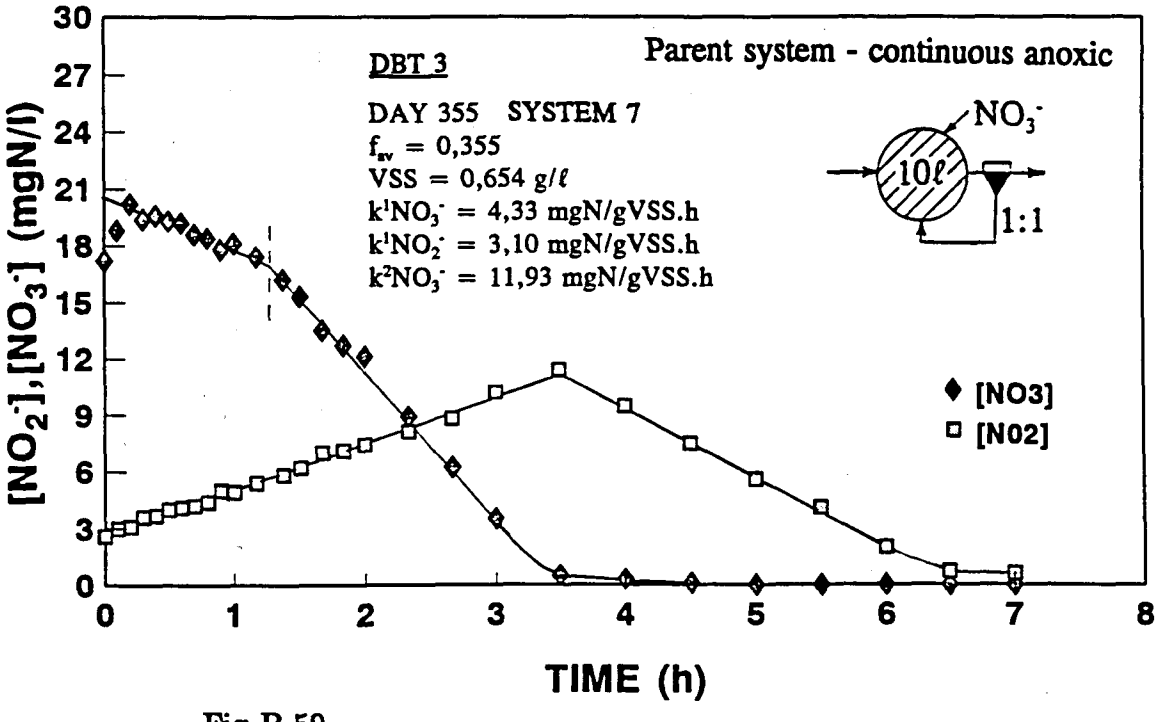


Fig B.59

Table B.82

DENITRIFICATION BATCH TEST 4 OPERATING CONDITIONS		
Objective: To determine the effect of an increase in length of exposure to anoxic conditions after steady-state aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 7 ; Day 364 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	2,0
Water volume	(ℓ)	0,3
Sewage volume	(ℓ)	0,7
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	663
VSS	(mg/ℓ)	565
F/M	(mgCOD/mgVSS	0,5
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	303

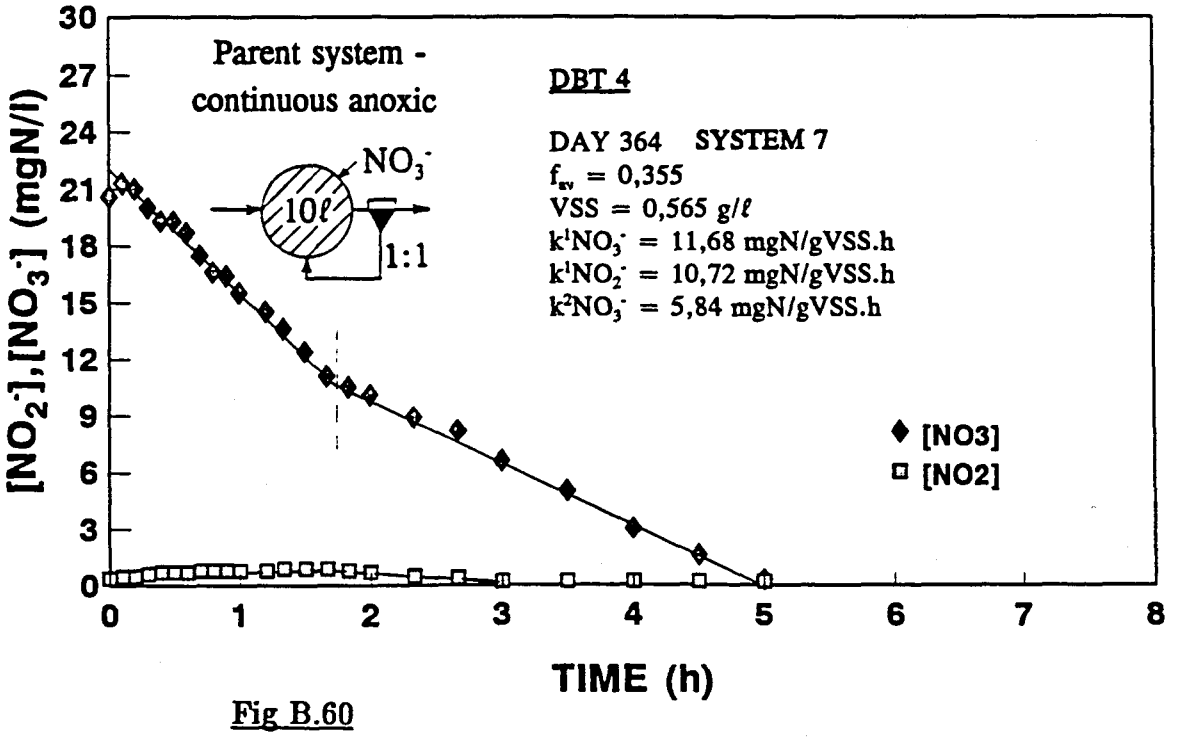


Fig B.60

APPENDIX B.5

ULTRAFILTRATION OF MUNICIPAL SEWAGE

As a means of obtaining the readily biodegradable and slowly biodegradable fractions of municipal sewage, (unsettled raw sewage from Mitchell's Plain treatment works, Cape Town) was subjected to ultrafiltration. Fig B.61 illustrates the experimental setup by which the sewage was fractionated. A set of typical experimental data illustrating the COD of the influent and filtrate during ultrafiltration and the portion of each of those fractions which passes 0,45μm is illustrated in Fig B.62.

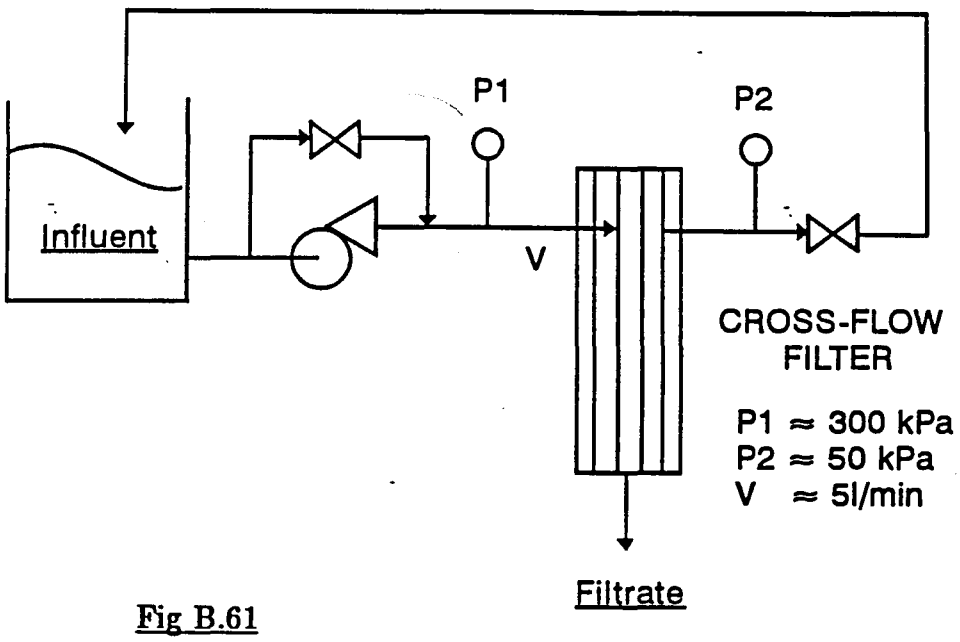


Fig B.61

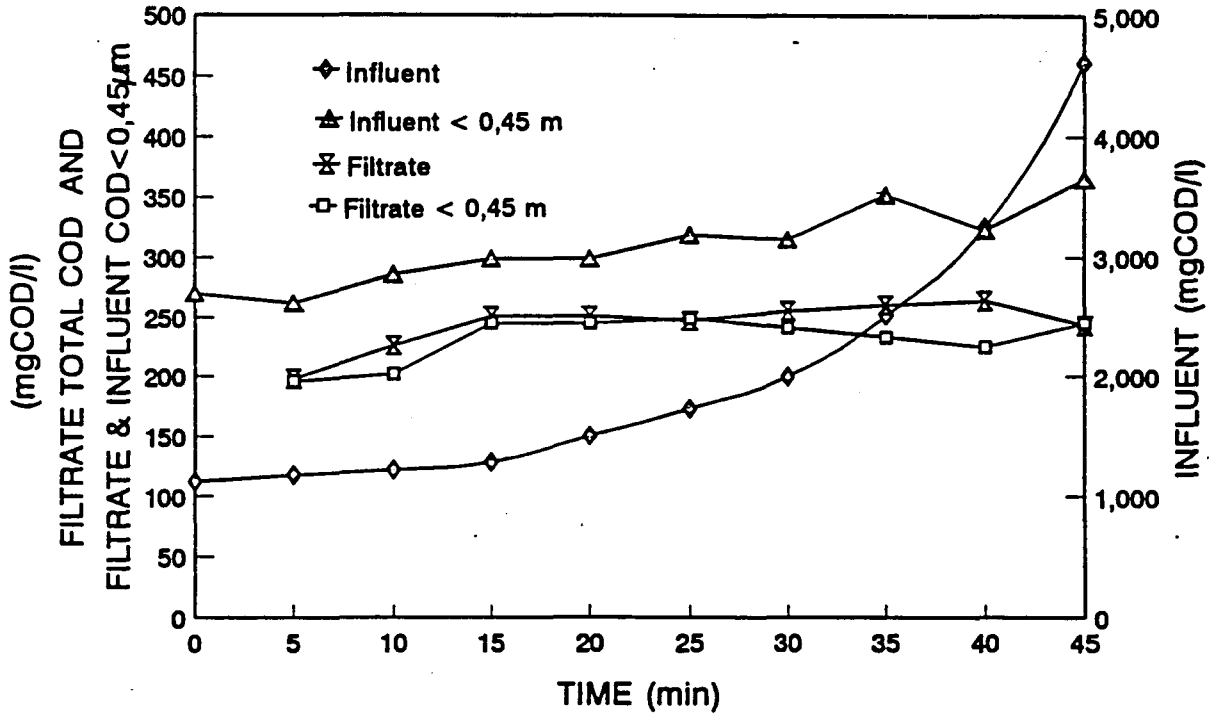


Fig B.62

APPENDIX C

A REVIEW OF RESEARCH INTO ELECTRON TRANSPORT PATHWAYS OF FACULTATIVE HETEROTROPHIC ORGANISMS

This review describes the research findings associated with identification of the structure, position with relation to the cytoplasmic membrane, and reactive mechanisms of the terminal electron transferring complexes, the nitrogen oxide reductases and the cytochrome oxidases of the electron transport pathway. From the research findings, an electron transport pathway is proposed as the one most representative of facultative heterotrophic organisms and is used throughout the report as such.

C.1 A METHODOLOGY FOR RESEARCH

C.2 CONSTITUTIVE AND ADAPTIVE PATHWAY COMPONENTS

C.3 NITRATE REDUCTION

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Anoxic pathway additions to aerobic pathways

Point of divergence from aerobic pathways of electron flow to nitrate reductase

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Nitrite reduction product identification

Orientation of nitrite reductase

C.5 NITRIC OXIDE REDUCTASE

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Conclusions

C.6 NITROUS OXIDE REDUCTASE

Orientation of nitrous oxide reductase

Subunit structure of nitrous oxide reductase

C.7 CYTOCHROME OXIDASE IN FACULTATIVE ORGANISMS

Orientation of cytochrome oxidases aa_3 and *o*

Conclusions

C.8 CLOSURE

C.1 A METHODOLOGY FOR RESEARCH

In elucidating the biochemical pathways employed by an organism, it is often useful to compare the physiology of the organism being studied with another functionally similar organism, the physiology of which is well established. This experimental methodology was followed by workers investigating the biochemical pathways of denitrifying organisms. To digress briefly; heterotrophic organisms found in activated sludge are categorized according to their respiratory function (that is, obligate aerobic, (O_2 as electron acceptor), facultative aerobic, (O_2 or NO_3^-/NO_2^- as electron acceptors), or anaerobic, (electron acceptor internally generated). Single cell organisms which have a low level of biochemical pathway organization and derive their energy from external electron donors are known as *prokaryotes.* Organisms with a considerably higher level of biochemical pathway organization are known as *eukaryotes*, and in their cellular composition have a nucleus, separated from the cytoplasm by a nuclear membrane and more importantly as far as this work is concerned, a mitochondrion (i.e. an internal energy generating organelle). A further difference between prokaryotes and eukaryotes is that prokaryotes can be facultative whereas eukaryotes are obligate aerobes. Thus, facultative heterotrophic organisms found in activated sludge are regarded as prokaryotes. The biochemistry of the aerobic respiratory pathways of the mitochondrion of eukaryotic organisms is well established. A cursory examination of the aerobic respiratory pathways of prokaryotic organisms indicates many similarities to mitochondrial pathways, especially with regard to the enzymatic components of the aerobic respiratory pathway.[1] As a consequence of the metabolic similarities of prokaryotes and eukaryotes, the well established biochemistry of the mitochondrial respiratory pathways has been used as a model for research into the aerobic respiratory pathways of the prokaryotic organisms.

A number of organisms have been selected as representative of the prokaryotic group and research has been concentrated on these. In this regard there are two facultative organisms that have received considerable research attention;

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[1] The similarities between prokaryotes and eukaryotes have given rise to what is known as the endosymbiotic theory for the evolutionary origin in the eukaryotic organism (Margulis, 1970). According to this theory, an aerobic bacterium evolved to a mitochondrion. A simple aerobic prokaryotic bacterium which could oxidize organic substrate with oxygen as electron acceptor, merged with an amoeboid cell which depended on fermentation for production of energy; the metabolism of the two organisms eventually became integrated, giving rise to the eukaryotic organism. The adaptive denitrifying components of the aerobic bacterium are considered to be a subsequent evolutionary feature.

Paracoccus denitrificans,[2] a facultative *denitrifier* (i.e. reduces nitrate to one of the gaseous nitrogen compounds, nitric oxide, nitrous oxide, or dinitrogen) and *Escherichia coli*, a facultative *nitrate reducer* (i.e. reduces nitrate to the ionic nitrogen oxide, nitrite. With regard to *Pa. denitrificans*, John and Whatley (1975) outline the aspects of the bacterium which have led to this situation –

> "It is demonstrated that Paracoccus denitrificans resembles a mitochondrion more closely than do other bacteria, in that it effectively assembles in a single organism those features of the mitochondrial respiratory chain and oxidative phosphorylation which are otherwise distributed at random among most other aerobic bacteria.".

Because of these features, and because it has the characteristic of being able to reduce nitrate to gaseous compounds, a considerable degree of research has been devoted to the denitrifying pathways of *Pa. denitrificans* and as a result its respiratory pathways have come to be regarded as typical of facultative denitrifying organisms.

Another organism which has been the subject of considerable experimental research regarding its respiratory pathways is the facultative organism *E. coli*. Although this organism can respire aerobically and anoxically, under anoxic conditions it has limited capability in that it can only reduce nitrate to nitrite and is termed a facultative nitrate reducer. *E. coli* has become the most widely studied of all bacteria as a consequence of its ubiquitous occurrence in nature.

The eukaryotic organism with its mitochondrion and the prokaryotic organisms, *E. coli* and *Pa. denitrificans* have been selected in the discussion above as examples of organisms exhibiting different respiratory metabolic behaviour: the eukaryotic mitochondrion for organisms with pathways resembling those of obligate aerobes, *E. coli* as representative of facultative aerobes with the ability to reduce nitrate to nitrite (*nitrate reduction*) and *Pa. denitrificans* as representative of facultative aerobes with the ability to reduce nitrate or nitrite to gaseous products, nitric oxide,

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[2] Throughout this review, when organisms are mentioned the first time, the full name is given and when the organism is mentioned subsequently, a form of the name is given in which the first letter of the genus is coupled with the full name of the species, e.g. *Bacillus licheniformis* – *B. licheniformis*. In cases where the first letter does not differentiate between genera, the first two letters are used, e.g. *Paracoccus denitrificans* – *Pa. denitrificans*, to distinguish from *Pseudomonas denitrificans* – *Ps. denitrificans*.

nitrous oxide or dinitrogen (denitrification). The respiratory pathways exhibited by these organisms embrace the major aerobic/anoxic respiratory pathways of facultative organisms in nitrogen removal activated sludge systems and the physiology of the respiratory biochemical pathways exhibited by each of these organisms is considered typical of the group to which each belongs. For this reason these organisms often were used in experimental work enquiring into the electron transport pathways of facultative heterotrophic organisms.

C.2 CONSTITUTIVE AND ADAPTIVE PATHWAY COMPONENTS

The respiratory pathways of the facultative organisms *Pa. denitrificans* and *E. coli* and the obligate aerobic mitochondrion in the eurkaryotes are similar both in function and components, but as noted earlier there is one notable difference. The mitochondrion is able to respire under aerobic conditions only, whereas *Pa. denitrificans* and *E. coli* can respire under aerobic and also under anoxic conditions with nitrate present. According to John and Whatley (1975), the components of the respiratory pathways of *Pa. denitrificans* and the mitochondrion which are similar and which develop under essentially all environmental conditions are: NADH, flavoprotein, iron–sulphur proteins, ubiquinone, cytochromes of the *b* and *c*–type and cytochrome aa_3. These are all components of the respiratory chain, but only some are enzymes. All these components are referred to by John and Whatley (1975) as *constitutive* components of the respiratory pathway, a reference to their presence under all environmental conditions. The difference between the respiratory pathways of *Pa. denitrificans* and a mitochondrion is the presence of the nitrogen oxide reductases in *Pa. denitrificans* which are not found in mitochondria. These are referred to as *adaptive* components, viewed by John and Whatley (1975) as *ad hoc* additions to the constitutive aerobic respiratory pathway, their development a consequence of growth of aerobic organisms under anoxic conditions.

In this thesis, considerable attention is devoted to the nature of the aerobic and denitrifying enzymatic components of the respiratory pathway, especially in determining whether these enzymes are constitutive or adaptive and the effect of oxygen or lack of oxygen on their formation and activity.

In microbiological work, enzymes that are present in nearly constant amounts under all environmental conditions are called *constitutive enzymes.* Enzymes which are adaptive by nature and are synthesized only in response to the presence of certain substrates (and electron acceptors) are usually called *inducible enzymes.* For both

constitutive and inducible enzymes, the mechanisms by which *enzyme activity* is switched on or off are called *activation* and *inactivation* respectively. For inducible enzymes, the mechanisms by which *enzyme synthesis* is halted or initiated are termed *repression* and *derepression* (or *induction*) respectively. As this review progresses and comes to discuss the presence and absence of enzymes (especially under conditions in which organisms switch between aerobic and anoxic respiration and *vice versa*), it will become clear that the description of enzymes as either constitutive or inducible (as occurs in pure culture work) is inadequate for describing the changes in enzyme concentration and activity in mixed culture applied research such as activated sludge. Consequently, many enzymes cannot be rigorously categorized as either constitutive or inducible. For example, although some enzymes may be present and active under all conditions, they are not strictly constitutive, in that, in the absence of the substrate which induces their synthesis, they persist in small amounts.

C.3 NITRATE REDUCTION

The enzyme complexes which reduce each of the nitrogen oxides, are referred to as reductases, and are named according to the respective nitrogen oxides they reduce (i.e. the nitrate–, nitrite–, nitric oxide–, and nitrous oxide reductases). Initial work on nitrate reducing and denitrifying organisms concentrated on isolating and purifying cellular matter with the aim of determining the chemical composition of the reductases, this process being referred to as characterization. Later work determined the arrangement and sequence of the enzyme complexes common to aerobic and dentrifying pathways and the nitrogen oxide reductases particular to the denitrification pathway. Historical aspects concerning the discovery of the nitrogen oxide reductases, their arrangement as part of the denitrification pathway and their function as biochemical catalysts of electron flow are discussed for each of the reductases in turn beginning with the enzymes catalyzing the least reduced nitrogen oxide, nitrate. The majority of research into enzymes of the denitrification pathway was conducted on nitrate reductase, a consequence of nitrate being the first choice of the nitrogen oxides as electron acceptor for most denitrifying and nitrate reducing organisms. Although the primary objective of early research was to establish the point of divergence of electron flow to nitrate reductase on the electron transport pathway, as a consequence of the sequential production and reduction of the other nitrogen oxides derived from nitrate reduction, it was inevitable that research was directed also to establishing the points of divergence of electron flow to the other nitrogen oxide reductases.

The early research

A major contribution to research into the products of denitrification resulted from research conducted in the 1940's–1950's when workers extracted enzymes from cells of denitrifying organisms which after purification formed gaseous nitrogen oxides from the reduction of nitrate and nitrite *in vitro*. These enzymes, and those of the aerobic respiratory pathway had first been documented by MacMunn in 1884 after the discovery that cells contain iron containing (haem) pigments, known today as cytochromes. The leading biochemists of the time dismissed these observations as experimental error and it was not until the early 1920's that Kielin, while examining a suspension of yeast under a microscope viewed a four-banded absorption spectra representing three different pigments (the fourth band being common to all) and proper recognition was accorded MacMunn's work. Kielin realized that these substances acted as catalysts in the oxidation process and named them cytochromes *a*, *b* and *c*, the alphabetical terminology still being used today. The intervening years to the 1940's saw development in techniques for the extraction and purification of all the respiratory pathway enzymes.

Workers studying nitrate reduction in the 1940's–1950's extracted nitrate reducing fractions from *E. coli*, other organisms and also from green plants (Sato 1956; Nason and Evans 1953; Nicholas and Nason 1957; Taniguchi *et al.*, 1956). Two nitrate reductases were obtained, referred to as Pichinoty's nitrate reductase 'B' which served an assimilatory function (i.e. assimilation of nitrogen into cellular matter) and Pichinoty's nitrate reductase 'A', an enzyme with a dissimilatory function (i.e. energy production). *The dissimilatory enzyme only is of interest in this review.*

Taniguchi and Itagaki (1960) were the first to characterize a dissimilatory nitrate reducing enzyme, that of *E. coli*, grown anoxically in the presence of nitrate. It was found to contain 1 atom of bound molybdenum and 40 atoms of bound iron. Subsequently, other investigators measured similar compositions for the nitrate reductase from *E. coli* (MacGregor *et al.*, 1974; Forget, 1974). Regarding denitrifying organisms, Lam and Nicholas (1969b) purified a nitrate reductase from *Micrococcus denitrificans* (now *Pa. denitrificans*) shown by isotopic labelling to contain molybdenum but no flavin or cytochrome and Forget (1971) used chemical determinations on nitrate reductase from the same organism to yield an enzyme containing 0,4 atoms of molybdenum (Mo) and 8 atoms of iron (Fe) per molecule. The involvement of molybdenum and iron in the nitrate reductase of *Pa. denitrificans* was further verified by Forget and DerVartanian (1972), using

electron paramagnetic resonance (EPR) techniques.

Once a general molecular composition of the enzyme nitrate reductase had been established, workers then attempted to determine the functional relationship between it and other parts of the respiratory chain, and especially its association with the aerobic components of the respiratory chain.

Anoxic pathway additions to aerobic pathways

It was suggested as early as 1886 (Gayon and Dupetit) that a functional relationship exists between the aerobic and nitrate reducing components of the respiratory pathways of some organisms, but the nature of this relationship was not specified. Allen and van Niel (1952) concluded that facultative organisms could be considered as primarily aerobic organisms with an adaptive denitrification faculty,

> "...all denitrifying bacteria known to date are equally capable of growing under aerobic conditions in the absence of nitrate,..."

From studies on nitrate respiration by *Pseudomonas stutzeri*, Spangler and Gilmour (1966) stated "There appeared to be no major shift in primary metabolic pathways when nitrate was used as the terminal hydrogen *(or electron)* acceptor in nitrate respiration as opposed to aerobic respiration with free molecular oxygen". (Our addition in italics). These workers did not consider facultative organisms as either aerobic or anoxic organisms, but as having a primary respiratory pathway common to both aerobic and anoxic respiration.

From experiments in which growth under aerobic conditions was somewhat greater than growth with nitrate under anaerobic conditions, Downey (1966) considered *Bacillus stearothermophilus* to be primarily an aerobic organism with an adaptive nitrate reducing respiratory pathway, "..... the organism possesses the ability to respire anaerobically with nitrate as an oxidant. *(electron acceptor)* To do so, an adaptive enzyme, nitrate reductase must be synthesized.... An organism possessing both means of respiration presumably alters its oxidative mechanisms to suit the availability of the oxidant." (Our addition in italics)

As a consequence, schemes for the respiratory electron transfer pathways of facultative organisms were based on schemes for aerobic respiration, adapted to nitrate respiration. However, a major cause of disagreement concerned the point of

divergence of the electron pathway to nitrate reduction from the aerobic respiratory pathway. This aspect is highlighted in the following review of the development of the respiratory electron transfer pathways of facultative organisms.

Point of divergence from aerobic pathways of electron flow to nitrate reductase

As a means of determining the cytochromes of the respiratory pathway from which electrons pass to nitrate reductase, most workers adopted a policy of growing the organisms under 3 sets of conditions; aerobic, anoxic (nitrate present), and anaerobic (nitrate and O_2 absent). A conclusion regarding the effect of environmental conditions on enzyme synthesis was made by comparing the type and level of cytochromes produced under each set of conditions, based on the hypothesis that the enzyme complexes which exhibit the greatest development are those which are the most immediate to the electron acceptors, nitrate and oxygen.

In one of the first electron transfer schemes published for facultative organisms, illustrated in Fig C.1, Fewson and Nicholas (1961b) proposed that in *Pseudomonas aeruginosa* the aerobic and nitrate reducing electron transport pathways branch from cytochrome *c*, because anoxically grown (with nitrate present) *Ps. aeruginosa* exhibited an increase in cytochrome *c* concentration and nitrate reductase activity. Although cytochrome *b* was present in the enzymes, Fewson and Nicholas (1961b) were reluctant to assign an electron transferring function to this component, and cited evidence against its involvement in either aerobic or anoxic respiration. Because all the electron transferring complexes of the pathway were considered to be common to aerobic and anoxic respiration, there was no need to consider the constitutive or adaptive nature of the aerobic and anoxic components of the respiratory pathway. Molybdenum (Mo) was found to be a constituent of the nitrate reductase system, and it was suggested that during nitrate reduction, the molybdenum atom undergoes a valency change, probably $Mo^{6+} \rightarrow Mo^{5+}$. With respect to the effect of anoxic conditions on the development of cytochrome oxidase, when cells of *B. stearothermophilus* were changed from aerobic to anoxic growth conditions, with nitrate present, oxygen respiration decreased by between 50 and 70%; the cytochrome spectra indicating the disappearance of cytochrome a_3 (Downey, 1966; Downey *et al.*,1969). This indicated that cytochrome a_3 is an inducible rather than a constitutive enzyme and led to the consideration that another cytochrome oxidase, a constitutive enzyme is synthesized under anoxic conditions which allows respiration to occur immediately upon the introduction of oxygen.

Lam and Nicholas (1967) and Newton (1967) found that anoxically grown *Ps. aeruginosa* contained a higher concentration of the *b*, *c* and *o* cytochromes[3] than did the same organism grown under aerobic conditions. From these results it was concluded that cytochromes *b* and *c* were directly involved in electron transfer in nitrate respiration and cytochromes *o* and a_3 are constituents of the aerobic pathway. Lam and Nicholas (1969a) came to the same conclusion regarding *Pa. denitrificans* grown aerobically and anoxically, further reinforcing the notion that both cytochromes *b* and *c* are an integral part of the anoxic electron transport pathway to nitrate.

In contrast, in studies on *Klebsiella aerogenes*, (formerly *Aerobacter aerogenes*), Knook *et al.* (1973) did not implicate cytochrome *c* but found that growth conditions that favoured the synthesis of respiratory nitrate reductase led to the greatest amounts of cytochrome *b* being formed. "The evidence ... suggests that cytochrome *b* is a carrier in the electron transport systems to both nitrate and oxygen; from cytochrome *b* branching occurs, with one branch linked to the respiratory nitrate reductase and one branch linked to oxidase systems containing the cytochromes *a*, *d* and *o*". This pathway is illustrated in Fig C.2. Specifically, they did not detect the presence of cytochromes of the *c*-type in extracts from the membranes and as a result did not include cytochrome *c* as a part of the respiration pathway of this organism. Thus, the divergence of the paths to nitrate reductase and oxidase at cytochrome *b* in *K. aerogenes* was different from the scheme of Fewson and Nicholas (1961b) for *Ps. aeruginosa* (Fig C.1), in which cytochrome *c* was shown to be the last common element.

John and Whatley (1975), in a scheme for *Pa. denitrificans* illustrated in Fig C.3, combined elements of both of the above schemes and also the findings of Lam and Nicholas (1967) and Newton (1967); the electron transport pathway included cytochromes *b* and *c*; the pathway to nitrate reductase branching at cytochrome *b* and the pathway to terminal oxidase (cytochrome aa_3) and the alternative terminal oxidase (cytochrome *o*) branching at cytochrome c_1c. In this scheme, a separate nitrite reducing enzyme, cytochrome *cd* was included, accepting electrons from a point different to nitrate reductase whereas the scheme of Knook *et al.* (1973) proposed the cytochrome *cd* complex as electron donor to both nitrate and nitrite.

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[3] It may seem surprising that cytochrome *o*, which is one of the enzymes associated with aerobic respiration, develops a higher concentration with anoxic than aerobic respiration. This is discussed in more detail in Section C.7.

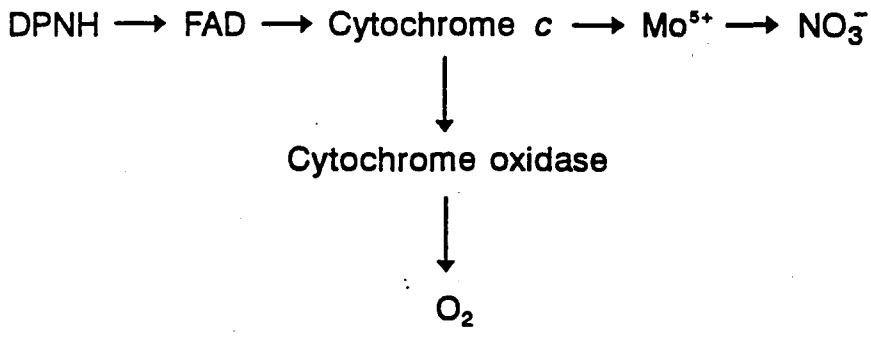

Fig C.1: Electron transport pathway (ETP) proposed by Fewson and Nicholas (1961b), for *Pseudomonas aeruginosa* in which cytochrome *c* is the branch point for electrons to nitrate reductase and cytochrome oxidase. [Abbreviations: DPNH – diphosphopyridine nucleotide (reduced); FAD – Flavin adenine dinucleotide; Mo – molybdenum.]

