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Aerobic denitrification – old wine in new bottles?

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The evidence concerning aerobic denitrification over the past 100 years has been reviewed and the conclusion reached that the denitrification systems of some bacteria are inhibited by oxygen, other species are capable of aerobic denitrification, or co-respiration of nitrate and oxygen. Possible mechanisms and ecological implications are discussed.

INTRODUCTION

The three pathways by which bacteria can reduce nitrate are shown in Fig. 1.

The assimilatory route to ammonium (1) occurs when there is not a more suitable source of nitrogen available, and ultimately results in the nitrogen from the nitrate being incorporated into the biomass. A superficially similar route to ammonium (2) is known as dissimilatory nitrate reduction. This serves as a source of respiratory energy and as a means of re-oxidizing reduced pyrimidine nucleotides in some fermentative species (Cole and Brown, 1980; Yordy and Ruoff, 1981; De Vries et al., 1982). In this case, the pathway can be active in the presence of ammonium, the enzymes differ from the assimilatory route and the nitrate nitrogen is excreted as ammonium. The third, and last, pathway is that in which nitrate is reduced through a sequence of nitrogen oxides to nitrogen gas (3). It is this last pathway, known as denitrification, which is important for the production of respiratory energy, and is the major focus of this review.

Gayon and Dupetit (1886) first introduced the term "denitrification" for a phenomenon which had, by then, been known for some years. There has, since then, been a large number of papers published on the subject, to the extent that as long ago as 1953 Kluyver commented "It seems a somewhat risky enterprise

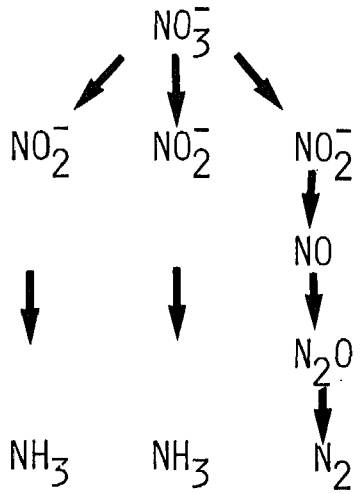

Fig. 1. The pathways of nitrate reduction available to bacteria. From left to right, assimilatory nitrate reduction (1), dissimilatory nitrate reduction (2), denitrification (3).

to make bacterial nitrate reduction the subject of a contribution to a modern symposium on bacterial metabolism. Most bacteriologists will consider the subject distinctly demoded, and they are fully satisfied with their knowledge of the process." However, even 30 years later there are still many points of interest and also of controversy which have not yet been resolved. One of these, the participation of nitric oxide as a free or bound intermediate in nitrate reduction to nitrogen is outside the scope of this review and those interested should consult other recent papers (e.g. Payne, 1981; Hollocher, 1982; Knowles, 1982).

Bréal (1892) began another controversy which continues to this day when, in a paper on the denitrifying ability of straw-based cultures he said, "Le ferment qui décompose les nitrates est aérobie; car si l'on remplit le flacon dont nous venons de nous servir avec l'oxygène, la réduction s'effectue encore aisement". (The ferment that decomposes the nitrates is aerobic; since, if one replenishes the oxygen in the flask which we have just used, the reduction proceeds easily again.) It is to the arguments surrounding the occurrence of aerobic denitrification that the focus of this review will be directed.

Historical perspective

It is not proposed to deal with the full history of denitrification. This has recently been reviewed (Payne, 1981), and in this account only papers relating to the problems surrounding aerobic denitrification will be discussed.

Despite fairly frequent reports of bacteria able to denitrify in the presence of measurable amounts of oxygen, it has often been stated that denitrification occurs only under anaerobic conditions, and that the presence of oxygen inhibits the activity of the denitrifying enzymes and suppresses their synthesis (see for example Bryan, 1981). This generalization is based on studies with pure cultures of a few well-known organisms. Another argument sometimes heard is that aerobic denitrification is "thermodynamically impossible". It should be stressed that the process is thermodynamically unfavourable when compared with oxygen respiration (Thauer et al., 1977) but certainly not impossible.

Many of the early reports of the occurrence of aerobic denitrification were dismissed, probably with reason, as examples of poor aeration or poor experimental technique. Most anaerobic experiments were done in completely filled, glass-stoppered bottles or by the use of alkaline pyrogallol. "Aerobic" samples, including those of Bréal in the paper previously referred to, were frequently just exposed to the air and not shaken or sparged with air. Typical culture vessels are shown in Fig. 2. Such procedures clearly resulted in an oxygen gradient in the culture, and it is thus likely that most of the observed denitrification occurred in the anaerobic areas of the medium. The dangers of relying on estimates of the degree of aerobiosis can be seen in Table 1. The cultures used in experiment A were described as having had "moderate aeration". These cultures involved 100-ml amounts of medium in 300-ml conical flasks which were incubated with

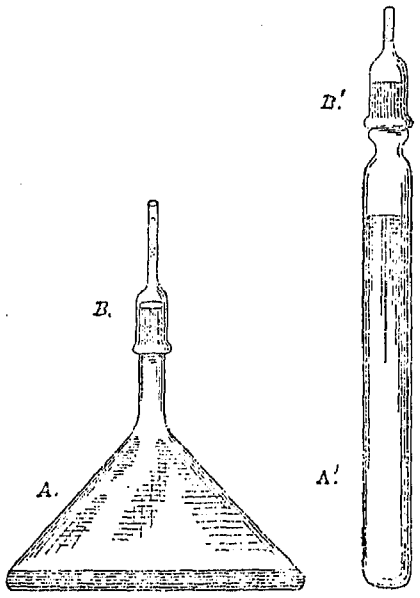

Fig. 2. Examples of culture vessels used to produce "aerobic" and "anaerobic" conditions in early denitrification studies (taken from Gayon and Dupetit, 1886).

