Filamentous organism bulking in nutrient removal activated sludge systems

Paper 10: Metabolic behaviour of heterotrophic facultative aerobic organisms under aerated/unaerated conditions

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Abstract

A model is outlined for the major biochemical respiratory mechanisms of a conceptualised 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 changes between these 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.

List of symbols

BT = batch test
COD = chemical oxygen demand
cs = cysteine
cyt = cytochrome
DBT = denitrification batch test
DO = dissolved oxygen
e^- = electron
ETP = electron transport pathway
FAD = flavin adenine dinucleotide - oxidised
$FADH_2$ = flavin adenine dinucleotide - reduced
FeS = iron sulphide
FMN = flavin mononucleotide
hs = histidene
IAND = intermittently aerated nitrification-denitrification
MLE = modified Ludzack-Ettinger
MUCT = modified University of Cape Town
NAD^+ = nicotinamide adenine dinucleotide - oxidised
NADH = nicotinamide adenine dinucleotide - reduced
NO = nitric oxide
NO_2^- = nitrite
NO_3^- = nitrate
N_2 = dinitrogen
N_2O = nitrous oxide
OUR = oxygen utilisation rate
RBCOD = readily biodegradable COD
SBCOD = slowly biodegradable COD
TCA = tricarboxylic acid
$\hat{\mu}_i$ = nitrifier specific growth rate
$\hat{\mu}_m$ = nitrifier maximum specific growth rate
2RND = two-reactor nitrification-denitrifcation

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Section I
A conceptual biochemical model

Introduction

In the nutrient removal activated sludge sewage treatment system, the microbial population is subjected to cycles of aerated and unaerated conditions. It can be assumed that the main portion of the microbial population that develops under such conditions will be the facultative heterotrophic organisms, which have a capacity for substrate utilisation under both aerated and unaerated conditions. Casey et al. (1999) presented a review of the biochemical respiratory pathways and mechanisms operative in heterotrophic facultative organisms under a variety of environmental conditions. The review established that a general similarity exists in biochemical pathway organisation between different facultative heterotrophic organism species. In a mixed culture such as activated sludge, these individual species are likely to give rise to a conjoined response which retains features commonly present in pure cultures but which may not be entirely representative of the response of any single species. In order to model the macroscopic behaviour of activated sludge it is necessary to conceptualise this conjoined response as that of a single surrogate heterotrophic facultative organism. It is the intention in this paper to develop a model for the respiratory biochemical mechanisms operative in such a conceptualised surrogate organism under aerated and unaerated conditions (and in changes between them), the biochemical mechanisms being based on those of individual species in pure culture.

Method for modelling aerobic and anoxic respiration by the conceptualised facultative heterotrophic organism

For the purposes of this model, the complexes which mediate proton and electron transport in the ETP and their arrangement about the cytoplasmic membrane can be conceptualised as illustrated in Fig. 2 of Casey et al. (1999). This ETP is a modification of that proposed by Ferguson (1982) for the facultative organism *Paracoccus* (*Pa.*) *denitrificans*, and incorporates the reduction of nitric oxide at nitric oxide reductase as an obligatory step. As

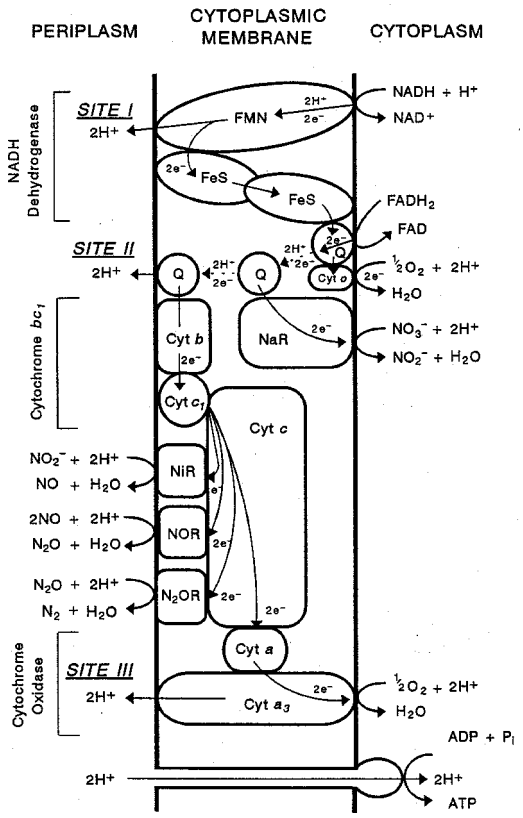


Figure 1

The ETP for a conceptualised facultative organism in which all of the electron transferring complexes are present and illustrated with respect to the cytoplasmic membrane.

described by Casey et al. (1999), the presence or not of nitric oxide was a contentious issue and many of the earlier ETPs did not incorporate nitric oxide. The association of the complexes of the ETP with the cytoplasmic membrane is illustrated in Fig. 1. This ETP is proposed as the operative one for the conceptualised facultative organism. It should be noted that these figures indicate all the enzyme complexes synthesised by facultative heterotrophic organisms, irrespective of whether the complexes are synthesised under only aerobic or only anoxic conditions (inducible) or are synthesised under both aerobic and anoxic conditions (constitutive). The pathway and the relationship of the enzymes to the cytoplasmic membrane 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 below in 8 parts:

- **Part 1** describes 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 TCA cycle) through the first three complexes of the ETP (NADH dehydrogenase, the FeS complexes, and ubiquinone).
- **Part 2** describes the biochemical processes associated with respiration under steady-state aerobic conditions.
- **Part 3** describes the biochemical processes associated with respiration under steady-state anoxic conditions.
- **Part 4** describes the biochemical processes associated with a change from steady-state aerobic to steady-state anoxic conditions.
- **Part 5** describes the biochemical processes associated with a change from steady-state 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 in which nitrate and nitrite are absent as a consequence of the process of denitrification.
- **Part 6** describes the biochemical processes associated with a change from steady-state anoxic to steady-state 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) for some time 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, which are the conditions encountered in single-sludge biological N and N&P removal systems.
- **Part 7** describes the biochemical processes associated with frequent changes between anoxic and aerobic conditions (such that steady-state aerobic and steady-state anoxic conditions are unlikely to develop), and examines the mechanism of inhibition of aerobic respiration by nitric oxide. These changes are similar to conditions encountered in single-sludge biological N and N&P removal systems.
- **Part 8** describes the role of aerobic mass fraction, RBCOD, and nitrite on the inhibition mechanisms.

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

Each of the eight 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 biochemical processes which precede these, such as those 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 by Casey et al. (1999) and are not repeated here. The role of NADH in the respiratory process is to serve as the link between electron and proton supply and the ETP. NADH transports protons and electrons generated in the substrate oxidation processes (principally the TCA cycle) to ETP complexes. These ETP complexes extrude the protons to the periplasm, indirectly resulting in the production of energy, and finally transfer the electrons 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 substrate oxidation (Lehninger, 1975) [*Note: As indicated in Casey et al. (1999), electrons supplied to the ETP can originate from NADH and/or $FADH_2$. However, for simplification, where mention is made of the source of electrons, it is assumed to be NADH*].

Detailed description of the eight parts of the biochemical model describing aerobic and anoxic respiration by the conceptualised facultative heterotrophic organism

Each of the eight parts of the biochemical model listed above is discussed in detail below.

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

The ETP complexes, NADH dehydrogenase, the FeS complexes, and ubiquinone are synthesised under aerobic and anoxic conditions and are modelled as illustrated in Figs. 2 of Casey et al. (1999) and Fig. 1. NADH transfers a pair of electrons and two protons (one originating from the cytoplasm) to the NADH dehydrogenase complex which comprises FMN and the FeS complexes. In this transfer two protons are removed from the cytoplasm and two protons are 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 respiration is introduced only in subsequent electron transfer steps and this difference is highlighted in the relevant parts 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. However, at DO concentrations < 0.5 mgO/ℓ, some of the biochemical mechanisms of the aerobic ETP are adversely affected. In the biochemical model the role of oxygen concentration in respiration is modelled by description of the biochemical mechanisms mediated under two ranges of DO concentration; DO ≥0.5 mgO/ℓ, and 0.2 < DO < 0.5 mgO/ℓ.

DO ≥ 0.5 mgO/ℓ

For DO ≥ 0.5 mg O/ℓ, the complexes of the ETP can be modelled as illustrated in Fig. 2. The nitrogen oxide reductases are present at a low (basal) level (Lam and Nicholas, 1969a). Both of the oxidases cytochrome *o* and cytochrome aa_3 are present; cytochrome aa_3 is synthesised to a greater extent than cytochrome *o*, and at these concentrations of DO, cytochrome *o* is synthesised to 30% of that to which it is synthesised under anoxic conditions (Sapshead and Wimpenny, 1972). For both of the oxidases, the active site (the site at which oxygen is reduced) is situated on the cytoplasmic side of the membrane which implies that oxygen crosses the membrane to the cytoplasmic side for reduction.

Oxygen enters the organism through the cell wall, and crosses the cytoplasmic membrane to the active sites of cytochrome *o* and cytochrome aa_3. 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* and the greater proportion 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:

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

At this point it should be stated parenthetically that the two oxidases have different reactive centres as described by Casey et al. (1999); the reactive centre of cytochrome aa_3 comprises two iron-histidine (Fe-hs) complexes, one copper-histidine (Cu-hs) complex and a copper-histidine-cyteine (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 implications with regard to aerobic respiration following a change from anoxic to aerobic conditions and these implications are highlighted in Part 6 below.

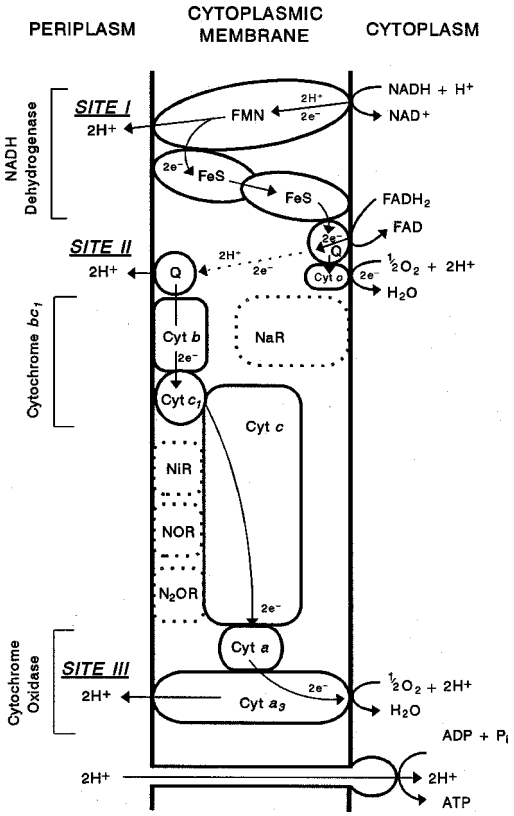


Figure 2

The ETP of the conceptualised facultative organism under aerobic conditions with dissolved oxygen (DO) ≥0.5 mgO/ℓ

Cytochrome aa_3 is referred to as the principal oxidase and cytochrome *o* as the alternative oxidase, because 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 II) 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 of that associated with the reduction of oxygen at cytochrome aa_3 because electrons pass only two proton-pumping sites for cytochrome *o* and three for cytochrome aa_3, and the passing of each proton-pumping site results in the formation of one ATP molecule, through the mechanisms of oxidative phosphorylation described by Casey et al. (1999).

