PHYSIOLOGICAL AND ECOLOGICAL ASPECTS OF AEROBIC DENITRIFICATION, A LINK WITH HETEROTROPHIC NITRIFICATION?

Lesley A. Robertson and J.G. Kuenen

Kluyver Laboratory for Biotechnology
Delft University of Technology
Delft
The Netherlands

INTRODUCTION

For about a century, the existence of aerobic denitrification was a subject of debate, if not controversy (Robertson & Kuenen, 1984a). However, as available technology (e.g. sensitive oxygen - analyzing equipment, chemostats, mass spectrometers) has improved, it has been possible to establish that a number of strains do indeed denitrify while simultaneously respiring oxygen. This is, of course, not true of all denitrifiers. Thiomicrospira denitrificans appears to be the most specialized as it can only tolerate oxygen in limiting concentrations. Other species, including most Paracoccus denitrificans strains, can grow under fully aerobic conditions, but only denitrify in the virtual absence of oxygen. These species fit the classical definition of denitrifiers. A third group is made up of strains which simultaneously utilize oxygen and denitrify - the aerobic denitrifiers. The members of this group do not show a uniform response to oxygen, denitrification by some of them is inhibited by relatively low amounts of oxygen (e.g. Hyphomicrobium X; Meiberg et al., 1980) while others continue to denitrify even at air saturation (e.g. Thiosphaera pantotropha). It is not unlikely that, as with electron donors, the environmental conditions and nutrient supplies determine whether specialist or versatile denitrifiers occur in any given ecosystem.

This paper aims to document some of the more recent reports of aerobic denitrification and to discuss some of the ecological and physiological pressures which might determine whether aerobic or specialist denitrifiers are selected in any situation.

THIOSPHAERA PANTOTROPHA

We first became interested in the possibility of aerobic denitrification six years ago when when we found that a new isolate, Thiosphaera pantotropha possessed constitutive denitrifying enzymes which were active, even in cultures grown at dissolved O_2 concentrations

Denitrification in Soil and Sediment, Edited by N. P. Revsbech and J. Sørensen, Plenum Press, New York, 1990

80-90% of air saturation (Robertson & Kuenen, 1983a; 1984b; Robertson et al., 1986). In batch culture, the simultaneous use of two electron acceptors conferred a higher growth rate than when either electron acceptor was supplied separately. During these experiments, it was also found that Tsa. pantotropha can oxidize ammonia to nitrite, provided that it is supplied with an organic substrate. Preliminary experiments using a mass spectrometer with labelled and unlabelled inorganic nitrogen compounds confirmed that N_2 rather than N_2O is produced by Tsa. pantotropha during denitrification. Moreover, cells supplied with $^{15}NH_4Cl$ and metabolizing endogenous reserves generated ^{15} $^{15}N_2$ (E.W.J. van Niel, L.A. Robertson & R.P. Cox, unpublished results).

The oxidation of ammonia to nitrite or nitrate in the presence of an organic substrate is known as heterotrophic nitrification (Verstraete, 1975; Killham, 1986) and has, for a long time, been dismissed as of little ecological importance because heterotrophic nitrifiers accumulate very little nitrite or nitrate. However, the activity of heterotrophic nitrifiers such as Tsa. pantotropha, which simultaneously denitrify the nitrite they produce, can be seriously underestimated unless complete nitrogen balances are made. Because nitrification and denitrification appear to be closely linked in Tsa. pantotropha as well as some of the other aerobic denitrifiers, the two phenomena will be discussed together.

It became very clear early in our studies that Tsa. pantotropha adjusted its behaviour to accommodate even small changes in its environment (e.g. raising the dissolved oxygen concentration from 80% air saturation to 90% air saturation can add an hour to the doubling time), and most subsequent experiments were therefore carried out in chemostats with pH and dissolved oxygen control. By running steady state, acetate-limited cultures at different dilution (growth) rates but with the dissolved oxygen concentration controlled at 80% air saturation, several factors immediately became apparent (Table 1):

1: The presence of nitrate or nitrite in the medium reduced the rate of nitrification, except when low amounts (5 mM) of nitrite were supplied, and the organism was able to denitrify all of it, thus maintaining an effective concentration of 0 in the culture medium.

2: Both the nitrification and denitrification rates increased as the growth (dilution) rate increased.

3: Thiosulphate inhibited nitrification.

4: Heterotrophic nitrification cannot be treated as a form of mixotrophy (the metabolism of an organic substrate and an inorganic energy supplement) because not only did the cultures not gain extra energy for growth from the reaction, but biomass yields from cultures with the highest nitrification rates were lower than any of the others, and only 60% of the yields expected.

Cultures run at different dissolved oxygen concentrations revealed that both the nitrification and denitrification rates increased as the dissolved oxygen fell, reaching a maximum at 25-30% of air saturation. Below this, the cultures not provided with nitrate or nitrite accumulated poly β-hydroxybutyrate (PHB) and started forming biolayers on all available surfaces, a general response of this organism to oxygen limitation.