Table 1. The results presented by Verhoeven (1956) to show the effect of different degrees of aeration on denitrification by different strains of bacteria. Section A: poorly aerated. Section B: vigorously aerated. See text

	N as % initial NO_3^- nitrogen					
	NO_3^- start	NO_3^- end	NO_2^-	Protein	NH_4^+	Gas products
A						
Bacillus megaterium	100	95.7	traces	4.3	0	0
Micrococcus denitrificans[1]	100	0	traces	14.8	0	85.2
Pseudomonas aeruginosa	100	0	traces	10.6	0	89.2
Bacillus licheniformis	100	0	traces	15.7	89.4	0
Bacillus licheniformis	100	33.8	4.5	10.9	54.8	0
B						
Bacillus megaterium	100	93.6	0	5.9	0	0
Micrococcus denitrificans[1]	100	72.3	0.2	16.6	4.2	6.9
Pseudomonas aeruginosa	100	72.6	0.2	14.5	9.6	3.1
Bacillus licheniformis	100	72.8	0.4	19.7	0.4	6.6
Bacillus licheniformis	100	87.0	0.4	8.5	0.7	3.4

[1]Now *Paracoccus denitrificans*.

Table 2. Summary of the experimental conditions described by Collins (1955)

Vessel number	Shape	Vol. (ml)	Liquid vol. (ml)	% filled
1	straight-sided bottle	1000	200	20
2	conical	1000	200	20
3	conical	500	200	40
4	round-bottomed	500	200	40
5	round-bottomed	490	200	41
6	round, anaerobic	500	200	40

a current of air passing over the surface of the liquid, but without shaking. The results in experiment B were obtained with cultures described as having had "excessive aeration". In this case, 50-ml amounts of medium were used in 300-ml conical flasks which were incubated on a shaker with air flowing over the surface of the liquid at twice the rate of flow used in the previous cultures.

As can be seen in Table 1, the degree of denitrification or dissimilatory nitrate reduction varied both with the experimental method and with the organism used. It should be noted that even in the cultures which received "excessive aeration", *Micrococcus* (now *Paracoccus*) *denitrificans* appeared to retain at least some of its denitrifying ability. The organism has now been established as having a denitrification system which is extremely sensitive to oxygen (John, 1977; Alefounder

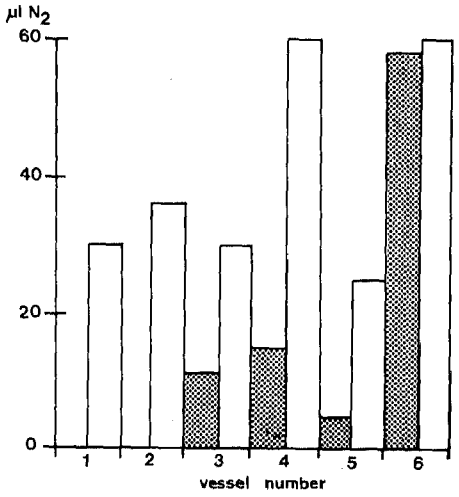

Fig. 3. The amount of gas produced by cells grown under the cultural conditions shown in Table 2, washed and resuspended in phosphate buffer, and then supplied with yeast autolysate and nitrate in Warburg manometers. Open bars *Pseudomonas aeruginosa* NCTC 6750, shaded bars *Ps. aeruginosa* T8. Nitrogen produced as µl nitrogen · h^{-1} mg^{-1} wet wt of cells (data from Collins, 1955).

et al., 1981). Aerobic conditions within a culture are dependent on the oxygen transfer coefficient for the culture as counteracted by the rate of oxygen uptake by the organisms. Thus, growth conditions sufficient to provide aerobiosis in one shaken culture may be inadequate for another, faster growing species. The results obtained in Table 1 thus emphasize that dissolved oxygen measurement is essential in experiments of this type. A paper (Collins, 1955) which is frequently quoted as showing that the shape of the culture vessel can determine the efficiency of aeration in a culture is another case where the results cannot be fully interpreted because the dissolved oxygen concentration is not known. Various strains and species of denitrifying bacteria were grown on a shaker in culture vessels of different shapes and sizes (summarized in Table 2). The denitrifying ability of the resulting biomass was then compared with that of a similar culture grown anaerobically. The results obtained with two strains of *Pseudomonas aeruginosa* are shown in Fig. 4. Since *Ps. aeruginosa* NCTC 6750 and some of the other organisms tested had no denitrifying ability when cultured in the bottle (vessel 1) and 1000-ml conical flask (vessel 2), it was assumed that the shape of the vessel was the determining factor. However, for efficient oxygen transfer the surface to volume ratio of the liquid is at least as important as the mixing efficiency. As all of the vessels received the same volume of liquid (200 ml) it appears that the ratio between the volume of the vessel and the volume of the culture liquid was of importance in these experiments. Unfortunately, a 1000-ml round-bottomed flask was not included in the study. That *Ps. aeruginosa* strain

Table 3. The fate of nitrate supplied to a *Pseudomonas* species under different degrees of aeration as found by Meiklejohn (1940)

Culture conditions	Time (days)	Total N	Protein N
1. 2-cm deep culture in cotton-plugged conical flask	0	237	–
	7	53	45
2. 2-cm deep culture in cotton-plugged conical flask with continuous air sparging	0	235	–
	7	66	66
3. anaerobic culture incubated in the presence of alkaline pyrogallol	0	222	–
	7	33	24

T8 retained about half of its denitrifying ability under all conditions further demonstrates the variability of the results obtained with different organisms which may have different growth characteristics such as growth rate or response to oxygen exposure.

