Denitrification of drinking water - A bioenergetic evaluation

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Abstract

An investigation into denitrification of groundwater, using fluidised bed reactors with ethanol as carbon source and electron donor included, *inter alia*, determination of the bioyield and the ethanol to NO_3^- ratio required to effect complete denitrification. The values measured for these parameters are in close agreement with other published data, Y_{net} equals 0.15g cells/g NO_3^- metabolised or 0.3 g.cells/g ethanol metabolised, and the g ethanol utilised/g NO_3^- removed is 0.5. However, theoretical investigation into the denitrification process indicates that the observed yield data are very much lower than those expected from theoretical considerations, i.e. an approximate expected theoretical anoxic yield of 0.69 g cells/g ethanol metabolised.

To investigate possible reasons for the discrepancy, a bioenergetic model was formulated. Application of the model to anoxic processes using reasonable assumptions based on expected anabolic and catabolic efficiencies also gives theoretical yield data which are much higher than those obtained experimently here and in other published data. To obtain agreement between theory and practice the catabolic efficiency has to be reduced to approximately one third of its expected value. Possible reasons for this discrepancy are proposed.

Introduction

Groundwater denitrification using fluidised bed (FB) reactors with ethanol as carbon source and electron donor, was studied at the Technion, Haifa, Israel. The experimental system and operating results regarding denitrification efficiency, NO_3^- and NO_2^- profiles, biomass profile and physical characteristics of the biofilm are given elsewhere (Green et al., 1993; 1994). This paper concentrates on the experimental results for both bioyield and the corresponding C and N removals and their relation to theoretical expectations which are based on bioenergetic considerations.

Experimental work

Material and methods

Reactors

FB reactors with a working volume of 8.9 ℓ (9 cm dia.) were used for the denitrification experiments. A constant temperature of 25°C was maintained.

Feeding solution

The reactors were fed with tap water enriched with NO_3^- (100 mg/ℓ as NO_3^-), ethanol (50 to 100 mg/ℓ) and phosphate (1 mg/ℓ).

Analysis

Nitrate was measured using the spectrophotometric screening method, according to *Standard Methods* (1989). Nitrite was analysed by the colorimetric method according to *Standard Methods* (1989) and ethanol was determined using an enzymatic method (Sigma kit). The total and volatile suspended solids (TSS and VSS) concentration was measured according to *Standard Methods* (1989). VSS was used as the measure for biomass concentration.

Sampling procedure

A short tube with a measured volume of 5.4 mℓ together with the accompanying valves were connected to the sampling ports. Reactor contents from a given sampling port height were released through the tube and subsequently trapped for measurement.

Excess biomass removal

Excess biomass was taken out manually once or twice a day (depending on the volumetric loading rate in the reactor), by draining from the reactor the portion of the biofilm-covered sand above a desired level. Stripping of the biomass from sand particles was performed by a high speed mixer and the clean sand was returned to the reactor.

Predominating bacteria

Pseudomonas spp. were found to be the dominating bacteria.

Results

Biomass yield

Biomass yield was determined at reactor operating conditions which gave almost zero nitrite and ethanol concentrations in the effluent (to ensure no ethanol by-product residuals). The biomass yield which is defined here as grams of cells produced per gram of NO_3^- or ethanol removed, was calculated based on NO_3^- and ethanol concentrations in the influent and effluent and on the biomass concentrations in the reactor and effluent (including biomass removal). The biomass concentration in the reactor was determined based on a weighted average of biomass concentrations along the reactor. The average biomass yield was found to be 0.15 g cells/g NO_3^- removed and 0.3 g cells/g ethanol removed.

Ethanol requirement

Minimal ethanol requirement for NO_3^- removal was studied at retention time of 4.5 min. Ethanol to NO_3^- mass ratio was decreased