Table 1. Correlation of chemostat yields (mg/L) with nitrification (NH_4^+ oxidation) and denitrification (reduction of NO_3^- supplied and NO_2^- supplied or produced from nitrification) rates (nmol^{-1} min^{-1} mg protein^{-1}). All cultures were substrate limited, and supplied with NH_4^+. Dissolved O_2=80% air saturation. D=0.04 h^{-1}. * indicates yield lower than expected. (Data from Robertson et al., 1988).

Additive	Nitrification	Denitrification	Yield
-	43	43	81*
NO_3^-	12	107	103
NO_2^- (limiting)	48	85	80*
NO_2^- (saturating)	25	98	115
$S_2O_3^{2-}$	21	21	145
$S_2O_3^{2-}$ / NO_3^-	6	36	120

In order to attempt to explain the behaviour of Tsa. pantotropha, and to help in the design of further experiments, a model based on the physiological data and preliminary cytochrome determinations was constructed (Fig. 1). A basic assumption is that, for an unknown reason, there is a bottleneck in the flow of electrons to oxygen via cytochrome aa_3, thus limiting the rate at which NADH can be reoxidized by this route. This bottleneck may be at the cytochrome c level. The somewhat greater degree of reduction in the cytochrome chain that such a

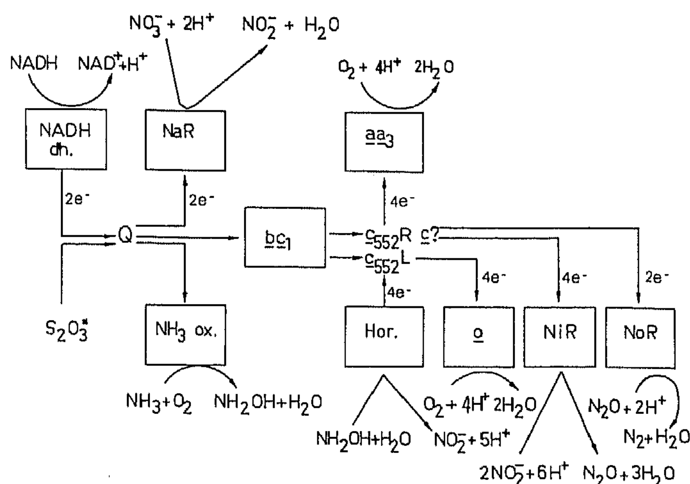

Figure 1. Schematic representation of the model used to describe the various potential routes for electron transport in Tsa. pantotropha. Cytochromes are underlined; NaR = nitrate reductase; NH_3 ox. = NH_3 monooxygenase; Hor. = hydroxylamine oxidoreductase; NiR = nitrite reductase; NoR = nitrous oxide reductase; c? indicates one or more additional cytochromes c; e = electrons; $S_2O_3^{2-}$ = thiosulphate oxidizing pathway (Robertson, 1988).

Table 2. Nitrogen balances from Tsa. pantotropha cultures growing on acetate with nitrate (7.5 mM) as the sole source of nitrogen. D = 0.04 h^{-1}. a = PHB synthesized; b = ammonia (7.5 mM) + nitrate (40 mM) cultures shown for comparison.

Dissolved oxygen (% air)	Nitrate lost (mM)	Nitrite formed (mM)	Protein (g mol^{-1} acetate)
80	1.3	0.03	5.9
50	0.8	0.05	4.3
33	1.8	0.45	3.6
23	6.3	0.02	2.7[a]
80	0.5	0.03	5.5
40	3.5	0.05	5.5
20	14.7	0.10	2.8
80[b]	13.4	0.02	5.2
0[b]	24.0	0.10	2.5

bottleneck would cause should then allow electrons to flow to the denitrifying pathway, thus allowing a faster rate of NADH oxidation. For simplicity, ammonia monoxygenase is shown as a part of the sequence. It should be noted that NAD(P)H rather than electron flow is involved in this reaction.

The ammonia monooxygenase of Tsa. pantotropha is NAD(P)H dependent (Robertson & Kuenen, 1988) and thus provides an additional means of regenerating reducing power, if necessary. However, since the use of this pathway does not result in energy generation (and may even cost the cell energy), denitrification is obviously more favourable. A certain amount of energy may, of course, be gleaned from the denitrification of nitrite produced during nitrification. The apparently inhibitory effect of nitrate or nitrite on nitrification may thus be two-fold. Firstly,

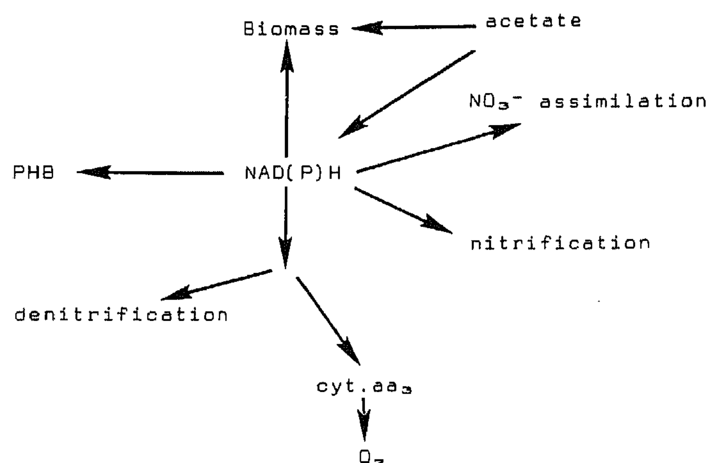

Figure 2. Simplified scheme showing the various possible options for NAD(P)H utilization available to Tsa. pantotropha (Robertson, 1988).