Despite their lack of dissolved oxygen measurements, a few early reports include experimental description complete enough to allow the results to be viewed with a certain degree of confidence. One such is the report by Meiklejohn (1940) in which the effect of various environmental effects on denitrification by two *Pseudomonas* species was measured. Table 3 summarizes one of the experiments.

It can be seen that under all three growth conditions virtually all of the nitrate not assimilated by the cells had disappeared from the medium. Moreover, as denitrification provides less energy than oxygen respiration (Koike and Hattori, 1975), the increase in yield with the increasing supply of air to the culture could be expected if the cells were altering the balance between oxygen and nitrate respiration according to oxygen availability. In contrast to Meiklejohn (1940), Marshall et al. (1953) supplied both ammonium and nitrate to their cultures. By using the ^{15}N form of either nitrogen compound in turn, they attempted to trace the fate of the ammonia and nitrate nitrogen atoms in sparged and unsparged cultures grown in shallow (25-mm) layers in conical vessels with cotton plugs. They found that when the ^{15}N was supplied in the ammonium, its disappearance from the medium was matched by its appearance in the biomass. However, if the ^{15}N was incorporated in the nitrate, it did not appear in the biomass as long as ammonium was present, but 97% of the nitrate disappeared from the medium. In common with the results of Meiklejohn, the biomass yield was higher in the sparged culture. Although, because of the lack of dissolved oxygen measurements, neither of these papers can be taken as evidence for fully aerobic denitrification, they certainly indicate that denitrification can proceed in cultures which are well-mixed, and which are being continuously supplied with air.

Even now, 30 years after these studies were done, and despite the considerable

improvements in the equipment available, investigations into the effect of oxygen on denitrification in which only the oxygen level measured was that in the gaseous phase and not in solution are still being published (see, for example Nakajima et al., 1984). This is especially undesirable when a rich medium, in which bacterial oxygen uptake can outstrip oxygen transfer from the gas to the liquid phases, is used without shaking or sparging (see, for example, Watahiki et al., 1983).

Some researchers tried to investigate oxygen inhibition of denitrification by measuring redox levels in their cultures. Korochinka (1936) found that *Ps. denitrofluorescens* (now *Ps. fluorescens*) continued to denitrify at rH values of 24–25, but that the rate was reduced at a rH value of 35. She concluded that the presence of air was not sufficient to stop denitrification. Kefauver and Allison (1957) used a complex apparatus in an attempt to ensure good aeration. Although they did not measure dissolved oxygen, they did measure redox and found, as did Elema (1932), a drop in the redox of the medium with the onset of nitrite reduction. However, during experiments in which they controlled the rH in suspensions of resting cells at values between + 100 and + 350 mV, they still obtained nitrite reduction. They concluded that the redox of the medium was not a controlling factor, and that their test species could use oxygen and nitrate simultaneously when less than 6% oxygen was present in the influent gas. Recently, Ottow and Fabig (1983) established that the rH level in cultures of *Moraxella* sp. and *Acinetobacter* sp. was related to the metabolic activity of the culture rather than the degree of anaerobiosis. Experiments relying on redox measurements in the growth medium will therefore not yield information regarding denitrification in the presence of measureable amounts of oxygen.

Oxygen inhibition

In the early days of the work on denitrification, one line of research was based on the view that the sole function of nitrate reduction was to provide "oxygen" for use in respiration (see for example, Cranston and Lloyd, 1930; Lloyd, 1931). If this was correct, it would be logical that in the presence of more readily accessible oxygen, there would be no requirement for nitrate reduction. It is now known that nitrate, nitrite and the other oxides of nitrogen serve as electron acceptors in their own right, and that the respiratory pathways to oxygen or to the nitrogen oxides are to some extent separate (see recent reviews such as Stouthamer, 1980, 1984; Payne, 1981; Knowles, 1982). Some species of bacteria are known to have denitrification systems which are very sensitive to the presence of oxygen, and the reasons for this inhibition are not yet completely clear. It is not practicable to deal with all of the papers demonstrating oxygen inhibition, but to provide a balanced view a few will be mentioned.

Jannasch (1960) used a very simple experiment to demonstrate that the denitri-

fying enzymes of *Ps. stutzeri* are inactivated on exposure to oxygen. In a mixed culture of *Ps. stutzeri* and a *Chlorella* species, nitrate disappeared from the culture as long as it was incubated in the dark. However, if the culture vessel was illuminated, denitrification stopped immediately. As the denitrification in a similar vessel containing a pure culture of *Ps. stutzeri* was unaffected by light, it was concluded that the oxygen generated during photosynthesis was inhibiting nitrate reduction by the pseudomonad.

Using electrodes to measure oxygen, nitrate and nitrite, John (1977) showed that *Pa. denitrificans* and *Escherichia coli* both ceased reducing nitrate immediately they were supplied with oxygen, and began again as soon as the oxygen was depleted.