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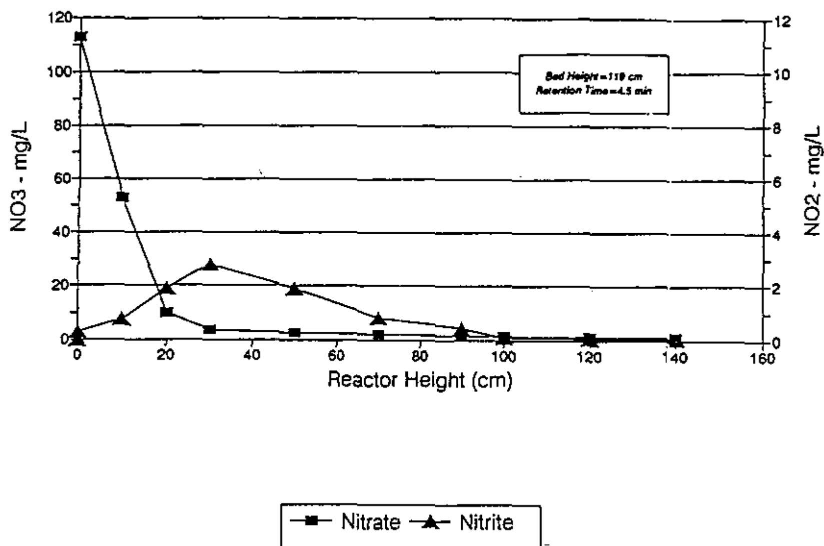


Figure 1
Nitrate and nitrite concentrations along the reactor at non-limiting conditions: ETOH:NO_3^-= 0.6

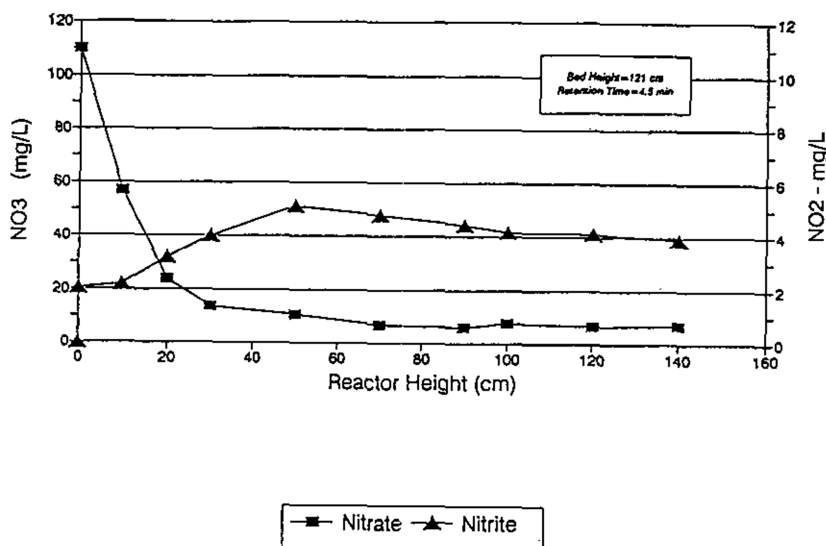


Figure 2
Nitrate and nitrite concentrations along the reactor at carbon limiting conditions: ETOH:NO_3^- = 0.45

gradually from 0.8 to 0.4 (g ethanol/g NO_3^-). To ensure steady state conditions, the reactors were operated for several days at each ratio of ethanol to NO_3^-. The results showed that NO_3^- and NO_2^- concentrations in the effluent were very close to zero when the ethanol to NO_3^- ratio was higher than 0.5. An increase in both NO_3^- and NO_2^- concentrations in the effluent was observed at ethanol to NO_3^- ratios of 0.5 (Figs. 1, 2). At this ratio of ethanol to NO_3^- (0.5 g/g), the NO_3^- removal rate decreased and NO_3^- and NO_2^- concentrations could be detected in the effluent.

Bioenergetic evaluation

Experimental results reported above for both bioyield and the corresponding C and N removals are in good agreement with most other research findings (McCarty et al., 1969; Christensen and Harremoes, 1977; Schroeder, 1977; Grady and Lim, 1980; Van der Hoek et al., 1987; Green et al., 1993 and 1994). However, these values are not in agreement with theoretical expectations based on bioenergetic considerations (Payne, 1981). Payne shows that from oxidative phosphorylation considerations alone one would expect a cell yield (g organism per electron equivalent of substrate metabolised) of 77% of the corresponding maximum aerobic yield. This value is rarely observed in practice. Literature indicates a broad spectrum of bioyield data varying from 1 to 2.5 g VSS/ electron equivalents of substrate removed, compared with an expected value of a mean aerobic value suggested by Payne (1981) of 3.3 g VSS/electron equivalent removed, i.e. an anoxic yield value of between 30 to 75% of aerobic value.