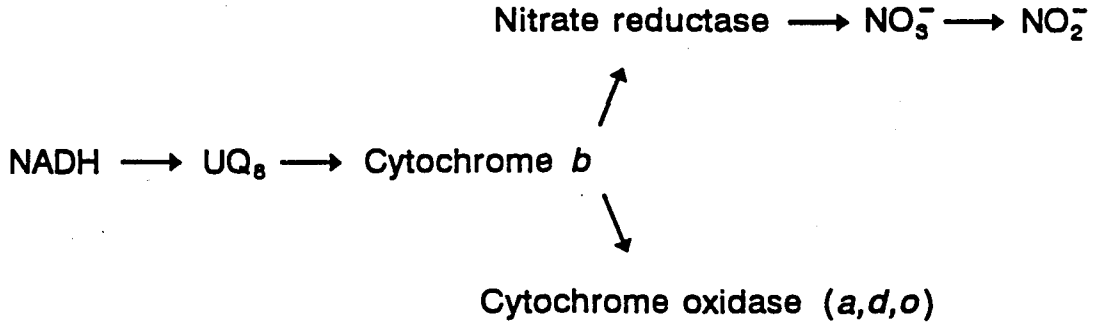

Fig C.2: Electron transport pathway (ETP) proposed by Knook *et al.* (1973) for *Klebsiella* (formerly *Aerobacter) aerogenes* in which cytochrome *b* is the branch point for electrons to nitrate reductase and cytochrome oxidase. [Abbreviations: NADH – nicotinamide adenine dinucleotide (reduced); UQ_8 – ubiquinone 8.]

In a scheme for aerobic respiration by *Pa. denitrificans*, Willison and John (1979) proposed that electron transport to oxygen originates from a ubiquinone–cytochrome *b* complex and electron flow is mediated by either cytochrome aa_3 or cytochrome *o*.

Stouthamer (1980) made two notable improvements to the scheme of John and Whatley (1975) for *Pa. denitrificans* by including nitrous oxide reductase as the third sequential nitrogen oxide reductase and indicating that the alternative cytochrome oxidase (cytochrome *o*) branches from the cytochrome *b* complex rather than the cytochrome c_1c complex as illustrated in Fig C.4.

Knowles (1982) proposed a more comprehensive model of the probable pathways of electron transport in *Pa. denitrificans* by including nitric oxide reductase; the scheme was constructed from the findings of Boogerd *et al.* (1980), John and Whatley (1975) and Porte and Vignais (1980) and is illustrated in Fig C.5. The branching points to the oxidases and the other nitrogen oxide reductases are discussed below. It should be noted that although Knowles (1982) presents cytochromes *b* and *c* as single entities, the cytochrome *b* and cytochrome *c* designations (cytochrome *b* and cytochrome *c* respectively) refer to the complexes of these enzymes which contain two types of cytochrome *b* and cytochrome *c*, more informatively designated by John and Whatley (1975) and Stouthamer (1980) as cytochrome $b\,^{562}_{565}$ and cytochrome c_1c. The benefit of indicating the individual enzymes of the cytochrome *b* and cytochrome *c* complexes is apparent in the electron transport scheme of Ferguson (1982) for *Pa. denitrificans* in which the individual enzymes cytochrome c_1 and cytochrome *c* of the cytochrome *c* complex are separated and allocated to different complexes.

Given the similarities between the respiratory processes of *Pa. denitrificans* and the mitochondrion, Ferguson (1982) considered it probable that the respiratory components of *Pa. denitrificans* have an organization similar to the scheme proposed by Kröger and Klingenberg (1970) for the respiratory processes of the mitochondrion, in which the components are organized into complexes as illustrated in Fig C.6.

This scheme has two features different to previous schemes for facultative organisms. One difference is the organization of cytochromes *b* and c_1 into the one complex, referred to as the cytochrome bc_1 complex. The other difference is that the

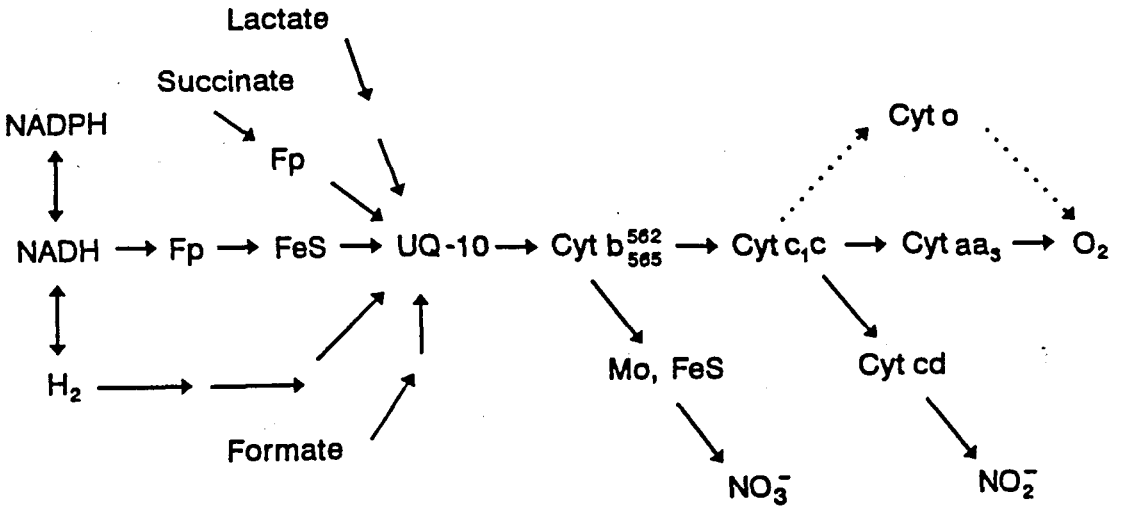

Fig C.3: Electron transport pathway (ETP) proposed by John and Whatley (1975) for *Paracoccus denitrificans* in which cytochrome *c* complex is the branch point for electrons to nitrite reductase and the oxidase cytochrome aa_3, cytochrome *b* is the branch point to nitrate reductase and an alternative oxidase, cytochrome *o* has a branch point from cytochrome *c*. [Abbreviations: NADPH – nicotinamide adenine dinucleotide phosphate (reduced); Fp – flavoprotein; FeS – iron sulphur centres; UQ–10 – ubiquinone 10; Cyt– Cytochrome; Mo – Molybdenum.]

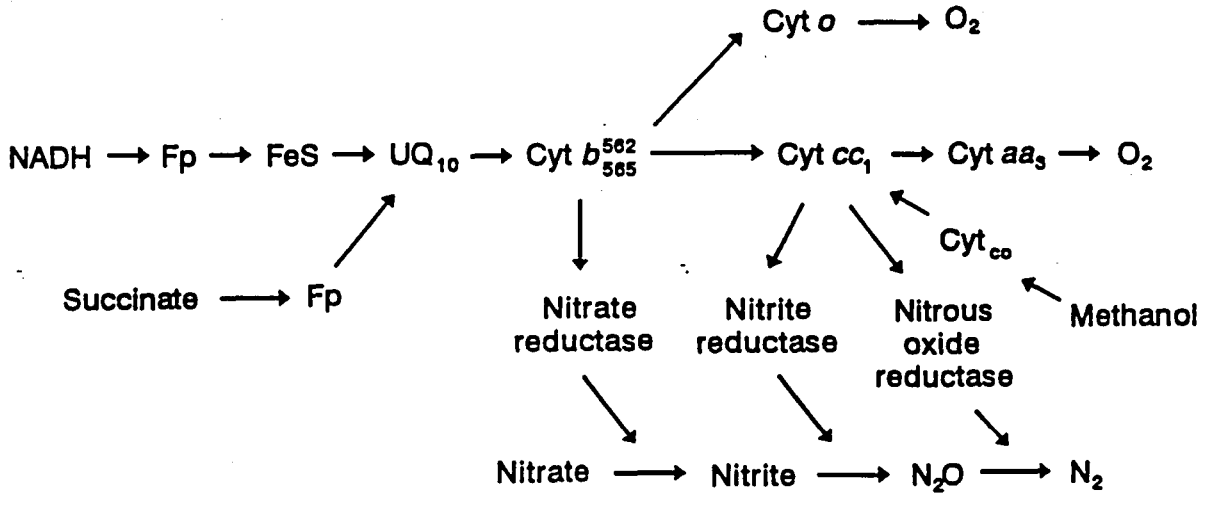

Fig C.4: Electron transport pathway (ETP) proposed by Stouthamer (1980) for *Paracoccus denitrificans* in which nitrous oxide reductase is included with an electron branch point from cytochrome cc_1. [Abbreviations: NADH – nicotinamide adenine dinucleotide (reduced); Fp – flavoprotein, FeS – iron sulphur centres; UQ_{10} – ubiquinone 10; Cyt – Cytochrome.]

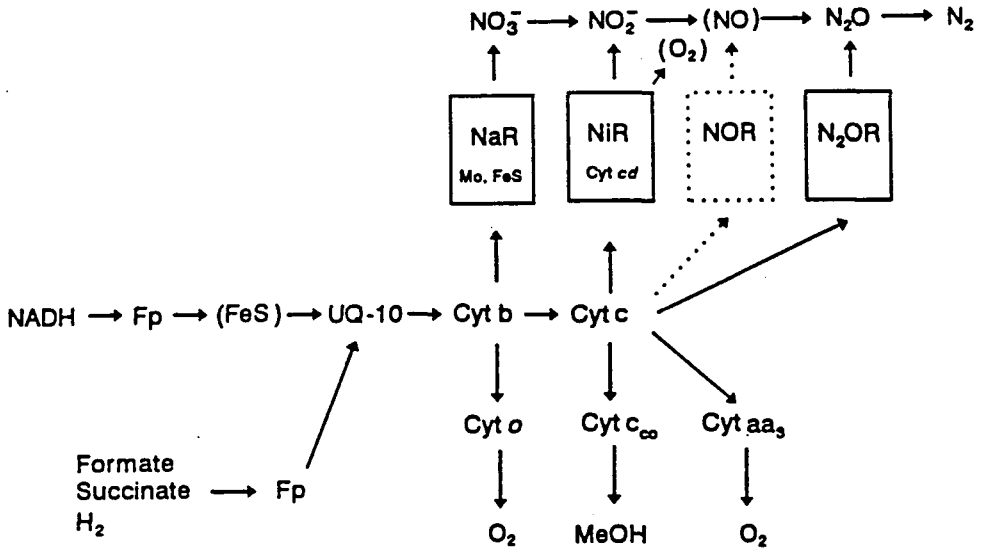

Fig C.5: Electron transport pathway (ETP) proposed by Knowles (1982) for *Paracoccus denitrificans*, in which nitric oxide reductase is included with an electron branch point from Cytochrome *c*. [Abbreviations: NADH – nicotinamide adenine dinucleotide (reduced); Fp – flavoprotein; FeS – iron sulphur centres; UQ-10 – ubiquinone 10; Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR nitric oxide reductase; N_2OR – nitrous oxide reductase; Mo – Molybdenum; MeOH – methanol.]

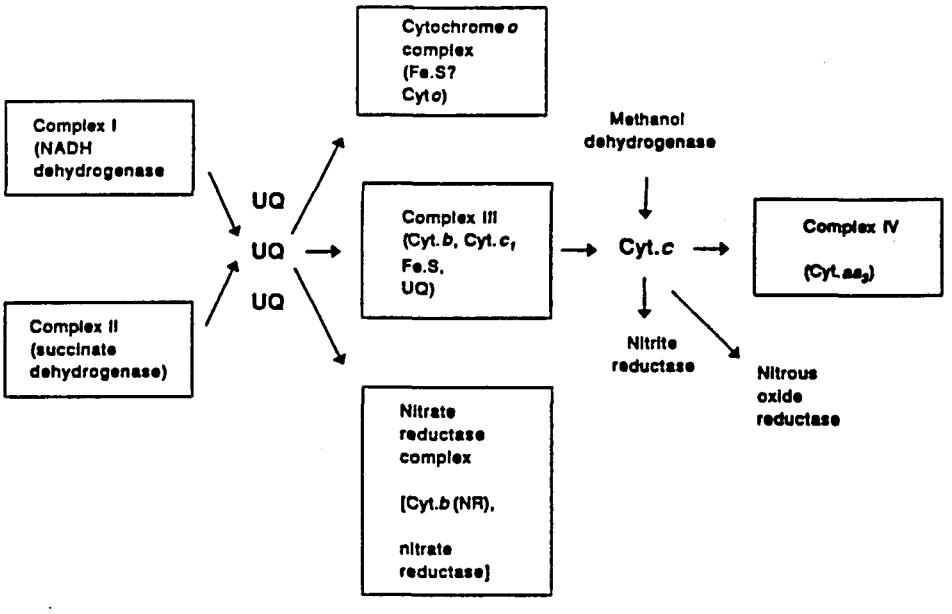

Fig C.6: Electron transport pathway (ETP) proposed by Ferguson (1982) for *Paracoccus. denitrificans* in which the components are organized into complexes and the electron branch point to the nitrate reductase, cytochrome *b*, and cytochrome *o* complexes is from ubiquinone. (Note that nitric oxide reductase is not included.) [Abbreviations: NADH – nicotinamide adenine dinucleotide (reduced); UQ – ubiquinone; Cyt – Cytochrome; NR – nitrate reductase.]

points of divergence to nitrate reductase and cytochrome *o* are at ubiquinone, which is shown as a homogeneous pool connecting the dehydrogenases with the cytochromes. Changing the point of divergence of electrons to nitrate reductase from cytochrome *b* to ubiquinone resulted from work conducted by Alefounder *et al.* (1981) with the inhibitory chemical antimycin A. The mode of action of inhibitors of the electron transport pathway is to restrict the flow of electrons between specific cytochromes. The point of action of antimycin A has conventionally been allocated to between the cytochrome *b* and cytochrome *c* complexes (Knobloch et al., 1971). However, the exact point of action of antimycin A is somewhat unclear owing to the schematic separation of cytochromes of the *c* complex (i.e. cytochrome *c* and cytochrome c_1) and the organization of cytochromes *b* and c_1 into one complex. The work of Alefounder et al., (1981) indicates that antimycin A restricts electron flow through the cytochrome bc_1 complex and as a result of the finding that antimycin A does not reduce the rate of nitrate reduction by *Pa. denitrificans* (John and Whatley, 1975), Ferguson (1982) proposed ubiquinone as the point of divergence to nitrate reductase. This proposal was supported by Kučera *et al.* (1984b) working with *Pa. denitrificans* and the inhibitors antimycin A and mucidin. They suggested that the observations of Lam and Nicholas (1969a) and John and Whatley (1970) that cytochrome *b* of the cytochrome bc_1 complex is oxidized following the addition of nitrate, is a consequence of an increase in the degree of ubiquinone reduction due to the flow of electrons from ubiquinone to the nitrate reductase complex. Kučera *et al.* (1984b) suggested that a more likely explanation for the oxidation of cytochrome *b* during nitrate reduction is that *nitrate reductase contains cytochrome b*, a suggestion motivated by analogy with the constituents of the nitrate reductase of *E. coli* (Ferguson, 1982) and by work conducted by Calder and Lascelles (1984) with *Pa. denitrificans.*

Experimental evidence supporting the organization of respiratory components into complexes in the manner of Ferguson (1982) was furnished by Berry and Trumpower (1985). They isolated from *Pa. denitrificans* a ubiquinol oxidase, containing a cytochrome bc_1 complex, and a cytochrome c–aa_3 complex. The complexes corresponded to those of the mitochondrial complexes, even to the point that inhibitors of electron flow through the mitochondrial bc_1 complex inhibited electron flow through the same complex of *Pa. denitrificans*, indicating the similarity in the pathways. Further work by Yang and Trumpower (1986) on the electron transferring function of the cytochrome bc_1 complex of *Pa. denitrificans* indicated that it is similar if not identical to the cytochrome bc_1 complex of

mitochondria. Parsonage *et al.* (1986) in studies on *Pa. denitrificans* using the inhibitor myxothiazol, a more powerful inhibitor of the cytochrome bc_1 complex than antimycin A, concluded similarly, that the electron transport pathways to nitrate reductase and cytochrome oxidase *o* branch at ubiquinone and not at the cytochrome bc_1 complex.

From the review presented above, the electron transport pathway proposed by Ferguson (1982) appears to be the most acceptable one to date. However, an inadequacy in the pathway is the omission of nitric oxide reductase. A scheme in which nitric oxide reductase is included is illustrated in the section describing nitric oxide reductase.

Location of nitrate reductase in the cytoplasmic membrane

Scholes and Smith (1968) and Lam and Nicholas (1969b) reported the cytochromes of the respiratory chain of *Pa. denitrificans* to be situated in or associated with the cytoplasmic membrane, but did not make a statement regarding the side of the membrane at which the active site is situated. Other workers reported that nitrate reductase is a transmembrane enzyme, indicating that it is exposed on both the periplasmic (outer) and cytoplasmic (inner) sides of the membrane (*K. aerogenes*, Weintjes et al., 1979; *E. coli*, Boxer and Clegg, 1975) but also did not allocate the active site of the enzyme to a particular side. Later work indicated that although nitrate reductase spans the membrane, the active site at which nitrate is reduced is on the cytoplasmic side of the membrane (*E. coli*, Kemp *et al.*, 1975; Jones *et al.*, 1978, Kristjansson and Hollocher, 1979; *Pa. denitrificans*, John 1977, Kristjansson *et al.*, 1978, Alefounder and Ferguson 1980; *Ps. denitrificans*, Kristjansson *et al.*, 1978, *Bacillus licheniformis*, Weintjes *et al.*, 1979).

A cytoplasmic placement for the active site of nitrate reductase necessitates a means for nitrate movement across the membrane from the periplasmic to the cytoplasmic side – an aspect discussed at a later stage in this section.

Subunit structure of nitrate reductase

In determining the point of divergence of electron flow to nitrate reductase, workers found that organisms grown under anoxic conditions developed a higher concentration of cytochrome *b* than did organisms grown under aerobic conditions. Having established that cytochrome *b* is not the enzyme from which the electron transport pathway to nitrate reductase diverges, nor is it one of the enzymes on the

oxidized side of the electron transport pathway to nitrate reductase, the reason for its increase under anoxic conditions in the presence of nitrate was perplexing and needed to be addressed. From work conducted on *E. coli*, Ruiz-Herrera and De Moss (1969) proposed that a *b*-type cytochrome, distinct from that involved in the aerobic electron transport pathway, functions as part of the nitrate reductase complex under anoxic conditions with nitrate present. Further support for this explanation was gained by workers examining the structure of nitrate reductase and the role of its subunits in catalyzing electron flow, and is discussed below.

Variation in the molecular weights reported for nitrate reductase result from different extraction and purification techniques (Stouthamer, 1976). These different techniques led also to different numbers of subunits being reported for nitrate reductase, not only between organisms, but also from the same organism (Hochstein and Tomlinson, 1988). Because of the important catalytic role of nitrate reductase, the composition and function of this enzyme is of considerable importance and it is imperative that the number and function of these subunits is well established in order to understand the mechanisms of electron transfer to nitrate.

The nitrate reductases of many denitrifying organisms are reported to consist of three polypeptides, referred to as subunits α, β and γ (*K. aerogenes*, van 't Riet and Planta, 1975; *Proteus mirabilis*, Oltmann *et al.*, 1976a; *B. licheniformis*, van 't Riet *et al.*, 1979; *Ps. denitrificans*, Ishizuka *et al.*, 1984; and *Pa. denitrificans*, Craske and Ferguson 1986). However, initial work on characterizing this enzyme from some denitrifying organisms had detected only two polypeptides, referred to as subunits α and β (*B. licheniformis* Weintjes *et al.*, 1979; *K. aerogenes*, Weintjes *et al.*, 1979, Abraham *et al.*, 1981; *Ps. aeruginosa*, Carlson *et al.*, 1982).

In a similar fashion, the nitrate reductase of *E. coli* was also initially reported as having two subunits (MacGregor *et al.*, 1974) and then later three subunits (Enoch and Lester, 1974; MacGregor 1975b; Morpeth and Boxer, 1985), the difference being attributed to the method used to release the membrane bound enzyme (Enoch and Lester, 1975; MacGregor, 1976; Lund and de Moss, 1976).

It was established that the number of nitrate reductase subunits detected from denitrifying organisms is also dependent on the extraction procedure used (Craske and Ferguson, 1986; Ishizuka *et al.*, 1984; van't Riet *et al.*, 1979) These workers suggested that the use of heat treatment extraction procedures as opposed to

extraction with detergents may lead to the loss of subunit γ, (the cytochrome *b* type subunit).

The detection of the third subunit, γ in the nitrate reductase complexes of *E. coli* and denitrifying organisms and its characterization as a *b*–type cytochrome, distinct from cytochrome *b* in the cytochrome bc_1 complex, can explain the previous observation of an increase in *b*–type cytochrome found in organisms grown under anoxic conditions. This increase, initially attributed to an increase in the *b*–type cytochrome of the cytochrome *b* complex, led to the assumption that the electron transport path to nitrate reductase diverged from this complex (see Figs C.2, C.3, C.4 and C.5).

Craske and Ferguson (1986), purified and characterized the nitrate reductase from *Pa. denitrificans*. It contained three subunits (designated α, β and γ) having similar molecular masses to the corresponding components of the *E. coli* nitrate reductase complex. Reporting on the purification of nitrate reductase from *Pa. denitrificans* and the identification of subunit γ, Craske and Ferguson (1986) indicate that the ready dissociation of the γ subunit from the enzyme during purification may be the reason that it has gone undetected in other studies on nitrate reductase from *Pa. denitrificans* and other denitrifiers. From spectrophotometric analysis it was concluded that the γ subunit is a *b*–type cytochrome. Ballard and Ferguson (1988) supplied further evidence in support of this finding. They demonstrated that the γ subunit contains two *b*–type haem (iron) centres. Hackett and Bragg (1982) had previously shown that nitrate reductase from *E. coli* also contains two *b*–type haem centres.

In summary, identification of a nitrate reductase specific *b*–type cytochrome has implications concerning the branch point to nitrate reductase in the electron transport chain, since the presence of a *b*–type cytochrome would obviate the need for the electron transport branch point to come from the cytochrome bc_1 complex, lending further support for the suggested branch point at ubiquinone.

<u>Role of nitrate reductase subunits in electron transfer</u>

The role of two of the subunits of nitrate reductase in electron transfer has been reasonably well established for *E. coli*. The γ subunit (containing cytochrome *b*) has a role in the association of nitrate reductase with electron donor complexes (Enoch and Lester, 1974) and is also essential for association of nitrate reductase

with the membrane (MacGregor, 1975c). It catalyzes the transfer of electrons from ubiquinol to the α subunit (Ingledew and Poole, 1984). Chaudhry and MacGregor (1983a) established that the iron–sulphur (FeS) and molybdenum centres of nitrate reductase in this organism are associated with the α subunit, and since EPR studies have implicated these centres in electron transfer, the catalytic site of the reductase is allocated to subunit α. The role of subunit β is not as well understood. Some work suggests that it is involved in membrane attachment (Ingledew and Poole, 1984; Stouthamer, 1988; Stewart, 1988), or that it has a role in regulating the synthesis of nitrate reductase (Ingledew and Poole, 1984), but it does not appear to be involved in electron transfer between subunits α and γ.

Stewart (1988), in a review of the work of MacGregor (1976), Stewart and MacGregor (1982) and Hackett and MacGregor (1981), concludes that subunits α and β are synthesized as soluble precursors (i.e. not bound to the membrane) to nitrate reductase formation and that the presence of subunit γ is essential for assembly of the reductases into the cytoplasmic membrane. (For the effect of oxygen on the assembly of the subunits into the membrane, see Appendix D).

Less work has been conducted on the subunits of nitrate reductase in denitrifying organisms than has been conducted on the subunits of nitrate reductase in the nitrate reducing organism *E. coli*. Craske and Ferguson (1986) suggest a mechanism for electron flow through nitrate reductase of *Pa. denitrificans* based on their own findings and also by analogy with the flow of electrons through the nitrate reductase of *E. coli* (Ingledew and Poole, 1984).

> "..... in the plasma membrane of *P. denitrificans* the γ subunit catalyzes electron transfer to the α and β subunits of nitrate reductase from ubiquinol which acts as a branch point in the respiratory chain."

Continuing the analogy with nitrate reductase of *E. coli* in which the iron–sulphur clusters and molybdenum are associated with the α subunit (Chaudhry and MacGregor, 1983b) the catalytic site of nitrate reductase of *Pa. denitrificans* is similarly allocated to subunit α, due to the finding that two or three iron–sulphur clusters and molybdenum which are the active centres of nitrate reductase in *E. coli* are also found in various denitrifiers (*Pa. denitrificans*, Forget, 1971; *B. licheniformis*, van 't Riet *et al.*, 1979; and *K. aerogenes*, van 't Riet *et al.*, 1975).

To summarize, electron flow in *Pa. denitrificans* would thus be, from ubiquinol via the cytochrome *b* component of the nitrate reductase complex (subunit γ) on the periplasmic side of the membrane to the active site (subunit α) of the complex at the cytoplasmic side of the membrane where nitrate would then be reduced to nitrite. Given the information available for nitrate reductase of *Pa. denitrificans*, the schematic representation of the orientation of the subunits and the membrane of Stewart (1988) for *E. coli* may also be representative of subunits of denitrifying organisms and is illustrated in Fig C.7.

Transport of nitrate to nitrate reductase

Mitchell (1968) proposed three systems for facilitated transport of ions across cell membranes. Applied to the transport of nitrate these can be described as:

(i) a uniport system in which nitrate crosses the membrane via a specific nitrate channel,

(ii) either an H^+/NO_3^- symport system in which a proton crosses the membrane together with a nitrate ion which because it is electroneutral is not influenced by the membrane electrical potential, or, a symport of nitrate with more than one proton which is promoted by the membrane electrical potential,

(iii) an electroneutral NO_3^-/NO_2^- antiport system in which a nitrate ion passing to the cytoplasmic side of the membrane is exchanged for a nitrite ion passing to the periplasm.

Most transport systems proposed for the movement of nitrate across the membranes are based on these three mechanisms or a modification of them. However, little direct evidence exists for these mechanisms, and mechanisms for the manner by which nitrate crosses the cytoplasmic membrane to reach the active site of nitrate reductase (subunit α) are entirely speculative. John (1977) proposed that in *Pa. denitrificans* a dedicated uniport nitrate carrier system permits nitrate entry across the membrane from the periplasmic side to the cytoplasmic side, since in uptake experiments with nitrate and chlorate, *Pa. denitrificans* could discriminate between nitrate and its experimental analogue, chlorate. Kristjansson *et al.* (1978) showed that nitrate uptake by *Pa. denitrificans* is accompanied by proton uptake on a 1:1 stoichiometric basis and Kristjansson and Hollocher (1979) suggested that a mechanism to explain this is a NO_3^-/NO_2^- antiport functioning either directly or as a H^+/NO_3^- antiport for nitrate uptake and a NO_2^-/H^+ antiport for nitrite release. Based on a consideration of the three systems of Mitchell (1968), Boogerd *et al.*

(1983a) proposed two uptake systems for nitrate. The first system initiates nitrate uptake and is dependent on the membrane electrical potential, alternatively called proton motive force (pmf). Nitrate crosses the membrane together with two or more protons via an H^+/NO_3^- symport system and is reduced to nitrite at the catalytic site of nitrate reductase at the α subunit located on the cytoplasmic side. The second nitrate uptake system is an NO_3^-/NO_2^- antiport which takes over nitrate uptake from the first system. In an exchange mechanism, one nitrite molecule produced from nitrate reduction passes back to the periplasm for each nitrate molecule crossing the membrane to the cytoplasm. The process is electroneutral and self perpetuating so long as nitrate is present.

Although present evidence points to a NO_3^-/NO_2^- antiport as a likely mechanism for nitrate and nitrite transport, the evidence is circumstantial and no physical evidence of such a system has yet been demonstrated. Consequently, Craske and Ferguson (1986) suggest that the nitrate reductase complex itself incorporates a nitrate–specific channel which provides access for nitrate to the active site of its reductase. In conclusion, it is apparent that more work needs to be done to elucidate the mechanisms of nitrate transport across the membrane to nitrate reductase. The effect of factors such as oxygen on the movement of nitrate across the membrane is discussed in Appendix D.

Regarding the active site of nitrate reductase, the mechanism of the catalytic action of nitrate reductase and the role of each of the subunits of the reductase have been documented in far greater detail for the nitrate reducing organism *E. coli* than for denitrifying organisms. For *E. coli*, a proposal for the manner by which the molybdenum component of the nitrate reductase complex acts as a catalyst in the reduction of nitrate to nitrite by coupled proton–electron transfer was outlined by Stiefel (1973) and is illustrated in Fig C.8. Molybdenum (Mo) in oxidation state (IV) and attached to a fully protonated ligand interacts with nitrate. The Mo atom changes from oxidation state (IV) to (VI), with the concomitant reduction of NO_3^- by two electrons. The atom XH becomes acidic and transfers its proton to nitrate which then splits to nitrite and hydroxide. It is presumed that the Mo in the (VI) state is then reduced by electron transfer from the FeS clusters in the enzyme, to regain its Mo (IV) state.

The section above concludes the review of the function and position of the nitrate reductase and covered aspects such as (1) the development of the ETP with

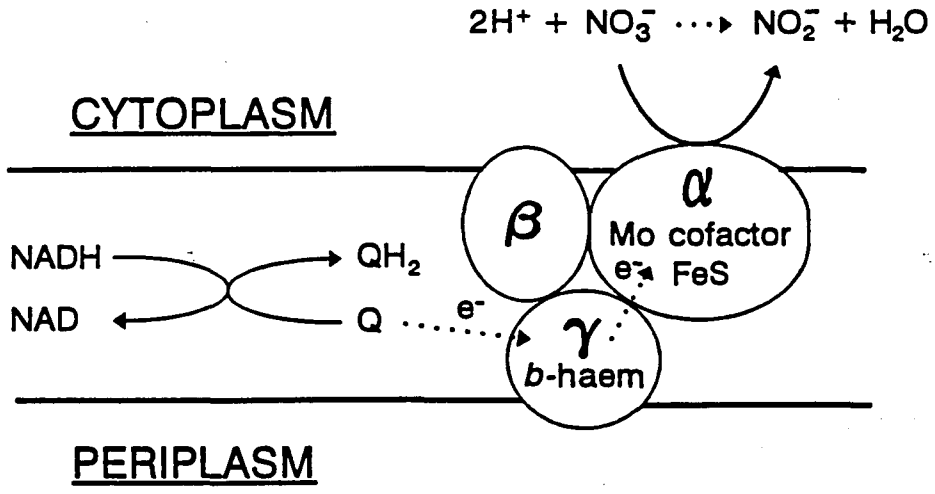

Fig C.7: The orientation and electron transferring function of the nitrate reductase subunits, α, β and γ in denitrifying organisms. [Adapted from Stewart (1988) for *Eschericia coli.*]

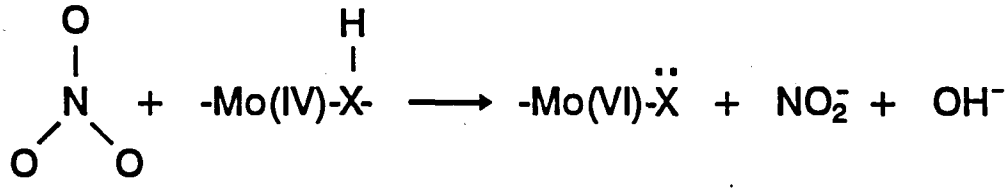

Fig C.8: Electron transferring mechanism of Molybdenum (Mo) in nitrate reductase. [From Stiefel (1973)]

emphasis on establishing the branch point of the electron flow to nitrate reductase, and (2) the structure of nitrate reductase and its orientation with respect to the cytoplasmic membrane, and (3) the catalytic action of the nitrate reductase in its electron transport function, and (4) mechanisms by which nitrate is transported across the membrane.