It has been shown that the different enzymes of the denitrification pathway in *Pa. halodenitrificans* respond to different concentrations of oxygen. Hochstein et al. (1984) grew this species to steady state in anaerobic chemostats, and then established steady states at different dissolved oxygen concentrations. They found that although with 5% oxygen in the influent gas there was no detectable dissolved oxygen in the culture, production of dinitrogen had been completely replaced by the production of N_2O. By the time the dissolved oxygen level became measureable (1 $nmol \cdot l^{-1}$ with an influent gas concentration of 7.5% oxygen), the products of nitrate reduction were nitrite and N_2O in approximately equal parts. When the culture was supplied with air, only a small amount of nitrite was produced, and the remainder of the nitrate was untouched.

Schulp and Stouthamer (1970) showed the gradual inactivation of nitrate reductase in anaerobically grown cells of *Bacillus licheniformis* when these were incubated in a buffer, on a shaker, in the presence of air. A similar culture which was not shaken did not lose its activity. Growing cultures began nitrate reduction after the dissolved oxygen in the medium had fallen to below 20% of air saturation, but cells grown at higher concentrations of oxygen did not reduce nitrate, and did not contain nitrate reductase. Similarly, cultures of *Thiobacillus denitrificans* growing on thiosulphate in a chemostat did not reduce nitrate while oxygen was present in the cultures, and cultures maintained at various levels of dissolved oxygen required an induction period the length of which was related to the amount of oxygen present in the growth medium (5 h at 88% of air saturation and 1 h at 30%) before they were able to reduce nitrite (Justin and Kelly, 1978). Finally, Alefounder et al. (1981) used a nitrate electrode and H_2O_2 to show that nitrate reduction by *P. denitrificans* stops immediately oxygen is added and resumes only when the available oxygen is exhausted.

Aerobic denitrification

Skerman et al. (1951) appear to have been the first to include the measurement of dissolved oxygen in their investigation of the effect of oxygen on denitrifica-

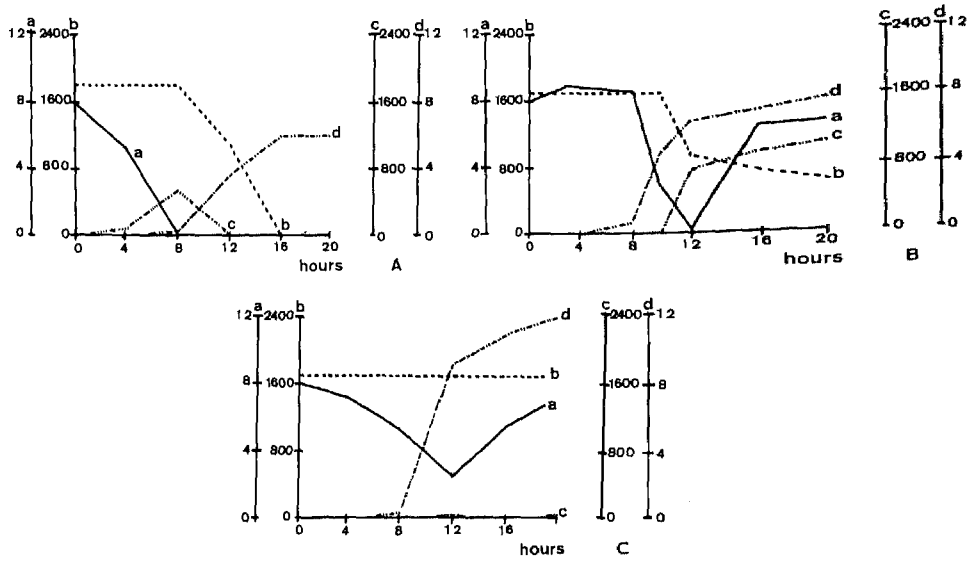

Fig. 4. Results of experiments using an oxygen electrode to measure the respiratory activity of a *Pseudomonas* species at different stirring rates in peptone yeast broth at 25 °C in the presence of nitrate. Nitrate and nitrite were determined colorimetrically. Curve a: dissolved oxygen (as ppm). Curve b: nitrate present (as ppm). Curve c: nitrite present (as ppm). Curve d: cell numbers determined from plate counts (1×10^9). Fig. 4A: Stirring rate 100 rpm. Fig. 4B: Stirring rate 300 rpm. Fig. 4C: Stirring rate 500 rpm (Data from Skerman et al., 1951).

tion. Some of their results are shown in Fig. 4. They varied the amount of oxygen supplied to their batch culture of a *Pseudomonas* sp. Nitrite appeared in the culture before the dissolved oxygen concentration reached 0, and in at least one case (see Fig. 4b), significant amounts of nitrate had been reduced before the dissolved oxygen became depleted. The increase in the level of dissolved oxygen present (Fig. 4b) as growth stopped serves as a reminder that oxygen was being supplied to these cultures continuously, and that oxygen and nitrate reduction must have been taking place simultaneously. Another indication of this is the increasing yield of cells as the ratio of oxygen to nitrate used increased, with the highest yield being obtained in the culture with the highest stirring rate (Fig. 4c), when little or no nitrate was reduced.