0.2 < DO < 0.5 mgO/ℓ

For conditions in which the DO is between 0.2 and 0.5 mgO/ℓ, the ETP can be modelled as illustrated in Fig. 1. 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 synthesised to a lower level and cytochrome *o*

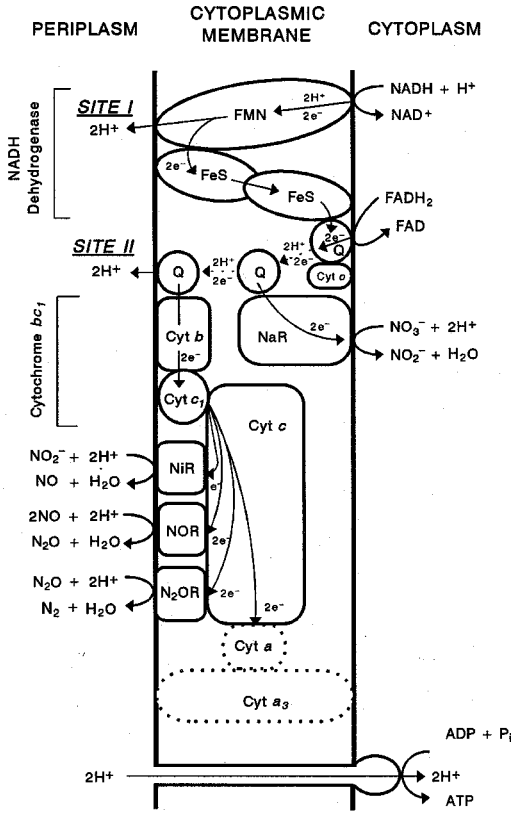


Figure 3

The ETP of the conceptualised facultative organism under steady-state anoxic conditions with nitrate and nitrite present

is synthesised to a higher level than under the higher DO conditions and each of the reductases is synthesised at a lower level than under conditions in which DO < 0.2 mgO/ℓ, and at a higher level than under conditions of DO > 0.5 mgO/ℓ. At the lower end of 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 that 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.

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 ETP of the facultative organism can be modelled as shown in Fig. 3. 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, 1969b; 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: with a proton (H^+), in a symport system which initiates nitrate reduction, or 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 (Boogerd et al., 1983a). The overall reduction of nitrate to dinitrogen in which 5 pairs of electrons are required is as follows:

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O \quad (2)$$

With nitrate present at nitrate reductase, two pairs of 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 at the catalytic site of nitrate reductase (the subunit) (Chaudhry and MacGregor, 1983) on the cytoplasmic side of the membrane (John, 1977; Alefounder and Ferguson, 1980). Reduction of nitrate (NO_3^-) to nitrite (NO_2^-) is as follows:

$$4H^+ + 4e^- + 2NO_3^- \rightarrow 2NO_2^- + 2H_2O \quad (3)$$

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). A second pair of electrons associated with NADH produced in the TCA cycle is passed via the NADH dehydrogenase complex to ubiquinone, the cytochrome bc_1 complex, cytochrome *c* and then to the active site of nitrite reductase, where nitrite, produced either extracellularly or from the reduction of nitrate, is reduced to nitric oxide (NO) as follows:

$$4H^+ + 2e^- + 2NO_2^- \rightarrow 2NO + 2H_2O \quad (4)$$

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). 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 the active site of nitric oxide reductase where nitric oxide produced from the reduction of nitrite is reduced to nitrous oxide (N_2O) as follows:

$$2H^+ + 2e^- + 2NO \rightarrow N_2O + H_2O \quad (5)$$

It should be noted that it is at this point in the denitrification pathway that the bond between the two nitrogen atoms is formed. 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). 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:

$$2H^+ + 2e^- + N_2O \rightarrow N_2 + H_2O \quad (6)$$

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

The description of the denitrification pathway above is 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 with complex interactions between the intermediates and the reductases.

Mechanisms controlling reduction of the denitrification intermediates

For conditions under which electron supply from NADH to the ETP is at a high level and continuous, a set of mechanisms control simultaneous denitrification of all the intermediates such that an equilibrium is attained in their reduction and formation. These mechanisms operate through activation and inhibition of the reductases. The mechanism of activation can be described as follows:

- Distribution of electrons between the nitrogen oxide reductases is dependent on the redox potentials established by each of the nitrogen oxides at their respective reductases. The redox potential at each reductase increases as the concentration of the associated nitrogen oxide increases at the reductase, thereby attracting electrons to the reductase and stimulating electron transfer from the reductase to the associated nitrogen oxide.

The mechanism of inhibition is best illustrated by considering the transfer from anaerobic (i.e. absence of nitrate and nitrite) conditions to anoxic conditions with nitrate only present.

- Upon nitrate becoming available, electron flow is to nitrate reductase and nitrate is reduced to nitrite. As the intracellular concentration of nitrite increases, the redox potential at nitrite reductase increases and a portion of the electron flow is directed to nitrite reductase and nitrite is reduced to nitric oxide. Thus, the formation of nitrite has an indirect inhibitory effect on nitrate reductase and a direct activating effect on its own reductase. Similarly, each nitrogen oxide has an indirect inhibitory effect on each reductase other than its own and a direct activating effect on its own reductase, and these mechanisms in combination act to control the intracellular levels of the nitrogen oxides.

Effect of electron supply on production of nitrogen oxide intermediates

The rate of electron supply to the nitrogen oxide reductases affects the intracellular accumulation of the nitrogen oxides. Low rates of electron supply (i.e. slowly biodegradable substrate - see Part 8 below), are insufficient to maintain low-level intracellular steady-state concentrations of all the nitrogen oxides. Under such conditions, electrons flow preferentially to nitrate reductase; the other intermediates, nitrite, nitric oxide, and nitrous oxide tend to accumulate. High rates of electron supply (i.e. readily biodegradable substrate), are sufficient to maintain low-level steady-state concentrations of the nitrogen oxides as described above.

Part 4 - Steady-state aerobic conditions switched to steady-state anoxic conditions

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. 2 to that described by Fig. 3, i.e. all of the nitrogen oxides and cytochrome *o* are synthesised and cytochrome aa_3 is degraded. [*Note: The term degraded is used in this approach to describe the reduction in levels of enzymes with time in the surrogate organism. Macroscopically, in activated sludge mixed culture, the degradation of cytochrome* aa_3 *for example would include die-off of those organisms with* aa_3 *and growth of those organisms without* aa_3] . The changes occur in the following manner: In the absence of oxygen the membrane becomes permeable to nitrate, and synthesis of the nitrogen oxide reductases is initiated. 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 resulting in the sequential formation from nitrate of nitrite, nitric oxide, nitrous oxide and then dinitrogen with time (or equivalently, with step-wise decrease in oxygen concentration).

Under anoxic conditions, cytochrome aa_3 is not synthesised, or is synthesised at a basal (low) level, and simultaneously with the formation of the nitrogen oxide reductases, the level of cytochrome *o* increases from the level established under fully aerobic conditions to an increased level under anoxic conditions. The implication of this increase in cytochrome *o* in the switch from aerobic to anoxic conditions will become apparent in Part 6 below when the changes in the ETP during a reverse switch (i.e. from steady-state anoxic to steady-state aerobic conditions) are discussed.

From the literature, it is unclear as to the period of time required for complete transformation of the ETP from that synthesised under steady-state aerobic conditions to that synthesised under steady-state anoxic conditions. However, from experiments conducted in this investigation (described in Section II below) it appears that a period of days rather than hours is necessary. Electrons which pass to the reductases are utilised for the reduction of the nitrogen oxides as described by Eqs. (2) to (6) and stimulates synthesis of the reductases. Immediately following a change from steady-state aerobic conditions to anoxic conditions with nitrate and nitrite present, electron transfer to nitrate and nitrite is limited by the level of synthesis of the reductases, since nitrate- and nitrite reductase and the other nitrogen oxide reductases are not synthesised under prior aerobic conditions. This results in an initial reduced growth rate of the organism under anoxic conditions. As the reductases are synthesised 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, as described in Part 3 above.

Part 5 - Anoxic conditions changed to anaerobic conditions

The 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 synthesis of the nitrogen oxide reductases which require the presence of the nitrogen oxides for maximum synthesis. Additionally, anaerobic conditions with substrate present ensures that any intracellularly accumulated nitrogen oxides have been removed. The relevance of this aspect to aerobic respiration will be appreciated in the following section (Part 6).

Part 6 - Steady-state anoxic conditions changed to steady-state aerobic conditions

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

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

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 discussed in the stepwise order in which they affect aerobic respiration after the transition.

(i) Changes in nitrogen oxide reductase activities

Oxygen inhibits the activities of the nitrogen oxide reductases in the order from the reductases for the least reduced nitrogen oxides to the reductases for the most reduced nitrogen oxides, i.e. nitrous oxide-, nitric oxide-, nitrite-, and nitrate reductases (Hochstein et al., 1984). Inhibition is 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 (e.g. nitrous oxide reductase activity is inhibited at a lower oxygen concentration than is nitric oxide reductase activity). Oxygen creates a redox potential at the oxidases sufficient to re-direct electrons to the oxidases from the reductases. As the concentration of oxygen increases, the redox potentials at the oxidases increase, from lower than the potentials at the lowest nitrogen oxide reductase (nitrous oxide) to greater than the potentials at the reductases for each of the more reduced nitrogen oxides, nitric oxide, nitrite and nitrate. In the presence of nitrate, this results in the successive accumulation of nitrous oxide, nitric oxide, and nitrite (Alefounder et al., 1981).

