

## Nitrification and denitrification by *Thiosphaera pantotropha* in aerobic chemostat cultures

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### Abstract

*Thiosphaera pantotropha* has been reported to denitrify aerobically and nitrify heterotrophically. However, recent evidence has indicated that these properties (particularly aerobic denitrification) have been lost. The occurrence and levels of aerobic denitrification and heterotrophic nitrification by *T. pantotropha* in chemostat cultures have therefore been re-evaluated. Only low nitrate reduction rates were observed: the apparent nitrogen loss was of the same order of magnitude as the combined error in the calculated nitrogen consumption. However,  $^{15}\text{N}$  mass spectrometry revealed low aerobic denitrification rates (about 10% of the rates originally published by this group). Heterotrophic nitrification rates were about a third of previous observations.  $\text{N}_2$  and  $\text{N}_2\text{O}$  were both produced from  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$ . Periplasmic nitrate reductase was present in aerobically grown cells.

**Keywords:** Aerobic denitrification; Heterotrophic nitrification; *Thiosphaera pantotropha*; Nitrogen balance; Mass spectrometry

### 1. Introduction

Nitrate, nitrite and ammonium are important pollutants in industrial and municipal waste water. Conventional methods for the biological removal of these compounds involve two discrete steps that are generally carried out in separate bioreactors [1,2]. Ammonium is oxidized to nitrite and/or nitrate with oxygen (nitrification) in an aerobic reactor. In a separate, anoxic reactor organic compounds and/or reduced sulphur compounds are used to reduce nitrate and nitrite to molecular nitrogen. The use of two differ-

ent reactors for denitrification and nitrification stems from the fact that denitrification has generally been assumed to be an anaerobic, oxygen-sensitive process [3–5]. Obviously, if denitrification was possible under aerobic conditions, this could lead to a substantial reduction in the complexity and costs of waste-water treatment plants.

Denitrification under fully aerobic conditions by the facultatively autotrophic bacterium *Thiosphaera pantotropha* was reported some time ago [6,7]. High denitrification rates were found, even in cultures in which the dissolved oxygen concentration was above 80% of air saturation [7]. In these studies, the release of gaseous nitrogen compounds from the cultures was not measured, and the denitrification rate was calculated from the amount of nitrogen that could

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not be recovered as dissolved nitrogen compounds or in biomass. Loss of nitrogen from the cultures was observed, even when ammonium was the only nitrogen compound added [7]. This was attributed to the simultaneous occurrence of two processes, heterotrophic nitrification (leading to the formation of nitrite from ammonium) and aerobic denitrification (which reduces the nitrite to dinitrogen gas).

Since the original observations [6], various research groups have used *T. pantotropha* as a model organism to study aerobic denitrification (e.g. [8–10]). However, in a recent study [11], it was reported that the high aerobic denitrification rates reported by Robertson and coworkers could not be reproduced. Van Niel [12] had earlier reported considerably lower aerobic denitrification rates than those described in the previous reports. Furthermore, recent experiments using  $^{15}\text{N}$ -labelled  $\text{NO}_2^-$  and  $\text{NO}_3^-$  with batch cultures had also indicated that aerobic denitrification rates by the strains of *T. pantotropha* currently available may now be much lower than originally reported [13]. However, other recent reports on experiments with resting cell suspensions [9,10] seem to confirm that *T. pantotropha* remains capable of high aerobic denitrification rates. All of these studies were carried out with cell lines sub-cultured from the original isolate (LMD 82.5, [14]) at different times. Because different cell lines behaved differently, they have been treated as different strains, with separate collection numbers.

This paper describes the reinvestigation of the ability of *T. pantotropha* cultures to carry out aerobic denitrification and heterotrophic nitrification, and to try to resolve the confusion outlined above. To this end, nitrogen balances were made for well-mixed (> 850 rpm) chemostat cultures.  $^{15}\text{N}$ -labelled  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$  were used with mass spectrometry

to directly identify and measure any production of gaseous denitrification products (i.e.  $\text{N}_2$  and  $\text{N}_2\text{O}$ ) under the carefully controlled conditions possible with continuous cultures.