In the case of ethanol as substrate (electron donor) and nitrate as both terminal electron acceptor and nitrogen source for synthesis, from energetic considerations one would expect a cell yield of 2.54 g VSS/electron equivalents of substrate metabolised, or 0.66 g VSS/g ethanol removed (each mole of ethanol yields 12 e^-). The yield value determined here and reported above for these reactants is 1.15 g VSS/electron equivalent of substrate removed, and in terms of nitrate removed - 0.15 g VSS/g NO_3^- removed. Clearly the observed bioyield is significantly less than the theoretical value.

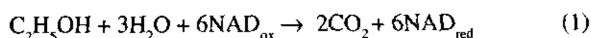
It is of interest to inquire into possible reasons for these discrepancies. The approach will be to formulate a bioenergetic model similar to that of McCarty (1971). The assumptions pertaining to anabolic and catabolic efficiencies will be varied until theory and observation are in agreement. This in turn allows speculation around factors giving rise to the lack of agreement between theory and practice.

Theoretical model

The metabolism of a cell can be divided into anabolic (cell synthesis) and catabolic (respiration) components. The approach will be to determine energy requirements for the anabolic component and match this with energy generated in the catabolic processes with due consideration to entropy changes (energetic efficiencies) associated with each of these.

In the metabolism it will be assumed that:

- Substrate is oxidised to electrons (and other by-products) and these are carried in the form of NAD in its reduced state, NAD_{red}, either to a synthesis site yielding cell material or to a terminal electron acceptor (respiration site) where bioenergy is conserved (in the form of ATP). Generation of NAD_{red} is effected by coupling NAD in the oxidised state, NAD_{ox}, into a substrate oxidation reaction, with each mole NAD_{ox} able to accept 2 electrons and 2 protons. For example, oxidation of ethanol to carbon dioxide yields 12 protons and 12 electrons, and thus 6 moles of NAD_{red} are generated, as follows:



$$C_2H_5OH + 3H_2O + 6NAD_{ox} \rightarrow 2CO_2 + 6NAD_{red} \qquad (1)$$

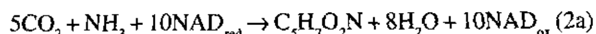
- Only a fraction of the energy released in respiration is available as bioenergy; this fraction constitutes the efficiency of the catabolic process. Similarly, only a fraction of this bioenergy will be conserved in raising the free energy level of "cellular building material" into new cell material; this fraction constitutes the efficiency of the anabolic process. Consequently, for the purposes of modelling one need only assume values for these efficiencies. This approach bypasses the need of explicitly dealing with ATP generation and its associated free energy of hydrolysis (a widely debatable value under physiological conditions). In this event ATP does not have to be considered explicitly as the intermediate energy carrier between respiration and synthesis. An overview of the model is set out in Fig. 3.

Anabolic model

Stoichiometry

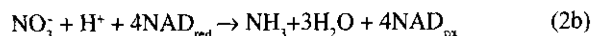
In this paper cell composition is modelled as $C_5H_7O_2N$ (Hoover and Porgess, 1952), which is the stoichiometric model commonly used in both waste-water treatment and denitrification systems (McCarty, 1971).

The general equation describing synthesis of cell material from NAD_{red}, CO_2 and NH_3 is formulated by balancing oxidation of NAD_{red} with reduction of CO_2 and NH_3 to new cell material ($C_5H_7O_2N$ with a donating capacity of 20 electrons), i.e.:



$$5CO_2 + NH_3 + 10NAD_{red} \rightarrow C_5H_7O_2N + 8H_2O + 10NAD_{ox} \qquad (2a)$$

Referring to Eq. (2a) nitrogen source for synthesis is NH_3, however, because NO_3^- is the N source it first must be reduced (via NAD reduced), i.e.:



$$NO_3^- + H^+ + 4NAD_{red} \rightarrow NH_3 + 3H_2O + 4NAD_{ox} \qquad (2b)$$

Ethanol required in the formation of cell material is determined by balancing the NAD_{red} required in the 2 synthesis reactions above with NAD_{red} generated in oxidation of ethanol (Eq. 1). In total 14 moles of NAD_{red} are required for synthesis, and thus 2.33 moles of ethanol need to be oxidised for synthesis alone. For any other substrate the stoichiometric mole requirement for synthesis is determined from the corresponding molar electron-donating capacity of the substrate.