A second example of denitrification in the presence of low, but significant concentrations of dissolved oxygen was reported by Meiberg et al. (1980). They found that with *Hyphomicrobium* X in chemostat cultures growing on dimethylamine, the induction of the denitrifying enzymes required the presence of nitrate, and also appeared to be linked to the growth rate. At a dilution rate of 0.05 h^{-1}, the "threshold concentration" of dissolved oxygen below which nitrate reductase was induced was about 28% of air saturation. At a dilution rate of 0.1 h^{-1}, this "threshold concentration" had fallen to about 9% and at μ^{max} (0.18

Table 4. Nitrate reduction and oxygen uptake by *Alcaligenes* sp. In all cases the initial dissolved oxygen concentration was 5 mg·l^{-1} (data from Krul, 1976)

Growth conditions	Oxygen uptake nmol·min^{-1} mg^{-1} protein	Nitrate reduction, oxygen present nmol·min^{-1} mg^{-1} protein	Nitrate reduction, oxygen exhausted nmol·min^{-1} mg^{-1} protein
1. aerobic ammonium as nitrogen source	146	6	43
2. anaerobic nitrate present	151	15	90
3. cells from 2 aerated for 24 h in N-free medium	81	6	39

h^{-1}) the enzymes were only induced if the cultures became virtually anaerobic. This derepression of the denitrifying enzymes at low growth rates may be ecologically significant with respect to soil denitrification.

Aquaspirillum magnetotacticum (Bazylinski and Blakemore, 1983*a*) presents an interesting example of denitrification in the presence of low concentrations of oxygen. This is a microaerophilic species with an absolute requirement for O_2. It does not grow anaerobically. However, cells grown in the presence of nitrate with between 0.2 to 1% oxygen (initial head-space concentration) reduced the nitrate to nitrogen with a transient appearance of N_2O, while at the same time consuming oxygen. Furthermore, if acetylene was included, nitrogen production was replaced by the generation of N_2O, a common phenomenon among denitrifying species (Payne, 1981). Cells cultured with ammonia only, did not produce any of the nitrogen oxides. It is clear that *A. magnetotacticum* is capable of true denitrification, despite its requirement for oxygen. It is perhaps of interest to note that growing cells of *A. magnetotacticum* are also capable of fixing atmospheric nitrogen (Bazylinski and Blakemore, 1983*b*).

The examples given so far have involved denitrification in the presence of reduced amounts of dissolved oxygen. However, at least two species have been shown to denitrify in the presence of much higher concentrations of oxygen.

Krul (1976) found that both aerobically and anaerobically grown cells of an unidentified species of *Alcaligenes* were able to reduce nitrate and oxygen at the same time. The reduction rates he obtained, by using oxygen and nitrate electrodes together to measure the respiratory activity of cells supplied with glycerol in a closed, thermostatically controlled cell, are summarized in Table 4. It can be seen that the rate of nitrate reduction occurring at the same time as oxygen uptake was much lower than when all of the oxygen had been utilized. Nitrate reductase is presumably constitutive in these cells since even the cells grown aerobically in the absence of nitrate were able to reduce nitrate immediately.

In a subsequent experiment, Krul and Veeningen (1977) found that cells

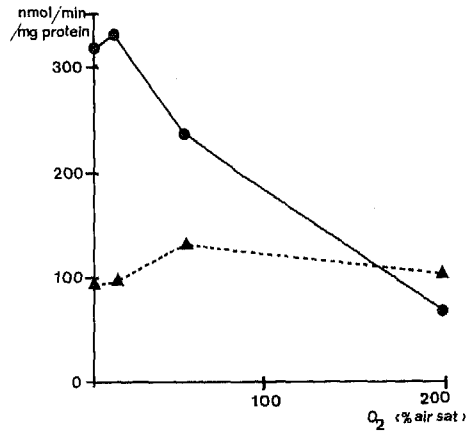

Fig. 5. The effect of the dissolved oxygen concentration on the oxygen uptake and nitrate reduction rates in *Alcaligines* strain 15 as measured using oxygen and nitrate electrodes (Data from Krul and Veeningen, 1977). ●, oxygen uptake; ▲, nitrate reduction.

grown aerobically (dissolved oxygen concentrations 53% and 200% of air saturation) in the presence of nitrate did not contain more nitrate reductase than similar cultures grown without nitrate. Fig. 5 shows the rates of oxygen uptake and anaerobic nitrate reduction obtained with cultures of this organism grown at different dissolved oxygen concentrations. It can be seen that even the cells grown at dissolved oxygen concentrations twice that of air saturation still contained 20% of their anaerobic nitrate-reducing capacity.

Our interest in aerobic denitrification first arose during studies on a isolate from a desulphurizing, denitrifying waste-water treatment system. It was found that aerobically grown cultures of the isolate, *Thiosphaera pantatropha* (Robertson and Kuenen, 1983*b*), were able to produce nitrogen in respirometric experiments immediately they were supplied with nitrate and substrate. A control organism, *Thiobacillus versutus* (Harrison, 1983), required the expected 3-4-h lag period before gas production began (Robertson and Kuenen, 1983*b*, 1984). This suggested that the enzymes of denitrification might be constitutive, but not necessarily always active, in *Ts. pantotropha*. However, when cultures were grown aerobically, a distinct difference in cultures with and without nitrate was found. Because of the problems associated with aerobic denitrification already detailed, cultures were grown in Kluyver flasks (Fig. 6) which were fitted with electrodes to measure dissolved oxygen. It was found that with mineral salts medium with a limited amount of substrate (e.g. 10mM acetate), the culture was well-mixed and free from clumps, and, when air was used for sparging, the dissolved oxygen level never fell below 80% of air saturation. The resulting growth curves are shown in Fig. 7, together with a growth curve obtained with anaerobically grown cells. The apparent differences in the yield indicated by the optical densities were