## 2. Materials and methods

### 2.1. Organisms

*Thiosphaera pantotropha* LMD 92.63 (obtained from Dr. Ferguson) was selected from a number of *T. pantotropha* cell-lines, all of which were sub-cultures from the original isolate (LMD 82.5) that had been maintained in different laboratories and on different media. This strain was selected because it showed the strongest aerobic denitrifying activity in preliminary experiments based on chemical nitrogen balances (of 9 strains tested, only 1 showed no activity at all, results not shown). This strain has been used in recent research reporting significant aerobic denitrification rates [8,13]. *T. pantotropha* LMD 89.149 (a strain that has been maintained in Delft) was used in a comparative experiment to establish the similarity (or lack thereof) in behaviour of different cell-lines (Table 1). Stock cultures were maintained at  $-70^\circ\text{C}$  with 30% (v/v) glycerol. Prior to further cultivation, samples from the frozen stock cultures were cultured on TY-agar (Oxoid Tryptone,  $1.6\text{ g l}^{-1}$ ; Difco Yeast Extract,  $3\text{ g l}^{-1}$ ; Difco agar,  $15\text{ g l}^{-1}$ ) plates. It has recently been proposed that *T. pantotropha* should be transferred to *Paracoccus denitrificans* [15]. This transfer appears to be valid according to the current taxonomic rules, but several authors, including ourselves, consider this transfer premature. *T. pantotropha* and *P. denitrificans* differ sufficiently in a number of respects, including their

Table 1

Nitrogen balances for continuous cultures of *T. pantotropha* LMD 92.63 and *T. pantotropha* LMD 89.149 grown at a dilution rate of  $0.14\text{ h}^{-1}$ .  $\text{O}_2$  = % air saturation. All other concentrations as mM N.  $\text{NO}_2^-$  was always  $< 0.10\text{ mM}$ .  $\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-$  represent the difference between the in- and out-flowing concentrations (and may be considered to be 'lost' nitrogen.  $\Delta\text{NH}_4^+$  has been corrected for N-assimilation

Strain (LMD)	$\text{O}_2$	$\text{NH}_4^+$ in	$\text{NH}_4^+$ out	$\Delta\text{NH}_4^+$	$\text{NO}_3^-$ in	$\text{NO}_3^-$ out	$\Delta\text{NO}_3^-$
92.63	85	$14.40 \pm 0.09$	$10.19 \pm 0.30$	0.34	$31.82 \pm 0.84$	$31.81 \pm 0.09$	0.01
92.63	85	$15.40 \pm 0.26$	$10.76 \pm 0.20$	0.86	$30.89 \pm 0.58$	$30.79 \pm 0.54$	0.10
89.149	85	$14.40 \pm 0.11$	$10.19 \pm 0.15$	0.26	$31.14 \pm 0.23$	$30.18 \pm 1.12$	0.96
92.63	0	$12.03 \pm 0.07$	$7.85 \pm 0.21$	0.58	$31.72 \pm 0.86$	$13.32 \pm 0.88$	18.4

physiology and antibody reaction, to have been considered separate species before the development of 16S RNA analysis as a taxonomic tool. Moreover, there are other bacteria with similar 16S RNA analyses, including a phototroph. Therefore, until the situation can be resolved (work currently in progress), the (uninformed) reader would not be served by renaming *T. pantotropha*.

## 2.2. Chemostat cultures

The first series of continuous cultures were grown in laboratory chemostats (Applikon, The Netherlands). The working volume was kept constant at 1.5 l using a peristaltic effluent pump coupled to a level sensor (Applikon, The Netherlands). Using this method, differences between the biomass concentration in samples taken directly from the culture and in samples taken from the culture effluent [16] were smaller than 1% in all cultures. The pH was automatically titrated with 1 M KOH or 1 M H<sub>2</sub>SO<sub>4</sub>. Cultures were sparged with air or nitrogen gas (0.5 l min<sup>-1</sup>), using Brooks mass flow controllers (5850 E, 0–10 l min<sup>-1</sup>), and stirred at 800 rpm. The dissolved-oxygen concentration in the cultures was monitored with a sterilizable oxygen electrode (Ingold, 341003003). The mineral medium [17] contained (g l<sup>-1</sup>): KH<sub>2</sub>PO<sub>4</sub>, 0.65; K<sub>2</sub>HPO<sub>4</sub>, 0.5; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.99; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; trace element solution, 2 ml. After sterilization, sodium acetate (20 mM) and potassium nitrate (32 mM) were added.