The end product of the catalytic action of nitrate reductase is nitrite and the following section reviews the next step in the denitrification chain, i.e. the enzymatic reduction of nitrite by the enzyme complex nitrite reductase.

C.4 NITRITE REDUCTASE

Research into the second step of the denitrification process, i.e. the reduction of nitrite, followed a pattern similar to that of research into the reduction of nitrate as outlined above. Nitrite reducing fractions were extracted from organisms respiring on nitrite under anoxic conditions and shown to have enzymatic characteristics *in vitro* with nitrite as electron acceptor. Extracts were purified and characterized, and the position on the electron transport chain at which electrons are passed to nitrite reductase was established. A major difference between research into nitrite reductase and nitrate reductase is that whereas the product of nitrate reduction (i.e. nitrite) was well established, the identity of the product of nitrite reduction (i.e. either nitrous oxide or nitric oxide) was the source of considerable argument. A further difference between research into the two reductases is that workers were able to draw an analogy between the nitrate reductase of *E. coli* and that of denitrifying organisms, but the absence of nitrite reduction in *E. coli* precluded this in an analysis of the nitrite reduction step of denitrifying organisms.

Respiratory nitrite reductase was first reported in cell free extracts of *Ps. aeruginosa* in 1939 by Yamagata. It has been found subsequently in *Thiobacillus denitrificans* (Baalsrud and Baalsrud, 1954), *Bacillus subtilus* (Najjar and Allen, 1954), *Ps. aeruginosa* (Yamanaka and Okunuki, 1963a), *Ps. stutzeri* (Chung and Najjar, 1956a), and *Ps. denitrificans* (Radcliffe and Nicholas, 1968).

Nitrite reductase was purified more than 600 fold from extracts prepared from actively reducing *P. aeruginosa*, by Walker and Nicholas (1961b) who identified a c-type cytochrome and another complex supposed to be a copper containing enzyme, the combination being able to reduce nitrite to nitric oxide.

Kodama (1970) studied the effect of nitrate and nitrite on the cytochrome system of cells of a denitrifying bacterium *Ps. stutzeri* grown under aerobic and anoxic conditions. Nitrite induced the synthesis of an enzyme identified as a *cd*–type cytochrome (formerly named a_2c) under anoxic conditions or semi–aerobic (low DO) conditions. Nitrate repressed a *c*–type cytochrome (referred to as c_{552}) under the same conditions.

A cytochrome complex containing a *c*–type component and a *cd* component was characterized by Newton (1967, 1969) in an extract from *Pa. denitrificans* grown anoxically in the presence of nitrate. These results were verified by Lam and Nicholas (1969c) who concluded that the enzymes extracted from *Pa. denitrificans* were probably of the *c* and *d* (a_2) types contained in the one complex. Using reduced cytochrome *c* as the electron donor the gaseous products of the purified nitrite reductase were measured and identified as containing more than 90% nitric oxide. (It is noteworthy that even at this early stage of research into nitrite reductase, it had been established that reduced cytochrome *c* could act as an electron donor to the cytochrome *cd* complex of nitrite reductase, thereby establishing the point at which electrons in the electron transport pathway are diverted to nitrite reductase (see Figs C.3, C.4 and C.5). Zumft *et al.* (1979) also showed that nitrite reductase in *Ps. aeruginosa* is of the *cd*–type and receives electrons from cytochrome *c*. Payne (1981) makes an interesting observation in describing two different avenues along which research into nitrite reductase developed. The Payne group recognized the existence of a specific nitrite reducing enzyme complex in denitrifiers and were concerned with characterizing the nitrite reducing fractions extracted from various organisms. Other groups (Horio *et al.*, 1961; Yamanaka *et al.*, 1962; Yamanaka and Okunuki, 1963b; Yamanaka, 1964; Wharton *et al.*, 1973 and Gudat *et al.*, 1973) working independently from the workers discussed above, were attempting to characterize a cytochrome oxidase isolated from *Ps. aeruginosa*. Some of the groups working with cytochrome oxidase realized that the oxidase isolated from *Ps. aeruginosa* possessed the general properties of cytochrome oxidase but also acted as a nitrite reductase (Yamanaka *et al.*, 1962; Yamanaka, 1964). The implications of this finding were not immediately apparent to some groups and so research into nitrite reductase continued along two different paths for some time before it was realized that essentially all nitrite reductase has cytochrome oxidase activity and that the complex which demonstrated oxidase activity was identical to the complex which reduced nitrite. This finding led to an interesting situation since under aerobic

conditions the possibility arises that both oxygen and nitrite could compete for nitrite reductase. However Payne (1981) indicates that the redox potential established at cytochrome oxidase precludes electron transfer to nitrite reductase.

Two types of nitrite reductase exist, the cytochrome *cd* complex discussed above and a copper–containing metallo–flavoprotein which also exhibits oxidase activity and has been isolated from *Achromobacter cycloclastes*, (Iwasaki and Matsubara, 1972; Iwasaki *et al.*, 1975), *Alcaligenes* sp. (Iwasaki *et al.*, 1963) and from strains of *Rhodospeudomonas sphaeroides*, (Sawada and Satoh, 1980).

Since no difference is apparent between these cytochromes, i.e. the *cd*–type and copper containing ones, in their activity, function, or position on the electron transport pathway, no distinction will be made between them when discussing the general function of nitrite reductase in the electron transport pathway, in the remainder of this review.

<u>Nitrite reduction product identification</u>

While agreement between groups was relatively uniform concerning characterization of the nitrite reductases for various denitrifying bacteria, an area of vigorous dispute emerged concerning the identity of the major product of the reduction of nitrite by nitrite reductase during denitrification, i.e. either nitric oxide (NO) or nitrous oxide (N_2O). This is of considerable importance since the identification of nitric oxide as a free obligatory intermediate would necessitate the existence of a specific nitric oxide reductase for its reduction.

The resolution of the question of whether nitric oxide is an intermediate was hampered by the finding that generally, nitric oxide production was detected from cell free extracts only, and not from whole cell suspensions (*Ps. stutzeri*, Najjar and Allen, 1954; *Bacillus licheniformis*, Najjar and Chung, 1956; *T. denitrificans*, Baalsrud and Baalsrud, 1954; *Ps. aeruginosa*, Walker and Nicholas, 1960). Some workers detected nitric oxide production in whole cell suspensions of denitrifying organisms (*Corynebacterium nephridii*, Renner and Becker, 1970; *Ps. denitrificans*, Radcliffe and Nicholas, 1968; *Pseudomonas perfectomarinus* – now *Pseudomonas perfectomarina*, Barbaree and Payne, 1967). This anomaly was partially resolved when Verhoeven (1956) demonstrated that whole cell suspensions of *Pa. denitrificans* and *Ps. stutzeri* did not produce extracellular nitric oxide under normal denitrifying conditions, but nitric oxide was produced by whole cell

suspensions of the same organisms under conditions adverse to normal metabolism, such as abnormally high concentrations of nitrite or the presence of cyanide, and also by "physiologically old cells". Voβwinkel and Bothe (1990) also found that nitric oxide was detected in gaseous products of *Pseudomonas* spp. reducing nitrate, only when the concentration of nitrite in the medium increased above a certain level.

It was concluded that such findings indicated that under normal denitrifying conditions, the end product of nitrite reduction, i.e. nitric oxide, is consumed as rapidly as it is produced whereas under the adverse conditions described above, nitric oxide is not consumed as rapidly, it accumulates, and is detected in the gaseous form. In the work of Verhoeven (1956), an exception to the finding that whole cell suspensions produced nitric oxide only under adverse denitrifying conditions, was *T. denitrificans*, which produced nitric oxide under normal denitrifying conditions. This may result from an intolerance of even intermediate concentrations of nitrite.

Many other research groups investigated bacterial nitrite reduction and the likely products of this process, and these groups have undertaken extensive investigations into the products of the reduction of nitrite, the main emphasis being on clarification of the identity of the true product of the reaction, i.e. either nitric oxide, nitrous oxide, both of these, or a complex of one of these nitrogen oxides. Mostly, groups acknowledged the existence of nitrous oxide as a true intermediate but many groups did not consider nitric oxide as being an intermediate, considering that nitrous oxide is generated by the reduction of nitrite. The groups engaged in this research whose work is reviewed here are referred to as the Tiedje, Hollocher and Payne groups respectively. In a comprehensive review of the role of nitric oxide in the denitrification pathway, Goretski and Hollocher (1990) concluded that three options exist to explain the status of nitric oxide in the denitrification step in which nitrite is reduced:

(i) The pathway between nitrite and nitrous oxide is one that entirely avoids the production of nitric oxide as a free intermediate.

(ii) Nitric oxide remains a largely enzyme–bound or otherwise compartmentalized intermediate.

(iii) Nitric oxide is a free intermediate between nitrite and nitrous oxide and is kinetically controlled to very low concentrations.

The Tiedje research group has favoured option (i). They assign the responsibility for the reduction of nitrite to nitrous oxide entirely to nitrite reductase. The scheme of Averill and Tiedje (1982) requires that nitrite traps an enzyme-bound nitroxyl ion (NO^+) to form bound N_2O_3 which is then reduced by two subsequent steps to nitrous oxide. These studies were conducted using Nitrogen-15 isotope labelling. Experiments using various isotopes of nitrogen were designed by the Tiedje group from which it was concluded that the hypothesized mechanism for nitrite reduction avoids the production of nitric oxide, (Aerssens *et al.*, 1986; Weeg-Aerssens *et al.*, 1987; 1988). (It is interesting that whereas this mechanism does not allow for the inclusion of NO as an intermediate, an early study by this group (Firestone *et al.*, 1979) using Nitrogen-13 isotope labelling concluded that nitric oxide did constitute part of the denitrification pathway, either as an intermediate or as a species in rapid equilibrium with an unidentified intermediate).

The complexity of determining the role of nitric oxide as an intermediate in denitrification, is highlighted by the efforts of the Hollocher group who over a number of years covered the spectrum of possible options. Initial experiments suggested that nitric oxide is not produced as an intermediate, i.e. option (i). Further experiments implicated nitric oxide as a membrane bound intermediate, i.e. option (ii). Later experiments confirmed NO as a free intermediate requiring its own reductase, i.e. option (iii). Details of these experiments and the organisms on which they were conducted are given below.

The majority of experiments conducted by this group used isotopically labelled Nitrogen-15 (^{15}N).

St John and Hollocher (1977) using gas chromatography and mass spectrometry to analyse isotopically labelled intermediates, found evidence for nitrous oxide but not nitric oxide as a free obligatory intermediate during denitrification of nitrite to dinitrogen by *Ps aeruginosa*.

Garber and Hollocher (1981) studied the role of nitric oxide in denitrification pathways in *Pa. denitrificans, Ps. stutzeri* and *Ps. aerofaciens* and ruled out the involvement of free nitric oxide as an obligatory intermediate in reduction of nitrite

to nitrous oxide.

These two sets of experiments implied either option (i) or (ii).

Further experiments by this group (Garber and Hollocher, 1982a, 1982b; Garber *et al.*, 1983) with *Ps. stutzeri*, *Pa. denitrificans*, *Ps. aerofaciens* and *Ps. fluorescenes* provided indirect evidence for a variety of intermediates, such as the free intermediate, nitroxyl (HNO) or the enzyme bound intermediate, nitrosyl (NO^+), thereby favouring either option (ii) or option (i).

Kim and Hollocher (1983), used gas chromatography, mass spectrometry and isotopic labelling with reductase from *Ps. aeruginosa* to favour option (ii) and concluded that nitrite is reduced to nitrous oxide via enzyme bound nitric oxide. This result was contradicted by the work of Goretski and Hollocher (1988), who trapped nitric oxide produced during denitrification using extracellular haemoglobin, to provide evidence for nitric oxide as a free intermediate from the reduction of nitrite. The only obstacle to an acceptance of option (iii) was the lack of evidence for the kinetic competence of nitric oxide as an intermediate in denitrification.

Prior to the work of Goretski and Hollocher (1988), the results of the Hollocher group had indicated that nitrous oxide forms the major product in the reduction of nitrite during denitrification. Subsequently, Hoglen and Hollocher (1989) reported these results to be erroneous, and attributed them to experimental artifacts, in which there was either an incomplete separation of nitrite reductase from nitric oxide reductase or a secondary chemical reduction of nitric oxide by the reducing system, both situations leading to a substantial reduction in the concentration of nitric oxide such that it could not be measured under steady state conditions.

Evidence for nitric oxide as a free intermediate in the denitrification pathway was provided by Goretski and Hollocher (1990) who demonstrated very low intracellular steady state concentrations of nitric oxide for 5 denitrifying bacteria and on the basis of this result the Hollocher group accepted option (iii), that nitric oxide is a free intermediate in the pathway between nitrite and nitrous oxide. Further, Goretski *et al.* (1990) concluded that nitric oxide reductase is a comparatively active enzyme and that the kinetically controlled very low steady–state concentrations of aqueous nitric oxide measured during denitrification, prevents nitric oxide achieving toxic concentrations.

The Payne group has consistently presented evidence in favour of option (iii). Payne *et al.* (1971) separated a nitrite–reducing complex from *Ps. perfectomarinus.* The specificity of the complex to nitrite reduction only was demonstrated by the fact that nitric oxide was the only gaseous product detected from the reduction of nitrite, and the complex failed to reduce nitrate, nitric oxide or nitrous oxide.

Even though the early evidence presented by the Payne group in favour of nitric oxide being a true intermediate appeared to be irrefutable, it was to a large extent disregarded by other workers. This lack of acceptance continued despite further evidence supplied by the Payne group in support of the formation of nitric oxide from nitrite reduction; Le Gall *et al.*, (1979) purified nitrite reductase from *T. denitrificans* which after reaction with nitrite formed nitric oxide.

In a number of reviews over the years, Payne has considered the evidence both for and against the formation of nitric oxide as a denitrification intermediate; (Payne, 1973; Payne, 1981), the conclusion in each case being that the weight of available evidence lends considerable support for nitric oxide formation from nitrite.

Similar evidence has come subsequently from many other research groups working with a great variety of organisms, e.g. *Ps. aeruginosa*, (Walker and Nicholas, 1961b; Yamanaka *et al.*, 1961; Shimada and Orii, 1975; Saraste and Kuronen, 1978; Kučera, 1989), *Pa. halodenitrificans*, (Grant and Hochstein, 1984), *T. denitrificans*, (Sawhney and Nicholas, 1978), *R. sphaeroides* forma sp denitrificans, (Sawada and Satoh, 1980), *Pa. denitrificans*, (Carr *et al.*, 1989; Carr and Ferguson, 1990b), *Ps. perfectomarina* (Zafiriou *et al.*, 1989).

Proper consideration of the available evidence suggests that nitric oxide should be assigned a role as a free intermediate in the denitrification chain, the only evidence which excludes it, being either hypothetical, or having been shown to be flawed by experimental artifact (i.e. ^{15}N isotope labelling techniques).

<u>Orientation of Nitrite Reductase</u>

Sawhney and Nicholas (1978) considered the nitrite reductase from *T. denitrificans* to be membrane bound, indicating that it is incorporated as a part of the membrane. However, they did not ascribe the reactive site of the reductase to either the periplasmic or cytoplasmic side of the membrane. Weintjes *et al.* (1979) also suggested nitrite reductase to be transmembrane in *K. aerogenes.*

Although Kristjansson *et al.* (1978) with *Pa. denitrificans* and Saraste and Kuronen (1978) with *Pseudomonas* sp. concluded that the active site of nitrite reductase was cytoplasmically oriented, other workers allocated the active site to the periplasmic side of the membrane in those and other organisms, i.e. *Pa. denitrificans*, Meijer *et al.* (1979b); Alefounder and Ferguson (1980); *Ps. aeruginosa*, Wood (1978); and *Desulfovibrio desulfuricans*, Steenkamp and Peck (1981). Boogerd *et al.* (1981) described a periplasmic orientation for nitrite reductase in *Pa. denitrificans* based on energetic considerations.

Alefounder and Ferguson (1980) suggest that the cytoplasmic location for nitrite reductase offered by Kristjansson *et al.* (1978) for *Pa. denitrificans* resulted from difficulties in analyzing the very complex pH changes associated with the addition of nitrite and nitrate to intact cells. The location of nitrite reductase with respect to the membrane is of importance concerning the movement of nitrite to the reductase and the movement of the product, nitric oxide away from it and will be discussed in detail below in the section concerning the effect of oxygen on denitrification.

C.5 NITRIC OXIDE REDUCTASE

A primary consideration in the determination of the existence of nitric oxide as a free intermediate in the process of denitrification is the consideration of the existence or not of a nitric oxide reducing fraction, since, if nitric oxide is the only or major product of nitrite reduction, a nitric oxide reductase is required for its conversion to nitrous oxide. As indicated above, the initial lack of support by many research groups for nitric oxide as an intermediate of the denitrification pathway, meant that little work was done by these groups on identification of a specific nitric oxide reductase.

In early work conducted on nitric oxide reductase, Baalsrud and Baalsrud (1954) showed that *T. denitrificans* converts nitric oxide to gaseous products and Verhoeven (1956) established the consumption of nitric oxide by cell extracts from anoxically grown cells of *Ps. stutzeri* and *Pa. denitrificans* but not in cell extracts from aerobically grown cells; however the enzyme responsible was not purified or characterized. Chung and Najjar (1956b) partially characterized a nitric oxide reductase from *Ps. stutzeri* and concluded that it was a flavoprotein containing both iron and copper, and Fewson and Nicholas (1961c) showed that a nitric oxide reductase purified from *Ps. aeruginosa* is a metallo–flavoprotein. Payne *et al.* (1971) and Cox and Payne (1973) established that a nitric oxide reducing fraction could be

extracted from *Ps. perfectomarinus* and that nitrous oxide was the only product detected from the reduction of nitric oxide and Pichinoty *et al.* (1979) isolated seven strains of *Bacillus* sp. which grew anoxically with nitric oxide as terminal electron acceptor.

Orientation of nitric oxide reductase

Zumft and Frunzke (1982) described a membrane bound nitric oxide reductase from *Ps. perfectomarinus* able to catalyse the reduction of nitric oxide to nitrous oxide, and similarly to Hoglen and Hollocher (1989) for *Pa. denitrificans* and Heiss *et al.* (1989), who purified a nitric oxide reductase from *Ps. stutzeri* containing *b*– and *c*–type haem did not determine the position of the active site of the reductase. In a comprehensive study of nitric oxide reductase from *Pa. denitrificans*, Carr *et al.* (1989) demonstrated that; (i) nitric oxide reductase is membrane bound, (ii) nitric oxide reductase is a discrete enzyme as opposed to being part of a nitrite reductase complex, (iii) the addition of Triton X–100 as a means of selectively inhibiting reduction of nitric oxide, led to detection of nitric oxide upon the reduction of nitrate, but nitric oxide was not detected upon the reduction of nitrate in the absence of Triton X–100, indicating that under normal denitrifying conditions, steady state concentrations of nitric oxide are maintained at very low levels.

In reviewing the topic, Zumft (1993) concluded that results from experiments concerned with proton stoichiometry during nitric oxide reduction indicated that the H^+ consuming (electron transferring) site of nitric oxide reductase is periplasmically oriented. A similar conclusion could be drawn from proton uptake experiments conducted by Ferguson (1988) and Stouthamer (1988). In contrast, from experiments concerned with generation of membrane potential during NO reduction it could be inferred that the active site of nitric oxide reductase is situated on the cytoplasmic site of the membrane (Zumft, 1993).

Subunit structure of nitric oxide reductase

Barbaree and Payne (1967) removed a sub–fraction rich in *c*–type cytochrome from the nitric oxide reducing fraction derived from *Ps. perfectomarinus* and the remaining fraction lost almost all capacity for reduction of nitric oxide. After return of the sub–fraction the capacity for nitric oxide reduction also returned.

Heiss *et al.* (1989) isolated a membrane–bound cytochrome *bc* complex from *Ps. stutzeri* which transformed nitric oxide to nitrous oxide. The enzyme complex

was different to the cytochrome bc_1 complex of denitrifying organisms, the immediate electron donor to cytochrome *c*.

Carr and Ferguson (1990a) characterized nitrite oxide reductase from *Pa. denitrificans* and showed also that it contains both *b* and *c*–type haems.

Effect of oxygen on nitric oxide reductase activity

Remde and Conrad (1991) conducted experiments on the production and consumption of nitric oxide by three denitrifying organisms, *Ps. aeruginosa, Ps.stutzeri* and *Azospirellum brasilense.* They concluded that nitric oxide reductase activity is inhibited by oxygen to a lesser extent than is nitrite reductase activity ensuring that concentrations of nitric oxide are maintained at low levels with the onset of aerobic conditions.

Conclusions

The combination of evidence from above, that nitric oxide is found as an intermediate in the reduction of nitrite and that a distinct nitric oxide reducing fraction has been purified (which catalyses the reduction of nitric oxide to nitrous oxide on the cytoplasmic side of the membrane), serves as sufficient evidence that nitric oxide is a true intermediate in the process of denitrification and will be considered as such in the remainder of this work. As a consequence, a modification can be made to the electron transport pathway for facultative organisms described by Ferguson (1982) (see Fig C.6). Nitric oxide is included as an obligate intermediate and nitric oxide reductase is sited accepting electrons from cytochrome c as illustrated in Fig C.9.

C.6 NITROUS OXIDE REDUCTASE

Since the greater part of interest in this review is concerned with nitric oxide and the enzymes prior to nitric oxide in the electron transfer pathway, work conducted on nitrous oxide reductase is included more for the sake of completeness than as a consequence of it having a special role in denitrification as far as bulking is concerned.

Prior to the 1960s the role of nitrous oxide had not been definitely established. Several workers discounted it as a normal intermediate in denitrification (Allen and van Niel, 1952; Sacks and Barker, 1952). Kluyver and Verhoeven (1954) concluded that nitrous oxide is produced as an intermediate by at least some bacterial species. However, as discussed earlier, some organisms do not execute the entire

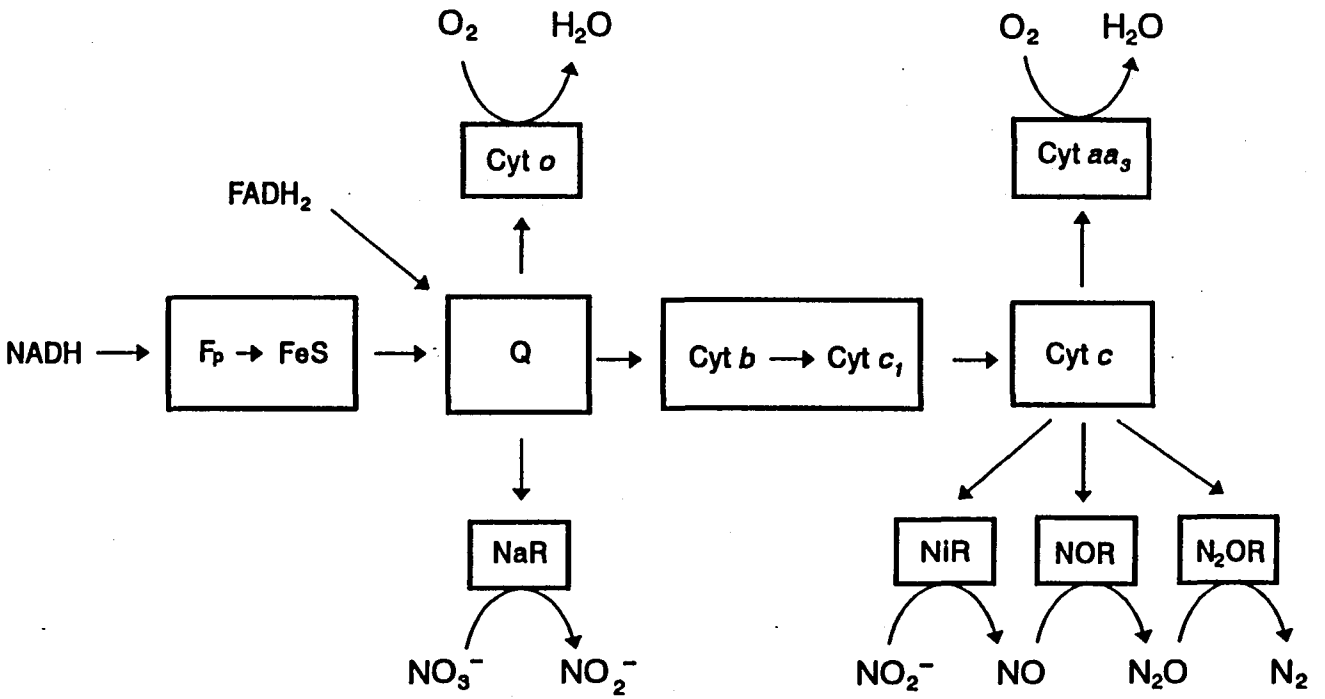

Fig C.9: Electron transport pathway (ETP) for facultative heterotrophic organisms in which the components are organized into complexes and nitric oxide reductase is included with the electron branch point from Cytochrome *c*. Adapted from Ferguson (1982). [Abbreviations: NADH – nicotinamide adenine dinucleotide (reduced); $FADH_2$ – flavin adenine dinucleotide (reduced); Fp – flavoprotein; FeS – iron sulphur centres; Q – ubiquinone; Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase.]

denitrification pathway, their final product being nitrous oxide. Renner and Becker (1970) established *C. nephridii* as such an organism, capable of reducing nitrate, nitrite and nitric oxide to nitrous oxide in the absence of nitrous oxide reductase. However in denitrifying organisms producing dinitrogen as a final product, the existence of a distinct nitrous oxide reductase in the denitrification pathway has rarely been in dispute (even in organisms which produce nitrous oxide under some conditions) and experiments designed to extract and purify nitrous oxide reductase have been conducted as a matter of course in denitrification research. Payne *et al.* (1971) extracted a nitrous oxide reducing fraction from *Ps. perfectomarinus* which, when fed all the nitrogen oxides separately, reduced nitrous oxide only and produced dinitrogen. Kristjansson and Hollocher (1980) purified nitrous oxide reductase from *Pa. denitrificans.*

Orientation of nitrous oxide reductase

Boogerd *et al.* (1981) have shown from proton translocation and energetic considerations that nitrous oxide reductase is located on the periplasmic side of the membrane in *Pa. denitrificans.*

Subunit structure of nitrous oxide reductase

Kristjansson and Hollocher (1981) ascertained that the enzyme does not contain either of the usual active metal centres, molybdenum or iron contained in the cytochrome elements of the other nitrogen oxide reductases (i.e. nitrate–, nitrite–, and nitric oxide reductase). However the cytochromes which comprise the enzymes may contain copper as an essential element, since cells of *Alcaligenes* sp. (Iwasaki *et al.*, 1981) and *Ps. perfectomarinus* (Matsubara *et al.*, 1982) did not synthesize nitrous oxide reductase during growth in copper deficient medium. Coyle *et al.*, (1985) characterized the reductase from the latter organism and found that it contained two identical subunits containing copper atoms, a finding supported by Riester *et al.* (1989) from experiments conducted with *Ps. stutzeri.*

A characteristic of the majority of organisms reviewed here which is easily overlooked in examining the anoxic respiratory pathways of these organisms (and especially *Pa. denitrificans* and *E. coli*) is that they are primarily aerobic organisms with well developed pathways and enzyme systems for the utilization of oxygen as terminal electron acceptor. As discussed at the start of this appendix, comparison of the respiratory pathway of *Pa. denitrificans* with that of the mitochondrion indicates that facultative organisms are first and foremost aerobic organisms, with

anoxic respiration a second choice mechanism. However the intrinsic nature of the aerobic and anoxic components of the respiratory pathway means that it is impossible to examine anoxic respiration without also examining aerobic respiration. For this reason, the enzymes which function under aerobic conditions and comprise a part of the respiratory chain are also reviewed here. Of particular importance is the complex of enzymes known as cytochrome oxidase which acts to transfer electrons to the terminal electron acceptor oxygen.

C.7 CYTOCHROME OXIDASE IN FACULTATIVE ORGANISMS

Anoxic conditions which result in the synthesis of the denitrifying reductases also affect the synthesis and activity of the oxidases. In facultative organisms, a cytochrome oxidase complex additional to cytochrome aa_3 is present. This complex is not present in obligate aerobes, but is always present in facultative organisms, whether grown under aerobic or anoxic conditions, i.e. it is a constitutive complex. The presence of this complex defines the organism as having a facultative capacity. Because of the critical importance of this oxidase in facultative organism behaviour, it is necessary to review the research which led to its discovery and subsequent inclusion as part of the ETP for facultative organisms. Initial work concerned with the effect of anoxic conditions on cytochrome oxidase was directed entirely at cytochromes of the *a*–type since at that time it was considered that no other oxidase existed. It was considered that oxidase activity in facultative organisms resulted from the same enzymes as oxidase activity in obligate aerobic organisms.

Scholes and Smith (1968) working with *Pa. denitrificans* showed that the content of *a*–type cytochromes is considerably lower in organisms grown anoxically (with nitrate present) than in organisms grown aerobically.

Downey *et al.* (1969) investigating the effect of anoxic growth conditions on the aerobic respiration rate of *B. stearothermophilus* found that a culture grown under anoxic conditions increased their level of nitrate reductase 5 to 10–fold over an aerobically grown culture but did not decrease their aerobic respiratory activity proportionally, retaining 30 to 40% of the activity of aerobically grown cells, under subsequent aerobic conditions. This indicated either that nitrate reductase and cytochrome oxidase activity are controlled by separate mechanisms or that a constitutive aerobic enzyme with a lower activity is synthesized under anoxic conditions.

Schulp and Stouthamer (1970) investigating the effect of anoxic conditions on *B. licheniformis* also found that the organisms retained 30 to 40% of their respiratory activity under subsequent aerobic conditions after growth under anoxic conditions even though after anoxic growth, no cytochromes of the *a*-type were present. This peculiar finding led to the assumption that an enzyme with oxidase activity develops under anoxic conditions which is capable of transferring electrons to oxygen under subsequent aerobic conditions. This enzyme was designated cytochrome *o* and is referred to as an alternative oxidase. Although cytochrome *o* had not been included in schemes for respiratory pathways of denitrifying organisms at that time, it had been detected in membrane extracts from the nitrate reducing organism *E. coli*, grown under both anoxic and aerobic conditions by Gray *et al.* (1966). Lam and Nicholas (1969c) found that a constitutive cytochrome oxidase is either the major or sole cytochrome oxidase which develops in *Pa. denitrificans* under limited oxygen conditions.

Sapshead and Wimpenny (1972) grew *Pa. denitrificans* under both aerobic and anoxic conditions with nitrate present. Under anoxic conditions, cytochrome *o* was the only oxidase synthesized, whereas under aerobic conditions both cytochrome aa_3 and cytochrome *o* were produced and three to four times more cytochrome *o* was synthesized under anoxic than aerobic conditions. This indicates that cytochrome *o* is constitutive and contrary to the results of John and Whatley (1975) with *Pa. denitrificans*, cytochrome aa_3 is not truly constitutive, but is an adaptive enzyme which may exist at very low concentrations under anoxic conditions.