(ii) 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 inhibited or prevented (John, 1977; Alefounder and Ferguson, 1980; Ku era et al., 1983; Noji and Taniguchi, 1987). This effect is specific to the movement of nitrate. Accepting that under steady-state anoxic conditions nitrate movement across the membrane occurs via an NO_3^-/NO_2^- antiport mechanism (John, 1977), it follows that impermeability of the membrane to nitrate movement results from an effect of oxygen in preventing operation of the antiport mechanism.

(iii) 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 (Newton, 1967; Lam and Nicholas, 1969b) but to only 30% of the level compared with anoxic conditions. It is proposed that synthesis of cytochrome aa_3 occurs at a rate equal to the rate of degradation of cytochrome *o*, such that the nett level of available cytochrome oxidase (cytochrome *o* + cytochrome aa_3) is at the least, maintained under aerobic conditions. [*Note: 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 under anoxic conditions it is likely that it is not only the level of cytochrome o that degrades, but also that "new" organisms have a significantly lower level of cytochrome o, yielding a lower nett level for the sludge mass with time, both causing a decreasing level of cytochrome o in the surrogate organism*]. Under aerobic conditions, electron flow to the oxidases is utilised for the reduction of oxygen and stimulates 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 a 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 to 8 h 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).

(iv) Interaction of nitric oxide with cytochrome o

Interaction of intracellular nitric oxide with cytochrome *o* prevents electron transfer to oxygen at the oxidase. Such interaction is possible only if nitric oxide (produced from the reduction of nitrite) is present 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 when conditions change to aerobic, because the other oxidase cytochrome aa_3 is not synthesised under the prior anoxic conditions. As described in Part 2 above, cytochrome *o* is different to cytochrome aa_3 in that the reactive centre of cytochrome o contains two iron (Fe) and one copper (Cu) atom whereas the reactive centre of cytochrome aa_3 comprises two Fe atoms and two Cu 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. [*Note: 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 aa_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. It is hypothesised that the physical conformation of the Fe-Cu couple does not lend itself to interaction with nitric oxide*]. 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. A description of the hypothesised interactions is given in Part 7 below.

Part 7: Exposure of organisms to frequent changes between anoxic and aerobic conditions

In Parts 1 to 6 described above, a conceptual model for facultative organisms is described for steady-state conditions and for changes from one steady-state condition to another. The objective in the formulation of this model is to apply it to the sludge mass which develops in N, and N&P removal systems. In such systems the sludge is alternately subjected to relatively short periods of aerated and unaerated conditions, for periods insufficient to achieve maximal production of the ETP enzyme systems compared with steady-state aerobic conditions (see Part 2 above), or steady-state anoxic conditions (see Part 3 above). The biochemical processes that

develop under alternately aerated/unaerated conditions are described here.

Consider an intermittently aerated system such as that described by Gabb et al. (1996) in which the aerobic mass comprises 35% of the total mass in the system. In such a system the heterotrophic sludge mass (modelled as the surrogate facultative organism mass) typically experiences an anoxic-aerobic cycle every 20 min. These cycles are considered to stimulate changes in the enzyme systems of the surrogate organism mass. For an organism subjected to alternating anoxic-aerobic conditions, in the light of the above, it is reasonable to assume that all of the reductases and the oxidases are synthesised, albeit not to the levels produced under the steady-state conditions. Because of the exposure to anoxic conditions for a high proportion of the intermittent aeration cycle (about 60 to70% of the time), all the reductases are synthesised at high levels and of the oxidases, cytochrome *o* is synthesised at a high level and cytochrome aa_3 at a low level. However, compared to steady-state anoxic conditions, the reductases and cytochrome *o* are synthesised at a lower level and cytochrome aa_3 at a higher level. Starting from the time at which the aerobic period begins, the changes in complexes and transport processes associated with the ETP are essentially similar to those described in Part 6 above, i.e.:

- The activities of the reductases are inhibited by oxygen resulting in the intracellular accumulation of nitric oxide and nitrite.
- Oxygen reduces the permeability of the cytoplasmic membrane such that movement of nitrate to the active site of its reductase on the cytoplasmic side of the membrane is restricted.
- Synthesis of the reductases and the oxidase cytochrome *o* are prevented and synthesis of cytochrome aa_3 is induced in the presence of oxygen. However, due to the short aerobic time period in the alternating anoxic-aerobic system described here, little change occurs in the levels of the reductases or oxidases during this period.
- Intracellularly accumulated nitric oxide interacts with the Fe-containing complexes of the oxidase cytochrome *o*, creating iron-nitric oxide (Fe-NO) complexes which are incapable of transferring electrons. Nitric oxide does not inhibit the oxidase cytochrome aa_3, but since this oxidase is present at a low level under the alternating anoxic-aerobic conditions, it does not provide a high flow of electrons to oxygen. [*Note: 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*]. Electrons, unable to be passed to cytochrome *o*, are available to the reductases. From redox potential considerations of the reactions for 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. Nitric oxide then meets one of two fates: reduction by nitric oxide reductase to form nitrous oxide, or reaction with any uninhibited cytochrome *o* on the cytoplasmic side of the membrane resulting in continued inhibition of electron transfer to oxygen (Carr and Ferguson, 1990) and continued re-direction of electrons to nitrite reductase. Immediately upon a change from anoxic to aerobic conditions, any nitrite that has accumulated intracellularly through inhibition of the reductases by oxygen will act as electron acceptor at nitrite reductase. However, because the active site of nitrite reductase is situated on the periplasmic side of the membrane, extracellular nitrite (produced through nitrification under aerobic conditions) can become intracellular and act as intracellularly produced nitrite, thereby contributing to the inhibition mechanism.

Part 8: The effect on inhibition of aerobic respiration due to magnitude of the aerobic mass fraction, availability of RBCOD, and concentration of nitrite

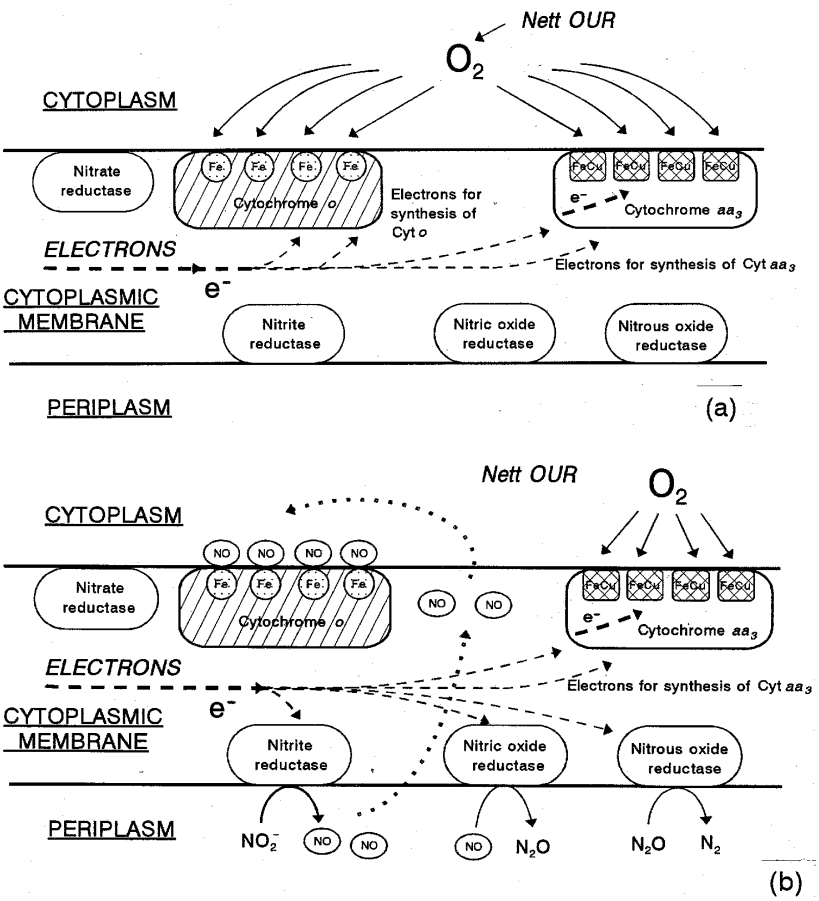
[*Note: In this analysis it is accepted that slowly biodegradable substrate is always present irrespective of the presence or absence of readily biodegradable substrate. Thus, when it is stated that readily biodegradable substrate is absent, it is implied that electrons are supplied by slowly biodegradable substrate*]. Changes in the three factors given above are of particular importance in the mechanism of inhibition of aerobic respiration. Many combinations of these factors are possible but the nett effect of only the two most important scenarios is described, i.e. anoxic-aerobic conditions in which nitrite is present under anoxic conditions and in which readily biodegradable substrate is present or absent under aerobic conditions.

Effect on inhibition of aerobic respiration under aerobic conditions of the concentration of nitrite under preceding anoxic conditions

In modelling the alternating anoxic-aerobic conditions described above it was assumed that nitrite was present throughout the anoxic period such that denitrification was not limited. For conditions in which the nitrite present is completely denitrified before the end of the anoxic period and start of the aerobic period, conditions are similar to those described in Part 5 in which anoxic conditions become anaerobic. Neither nitric oxide nor nitrite is accumulated intracellularly and at the start of aerobic conditions, inhibition of aerobic respiration is not induced - electrons are transferred to oxygen via cytochrome aa_3 and *o*. The effect on inhibition of the presence and absence of nitrite is shown diagrammatically in Figs. 4a and b respectively. A description of the movement of electrons, oxygen and the nitrogen oxide intermediates and the interaction between these and the reductases was given in Part 7 above.

Effect on inhibition of aerobic respiration of the rate of supply of electrons

(i) *Readily biodegradable substrate present under anoxic conditions:* In modelling the alternating anoxic-aerobic conditions described above it was assumed that the rate of supply of electrons to the facultative organism mass was due to the presence of slowly biodegradable substrate (i.e. slow rate of supply). As described in Part 3 above , with slowly biodegradable substrate under anoxic conditions with nitrate present, electrons flow preferentially to nitrate reductase where the greatest redox potential develops and nitrite, nitric oxide, and nitrous oxide accumulate. Under subsequent aerobic conditions, aerobic respiration is inhibited by the accumulated nitric oxide. With readily biodegradable substrate present under anoxic conditions, the rate of supply of electrons is sufficient to maintain low levels of all the denitrification intermediates and inhibition is not induced at the start of subsequent aerobic conditions.