The second series of continuous cultures (to be used for the mass spectrometry) were grown in similar equipment, but using the medium originally described by Robertson et al. [7] for the continuous cultivation of *T. pantotropha*, but with lower concentrations of phosphate and Mg<sup>2+</sup> to eliminate any possibility of precipitates forming. This contained (g l<sup>-1</sup>) K<sub>2</sub>HPO<sub>4</sub>, 0.4; KH<sub>2</sub>PO<sub>4</sub>, 0.15; NH<sub>4</sub>Cl, 0.4; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; and 2 ml trace element solution. 20 mM acetate or 5 mM butyrate (see text) were supplied as substrates. When appropriate, KNO<sub>3</sub> and KNO<sub>2</sub> were added to the medium at concentrations shown in the text. The trace element solution [18] contained (g l<sup>-1</sup>) EDTA, 50; ZnSO<sub>4</sub>, 2.2; CaCl<sub>2</sub>, 5.5; MnCl<sub>2</sub>·4H<sub>2</sub>O, 5.06; FeSO<sub>4</sub>·7H<sub>2</sub>O, 5.0; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 1.1; CuSO<sub>4</sub>·5H<sub>2</sub>O, 1.57; CoCl<sub>2</sub>·6H<sub>2</sub>O, 1.61. The working volume for these

experiments was 1.0 l, and the pH was maintained at 8.1 by auto-titration with 1 M NaOH or 0.5 M H<sub>2</sub>SO<sub>4</sub>. The cultures were sparged with mixtures of air and helium to give the desired dissolved oxygen concentrations at sufficiently high stirring speeds (850 rpm) to ensure homogeneity. To minimize the risk of biofilm formation on the walls of the culture vessels, cultures were only run for two-week periods. Before cleaning, the glass was carefully checked for wall growth. This was not observed, except in one case (results not included here) where a fault had left the culture oxygen- rather than substrate-limited. As is usual with substrate-limited cultures of this organism, frequent checks with phase contrast microscopy showed only single cells and pairs. Clumps of a size to permit the formation of anoxic microsites (> 20 μm) were never observed. Similar results were obtained with the two slightly different media described here and with the original (higher phosphate and Mg<sup>2+</sup>) medium. The purity of cultures was routinely checked by plating on TY agar.

## 2.3. Dry weight

The dry weight of cell suspensions was determined by filtering culture samples over pre-weighed nitrocellulose filters (pore diameter 0.45 μm, Schleicher and Schüll, Dassel, Germany). The cells were washed three times with demineralized water and dried to constant weight in a microwave oven (15 min at 180 W output).

## 2.4. Mass spectrometry

Continuous cultures were grown to steady state at the desired dissolved oxygen concentration in medium containing appropriate nitrogen compounds for the experiment (i.e. with NH<sub>4</sub><sup>+</sup> alone, NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup>, or NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>). The mass spectrometer (Prima 600, VG Instruments) was then brought on-line and background readings for the different masses taken. Once the system had stabilised, <sup>15</sup>NH<sub>4</sub><sup>+</sup>, <sup>15</sup>NO<sub>3</sub><sup>-</sup> or <sup>15</sup>NO<sub>2</sub><sup>-</sup> were added (see Table 3 for concentrations), and the changes in the concentrations of components in the off-gas were monitored. Gas flow rates were determined using a wet gas meter (Schlumberger, Germany). Gas production rates were obtained after subtracting the background readings.