Energy considerations

The anabolic reaction(s) above requires an input of bioenergy (ATP). An estimate of this requirement can be made from the change in the free energy between products and reactants. However, in this regard, the standard free energy of formation of cell material is unknown. A value for this can be obtained from

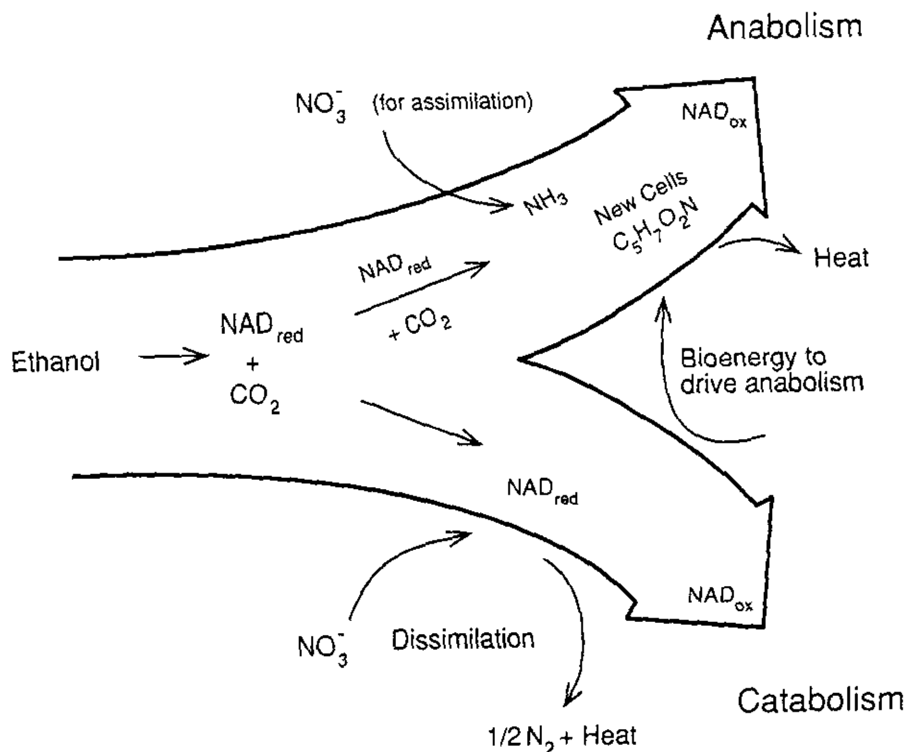


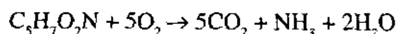
Figure 3
Schematic description of the model

TABLE 1
STANDARD FREE ENERGY OF FORMATION (ΔG_F^o) DATA OF COMPOUNDS RELEVANT FOR THE DENITRIFICATION MODEL

Compound	ΔG_F^o (Kcal/mole)
Acetic acid - ionised	-88 3
- unionised	-94 8
Propionic acid (unionised)	-91.7
Pyruvic acid (ionised)	-113.3
Ethanol	-43.4
Glucose	-217.0
NH_3 (aq)	-6.4
NO_3^-	-26.3
HCO_3	-140.3
H_2O	-56.7
H^+ (at pH 7)	-9.6
(NAD_{red}-NAD_{ox})*	-5.0
Protoplasm (Cell material: $C_5H_7O_2N$)**	+77.0

* From Lehninger (1975)
** Determined in the text

observed data on enthalpy of combustion of cell material; this was found to be (-)5.3 Kcal/g of dry cell material (Morowitz, 1963). Recognising that cell material is composed of approximately 90% volatile material, the enthalpy of combustion of the volatile component is (-)5.9 Kcal/g of volatile cell material. Morowitz shows that the entropy component of the enthalpy of cell combustion is negligible, so that the free energy of oxidation of cell material (to CO_2, NH_3 and H_2O) is (-)5.9Kcal/g of volatile cell material. Therefore, the free energy of oxidation of 1 mole of $C_5H_7O_2N$ (i.e. 113 g) is (-)667 Kcal. The free energy formation of cell material is then determined from the combustion reaction below and the standard free energy data listed in Table 1:



$$C_5H_7O_2N + 5O_2 \rightarrow 5CO_2 + NH_3 + 2H_2O$$

giving a standard free energy formation for cell material equal to (+)77 Kcal/mole.