Figures 4a and b

Model of interactions about the cytoplasmic membrane of a conceptualised facultative organism grown under alternating anoxic-aerobic conditions, illustrating electron transfer to oxygen under aerobic conditions in the (a, top) absence of nitrite and (b, bottom) presence of nitrite, illustrating the mechanisms resulting in inhibition of aerobic respiration

(ii) *Readily biodegradable substrate present under aerobic conditions:* In modelling alternating anoxic-aerobic conditions with electrons supplied by slowly biodegradable substrate, nitric oxide that accumulates under anoxic conditions inhibits electron transfer to cytochrome *o* under subsequent aerobic conditions and electrons are redirected to nitrite reductase as described in Part 7 above. However, if readily biodegradable substrate is present under aerobic conditions, the supply of electrons is sufficiently high to maintain low levels of intracellular nitrite and nitric oxide, and cytochrome aa_3 is not inhibited to the same extent. Additionally, the high rate of electron supply allows increased synthesis of the uninhibited oxidase cytochrome *o*, with concomitant progressively increasing uninhibited aerobic respiration. The effect on inhibition of the presence of readily biodegradable substrate is illustrated in Figs. 5a to d.

Effect of magnitude of aerobic mass fraction on inhibition of aerobic respiration

For facultative organisms subjected to alternating anoxic-aerobic conditions with a larger aerobic mass fraction than the 35% described above, the reductases and cytochrome *o* are synthesised at lower levels and cytochrome aa_3 is synthesised at a higher level, which results in a lower aerobic inhibition since a greater proportion of electrons pass to cytochrome aa_3 which is not inhibited by nitric oxide. For alternating anoxic-aerobic conditions with a smaller aerobic mass fraction than the 35% described above, the reductases and cytochrome *o* are synthesised at higher levels and cytochrome aa_3 is synthesised at a lower level. For conditions in which nitrite is available, aerobic respiration is inhibited to a greater extent since a greater proportion of electrons would pass to cytochrome *o* (high level of synthesis) than to cytochrome aa_3 (low level of synthesis). However, as the aerobic mass fraction becomes smaller, the contribution of aerobic respiration to total respiration (aerobic + anoxic) becomes less significant. At a significantly reduced aerobic mass fraction, conditions approach those described in Part 3 for steady-state anoxic conditions.

Energetic yield associated with inhibition of aerobic respiration

Ekama et al. (1996) showed 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 six protons were transferred across the membrane, resulting in the production of three ATP; for each pair of electrons reaching oxygen at cytochrome *o*, two proton-pumping sites were passed, at which a total of four protons were transferred across the membrane resulting in the production of two 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 four protons were transferred across the membrane resulting in the production of two ATP. From this it can be concluded that a lower energetic yield results from inhibited aerobic respiration compared with uninhibited aerobic respiration. In the inhibited case, a proportion of electrons is re-directed to the nitrogen oxide reductases which results in the production of about two-thirds of the energy had the electrons passed to cytochrome aa_3 during uninhibited aerobic respiration.

Proposals and implications of the biochemical model

The conceptual model described above was formulated by representing the facultative heterotrophic organism mass from activated sludge as a single facultative heterotrophic organism. Proposals and implications of the model listed below provide specific aspects which can be tested experimentally using the facultative heterotrophic organism mass harvested from activated sludge systems subjected to various environmental factors, viz. magnitude of aerobic mass fraction, concentration of nitrite and nitrate and rate of supply of electrons from RBCOD (high rate) and SBCOD (low rate).

(1) Effect of nitrite concentration under anoxic conditions, on induction of inhibition of respiration under subsequent aerobic conditions, with low rate of supply of electrons

- 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 accumulation inhibits utilisation of oxygen under subsequent aerobic conditions.

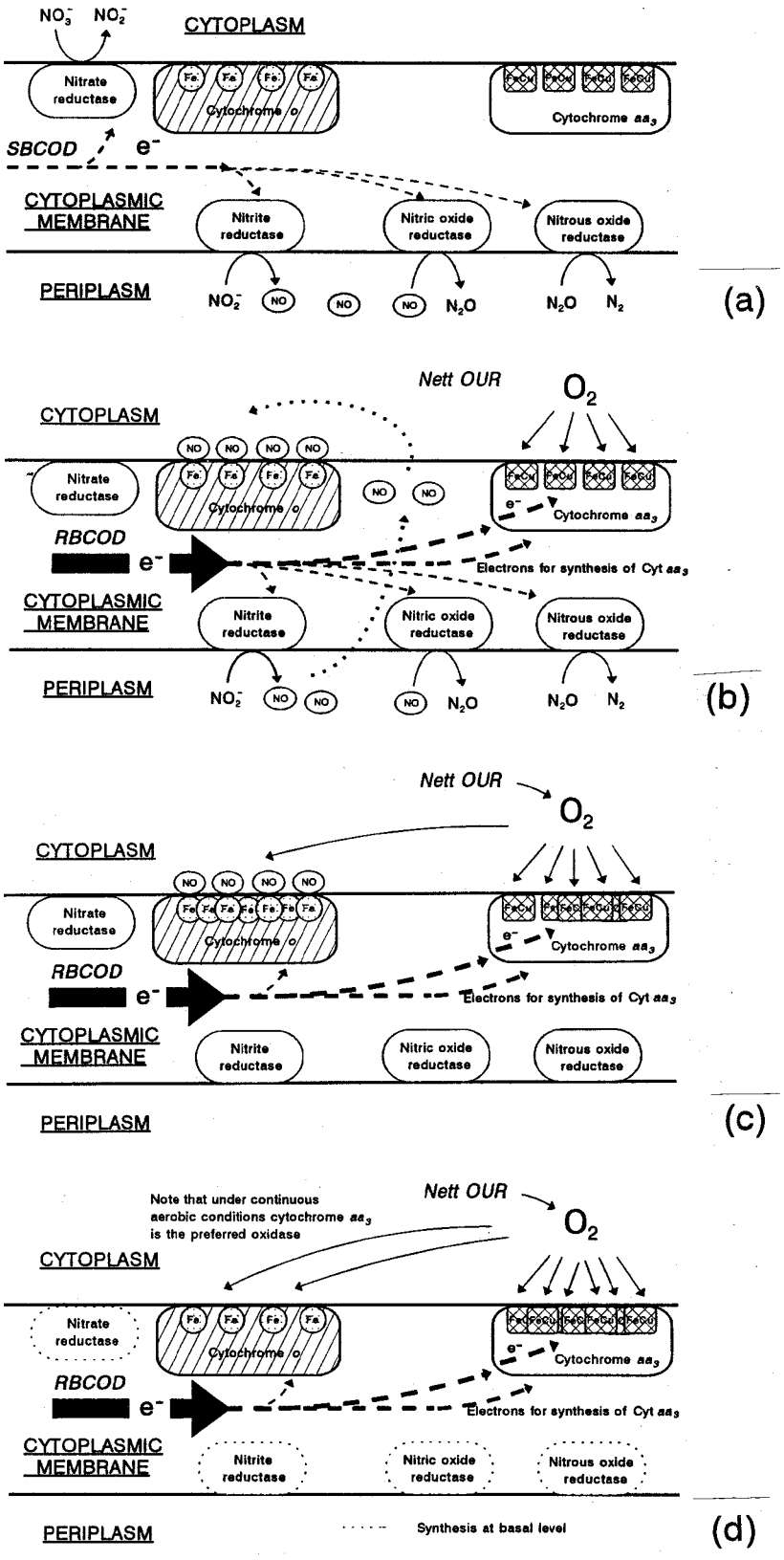
- With nitrite absent under anoxic/anaerobic conditions, no accumulation of nitric oxide occurs and aerobic respiration will not be inhibited under subsequent aerobic conditions, with low rate of supply of electrons.
- The extent of inhibition of oxygen utilisation under aerobic conditions (with low rate of supply of electrons) is proportional to the level of nitric oxide accumulated intracellularly under prior anoxic conditions, which in turn is proportional to the concentration of extracellular nitrite under the same anoxic conditions.

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

- Even if inhibition is not induced under preceding anoxic conditions, 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 aerobic denitrification of nitrite is proportional to the concentration of nitrite under aerobic conditions.
- The extent of inhibition of aerobic respiration induced by nitrite under aerobic conditions is less than the extent of inhibition of oxygen utilisation that would be induced by the same nitrite concentration under preceding anoxic conditions.
- With nitrate added or present under aerobic conditions, inhibition of aerobic respiration will not be induced due to impermeability of the membrane to nitrate movement to its reductase.

(3) Effect of rate of supply of electrons under aerobic condition on induction and relief of inhibition of aerobic respiration

- With only slowly biodegradable substrate present under aerobic conditions, nitrite (either added or present under preceding anoxic conditions) is denitrified under the aerobic conditions and inhibition of respiration will be induced.
- If inhibition is present at the start of aerobic conditions (due to the reduction of nitrite under preceding anoxic conditions), the presence of readily biodegradable substrate under the aerobic conditions will relieve inhibition.
- With readily biodegradable substrate present under aerobic conditions, nitrite added or present will not result in accumulation of nitric oxide and inhibition of aerobic respiration will not be induced.



Figures 5a to d

Model of interactions about the cytoplasmic membrane of a conceptualised 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 and c) aerobic conditions and (d) continuous aerobic conditions with nitrite present and readily biodegradable substrate (RBCOD) as electron source

- Under aerobic conditions, inhibition, which is relieved with readily biodegradable substrate will be reinduced when the readily biodegradable substrate has been exhausted and only slowly biodegradable substrate and nitrite is 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 systems).

(5) Inhibition of aerobic respiration results in aerobic denitrification

- If conditions are such that inhibition of aerobic respiration is induced, then nitrite reduction, but not nitrate reduction will predominantly take place, since nitrate cannot gain access to its reductase under aerobic conditions due to impermeability of the cytoplasmic membrane to nitrate movement.

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

- For anoxically grown sludge subjected to aerobic conditions, the potential for inhibition of aerobic respiration will decrease with continued exposure to aerobic conditions as a consequence of decreased synthesis of the nitrogen oxide reductases and increased synthesis of the uninhibited oxidase cytochrome aa_3.
- For aerobically grown sludge subjected to anoxic conditions, potential for inhibition of aerobic respiration will increase with continued exposure to anoxic conditions as a consequence of increased synthesis of the nitrogen oxide reductases and the inhibited oxidase cytochrome *o*, 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 as a consequence of decreased synthesis of the nitrogen oxide reductases.