Because of either high background levels (e.g.  $^{14,14}\text{N}_2$ ) or overlapping cracking patterns (e.g. mass 30), some mass readings in such an experimental system are less reliable than others. Within the limits of the experimental set-up, masses 29 ( $^{14,15}\text{N}_2$ ), 44 ( $^{14,14}\text{N}_2\text{O}$ ) and 46 ( $^{15,15}\text{N}_2\text{O}$ ) were deemed to be the most reliable. Possible confusion at mass 44 with  $\text{CO}_2$  was prevented by the use of steady state cultures where the  $\text{CO}_2$  concentration would remain constant, and included in the background readings. The other 3 gases ( $^{14,14}\text{N}_2\text{O}$ ,  $^{15,15}\text{N}_2$ ,  $^{14,15}\text{N}_2\text{O}$ ) were calculated on the basis of the 3 observed masses and the formula  $a^2 + 2ab + b^2$ , where  $a$  and  $b$  were the different concentrations of  $^{14}\text{N}$  and  $^{15}\text{N}$  in the media [13,19]. NO was never detected.

### 2.5. Measurement of nitrate and nitrite reduction rates

Culture samples were centrifuged (10 min at  $10\,000 \times g$ ,  $4^\circ\text{C}$ ), washed and re-suspended in mineral medium without acetate. Washed cell suspensions were transferred to a gas-tight, stirred and thermostatically controlled ( $30^\circ\text{C}$ ) reaction chamber equipped with butyl-rubber septa, similar to that described by Gommers et al. [20], except that a magnetic bar was used as a stirrer in place of the floating stirrer. After closing the gas-tight reaction chamber, the cell suspension was sparged with nitrogen gas by means of 2 needles through the septum. After injection of the electron acceptor (3.5 mM potassium nitrate), two samples were taken at  $t = 0$  and  $t = 5$  min to determine the endogenous reduction rate. Subsequently, the electron donor (2 mM sodium acetate) was injected. At regular intervals, samples were taken and analysed for acetate, nitrate and nitrite.

### 2.6. Periplasmic-nitrate-reductase assay

An aerobic, batch-grown cell suspension (50 ml) containing 20 mM nitrate was washed twice and resuspended in a 0.85% NaCl solution. 1 ml suspension was added to a reaction chamber containing 5 ml HEPES (50 mM, pH 8.0), 3.55 ml demineralized water, 0.1 ml Na-EDTA (10 mM), 0.2 ml  $\text{KNO}_3$  (1 M) and 0.1 ml methyl viologen (400 mM). The suspension was continually sparged with argon. The

reaction was started by adding 30  $\mu\text{l}$  dithionite (500 mM in 0.5 M  $\text{NaHCO}_3$ ). Nitrate reduction was measured with a nitrate-electrode (Radiometer, Denmark).

### 2.7. Analytical procedures

Acetate was determined by HPLC using an HPX-87H column ( $300 \times 7.8$  mm, Bio-Rad, USA), enzymatically, using a commercial test kit (Boehringer: cat.no. 148261), or from Total Organic Carbon (TOC) determinations using a Beckman TOCA Master 915-B.

Nitrite was measured colorimetrically as described by Griess-Romijn van Eck [21]. Nitrate was determined by HPLC using a Nucleosil 10 anion column (Chrompack) and colorimetrically, using salicylic acid [22]. Ammonia and hydroxylamine were determined colorimetrically as described by Fawcett and Scott [23] and Frear and Burrell [24], respectively. For calculation of nitrogen present in biomass, an elemental composition of *T. pantotropha* biomass of  $\text{CH}_{1.81}\text{O}_{0.54}\text{N}_{0.25}$  was used [25]. All statistics shown are standard deviations of at least 2 sets of triplicate measurements taken on different days.