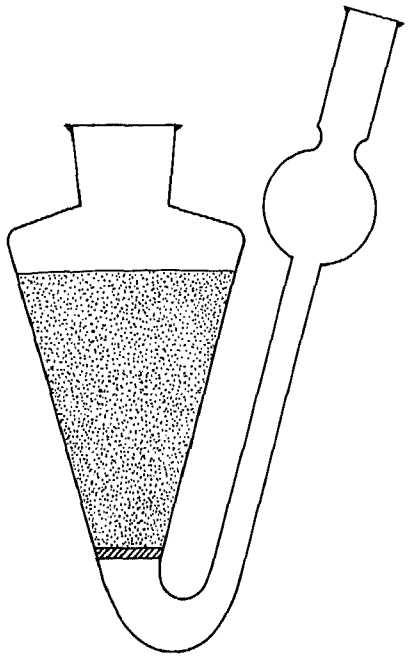

Fig. 6. Outline diagram of a Kluyver flask. Air or any selected gas can be blown through a sterile filter on the side-arm at a flow rate sufficient to keep the culture homogeneously suspended. For the experiments described in this paper, the flask was fitted with an oxygen electrode.

confirmed by protein determinations. Analysis of the spent growth medium showed that ammonium, the nitrogen source for growth, was still available, but that the concentration had fallen. Nitrate had not only disappeared from the anaerobic cultures but also a substantial amount had gone from the aerobic culture. It could be calculated that of the acetate respired about 50% must have been oxidized through nitrate in the fully aerobic culture. The differences in the yields obtained from the three growth conditions clearly confirmed the simultaneous utilization of nitrate and oxygen in the culture. The results of these experiments provide both qualitative and quantitative evidence of aerobic denitrification at high dissolved oxygen concentrations.

Further evidence was obtained from the total oxygen uptake by aerobically grown cells in the presence and absence of nitrate and two denitrification inhibitors, antimycin a and cyanide, which was measured using a thermostatically controlled cell incorporating a Clark-type oxygen electrode. The results are shown in Fig. 8a. That the inhibitors were indeed effective in this species was confirmed by anaerobic respirometry (Fig. 8b) using cells from the same batch of cells as those used in the oxygen uptake experiments.

Chemostat studies at sub-maximal growth rates (0.015–0.175 h^{-1}) have con-

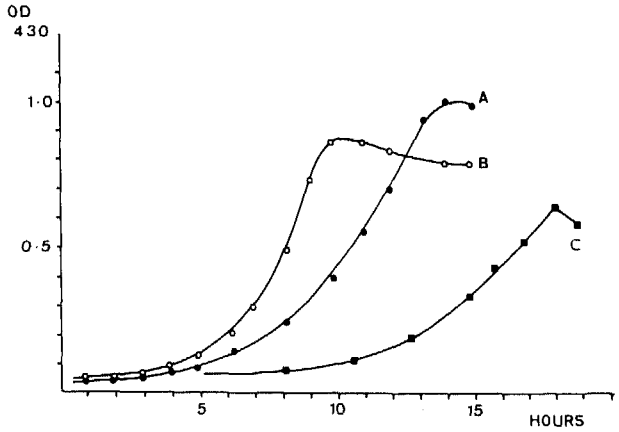

Fig. 7. Growth curves obtained with *Thiosphaera pantotropha* when grown: A, aerobically with acetate; B, aerobically with acetate and nitrate; C, anaerobically with acetate and nitrate. All cultures received ammonium as a nitrogen source, and the dissolved oxygen level in the aerobic cultures never fell below 80% of air saturation. The final protein concentrations (measured at the end of the onset of the stationary phase) were 81, 60 and 40 $mg \cdot l^{-1}$, respectively, confirming the apparent differences in the yields indicated by the optical densities. Clumping of the cells did not occur. OD, optical density at 430 nm. Acetate and nitrate concentrations 10 and 20 mM, respectively. Figure and data from Robertson and Kuenen, 1984.

firmed the disappearance of nitrate from cultures grown with ammonium as a nitrogen source and under controlled (more than 75% of air saturation) levels of dissolved oxygen. Additionally, nitrate and nitrite reductase have been found in these cultures (Robertson and Kuenen, to be published).

A possible physiological explanation for aerobic denitrification by *Ts. pantotropha* is that in this constitutive denitrifier the branch of the respiratory pathway to oxygen has a (rate-limiting) bottle-neck. Addition of nitrate to the aerobic culture would then allow the observed faster growth rate (Robertson and Kuenen, 1983*a*, 1984). An alternative explanation will be discussed in the following paragraphs.

Possible mechanisms of regulation of the denitrification capacity

As yet, the mechanism by which oxygen affects denitrification in many bacteria is not fully understood, and it is not yet clear if one or several mechanisms are involved. In some species, anoxia is alone sufficient to induce nitrate reductase, but in others the presence of nitrate is required. Again, in some species the denitrifying enzymes appear to be inactivated by oxygen, whereas in others synthesis is repressed but the existing enzymes only gradually disappear (Payne, 1981; Knowles, 1982).