We are now in a position to determine the energy requirement for the synthesis reaction, Eq.(2a). Substituting the relevant free energy data (Table 1) and using the free energy of formation of cell material determined above, one obtains the energy requirement to form one mole of cell material from NAD_{red}, CO_2 and NH_3. This value equals (+)147 Kcal/mole of cell material, the positive sign indicating an uphill reaction requiring an input of bioenergy.

The value above for anabolic reaction does not reflect the total energy to be supplied to drive this synthesis reaction for the following reasons:

- There may be either an energy requirement or energy release in converting NO_3^- (the supplied form of nitrogen) to NH_3, the form of N incorporated into new cell material (see Eq. 2b). From the free energy data listed in Table 1, this conversion process is an exergonic reaction (free energy change = (-)121 Kcal) so that energy (ATP) could possibly be conserved. However, the literature survey gives no indication of whether cells are able to conserve ATP in this reaction. In the model presented here this reaction is considered to be spontaneous with no conservation of energy in the form of ATP. This assumption leads to an over-estimate of the energy required for synthesis. However, should biochemistry indicate otherwise the model can easily be adjusted to account for this energy component.
- Energy associated with the generation of 14 NAD_{red} from 2.33 moles of ethanol, Eq.(1), can be assessed from the standard free energy data in Table 1. This free energy change is (-) 7 Kcal/ 14 moles of NAD_{red} generated, a slightly exergonic reaction so that in this example it is considered negligible. However, it is important to note that there are processes where this component to anabolic energy requirements is highly significant, for example in the generation of NAD_{red} from ammonia by nitrifying autotrophs.
- Energy supplied to biosynthesis reactions is usually effected by coupling ATP into the reaction. In all such natural reactions there is an associated heat loss giving rise to a reduced efficiency. Examination of ATP requirements in documented synthesis reactions (e.g. reverse glycolysis and amino acid synthesis) indicates an efficiency of close to 60%. A similar value was used by McCarty (1971), consequently this value is selected to describe the efficiency of the anabolic process.

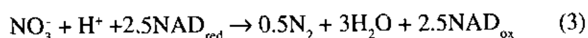
Summarising the anabolic reaction in a denitrifying environment with ethanol as substrate:

- For the formation of one mole of biocell material, 147/ 0.60 = 245 Kcal are required at 60% anabolic efficiency.
- Twenty eight (28) electron equivalents of substrate (i.e., 14 NAD_{red}) are incorporated into biomass synthesis reactions, Eqs. (2a) and (2b), i.e. 2.33 moles of ethanol enter into the synthesis reaction.
- One mole of NO_3^- is denitrified to NH_3 (for assimilation purposes) in the generation of one mole of biomass.

(Note that the last two above are independent of anabolic efficiency).

Catabolic model

The anabolic model above establishes the energy which must be supplied from catabolism to drive the synthesis reaction (220 Kcal/mole). In biosystems the catabolic energy is released and captured in a series of controlled redox reactions. In our model the electron supply to the catabolic redox reaction is via NAD_{red} and the terminal electron acceptor is NO_3 (and its intermediates) forming N_2, i.e.,



$$NO_3^- + H^+ + 2.5NAD_{red} \rightarrow 0.5N_2 + 3H_2O + 2.5NAD_{ox} \qquad (3)$$

The free energy change for this reaction is determined from relevant data listed in Table 1: (-)123 Kcal. A fraction of this will be captured (as ATP the energy carrier) and the remainder lost as heat. From theoretical considerations (Payne, 1981) one would expect that approximately 60% of the free energy is captured. This would lead to the capture of 74 Kcal in the above reaction. Recognising that the equation involves the transfer of 5 electron equivalents, the free energy conserved in the above reaction would be 15 Kcal/electron equivalent and the corresponding NO_3^- reduction 0.2 moles N/electron equivalent transferred. It is now possible to estimate catabolic substrate requirement and NO_3^- reduced (to N_2) to drive the anabolic reaction.

To supply 245 Kcal one therefore requires 16.3 electron equivalents to be catabolised to generate 1 mole of cell material at 60% anabolic and catabolic efficiencies and the corresponding NO_3^- reduced is 3.3 moles.

There are serious uncertainties regarding the efficiency of energy captured (catabolism). Payne (1981) implicitly ascribes variations in aerobic biogrowth between different pure cultures operating on the same substrate to fluctuations in catabolic efficiencies of between 30 and 65%.