## 3. Results

### 3.1. Aerobic denitrification and heterotrophic nitrification in substrate-limited chemostat cultures

Table 1 shows the fate of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  in acetate-limited continuous cultures of *T. pantotropha* LMD 92.63. As well as duplicate experiments done at dissolved oxygen concentrations of 85% air saturation, a replicate experiment with *T. pantotropha* LMD 89.149 was included for comparison. Little or no  $\text{NO}_3^-$  was lost from the aerobic cultures of *T. pantotropha* LMD 92.63. The *T. pantotropha* LMD 89.149 culture appeared to lose more  $\text{NO}_3^-$ . However, the apparent  $\text{NO}_3^-$  losses were all smaller than the standard deviations of the measurements (Table 1).  $\text{NH}_4^+$  losses from the 3 cultures were different, but were also all close to the standard deviations of the measurements. In earlier experiments with similar chemostat cultures of *T. pantotropha* LMD 82.5,

Table 2

Chemical nitrogen balances for *Thiosphaera pantotropha* LMD 92.63 cultures growing in substrate-limited continuous cultures where  $\text{NH}_4^+$  was the sole nitrogen compound provided. Dilution rate =  $0.06 \text{ h}^{-1}$ , stirring speed 850 rpm.  $\text{O}_2$  = % air saturation. All values as mM N.  $\Delta\text{NH}_4^+$  represents the difference between the in- and out-flowing medium, corrected for biomass assimilation and  $\text{NO}_2^-$  accumulation ( $\text{NH}_4^+$  'lost')

$\text{O}_2$	$\text{NH}_4^+$ in	$\text{NH}_4^+$ out	$\text{NO}_2^-$ out	$\Delta\text{NH}_4^+$	Nitrification rate $\text{nmol min}^{-1} \text{ mg dry wt}^{-1}$
Substrate = acetate					
50	8.28( $\pm 0.14$ )	5.10( $\pm 0.28$ )	0	1.46	8
25	8.28( $\pm 0.14$ )	4.94( $\pm 0.12$ )	0	1.62	8.9
1	8.40( $\pm 0.05$ )	2.74( $\pm 0.54$ )	0.01	4.18	27.0
Substrate = butyrate					
> 90	8.32( $\pm 0.08$ )	4.83( $\pm 0.54$ )	0	-0.13	0
19	8.32( $\pm 0.08$ )	4.10( $\pm 0.49$ )	0.03	0.23	0.6
5	8.32( $\pm 0.08$ )	4.33( $\pm 0.17$ )	0.02	0.10	0.2

losses of 16.6 mM  $\text{NO}_3^-$  and 1 mM  $\text{NH}_4^+$  had been reported [7].

The fate of  $\text{NH}_4^+$  as the sole nitrogen source in substrate-limited continuous cultures of *T. pantotropha* LMD 92.63 can be seen in Table 2. It can be seen that a significant amount of  $\text{NH}_4^+$  (in excess of the experimental errors) was 'lost' from the acetate-based cultures, and that this increased dramatically as the dissolved oxygen approached limiting

concentrations. However, it was only at the lowest dissolved oxygen concentration that the nitrification/denitrification rate approached previously found levels (at a similar dilution rate and 80% air saturation,  $28 \text{ nmol min}^{-1} \text{ mg biomass}^{-1}$ ). As it had been reported that butyrate was a more effective substrate for aerobic denitrification by *Paracoccus denitrificans* than acetate [26], some cultures were run with 5 mM butyrate rather than 20 mM acetate. There was

Table 3

$\text{N}_2\text{O}$  and  $\text{N}_2$  production by *Thiosphaera pantotropha* LMD 92.63 cultures growing in substrate-limited chemostat cultures at different dissolved oxygen concentrations after the addition of a  $^{15}\text{N}$ -labelled compound. Stirring speed between 850 and 980 rpm. <sup>a</sup> = 5 mM  $^{14}\text{NO}_2^-$  was also added to bring the final concentration to 10 mM. The error in the measurements was around or below  $\pm 10\%$