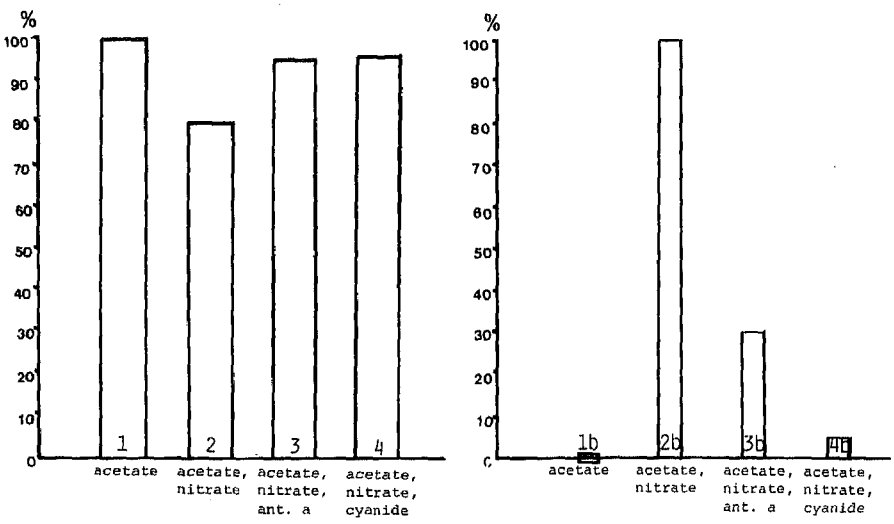

Fig. 8. The effect of nitrate and denitrification inhibitors on total oxygen uptake and nitrogen production by washed cells of *Thiosphaera pantatropha* which had been aerobically grown in the presence of nitrate. Bars 1–4 show oxygen uptake, as measured with a Clark-type oxygen electrode. Bars 1b–4b show nitrogen production as measured by anaerobic respirometry. In both cases, the gas volumes obtained from the "untreated" samples (i.e. the aerobic sample receiving acetate alone, and the anaerobic sample receiving only acetate and nitrate) were taken as 100%.

Much of the work on oxygen inhibition has been done with *Pa. denitrificans* (Alefounder and Ferguson, 1980; Alefounder et al., 1983; Boogerd, 1984; Stouthamer, 1984) and it appears that two factors may be interacting in the inhibition of denitrification by oxygen in this species. The redox level in the cytochrome chain has been shown to control the flow of electrons to the different cytochromes, and thus to determine whether or not electrons are available for denitrification (Kučera and Dadák, 1983). However, it has also been shown that the cell membrane alters its permeability to nitrate in response to dissolved oxygen (Alefounder et al., 1984). Since the dissimilatory nitrate reductase is located on the inside of the cell membrane (Stouthamer, 1980), a permeability barrier between the enzyme and its substrate would be a very effective controlling factor.

The dissimilatory nitrite reductase is located on the outside of the membrane (Stouthamer, 1980, 1984; Boogerd, 1984) and therefore lack of access cannot provide the full explanation. However, it is known that nitrite inhibits oxygen uptake, thus altering the redox level of the cytochrome chain (Kučera and Dadák, 1983; Kučera et al., 1984). It thus seems reasonable to suppose that once nitrite is present in the cell, whether produced by nitrate reduction or by some other means, oxygen uptake would be inhibited sufficiently to permit electron

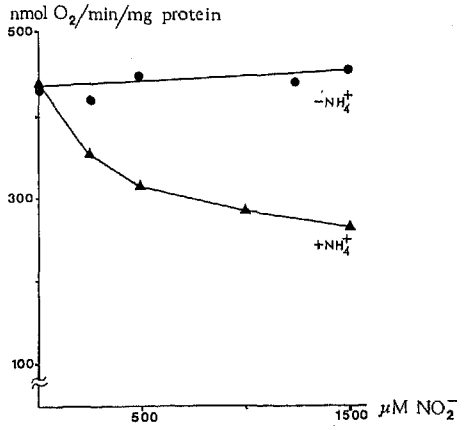

Fig. 9. The effect of increasing concentrations of nitrite on the rate of oxygen uptake by *Thiosphaera pantotropha* in the presence and absence of ammonium. Aerobically grown cells were washed and resuspended in phosphate buffer. The sample was then divided into two aliquots and NH_4Cl (0.3 $g \cdot l^{-1}$) was added to one. Oxygen uptake, using thiosulphate as the energy source, was measured with a Clark-type electrode in a closed, thermostatically controlled cell. Nitrite was added 5 min before the thiosulphate. ●, without ammonium; ▲, with ammonium.

flow to nitrite reductase and the other enzymes of denitrification. This hypothesis might also provide an alternative, or supplementary, explanation for the results obtained with the aerobic denitrifier, *Ts. pantotropha* as discussed in the preceding paragraph. Under aerobic conditions this species produces nitrite from ammonium in the presence of another energy source, a process known as heterotrophic nitrification (Robertson and Kuenen, 1984). This apparently widespread phenomenon is, as yet, little understood, but may be linked to denitrification as it has been found in many denitrifiers (Castignetti and Hollocher, 1984). Fig. 9 shows the effect of nitrite on the oxygen uptake of *Ts. pantotropha* in the presence and absence of ammonium when provided with thiosulphate as a source of energy. It can be seen that in the absence of ammonium the nitrite had little or no effect, but when ammonium was present the nitrite substantially inhibited the uptake of oxygen. This might be explained if one assumes that in the presence of ammonium the intracellular nitrite concentration rose because heterotrophic nitrification continued but the nitrite reductase was inadequate to cope with both the internally produced and externally supplied nitrite. Furthermore, nitrite was always present in preparations of cells which had been grown with ammonium as the nitrogen source, washed and ultrasonically disrupted. A rough estimation, from preliminary results based on the amount of nitrite present per mg protein, indicated that the internal concentration of nitrite was about 0.5 mM. From the curves in Fig. 9 it can be estimated that this would slow the rate of oxygen uptake by about 20% which would probably be sufficient to reduce the redox level of the cytochrome chain and permit electron flow to

the denitrifying enzymes regardless of any oxygen present. As yet, the phenomenon of heterotrophic nitrification is not well understood, and therefore it is not possible to say whether aerobic denitrification is a mechanism for, perhaps, detoxifying the nitrite produced. Work is currently in progress to discover whether other aerobic denitrifiers are also heterotrophic nitrifiers, and vice versa.