Cell yield

Conventionally cell yield is defined in one of two ways depending on the objectives: cell mass produced per unit substrate either metabolised (Y_{met}) or catabolised (Y_{cat}). In the case of cell mass produced per substrate metabolised, the unit substrate metabolised can be expressed as grams substrate, moles substrate, electron equivalents, COD, etc. In the case of cell mass produced per substrate catabolised the unit substrate catabolised can be expressed in units of oxygen consumed, electron equivalents donated to terminal acceptor, nitrate reduced to nitrogen, etc. It is possible to convert between Y_{met} and Y_{cat} provided that the electron donating capacity per mass of cell material is known. In terms of the model described here: molecular weight of $C_5H_7O_2N$ equals 113 g and electron donating capacity equals 20.

Based on the calculated energy required for anabolism, 245 Kcal/mole cell mass, at 60% efficiency and catabolic energy yield of 15 Kcal/electron equivalent metabolised, Y_{met} and Y_{cat} can be determined:

Y_{met}

a: In terms of electron equivalents metabolised

electron equivalents anabolised = 2.33*12 = 28 (see section: **Anabolic model**)
electron equivalents catabolised = 245/15 = 16.3 (see section: **Catabolic model**)
electron equivalents metabolised = 44.3

Y_{met} = 113/44.3 = 2.55 g cells/electron metabolised.

b: In terms of moles ethanol (electron donating capacity=12)

Y_{met} = 2.55*12 = 30.6 g cells/mole ethanol metabolised, or 0.67 g cells/g ethanol metabolised.

c: In terms of NO_3 reduced (both assimilatory and dissimilatory)

moles NO_3^- reduced to NH_3 for anabolism = 1 mole/mole cells generated
moles NO_3^- reduced to N_2 for catabolism (electron accept ing capacity of NO_3^- = 5) = 16.3/5 = 3.26.
Thus, mole NO_3^- removed = 4.26, and
Y_{met} = 113/4.26 = 26.5 g cells/mole NO_3 or 0.43 g cell/g NO_3^-

Y_{cat}

d: In terms of electron equivalents catabolised

electron equivalents catabolised = 245/15 = 16.3 (see section : **Catabolic model**)
Y_{cat} = 113/16.3 = 6.93 g cells/electron catabolised.

e: In terms of moles ethanol (electron donating capacity=12)

Y_{cat} = 6.93*12 = 83.2 g cells/mole ethanol catabolised, or 1.81 g cells/g ethanol catabolised.

f: In terms of NO_3^- reduced (dissimilatory)

moles nitrate reduced for catabolism (electron accepting capacity of NO_3^- = 5) = 16.3/5 = 3.26,
Y_{cat} = 113/3.26 = 34.7 g cells/mole NO_3^- reduced to N_2, or 0.56 g cell/g NO_3^-.

Comparison of experimental and literature results for cell growth on ethanol and NO_3^- with theoretical data determined above for 60% efficiency for anabolism and catabolism gives:

(1) Cell yield

g cell/g NO_3^- metabolised

Experimental:	0.15
Theoretical (from c):	0.43
Literature:	0.1 - 0.22

g cells/g ethanol metabolised

Experimental:	0.3
Theoretical (from b):	0.67

(2) Ethanol to NO_3^- ratio

g ethanol required per g NO_3^- removed

Experimental:	0.5
Theoretical (from c/b):	0.64
Literature:	0.37 - 0.6

The comparison above shows that while the experimental and literature values are in good agreement, the theoretical data are significantly different. It would appear that the theoretical results grossly under-estimate the NO_3^- to be reduced into N_2 to supply the

TABLE 2
CELL YIELD, Y_{met}, AND ETHANOL TO NO_3 REMOVED FOR A VARIABLE CATABOLIC EFFICIENCY AND CONSTANT SYNTHESIS (ANABOLIC) EFFICIENCY OF 60%

a	b	c	d	e	f	g
catabolic efficiency[2]	energy captured/e^-eq. catabolised[3]	e^- eq. catabolised/ mole biomass[4]	NO_3/mole biomass[5]	Ethanol metabolised/ mole biomass[6]	g ethanol/g NO_3 removed[7]	Y_{met}[8]
(%)	(Kcal/e^-eq.)		(mole/mole)	(g/mole)		g cell/g NO_3^-
60	14.7	16.7	4.34	3.73	0.64	0.42
50	12.3	19.9	5.0	4.0	0.59	0.37
40	9.8	25.0	6.0	4.42	0.55	0.30
30	7.4	33.3	7.7	5.11	0.49	0.24
20	4.9	50.0	11.0	6.5	0.44	0.17