Section II
Experimental testing of the conceptual biochemical model

Introduction

In Section I a conceptual model was formulated for the biochemical mechanisms of aerobic-facultative heterotrophic organisms subjected to various environmental conditions. The model was formulated from a literature review which highlighted the significant biochemical mechanisms of aerobic facultative organisms, especially with regard to interaction between the aerobic and anoxic respiration pathways of those organisms; hypotheses regarding the kinetics of denitrification under aerobic and anoxic conditions with readily biodegradable substrate present and absent; and results of 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 process phenomena such as nitrification, aerobic substrate utilisation 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, by monitoring changes in the reactants and products of these processes. Consequently, the experimental programme which follows below should be seen in the light of an endeavour to accumulate a body of circumstantial evidence to test the proposals and implications of the hypothesised biochemical model.

It is important to note that the implications listed under statements (1), (2), and (3) below are for organisms exposed to frequent alternating anoxic-aerobic conditions (such that both aerobic and anoxic respiratory enzymes are synthesised). Therefore, the experimental work to test the implications was conducted on activated sludge developed under such conditions, i.e. a 2RND (MLE) system (System 5, Lakay et al., 1999) with 3:1 a-recycle and 1:1 s-recycle, unless otherwise stated. For the implications listed under statement (7) below, work to test these was conducted on sludge developed under steady-state aerobic (completely aerobic) and steady-state anoxic (completely anoxic) periods in an IAND system (System 1, Lakay et al., 1999).

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. However, only a sample of those batch tests are described in this paper. The numbering system for the batch tests from the original test program has been retained and as a consequence the numbers for the batch tests in this paper are not sequential. Each of the selected batch test experiments is described below with an explanation of the relevance of the results to the hypothesis. The conditions and results for all of the batch tests from the original experimental program are described by Casey et al. (1994).

(1) Respiration under aerobic conditions which follow an anoxic period 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 present at the start of aerobic conditions. Four aerobic batch tests (BT2, BT4, BT5, BT11) were conducted on sludge harvested from the 2RND system, which was held anoxic for a 2 h period and then aerated as indicated in Fig. 6. Different concentrations of nitrite were added to each batch test during the anoxic period 1 h before aeration commenced, and sewage (as a source of RBCOD) was added at the start of the aeration period. The first test (Batch Test 2, referred to as 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) at the start of aerobic conditions. [*Note: The experimental procedure for conducting these tests and the reasons for the procedure are described by Casey et al. (1994)*].

Figure 6 indicates that the larger the concentration of nitrite present at the onset of aerobic conditions, the greater the degree of inhibition of oxygen utilisation. [*Note: The measure of the degree of inhibition is obtained by calculating the fraction the initial specific oxygen utilisation rate ($\hat{\mu}_i$) is of the maximum specific*

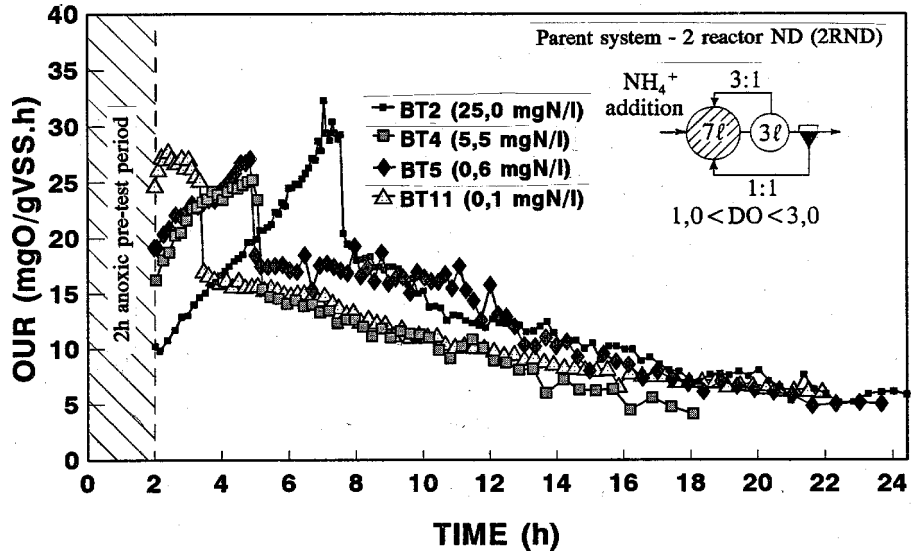


Figure 6
OUR (in mgO/gVSS·h) with time (h) for Batch Tests (BT) 2, 4, 5 and 11 with different concentrations of nitrite present at the onset of aerobic conditions conducted on sludge harvested from System 5 of Lakay et al. (1999) (see their Fig. 7) on Days 227 (BT2), 233 (BT4), 240 (BT5) and 280 (BT11)

oxygen utilisation 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 utilised completely (indicated by a precipitous drop in OUR) before the $\hat{\mu}_m$ is reached]. 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, the following conclusions can be drawn:

- Nitrite present with slowly biodegradable substrate under anoxic conditions, and at the start of subsequent aerobic conditions results in inhibition of aerobic respiration.
- With nitrite absent under anoxic conditions and at the start of subsequent aerobic conditions, aerobic respiration is not inhibited.
- 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) The effect of the presence of nitrite and nitrate under aerobic conditions on aerobic respiration

Effect on aerobic respiration of denitrification of nitrite under aerobic conditions

A fundamental aspect of the model is that inhibition under aerobic conditions results from nitric oxide produced by the aerobic denitrification of nitrite. The periplasmic orientation of nitrite reductase would suggest that nitrite has access to its reductase under aerobic conditions, but it was uncertain whether aerobic denitrification could occur at a sufficienty high rate to produce nitric oxide in quantities that would inhibit oxidase activity. The series of experiments described below investigated two aspects of aerobic denitrification of nitrite - the mechanism of aerobic denitrification of nitrite to nitric oxide, and the time period required for the mechanism to produce sufficient nitric oxide to be inhibitory. In Fig. 7, 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 sewage (i.e. 60, 30, 15 and 5 min respectively). The sludge in these tests was pretreated with a 2 h anoxic-anaerobic period to ensure that no extracellular nitrite or intracellular nitric oxide was present at the end of the start of aerobic conditions. Nitrite was added with the objective of ensuring that at the time of addition of sewage, each of the tests had similar concentrations of nitrite. For batch tests BT13, BT14, BT15 and BT16, 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.

A number of characteristics of the batch tests need to be described in terms of the biochemical and microbiological aspects of the model:

- The anoxic-anaerobic pretreatment conditions would ensure the absence of nitrite and intracellular nitric oxide at the start of the tests,
- The 2 h aerobic period with nitrite and SBCOD, but not RBCOD available, would result in an accumulation of nitric oxide,
- The addition of sewage (containing RBCOD) would ensure a rapid supply of electrons which would allow the reduction of intracellularly accumulated nitric oxide, thereby relieving inhibition of aerobic respiration, noted in the batch tests by an increase in maximum specific OUR with time.

From the results of the batch tests, the following conclusions can be drawn:

- Denitrification of nitrite occurs under aerobic conditions and causes inhibition of aerobic respiration.
- For a given concentration of nitrite, the longer the period for aerobic 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 min before the addition of feed, the periods required for relief of inhibition were approximately 2, 3¼, 3, 4½ h respectively, a generally increasing trend with increase in time. (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 may not be achieved with sewage containing a small fraction of readily biodegradable substrate).

Effect on aerobic respiration of denitrification of nitrate under aerobic conditions

The 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 to nitrate, restricting the movement of nitrate to its reductase. This proposal was tested experimentally as BT12 (see

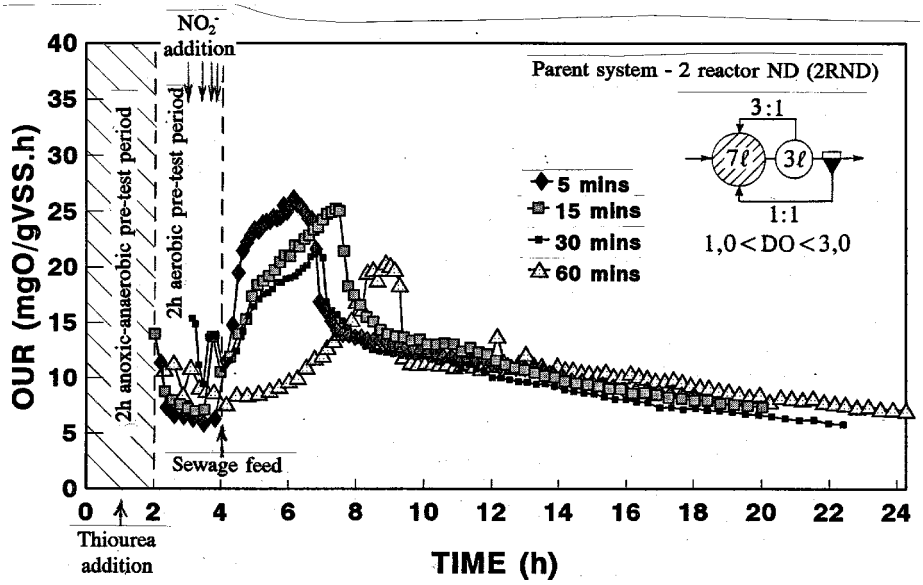


Figure 7
OUR (in mgO/gVSS·h) with time (h) for Batch Tests (BT) 13, 14, 15 and 16 with nitrite addition under aerobic conditions at different time periods before the addition of a substrate conducted on sludge harvested from System 5 of Lakay et al. (1999) (see their Fig. 7) on Days 285 (BT13), 330 (BT14), 333 (BT15) and 334 (BT16)

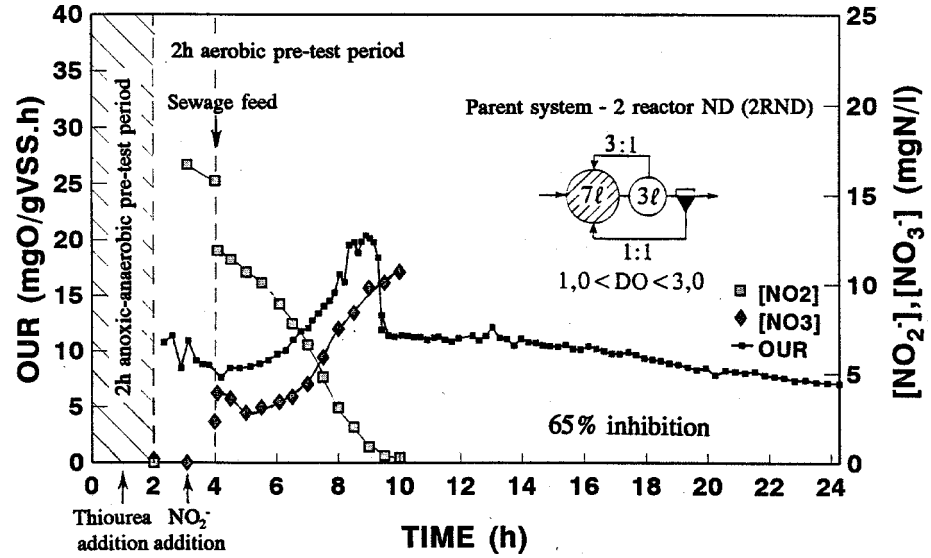


Figure 8
OUR (■, in mgO/gVSS·h) and nitrate (◆) and nitrite (□) concentrations with time (h) for Batch Test 13 with nitrite added under aerobic conditions in the absence of readily biodegradable substrate conducted on sludge harvested from System 5 of Lakay et al. (1999) (see their Fig. 7) on Day 285.