	Dilution rate ( $\text{h}^{-1}$ )	Dissolved $\text{O}_2$ (% air)	$^{15}\text{N}$ added (mM)	$\text{N}_2$	$\text{N}_2\text{O}$	Total	% Gas as $\text{N}_2$
				$\text{nmol N min}^{-1} \text{ (mg dry wt)}^{-1}$			
<b>A: <math>\text{NH}_4^+</math> as sole Nitrogen compound in growth medium</b>							
Acetate as substrate							
1	0.06	50	$\text{NH}_4^+$ (5)	0.6	4.4	5.0	12
2	0.06	25	$\text{NH}_4^+$ (5)	1.2	3.9	5.1	28
3	0.07	47	$\text{NH}_4^+$ (7.5)	1.0	12.7	13.7	7
4	0.07	< 1	$\text{NH}_4^+$ (7.5)	2.1	19.3	21.4	10
5	0.07	85	$\text{NO}_2^-$ (10)	0.1	3.8	3.9	3
6	0.06	25	$\text{NO}_2^-$ (5 <sup>a</sup> )	0.9	3.1	4.0	23
Acetate and thiosulphate as substrates							
7	0.07	34	$\text{NH}_4^+$ (7.5)	0.5	8.1	8.6	6
<b>B: <math>\text{NH}_4^+</math> and <math>\text{NO}_3^-</math> both present in growth medium</b>							
Acetate as substrate							
8	0.07	95	$\text{NO}_3^-$ (10)	0	4.4	4.4	0
9	0.07	25	$\text{NO}_3^-$ (10)	0.4	9.1	9.5	4
10	0.07	20	$\text{NO}_3^-$ (10)	0.7	13.2	13.9	5
11	0.1	85	$\text{NH}_4^+$ (7.5)	0.05	2.2	2.25	2
12	0.1	55	$\text{NO}_2^-$ (10)	0.7	11.1	11.8	6

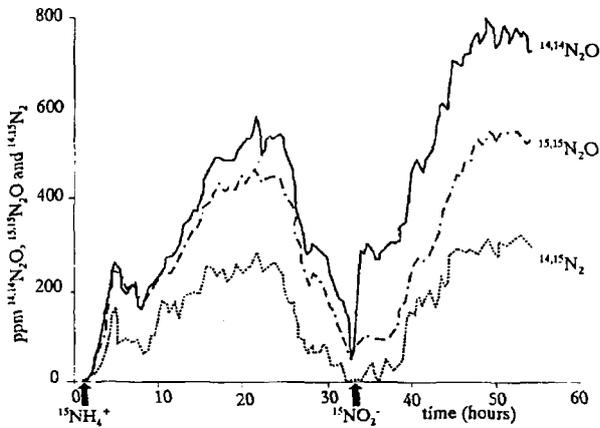


Fig. 1.  $N_2$  and  $N_2O$  production by a steady-state, acetate-limited, continuous culture of *Thiosphaera pantotropha* LMD 92.63 after pulse addition of 5 mM  $^{15}NH_4^+$ , and subsequently 10 mM  $NO_2^-$  (5 mM  $^{14}NO_2^-$  and 5 mM  $^{15}NO_2^-$ ). Dissolved  $O_2$  = 25% air saturation;  $D = 0.06 h^{-1}$ ; stirrer = 800 rpm.

little or no  $NH_4^+$  lost from the butyrate cultures, even with a low (5% air saturation) dissolved oxygen concentration.

In the experiments discussed above, and in previous work on aerobic denitrification by growing cultures of *T. pantotropha* [6,7,11], aerobic denitrification rates have always been calculated from the 'gap' in nitrogen balances, including only soluble nitrogen compounds and biomass. As illustrated by Tables 1 and 2, some nitrogen appeared to be lost from most

of the cultures. However, in many of the cultures, the combined error in the calculated nitrogen consumption was of the same order of magnitude as the apparent nitrogen losses (Tables 1 and 2). Recent experiments [13] using batch cultures of several heterotrophic nitrifiers (including *T. pantotropha* LMD 92.63) indicated that  $^{15}N$  mass spectrometry should also be sensitive enough to detect nitrogen losses from *T. pantotropha* continuous cultures. Therefore, the possibility that low rates of heterotrophic nitrification and aerobic denitrification might have been obscured by these analytical errors was investigated using mass spectrometric analysis of off-gases from continuous cultures supplemented with  $^{15}N$ -labelled compounds.