Orientation of cytochrome oxidases aa_3 and *o*

The branching point to cytochrome aa_3 on the electron transport pathway for obligate aerobic organisms is considered to be at cytochrome *c*. Cytochrome *c* also serves as the branch point for cytochrome aa_3 in facultative denitrifying organisms as indicated in Fig C.9. However, the branching point proposed for the alternative oxidase, cytochrome *o* has changed as research into the ETP of facultative organisms progressed; from cytochrome *c* to cytochrome *b* and then to ubiquinone, in a manner similar to that in which the proposed branching point to nitrate reductase changed between these same complexes. To recap, Knobloch *et al.* (1971) and Jones *et al.* (1978) proposed cytochrome *c* as the immediate electron donor to cytochrome *o* and John and Whatley (1975) assigned the branch point to cytochrome *o* as the cytochrome c_1c complex (see Fig C.3). Van Verseveld and

Stouthamer (1978) and Stouthamer (1980) offered evidence based on work conducted on *Pa. denitrificans* with the inhibitor antimycin A that the branching point for cytochrome *o* is not at cytochrome *c* but at cytochrome *b* (see Fig C.4). These workers considered that the site of action of antimycin A was between cytochrome *b* and cytochrome *c*, and that it halted electron flow between these two cytochromes. Ferguson (1982), argued against cytochrome *c* as electron donor to cytochrome *o*, one reason being that Willison and John (1979) had shown that a mutant of *Pa. denitrificans* which lacked cytochrome *c* still was able to grow aerobically, due to the presence of an alternative respiratory pathway terminating in a "cytochrome *o*-like oxidase". Ferguson (1982) also argued against cytochrome *b* as electron donor to cytochrome *o*, because electron flow to cytochrome *o* is insensitive to antimycin (Alefounder *et al.*, 1981) and antimycin is a powerful inhibitor of electron flow through the cytochrome bc_1 complex. For these reasons, ubiquinone was proposed as the immediate electron donor to cytochrome *o* and is indicated as such in Fig C.6.

The question then arises as to why different oxidases develop under different environmental conditions. For an organism subjected to alternating aerobic and anoxic periods, the production of the constitutive enzyme cytochrome *o* under anoxic conditions would permit it to immediately begin respiring aerobically after it has been subjected to prolonged anoxic periods (during which time the aerobically inducible enzyme cytochrome aa_3 is degraded to very low or insignificant levels). Because aerobic respiration is energetically more favourable than anoxic respiration (see Chapter 4), a facultative organism possessing such an alternative oxidase would be placed at a competitive advantage against a facultative organism which did not possess this oxidase. The reason for the use and synthesis of cytochrome oxidase aa_3 can also be explained on an energetic basis – a greater energetic yield is achieved through the use of cytochrome aa_3 than cytochrome *o* and this aspect is described in in Chapter 4.

Conclusions

In facultative denitrifying organisms, two types of cytochrome oxidase are synthesized; an inducible cytochrome aa_3 complex which develops under aerobic conditions only, and a constitutive cytochrome *o* complex which is present under both anoxic and aerobic conditions. In the electron transport pathway, electrons branch to cytochrome aa_3 from cytochrome *c* and to cytochrome *o* from ubiquinone.

C.8 CLOSURE

The primary objective of this Appendix – to indicate the chronological research findings in the identification and placement of the various electron transferring complexes of the ETP– has resulted in the identification of an ETP which is used throughout the report as the archtypical one for facultative organisms. Such an ETP is of critical importance in the development of the biochemical model for facultative organism respiration in Chapter 5.

APPENDIX D

MECHANISMS OF REGULATION OF ANOXIC AND AEROBIC RESPIRATION

This review serves as an extended version of the review of the mechanisms of regulation of anoxic and aerobic respiration in Part II of Chapter 4. The mechanisms described here form the basis for the biochemical model of facultative organism respiration described in Chapter 5 and as such are of critical importance to the investigation. For this reason it was considered essential to provide a resource for the reader whereby they could evaluate the research findings pertinent to specific areas of the model and make an independent assessment of the information. The research findings are presented from a historical perspective in order that the significant findings are placed in context with other work of the time.

D.1 INTRODUCTION

D.2 MECHANISMS OF REGULATION OF ANOXIC RESPIRATION

Regulation by oxygen of the synthesis of denitrifying enzymes (aerobic growth conditions changed to anoxic conditions)

- *Organisms requiring both the absence of oxygen and the presence of nitrate for induction of nitrate reductase synthesis*
- *Organisms requiring only the absence of oxygen for induction of nitrate reductase synthesis*
- *Regulation of the synthesis of the denitrifying enzymes by oxygen and nitrate at the genetic level*
- *Development of denitrifying enzymes in activated sludge organisms grown in pure culture under aerobic conditions and transferred to anoxic conditions*

Mechanisms for regulation of synthesis and activity of denitrifying enzymes under anoxic conditions (anoxic growth conditions)

- *Nitrate reduction*
- *Nitrite reduction*
- *Nitric oxide reduction*
- *Nitrous oxide reduction*

A pattern in the inhibition of activity in the interactions between denitrification intermediates and reductases

- *Mechanism of inhibition of activity*

Regulation by oxygen of the activities of the denitrifying enzymes (anoxic growth conditions changed to aerobic conditions

- *Inactivation of the nitrogen oxide reductases through direct interference by oxygen*
- *Inhibition by oxygen of electron flow to the nitrogen oxide reductases as a result of changes in the redox potential of components of the electron transport pathway*
- *Prevention by oxygen of the insertion of the nitrate reductase subunits into the cytoplasmic membrane*
- *An effect of oxygen in changing the permeability of the cytoplasmic membrane, thereby regulating the movement of nitrate to nitrate reductase*

D.3 MECHANISMS OF REGULATION OF AEROBIC RESPIRATION

The mechanism of inhibition by nitrite (NO_2^-) of oxygen utilization

Nitric oxide (NO) as the inhibitory species

Which of the cytochrome oxidases (cytochrome aa_3 or cytochrome *o*) is inhibited by nitric oxide?

Explanation for the development of the alternative oxidase cytochrome *o*

D.4 AEROBIC DENITRIFICATION

Introductory remarks

Defining "aerobic conditions"

Aerobic denitrification in which the concentration of dissolved oxygen was not measured

Experimental artifacts responsible for aerobic denitrification

Aerobic denitrification in which the concentration of dissolved oxygen was measured

D.5 CLOSURE

D.1 INTRODUCTION

This review is primarily concerned with the mechanisms by which heterotrophic facultative organisms switch from the use of oxygen to nitrogen oxides as electron acceptor and vice–versa. Under aerobic conditions, facultative organisms use oxygen (O_2) as electron acceptor and under anoxic conditions the nitrogen oxides, nitrate (NO_3^-) and nitrite (NO_2^-).

As demonstrated in the preceding review of the biochemistry of facultative organisms, the aerobic and anoxic electron transport pathways are associated through many common electron transporting complexes. This review investigates the interactions between the aerobic and anoxic respiratory complexes during changes from aerobic to anoxic conditions and vice–versa, and examines the factors which initiate, regulate and terminate each of aerobic and anoxic respiration.

Although both anoxic and aerobic respiration are investigated, the majority of work investigates the effect of oxygen on anoxic respiration. As will become evident as the review progresses, the mechanisms of regulation of aerobic respiration originated from work conducted on mechanisms of regulation of anoxic respiration.

As an introduction to the mechanisms of regulation of anoxic and aerobic respiration, it is interesting to examine the course of change in perception regarding the respiratory process of denitrification, starting with the first observations of the loss of nitrogen from soils in the late 1800s which were attributed to chemical processes to the realization that the process is biological and nitrate and nitrite act as electron acceptors.

Because it was recognized that nitrate was a plant fertilizer, the loss of nitrate as gaseous nitrogen drew scientific attention in the late 1800s and steps were taken to investigate means of limiting its loss from soil. It was recognized also that proper aeration of soil limited nitrogen loss and consequently early experimental work was concerned primarily with the effect of oxygen on limiting the process of denitrification.

In the first systematic study of denitrification in 1867, Smith described the disappearance of the nitrogen from nitrate in organic–rich waters as being in one of the gaseous forms, ammonia or nitric oxide. Schloesing (1868) suggested that the liberation of nitrogenous gas from wine and tobacco juice was a result of the

reduction of nitrate, and not the oxidation of ammonia and this result was confirmed by Pasteur in the same year. However the conversion of nitrate to gaseous nitrogen forms was ascribed by all these workers to chemical and not microbiological processes.

Schoenbein (1868) working with soil denitrification was the first to attribute the loss of nitrate as gaseous products to micro-organisms and Meusel (1875) supported this proposal with a mechanism by which nitrate served as an oxidant, the atoms of nitrate being used for combustion in the same way that oxygen is used under aerobic conditions. With the wisdom of hindsight, it seems strange that it took a number of years to implicate bacteria in the *loss* of nitrate from soil, given that the *production* of nitrate in soil (i.e. nitrification) was already considered a bacterial process. Undoubtedly, one of the reasons for reluctance to accept denitrification as a bacterial process was that it was difficult to imagine that the aerobic environment required for the production of nitrate through nitrification would also be a suitable environment for the loss of nitrate through denitrification. This anomaly was partially resolved by the hypothesis that aerobic and anoxic microsites could exist side by side in soil, permitting the existence of both processes.

Although the majority of experiments concerned with the loss of nitrogen were conducted with soils, the term "denitrification", coined by Gayon and Dupetit (1882) originated from work conducted on the bacterial removal of nitrogen from sewage. In one of the first microbiologically correct series of experiments, these workers observed the biological production of nitrite, nitrous oxide and dinitrogen in nitrified sewage passed through sand columns in the absence of oxygen. Additionally, they noted that a fraction of the nitrate nitrogen was assimilated into the cells of the denitrifying bacteria.

At this point it is appropriate to indicate the dilemma facing microbiologists investigating denitrification. Whereas agronomists of the time required knowledge of the mechanisms of denitrification in order to try to limit the loss of nitrogen from soils, sanitary engineers saw the benefits of the process for the removal of nitrogen from sewage and sought to exploit it to the maximum.

In a major accomplishment in 1886, Gayon and Dupetit isolated pure cultures of two strains of denitrifying bacteria, but as with other investigators of the same period assumed that the removal of nitrate in the form of a more reduced gaseous

nitrogen oxide was a consequence of the use for normal aerobic respiration of the oxygen atoms separated from nitrate. This misconception was further entrenched following the work of Weissenberg (1902), the first worker to make the assertion that in situations in which molecular oxygen was depleted, denitrification was then effected by normal aerobic bacteria that used the oxygen in nitrate in lieu of molecular oxygen. It was not until the work of Kluyver and Donker (1926) and Barritt (1931) that the perception of the role of nitrate and nitrite in denitrification changed, from one in which the oxygen atoms are removed and used in the normal aerobic respiratory pathway, to one in which the nitrate or nitrite ion act as an electron acceptor and is reduced.

Subsequent work in denitrification was oriented toward examining aspects concerning the nitrogen oxide intermediates of the denitrification pathway, firstly with regard to the existence or not of nitrous oxide and nitric oxide, secondly to establish the presence of the enzymes which transfer electrons from substrates to each of the intermediates and thirdly to examine the effect of oxygen on the function of the enzymes.

Descriptions of the inhibitory effect of oxygen on denitrification remained qualitative until the mid 1900s because of the lack of technology to accurately measure oxygen levels. Sacks and Barker (1949), Marshall *et al.* (1953), Kluyver and Verhoeven (1954) and Collins (1955) concluded or accepted that oxygen affects nitrate reduction or denitrification in one or two ways; by suppressing the formation of the enzyme systems and/or by directly interfering with the action of the enzyme systems after formation in the bacteria. Although these workers observed and in some cases could measure the effect of oxygen on denitrification, a lack of knowledge concerning the components of the electron transport pathways to nitrate and oxygen prevented them from making hypotheses concerning the exact mechanism.

With advances in the purification (i.e. separation from cellular matter) and characterization (i.e. analysis of structure and reactivity) of the nitrogen oxide (i.e. NO_3^-, NO_2^-, NO, N_2O) reductases along with the correct orientation of these reductases in the denitrification pathway (see Appendix C), later workers were able to propose mechanisms for inhibition of denitrification by oxygen.

D.2 MECHANISMS OF REGULATION OF ANOXIC RESPIRATION

It is generally accepted that inhibition by oxygen of denitrification occurs in two ways; oxygen affects both the *synthesis* and the *activity* of denitrifying enzymes. *Synthesis* refers to the formation of the enzyme by the cell i.e. genetic expression for protein composition, incorporation of the protein subunits into the membrane, and continued production of the enzyme. *Activity* refers to the ability of the enzyme to pass electrons to the electron acceptor specific to that enzyme.

In the investigations described below into the effect of oxygen on denitrifying enzymes, workers examined two situations, (i) growing the organisms under aerobic conditions and transferring them to anoxic conditions and, (ii) growing the organisms under anoxic conditions and transferring them to aerobic conditions. This is an exceedingly fortuitous approach as far as this investigation is concerned, because, in activated sludge systems removing nitrogen and phosphorus, or nitrogen only, organisms are subjected to exactly these conditions, in moving between aerated and unaerated zones.

The manner by which *synthesis* of enzymes is affected by oxygen was usually determined in experiments in which organisms were grown under aerobic conditions under which little or no denitrifying enzymes developed, the conditions were then changed to anoxic, the subsequent development of the enzymes being monitored. The manner by which the *activity* of the denitrifying enzymes was affected by oxygen was determined by growing the organisms under anoxic conditions, such that their denitrifying enzymes were fully developed and then changing the conditions to aerobic and measuring the extent to which denitrification is affected through inactivation of the enzymes. In many facultative organisms, the denitrifying enzymes are not constitutive under aerobic conditions, and are degraded with time. Although changing the growth conditions from anoxic to aerobic provided information on the activity of the enzymes, by monitoring such systems over longer periods, workers gained information concerning the effect of oxygen on the degradation and synthesis of enzyme systems.

The approach of this review follows the general experimental approach of investigations into the effect of oxygen on denitrification. The mechanisms hypothesized as being responsible for this effect are described below and are reviewed in that order.

(1) Regulation by oxygen of the *synthesis* of the denitrifying enzyme systems.

(2) Regulation by oxygen of the *activity* of the denitrifying enzymes by:

(i) direct interference with their action irrespective of whether they are constitutive or partially formed inducible enzymes.

(ii) diverting the flow of electrons from one respiratory system (anoxic) to another (aerobic) or vice–versa via the redox state of components of the respiratory system.

(iii) prevention of the insertion of the nitrate reductase subunits into the cytoplasmic membrane.

(iv) affecting the permeability of the cytoplasmic membrane, thereby regulating nitrate movement to its reductase.

<u>Regulation by oxygen of the synthesis of denitrifying enzymes</u>

(Aerobic growth conditions changed to anoxic conditions)

In Appendix C it was established that the enzymes of the denitrification pathway are absent or at low levels under aerobic conditions. In this Appendix, the conditions required to overcome repression of the denitrification enzymes and induce their synthesis are described.

In studies conducted on various facultative aerobic organisms grown under aerobic conditions and then subjected to anoxic conditions, some differences have been reported concerning the conditions required for the development of the various denitrifying complexes of the respiratory chain. The major difference between organisms concerned the requirement or not for the presence of the nitrogen oxides for induction of the denitrifying enzymes.

The majority of research has been concerned with the induction of nitrate reductase. Little information is available with regard to the effect of oxygen and the nitrogen oxides on the induction of nitrite–, nitric oxide–, and nitrous oxide reductase, and where reference is made to the reductases other than that for nitrate, it is usually as a consequence of work conducted on nitrate reductase.

Organisms requiring both the absence of oxygen and the presence of nitrate for induction of nitrate reductase synthesis

- *Bacillus stearothermophilus (B. stearothermophilus)*

Downey (1966) reported that nitrate was required for growth under anoxic conditions for *B. stearothermophilus* and that although oxygen at *low* levels did not retard *induction* of the enzymes, enzyme synthesis was considerably lessened by aeration. In further work by Downey *et al.* (1969), on the same organism, grown aerobically with nitrate, low levels of nitrate reductase were reported. When the culture was switched to anoxic conditions, no immediate denitrification was exhibited but after a brief lag period (≈ 1 hr) growth of the organism and production of nitrate reductase increased and denitrification was established. Nitrite reductase formation in the organism was found to be more sensitive to oxygen than nitrate reductase formation and under anoxic conditions was induced sequentially to nitrate reductase after the addition of nitrate.

- *Klebsiella aerogenes (K. aerogenes)*

Pichinoty and d'Ornano (1961 b,c) found that for *K. aerogenes*, aeration repressed production of the nitrate reducing enzyme and under anoxic conditions nitrate was required to induce its formation.

- *Paracoccus denitrificans (Pa. denitrificans)*

Lam and Nicholas (1969a) studied aerobic and anoxic respiration in *Pa. denitrificans* (formerly *Micrococcus denitrificans*) and found that cells grown aerobically and containing very little nitrate reductase, and no nitrite reductase, when transferred to anoxic conditions containing NO_3^- developed rapid nitrate reductase activity within 2–4 hours and developed rapid nitrite reductase activity within 4–6 hours. The result indicated that for *Pa. denitrificans*, nitrate reductase formation required both derepression (the absence of O_2) and specific induction (the presence of NO_3^-), but regarding the formation of nitrite reductase, no conclusions regarding the necessity for induction by NO_2^- could be made because nitrite is formed as a consequence of nitrate reduction and its presence may have induced nitrite reductase formation. To determine the requirement for NO_2^- in induction of nitrite reductase, cells of *Pa. denitrificans* grown aerobically and transferred to anoxic conditions containing NO_2^- but not NO_3^- exhibited high nitrite reductase activity but low nitrate reductase activity even after 30 hours. These results indicated that nitrate– and nitrite reductase formation require both the absence of oxygen and the presence of NO_3^- and NO_2^- respectively.

It is not only the cytochromes contained in the nitrogen oxide reductases whose synthesis is altered in the absence of oxygen. In studies conducted on nitrite reductase of *Pa. denitrificans*, Lam and Nicholas (1969c) examined the effect of oxygen on the formation of that enzyme and on the central cytochrome *c*. The formations of nitrite reductase and cytochrome c were repressed by oxygen and in cells grown with decreasing concentrations of oxygen, the concentration of cytochrome c increased proportionally with that of nitrite reductase activity. (Given the benefit of knowledge gained since these experiments were conducted, cytochrome *c* is directly oxidized by nitrite reductase during electron transfer in the denitrification pathway, and it is understandable that nitrite reductase activity could be proportional to the concentration of cytochrome *c* present).

Results of work concerned with the effect of a lack of oxygen on cytochrome development was reported by Sapshead and Wimpenny (1972) who found that *Pa. denitrificans* grown anoxically with nitrate did not synthesize the aerobic enzyme cytochrome aa_3 but had a higher content of the *cd*-type cytochromes than cells grown under aerobic conditions. Levels of cytochromes (i.e. types cytochrome aa_3 and cytochrome *cd*) grown under low concentrations of oxygen were intermediate between those which developed in bacteria grown under aerobic conditions and under anoxic conditions with nitrate. This led the authors to conclude that oxygen is of greater importance than nitrate in determining the type and amount of cytochromes present in cells grown under various concentrations of oxygen.

The studies on *Pa. denitrificans* reviewed above illustrate the importance of both the presence of nitrate and the absence of oxygen in inducing the formation of nitrate reductase. The concentration of DO is of primary importance in this regard, since a high concentration of oxygen (i.e. saturation conditions) invariably leads to suppression of the formation of the enzyme irrespective of the presence or absence of nitrate. However, as the level of DO falls, the relative importance of nitrate in induction of enzyme formation increases such that at a very low DO or under anoxic conditions the presence of nitrate is of primary importance.

- *Escherichia coli (E. coli)*

In studies conducted on *E. coli*, Showe and de Moss (1968) concluded that under aerobic (oxygen saturation) conditions synthesis of the nitrate reducing enzyme was independent of the presence or absence of nitrate since it was synthesized at a very

low (basal) level irrespective of the presence or absence of nitrate. Under aerobic conditions, the synthesis of nitrate reductase was repressed to its basal level. However, at concentrations of oxygen lower than saturation but at which enzyme formation was still completely repressed, the presence of nitrate induced the formation of nitrate reductase. The authors concluded that the rate of synthesis of the nitrate reducing enzyme in *E. coli* could be increased by

(i) derepression, i.e. a shift from aerobic to anoxic growth,
or
(ii) induction, i.e. the addition of nitrate to anoxic growth conditions,
or
(iii) a combination of the two situations above, i.e. a shift from aerobic conditions to anoxic conditions with nitrate present.

In each of the above transitions, synthesis of nitrate reductase was marked by two *rates, an initial rapid rate followed by a slower rate, the significance of which is described below. Concerning the levels of nitrate reducing enzyme in studies conducted with E. coli* by Showe and de Moss (1968), it was found that in highly aerated cultures, enzyme levels were 2–4% of those observed under anaerobic conditions in the absence of nitrate. Enzyme levels increased under anaerobic conditions but with nitrate present were 20 times greater than in the absence of nitrate. (Thus, the enzyme levels were between 500–1000 times greater under anoxic conditions with nitrate present, than under aerobic conditions). The researchers concluded that nitrate reductase synthesis was controlled by a redox–sensitive repressor, sensitive not only to nitrate, but also to the effective intracellular redox potential. This redox potential is a function not only of the potential oxidants (e.g. O_2, NO_3^-) and reductants (e.g. NADH) in the cell, but also of the catalytic capabilities of the cell for electron flow, (i.e. the catalytic components of the denitrification pathway preceding nitrate reductase). This aspect of redox control in denitrification is discussed further in a later section which investigates the role of redox potential of components of the denitrification pathway in the activity of the nitrogen oxide reductases. (It is interesting to note that Wimpenny and Cole (1967) in studies on *E. coli* argued for the controlling factor in nitrate reductase formation as being the redox potential of the medium). In a review of control of synthesis of nitrate reductase, Stewart (1988) suggests that the results of Showe and De Moss (1968) indicate that control by nitrate reductase and oxygen are separate, but the effect of oxygen is dominant. Ruiz–Herrera and

Salas–Vargas (1976) examined also the effect of oxygen and nitrate on nitrate reductase synthesis. They confirmed the results of Showe and De Moss (1968) that synthesis of nitrate reductase is biphasic but concluded that the redox sensitive repressor did not affect the synthesis of the enzyme itself but affected messenger ribo–nucleic acid (mRNA) synthesis, since in a shift from aerobic to anoxic conditions with nitrate present the synthesis of mRNA was also biphasic.

- *Alcaligenes faecalis (A. faecalis)*

Concerning the requirements for the induction of nitrite reductase in the denitrifying bacterium *A. faecalis*, Kakutani *et al.* (1981) demonstrated that nitrite reductase was only partly derepressed by low oxygen concentration, and full induction of nitrite reductase required both low oxygen concentration and the presence of nitrate or nitrite, but most probably nitrite as the major inducer.

- *Flavobacterium* sp.

Firestone and Tiedje (1979) investigated the synthesis of the denitrifying enzymes nitrate– and nitrous oxide reductase in a *Flavobacterium* sp. transferred from aerobic to anoxic conditions. Enzymes were synthesized sequentially in the same order as the denitrification sequence, i.e. from nitrate reductase to nitrous oxide reductase, sequentially. The synthesis of nitrate reductase before nitrous oxide reductase led to an initial high level of N_2O which decreased with time as a result of the sequential formation of nitrous oxide reductase. The authors concluded that the formation of nitrous oxide reductase was induced either by NO_3^- or by the N_2O produced by reduction of NO_3^- but could not conclude which was responsible. These workers did not make mention of the other denitrifying enzymes, nitrite– and nitric oxide reductase.

- *Pseudomonas* sp.

As an example of the diversity of conditions which lead to induction of the reductases, Aida *et al.* (1986) found that the conditions necessary for induction of nitrate reductase and nitrous oxide reductase in a denitrifying *Pseudomonas* sp. were neither aerobic nor anoxic but microaerobic, irrespective of the presence or absence of nitrate or nitrous oxide. The authors concluded that the dependence on microaerobiosis for the formation of these reductases was due to an inability to produce energy anoxically until the anoxic respiratory enzymes were formed.

Organisms requiring only the absence of oxygen for induction of nitrate reductase synthesis

For the organisms described above (grown under aerobic conditions and transferred to anoxic conditions), initiation of nitrate reductase synthesis required both the absence of oxygen (derepression) and the presence of nitrate (induction). However, for other organisms (also grown under aerobic conditions and transferred to anoxic conditions) initiation of nitrate reductase synthesis required only repression by the absence of oxygen. Induction by nitrate was not required. As mentioned above, for the experiments described below, the necessity for the presence of nitrogen oxides other than nitrate in the induction of their respective nitrogen oxide reductases was not usually tested directly, but originated as a consequence of experiments with nitrate and nitrate reductase.

- *Bacillus licheniformis* (*B. licheniformis*)

Schulp and Stouthamer (1970) found that nitrate reductase formation was induced under anoxic conditions in the absence of nitrate in *B. licheniformis*, although the addition of nitrate did cause a 2½ fold increase in nitrate reductase activity.

- *Haemophilus parainfluenzae* (*H. parainfluenzae*)

Sinclair and White (1970) also found that nitrate was not necessary for the development of nitrate and nitrite reductase activities in *H. parainfluenzae* at low oxygen concentrations, although the activities of enzymes in cells grown at low oxygen concentrations with nitrate absent were about half those in cells grown at low oxygen concentrations with nitrate present.

- *Proteus mirabilis* (*P. mirabilis*)

In experiments conducted on *P. mirabilis*, de Groot and Stouthamer (1970b) found that nitrate reductase was subjected to repression and inactivation by oxygen, but when a culture of the organism growing aerobically was shifted to anoxic conditions, the formation of the enzyme was derepressed even in the absence of nitrate.

- *Pseudomonas perfectomarinus* (*Ps. perfectomarinus*)

Payne *et al.* (1971) grew *Ps.perfectomarinus* aerobically and observed the repression of denitrifying enzymes. Under conditions in which oxygen was absent, the bacteria synthesized all the denitrifying enzymes (i.e. separate nitrate, nitrite, nitric oxide and nitrous oxide reducing fractions) within 3 hours, even in the absence of the nitrogen oxides. However the authors did not state to what proportion of the

maximum attainable level the enzymes were synthesized, except that, when given a 3h anoxic incubation period, the aerobically grown bacteria were able to reduce nitrite at only 40% of the rate exhibited by bacteria grown anoxically with nitrate. Payne (1973) compared the effect of oxygen on the nitrogen oxide reductases, finding that nitrous oxide reductase is less strongly repressed by oxygen than the other reductases.

- *Pseudomonas stutzeri* (*Ps. stutzeri*)

Because the organisms described above were found not to require nitrate to induce formation of nitrate reductase, greater attention was paid to the derepressive effect of the absence of oxygen on the formation of nitrate reductase than the other nitrogen oxide reductases. Kodama (1970) investigated the effects of aerobic, semi-aerobic (low concentrations of dissolved oxygen) and anoxic conditions on the cytochrome systems of *Ps. stutzeri* with respect to the effect of nitrate and nitrite on their respective cytochromes and other cytochromes of the denitrification pathway. It was found that under aerobic conditions, synthesis of all the denitrification cytochrome components was repressed, regardless of the presence or absence of nitrate or nitrite. Under semi-aerobic conditions, cytochrome *c* and cytochrome *b* of main denitrification pathway increased in the absence of nitrate and nitrite. Under anoxic or semi-aerobic conditions, nitrite induced nitrite reductase, while nitrate repressed synthesis of this enzyme.

From these results it can be concluded that although many organisms required only the absence of oxygen to induce formation of nitrate reductase, maximum levels of the enzyme were synthesized only in the presence of nitrate.

Although the greater part of this review has been dedicated to examination of the biochemical and genetic mechanisms by which oxygen and nitrogen oxides regulate formation of denitrifying enzymes, of more interest to this investigation is how these mechanisms apply to denitrifying organisms found in activated sludge.

Regulation of the synthesis of the denitrifying enzymes by oxygen and nitrate at the genetic level

In the section above describing the relationships between the denitrification intermediates and reductases, it was difficult to determine from the researchers work whether the action of the intermediates resulted in changes in the activity or synthesis of the reductases. Since the nett result of these mechanisms is determined

by measuring the rate of reduction of the nitrogen oxide intermediates, the effect of the intermediates on the nitrogen oxide reductases was attributed to changes in enzyme activity.

Little experimental work has been conducted on the effect at the genetic level of the nitrogen oxide intermediates on enzyme synthesis – most work has been concerned with hypothetical mechanisms for this effect and has been directed at nitrate reductase only.

In the foregoing analysis of the effect of oxygen and nitrogen oxides on the synthesis of the denitrifying enzymes, the development or lack of development of these enzymes in the presence of oxygen and absence of nitrate was referred to simply as induction or repression of formation. Because these means of regulation could not be explained using simple biochemical mechanisms, researchers were led to investigate these phenomena at the level of genetic expression of the reductases. The organism *E. coli* is one of the most studied organisms at a biochemical level particularly as far as nitrate reduction is concerned. As a result it has lent itself well to genetic studies concerned with oxygen regulation of nitrate reduction.

Several studies have concluded that control of nitrate reductase synthesis by nitrate and oxygen is at the stages of transcription and translation of gene expression. Many different mechanisms have been proposed by which such control could be executed. Ruiz–Herrera and Salas–Vargas (1976) interpreted results gained with *E. coli* shifted between aerobic and anoxic conditions as evidence that oxygen (and possibly nitrate) affect simultaneously the transcriptional and translational processes involved in nitrate reductase synthesis. In a review of nitrate respiration, Stewart (1988) concluded that biochemical pathway studies with *E. coli* such as those by Showe and de Moss (1968) indicated that nitrate and oxygen are independent regulators of nitrate reductase gene expression.

Concerning the role of nitrate, work by Stewart (1982) and Li and de Moss (1987) provided evidence that nitrate acts to induce transcription of the genes regulating nitrate reductase synthesis.

Concerning the role of oxygen in repression of nitrate reductase synthesis, Stewart (1988) states that a single regulatory protein which responds to changes in redox states induced by the presence or absence of oxygen could affect nitrate reductase

gene expression. Kaprálek *et al.* (1982) found that oxygen controlled the synthesis of nitrate reductase in *E. coli* at two sites. Oxygen appeared to inhibit the synthesis of nitrate reductase at the level of transcription and in addition, later steps (possibly translation) were found to be blocked.

To explain the effects of oxygen and nitrate at the level of genetic control of nitrate reductase synthesis, Stewart (1988) concluded that two different mechanisms are required, one for enzyme induction by nitrate and one for enzyme repression by oxygen. Enzyme induction and repression are controlled by the action of specific regulatory proteins called *activators* and *repressors* respectively, which allow or prevent mRNA synthesis (transcription) by binding to DNA sequences called *operators* located near the beginning (i.e. the *promoter*) of the structural genes which specify the synthesis of the enzyme. The mechanism by which the regulatory proteins become either activators or repressors is a result of the affinity of the regulatory proteins for the operators which in turn is a consequence of the effect of the activator molecule (nitrate) or the repressor molecule (oxygen) on the regulatory proteins. Since oxygen and nitrate have opposite effects on enzyme synthesis (inductive and repressive respectively) their effect on the regulatory proteins is also different.

- *Induction and repression of enzyme synthesis*:
  Aerobic conditions: Stouthamer (1988) reviews mechanisms for the regulation and induction of synthesis of nitrate reductase in which nitrate acts as inducer and both oxygen and nitrate act as repressors of nitrate reductase synthesis. The regulatory gene for nitrate reductase synthesis specifies a regulatory protein which normally acts to repress nitrate reductase synthesis. The regulatory protein operates differently in the presence and in the absence of oxygen and is called a repressor. When oxygen is present, it binds to the regulatory protein which undergoes a conformational change enabling it to bind to the operator sequence of the DNA and thereby inhibit the binding of RNA polymerase to the promoter. In that condition the structural gene for nitrate reductase synthesis cannot be specified.