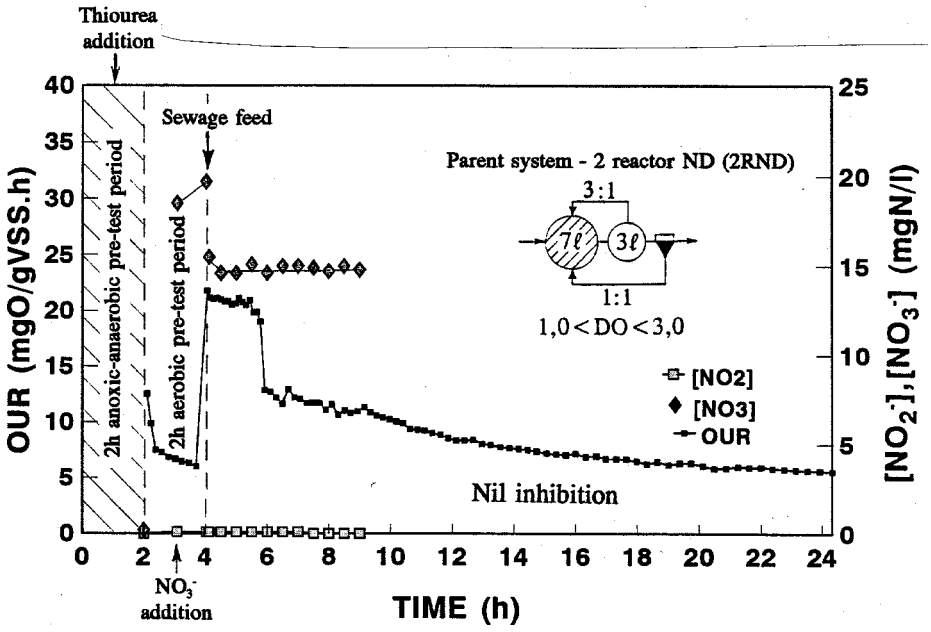


Figure 9
OUR (■, in mgO/gVSS·h) and nitrate (◆) and nitrite (□) concentrations with time (h) for Batch Test 12 with nitrate added under aerobic conditions in the absence of readily biodegradable substrate demonstrating no induction of inhibition conducted on sludge harvested from System 5 of Lakay et al. (1999) (see their Fig. 7) on Day 284.

Fig. 9) and was compared with the nitrite denitrification results from BT13, details of which are given in Fig. 8. To test aerobic denitrification of nitrate, the same experimental conditions were applied as for BT13, except that nitrate instead of nitrite was added under aerobic conditions 1 h before the addition of sewage (BT12, see Fig. 9). The concentration of nitrate after addition of sewage (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 sewage addition in BT5, shown in Fig. 8. As indicated in Fig. 9, addition of nitrate under aerobic conditions did not result in inhibition of OUR since there was no initial period of OUR increase following sewage addition. From this result it can be concluded that nitrate is not denitrified to the intermediates NO_2^-, NO, and N_2O with SBCOD under aerobic conditions.

(3) Effect of rate of electron supply under aerobic conditions on induction and relief of inhibition of aerobic respiration

With slowly biodegradable substrate, aerobic denitrification of nitrite induces inhibition of respiration

In (2) above it was described that for nitrite added under aerobic conditions, inhibition of aerobic respiration is induced (Batch Tests 13 to 16). In these tests, nitrite was added under aerobic conditions with only slowly biodegradable substrate 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 relieves inhibition of respiration induced by denitrification of nitrite with slowly biodegradable substrate under preceding anoxic conditions

In (1) above it was described that nitrite addition under anoxic conditions induces inhibition of aerobic respiration under subsequent aerobic conditions (Batch Tests 2, 4, 5 and 11). In these tests, nitrite was added under anoxic conditions with only slowly biodegradable substrate 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, inhibition is not induced

From the foregoing batch tests it is apparent that addition of RBCOD relieves the inhibition effect, reflected in a steadily increasing maximum specific OUR following the addition of sewage. 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 and produce nitric oxide, rather than to nitric oxide reductase to reduce it; the increased nitric oxide concentration inhibits aerobic respiration.

To examine this aspect more directly, two further aerobic batch tests were conducted and compared with BT13, i.e. BT 17 and BT1. In BT13 (Fig. 8) nitrite was added 1 h before sewage RBCOD was added. In BT12 (Fig. 10), nitrite was added 1 h after sewage RBCOD was added, and in BT1 (Fig. 11), no nitrite was added, but sewage RBCOD was added. Inhibition was noted in only one case, i.e. BT13, where nitrite was added in the absence of sewage RBCOD. This indicated that inhibition is induced by nitrite denitrification (when a slow rate of supply of electrons (SBCOD) exists.

It could be argued that in Batch Test 17 inhibition was induced, but also that the decrease in OUR due to inhibition was offset by a larger increase in OUR due to nitrification. However, this does not appear to be the case, given that for BT13 (Fig. 8) the addition of nitrite in the absence of readily biodegradable substrate had a constant oxygen requirement (for nitrification) of about 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 indicate that the increase in OUR following the addition of nitrite was due to nitrification only, not the nett result of inhibition and nitrification.

(4) Inhibition relieved with readily biodegradable substrate will be re-induced in the absence of readily biodegradable substrate

From the model, in the presence of a fast rate of supply of electrons (RBCOD available), inhibition is relieved, and in the absence of a fast rate of supply of electrons (only SBCOD available), inhibition is induced in the presence of nitrite. In the foregoing batch tests in which inhibition is induced with slowly biodegradable substrate and relieved with readily biodegradable substrate, it is unclear as to whether relief is permanent or temporary. To examine this aspect, Batch Tests 10 and 11 were conducted (see Fig. 12), in which sewage was added to sludge from which nitrite and nitrate had been removed through the initial 6 h pretest period. The OUR profile illustrated in Fig. 12 indicates no inhibition as expected (similar to Fig. 11) and indicates that the RBCOD was completely utilised after 2 h. About 20 h after sewage addition, nitrite was added at a concentration of 6.0 mgNO_2^--N/ℓ and 5 min later sewage was added. The presence of nitrite and slowly biodegradable substrate, but absence of readily biodegradable substrate in the 5 min period prior to sewage addition, induced inhibition, which took 4½ h 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 of 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 a fast rate of supply of electrons (readily biodegradable substrate) and inhibition occurs or is induced in the presence of a slow rate of supply of electrons (slowly biodegradable substrate) with nitrite present, irrespective of whether the sludge has previously been relieved of, or was free of inhibition.

(5) Inhibition of respiration is a general mechanism applicable to all sludges subjected to alternating anoxic-aerobic conditions irrespective of system configuration

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 IAND system were each subjected to the same conditions as described in Statement 1 above and illustrated in Fig. 6.

Similar results were measured in sludges from MUCT and IAND systems, indicating that inhibition of aerobic respiration can be stimulated in sludges from different N removal configurations.

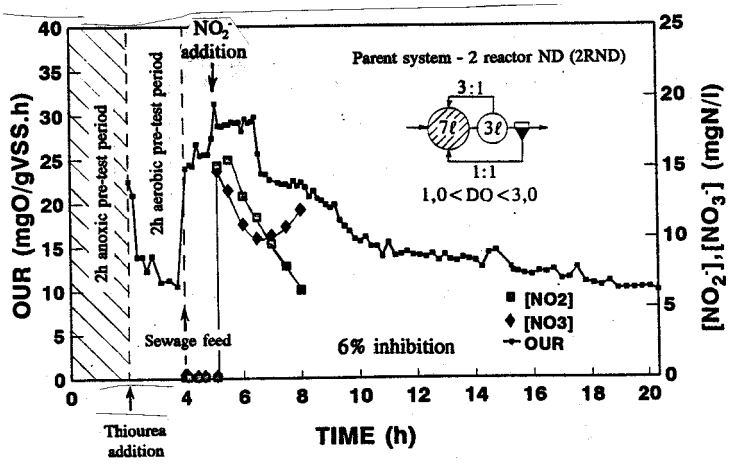


Figure 10

OUR (■, in mgO/gVSS·h) and nitrate (◆) and nitrite (□) concentrations with time (h) for Batch Test 17 with nitrite added under aerobic conditions in the presence of readily biodegradable substrate demonstrating little inhibition conducted on sludge harvested from System 5 of Lakay et al. (1999) (see their Fig. 7) on Day 335.

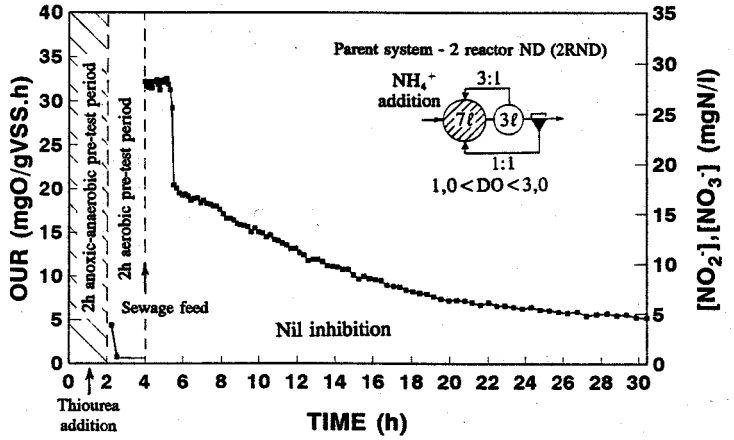


Figure 11

OUR (■, in mgO/gVSS·h) and nitrate (◆) and nitrite (□) concentrations with time (h) for Batch Test 1 with nitrate and nitrite absent before addition of substrate demonstrating no induction of inhibition conducted on sludge harvested from System 5 of Lakay et al. (1999) (see their Fig. 7) on Day 225.