both ammonia and hydroxylamine oxidation are inhibited by nitrite (Robertson & Kuenen, 1988) and, secondly, there is less need for nitrification when denitrification can occur. Similarly, thiosulphate probably has a double effect. As with the autotrophic nitrifiers, it inhibits ammonia monooxygenase (Robertson & Kuenen, 1987), but growth on thiosulphate also results in the synthesis of additional cytochromes c (Robertson, 1988) which may thus overcome our postulated bottleneck. Two other methods of dealing with its redox problems are available to Tsa. pantotropha. Firstly, the assimilatory nitrite reductase requires NADH, and thus during nitrate or nitrite assimilation in the absence of ammonium any surplus NADH could be consumed. Aerobic denitrification rates in cultures where nitrate was the sole source of nitrogen were indeed found to be only 10% of those obtained when ammonia was also present (Table 2). The final option may be summarized as "if all else fails, make PHB". Figure 2 summarizes the different ways by which Tsa. pantotropha can oxidize NADH.

NITRIFICATION AND DENITRIFICATION BY OTHER STRAINS

It was obviously of great interest to discover whether Tsa pantotropha was unique, or whether other bacteria shared its capacity for heterotrophic nitrification and aerobic denitrification. In 1984, Castignetti & Hollocher described their finding that a number of denitrifying soil bacteria were also capable of heterotrophic nitrification. However, many of these strains did not accumulate very much nitrite, and it therefore seemed possible that they were also denitrifying. Several strains were therefore checked in batch culture and, as with Tsa. pantotropha, were found to grow more rapidly with nitrate and oxygen than on a single electron acceptor (Table 3). Like Tsa. pantotropha, and unlike the Pa. denitrificans control strain, the protein yield of these batch cultures was lower in the oxygen/nitrate cultures, and a substantial amount of nitrate has disappeared. Chemostat experiments with two of these strains showed that both were capable of aerobic denitrification, but that their behaviour was slightly different from that of Tsa. pantotropha and from each other. Alcaligenes sp. nitrified and denitrified efficiently at lower dissolved oxygen concentrations, but accumulated nitrification and denitrification intermediates in increasing quantities as the dissolved oxygen was increased (figure 3). A strain from the heterogenous group known as "Pseudomonas denitrificans", on the other hand, was as insensitive to dissolved oxygen as Tsa. pantotropha, but needed to induce its dissimilatory nitrate reductase. The remainder of its denitrifying pathway was constitutive. Unlike Tsa. pantotropha, nitrate in the medium did not reduce the rate of nitrification by "Ps. denitrificans".

There were two other major points of similarity between these nitrifier/denitrifiers. All of them possessed the copper-based nitrite reductase rather than cytochrome cd (Kuenen & Robertson, 1987; Robertson et al., 1989a; 1989b) and, until recently, it appeared that all aerobic denitrifiers had this enzyme. This will be discussed further below. With one exception, all of them were able to induce a significant thiosulphate - oxidizing capacity after batch culture on a mixture of acetate and thiosulphate (Table 4). In fact, "Ps. denitrificans" was able to grow autotrophically on thiosulphate in a chemostat, the other strains have yet to be checked in this way.

It is not yet clear whether the possession of such diverse abilities as nitrification and thiosulphate oxidation are coincidental

Table 3. Comparison of the maximum specific growth rates (μ_{max}), protein concentrations and nitrate reduction obtained from aerobic or anaerobic batch cultures of bacteria known to be capable of heterotrophic nitrification. Ammonia was the nitrogen source. The dissolved oxygen concentration was kept above 80% of air saturation. (Adapted from Robertson et al., 1989A). Pseudomonas sp. LMD 84.60 is the strain formerly known as Ps. denitrificans. Pa. denitrificans LMD 22.21 was the negative control.

Organism	μ_{max} (h^{-1})			Protein (mg/l)		NO_3^-
	O_2	O_2/NO_3^-	NO_3^-	O_2	O_2/NO_3^-	mM
Pseudomonas sp. LMD 84.60	0.1	0.41	0.15	78	60	5.0
A. faecalis LMD 84.59	0.17	0.25	0.07	30	14	4.1
Ps. aureofaciens LMD 37.26	0.19	0.21	0.07	66	66	5.0
T. pantotropha LMD 82.5	0.28	0.34	0.25	81	60	5.5
Pa. denitrificans LMD 22.21	0.28	0.28	nd.	92	88	<1.0

nd. = not determined

or, in some way, linked. That not all thiosulphate-oxidizing bacteria fall into this group is illustrated by the fact that our control strain of Pa. denitrificans can grow autotrophically on thiosulphate, but does not nitrify to any significant degree and is not an aerobic denitrifier. Two indirect pieces of evidence suggest that there might be a link. Firstly, the preliminary cytochrome studies with Tsa. pantotropha indicated that a particular cytochrome c is common to both nitrification and thiosulphate oxidation. Secondly, the apparent ecological niche for a versatile denitrifier (and all of the aerobic denitrifiers thus far tested have also been heterotrophic nitrifiers) is where the dissolved