  Anoxic conditions: In the absence of oxygen, the regulatory protein does not undergo a conformational change and thus is unable to bind to the operator sequence of the DNA. The promoter is not inhibited in binding to RNA polymerase and the structural gene for nitrate reductase synthesis can be

specified. Nitrate reductase is then synthesized. In the absence of oxygen, a second regulatory mechanism for enzyme synthesis (autogenous regulation) is functional and operates according to the presence and absence of nitrate.

The mechanism of control of enzyme induction by autogenous regulation was described by Pateman and Cove (1967) who identified a gene which specifies the regulatory protein described above. By itself, this protein functions as an activator for a group of genes which includes the gene for nitrate reductase synthesis. Concerning the mode of action of the regulatory protein, there are two conditions to consider; the absence of nitrate and the presence of nitrate.

(1) In the absence of nitrate, free nitrate reductase converts the protein through a change in physical form (conformation) from an activator to a repressor for a specific group of genes – one of the genes specifies nitrate reductase synthesis and with that gene repressed, the synthesis of nitrate reductase is prevented or reduced.

(2) In the presence of nitrate, nitrate reductase is bound to the nitrate molecule and cannot convert the regulatory proteins from an activator to a repressor of gene specification. Consequently, the gene is activated and nitrate reductase is synthesized. The concentration of nitrate decreases as a result of oxidation by the additional nitrate reductase and free nitrate reductase is then available to convert the regulatory protein from an activator to a repressor, i.e. a return to state (1). Thus, as intracellular nitrate concentrations increase or decrease due to corresponding changes in extracellular nitrate concentrations, nitrate reductase synthesis will be activated or repressed to accommodate these changes. These models were hypothesized to explain the regulation of nitrate reductase synthesis only although Cove and Pateman (1969) proposed that in *Aspergillus nidulans* nitrate reductase acts by this mechanism to control the rate of synthesis of nitrate reductase as well as the rate of synthesis of nitrite reductase. Due to the apparent complexity of interactions between the many intermediates and reductases, mechanisms for synthesis of the other nitrogen oxide reductases have not yet been proposed. However it would seem likely that the absence of oxygen is also responsible for derepression of synthesis of the other nitrogen oxides, since Payne (1973) found with *Ps. perfectomarina* that synthesis of all the denitrifying enzymes began simultaneously when an

aerobic culture of the organisms was shifted to anoxic conditions.

Development of denitrifying enzymes in activated sludge organisms grown in pure culture under aerobic conditions and transferred to anoxic conditions

This review thus far has been limited to specific types of bacteria in its examination of the conditions which repress and initiate denitrifying enzyme formation. Generally the same bacteria are selected for study by different research groups because of the benefit of accumulated knowledge. Because of uncertainty that the features described for these organisms are found in activated sludge organisms generally, an element of caution must be introduced when applying observations made for bacteria grown in pure culture to organisms which develop in activated sludge mixed culture. This is not only for the reason that very few of the organisms common to activated sludge have been reviewed here, but also because organisms in mixed culture behave differently to those in pure culture. Consequently, if it can be shown that bacteria isolated from activated sludge show a similar denitrification behavioural pattern to the specific bacteria studied, then we can be more confident in applying the denitrification behaviour of specific bacteria denitrification behaviour to a mixed culture like activated sludge. Some very significant work has been conducted on denitrifying organisms taken from activated sludge.

In this section, results are reviewed of two sets of experiments concerning the effect of oxygen on denitrification by organisms in pure culture isolated from activated sludge.

The first set of experiments was conducted in two parts.

- In the first part, Krul and Veeningen (1977) examined the effect of aerobic cultivation on nitrate reductase formation for 24 denitrifying bacteria isolated from aerobic and denitrifying sludges and grow in laboratory-scale systems under aerobic (DO $>$ 4.0 mgO/ℓ) and anoxic (DO = 0 mgO/ℓ conditions.

  The effect of aerobic conditions on the formation of nitrate reductase was examined by growing the bacterial cultures under aerobic conditions and measuring the rate of nitrate reduction of cell-free extracts of the bacteria under anoxic conditions. The maximum possible synthesis of nitrate reductase was determined by growing the bacterial cultures under anoxic conditions and measuring the rate of nitrate reduction of cell-free extracts of the bacteria under

similar separate anoxic conditions. The authors assumed that the rate of nitrate reduction was due to the degree of synthesis of nitrate reductase. The formation of nitrate reductase after growth under aerobic conditions varied from total repression in some bacteria to near non-repression in other bacteria. Although not stated it is implied that the authors considered that testing cell-free extracts rather than whole-cell suspension ensured that the rate of nitrate reduction was a function of the level of nitrate reductase only and not a function of nitrate reductase activity which, as will be discussed in a later section, is affected by the presence of other components of the bacterial cell which restrict the movement of nitrate to its reductase.

- In the second part of the first set of experiments conducted by Krul and Veeningen (1977), the bacterial type which exhibited the greatest nitrate reduction rate after growth at DO > 2,0 mgO/ℓ was tested for the effect of DO on nitrate reductase synthesis by growing that bacterial type at four different concentrations of DO, ranging from anoxic conditions to conditions with a DO concentration > 15 mgO/ℓ. The rate of nitrate reduction decreased as the concentration of DO increased; the nitrate reduction rate by cell-free extracts grown under anoxic conditions was five times the rate of cell-free extracts of the same organism grown under aerobic conditions at a DO concentration > 15 mgO/ℓ.

In the second set of experiments, Simpkin and Boyle (1988) conducted similar tests to those described above, the major difference being that rather than determining the activity of nitrate reductase from activated sludge organisms grown in pure culture, the activity was determined of nitrate reductase in the complete organism mass of activated sludge grown under various aerobic/anoxic conditions. As in the determinations of Krul and Veeningen (1977), the workers equated the rate of nitrate reduction in cell-free extracts of the sludge mass with the degree of synthesis of nitrate reductase. The maximum synthesis of nitrate and nitrite reductases (potential nitrate and nitrite reductase activity) was measured by examining the rates of nitrate and nitrite reduction under anoxic conditions of sludge grown under various aerobic/anoxic conditions (from < ½ hour to 4 hours anoxic period in a 6 hour cycle), the sample then held anoxic for 3 hours with nitrate present. The synthesis of nitrate and nitrite reductases under aerobic conditions (expressed nitrate and nitrite reductase activity) was measured by examining immediately after sampling the rates of nitrate and nitrite reduction under anoxic conditions with

nitrate present.

The results of the experiments indicated that the synthesis of nitrate and nitrite reductases were no more than 50% repressed in all of the anoxic/aerobic systems and the authors concluded that the levels of nitrate and nitrite reducing enzymes did not appear to go through significant levels of decay and formation in organisms passing through aerobic and anoxic cycles in an activated sludge system. However a difference of 50% in the degree of enzyme synthesis would appear to be quite significant if the rate of nitrate reduction is directly related to the degree of enzyme synthesis. A problem with the experimental method which may have led to measurement of lower potential reductase activity was that in measuring this parameter the sludge was held anoxic for only 3 hours after sampling. It is likely that some of the organisms contained in the sludge mass required a considerably longer period to synthesize their nitrate and nitrite reductase enzymes to the maximum levels. The authors conclusions regarding the experimental results were that the changes in synthesis of nitrate and nitrite reductases between aerobic and anoxic conditions were not sufficient to account for the changes in the rate of nitrate and nitrite reduction between aerobic and anoxic conditions. They concluded that the changes in the rate of nitrate and nitrite reduction measured in their experiments could be accounted for only by assuming that oxygen affects the activity rather than the synthesis of the nitrate and nitrite reductases.

Considering the results of Krul and Veeningen (1977) and Simpkin and Boyle (1988) with activated sludge, it is apparent that nitrate and nitrite reductase synthesis in activated sludge organisms is affected sufficiently by exposure to aerobic conditions to influence the rate of nitrate and nitrite reduction in anoxic/aerobic activated sludge systems.

The work of both research groups demonstrates that variations in DO concentration from aerobic to anoxic levels influence a mixed culture of organisms in a similar way to that observed above for specific organisms in pure culture, if not so much in degree then at least in trend. Consequently, in so far as denitrification behaviour is concerned it is reasonable to accept the results of the bacterial pure culture studies as providing a framework within which to assess experimental results obtained with mixed culture bacterial populations.

Mechanisms for regulation of synthesis and activity of denitrifying enzymes under anoxic conditions (anoxic growth conditions)

The work above demonstrates the control exerted by oxygen on the synthesis of denitrifying enzymes when organisms grown under aerobic conditions are exposed to anoxic conditions. It is important to consider the mechanisms which control the activity of denitrification enzymes under anoxic conditions.

Under anoxic conditions and when substrate is not limiting, the denitrification rate of an organism is a function of the level of denitrifying enzyme present, the activity of the enzyme and the amount of nitrogen oxide available for reduction. It was shown that synthesis of a nitrogen oxide reductase is stimulated by its own nitrogen oxide, i.e. the presence of nitrate stimulates the synthesis of nitrate reductase. This occurs under conditions in which an organism is switched from aerobic to anoxic conditions with a nitrogen oxide present, and also under continuous anoxic conditions with a nitrogen oxide present. A factor which has not yet been considered is the effect (inhibitory or productive) of the intermediates of the process of denitrification, on the activity of enzymes which are not specific for the reduction of those intermediates, e.g. the inhibitory effect of nitrite on the activity of nitrate reductase. Because there are four denitrification intermediates including nitrate, and a reductase specific to each of these intermediates, the number of possible interactions between intermediates and reductases is considerable. Consequently, analysis of the literature is difficult and is made more complex by contradictory results between workers. The manner in which each of the reductases is affected by each of the intermediates is considered, except for cases where these interactions have not been documented.

Nitrate Reduction

Fewson and Nicholas (1961b) and Lam and Nicholas (1969b), working with *Pa. denitrificans* found that nitrate induced the formation of nitrate reductase. However they were unable to come to a conclusion regarding the effect of nitrate on the activity of nitrate reductase because of uncertainty regarding the effect of the other intermediates of denitrification formed by the reduction of nitrate. There is some disagreement between workers concerning the effect of nitrite on the activity of nitrate reductase. Kodama *et al.* (1969), De Groot and Stouthamer (1970a) and Kučera *et al.* (1983a) worked with *Ps. stutzeri*, *P. mirabilis* and *Pa. denitrificans* respectively, and found that the presence of nitrite had an inhibitory effect on the organisms ability to reduce nitrate. However, Showe and de Moss (1968) found that

nitrite did not repress nitrate reductase synthesis in *E. coli* and Meijer *et al.* (1979a) and Alefounder *et al.* (1983) found that nitrite did not inhibit the reduction of nitrate in *Pa. denitrificans*, the latter workers showing that nitrite stimulated nitrate reductase activity.

The intermediate about which there was uniform agreement concerning its inhibitory action was nitrous oxide. Alefounder *et al.* (1982), Kučera *et al.* (1983a) and Alefounder *et al.* (1983) all worked with *Pa. denitrificans* and found that nitrous oxide significantly inhibited nitrate reduction, and that the addition of antimycin or acetylene overcame this inhibition by preventing electron flow to nitrous oxide. This result indicated that a competition exists between nitrous oxide and nitrate for electron flow to their respective reductases. The inhibitory action of nitrous oxide on nitrate reductase was removed by the addition of nitrite, most probably through partitioning electron flow away from nitrous oxide reductase to the nitrogen oxide reductases earlier in the denitrification pathway, i.e. nitrate and nitrite reductase.

Nitrite Reduction

Lam and Nicholas (1969c), Kodama (1970) and Justin and Kelly (1978) found that nitrite induced the formation of nitrite reductase in *Pa. denitrificans*, *Ps. stutzeri and Thiobacillus denitrificans* respectively. There is some disagreement concerning the effect of nitrate on nitrite reductase activity. Payne *et al.* (1971) report that nitrate did not repress the rate of nitrite reduction in fractions from *Ps. perfectomarinus*, but Kodama *et al.* (1969) and Kodama (1970) reported that nitrate did inhibit the rate of nitrite reduction in *Ps. stutzeri*, in that nitrate was reduced by its reductase in preference to the reduction of nitrite by its reductase. Although Kakutani *et al.* (1981) with *A. faecalis* found that nitrate induced synthesis of the reductase, it was considered that this effect resulted from nitrite produced by reduction of nitrate, because nitrate failed to induce nitrite reductase synthesis in a nitrate reductase deficient mutant. It is likely that apparent induction of nitrite reductase by nitrate in other organisms (*Pa. denitrificans*, Newton, 1969; *Ps. stutzeri*, Kodama *et al.*, 1969, Kodama, 1970; *Achromobacter cycloclastes* [1], Iwasaki and Matsubara, 1972) was also due to nitrite produced from nitrate reduction. Shapleigh *et al.* (1987) found that the reduction of NO_2^- was inhibited by NO (which accumulated as a result of the inhibition by Triton X-100 of the reduction of NO to N_2O) in nitrite reducing extracts from *Achromobacter cycloclastes*, *Pa. denitrificans*, *Ps. aeruginosa* and *Ps. perfectomarinus*. Alefounder

[1]Unsure taxonomic classification.

et al. (1983) found that the addition of nitrous oxide to cultures of *Pa. denitrificans* strongly inhibited the reduction of added nitrite.

Nitric Oxide Reduction

Because of reluctance by many research groups to accept either nitric oxide as an intermediate in the pathway of denitrification or a specific nitric oxide reductase, little consideration had been given to the effect of the intermediates of denitrification on the reduction of nitric oxide. Payne and Riley (1969) showed that nitrate has an inhibitory effect on the activity of a nitric oxide reducing fraction from *Ps. perfectomarinus.* Payne *et al.* (1971) working on the same organism found also that nitrite inhibited nitric oxide reduction but that four to five times as much nitrite as nitrate was required under identical conditions to achieve the maximum inhibition of activity which was 60% of the total activity. Frunzke and Zumft (1986) found that *Ps. perfectomarina* grown with NO_3^- had a rate of NO reduction at least 3 times higher than when grown with N_2O, indicating that the activity of nitrogen oxide reductase is stimulated by the presence of nitrogen oxides which result in the generation of the nitrogen oxide specific to that reductase.

Nitrous Oxide Reduction

Payne *et al.* (1971) suggested that when nitric oxide is present in considerably greater excess than nitrous oxide, then nitrous oxide reduction is inhibited in an extract from *Ps. perfectomarinus.* Frunzke and Zumft (1986) found also that in *Ps. perfectomarina* nitrous oxide respiration was inhibited by nitric oxide.

A pattern in the inhibition of activity in the interactions between denitrification intermediates and reductases

A measure of caution is advised by Carlson and Ingraham (1983) in making generalizations regarding the effect of the intermediates of denitrification on each of the denitrification steps. Although comparisons of the denitrification behaviour of *Ps. stutzeri, Ps. aeruginosa* and *Pa. denitrificans* indicated a pattern in the *manner* in which the denitrification intermediates affect each reduction step, there are differences in the *degree* to which the denitrification intermediates affect each step. In particular, nitric and nitrous oxide reduction by *Ps. aeruginosa* was considerably more sensitive to inhibition by nitrite than either of the other two organisms tested, indicating the danger in using the behaviour of one denitrifying organism as representative of all denitrifying organisms. The results described above for the interactions between denitrification intermediates and reductases are presented in

Table 4.1. A pattern is apparent in the manner by which the denitrification intermediates affect the activity of the nitrogen oxide reductases.

Regarding the effect of the immediate and sequential products of denitrification on the activity of the reductases, a mechanism of inhibition of reduction via product formation occurs for each of the nitrate, nitrite, nitric oxide and nitrous oxide reductases, i.e, nitrite and nitrous oxide inhibit nitrate reduction, nitric oxide and nitrous oxide inhibit nitrite reduction, and nitrous oxide inhibits nitric oxide reductase.

Regarding the effect of the specific and the preceding intermediates on the denitrifying reductases, two cases are evident;

(1) For the reductases reducing the *ionic* nitrogen oxides, i.e. nitrate and nitrite reductase, the nitrogen oxide specific to that reductase has an inductive effect on the reductase, i.e. nitrate induces nitrate reductase activity, and nitrite induces nitrite reductase activity. Nitrite has an inactivating effect on nitrate reductase and nitrate has little effect on nitrite reductase.

(2) For the reductases reducing the *gaseous* nitrogen oxides, i.e. nitric oxide and nitrous oxide reductase, the nitrogen oxide specific to that reductase has an activating effect on the reductase whereas the preceding intermediates have an inactivation (inhibitory) effect on the reductase, i.e. nitric oxide and nitrous oxide activate nitric oxide reductase and nitrous oxide reductase respectively, but nitrate and nitrite inactivate nitric oxide reductase, and nitrite and nitric oxide inactivate nitrous oxide reductase.

Mechanism of inhibition of activity

The results indicate that inhibition of reductase activity occurs through competition for available electrons by terminal electron acceptors. As one intermediate builds up, the redox potential at the reductase specific to that intermediate is such that electron flow increases to the reductase until the concentration of that intermediate decreases as a result of reduction. For example, during the reduction of nitrate, the intracellular concentration of nitrite increases and electron flow then increases to nitrite reductase. The intracellular redox potential established by the build up of nitrite appears to have an inhibitory effect on the activity of nitrate reductase. The objective in examining the effect of denitrification intermediates on the nitrogen

oxide reductases was to apply the experimental results to practical situations in which denitrifying organisms in activated sludge are exposed to different concentrations of nitrate and nitrite under anoxic conditions. A problem with the experimental results is that they come from artificially manufactured situations in which the reductase to be studied is isolated from the organism and the inhibitory effects of various intermediates are tested, often at very high concentrations. This inclines one to view the denitrification pathway as a sequential step–wise process in which nitrate is completely reduced to nitrite which is then completely reduced to nitric oxide and so on to dinitrogen. However, in practice, denitrification occurs by the simultaneous reduction of nitrate, nitrite, nitric oxide and nitrous oxide, in which the inhibitory and inductive mechanisms described above ensure that none of the intermediates develop to high levels.

The simultaneous reduction of the nitrogen oxides was noted in studies with *Pa. denitrificans* by many workers, (John, 1977; Garber and Hollocher, 1981; Alefounder *et al.*, 1981, 1982, 1983; Ferguson, 1982; Boogerd *et al.*, 1981; Kučera *et al.*, 1983a; Kučera *et al.*, 1986a).

Regulation by oxygen of the activities of the denitrifying enzymes (anoxic growth conditions changed to aerobic conditions)

In the above work, mechanisms were described by which oxygen represses the synthesis of denitrifying enzymes. It is known that oxygen regulates denitrification by two mechanisms; through repression of nitrogen oxide synthesis, and through inhibition of nitrogen oxide activity.

This section examines the mechanisms involved in changes in activities of denitrifying enzymes when bacteria, grown under anoxic conditions in the presence of one of the nitrogen oxides (such that the denitrifying enzymes are developed to their maximum level and activity), are transferred to aerobic conditions. It is generally accepted that immediately upon exposure to aerobic conditions, denitrification ceases or decreases to a small fraction of the denitrification rate under anoxic conditions. Since it is improbable that such a rapid change in nitrogen oxide reduction could result from changes in the levels of denitrifying enzymes, the effect of oxygen in preventing or inhibiting denitrification is attributed to its effect on the activities of the nitrogen oxide enzymes, i.e. their electron transferring ability.

Other than by directly measuring the level of denitrifying enzymes it is impossible to determine whether a reduced denitrification rate in the presence of oxygen is a result of repression of enzyme synthesis or inhibition of enzyme activity. Tiedje (1985) concludes that enzyme activities are inhibited at lower concentrations of oxygen than enzyme synthesis. Thus, under conditions in which the concentration of dissolved oxygen increases, enzymes are firstly inactivated at the lower concentrations of oxygen and then synthesis is repressed at higher concentrations. This behaviour has been established for two organisms, *Hyphomycrobium* X (Meiberg *et al.*, 1980) and *Thiobacillus denitrificans* (Justin and Kelly, 1978). This mechanism would have the benefit that under conditions of increasing oxygen concentration, enzyme synthesis continues while enzyme activity declines such that in the event of a rapid decrease in oxygen concentration in which nitrate is present, denitrification can resume immediately.

Regarding the effect of concentrations of oxygen which affect enzyme activity only, the different denitrifying enzymes exhibit different sensitivities to the same dissolved oxygen concentration.

Hochstein *et al.* (1984) conducted experiments with *Pa. halodenitrificans* exposed to shifts from low to high oxygen concentrations and established that sensitivity to oxygen was different for the different denitrifying enzymes. Although the workers were not emphatic that the sensitivity to oxygen was a result of inhibition of activity, they considered that the results were indicative of inhibition of activity rather than repression of synthesis.

The order of increasing sensitivity to dissolved oxygen is the same order as the denitrification steps for the enzyme from least reduced nitrogen oxide, i.e. nitrate reductase to the enzyme for the most reduced nitrogen oxide, nitrous oxide reductase. Thus, under conditions of increasing oxygen concentration, but at concentrations at which enzyme synthesis is not repressed, the nitrogen oxides which appeared sequentially were; dinitrogen, nitrous oxide and then nitrite. It is apparent that Hochstein *et al.* (1984) did not consider nitric oxide as a free intermediate in denitrification and because of its intracellular production and reduction did not observe it and did not include it in the above analysis. However for the sake of this work, it will be considered that nitric oxide reductase is affected by oxygen also and is affected in the order of denitrification, i.e. nitric oxide reductase is less sensitive than nitrous oxide reductase but more sensitive than

nitrite reductase.

The inactivation of the denitrifying enzymes (and nitrate reductase in particular), under aerobic conditions is hypothesized to occur by one or a combination of the following four mechanisms;

- oxygen interferes directly with the enzymes thereby preventing exchange of electrons from the reductases to the appropriate electron acceptors,
- extracellular oxygen affects the redox potential of the enzyme complexes of organism, thereby affecting the regulation of the flow of electrons to the terminal electron acceptors,
- oxygen prevents insertion of components of nitrate reductase into the cytoplasmic membrane, thereby preventing transport of electrons from the electron transferring complexes to nitrate reductase,
- oxygen affects the permeability of the cytoplasmic membrane, thereby regulating the movement of nitrate to nitrate reductase, which in turn prevents electron transport from nitrate reductase to nitrate.

Inactivation of the nitrogen oxide reductases through direct interference by oxygen
An obvious mechanism by which oxygen could inactivate the nitrogen oxide reductases is through direct attachment to, or reaction with the reductases, in a manner which prevents the transfer of electrons to the nitrogen oxide electron acceptors. However, in research into this effect, no such mechanism has been found and as proof that oxygen does not act in this manner, workers have considered it sufficient to demonstrate that oxygen inhibits nitrogen oxidase reduction in a manner other than through direct interference with the reductases.

Payne (1973) suggested that inactivation of nitrogen oxide reductase results from oxygen acting as an electron acceptor rather than from an effect of oxygen *per se*.

Stouthamer (1988) excluded the possibility that inactivation occurs through the direct interference by oxygen, on the basis of the following experimental observations:

- Alefounder *et al.* (1981) and Kučera *et al.* (1981) working independently, with *Pa. denitrificans*, used an artificial electron acceptor, ferricyanide, (it accepts electrons from cytochrome *c*, Boogerd *et al.*, 1981, van Verseveld *et al.*, 1981) to

mimic the effect of oxygen in preventing nitrate reduction by ensuring the transfer of electrons from cytochrome *c* to cytochrome oxidase aa_3. In the presence of ferricyanide, the addition of antimycin A (which inhibits electron flow to cytochrome *c* and therefore also to ferricyanide; Alefounder *et al.*, 1981, Alefounder and Ferguson, 1982) initiated nitrate reduction. Because ferricyanide and oxygen both accept electrons from cytochrome *c* (for oxygen, via cytochrome oxidase) the experimental results demonstrate that oxygen does not inhibit nitrate reduction by direct action but acts by preventing or inhibiting electron movement to nitrate reductase.

- Kučera *et al.* (1981) showed that for *Pa. denitrificans*, nitrate reduction under aerobic conditions proceeded at a rate considerably lower than nitrate reduction under anoxic conditions. After the addition of hydroxylamine (which inhibits electron transport to cytochrome oxidase), the rate of nitrate reduction increased to a rate similar to that measured under anoxic conditions. This result indicates that the electron transfer pathway to cytochrome oxidase is the preferred route, but in situations in which electron transport to cytochrome oxidase is inhibited, electron flow is enhanced to nitrate reductase.

- Kučera *et al.* (1983a) showed that nitrite and nitrous oxide and Alefounder *et al.* (1983) showed that nitrous oxide,[2] act on *Pa. denitrificans* in a manner similar to oxygen, by inhibiting nitrate reductase activity, this activity resuming upon the addition of antimycin A. Antimycin A inhibits electron flow to the part of the electron transfer chain comprising cytochrome *c* (Alefounder *et al.*, 1981, Alefounder and Ferguson, 1982), thereby decreasing the rate of electron flow to nitrite reductase and nitrous oxide reductase and increasing the rate of electron flow to nitrate reductase via ubiquinone.

In a review of these results, Stouthamer (1988) concluded that molecular oxygen does not act directly on nitrate reductase to inhibit its activity but acts by redirecting the flow of electrons in the electron transport chain from nitrate reductase to terminal oxidase, most probably through changes in the reduction/oxidation (redox) state of one or more of the components of the respiratory electron transport pathway.

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[2]The authors discuss the reasons as to why they found that nitrite did not inhibit nitrate reductase activity, but rather stimulated its activity, in contradiction to the results of Kučera *et al.* (1983a).

Inhibition by oxygen of electron flow to the nitrogen oxide reductases as a result of changes in the redox potential of components of the electron transport pathway

In early work on the effect of oxygen on nitrate reductase, considerable variation was reported in the degree to which oxygen affected the activity of the nitrogen oxide reductases. However, irrespective of the degree to which oxygen affected this activity, workers such as Stickland (1931) concluded that the manner by which oxygen affects the flow of electrons to nitrate via nitrate reductase, is by redirecting the electrons to oxygen via cytochrome oxidase, but because of a lack of understanding concerning the complexes comprising the aerobic and anoxic pathways they did not propose specific mechanisms as to how this could occur. Workers such as Fewson and Nicholas (1961b) noted a loss of nitrate reductase activity in *Ps. aeruginosa* under aerobic conditions and attributed it to the diverting of electrons away from the denitrifying pathway and nitrate reduction, and to the aerobic pathway and the reduction of oxygen.

In determining the control exerted by oxygen on the denitrifying enzymes of *K. aerogenes*, van't Riet *et al.* (1968) subjected a culture of the organisms to a shift from anoxic conditions to aerobic conditions and noted an immediate cessation of nitrite production from nitrate reduction. They suggested that oxygen inactivates nitrate reductase and represses its further formation but did not propose a mechanism to explain either inactivation or repression. However, further work by van't Riet *et al.* (1972) with *K. aerogenes* implicated a component or components of the respiratory chain as being responsible for the withdrawal of electrons from denitrifying enzymes under aerobic conditions. This implication arose from experiments in which purified nitrate reductase from *K. aerogenes* was not inactivated by oxygen whereas cell free, nitrate reducing extracts (which besides the nitrate reducing fraction also contained other electron transferring components of the respiratory pathway still associated with the cytoplasmic membrane) from the same organism were inactivated.

In similar experiments, Pichinoty and d'Ornano (1961c) working with the same organism also did not observe inactivation of purified nitrate reductase by oxygen. The inactivation of enzymes under aerobic conditions was noted by de Groot and Stouthamer (1970a) with *P. mirabilis*. The authors assessed nitrate reductase activity by examining (i) the rate of nitrate reduction of cell-free extracts of the culture under anoxic conditions and (ii) the rate of nitrite accumulation of whole-cell suspensions of the culture under anoxic conditions. After shift of the

culture from anoxic to aerobic conditions, the formation of nitrite stopped immediately even though the activity of the nitrate reductase of cell-free extracts remained high. The authors concluded that under aerobic conditions nitrate reductase is inactivated, its formation is repressed and electron transfer occurs preferentially to oxygen.

In a review of the effect of oxygen on the synthesis and activity of nitrate reductase, Stouthamer (1976) suggests that inactivation and repression of nitrate reductase by oxygen is not a general rule since Schulp (1972) found that when a culture of *Bacillus licheniformis* was shifted from anoxic to aerobic conditions, nitrate reductase formation ceased immediately but nitrite formation continued for another 45–60 minutes. This indicated that for this organism, oxygen did not inactivate nitrate reductase immediately as was the case for the organisms discussed above, but initially repressed enzyme formation, the enzyme being inactivated with time.

Unfortunately, it appears that the wealth of research produced between 1960 and 1970 did not continue into the next decade, the impetus being regained only in the 1980s, perhaps in view of the fact that the centenary of the discovery of the biological nature of denitrification was due in that decade. The 1970s saw a period of consolidation in the field of denitrification, resulting in research in the following decade being devoted to determining which components of the respiratory chain regulate the flow of electrons between cytochrome oxidase and the nitrogen oxide reductases and the mechanism by which this regulation occurs.

Research concerning the particular components of the electron transport pathway that could control the flow of electrons to nitrate reductase and cytochrome oxidase was limited by uncertainty regarding the arrangement of the terminal reductase and oxidase complexes with respect to other components of the pathway. Since it is probable that the point of divergence of electrons to either nitrate or oxygen would also be the point at which control could be executed, then as knowledge was gained regarding the position of the branching point of electrons to nitrate reductase and cytochrome oxidase, so the position in the pathway proposed to exert control over electron flow also changed. The chronological development of knowledge concerning the electron transport pathway is presented in detail in Appendix C. To briefly recap on significant developments; the scheme of Knook *et al.* (1973) (see Fig C.2) had a branching point common to nitrate reductase, nitrite reductase and cytochrome oxidase at cytochrome *b*. John and Whatley (1975) proposed the

branching point to nitrate reductase at cytochrome *b* and the branching point to nitrite reductase and the cytochrome oxidases at cytochrome *c* (see Fig C.3). Stouthamer *et al.* (1980) included nitrous oxide reductase accepting electrons from the cytrochrome cc_1 complex (see Fig C.4). Later schemes by Vignais *et al.* (1982), Whatley (1981) and Knowles (1982) established the electron pathway branching to nitrate reduction and to terminal oxidase at the bc_1 complex or from cytochrome *b* and cytochrome *c* respectively (see Fig C.5). In a more recent scheme, Ferguson (1982) proposed that nitrate reductase gains electrons from ubiquinone rather than from the cytochrome *bc* complex, cytochrome oxidase gains electrons from cytochrome *c* (see Fig C.6) and alternative cytochrome oxidase (cytochrome *o*) gains electrons from ubiquinone. It is important in the following review of the involvement of different components of the electron transport pathway in the control of electron flow, that the state of knowledge of the components of the pathway at the time of the research be taken into account. It may be necessary to refer to the electron transport scheme applicable at the time to understand the research.

- *Redox control of electron flow to nitrate reductase exerted by cytochrome b:*

Prior to the scheme of Ferguson (1982), other schemes for the denitrification pathway (Knook *et al.*, 1973; John and Whatley, 1975; Stouthamer, 1980; Knowles, 1982) considered the branch point to nitrate reductase to be situated at cytochrome *b*. In such schemes cytochrome *b* could have a controlling function over the flow of electrons to nitrate reductase. Alefounder *et al.* (1983) considers this unlikely given an acceptance of the scheme of Ferguson (1982) (see Fig C.6), in which cytochrome *b* occurs in the respiratory chain after the branch point to nitrate reductase. It would then be difficult to envisage that nitrate reductase could be sensitive to the redox state of a component on the oxidizing side of the branch point to nitrate reductase. Also, nitrate reduction is inhibited both when electron flow is through cytochrome *b* (i.e. in the presence of added nitrous oxide which diverts electrons away from nitrate reductase to nitrous oxide reductase) (Kučera *et al.*, 1983a; Alefounder *et al.*, 1983).