Concluding remarks

From the evidence presented here, it is obvious that many facultatively anaerobic denitrifiers are capable of the simultaneous utilization of oxygen and nitrate (or another nitrogen oxide). The observation that "an important aspect of bacterial metabolism that seems to be little appreciated is the fact that an organism may use two alternative respiratory mechanisms at the same time, provided that conditions are satisfactory for both" (Skerman et al., 1951) is still true today. In this sense all of these bacteria are able to denitrify "aerobically". The important difference between them is the concentration of oxygen at which the denitrification capacity is reduced or shut down completely, a concentration which can vary enormously between different organisms. Since the term "aerobic denitrification" tends to imply denitrification in spite of the presence of oxygen rather than the simultaneous use of oxygen and nitrate, and because it has been the subject of so much dissention, the term "co-respiration" to describe this form of metabolism might be more appropriate. We feel that from a physiological point of view the important question is whether oxygen is saturating the terminal cytochrome oxidase or not. This occurs at concentrations in the order of 1–2% of air saturation (2-4 micromoles). In instances where the level of oxygen is above this threshold, as was the case with many of the experiments described here, then it appears justified to speak of "aerobic denitrification". 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 1% of oxygen). Can aerobic denitrification be rationalized, or even justified, in ecological terms? The obvious conceptual problem is that, because of the lower yield, it seems inefficient for an organism to co-respire oxygen and nitrate. In ecological 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. However, this reasoning depends on the implicit assumption that these species exist in the same niche in which aerobic and anaerobic conditions alternate in a time-span favourable for efficient growth leading to a maximal yield. However, not all niches are the same and growth yield is not the only property important for survival. The specific growth rate under the relevant conditions and the ability of the organism to respond efficiently and rapidly to a changing environment (i.e. the reactivity of the organism (Kuenen and Beudeker, 1982; Leegwater, 1983) are also of great importance. A

number of recent ecophysiological studies have addressed this type of question with regard to substrate specificity and/or alternating mixed substrate utilization (Rittenberg, 1969; Gottschal and Kuenen, 1980; Kuenen and Gottschal, 1982; Kuenen and Robertson, 1984*a*, *b*). Whether a parallel can be drawn remains to be seen. Certainly, a species which did not need to reinduce its denitrifying system after a brief exposure to oxygen, or which could continue to denitrify while taking advantage of the extra energy provided by limiting amounts of oxygen would be able to out-compete other species not possessed of these properties. That is to say, a denitrifier living in a environment where long periods of anaerobic conditions are interspersed by short periods of aerobiosis should not inactivate its denitrifying system too readily since this would result in insufficient or no denitrifying capacity when anaerobic conditions were restored. It is our current view that *Pa. denitrificans* is a typical example of a species which grows efficiently under long term fluctuations of aerobiosis. *Ts. pantotropha* (Robertson and Kuenen, 1983*b*), a species which has much in common with *Pa. denitrificans* but which has a constitutive denitrifying system, would thrive in similar environments when the aerobic periods were short. Experiments are in progress to prove this hypothesis. Indeed, constitutive denitrifiers have been isolated from semi-natural and natural sources and provide indirect evidence to support this contention. *Ts. pantotropha* was itself isolated from a waste-water treatment system receiving very low amounts of oxygen as well as nitrate, and *Thiomicrospira denitrificans* (Timmer-Ten Hoor, 1975), a species only able to tolerate oxygen at levels below current detection thresholds, was found in sulphide-containing mud in which little or no oxygen would occur. Other "aerobic denitrifiers" have also been isolated (e.g. Krul, 1976; Krul and Veeningen, 1977; Meiberg et al., 1980; Robertson and Kuenen, 1983*a*) and their activities may account for some of the nitrogen losses from aerobic treatment plants, and may even explain the occurrence of levels of denitrifying enzymes in well-aerated soils (Tiedje et al., 1982).

Other types of bacteria are being found to use more than one sort of electron acceptor. As mentioned in the introduction, some fermentative species can obtain respiratory energy from the reduction of nitrate to nitrite. At least one of these, a species of *Klebsiella,* has been shown to use oxygen and nitrate simultaneously if grown at dissolved oxygen tensions below 15 mm Hg (Dunn et al., 1979). Another species of *Klebsiella* from the same source as *Ts. pantotropha* also utilized nitrate at dissolved oxygen concentrations up to 80% of air saturation (Robertson and Kuenen, unpubl. results), and it is clear that the phenomenon of co-respiration is not limited to the true denitrifiers. Whether or not the term can be extended to species which use, for example, sulphate instead of oxygen but can reduce nitrate via nitrite to ammonia, such as *Desulfovibrio desulfuricans* (Liu and Peck, 1981; Steenkamp and Peck, 1981) remains to be seen.

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