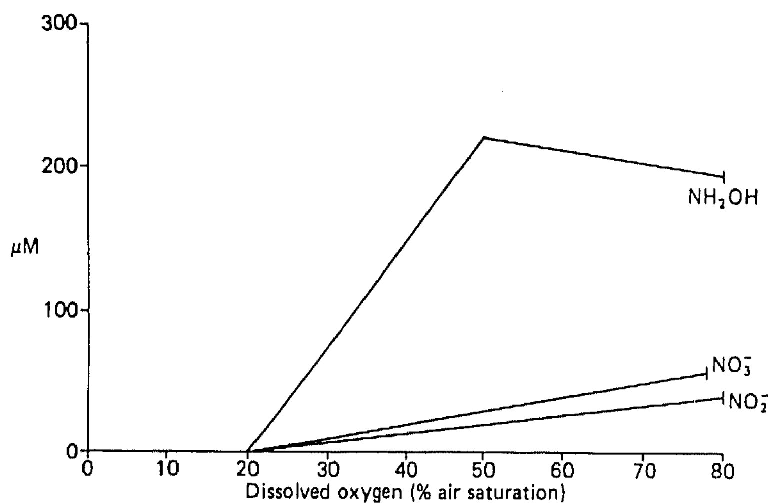

Figure 3. The gradual accumulation of oxidized nitrogen compounds in acetate-limited chemostat cultures of Alcaligenes sp. as the dissolved oxygen was increased. Ammonium was the sole source of nitrogen. (Kuenen & Robertson, 1987).

Table 4. Ammonia disappearance and thiosulphate oxidation rates obtained with aerobic, heterotrophic batch cultures. NH_3 was the sole source of nitrogen, and 5 mM acetate and 10 mM thiosulphate were supplied as substrates. The NH_3 disappearance figures have been corrected for assimilated nitrogen. The Pseudomonas sp. was grown in a chemostat (20 mM acetate and 5 mM thiosulphate). * = N-limited, nd = not determined.

Organism	NH_3 (mM)	Oxygen uptake rate (nmol/min/mg protein)
Pseudomonas sp. LMD 84.60	nd	615
A. faecalis LMD 84.59	-3.6*	598
A. faecalis S6 LMD 60.48	-2.7	0
Ps. aureofaciens LMD 37.26	-2.9	652
Ps. fluorescens LMD 60.46	-4.6	109
Ps. fluorescens LMD 60.44	-3.3	128
Ps. fluorescens LMD 60.48	0	0
Ps. fluorescens LMD 60.71	-1.2	0

oxygen concentration is limiting, or fluctuates. This adequately describes life at aerobic/anaerobic interfaces, where reduced sulphur compounds can also be expected to occur.

REPORTS OF AEROBIC DENITRIFICATION IN THE LITERATURE

It is neither practical or desirable to review the historical arguments about aerobic denitrification here, the interested reader will find this subject extensively covered in Robertson & Kuenen (1984a). However, since this review was published, other accounts of aerobic denitrification have appeared, and it is appropriate to consider them here.

The Nitrosomonas species described by Poth (1986) was shown to denitrify at a very low rate (less than 1 nmol min^{-1} mg $protein^{-1}$) under "oxygen stress" (concentration not reported). This strain, like Aquaspirillum magnetotacticum (Bazylinski & Blakemore, 1983), requires oxygen for growth, and might thus be described as obligately aerobic denitrifiers. However, it should be remembered that all denitrifiers are able to denitrify under oxygen limitation.

In a recent paper, Strand et al. (1988) examined aerobic denitrification by chemostat-grown cultures of a non-flocculating strain of Zooglea ramigera at a range of dissolved oxygen concentrations. As with Tsa. pantotropha the rate of denitrification by Z. ramigera achieved its maximum at an oxygen concentration somewhat lower than 50% of air saturation. Strand et al.. (1988) also measured ammonia levels in their cultures and it appears from the data provided that ammonia was disappearing from their cultures at a rate about half that found with similar cultures of Tsa. pantotropha. It would therefore appear that this strain also combines heterotrophic nitrification and aerobic denitrification.

As mentioned above, modern techniques, especially the use of ^{15}N-labelled compounds and mass spectrometry, have recently been used (Degn et al., 1985; Lloyd et al., 1986), to analyse the products of