- *Redox control of electron flow to nitrate reductase exerted by cytochrome cd:*

Experiments conducted by Kučera *et al.* (1981) with ferricyanide and hydroxylamine led the authors to suggest that the redox state of cytochrome *cd* (nitrite reductase) which has cytochrome oxidase activity in the presence of oxygen and gains electrons from cytochrome *c* might play an important role in the control of nitrate reduction

by oxygen by abstracting electrons and inhibiting flow to nitrate reductase. After substituting ferricyanide (which gains electrons from cytochrome *c*) as electron acceptor for oxygen under anoxic conditions with nitrate present, nitrate reduction ceased. After addition of hydroxylamine which inhibits the oxidases of aerobically grown cells (Lam and Nicholas, 1969a), nitrate reduction proceeded under aerobic conditions at the rate measured under anoxic conditions. However, Alefounder *et al.* (1983) considered inhibition of nitrate reductase by control of electron flow to the oxidases via the redox state of cytochrome *cd* to be improbable for 3 reasons:

(i) Spheroplasts (intact cells with cell wall removed) of *Pa. denitrificans* depleted in cytochrome *cd* continued to be inhibited in nitrate reduction by oxygen (Alefounder and Ferguson, 1980) indicating either that control of inhibition is exerted at a position on the pathway other than cytochrome *cd*, or that another control mechanism other than control of electron flow is responsible.

(ii) No gradual loss of inhibition of nitrate reductase by oxygen was observed when the dissolved oxygen concentration fell below that of the maximum saturation coefficient of nitrite reductase (John 1977, Timkovich *et al.*, 1982).

(iii) Willison and John (1979) found that a cytochrome *c* deficient mutant (such that electron flow could not proceed from cytochrome *c* to cytochrome *cd*), was also inhibited in nitrate reduction by oxygen, indicating that the redox potential between cytochrome *c* and cytochrome *cd* does not have a role in inhibition of nitrate reduction by oxygen.

These three reasons, individually, and in combination, precluded a role for cytochrome *cd* in controlling electron flow to nitrate reductase in the presence of oxygen.

- *Redox control of electron flow to nitrate reductase exerted by ubiquinone:*

Alefounder *et al.* (1981) proposed the pool of ubiquinone as a candidate for the controlling factor in electron flow between nitrate reductase and cytochrome oxidase. The pool of ubiquinone comprises both reduced ubiquinone, simply called ubiquinone and oxidized ubiquinone, called ubiquinol. They hypothesized that the concentration of dissolved oxygen and the K_m of the oxidase affect the rate of electron transfer to oxygen thus affecting the extent of reduction of the pool of ubiquinone. A low extent of reduction of ubiquinone resulting from a high rate of

electron flow to cytochrome oxidase aa_3, would permit a low rate of electron flow to nitrate reductase. Conversely, a high extent of reduction of ubiquinone resulting from a low rate of electron flow to cytochrome oxidase aa_3 would permit a high rate of electron flow to nitrate reductase. The extent of reduction of ubiquinone could then directly or indirectly operate the switch for controlling nitrate reduction by regulating the movement of electrons or nitrate to nitrate reductase.

The scheme above has considerable appeal in the light of the respiratory electron pathway proposed by Ferguson (1982) in which ubiquinone is the last common component in electron flow to nitrate reductase and terminal oxidase.

Although no direct experimental evidence has been obtained to implicate ubiquinone as having a controlling function, Alefounder *et al.* (1983) found indirect evidence to suggest that following a transition from aerobic to anoxic conditions in which nitrate reduction was initiated, the extent of reduction of ubiquinone was increased. They concluded that relatively small changes in the oxidation state of ubiquinone could control the extent of nitrate reduction by control of electron flow to nitrate.

Because of the difficulty in measuring the redox state of intracellular components of the respiratory chain, there is little experimental evidence to support the hypothesis, but conversely little experimental evidence exists to disprove it.

Prevention by oxygen of the insertion of the nitrate reductase subunits into the cytoplasmic membrane

Stewart (1988), in reviewing the regulation of nitrate reductase activity by oxygen in *E. coli*, considers that in a shift from anoxic to aerobic conditions in which nitrate reductase cannot function in electron transport, nitrate reductase (and other anoxic respiratory enzymes) cannot be incorporated into the membrane. This hypothesis is based on the following three findings.

- De Groot and Stouthamer (1970b), showed that the formation of reductases is repressed under conditions when electron transfer to the reductases cannot occur. For example, under aerobic conditions in which electron flow is to terminal oxidase only, the formation of nitrate reductase would be repressed.

- MacGregor (1976) found that a function of the cytochrome *b* (γ) subunit of nitrate reductase is to associate the nitrate reductase complex with the

cytoplasmic membrane.

- Hackett and MacGregor (1981) found that the incorporation of nitrate reductase into the membrane is blocked immediately by a shift from anoxic to aerobic growth. These workers found that subunits α and β of nitrate reductase are synthesized in the cytoplasm but can only be inserted into the membrane in the presence of a third component, subunit γ. In *E. coli*, the synthesis of subunit γ occurs only under conditions which induce nitrate reductase synthesis, i.e. the absence of oxygen and the presence of nitrate. When *E. coli* was grown under anoxic conditions with nitrate present, all three subunits were synthesized in the cytoplasm and inserted into the membrane. When the organism was shifted to aerobic growth, with or without nitrate, the insertion of subunits α and β ceased immediately. Following a shift from anoxic conditions in the presence of nitrate to anoxic conditions without nitrate present, the insertion of subunits α and β also ceased after a brief period, indicating that nitrate is a requirement for nitrate reductase formation in *E. coli*. After insertion into the membrane, nitrate reductase did not degrade under aerobic conditions even though it was not functional, indicating that regulation of nitrate reductase levels is not achieved by turnover. Since nitrate reductase activity is lost rapidly after a shift from anoxic to aerobic growth it can be assumed that regulation of nitrate reductase activity is not achieved by regulation of enzyme synthesis.

An effect of oxygen in changing the permeability of the cytoplasmic membrane, thereby regulating the movement of nitrate to nitrate reductase

As outlined above, there appears to be considerable evidence to support mechanisms for the inactivation of nitrate reductase in which either the redox state of one of the components near the branching point to nitrate reductase and cytochrome oxidase controls the flow of electrons between the terminal electron acceptors, or, oxygen prevents the insertion of the nitrate reductase subunits into the cytoplasmic membrane. On the basis of theoretical arguments, inhibition of nitrate reduction could occur by preventing either electrons or nitrate from reaching the site of nitrate reductase. Experimental results indicate that mechanisms in which electrons are prevented from reaching nitrate reductase do not provide a completely adequate explanation for the manner by which oxygen exerts control over nitrate reduction. Such experiments indicated that the presence of oxygen affects the permeability of the membrane across which nitrate must pass to gain access to its reductase. These experiments usually approached the problem in one of two ways. In one approach,

the organisms were grown under anoxic conditions (such that all denitrifying components of the respiratory chain were present); the effect of oxygen on nitrate movement across the membrane was then investigated by gradually introducing oxygen into the anoxic environment and monitoring changes in reduction of the nitrogen oxides with time. In another approach, organisms were grown under anoxic conditions, and rapidly transferred to aerobic conditions under which denitrification did not proceed, or proceeded at a considerably reduced rate. The factors required to initiate denitrification (or nitrate reduction) under aerobic conditions by effecting changes in the cytoplasmic membrane were then determined.

- *Intact cells versus inside–out membrane vescicles*

John (1977) investigated the effect of oxygen on the reduction of nitrate by whole cells and with membrane vescicles. In the first test, intact cells of anoxically grown *Pa. denitrificans* having nitrate reductase and cytochrome oxidase activities were placed under aerobic conditions with nitrate present. Nitrate reduction commenced only when all the oxygen present had been consumed. The inhibitory effect of oxygen on nitrate reduction was shown repeatedly by reintroducing oxygen in the presence of nitrate such that nitrate reduction ceased. In the second experiment, inside out membrane vescicles of anoxically grown *Pa. denitrificans* with nitrate reductase and cytochrome oxidase activities (in which access by nitrate to nitrate reductase was not prevented by the cytoplasmic membrane) were subjected to the same aerobic conditions as in the first experiment. The membrane vescicles reduced oxygen and nitrate simultaneously but when the oxygen had been consumed there was little increase in the rate of nitrate reduction. Thus, in intact cells, electrons are used preferentially by oxygen, but in membrane vescicles, electrons were distributed between both nitrate reductase and cytochrome oxidase indicating that under aerobic conditions the cytoplasmic membrane plays an important role in the regulation of nitrate reduction.

- *Chemical permeabilization of cytoplasmic membrane*

Alefounder and Ferguson (1980) also implicated the cytoplasmic membrane in the inhibition of nitrate reduction. Spheroplasts of *Pa. denitrificans* treated with Triton X–100 (a chemical which permeabilizes the cytoplasmic membrane allowing the movement of nitrate to nitrate reductase), reduced nitrate and oxygen simultaneously under aerobic conditions. The increased flow of electrons to nitrate reductase due to the availability of nitrate, led to a decrease in the flow of electrons to the oxidases, the rate of oxygen reduction being only half the rate measured in

the absence of Triton X–100.

Kučera *et al.*, (1983b) working with the same organism under the same conditions confirmed that under aerobic conditions intact cells did not reduce nitrogen, but under aerobic conditions in the presence of Triton X–100, nitrate reductase activity increased to the same level as that found for intact cells under anoxic conditions, indicating a role for the cytoplasmic membrane in control of nitrate reduction under aerobic conditions. As a part of this latter investigation, the concentration of nitrite in the medium was monitored and a surprising finding was that as the concentration of nitrite increased, the rate of nitrate reduction also increased. To clarify the causes of this, the effect of the permeability of the cytoplasmic membrane on the rate of nitrite reduction under aerobic conditions was investigated.

The results of the investigation into the relationship between the concentration of nitrite and the rate of nitrate reduction demonstrated that following the process of permeabilization, when a certain nitrite concentration was attained as a result of nitrate reduction under aerobic conditions, electron flow to oxygen was inhibited and electron flow to the nitrogen oxides including nitrate increased.

This result is of considerable interest and importance to this review and further developments in this area are reviewed in Section D.3.

- *Additional electrons supplied to electron transport pathway*

It was suggested above that the state of oxidation of ubiquinone controls the flow of electrons between nitrate reductase and cytochrome oxidase. Alefounder *et al.* (1983) suggested that in the light of the finding that control of nitrate reduction by oxygen occurs by control of nitrate movement, the movement of nitrate also could be controlled by the extent of oxidation of ubiquinone. The authors designed experiments to show that control of nitrate reduction is not due to restriction on the movement of electrons to nitrate, thereby favouring the mechanism that control is exerted on the movement of nitrate to its reductase by the membrane. This mechanism is based on the assumption that under aerobic conditions, electrons flow exclusively to oxygen and not to nitrate. The authors used the chemical durohydroquinone as an additional source of electrons in an attempt to promote electron flow to nitrate under aerobic conditions by exceeding the normal electron flow requirements to oxygen. (The exact point at which durohydroquinone feeds electrons into the denitrification transport chain is not known but is considered to

act in a manner similar to reduced ubiquinone and donate electrons to nitrate reductase, cytochrome *o* and the cytochrome bc_1 complex). It was found that the addition of durohydroquinone did not result in nitrate reduction under aerobic conditions and the authors concluded that a good case could be made for control being exerted on nitrate movement across the cytoplasm rather than on preferential flow of electrons to oxygen.

Although the majority of work with this technique was conducted on the bacterium *Pa. denitrificans*, other workers investigated the effect of oxygen on the movement of nitrate in other organisms using different techniques.

- *Use of a transmembrane nitrate carrier*

Noji and Taniguchi (1987) studied the control of nitrate reduction in *E. coli* and concluded that control is primarily due to restriction of nitrate movement to its reductase on the cytoplasmic side of the membrane. Under aerobic conditions nitrate reduction was absent but under aerobic conditions in the presence of benzyl or heptyl viologen radicals (reported to be trans-membrane nitrate carriers, or ionopores) nitrate reduction increased. The workers suggested that oxygen may interact at a specific nitrate transport site in the electron transport chain especially if nitrate and nitrite movement across the membrane occurs by means of a nitrate/nitrite antiport system as suggested by Jones *et al.* (1980) and Boogerd *et al.* (1983a). However they also presented an alternative explanation for the impermeability of the membrane to nitrate under aerobic conditions; the transport mechanism for nitrate across the membrane is stimulated by the absence of oxygen rather than inhibited by the presence of oxygen.

- *Intact cells versus cell extracts*

Hernandez and Rowe (1987) studied the effect of oxygen at various concentrations on nitrate respiration in *Ps. aeruginosa* and found the inhibitory effect to be a maximum at 0,2% of oxygen saturation. Their results indicated the cause of inhibition to be at the level of nitrate uptake since inhibition was observed in whole cell preparations but not in cell extracts in which the cytoplasmic membrane was destroyed. An important finding in this work was that nitrite uptake by whole cells was not inhibited by the low concentrations of oxygen which inhibited nitrate reduction, and at higher concentrations of oxygen, nitrite reduction by whole cells was only partially inhibited. These results can be explained by (i) the periplasmic orientation for nitrite reductase (thereby eliminating the necessity for extracellular

nitrite to cross the membrane to attain the site of nitrite reductase) and (ii) the results of Kawakami *et al.* (1985) in which oxygen only partially inhibited nitrite reductase in *Pa. halodenitrificans.* The workers suggested that because only nitrate and not nitrite transport was controlled by the cytoplasmic membrane, then regulation of the denitrification process at the level of nitrate transport represented a primary mechanism by which the entire pathway could be regulated. However it should be noted that the regulation of nitrate movement would be a regulatory mechanism for the whole denitrifying pathway only if the sole source of nitrite is that which arises from nitrate reduction. If nitrite is available from another source, e.g. nitrification of ammonium (as occurs in activated sludge), then the significant difference in effect of oxygen on nitrate and nitrite reduction has important consequences, since it implies that nitrite could be reduced in the presence of oxygen (i.e. aerobic denitrification of nitrite).

In testing the inhibition of nitrate transport by oxygen as a general regulatory mechanism for nitrate respiration in nitrate reducing organisms, and denitrifying organisms, Hernandez and Rowe (1988) found that nitrate transport was completely and reversibly inhibited by oxygen in 11 of the 12 species tested. Inhibition was reversed by exposing the organisms to anoxic conditions. Nitrate reduction by cell–free extracts of the same organisms (in which the cytoplasmic membrane was destroyed) was not affected or was only partially affected by oxygen, providing further evidence that oxygen inhibits nitrate reduction through an effect on the cytoplasmic membrane, thereby limiting nitrate uptake.

The authors suggested that an organism which could control nitrate reduction by control of nitrate uptake rather than by inactivation of nitrate reductase by control of electron flow would have an advantage, for the following reasons:

- It would be energetically wasteful to have nitrate uptake continue in the presence of oxygen if control were exerted on electron flow, since once nitrate gained access to the cytoplasmic side it could not be utilized.

- Because transfer of an electron pair from substrate to oxygen provides more energy than the equivalent transfer to nitrate, it would be advantageous for an organism to be able to respond to transient changes in oxygen concentration by regulation of nitrate transport and by diverting electron flow from one respiratory system (anoxic) to another (aerobic) without inactivating the

denitrifying enzymes, since reversal of the inactivation may require anoxic conditions.

In conclusion, the importance of the orientation of nitrate reductase with the cytoplasmic membrane, with respect to the effect of oxygen on nitrate reduction is demonstrated in an interesting piece of work conducted by Bell *et al.* (1990) on the organism *Thiosphaera pantotropha.* In this organism a periplasmic nitrate reductase develops under both aerobic and anoxic conditions while the usual cytoplasmically oriented nitrate reductase develops under anoxic conditions only (as is the case for most facultative denitrifiers). The organism has the unusual ability to reduce nitrate at high rates under aerobic conditions and this ability is attributed to the periplasmically oriented nitrate reductase developed under aerobic conditions, an orientation which allows nitrate reduction to occur without the necessity for nitrate to cross the cytoplasmic membrane to be reduced. For all other organisms studied in this review, nitrate reductase is situated on the cytoplasmic side of the membrane and under aerobic conditions nitrate reduction does not occur, or occurs only at very low rates. Under anoxic conditions in the presence of nitrate, organisms with a cytoplasmic placement of nitrate reductase reduce nitrate at high rates. Thus, it appears that oxygen affects the permeability of the cytoplasmic membrane; under aerobic conditions the cytoplasmic membrane restricts the movement of nitrate to the cytoplasmic side, thereby inhibiting nitrate reduction. This conclusion is supported by the finding that nitrite reduction is inhibited only partially by oxygen as a consequence of the periplasmic orientation of nitrite reductase.

D.3 MECHANISMS OF REGULATION OF AEROBIC RESPIRATION

Introduction

In Appendix C the characteristics and the function of terminal cytochrome oxidase were outlined. It was shown that two types of cytochrome oxidase are synthesized by denitrifying, facultative organisms. These are the enzyme complexes, cytochrome aa_3 and cytochrome *o*; the former is an inducible enzyme synthesized by organisms under aerobic conditions only and the latter is a constitutive enzyme present under both aerobic and anoxic conditions.

It was shown above, that enzymes can be regulated in two ways; by regulation of the synthesis of the enzyme, and by regulation of the activity of the enzyme. In the case of denitrifying enzymes these two aspects are affected by oxygen, nitrate, and the intermediates of the process of denitrification (NO_2^-, NO and N_2O). Since the

respiratory chain of facultative organisms comprises both denitrifying reductases and cytochrome oxidases arranged in close proximity about the cytoplasmic membrane, it is likely that the factors which affect the denitrifying enzymes (i.e. oxygen, nitrate and the denitrifying intermediates) will also affect the cytochrome oxidases. The effect of the presence of aerated and unaerated conditions on the synthesis and activity of the cytochrome oxidases (cytochrome aa_3 and cytochrome *o*) was investigated in Appendix C, but an aspect not covered was the effect of the nitrogen oxides on the activity of the oxidases.

Research into the effect of the nitrogen oxides on the activity of cytochrome oxidase resulted from work designed to determine the mechanism by which oxygen affects nitrate respiration. In investigating the effect of oxygen on nitrate respiration it was shown that oxygen alters the permeability of the cytoplasmic membrane which affects the movement of nitrate and nitrite across the membrane. These experiments implicated nitrite as having a role in the control of the reduction of oxygen. The significance of that result to this investigation was noted, and this aspect is discussed in greater detail below, the anomaly being that although the initial part of this review was concerned with the effect of oxygen on nitrogen oxide reduction, the development of the review has led to the finding that the nitrogen oxides affect the reduction of oxygen.

Before reviewing the work concerned with the effect of the nitrogen oxides on cytochrome oxidase, it is instructive to outline the course of the review thus far.

Nitrate reduction is limited or completely stopped by the addition of oxygen. It was shown that this phenomenon is dependant on the redox state of the components of the respiratory chain at the electron branching sites and was explained as being due to competition by terminal oxides and reductases for a constant limited flux of electrons from ubiquinone. However this mechanism was insufficient to explain the result that, cultures of *Pa. denitrificans* treated with the detergent Triton X–100 (designed to increase the permeability of the cytoplasmic membrane) reduced nitrate in the presence of oxygen. It was then suggested that nitrate reduction is regulated by the presence of oxygen, which affects the permeability of the cytoplasmic membrane thereby controlling the accessibility of nitrate to its reductase. In experiments which examined this suggestion and confirmed it as a mechanism for control of nitrate reduction by oxygen, an additional finding was that in cells in which the cytoplasmic membrane was permeabilized an increase in nitrite

concentration from nitrate reduction under aerobic conditions was responsible for a decrease in oxidase activity. Investigation of this mechanism forms the basis of the following sections.

The mechanism of inhibition by nitrite (NO_2^-) of oxygen utilization

The aspect of nitrite inhibition of oxygen reduction, was investigated by Kučera and Dadak (1983) with anoxically grown cells of *Pa. denitrificans* subjected to aerobic conditions in the presence of an uncoupler, a chemical which reduces the transmembrane potential of the cytoplasmic membrane, allowing access of nitrite to the cytoplasmic side. As a means of determining the distribution of electrons between cytochrome oxidase and nitrite reductase, the redox potentials of the two enzyme complexes were measured. In the presence of the uncoupler and a high concentration of nitrite when the membrane potential was lowered, electron flow to cytochrome oxidase decreased and electron flow to nitrite reductase increased as a result of nitrite gaining access to the inhibitory site of cytochrome oxidase. The manner by which nitrite interfered with cytochrome oxidase was not specified by the authors.

A problem concerning the above experiment is that it is not clear as to whether electron flow to cytochrome oxidase was inhibited as a result of the direct interaction of nitrite with cytochrome oxidase at a specific inhibitory site, or whether the initiation of nitrite reduction led to a withdrawal of electrons from the electron transport branch of cytochrome oxidase.

A mechanism was suggested by Rowe *et al.* (1979) by which nitrite could inhibit oxidase activity in *Ps. aeruginosa.* Nitrite apparently exerted its effect by oxidizing the ferrous (Fe^{2+}) ion of cytochrome oxidase to the ferric (Fe^{3+}) form, thereby preventing the transfer of electrons to oxygen. Also, Yang (1985) investigated the inhibitory effect of nitrite on cytochrome oxidase in *Ps. aeruginosa.*

The addition of nitrite at concentrations of 1–5 M inhibited oxidase activity but interestingly, nitrate, at concentrations much higher than that of nitrite had little or no inhibitory effect. No explanation was offered for the lack of inhibition by nitrate, but it is likely that the explanation given above for the prevention of nitrate reduction under aerobic conditions applies; the presence of oxygen affects the permeability of the cytoplasmic membrane, thereby preventing the movement of nitrate to nitrate reductase situated on the cytoplasmic side, and preventing the

production of the inhibitory species nitrite, whereas the presence of oxygen does not affect the reduction of nitrite, because nitrite reductase is situated on the periplasmic side of the membrane and nitrite is not required to pass across the membrane to be reduced. However, this mechanism implies that nitrite itself is not the inhibitory species, because cytochrome oxidase is situated on the cytoplasmic side and the only means by which extracellular nitrite could affect the oxidase (assuming that under aerobic conditions nitrite cannot cross the membrane) is if a product of nitrite reduction, i.e. nitric oxide, crosses the membrane to its reductase on the cytoplasmic side and in the process interacts with cytochrome oxidase.

Kučera *et al.* (1986b) investigated the role of nitrite in inhibiting oxidase activity in *Pa. denitrificans* with regard to whether nitrite abstracts electrons from the electron transport pathway or whether it acts directly on cytochrome oxidases, by using methods which avoided the complicating effect of permeabilizing the membrane. They investigated the effect on oxidase and nitrite reductase activity of electron flow to cytochrome oxidase and nitrite reductase, using the artificial electron donor ascorbate and a chemical TMPD[3]. TMPD is referred to as a mediator as a consequence of its role in increasing the flow of electrons to cytochrome oxidase and nitrite reductase, such that electrons are available to nitrite reductase under aerobic conditions. The uptake of oxygen by anoxically grown *Pa. denitrificans* (such that the nitrite reducing component of the respiratory chain was present), was tested under aerobic conditions in medium to which ascorbate and TMPD were added, and in which nitrite was either present or absent. Under aerobic conditions, in the absence of nitrite, oxidase activity increased with increased TMPD concentration in the expected hyperbolic fashion. Under aerobic conditions in the presence of nitrite, oxidase activity initially increased with increased TMPD concentration, but decreased above a certain concentration of TMPD. *This indicated that the reduction of nitrite inhibited oxidase activity through the production of an unidentified agent.* This conclusion was reinforced by showing that a culture of *Pa. denitrificans* lacking nitrite reductase activity (i.e. grown aerobically such that nitrite reductase formation was repressed) exhibited an increased and uninhibited oxidase activity with increased TMPD concentration in the presence of nitrite under aerobic conditions. The authors concluded that it was not the presence of nitrite *per se* which inhibited oxidase activity but an unidentified inhibitory agent, possibly one of the denitrification intermediates, either nitric oxide or nitrous oxide, which result from the reduction of nitrite. Nitric oxide was implicated as the intermediate

[3] (N,N,N'–N'–tetramethyl–*p*–phenylenediamine)

responsible for inhibition of cytochrome oxidase after it was shown that cytochrome oxidase could be inhibited by the addition of nitric oxide but not by the addition of nitrous oxide.

Support for the finding that inhibition of terminal oxidase activity is due not to nitrite was presented by the Ferguson group. Parsonage *et al.* (1985) concluded from work on *Pa. denitrificans* with a permeabilizing agent and an uncoupler that the inhibitory species is a reaction product or an intermediate of the nitrite reductase reaction and is due not to nitrite alone. This conclusion arose as a corollary to the following findings;

- In cultures treated with the permeabilizing agent Triton X–100 or with an uncoupler which allows the movement of ions across the cytoplasmic membrane, very low concentrations of nitrite strongly inhibited electron flow to oxygen, whereas in cultures not treated with either of these two chemicals, similar low concentrations of nitrite did not inhibit electron flow to oxygen.

- With inside–out vesicles (in which periplasmically situated nitrite reductase was on the inside of the vesicle, reducing access of nitrite to it) treated with Triton X–100 or an uncoupler, low concentrations of nitrite did not inhibit electron flow to oxygen.

- In aerobically grown cultures (in which nitrite reductase was absent) treated with Triton X–100 or an uncoupler, low concentrations of nitrite did not inhibit electron flow to oxygen.

These results indicated that it was not nitrite *per se*, but a product of the catalytic activity of nitrite reductase that was responsible for the inhibition of electron flow to oxygen.

The Ferguson group considered two possible products or intermediates of nitrite reduction, nitric oxide (NO) and nitroxyl anion (NO^-) which could inhibit electron transport to oxygen by reacting with haem (Fe) centres. Nitric oxide was rejected as an inhibitory species on the grounds that, although nitric oxide is known to have a high affinity for oxygen, no oxygen consumption was measured during periods of inhibition as a result of the reaction of nitric oxide with oxygen. Because inhibition required either collapse of proton motive force by an uncoupler or permeabilization

of the membrane, it was considered that the inhibitory species should be negatively charged and the authors proposed the nitroxyl anion (NO^-).

Nitric oxide (NO) as the inhibitory species

It is evident that there was considerable uncertainty regarding the identity of the species responsible for inhibition of oxygen utilization. As indicated above, nitrite was initially assigned the role of the inhibitory species, with speculation that another species, produced as a product of denitrification of nitrite, was also inhibitory. Because of the lack of knowledge concerning the denitrification pathway and identity of nitrogen oxides formed from nitrite, there was an inability to specify exactly which nitrogen oxide was responsible.

This section explains the work which directly demonstrated or indirectly ascribed an inhibitory role to nitric oxide.

It is unfortunate that many of the research groups intent on identifying the intermediate ignored an important piece of work which had been conducted some time previously. In that work, Krul (1976) conducted experiments to determine the effect of nitrate, nitrite and nitric oxide on the oxygen utilization rate of an *Alcaligenes* sp. isolated from activated sludge. Because of the particular relevance of those experiments to this thesis, they are described in some detail.

Activated sludge was grown anoxically with nitrate present, and an organism, *Alcaligenes* sp. was isolated and then grown in pure culture under anoxic conditions with nitrate present.

The oxygen utilization rate of the culture was tested under two sets of conditions. Under the first set of conditions, the organism was subjected to a 24 hour period of aerobic pretreatment before testing for the effect on the utilization of oxygen of the added nitrogen oxides, nitrate, nitrite and nitric oxide. The addition of nitrate inhibited the rate of oxygen utilization only when the dissolved oxygen concentration was less than 0,5 mgO/ℓ, nitrite inhibited the rate at less than 2,0 mgO/ℓ only and nitric oxide inhibited the rate at less than 4,0 mgO/ℓ only. When added nitric oxide inhibited the utilization of oxygen, reduction of nitrate and nitrite was initiated at a rate equal to that at which they were reduced under anoxic conditions. Under the second set of conditions in which the organism was grown anoxically with nitrate present but without aerobic pretreatment, the utilization

rate of oxygen was not inhibited by the addition of nitrate or nitrite, and was inhibited only slightly by the addition of nitric oxide. From these experiments it was concluded that inhibition resulting from the addition of nitrate, nitrite or nitric oxide, was induced as a consequence of the formation of nitric oxide, the added nitrate and nitrite being reduced in turn to nitric oxide. The reason for the greater degree of inhibition measured for organisms subjected to 24 hour aerobic pretreatment than for organisms without pretreatment, was a result of the effect of aerobic conditions on the synthesis of the nitrogen oxide reductases. Lowered synthesis of nitric oxide reductase would result in a decreased rate of removal of nitric oxide of organisms subjected to aerobic pretreatment. This is supported by findings described earlier which indicated that aerobic conditions affect the activity of nitric oxide reductase to a greater degree than they do nitrite and nitrate reductase, leading to an accumulation of nitric oxide during subsequent denitrification of nitrate and nitrite.

A similarity was noted between the aerobic pretreatment/anoxic growth with nitrate conditions to which the inhibited *Alcaligenes* sp. was subjected, and the unaerated/aerated cycles to which activated sludge is subjected in N and N & P removal configurations. This similarity provided the basis for the hypothesis which explains the proliferation of filaments in aerated/unaerated systems. A fundamental aspect of the hypothesis is the inhibition of oxygen utilization of organisms in the sludge by nitric oxide, and in order that the hypothesis has credibility, it is essential that the mechanism of inhibition of oxygen utilization by nitric oxide is well established.

The remainder of this section is devoted to verification that nitric oxide is inhibitory to utilization of substrate under aerobic conditions.

In the work of the Kučera group (i.e. Kučera *et al.*, 1986b), the inhibition of oxidase activity was ascribed initially to the interaction of nitrite with cytochrome oxidase, and then to the interaction of nitric oxide with cytochrome oxidase.

Work by Kučera *et al.* (1987b) on cultures of *Pa. denitrificans* which followed the work of Kučera *et al.* (1986b) attempted to conclusively identify the inhibitory agent. The researchers added an uncoupler to an aerobic suspension of anoxically grown (with nitrite) *Pa. denitrificans* in order to induce aerobic denitrification of NO_2^-. From previous work it was suspected that the inhibitory species produced

from the reduction of NO_2^- was NO and attention was directed at establishing this. Since it was known that NO reacts with the haem (Fe^{3+}) centre of ferricytochrome C to form a complex which can be identified by spectroscopic means, ferricytochrome C was added to the suspension to react with any NO produced. The workers found that aerobic denitrification of NO_2^- was accompanied by the formation of a substance identified spectroscopically to absorb at the same wavelength as a complex of ferricytochrome C–NO, the NO component of the newly formed complex having most likely originated from the reduction of NO_2^-. *From these results, the workers concluded that NO originates from the reduction of NO_2^- during denitrification and contributes significantly to the inhibition of oxidase activity.*

Although Kučera *et al.* (1987b) accepted nitric oxide as inhibitory, they did not exclude nitrite as also having an inhibitory role. Kučera *et al.* (1987b) state "... it is shown that besides an increased inhibition of terminal oxidase brought about by NO_2^- anion another potent inhibitor originating in the course of nitrite reductase reaction affects the division of electron flow between oxygen and nitrite." The authors suggested that should nitrite contribute to the inhibition of the oxidases, then a condition necessary for inhibition by aerobic reduction of nitrite is a sufficiently high extracellular concentration of nitrite to produce a high intracellular nitrite concentration.