(6) Inhibition of aerobic respiration results in aerobic denitrification

Batch tests which demonstrate aerobic inhibition have an associated nett loss of NO_x unaccounted for by nitrification of nitrite to nitrate. For batch tests in which inhibition was not induced, all nitrite which disappeared, reappeared as nitrate through nitrification. Typically, sludges demonstrating inhibition of aerobic respiration show an initial rapid decrease in nitrite concentration due to denitrification, followed by an increase in nitrate concentration due to nitrification of nitrite (nitrification of ammonium to nitrite was inhibited by thiourea). For Batch Test 20, illustrated in Fig. 13 and conducted on sludge from an IAND System, in the first 7 h after feedings, 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 to dinitrogen. In Fig. 13, 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 utilised. Over the 4¼ h in which readily biodegradable substrate was present, approximately 1.4 $mgNO_3^-$-N/ℓ disappeared and approximately 0.9 $mgNO_2^-$-N/ℓ appeared, suggesting that 0.5 $mgNO_3^-$-N/ℓ was denitrified to dinitrogen. Since 1.5 mgN/ℓ in total was denitrified during the first 7 h after feeding, 1.0 $mgNO_2^-$-N/ℓ was denitrified during the same period. It appears that under aerobic conditions, for a sludge demonstrating 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 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

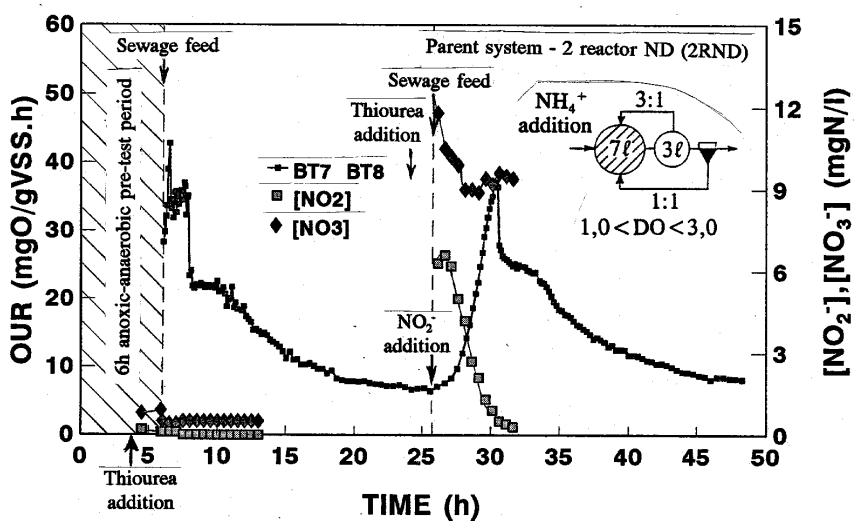


Figure 12

OUR (■, in mgO/gVSS·h) and nitrate (◆) and nitrite (□) concentrations with time (h) for Batch Tests (BT) 7 and 8 with inhibition relieved and then induced in the same sludge conducted on sludge harvested from System 5 of Lakay et al. (1999) (see their Fig. 7) on Days 252 (BT7) and 253 (BT8).

in statement (2) above, 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 these results it appears that the membrane is not completely impermeable to nitrate; some nitrate can pass across it to nitrate reductase.

(7) Synthesis of aerobic and anoxic pathways enzymes under steady-state aerobic and steady state anoxic conditions

The conceptual model describes the effect of steady-state aerobic and anoxic conditions on the synthesis of the enzymes of the aerobic and anoxic electron transport pathways. The model proposes that facultative organisms grown under aerobic conditions are unable to denitrify immediately when placed under anoxic conditions; and facultative organisms grown under anoxic conditions and then subjected to aerobic conditions can utilise substrate immediately under the aerobic conditions as a consequence of the development of constitutive cytochrome *o* under anoxic conditions.

Sludge in a single reactor system (System 1 - Lakay et al., 1999) was maintained under continuous anoxic conditions (3 sludge ages) and continuous aerobic conditions (3 sludge ages). To examine the effect of aerobic and anoxic conditions on the synthesis of aerobic and denitrifying enzymes, aerobic inhibition batch tests were conducted periodically on the sludge from the system during the anoxic and aerobic conditions. Since inhibition is a

Figure 13 (right)
Changes in nitrate (◆, mgNO_3^--N/ℓ) and nitrite (□, mgNO_2^--N/ℓ) concentration with time after feeding for Batch Test 20 conducted on sludge harvested from System 6 of Lakay et al. (1999) (see their Fig. 8) on Day 300.

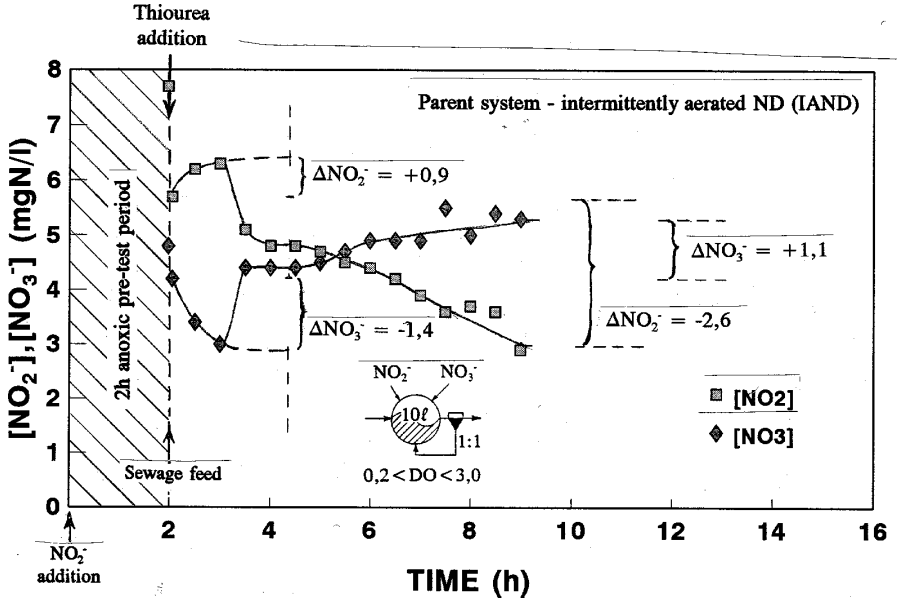
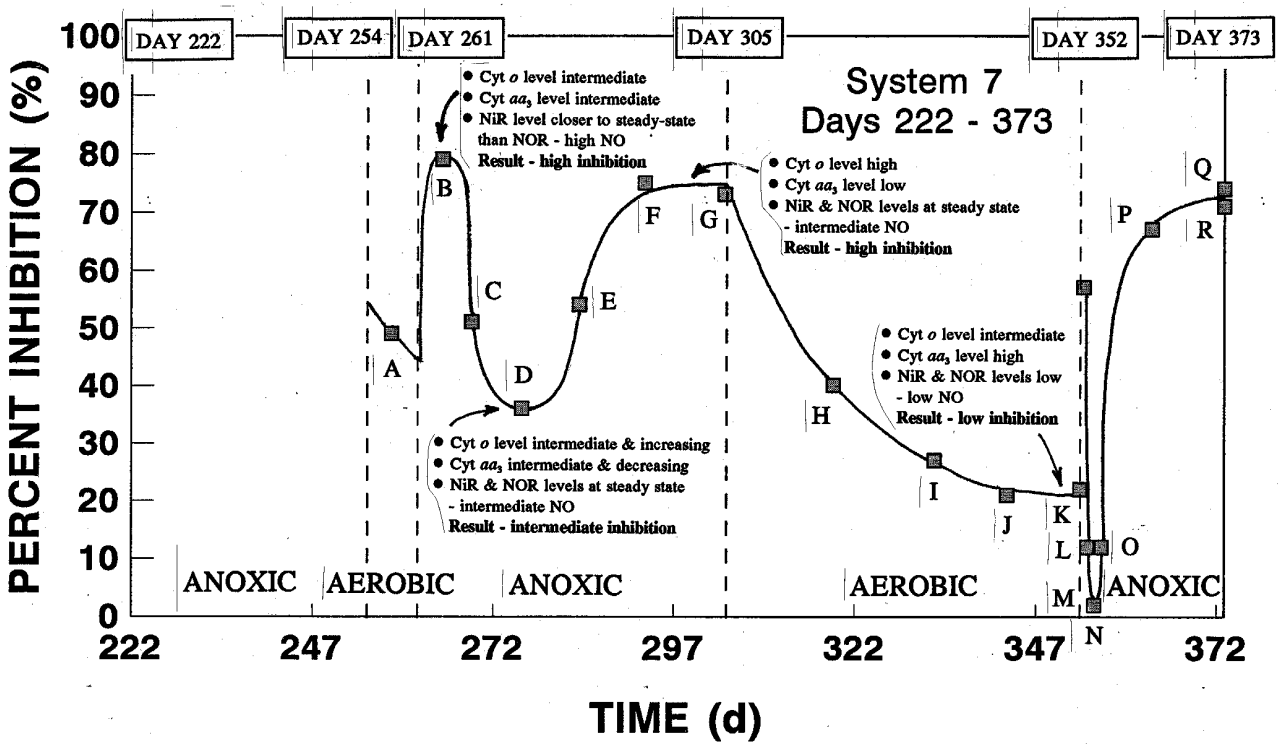
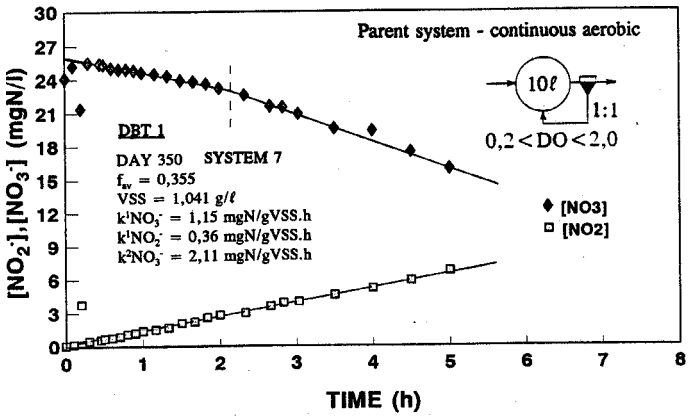
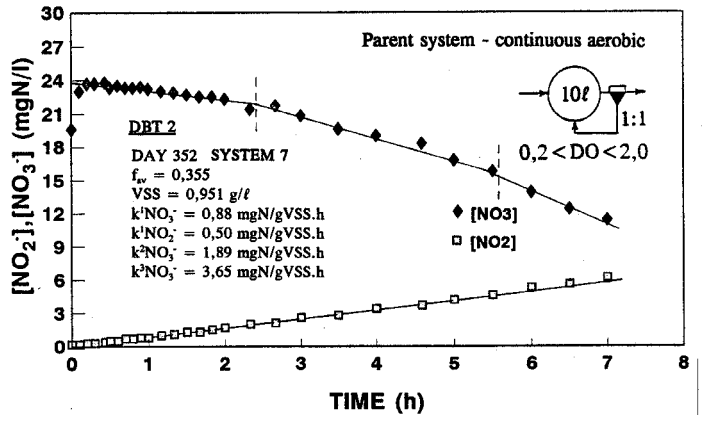


Figure 14 (bottom)
Percentage aerobic inhibition with time resulting from changes between aerobic and anoxic conditions as measured in 18 aerobic batch tests (BTs A to R) on sludge from System 1 of Lakay et al. (1999) (see their Fig. 3) on Days 258 (A), 265 (B), 269(C), 276 (D), 284 (E), 293 (F), 304 (G), 319 (H), 333 (I), 343 (J), 353 (K), 353 (L), 354 (M), 355 (N), 356 (O), 363 (P), 373 (Q) and 373 (R).