denitrification (aerobic or otherwise) in more detail. Lloyd et al. (1987) used this technique to investigate the persistence of denitrification in anaerobically-grown batch cultures when incubated under aerobic conditions, and came to the conclusion that the phenomenon is very widespread among bacteria. They found that strains of Paracoccus denitrificans, Ps. aeruginosa, Ps. stutzeri, Propionibacterium thoenii and newly isolated Pseudomonas species all continued to denitrify, even when the dissolved oxygen concentration was equivalent to air saturation. In most of the strains, the rate of N_2 evolution was low (0.13-0.40 nmol min^{-1} mg $protein^{-1}$), but Ps. stutzeri and Pr. thoenii produced N_2 at significantly higher rates (3.42-5.61 nmol min^{-1} mg $protein^{-1}$). This is, of course, still considerably lower (10-100 times, depending on the conditions) than the denitrification rates found with chemostat cultures of Tsa. pantotropha. All of the strains also produced small amounts of N_2O. Davies et al. (1989) then studied two of these strains, Pa. denitrificans (NCIB 8944) and Ps. aeruginosa (PA0129) in greater detail, growing the cells both aerobically and anaerobically, and then measuring denitrification under aerobic and anaerobic conditions. As might be expected, the anaerobically grown, anaerobically tested suspensions gave the highest denitrification rates. As the dissolved oxygen increased, N_2O began to appear as well as N_2. In order to completely inhibit N_2 production, extremely high (360 μM dissolved oxygen concentrations were required, and even then N_2O production continued. The position of Pa. denitrificans as a typical classical denitrifier is further complicated by results published by Kawakami et al. (1985). The authors showed that N_2O production in the presence of acetylene by anaerobically grown cells was only partially inhibited by oxygen, even at 50% of air saturation. N_2O production continued at reduced levels for periods of at least 4 hours. Moreover, it took approximately 1 hour for inhibition by oxygen to reach its maximum. In acetate-limited, steady state chemostat culture, the strain of Pa. denitrificans (LMD 22.21) used as a reference culture during our studies did not denitrify nitrate supplied in the medium aerobically, although the nitrogen balance made for the culture indicated that it was nitrifying (and denitrifying the product) at a low rate (6 nmol min^{-1} mg $protein^{-1}$, compared with 18 nmol min^{-1} mg $protein^{-1}$ obtained with Tsa. pantotropha under the same conditions). These observed differences in the denitrifying abilities of Pa. denitrificans are probably due to strain variation, perhaps resulting from the original enrichment or maintenance conditions. We have recently gained evidence that a variant of Tsa. pantotropha appeared in cultures which were not maintained on nitrate-containing medium. This variant had lost much of its ability to denitrify externally supplied nitrite or nitrate under aerobic conditions, but continued to simultaneously nitrify and then denitrify the product.

The sequential inhibition of the denitrifying enzymes by increasing amounts of oxygen was shown for Pa. halodenitrificans (Hochstein et al., 1984). Chemostat cultures and gas chromatography were used to demonstrate that as oxygen was supplied to the cultures, the amount of N_2 produced by the cells fell and the N_2O level increased. With 5% oxygen in the influent gas (and the dissolved oxygen undetectable), only N_2O was produced. With an oxygen supply greater than 5%, dissolved oxygen became detectable, the N_2 production began to fall, and nitrite began to accumulate. At air saturation, N_2O production had stopped and only trace amounts of nitrite were observed.

Our original hypothesis that the separation between classical and aerobic denitrifiers might be dependent on which nitrite reductase

(cytochrome cd or the copper-based enzyme) was present may need to be revised in view of the observations with the two Paracoccus strains described above. Further evidence that this idea is not completely correct came from the work of Korner & Zumft (1989) in which they examined the synthesis of the denitrifying enzymes in chemostat cultures of Ps. stutzeri (which has cytochrome cd) under different oxygen regimes. When supplied with nitrate in the medium, Ps. stutzeri contained significant amounts of nitrate and N_2O reductase at 50% air saturation, and the N_2O reductase was even detectable at air saturation. Nitrite reductase began to appear at about 25% air saturation, and reached its maximum just before the onset of complete anaerobiosis. It now seems more likely that the capacity for aerobic denitrification is determined by the redox state of the cytochrome chain, and the various factors which affect this.

Aerobic denitrification (of nitrite) was recently shown for Rhizobium "hedysari" strain HCNT1 (Casella et al., 1988). It seems that relatively low (10-75 µM) concentrations of nitrite inhibited oxygen uptake by bacteroids of this strain. Oxygen uptake began once the nitrite had been reduced. However, rather than having a respiratory function, the authors suggest that this form of "aerobic denitrification" is a means of protecting nitrogenase (which is also very sensitive to nitrite) from nitrite generated by the nitrate reductase of the host plant.

Observations in the field and in natural samples were made by Trevors (1985), Trevors & Starodub (1987) and Rönner & Sörensson (1985). They showed denitrification in the presence of reduced oxygen concentrations in soil samples, freshwater sediments and water from the deeper areas of the Baltic, respectively. In all three cases, denitrification was measured as N_2O production after acetylene treatment. Trevors & Starodub (1987) showed that the initial availability of nitrate was more important for the onset of denitrification than the degree of anoxia. During studies of N_2O and NO production (without acetylene) by common soil bacteria, Anderson & Levine (1986) found that Rhizobium japonicum and Pseudomonas fluorescens only continued to generate these gases up to a dissolved oxygen concentration of 5% air saturation, but that their strain of Alcaligenes faecalis continued to do so at air saturation and, in other words, must be an aerobic denitrifier. Stimulation of denitrification by the presence of oxygen was shown by Abou Seada & Ottow (1985) for Aeromonas "denitrificans", Azospirillum lipoferum and Bacillus licheniformis. They attributed this phenomenon to oxygen- stimulated mineralisation rates, and considered that the rate of mineralisation in soil was a more important controlling factor in denitrification than anaerobiosis. Unfortunately, only headspace analysis of the oxygen concentration was used during these experiments, and it is therefore not possible to relate the results to those from Trevors and Rönner & Sörensson, mentioned above.