Although the authors concluded that inhibition of oxidase activity was principally due to nitric oxide, they did not elaborate as to the mechanism by which nitric oxide interacted with cytochrome oxidase in order to inhibit its activity nor did they speculate as to the existence or orientation of a specific nitric oxide reductase. In considering the weight of evidence from other groups favouring NO as a free intermediate in the process of denitrification, it is interesting that until the finding that nitric oxide inhibited oxidase activity, the Kučera group had not accepted nitric oxide as an intermediate of the denitrification process. This was somewhat unusual, since it was shown in Appendix C that although many research groups also initially resisted accepting NO as an intermediate in denitrification, by the mid 1980's most groups had either proven or accepted such.

As described above, the Ferguson group also considered initially that nitrite was inhibitory but then concluded that the inhibitory species was either nitric oxide or nitroxyl. One of the objections raised by the Ferguson group for assigning a role to

nitric oxide was that, because of its extremely reactive nature, it would be present only in very small and unmeasurable quantities, if at all, and certainly not in quantities sufficient to inhibit cytochrome oxidase (Parsonage *et al.*, 1985). The Ferguson group opted for nitric oxide as the inhibitory species only after Carr and Ferguson (1990b) showed that *Pa. denitrificans* possessed a separate nitric oxide reductase that was active in the presence of oxygen and maintained the steady-stage dissolved nitric oxide concentration sufficiently low that reaction with oxygen was insignificant but sufficiently high that reaction with the active site of cytochrome oxidase would inhibit oxygen reduction.

It is interesting that in the work described above which concluded that nitric oxide inhibits oxidase activity, no mechanism was described for the inhibitory action of nitric oxide. However in some related work on the inhibition of respiratory processes by nitrite and nitric oxide with a photodenitrifier and a pathogenic organism, mechanisms were proposed and may have application to inhibition of respiration in facultative denitrifying organisms. Satoh (1984) working with the photodenitrifier *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* proposed that nitric oxide inhibited electron transfer through the cytochrome bc_1 complex. However, since the reduction of nitric oxide also requires the transfer of electrons through the cytochrome bc_1 complex it is improbable that nitric oxide would act such that its own reduction would be inhibited.

Reddy *et al.* (1983) investigated nitrite inhibition of *Clostridium botulinum.* Nitrite, added to cells of the organism, formed nitric oxide which reacted with iron-sulphur enzymes of the respiratory electron transferring complexes forming iron-nitric oxide complexes thereby inhibiting electron transfer.

Inhibition of electron transfer (apparently by the same mechanism) also occurred after the addition of nitric oxide, Salerno *et al.* (1976), providing further evidence that the inhibitory species is nitric oxide and not nitrite.

Because early work on the inhibition of oxidase activity considered that nitrite alone was responsible for the inhibition of oxidase activity, the function of the cytoplasmic membrane was accorded considerable significance due to the orientations of nitrate reductase on the cytoplasmic side and nitrite reductase on the periplasmic side of the membrane. The only source of nitrite which could interact with cytochrome oxidase, which is situated on the cytoplasmic side, is that which arises from the

reduction of nitrate on the cytoplasmic side. Given the subsequently discovered significance of nitric oxide in oxidase inhibition, it would seem reasonable that the orientation of a specific nitric oxide reductase with respect to the membrane also is of importance.

In Appendix C it was established that both nitric oxide reductase and cytochrome oxidase lie on the cytoplasmic side of the membrane. Such orientations lend support to the hypothesis that nitric oxide affects oxidase activity, since in crossing from the periplasmic side (where it is formed by the reduction of nitrite) to the cytoplasmic side (where it is reduced to nitrous oxide), nitric oxide has access to and can interact with cytochrome oxidase.

Which of the cytochrome oxidases (cytochrome aa_3, or cytochrome *o*) is inhibited by nitric oxide?

In Appendix C it was noted that in facultative denitrifying organisms, two different cytochrome oxidases develop, cytochrome *o* and cytochrome aa_3. Cytochrome *o* is a constitutive enzyme, synthesized under both aerobic and anoxic conditions, but cytochrome aa_3 is an inducible enzyme which is synthesized under aerobic conditions only.

Little information exists as to which of these cytochromes is inhibited by nitric oxide but two factors implicate cytochrome *o* as the oxidase subject to inhibition. The first factor concerns the environmental conditions under which cytochrome *o* and cytochrome aa_3 are synthesized and the conditions under which aerobic respiration is inhibited. Aerobic respiration is inhibited under aerobic conditions after growth under anoxic conditions with nitrate or nitrite present. Cytochrome *o* is the only oxidase synthesized under anoxic conditions (Lam and Nicholas, 1969a) and as such is the only oxidase present and available for inhibition under the subsequent aerobic conditions. On the basis of such an analysis, Yang (1982) concluded that cytochrome *o* of *Ps. aeruginosa* was inhibited by nitrite, and for a similar reason, Kučera *et al.* (1983b) considered that nitrite interacts mainly with cytochrome *o* in anoxically grown *Pa. denitrificans.* Although subsequent research has found that the inhibitory species is not nitrite but nitric oxide, the reasons for concluding that the inhibitory species affects cytochrome *o* and not cytochrome aa_3 are still relevant. Also, Casella *et al.* (1986) studied nitrite reduction in a *Rhizobium* "*hedysari*" strain and concluded that inhibition of aerobic respiration under aerobic conditions following growth under limited aeration conditions,

resulted from inhibition by nitrite of cytochrome *o*, the only oxidase synthesized under limited aeration conditions. Further, cultures of the organism grown aerobically such that cytochrome aa_3 was synthesized, were not inhibited by nitrite, even at excessive concentrations, indicating that nitrite does not inhibit cytochrome aa_3. (However, from this experiment it could not be concluded that nitric oxide does not inhibit cytochrome aa_3, since under aerobic growth conditions nitrite reductase, which produces nitric oxide from nitrite is not synthesized). The second factor which implicates cytochrome *o* as the oxidase subject to inhibition is a difference in reactive (electron-transferring) centres between cytochromes *o* and aa_3. The reactive centre of cytochrome aa_3 includes two iron-histidine (Fe-hs) complexes, one copper-histidine (Cu-hs) complex and a copper-histidine-cysteine (Cu-hs-cs) complex organized as two Fe-Cu couples. The reactive centre of cytochrome *o* comprises two Fe-hs complexes and one Cu-hs complex organized as an Fe-Cu couple, one of the Fe-hs complexes remaining uncoupled (Babcock and Wikström, 1992). The Fe-containing complexes have a propensity to react with nitric oxide to form iron-nitric oxide (Fe-NO) complexes (Wood *et al.*, 1981; Reddy *et al.*, 1983) which are incapable of transferring electrons to oxygen. From this evidence it is probable that nitric oxide interacts with the uncoupled Fe-hs complex of cytochrome *o* but not with the Fe-Cu complexes of cytochrome aa_3.

<u>Explanation for the development of the alternative oxidase cytochrome *o*</u>

As discussed earlier and from an examination of the proton pumping sites passed by electrons moving to either of the oxidases as indicated in Fig C.9, it is more beneficial energetically for oxygen to serve as electron acceptor at cytochrome aa_3 than at cytochrome *o*. Considering that the only other apparent difference between the oxidases is that cytochrome *o* is synthesized under anoxic conditions under in which oxygen is not present, the advantage afforded an organism possessing cytochrome *o* is not immediately apparent.

The advantage of a constitutive enzyme is that under conditions which change between aerobic and anoxic the organism can make immediate use of the electron acceptor specific to that condition without firstly synthesizing the enzyme. The disadvantage of constitutive enzymes is that if the conditions for which that enzyme is specific, occur infrequently, considerable energy is expended in the maintenance of the enzyme without any energetic return from its use.

Using this analysis of the advantages and disadvantages of constitutive and

inducible enzymes as a basis, an explanation is proposed for the development of a constitutive and an inducible oxidase in facultative denitrifying organisms.

Consider a facultative organism which does not possess a constitutive cytochrome oxidase subjected to aerobic/anoxic periods. After growth under anoxic conditions in which cytochrome aa_3 is repressed, the organism would not have a means of respiring under aerobic conditions with oxygen as electron acceptor until the inducible enzyme cytochrome aa_3 is synthesized. During the period of synthesis of cytochrome aa_3 and until it is fully operative, such an organism is placed at a disadvantage in competition with an organism which possesses a constitutive cytochrome oxidase. Therefore, the presence of a constitutive cytochrome oxidase such as cytochrome *o* (even though it has a lower yield than cytochrome aa_3 in accepting the same number of electrons) would allow a facultative organism switched to aerobic conditions after a long anoxic period, the immediate use of oxygen until the level of cytochrome aa_3 induced under aerobic conditions yields a greater energetic return than the constitutive cytochrome *o*.

D.4 AEROBIC DENITRIFICATION

Introductory remarks

In Chapter 5, a biochemical mechanism is outlined in which the reduction of nitrite under aerobic conditions leads to the inhibition of oxygen reduction by nitric oxide and the continued reduction of nitrite under aerobic conditions. This mechanism is dependent on the reduction of nitrite and not nitrate, because nitrate reduction in many organisms is inhibited by oxygen, which changes the permeability of the cytoplasmic membrane, across which nitrate must pass to its reductase. However, most work published on aerobic denitrification is concerned with the reduction of nitrate, not nitrite, leading one to suspect that many reported cases of aerobic denitrification of nitrate occur by a mechanism different to aerobic denitrification of nitrite through the inhibition of oxidase activity by nitric oxide. This mechanism for aerobic denitrification of nitrate is based on a tolerance of oxygen by denitrifying organisms and evidence toward this is given below. The following section reviews this mechanism but before doing so it is necessary to examine some other aspects of aerobic denitrification. These include; (i) the difficulty in determining whether supposedly aerobic experimental conditions in which nitrate disappears are truly aerobic, (ii) cases reported as aerobic denitrification but in which the concentration of dissolved oxygen was not measured, and (iii) cases of aerobic denitrification in which the concentration of oxygen was measured are discussed and instances of

nitrite reduction are noted. The review then outlines the variation in results between organisms as well as the variation in results between researchers working with the same organism.

Defining "aerobic conditions"

In reviewing the results of the experiments investigating aerobic denitrification it is difficult to determine whether the conditions under which denitrification is reported are truly aerobic. One reason is that it is difficult to *define* the concentration of oxygen that constitutes an aerobic environment and the degree of exclusion of oxygen that constitutes an anaerobic or anoxic environment. Another reason is that it is difficult to *measure* very low concentrations of oxygen and especially to be sure that anoxic sites do not exist in situations in which low concentrations of oxygen are measured in the liquid or the medium surrounding the cultures.

Regarding the definition of aerobic and anoxic environments, many early workers in the field of denitrification regarded the total exclusion of oxygen as a prerequisite for denitrification, but other workers were not so rigorous in their requirements for anaerobiosis.

Regarding the measurement of concentration of oxygen, in some experiments the concentration of oxygen was not measured either in the headspace above the culture or in the culture medium but conditions were assumed to be aerobic after sparging the culture with air or after vigorous shaking or stirring. In the first reports of aerobic denitrification the workers did not measure the concentration of dissolved oxygen in the liquid or medium, but measured the partial pressure of oxygen in the headspace above the liquid or medium and considered that physically vigorous aeration would ensure aerobic conditions in the liquid or medium, at most to the degree of the partial pressure of oxygen in the headspace. This rather qualitative approach to ensuring an aerobic environment was compounded by the technical inability in early experiments to measure the concentration of dissolved oxygen. In later reports of aerobic denitrification, although the concentration of dissolved oxygen in the bulk liquid was measured it was not certain that the concentration of dissolved oxygen measured in the bulk liquid was the same as that on the inside of a microbiological culture in which anoxic microsites could exist. Because of the lack of quantitative data in these experiments the means of comparing the effect of oxygen on denitrification between organisms is to summarize the important aspects of the experiments described by the authors. In most of these experiments, the

workers either grew the organism cultures under anoxic conditions with nitrate present and then introduced oxygen, examining the effect of different levels of oxygen on nitrate reduction, or, grew the organism cultures under aerobic conditions, decreasing the concentration of oxygen to determine the concentration at which nitrate reduction began. This experimental procedure is similar to that described in Chapter 5 for experiments used to examine the enzymes synthesized under aerobic and anoxic conditions.

Aerobic denitrification in which the concentration of dissolved oxygen was not measured

The first quantitative approach to investigations into the effect of oxygen on denitrification (Stickland, 1931) determined the influence of oxygen at various partial pressures (in which the level of oxygen was measured in the gaseous phase only) on nitrate reduction by *E. coli* . It was found that a 21% inhibition of nitrate reduction was caused by as little as 0.4% oxygen and that 93% inhibition was caused by 3.8% oxygen. In a report often quoted as demonstrating aerobic denitrification, Meiklejohn (1940) examined the fate of nitrate (the only source of nitrogen supplied to the culture) under different amounts of aeration and found that under a decreasing supply of oxygen, the cell yield decreased. Given that denitrification provides less energy than aerobic respiration for the same amount of substrate used, this result indicated that, the two *Pseudomonas* species tested increased their degree of nitrate respiration as the oxygen availability decreased, implying that nitrate reduction occurred in the presence of oxygen. Although the experimental routine was quite rigorous the concentration of dissolved oxygen in the culture medium itself was not measured. Sacks and Barker (1949) measured the influence of oxygen on both nitrate reduction and nitrite reduction in *Ps. denitrificans.* Previous reports had investigated the influence on nitrate reduction only. They demonstrated that nitrate and nitrite were reduced under aerobic conditions, albeit at rates considerably lower than under anoxic conditions, and that reduction of oxygen occurred simultaneously although the concentration of oxygen in the medium was not measured. These workers concluded that oxygen affects nitrate reduction and denitrification in two ways, by suppressing the formation of the enzyme systems and by directly interfering with the action of the enzyme systems.

Marshall *et al.* (1953) also worked with *Ps. denitrificans* and reported the complete denitrification of nitrate to gaseous products in aerated cultures. Reports of aerobic

denitrification such as these, in which the level of dissolved oxygen was not measured are always subject to a degree of scepticism because it is uncertain as to whether the transfer of oxygen from the gaseous phase to the liquid phase is slower than the rate of oxygen utilization in the liquid phase; if this is the case, then the liquid phase is essentially anoxic.

Experimental artifacts responsible for aerobic denitrification

Collins (1955) investigated the effectiveness of aeration in laboratory grown cultures by using various flask shapes for the growth of denitrifying organisms and illustrated the effect of that factor on aeration. From that work it was concluded that many reports of aerobic denitrification may have been due to inadequate aeration, reflecting the existence of anaerobic micro-environments. In demonstrating that the degree of nitrate reduction under aerobic conditions varied with the manner of aeration and the type of organism used, Verhoeven (1956) also found that experimental artifacts could influence results. It was concluded that reports of aerobic denitrification in which the concentration of dissolved oxygen was not measured [such as that by Meiklejohn (1940) with *Pseudomonas* sp. species] could be criticized with some justification.

Aerobic denitrification in which the concentration of dissolved oxygen was measured

One of the first investigations to measure the level of dissolved oxygen in studies into aerobic denitrification was conducted by Skerman *et al.* (1951) with a culture of *Pseudomonas* sp. Concentrations of nitrate and nitrite were also measured and it was concluded that oxygen and nitrate were used simultaneously. (Disappearance of nitrate was not due to consumption for assimilatory purposes because workers of the time were aware of the assimilatory nitrogen requirement of denitrifying organisms and accommodated this by supplying sufficient ammonium in the growth medium).

Because of the influence on aerobic denitrification of the artifacts described above, and in order to ensure that reported examples of aerobic denitrification were credible, almost all subsequent investigations in this field (and those reviewed below) measured concentrations of oxygen, nitrate and nitrite in the medium.

Variability between organisms in their sensitivity to oxygen is apparent in comparing the above results in which it was concluded that organisms reduce nitrate and nitrite under aerobic conditions, with those of John (1977) with *Pa. denitrificans* and *E. coli* in which the presence of oxygen prevented the

reduction of nitrate and nitrite. Concentrations of nitrate, nitrite and dissolved oxygen were monitored continuously during respiration by these organisms and it was found that for both organisms nitrate and nitrite reduction rapidly ceased upon aeration and recommenced only when all the oxygen had been consumed.

In determining whether aerobic denitrification is a general phenomena among all denitrifying organisms it is tempting for workers to ascribe the characteristics of the specific denitrifying organism under investigation to all denitrifying organisms. The error in such an assumption has been highlighted by research which illustrates the variation between organisms in the effect of oxygen on their rates of nitrate reduction and denitrification. These results indicate a variation from total cessation of denitrification in the presence of very small amounts of oxygen to continued denitrification (but at significantly reduced rates) under saturation aeration conditions. This aspect, i.e. a variation in tolerance by organisms to the effect of oxygen on denitrification, is the factor described at the beginning of this section as responsible for the reports of aerobic denitrification of nitrate, as distinct from the aerobic denitrification of nitrate resulting from the inhibition of oxidase activity by nitric oxide. The remainder of this review describes the enormous variation in results reported by different workers for the effect of oxygen on denitrification, not only for different organisms but also for the same organism. Such diversity in denitrification behaviour in the presence of oxygen was evident in organisms isolated from denitrifying activated sludge by Mechsner and Wuhrmann (1963). Some isolates required strict anoxic conditions for denitrification while others continued to denitrify at dissolved oxygen levels as high as 153 mmHg ($\approx$ 2.0 mgO/ℓ). Although some organisms continued to denitrify at high levels of dissolved oxygen, even in these organisms the rate of denitrification decreased with increasing oxygen concentration.

Hochstein *et al.* (1984) reported denitrification by *Pa. halodenitrificans* to take place over a broad range of oxygen concentrations with the apparently simultaneous occurrence of oxygen respiration. Lloyd *et al.* (1987), and Davies *et al.* (1989) provided evidence for aerobic denitrification in an array of organisms in the presence of concentrations of oxygen greater than air saturation. Strand *et al.* (1988) isolated a strain of *Zooglea ramigera* from activated sludge and measured significant nitrate reduction at a dissolved oxygen concentration of 8 mgO/ℓ. Hernandez and Rowe (1987) also found a high sensitivity to oxygen by nitrate respiring *Ps. aeruginosa*, the inhibitory effect attaining its maximum at about 0.2% of oxygen saturation

(i.e. ≈ 0,1 mgO/ℓ). As discussed earlier, Hernandez and Rowe (1988) found that 11 out of 12 widely divergent denitrifying bacterial species ceased nitrate reduction upon exposure to oxygen and only recommenced after all the oxygen had been consumed. The exception, *Enterobacter aerogenes*, continued to reduce nitrate at a concentration close to 10% of the dissolved oxygen saturation concentration. Such diverse findings illustrate why controversy has dogged research in the field of aerobic denitrification.

The controversy as to whether aerobic denitrification is a general mechanism among denitrifying organisms was further compounded by the work of Robertson and Kuenen (1983, 1984a,b, 1990) who isolated the organism *Thiosphaera pantotropha* from a nitrogen removal waste–water treatment system. The organism has the ability to reduce nitrate and oxygen simultaneously and to nitrify heterotrophically. Reduction of nitrate under aerobic conditions is attributed to a constitutive nitrite reducing enzyme system as opposed to the inducible nitrate reducing enzyme system of other denitrifiers. The authors did not propose these characteristics as being general among denitrifiers but the results do highlight the considerable diversity and variability amongst denitrifying organisms. They suggest that differences in the reported denitrifying ability of the same organism such as with *Pa. denitrificans* and *Ps. aeruginosa* may result from research groups experimenting with different strains of the same organism.

Aerobic denitrification was noted by Krul (1976) in activated sludge, and in that work, which was discussed earlier it was noted that the rate of nitrate reduction in activated sludge under aerobic conditions was about 20% of the rate in the absence of oxygen. (The concentration of dissolved oxygen under conditions described as aerobic was not stated by the authors). This reference appears to be the only work which links aerobic denitrification of nitrate and nitrite to the effect of nitric oxide in inhibiting aerobic respiration.

D.5 CLOSURE

The objective in reviewing reports of aerobic denitrification was to establish whether the hypothesized mechanism for aerobic denitrification has support. The review established that there is a great diversity among organisms in denitrification behaviour under aerobic conditions. Some organisms ceased denitrifying at very low concentrations of oxygen whilst others continued to denitrify at concentrations of oxygen near air–saturation. For organisms which denitrified under aerobic

conditions, it could not be determined whether denitrification occurred as a result of the inhibition of aerobic respiration or whether the organisms had a tolerance of oxygen, reflected by a decreased denitrification rate with increase in oxygen concentration.

APPENDIX E

NITRIFICATION

Appendix E reviews the biochemical mechanisms associated with oxidation of ammonium/ammonia (NH_4^+/NH_3) to nitrite (NO_2^-) by *Nitrosomonas* and the sequential oxidation of nitrite to nitrate (NO_3^-) by *Nitrobacter*. The effect of unfavourable conditions on these organisms is analysed, in particular the conditions which give rise to high concentrations of nitrite in mixed cultures of the two organisms.

E.1 INTRODUCTION

E.2 MECHANISMS OF NITRIFICATION

Oxidation of ammonium/ammonia to nitrite by *Nitrosomonas*

(i) The initial reacting chemical species
(ii) The intermediates in the oxidation step to nitrite
(iii) The source of oxygen for oxidation
(iv) The enzyme complexes associated with the two oxidation steps to nitrite
(v) Oxygen limitation affects products of ammonia oxidation

Oxidation of nitrite to nitrate by *Nitrobacter*

(i) The source of oxygen for oxidation of nitrite (produced by Nitrosomonas) to nitrate
(ii) The enzymes associated with the oxidation of nitrite to nitrate
(iii) NADH formation in assimilative reactions
(iv) Nitrobacter are facultative autotrophs
(v) Presence or absence of NO_2^- and organic matter determines autotrophic or heterotrophic growth

E.3 CONDITIONS AFFECTING NITRIFICATION BY *NITROSOMONAS* AND *NITROBACTER*

E.4 CLOSURE

E.1 INTRODUCTION

The process of nitrification has been reviewed for two reasons: Firstly, in nitrogen (N) and nutrient (N & P) removal biological wastewater treatment systems in which nitrification is a major and essential process, bulking is a common problem, and secondly, the process of denitrification which has been implicated in bulking (see Chapter 6) requires the endproducts of nitrification (nitrate and nitrite) as reactants.

In the early 1800s Sir Humphrey Davey suggested that the presence of nitrate in soils resulted from the oxidation of ammonia. However the oxidation process was considered to be chemical until Pasteur proposed that it was microbiologically mediated. This proposal gained considerable acceptance from the experiments of Schloesing and Müntz (1877) which demonstrated that no further nitrification was measured in sewage effluent subjected to processes which destroyed the microorganisms, i.e. heat treatment or the addition of chloroform.

Winogradsky (1890) firmly established the biological nature of nitrification after isolating and describing different bacteria that oxidize ammonium (NH_4^+) to nitrite (NO_2^-), and then nitrite to nitrate (NO_3^-), naming the bacteria *Nitrosomonas* and *Nitrobacter* respectively. The concept of chemoautotrophic growth[1] developed from his realization that the organisms did not require reduced carbon compounds but could utilize carbon dioxide as their source of carbon and could obtain energy from the oxidation of ammonium.

Little more significant work was conducted in the field until the 1960s when improvements in technology allowed the extraction and characterization of the enzymes responsible for production of the nitrite and nitrate, the endproducts of the two stage oxidation process.

E.2 MECHANISMS OF NITRIFICATION

The two steps of the oxidation process for the conversion of ammonium to nitrate are conventionally written as shown in Eqs E.1 and E.2.

$$NH_4^+ + \frac{3}{2}\,O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad \textit{Nitrosomonas} \qquad \text{(E.1)}$$

[1] Growth with inorganic material as the sources of carbon and energy:– for a description of organism categorization see Chapter 4.

$$NO_2^- + \frac{1}{2} O_2 \rightarrow NO_3^- \qquad \textit{Nitrobacter} \qquad (E.2)$$

Although these equations describe the essential aspects of the two step nitrification process, they are neither completely accurate nor adequate for describing the intermediates produced in each step. The two steps of nitrification are discussed separately.

Oxidation of ammonium/ammonia to nitrite by *Nitrosomonas*

In the reaction described by Eq E.1, four aspects are of interest: (i) the initial reacting species as either ammonium (NH_4^+) or ammonia (NH_3), (ii) the intermediates produced in the oxidation of ammonium to nitrite, (iii) the source of oxygen as either water (H_2O) or molecular oxygen (O_2), (iv) the enzymes associated with the oxidation of ammonium to nitrite and, (v) the effect of environmental conditions on the oxidation of ammonium to nitrite.

(i) The initial reacting chemical species: *Nitrosomonas* are conventionally considered to use the NH_4^+ ion for oxidation to nitrite because the compound exists mostly in the cationic form at pH values optimal for oxidation ($\approx$ 7,8). However Suzuki (1974), in studies on *Nitrosomonas europaea* concluded that NH_3 is the form used in the oxidation process. Also, Drozd (1976) argued that *Nitrosomonas* uses the NH_3 form because in growing cultures, the pH in the growth medium decreased rapidly, suggesting that a proton remains on the exterior of the cell wall, in the medium.

(ii) The intermediates in the oxidation step to nitrite: Based on the postulate of Kluyver and Donker (1926) that hydroxylamine (NH_2OH) is an intermediate of ammonia oxidation, Lees (1952) established that hydroxylamine can be oxidized to nitrite by cells of *Nitrosomonas.* Hoffman and Lees (1953) using hydrazine, an inhibitor of hydroxylamine oxidation, found that cells of *Nitrosomonas* oxidizing ammonia, generated large quantities of hydroxylamine, establishing that compound as an intermediate. The stoichiometric equations describing the oxidation of ammonia to nitrite are as follows:

$$2H^+ + 2e^- + O_2 + NH_3 \rightarrow NH_2OH + H_2O \text{ [2]} \quad \text{(E.3)}$$

Ammonia monooxygenase (AMO)

$$H_2O + NH_2OH \rightarrow 4e^- + 4H^+ + HNO_2 \text{ [3]} \quad \text{(E.4)}$$

Hydroxylamine oxidoreductase (HAO)

$$2H^+ + \tfrac{1}{2} O_2 + 2e^- \rightarrow H_2O \quad \text{(E.5)}$$

Cytochrome oxidase

(iii) The source of oxygen for oxidation: It is conventional to consider molecular oxygen (O_2) as this source for both steps in the oxidation of ammonia to nitrite. In the first oxidation step, molecular oxygen serves as the source of the oxygen atom for hydroxylamine (Eq E.3). In the second oxidation step, the oxygen atom of water serves as the second oxygen atom in the formation of nitrite, shown in the associated nitrous acid (HNO_2) and not the dissociated ($NO_2^- + H^+$) nitrite ion form (Eq E.4). Thus in the two–step oxidation of ammonia to nitrite, molecular oxygen has a dual role; (i) to donate an oxygen atom in the first oxidation step in the formation of hydroxylamine and, (ii) as an electron acceptor for two of the electrons produced in the second oxidation step (the other two electrons are returned to AMO for the formation of hydroxylamine). Evidence in support of these mechanisms is as follows: Rees and Nason (1966) using isotopically labelled oxygen atoms in molecular oxygen and in water determined that one, but not both of the oxygen atoms in nitrite is derived from molecular oxygen, the other presumably derived from water; subsequently, Dua *et al.* (1979), and Hollocher *et al.* (1981), also using labelled isotopes, established that the oxidation step of ammonia to hydroxylamine involved incorporation of oxygen from molecular oxygen. Based on these results, it was concluded that oxygen incorporated in the oxidation step of hydroxylamine originates from water, a conclusion verified by Andersson and Hooper (1983) using isotopically labelled oxygen and nitrogen.

(iv) The enzyme complexes associated with the two oxidation steps to nitrite: Two groups of enzymes separately catalyze the reactions; an ammonia oxygenase catalyzing the oxidation of ammonia to hydroxylamine and an hydroxylamine

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[2] Ammonia is shown in its dissociated (NH_3) and not its associated (NH_4^+) form for the reasons given above.

[3] In practice, a quantity of N_2O appears as an endproduct; the conditions under which this occurs are discussed at a later stage.

oxidoreductase catalyzing the oxidation of hydroxylamine to nitrite.

Ammonia monooxygenase (AMO): The ammonia oxygenase has not yet been isolated and characterized, and therefore it has not been directly demonstrated that it is a water producing monoxygenase (in which only one atom of molecular oxygen is incorporated into hydroxylamine) and not a dioxygenase (in which both atoms of molecular oxygen are incorporated into a nitrogen compound) (Hooper, 1989). However, indirect evidence from isotopic labelling experiments indicates that the enzyme is a monooxygenase (Andersson and Hooper, 1983). Although it is unclear as to whether the site of ammonia oxidation is on the cytoplasmic or periplasmic side of the membrane, it is hypothesized to reside on the cytoplasmic side, the benefit of which is the removal of protons from the cytoplasm and development of a transmembrane potential for the generation of proton motive force (pmf) for the production of energy. The position of the enzyme complexes with respect to the membrane and details of electron transport between the complexes is illustrated in Fig E.1. At the active site of AMO on the cytoplasmic side of the membrane, NH_3 is converted to NH_2OH as described by Eq E.3. NH_2OH passes across the membrane to the periplasmic side. Although the reaction assists in the generation of pmf, no evidence exists that energy transduction is directly coupled to the oxidation of NH_3 to NH_2OH at AMO.

Hydroxylamine oxidoreductase (HAO): The name of this enzyme implies that it has two functions; oxidation, and reduction. In this section we are concerned only with its oxidative function. Its reductive function is described when dealing with conditions in which dissolved oxygen is limiting. The oxidative function of the multiheme enzyme complex hydroxylamine oxidoreductase (HAO) is to oxidize hydroxylamine to nitrite. HAO is an extremely complex enzyme, both in structure and function (Hooper *et al.*, 1978). It contains approximately 24 *c*-type haems which interact with each other in a manner which indicates that they share electrons. It also contains a complex, P460 which includes iron centres in the Fe^{2+} state. At the active site of HAO, situated in the periplasmic space, NH_2OH is converted to nitrous acid (HNO_2) as described by Eq E.4. The sequence of oxidation–reduction reactions involved in the oxidation of hydroxylamine and transfer of electrons to oxygen are illustrated in Fig E.1. Hooper (1989) proposed that at the active site of HAO, NH_2OH is successively dehydrogenated to NO^+ as follows:

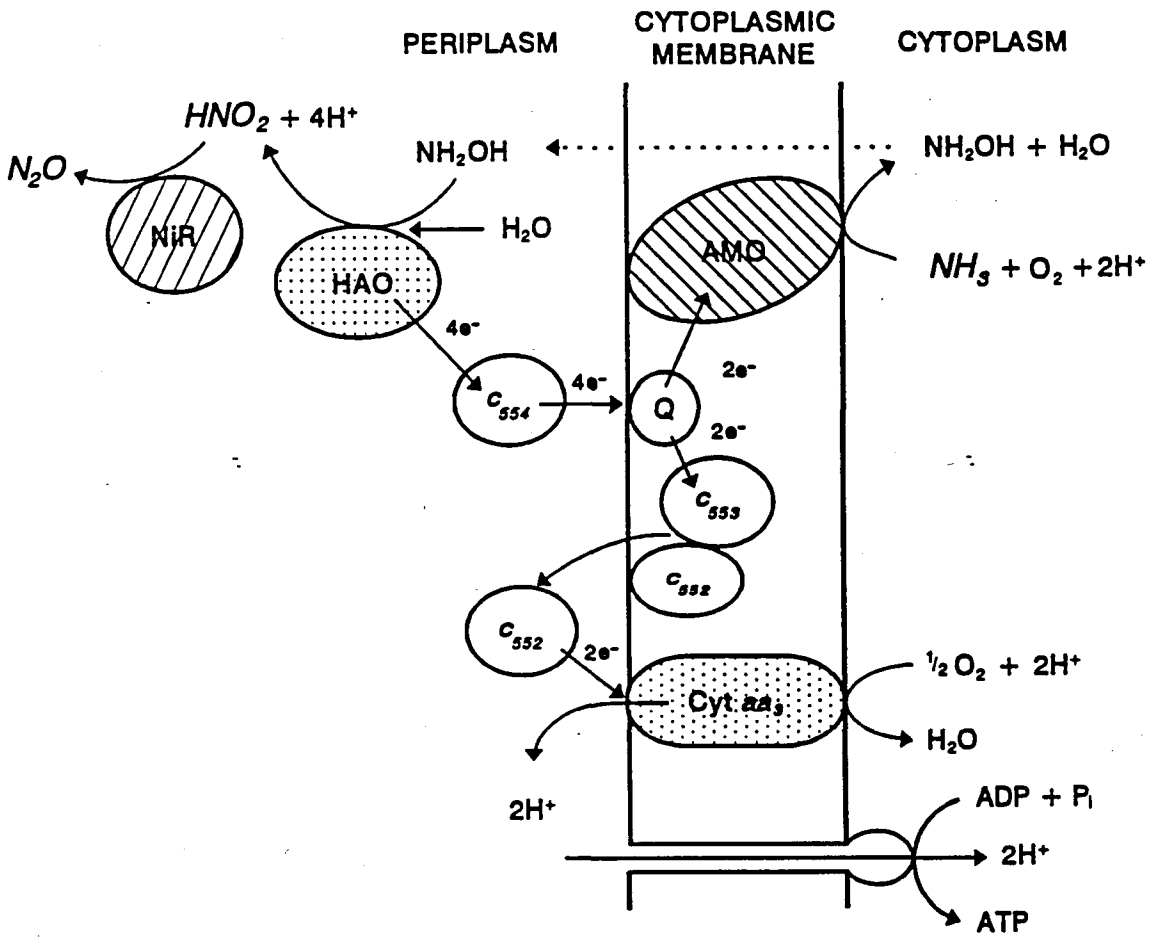

Fig E.1: Arrangement of enzymes of nitrogen oxidation and reduction, and electron transport in membranes of *Nitrosomonas* illustrating that the oxygen molecule incorporated in oxidation of ammonium (NH_3) originates from molecular oxygen (O_2) and the oxygen molecule incorporated in oxidation of hydroxylamine (NH_2OH) originates from water (H_2O). Adapted from Hooper (1989).