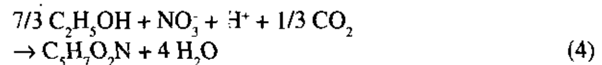
[1] Anabolic energy requirements at 60% efficiency = 147/0.6 = 245 Kcal/mole, from Eq.(2a)
[2] Assumed catabolic efficiency, ß, per cent
[3] From Eq. (3), giving energy release of 25 Kcal/e^- catabolised (see "catabolic model"), i.e., energy captured/(e^- eq catabolised) = 25*ß*/100
[4] From catabolic energy required/energy captured per e^- equivalent catabolised = 245 (from superscript 1)/column(b)
[5] Moles NO_3^- reduced to N_2 plus moles nitrate reduced to NH_3 (assimilation) = [column (c)/ (e^- eq to reduce 1 mole NO_3^- to N_2)] + [moles NO_3^- reduced to NH_3 (i.e.1)]
[6] [e^- eq catabolised column (c) + e^- eq into anabolism (i.e. 28)]/ [e^- eq released in oxidising 1 mole ethanol (i.e.12)]
[7] [column (e) x 46]/[column (d) x 62]
[8] Y_{met}= 113 g cells/[column(d) x 62]

required anabolic energy, i.e. the theoretical catabolic energy generation is badly over-estimated.

With regard to the over-estimate of the theoretical catabolic requirement for cell yield, this can be reassessed in terms of the model, simply by adjusting catabolic efficiency. Theoretical yield values vs. catabolic efficiency with a constant anabolic efficiency of 60% are listed in Table 2. The constant anabolic efficiency is based on application of this type of model to heterotrophic and autotrophic aerobic processes, for which we found very good agreement between theoretical and measured data published. The principal difference between aerobic and anoxic heterotrophic theoretical yield data occurs in the catabolic energy transformations. According to Payne (1981) for aerobic systems the ATP generated/electron equivalent transferred to the terminal electron acceptor is approximately 1.5 times that in anoxic systems.

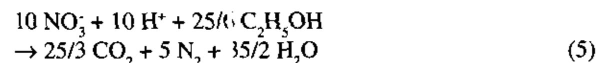
Interpolating data listed in Table 2, agreement between experimental and theoretical metabolic yield data is obtained in catabolic efficiency of about 20%. In the following section some possible reasons for this low efficiency are given. The corresponding stoichiometric anabolic, catabolic and metabolic reactions for this condition are set out below:

- Stoichiometric anabolic equation: The overall anabolic equation for generation of one mole cell material is obtained from Eqs. (1), (2a) and (2b) as:



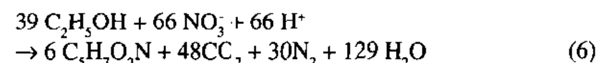
$$7/3\ C_2H_5OH + NO_3^- + H^+ + 1/3\ CO_2 \rightarrow C_5H_7O_2N + 4\ H_2O \quad (4)$$

- Stoichiometric catabolic equation: The overall catabolic equation to supply the anabolic energy requirements and assuming 60% anabolic efficiency and 20% catabolic efficiency is obtained from Eq. (3) and data listed in Table 2 as:



$$10\ NO_3^- + 10\ H^+ + 25/6\ C_2H_5OH \rightarrow 25/3\ CO_2 + 5\ N_2 + 35/2\ H_2O \quad (5)$$

- Stoichiometric metabolic equation: The overall metabolic equation in the generation of cell material (6 moles) from ethanol and nitrate is obtained by adding the stoichiometric anabolic and catabolic equations above giving:



$$39\ C_2H_5OH + 66\ NO_3^- + 66\ H^+ \rightarrow 6\ C_5H_7O_2N + 48CO_2 + 30N_2 + 129\ H_2O \quad (6)$$

Discussion

The work reported in this paper arose from an investigation into the denitrification of groundwater, using fluidised bed reactors with ethanol as carbon source and electron donor. Aspects of this

investigation included, *inter alia*, determination of the bioyield and the ethanol to nitrate ratio required to effect complete denitrification. The values measured for these parameters are in close agreement with other published data, that is Y_{met} = 0.3 g cells/g ethanol metabolised, and the g ethanol utilised/g NO_3^- removed is 0.5. However, theoretical investigation into the denitrification process indicates that the observed yield data are very much lower than those expected from theoretical considerations, i.e. an approximate expected anoxic yield of 0.69 g cells/g ethanol metabolised.