It is clear that a number of denitrifying bacteria are capable of simultaneously respiring oxygen and nitrate (or another nitrogen oxide), the phenomenon known as aerobic denitrification. As has been shown, especially with the Pa. halodenitrificans experiments (Hochstein et al., 1984), most denitrifying bacteria are able to do so "aerobically". The important difference is the concentration of oxygen at which either denitrification activity or the synthesis of the denitrifying enzymes is reduced or shut down completely, a concentration which can vary enormously between different organisms. From a physiological point of

view the important question is whether or not oxygen is saturating the terminal cytochrome oxidase. This generally occurs at concentrations in the order of 1-2% of air saturation (2-4 micromoles/litre). If the level of dissolved oxygen is above this threshold, the term "aerobic denitrification" is justified. From an ecological or environmental point of view, aerobic denitrification means that the process occurs at detectable levels of air saturation (in common practice, around 5% of of air saturation).

Can aerobic denitrification be rationalized, or even justified, in ecological terms? The obvious conceptual problem is that, because of the lower biomass yield of denitrifying cultures, it seems inefficient for an organism to co-respire oxygen and nitrate. In biochemical terms this might indeed be a disadvantage and, at first glance, it would appear that evolutionary pressure would act against the maintenance of such a trait in a species. The same could be said of heterotrophic nitrification, where a high nitrification rate is linked to a low yield. This reasoning depends on the implicit assumption that these species exist in a niche in which steady state aerobic and anaerobic conditions exist, or where aerobiosis and anaerobiosis alternate over periods favourable for maximal yield. However, these conditions are usually not fulfilled in nature. The specific growth rate under the relevant conditions and the ability of the organism to respond efficiently to a changing environment (i.e. the reactivity of the organism (Kuenen & Beudeker, 1982; Leegwater, 1983)) are of as great importance to the survival of the organism as biomass yield.

A number of recent ecophysiological studies have considered these aspects of electron donor specificity and/or alternating mixed substrate utilization (Rittenberg, 1969; Gottschal & Kuenen, 1980; Kuenen & Gottschal, 1982; Kuenen & Robertson, 1984A; 1984B). It seems that some parallels can be drawn between these experiments and electron acceptor utilization. Certainly, a species which did not need to re-induce its denitrifying system after exposure to oxygen, or which could continue to denitrify while taking advantage of the extra energy provided by limiting amounts of oxygen, would have a selective advantage over other, less versatile species. In addition, a denitrifier living in an

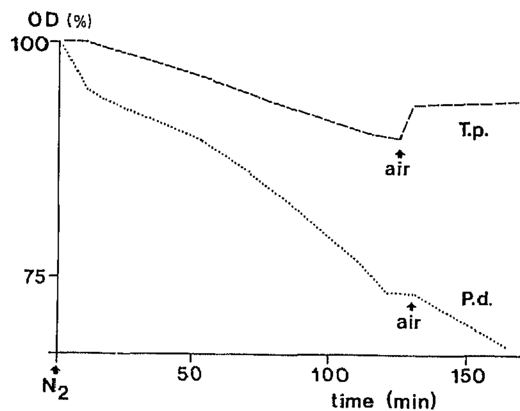

Figure 4. The outcome of switching steady-state, acetate-limited chemostat cultures of Tsa. pantotropha (T.p.) and Pa. denitrificans (P.d.) from aerobiosis (80% air saturation) to anaerobiosis (Robertson, 1988).

environment where long periods of anaerobic conditions are interspersed by short periods of aerobiosis would be at a disadvantage if it inactivated its denitrifying system too readily, because this would result in a lack of sufficient denitrifying capacity when anaerobic conditions were restored.

Another of the more obvious situations in which a versatile denitrifier might have a selective advantage over a specialist is under fluctuating oxygen levels. An experiment during which cultures of Tsa. pantotropha and of a specialist strain of Pa. denitrificans were grown to steady state at 80% air in an acetate-limiting medium with ammonia and nitrate showed that Tsa. pantotropha adjusted rapidly to the sudden onset of anaerobiosis, while Pa. denitrificans washed out with the dilution rate. When the air supply was equally suddenly restored, Tsa. pantotropha adjusted smoothly again, but Pa. denitrificans continued to wash out (figure 4). This was found to be due to the accumulation of up to 14 mM nitrite in the medium. 10 mM was sufficient to inhibit the oxygen uptake rate of these cultures by 50%. It is clear that Pa. denitrificans induced its nitrate reductase first, and was unable to readjust to the return of oxygen. If the anaerobic period was extended, the nitrite level eventually fell, and the culture was able to recover. These results emphasize the advantages which flexibility with regard to electron acceptors can confer under changing conditions. Tsa. pantotropha was isolated from a waste water treatment system receiving nitrate as well as intermittent amounts of oxygen. Other aerobic (i.e. constitutive) denitrifiers have been isolated from wastewater treatment systems (e.g. Krul, 1976; Krul & Veeningen, 1977; Meiberg et al., 1980; Robertson & Kuenen, 1983), and their activities may account for some of the nitrogen losses reported to occur in aerobic treatment plants. They may even explain the occurrence of levels of denitrifying enzymes in well aerated soils (Tiedje et al., 1982).