[Abbreviations: AMO – ammonium monooxygenase; HAO – hydroxylamine oxidoreductase; NiR – nitrite reductase; Q – Ubiquinone; Cyt – Cytochrome; ADP – adenosine diphosphate; ATP – adenosine triphosphate].

$$NH_2OH \rightarrow HNO + 2e^- + 2H^+ \quad \text{(E.6)}$$

$$NHO \rightarrow NO + e^- + H^+ \quad \text{(E.7)}$$

$$NO \rightarrow NO^+ + e^- \quad \text{(E.8)}$$

Nitrosyl (NO^+) is then hydrolysed via the formation of a ferrous nitrosyl (Fe–NO) derivative as follows:

$$Fe(II)\text{–}NO \Leftrightarrow Fe(II) + HNO_2 + H+ \quad \text{(E.9)}$$

The reaction described by Eq E.9 is based on an analogy with a model for nitrite reductase (Averill and Tiedje, 1982). Recently this model has lost favour and consequently it is unclear as to the mechanism of formation of HNO_2.

The 2 pairs of electrons from HAO, transferred to cytochrome c_{554} are passed to ubiquinone. From ubiquinone one pair of electrons are returned to AMO and the other pair are passed to a series of *c*–type cytochromes (c_{553} and c_{552}) situated either in the membrane or in the periplasm. From these cytochromes, a pair of electrons are passed to cytochrome aa_3 oxidase (Hooper, 1989).

Cytochrome oxidase: As described above, molecular oxygen serves not only as a source of oxygen atoms for oxidation of ammonia, but also as an electron acceptor for the pair of electrons transferred from ubiquinone to the *c*–type cytochromes. It is suggested by analogy with other cytochrome aa_3 terminal oxidases (Solioz *et al.*, 1982), that for oxygen acting as terminal electron acceptor, a pair of electrons are accepted from the *c*–type cytochromes, 2 protons are taken up from the cytoplasm, and in the process, 2 protons are pumped from the cytoplasm to the periplasm (Hooper, 1989); the uptake of 2 protons from the cytoplasm and transferal of 2 other protons to the periplasm results in the establishment of proton motive force, the first step in the production of ATP. These reactions are illustrated in Fig E.1.

Adenosine triphosphatase (ATPase): Establishing a proton motive force is the first step in a mechanism for the association of electron transfer with synthesis of the high energy molecule adenosine triphosphate (ATP), from adenosine diphosphate (ADP) (phosphorylation). The mechanism by which the transfer of electrons to oxygen at cytochrome oxidase is coupled to phosphorylation is referred to as

oxidative phosphorylation (Mitchell, 1979). The nett positive charge established by the utilization of protons to form H_2O in the reduction of oxygen (Eq E.5) promotes the flow of protons back to the cytoplasm via a protein called ATPase, which results in the formation of ATP from ADP (see Fig E.1). The mechanism is described fully in Appendix D in dealing with the mechanisms of coupling energy production with denitrification.

(v) Oxygen limitation affects products of ammonia oxidation: It is often noted that products additional to NO_2^- such as nitrous oxide (N_2O) and nitric oxide (NO) are produced in soils and sediments containing nitrifying organisms (Yoshida and Alexander 1970, 1971; Jorgenson *et al.*, 1984). Elkins *et al.* (1978) and Kaplan *et al.* (1978) proposed that increased production of N_2O during nitrification results from low concentrations of dissolved oxygen. On the basis of this proposal Goreau *et al.* (1980) examined the endproducts of the ammonia-oxidizing bacterium *Nitrosomonas* sp. at oxygen concentrations ranging from ≈ 7,0 to ≈ 0,2 mgO/ℓ. At high concentrations of oxygen, the yield of N_2O was ≈ 0,3% that of NO_2^-. At low concentrations of oxygen, the rate of production of NO_2^- decreased 7-fold and the rate of production of N_2O increased 4-fold, such that at concentrations of oxygen of ≈ 0,2 mgO/ℓ, the yield of N_2O was nearly 10% that of NO_2^-.

Lipschultz *et al.* (1981) examined the end products of the ammonia-oxidizing bacterium *Nitrosomonas europaea* at oxygen concentrations ranging from ≈ 2,0 to ≈ 0,2 mgO/ℓ. With decreasing concentration of dissolved oxygen, the endproduct ratio N_2O/NO_2^- increased 2-fold, the ratio NO/NO_2^- increased 7-fold and the ratio NO/N_2O decreased 2-fold, i.e. as the concentration of oxygen decreased, the proportion of NO_2^- decreased, the proportions of NO and N_2O increased and the proportion of N_2O relative to NO increased.

Nitrite reductase: The mechanisms which produce increased proportions of N_2O and NO under conditions of low DO concentration are unclear, but it is apparent that under such conditions, *Nitrosomonas* has a capacity to reduce NO_2^- or NH_2OH to N_2O and NO, in reactions analogous to those found in denitrifying organisms under similar conditions.

From experiments with purified HAO, it became apparent that under conditions of low DO concentration, the first oxidation step (the oxidation of ammonia to hydroxylamine) continues functioning, but the second step (the oxidation of

hydroxylamine to nitrite) is altered.

In experiments to elucidate the mechanisms involved in the reduction of hydroxylamine in the absence of oxygen, Falcone *et al.* (1963) found that under anaerobic conditions, no NO_2^- was formed and N_2O was the only product of the reduction of NH_2OH by purified HAO derived from *Nitrosomonas europaea.* Andersson (1964) also observed no NO_2^- from cell-free extracts of *Nitrosomonas* reducing NH_2OH under anaerobic conditions, but measured equivalent amounts of N_2O and NO. Hooper (1968) partially characterized a nitrite reductase from cell-free extracts of *Nitrosomonas* which under anaerobic conditions reduced hydroxylamine to a mixture of N_2O and NO. Further work on the separation and characterization of reductase from the hydroxylamine oxidoreductase complex by Ritchie and Nicholas (1972, 1974) established that the nitrite reductase is a copper containing protein and depends for full activity on interdependence with hydroxylamine oxidase such that under aerobic conditions, the reduction of NH_2OH results in the formation of NO_2^- and N_2O, and under anaerobic conditions, N_2O and NO are produced. It is interesting that in early research into denitrification, characterization of nitrite reductase also yielded N_2O and NO as endproducts, but subsequent studies found that two separate enzyme complexes could be characterized, a nitrite reductase complex which reduces NO_2 to NO, and a nitric oxide reductase which reduces NO to N_2O. It is suggested that an analogous situation is present for ammonia reducing organisms; a nitrite reductase and a nitric oxide reductase which produce NO and N_2O respectively. Continuing the analogy with denitrification concerning the effect of oxygen on nitrite/nitric oxide reductase, under conditions of high DO, the reductase does not function and NH_2OH produced at AMO from the oxidation of NH_3 is converted to HNO_2 at HAO and is released from the organism as NO_2^-. Under conditions of low DO, the reactions described above for the oxidation of NH_3 to NO_2^- are mediated but the product HNO_2 is reduced to a mixture of NO and N_2O at nitrite/nitric oxide reductase. Because of the importance of atmospheric conversion of N_2O to NO (a known ozone depletent), (McElroy and McConnell, 1971; Nicolet and Peetermans 1972; Crutzen, 1971), increased attention is being given to sources of naturally occurring N_2O other than from the denitrifying bacteria, in particular the ammonia oxidizing nitrifiers.

<u>Oxidation of nitrite to nitrate by *Nitrobacter*</u>

In the reaction described by Eq E.2, two aspects are of interest; (i) the source of oxygen from either H_2O or O_2, (ii) the enzymes associated with the oxidation of

NO_2^- to NO_3^- and, (iii) the effect of environmental conditions on the oxidation of NO_2^- to NO_3^-.

(i) The source of oxygen for oxidation of nitrite (produced by <u>Nitrosomonas</u>) to nitrate: Conventional stoichiometric reactions indicate molecular oxygen as the oxygen source for this reaction. Aleem *et al.* (1965) using isotopically labelled oxygen atoms in molecular oxygen and water determined that the majority of oxygen atoms in NO_3^- originate from water. The electrons produced during this reaction pass through a series of electron transferring cytochromes and in the final transfer oxygen acts as terminal electron acceptor. To confirm their finding that the oxygen for the oxidation of NO_2^- originates from water and not molecular oxygen, Aleem *et al.* (1965) grew *Nitrobacter* in the absence of oxygen, using ferricyanide as terminal electron acceptor; oxidation of NO_2^- was observed. These results were confirmed by Kumar *et al.* (1983) and Hollocher (1984), with *Nitrobacter agilis* and by Dispirito and Hooper (1986) with *Nitrobacter winogradskyi*, all three groups using isotopic labelling techniques.

The stoichiometric equations describing the oxidation of NO_2^- to NO_3^- and the concomitant reduction of oxygen are as follows:

$$H_2O + HNO_2 \rightarrow HNO_3 + 2H^+ + 2e^- \quad \text{(E.11)}$$

Nitrite dehydrogenase

$$2H^+ + 2e^- + \tfrac{1}{2} O_2 \rightarrow H_2O \quad \text{(E.12)}$$

Cytochrome oxidase

(ii) The enzymes associated with the oxidation of nitrite to nitrate: It was stated above that the electrons originating during the oxidation of NO_2^- pass through a series of electron transferring cytochromes to oxygen. The enzymes associated with the first part of the sequence (the oxidation of NO_2^-) are described as nitrite oxidase, or nitrite dehydrogenase, and have been shown by Faull *et al.* (1969) in work on *Nitrobacter agilis* to have two different activities, a nitrite oxidase ($NO_2^- \rightarrow NO_3^-$) and a nitrate reductase ($NO_3^- \rightarrow NO_2^-$) activity. The results were interpreted as indicative of the existence of two separate enzymes. Similar two-enzyme activities

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[4] Nitrite and nitrate are shown in their associated (HNO_2, and HNO_3) forms and not in their dissociated ($NO_2^- + H^+$) forms as a consequence of the higher affinity of NO_2^- for nitrite dehydrogenase at lower pH (O'Kelly *et al.*, 1970).

have been found for *Nitrobacter agilis* (Tanaka *et al.*, 1983) and *Nitrobacter hamburgensis* (Sundermeyer–Klinger *et al.*, 1984); considered as nitrite dehydrogenase activity for electron transfer in the presence of oxygen, and nitrate reductase activity in the absence of oxygen. The enzyme complexes which mediate the oxidation of NO_2^- in the presence of oxygen are described below. The enzyme complexes which mediate the reaction of NO_2^- in the absence of oxygen are described in (v) below.

Nitrite dehydrogenase: The nitrite dehydrogenase contains at least 2 different *c*–type cytochromes (Ingledew *et al.*, 1974), cytochrome a_1 (Hooper, 1989), and a cytochrome aa_3 oxidase (Yamanaka *et al.*, 1981).

In a review of electron transport to nitrite dehydrogenase and of ATP synthesis, (Hooper, 1989) emphasized that considerable uncertainty exists concerning the mechanism by which NO_2^- is oxidized to NO_3^-. Three possible schemes have been proposed and the most likely of the three (an adaptation of that proposed by Ferguson, 1982, and Aleem and Sewell, 1984) is shown in Fig E.2. Nitrite crosses the membrane via an NO_3^-/NO_2^- antiport mechanism analogous to that proposed by Boogerd *et al.* (1983a) for movement of NO_2^- and NO_3^- across the membrane in denitrifying organisms. Nitrite dehydrogenase, situated on the cytoplasmic side of the membrane, oxidizes NO_2^- to NO_3^- at cytochrome a_1 as described by Eq E.11. The oxygen atom originates from water and 2 protons and a pair of electrons are formed. The pair of electrons are transferred to a *c*–type cytochrome situated on the periplasmic side of the membrane and then to a cytochrome aa_3 oxidase complex.

Cytochrome oxidase: At the active site of cytochrome aa_3, molecular oxygen combines with 2 protons and the pair of electrons from cytochrome *c* (liberated during oxidation of nitrite) to form water, in the process of which 2 protons from the cytoplasm are pumped to the periplasm.

Adenosine triphosphatase (ATPase): The oxidation of one molecule of NO_2^- to NO_3^- results in a nett change in proton gradient resulting from the 2 protons removed from the cytoplasm and the 2 protons pumped to the periplasm at cytochrome aa_3.[5]

[5] The reason for the nett change in proton gradient with respect to the periplasm is that 2 protons are produced in the cytoplasm (–2), 2 protons are consumed in the reduction of O_2 (+2), and 2 protons are pumped to the periplasm.

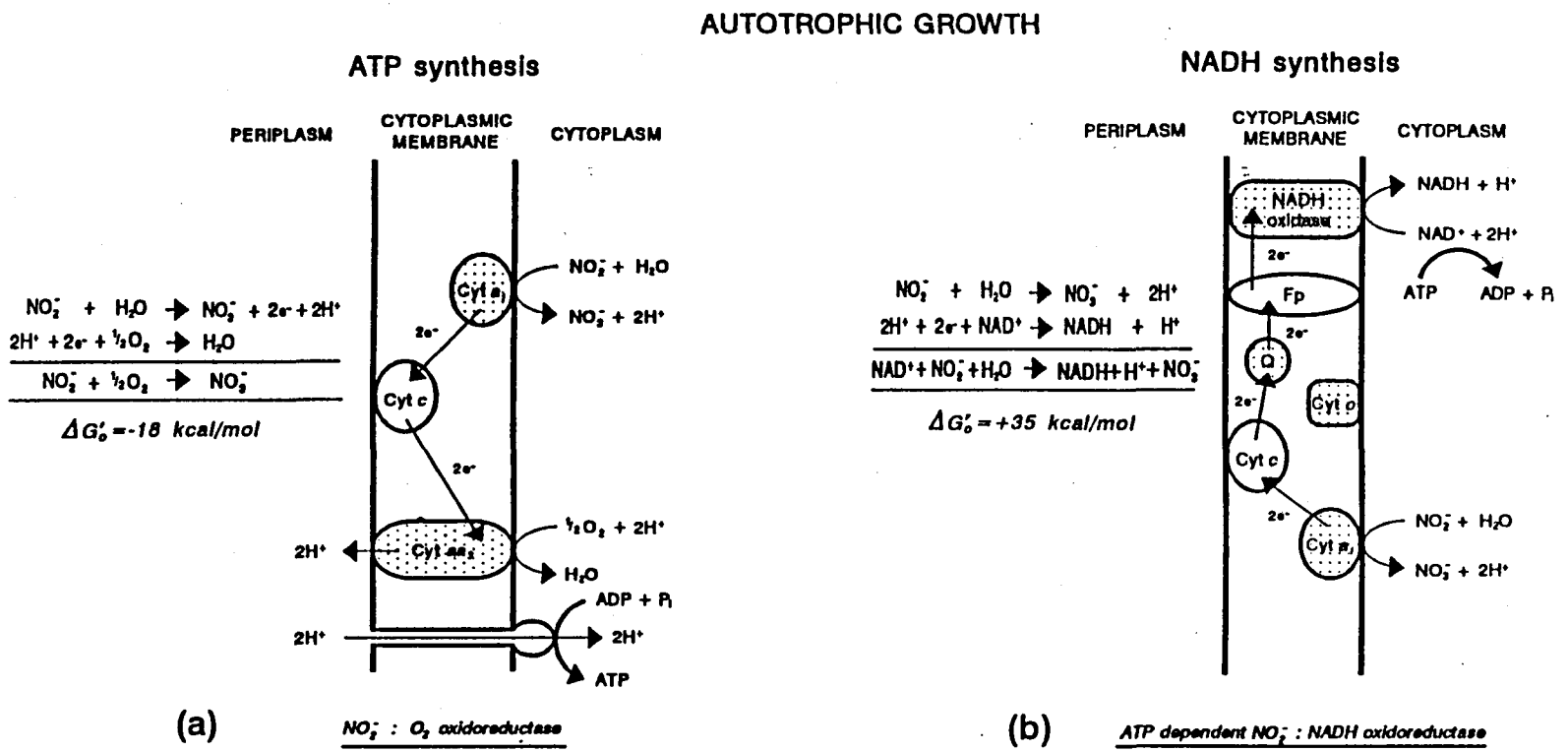

Fig E.2: Hypothesized arrangement of enzymes and electron transport under autotrophic growth conditions, illustrating (a) ATP synthesis through oxidation of nitrite (NO_2^-) to nitrate (NO_3^-) with molecular oxygen as the source of oxygen, and (b) NADH synthesis through reduction of nitrite to nitrate. Adapted from Ferguson (1982), and Aleem and Sewell (1981).

[Abbreviations: ADP – Adenosine diphosphate; ATP – Adenosine triphosphate; Cyt – Cytochrome; F_p – Flavoprotein; NAD^+ – nicotinamide adenine dinucleotide (oxidized); NADH – nicotinamide adenine dinucleotide (reduced); Q – Ubiquinone].

This is the first step in the synthesis of ATP from ADP through phosphorylation of ADP by an ATPase. However it is unclear whether this mechanism is operative since Hollocher *et al.* (1982) were unable to show that proton pumping accompanies NO_2^- oxidation.

(iii) NADH formation in assimilative reactions: The mechanism described in Fig E.2a is an endogonic (energy producing) reaction by which *Nitrobacter* produces ATP, which can then be used in the endergonic (energy consuming) reaction for the assimilation of CO_2 (Calvin–Benson cycle) which proceeds under both aerobic and anoxic conditions. For assimilative mechanisms, autotrophic organisms require not only ATP but also NADH (Aleem *et al.*, 1965). Figure E.2b illustrates the mechanism by which NADH is formed during the oxidation of NO_2^-. The series of enzyme complexes is known as ATP dependent NO_2^-: NADH oxidoreductase (Aleem and Sewell, 1981). Because water and not molecular oxygen is the source of oxygen atoms, it is assumed that this anabolic reaction can occur under aerobic and anoxic conditions. Nitrite is oxidized to NO_2^-, with water as the source of oxygen. A pair of electrons generated in the reaction pass sequentially through the electron transferring complexes, cytochrome *c*, ubiquinone, and flavoprotein to NADH oxidoreductase where NADH is synthesized at the expense of ATP formed by the mechanisms described in Fig E.2a.

(iv) <u>Nitrobacter</u> are facultative autotrophs: The metabolic process mediated by *Nitrobacter* is complicated by the finding that the organisms are not strict autotrophs, but are facultative autotrophs; i.e. can grow also as heterotrophs, (pyruvate as substrate, with NO_3^- as electron acceptor) or as mixotrophs (simultaneous autotrophic and heterotrophic growth) (Smith and Hoare, 1968; Bock, 1976; Feitag *et al.*, 1987). Figures E.3a and E.3b illustrate the mechanisms by which heterotrophic growth occurs under anoxic and aerobic conditions respectively. Under anoxic conditions, NADH is dehydrogenated at NADH dehydrogenase and the resulting electrons pass through an ETP in which NO_3^- acts as terminal electron acceptor and is reduced to NO_2^- with the formation of ATP (Aleem and Sewell, 1981) as illustrated by Fig E.3b. The group of enzyme complexes which mediate these reactions is known as NADH: NO_3^- oxidoreductase. In this group of enzymes, 2 energy conserving sites (Sites I and II) exist, at which 2 protons are pumped across the membrane for the transfer of a pair of electrons from NADH dehydrogenase to NO_3^- in the first step toward ATP synthesis. Under aerobic conditions, NADH is dehydrogenated at NADH dehydrogenase, and the resulting

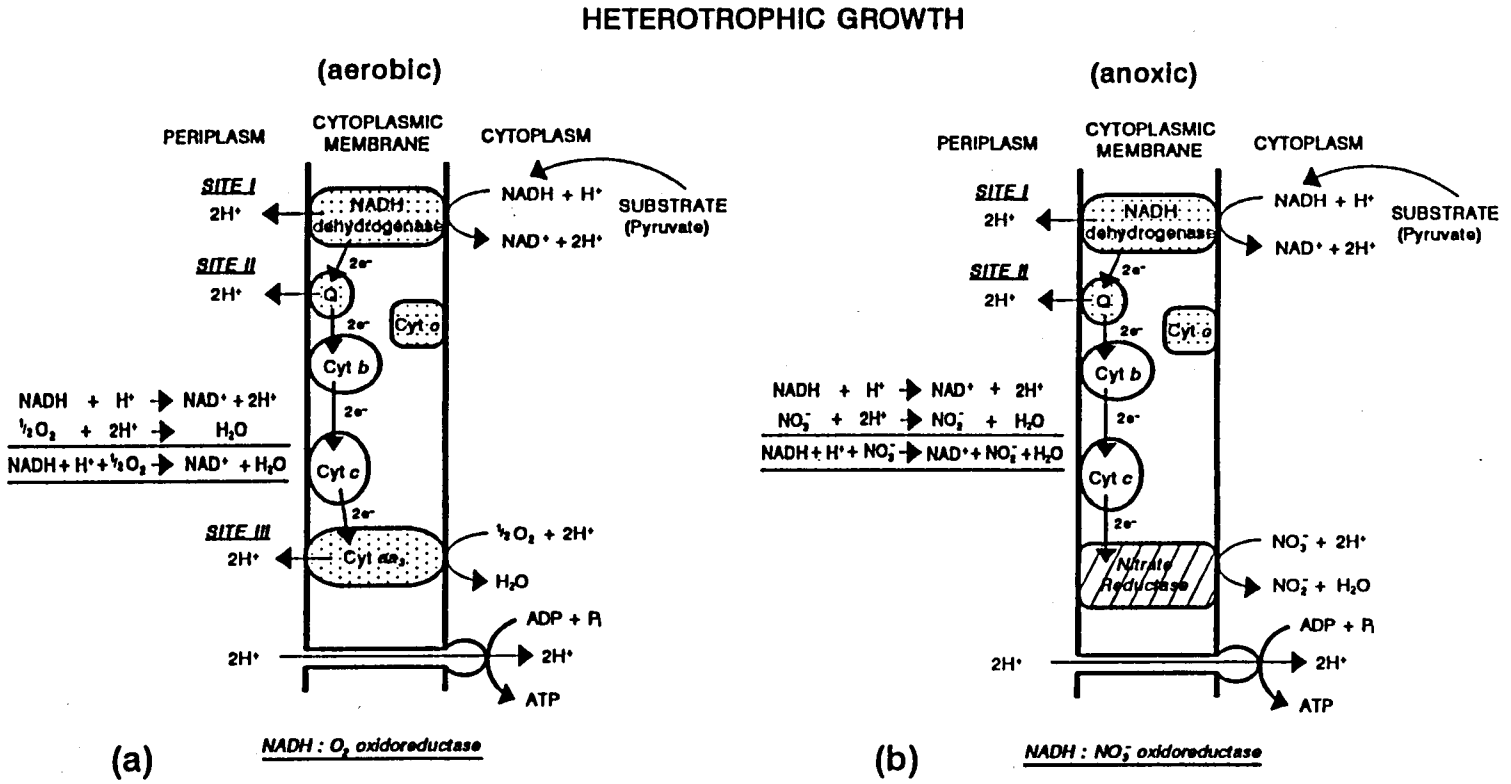

Fig E.3: Hypothesized arrangement of enzymes and electron transport under heterotrophic growth conditions, illustrating (a) ATP synthesis with molecular oxygen (O_2) as electron acceptor (aerobic conditions), and (b) ATP synthesis with nitrate (NO_3^-) as electron acceptor (anoxic conditions). Adapted from Sewell and Aleem (1979) and Aleem and Sewell (1981).

[Abbreviations: ADP – adenosine diphosphate; ATP – adenosine triphosphate; Cyt – Cytochrome; Q – Ubiquinone].

electrons pass through an ETP in which oxygen is reduced to water with the formation of ATP (Sewell and Aleem, 1979) as illustrated by Fig E.3b. The group of enzyme complexes which mediate these reactions are known as NADH: O_2 oxidoreductase. In this group of enzymes 3 energy conserving sites (Sites I, II and III) exist at which 2 protons are pumped across the membrane for the transfer of a pair of electrons from NADH dehydrogenase to oxygen in the first step toward ATP synthesis.

(v) Presence or absence of NO_2^- and organic matter determines autotrophic or heterotrophic growth: It is unclear from the literature as to the conditions or factors which determine whether *Nitrobacter* grows autotrophically or heterotrophically. It is proposed that under aerobic conditions with NO_2^- present, autotrophic growth occurs. Under aerobic conditions with NO_2^- absent and organic matter present aerobic heterotrophic growth occurs, and under conditions in which oxygen is absent, anoxic ($NO_3^- \rightarrow NO_2^-$) heterotrophic growth occurs. For each condition, both anabolic and catabolic processes occur. For all conditions, the anabolic reactions are described by Fig E.2b. For aerobic conditions with NO_2^- present, for aerobic conditions with NO_2^- absent and organic matter present, and for anoxic conditions with NO_3^- present, the catabolic reactions are described by Figs E.2a, E.3a and E.3b respectively. Concerning the yields associated with each condition, Steinmüller and Bock (1977) with *Nitrobacter agilis* showed that anoxic heterotrophic growth with NO_3^- as electron acceptor is slower than autotrophic growth oxidizing NO_2^-. In these studies, nitrite oxidase was repressed 90% in 10 days, after transition from aerobic autotrophic growth, with NO_2^- present, to aerobic heterotrophic growth with pyruvate present. For heterotrophic growth under anoxic conditions, cell yield was $\approx {}^2/_3$ that under aerobic conditions (Aleem and Sewell, 1981), a ratio similar for facultative heterotrophic growth under the same sets of conditions, and a consequence of the availability of 2 proton pumping sites under anoxic conditions and 3 proton pumping sites under aerobic conditions.

E.3 CONDITIONS AFFECTING NITRIFICATION BY *NITROSOMONAS* AND *NITROBACTER*

A number of conditions affect the growth of *Nitrobacter* and *Nitrosomonas*. These include temperature, pH and inhibitory substances. However in the experimental work conducted in the body of this thesis, these three conditions are controlled or eliminated and as a result do not affect nitrification. Two parameters which affect nitrification and which are present in the experimental work are (i) the

concentration of dissolved oxygen and, (ii) the presence of unaerated periods. Because nitrifiers obtain the majority of their energy under aerobic conditions, their growth is affected by the concentration of dissolved oxygen and the length of time of exposure to anaerobic or anoxic conditions. It is also not unreasonable to suppose that the two nitrifying organisms, *Nitrosomonas* and *Nitrobacter* not only are affected by the concentration of oxygen and the length of time of exposure of the organisms to unaerated conditions but are affected differently by these changes.

(i) The concentration of DO varies in nitrogen and nutrient removal systems either within the reactor (in an intermittently aerated configuration), or between reactors (in an anoxic–aerobic two– or multi–reactor configuration): In a review of the effect of changes in DO on *Nitrosomonas* and *Nitrobacter*, Painter (1970) makes note of the work of Ulken (1963) in which *Nitrobacter* was considered more sensitive to oxygen depletion than *Nitrosomonas*. Schöberl and Engel (1964) found that at 30°C, the growth of *Nitrobacter* was reduced at a DO of 2,0 mgO/ℓ but for *Nitrosomonas*, growth was not affected until the DO fell to 0,9 mgO/ℓ. It is generally accepted that under completely aerobic conditions in which neither NH_3 nor oxygen is limiting and with long residence times, the rate of oxidation by *Nitrosomonas* is frequently the limiting step in nitrification, with the result that the NO_2^- concentration in the medium is essentially nil. However, under conditions in which the dissolved oxygen concentration is below that at which the growth of *Nitrobacter* is affected (DO < 2,0 mgO/ℓ), but above that at which *Nitrosomonas* is affected (DO > 0,9 mgO/ℓ), it is envisaged that the NO_2^- concentration would increase as a result of its formation by *Nitrosomonas* and non-oxidation by *Nitrobacter*.

(ii) In anoxic–aerobic systems, unaerated conditions affect the performance of Nitrosomonas and Nitrobacter under subsequent aerated conditions: McCarty (1964) calculated yield coefficients of 0,29 mgVSS/mg NH_4^+–N for *Nitrosomonas* and 0,084 mgVSS/mgNO_2–N for *Nitrobacter* which equates to proportions of the nitrifier population for *Nitrosomonas* and *Nitrobacter* of 77,5% and 22,5% respectively under steady state aerobic conditions (Gee *et al.*, 1990). Because of the smaller population *of Nitrobacter*, exposure of nitrifier populations to long unaerated periods followed by exposure to aerated periods would lead to the *Nitrosomonas* population reaching steady state numbers under aerobic conditions prior to the *Nitrobacter* population. Thus, at the start of aerobic conditions after anoxic conditions and until steady–state population numbers develop, the disproportion between numbers of

Nitrosomonas and *Nitrobacter* would lead to an increase in the concentration of NO_2^-. This aspect of nitrification behaviour is examined in experiments conducted in the body of this report (see Chapter 3).

E.4 CLOSURE

The mechanisms of oxidation of ammonium to nitrite by *Nitrosomonas*, and oxidation of nitrite to nitrate by *Nitrobacter* are described in detail. Although the mechanisms themselves do not play a role in either the biochemical model for facultative organisms, or the bulking model, a consequence of the nitrification mechanisms – a high concentration of nitrite in alternating anoxic–aerobic systems – does play a role in the bulking model. While conducting the review, it was apparent that although a considerable quantity of research has been conducted into the biochemical mechanisms of nitrification, the information has not been sufficiently analyzed and organized, and it is hoped that this review may serve in some way, if not to remedy that situation, then to initiate such a process.

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