To investigate possible reasons for discrepancies, a bioenergetic model was formulated. Though the model is crude, it should indicate, from energy considerations and assumptions regarding energy conservation, the partitioning of energy between anabolism and catabolism. Indeed this proved to be the case in applying the model to aerobic processes.

Application of the model to anoxic processes using reasonable assumptions based on expected anabolic and catabolic efficiencies gives theoretical yield data close to those predicted by Payne (1981). To obtain agreement between theory and practice one has to reduce the catabolic (or anabolic) efficiency to approximately one third of its expected value. We can now speculate on possible reasons for this discrepancy:

- **Nutrient deficiency conditions**: Since the experimental system was operated on groundwater, one may expect nutrient deficient conditions. Such conditions often manifest with an organism response of either generation of extracellular polysaccharides, or some other futile method of ATP utilisation. With regard to generation of extracellular polysaccharides this results in an apparent increase in cell yield because the free energy associated with polymer generation is lower than for cell synthesis. Clearly this aspect cannot account for the lower cell yield. With regard to the futile method of ATP utilisation, e.g. uncoupling, this indeed would result in both lower catabolic efficiency and lower bioyield. Moreover, nutrient deficiency can be discounted, based on other laboratory investigations where nutrients were non-limiting, but similar yield data to that observed here were found.
- **Substrate type**: One could hypothesise that ethanol follows a different pathway in oxidative phosphorylation leading to lower than normal ATP generation per electron equivalent transferred to reduce nitrate to nitrogen. This may be the case; however, other data reported in literature using other substrates (for example acetic acid - Mateju et al., 1992) give similar results to ethanol.

  As mentioned earlier, though ethanol is a compound that is readily oxidised for bio-energy (catabolism), possibly its conversion into protoplasm (anabolism) is a relatively slow process. Perhaps sufficiently slow that the anabolic reaction is the limiting step in the metabolism. In this event one may postulate that to maintain a sufficiently high nutrient uptake rate (in a system with potentially nutrient deficient conditions) the organisms need to maintain a low ATP energy potential - this could be effected via a futile cycle in which ATP energy is simply released as heat without performance of work.
- **Toxicity**: Reduction of NO_3^- to N_2 occurs with a number of intermediate substances including NO_2^-, NO and N_2O. It is possible that one or more of these substances may have an adverse effect on cell metabolism. For example, Krul (1976) shows that NO represses oxidase activity which in turn has important effects on the denitrification process within activated sludge systems (Casey et al., 1992), *inter alia*, continued nitrite denitrification occurs on recycle to the aerobic zone with concomitant decrease in oxygen utilisation rate. Nitrite has been reported as an uncoupling agent for a number of denitrifying species (see Payne, 1981). However, Stouthamer (1991), referring to data of Rake and Eagon (1980) argues against this supposition, but points out that the role of NO coupled with NO_2^- requires further investigation. Indeed, the occurrence and non-occurrence of membrane-bound NO in the denitrification process is not understood. It is, however, possible to speculate on the role of the NO_2^- and NO to explain both the occurrence of aerobic NO_2^- reduction in denitrifying activated sludge systems and the apparent low cell yield value observed in anoxic systems. This requires accepting that NO_2^- causes a reduction in ATP synthesis by stimulating proton leakage across the ATP generating membrane. This leakage would explain the observed catabolic inefficiency. As far as the denitrifying activated sludge process is concerned, protection against such NO_2^- toxicity in the aerobic zone could be achieved by aerobically denitrifying NO_2^- to nitrogen oxides with the intermediate species NO repressing oxidase activity thereby directing electrons away from oxygen towards improved nitrite reduction, thus, overcoming the NO_2^- toxicity effect.

It is not possible to state categorically whether any of the above factors are in fact the cause of the apparent energy loss in an anoxic system operating on ethanol as C and e^- source. Investigating these factors is outside the scope of an engineering project and requires further in-depth investigation by the microbiological fraternity.

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