SUMMARY

Although "aerobic denitrification" has been a subject of some controversy for over a century, and had largely come to be discredited, reports of the simultaneous use of oxygen and denitrification by different species of bacteria have recently become more common. Research with some strains (e.g. Thiosphaera pantotropha) has revealed that bacteria simultaneously using two electron acceptors (i.e. nitrate and oxygen) grow more rapidly than when only a single electron acceptor is available. Further tests have indicated that there might be a link between aerobic denitrification and a form of nitrification which requires rather than generates energy, and is therefore known as heterotrophic nitrification. Heterotrophic nitrification (when defined as the oxidation of reduced nitrogen compounds during heterotrophic metabolism) is relatively common, being found in bacteria from different genera as well as fungi and even tissue from rat livers. Bacterial heterotrophic nitrification has tended to be under-estimated as, in many cases, the heterotrophic nitrifiers were simultaneously converting some of the nitrite (or other nitrogen oxide) to gaseous products (i.e. denitrifying). The relative importance of these strains within the nitrogen cycle may thus require some reconsideration. This paper reviews recent research into heterotrophic nitrification and aerobic denitrification, and presents a preliminary model which, if verified, will provide at least a partial explanation for the simultaneous occurrence of nitrification and denitrification in some bacteria. Finally, the ecological impact of bacteria capable of simultaneous nitrification and denitrification is considered.

REFERENCES

Abou Seada, M.N.I. and Ottow, J.C.G.,1985, Effect of increasing oxygen concentration on total denitrification and nitrous oxide release from soil by different bacteria. Biol. Fert. Soils 1:31-38.

Anderson, I.C. and Levine, J.S., 1986, Relative rates of NO and N_2O production by nitrifiers, denitrifiers and nitrate respirers. Appl. Env. Microbiol. 51:938-945.

Bazylinski D.A. and Blakemore R.P., 1983, Denitrification and assimilatory nitrate reduction in Aquaspirillum magnetotacticum. Appl. Env. Microbiol. 46:1118-1124.

Casella, S., Shapleigh, J.P., Lupi, F. and Payne, W.J., 1988, Nitrite reduction in bacteroids of Rhizobium "hedysari" strain HCNT1. Arch. Microbiol. 149:384-388.

Castignetti, D., and Hollocher, T.C., 1984, Heterotrophic nitrification among denitrifiers. Appl. Env. Microbiol. 47:620-623.

Davies, K.J.P., Lloyd, D. and Boddy, L., 1989, The effect of oxygenon denitrification in Paracoccus denitrificans and Pseudomonas aeruginosa. J. Gen. Microbiol. 135:2445-2451.

Degn, H., Cox, R.P. and LLoyd, D., 1985, Continuous measurement of dissolved gases in biochemical systems with the quadrupole massspectrometer. Methods Biochem. Anal. 31:166-194.

Gottschal, J.C. and Kuenen, J.G., 1980, Selective enrichment of facultatively chemolithotrophic Thiobacilli and related organisms in continuous culture. FEMS Microbiol. Letts. 7:241-247.

Hochstein L.I., Betlach M. and Kritikos G., 1984, The effect of oxygen on denitrification during steady-state growth of Paracoccus halodenitrificans. Arch. Microbiol. 137:74-78.

Kawakami, Y., Pacaud, B. and Nishimura, H., 1985, Inhibition of denitrification by oxygen in Paracoccus denitrificans. J. Ferment. Technol. 63:437-442.

Killham, K., 1986, Heterotrophic nitrification. IN Nitrification J.I. Prosser (editor) IRL Press. pp.117-126.

Korner, H. and Zumft, W.G., 1989, Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of Pseudomonas stutzeri. Appl. Env. Microbiol. 55:1670-1676.

Krul, J.M., 1976, Dissimilatory nitrate and nitrite reduction under aerobic conditions by an aerobically and anaerobically grown Alcaligines sp. and by active sludge. J. Appl. Bacteriol. 40:245-260.

Krul, J.M. and Veeningen, R., 1977, The synthesis of the dissimilatory nitrate reductase under aerobic conditions in a number of denitrifying bacteria isolated from active sludge and drinking water. Water Res. 11:39-43.

Kuenen, J.G. and Beudeker, R.F., 1982, Microbiology of Thiobacilli and other sulphur oxidizing autotrophs, mixotrophs and heterotrophs. Phil. Trans. Royal Soc. London. Ser. B. 298:473-497.

Kuenen, J.G. and Gottschal, G.C., 1982, Competition among chemolithotrophs and methylotrophs and their interactions with heterotrophic bacteria. IN Microbial Interactions and Communities vol 1. A.T. Bull and J.H. Slater. (editors) Academic Press. pp.153-187.

Kuenen, J.G. and Robertson, L.A., 1984A. Interactions between obligately and facutatively chemolithotrophic sulphur bacteria. IN Continuous Culture 8 Biotechnology, medicine and the environment. A.C.R. Dean, D.C. Ellwood and C.G.T. Evans (editors) Ellis Horwood Ltd. pp.139-158.