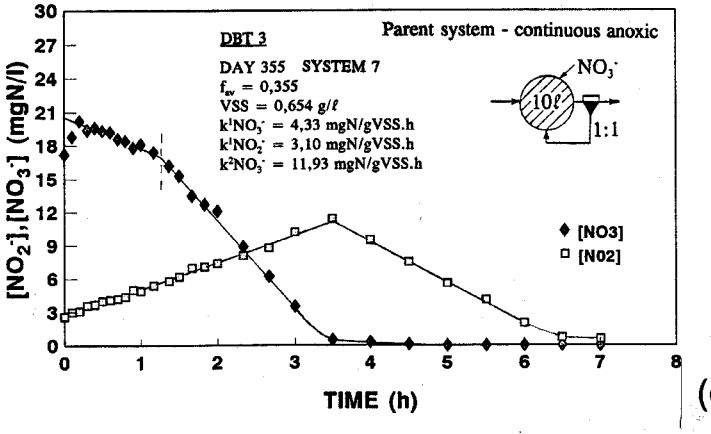




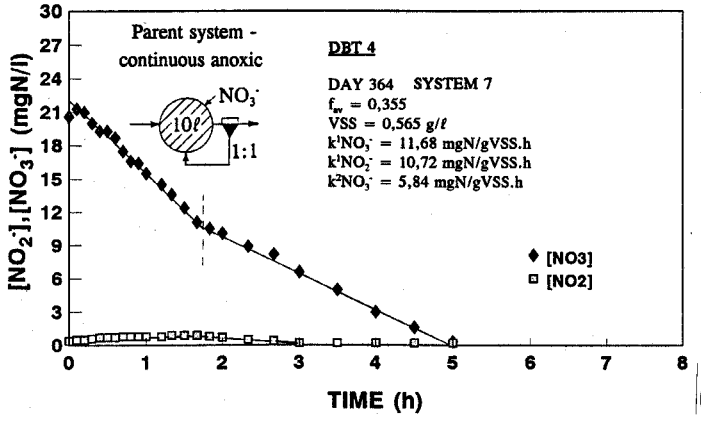
(a)



(b)



(c)



(d)

function of the level of the nitrogen oxide reductases (in particular nitrite reductase and nitric oxide reductase) and the oxidases, measurement of the level of inhibition provides 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 changes were made from aerobic to anoxic conditions and *vice versa*.

Figure 14 illustrates the degree of inhibition measured in 18 aerobic Batch tests A to R conducted on sludge from System 1 (see Lakay et al., 1999) 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 in Fig. 14 to illustrate the conditions to which the sludge was subjected prior to the first aerobic period. Two periods in Fig. 14 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. The long anoxic period (Days 261 - 305) resulted in steady-state conditions and maximum synthesis of the denitrifying enzymes would be expected by Day 306. Similarly, the long aerobic period (Days 306 - 352) resulted in steady-state conditions and maximum synthesis of the aerobic enzyme cytochrome aa_3 would be expected by 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 resulted from interaction of cytochrome *o* (synthesised 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:

- With continuous exposure to aerobic conditions, cytochrome aa_3 (which is not inhibited by nitric oxide) is synthesised to its maximum level, and, cytochrome *o* (which is strongly inhibited by nitric oxide) degrades to its minimum level, and the greater flow of electrons pass to cytochrome aa_3.
- During the aerobic period, the denitrifying reductases degrade with time, so that lower concentrations of nitric oxide are produced. In combination, a low concentration of nitric oxide, a low level of cyto-

Figure 15

Change in denitrification rate of nitrate and nitrite with time resulting from changes in operating conditions from fully aerobic to fully anoxic as measured in four anoxic batch tests (DBT 1 to 4) on sludge harvested from System 1 of Lakay et al. (1999) (see their Fig. 3) on Days 350 (DBT1), 352 (DBT2), 355 (DBT3) and 364 (DBT4).

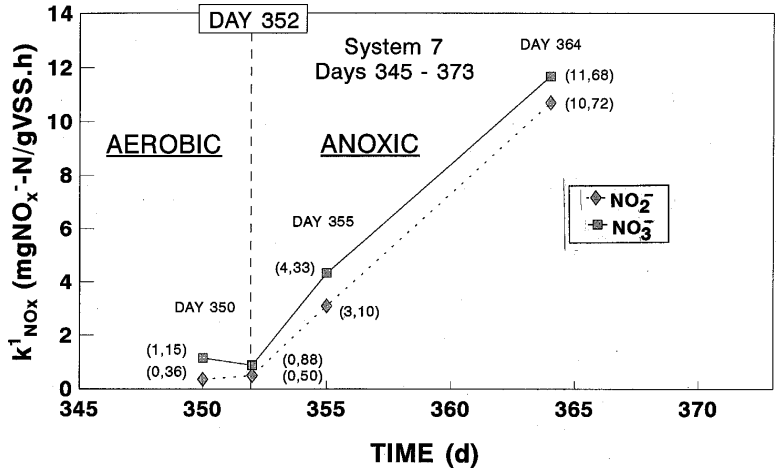


Figure 16
Rates of denitrification of nitrate (■, k^1_{NO3-}) and nitrite (◆, k^1_{NO2}) with time for a change from fully aerobic to fully anoxic operating conditions for sludge harvested from System 1 of Lakay et al. (1999) (see their Fig. 3) calculated from the results in Fig. 15.

chrome *o* and a high concentration of cytochrome aa_3 results 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. 14. During both periods, inhibition initially increased, then decreased, and then increased again to similar high steady-state values (≈72%). The difference in rates of increase and decrease in level of inhibition between the two anoxic periods may be a consequence of the length of the preceding aerobic period.

The overall increase in inhibition with time results from an increase in the level of nitrite reductase which produces nitric oxide, combined with an increase in the level of cytochrome *o*, and a reduction in the level of cytochrome aa_3.

Exposure of aerobically grown sludge to anoxic conditions will increase the denitrification potential of the sludge

Anoxic denitrification batch tests (DBT) were conducted on sludge from System 1 on Day 350 (DBT1) and Day 352 (DBT2) when System 1 was operated under aerobic conditions, and on Day 355 (DBT3) and Day 364 (DBT4) when it was operated under anoxic conditions. The results of the batch tests are illustrated in Fig. 15. For 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_{NO3-}, k^2_{NO3-}, and k^3_{NO3-} and the nett rate of nitrite reduction is labelled k^1_{NO2-}.[*Note: The k^1_{NO3-}, k^2_{NO3-} and k^3_{NO3-} rates to indicate the first, second and third rates of nitrate reduction must not be confused with the first, second and third rates of nitrate reduction K_1 , K_2, K_3 or K'_1 , K'_2, K'_3 employed in wastewater research to denote nitrate reduction with RBCOD, SBCOD in the primary anoxic reactor and endogenous SBCOD in the secondary anoxic reactor respectively and therefore are given lower case k symbols*]. The increase in nitrate concentration during the first 20 min 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_{NO3-} and k^1_{NO2-} from DBT1 to DBT4 are plotted in Fig. 16 and are discussed below.

Nitrate reduction: For sludge developed under aerobic conditions, the initial rates of nitrate reduction (k^1_{NO3-}) were very low for DBT1 (1.15 mgN/gVSS·h) and DBT2 (0.88 mgN/gVSS·h). On Day 352, System 1 was made anoxic, and the value of k^1_{NO3-} increased from 0.88 mgN/gVSS·h to 4.33 mgN/gVSS·h after 3 d of anoxic conditions, and to 11.68 mgN/gVSS·h after 15 d. This resulted from increased synthesis of nitrate reductase with exposure to anoxic conditions.

Nitrite reduction: Figure 16 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_{NO2-}) 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, rates of nitrite reduction were very low for DBT1 (k^1_{NO2-} = 0.36 mgN/gVSS·h) and DBT2 (k^1_{NO2-} = 0.50 mgN/gVSS·h). This is a result of the non-synthesis of nitrite reductase under aerobic conditions. Under anoxic conditions, the nitrite denitrification rate k^1_{NO2-} increased from 0.50 mgN/gVSS·h to 3.10 mgN/gVSS·h after 3 d of anoxic conditions and to 10.72 mgN/gVSS·h after 15 d. A noteworthy feature of Fig. 15 is that for sludge grown under anoxic conditions and subjected to anoxic batch tests (DBT3 and DBT4), nitrite production from nitrate reduction was considerably greater 3 d after a change from aerobic to anoxic conditions (3.7 $mgNO_2^-$-N/gVSS·h in DBT3) than it was 12 d after the change (1.1 $mgNO_2^-$-N/gVSS·h in DBT4). This indicated that after changing System 1 from aerobic to anoxic conditions, nitrate reductase synthesis was initially more rapid than nitrite reductase synthesis and nitrite accumulated, but as nitrite reductase synthesis increased, nitrite was reduced more rapidly and did not accumulate to the same extent. The results are in agreement with the proposals 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 nitrate, nitrite, nitric oxide and nitrous oxide.

Closure

In Section I of this paper, a conceptual model was outlined for the major biochemical respiratory mechanisms mediated when a heterotrophic facultative organism (representing the heterotrophic facultative mass of activated sludge) is subjected to steady-state aerobic, steady-state anoxic and changes between steady-state aerobic and steady-state anoxic conditions. In Section II of this paper the major proposals and implications of the model were tested with activated sludge in aerobic 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.

Acknowledgements

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