Kuenen, J.G. and Robertson, L.A., 1984B. Competition among

chemolithotrophic bacteria under aerobic and anaerobic conditions. IN Current Perspectives in Microbial Ecology. M.J. Klug and C.A. Reddy (editors, American Society for Microbiology. pp.306-313.

Kuenen, J.G. and Robertson, L.A., 1987, Ecology of nitrification and denitrification. IN The Nitrogen and Sulphur Cycles J.A. Cole and S. Ferguson (editors) Cambridge University Press. pp.162-218.

Leegwater, M.P.M., 1983, Microbial reactivity: its relevance to growth in natural and artificial environments. PhD Thesis, University of Amsterdam.

Lloyd D., Boddy, L. and Davies, K.J.P., 1987, Persistance of bacterial denitrification capacity under aerobic conditions: the rule rather than the exception. FEMS Microbiol. Ecol. 45:185-190.

Lloyd, D., Davies, K.J.P. and Boddy, L., 1986, Mass spectrometry as an ecological tool for in situ measurement of dissolved gases in sediment systems. FEMS Microbiol. Ecol. 38:11-17.

MacFarlane, G.T. and Herbert, R.A. ,1984, Dissimilatory nitrate reduction and nitrification in estuarine sediments. J. Gen. Microbiol. 130:2301-2308.

Meiberg, J.B.M., Bruinenberg P.M., Harder W. ,1980, Effect of dissolved oxygen tension on the metabolism of methylated amines in Hyphomicrobium X in the absence and presence of nitrate: evidence for "aerobic denitrification". J. Gen. Microbiol. 120:453-463.

Poth, M., 1986, Dinitrogen production from nitrite by a Nitrosomonas isolate. Appl. Env. Microbiol. 52:957-959.

Rittenberg S.C., 1969, The roles of exogenous organic matter in the physiology of chemolithotrophic bacteria. Adv. Microb. Physiol. 3: 151-196.

Robertson, L.A., 1988, Aerobic denitrification and heterotrophic nitrification in Thiosphaera pantotropha and other bacteria. PhD thesis. Delft University of Technology.

Robertson, L.A. and Kuenen, J.G., 1983a, Thiosphaera pantotropha gen. nov. sp. nov., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. J. Gen. Microbiol. 129:2847-2855.

Robertson, L.A. and Kuenen, J.G., 1983b, Anaerobic and aerobic denitrification by sulphide oxidizing bacteria from waste water. IN Anaerobic Waste Water Treatment W.J. van den Brink (editor) pp.3-12.

Robertson, L.A. and Kuenen, J.G., 1984a, Aerobic denitrification - old wine in new bottles? Antonie van Leeuwenhoek 50:525-544.

Robertson, L.A. and Kuenen, K.G., 1984b, Aerobic denitrification: a controversy revived. Arch. Microbiol. 139:351-354.

Robertson, L.A. and Kuenen, J.G., 1988, Heterotrophic nitrification in Thiosphaera pantotropha - oxygen uptake and enzyme studies. J. Gen. Microbiol. 134:857-863.

Robertson, L.A., Cornelisse, R., de Vos, P., Hadioetomo, R. and Kuenen, J.G., 1989a, Aerobic denitrification in various heterotrophic nitrifiers. Antonie van Leeuwenhoek 56:289-299.

Robertson, L.A., Cornelisse, R., Zheng, R. and Kuenen, J.G., 1989b, The effect of thiosulphate and other inhibitors of autotrophic nitrification on heterotrophic nitrifiers. Antonie van Leeuwenhoek 56: 301-309.

Robertson, L.A., van Kleeff, B.H.A. and Kuenen, J.G., 1986, A microcomputer-based method for semi-continuous monitoring of biological activities J. Microbiol. Methods 5:237-242.

Robertson, L.A., van Niel, E.W.J., Torremans, R.A.M. and Kuenen, J.G., 1988, Simultaneous nitrification and denitrification in aerobic chemostat cultures of Thiosphaera pantotropha. Appl. Env. Microbiol. 54:2812-2818.

Rönner, U. and Sörensen, F., 1985, Denitrification rates in the low-

oxygen waters of the stratified Baltic proper. Appl. Env. Microbiol. 50:801-806.

Strand, S.E., McDonnell, A.J. and Unz, R.F., 1988, Oxygen and nitrate reduction kinetics of a nonflocculating strain of *Zooglea ramigera*. Antonie van Leeuwenhoek 54:245-255.

Tiedje, J.M., Sexstone, A.J., Myrold, D.D. and Robinson, J.A., 1982, Denitrification: ecological niches, competition and survival. Antonie van Leeuwenhoek 48:569-583.

Trevors, J.T., 1985, The influence of oxygen concentrations on denitrification in soil. Appl. Microbiol. Biotechnol. 23:152-155.

Trevors, J.T. and Starodub, M.E., 1987, Effect of oxygen concentration on denitrification in freshwater sediment. J. Basic Microbiol. 27:387-391.

Verstraete, W., 1975. Heterotrophic nitrification in soils and aqueous media. Izvestija Akademii Nauk SSSR Ser. Biol. 4:541-558.