### 3.2. Direct measurement of gaseous N-compounds by mass spectrometry

After the measurements shown in Table 2 were completed, these cultures were used for the mass spectrometry experiments. Additional short-term experiments were carried out immediately after a steady state had been reached. With the exception of the cultures grown at 95% air saturation,  $N_2$  and  $N_2O$  were produced by all of the cultures, regardless of whether  $^{15}NH_4^+$  or  $^{15}NO_2^-$  was added (Table 3). Fig. 1 shows the results obtained with the culture grown

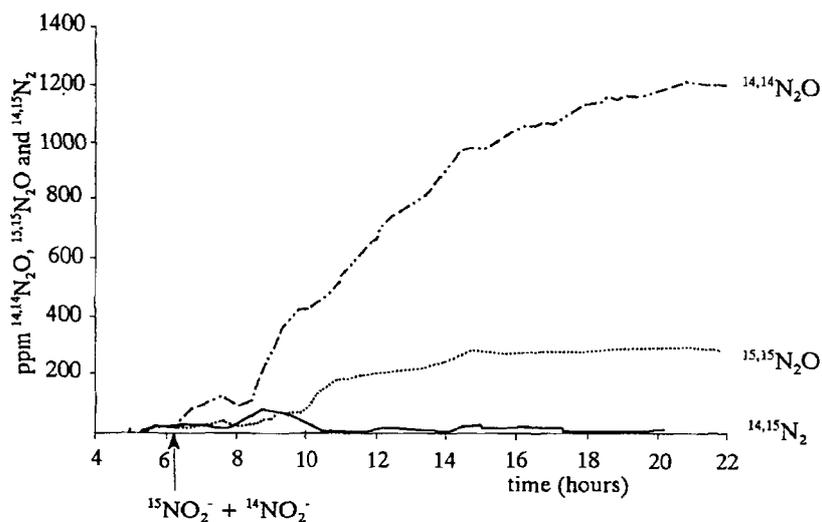


Fig. 2.  $N_2O$  production by a steady-state, butyrate-limited continuous culture of *Thiosphaera pantotropha* LMD 92.63 after a pulse addition of 10 mM  $NO_2^-$  (5 mM  $^{14}NO_2^-$  and 5 mM  $^{15}NO_2^-$ ). Dissolved  $O_2$  = > 95% air saturation;  $D = 0.06 h^{-1}$ ; stirrer = 800 rpm.

on acetate at 25% air saturation. The eventual fall in labelled gases was, of course, due to the  $^{15}\text{NH}_4^+$  being diluted and finally washed out by in-flowing medium. The cultures grown on butyrate and acetate at 95% air saturation did not produce  $\text{N}_2$ , only  $\text{N}_2\text{O}$  (Fig. 2 and Table 3, (line 8), respectively). The culture grown on butyrate at an oxygen concentration of 95% air saturation (Fig. 2) is a good illustration of how experimental error in chemical determinations can cause falsely negative (or positive) results, as mentioned above. The level of  $\text{N}_2\text{O}$  production calculated from the mass spectrometry data ( $0.9 \text{ nmol N min}^{-1} \text{ mg dry weight}^{-1}$ ) is equivalent to a loss of  $0.75 \text{ mM NH}_4^+$  from the culture. Such a loss easily 'disappears' within the standard deviation in the  $\text{NH}_4^+$  and biomass determinations (Table 2).

As can be seen from Table 3, gas production was affected by the dissolved oxygen, the growth rate, and  $\text{NH}_4^+$  concentration. The highest gas production rates, all other factors being equal, were found at the lower dissolved oxygen concentrations (e.g. compare lines 3 and 4). Similarly, the percentage of  $\text{N}_2$  in the gas mixture was inversely related to the dissolved oxygen concentration, decreasing as the oxygen level rose. However, the change in this ratio was due more to changes in the  $\text{N}_2\text{O}$  production rate than the rate of  $\text{N}_2$  production, perhaps suggesting that  $\text{N}_2\text{O}$  reductase was only minimally active. This effect was most pronounced in the cultures grown with  $\text{NH}_4^+$  as the sole N-compound (group A), but is still apparent in those grown with  $\text{NO}_3^-$  also present (compare lines 8, 9 and 10). A higher  $\text{NH}_4^+$  pulse gave higher gas production rates (compare lines 1 and 3). This is probably because the ammonia monooxygenase in this organism appears to use  $\text{NH}_3$  rather than  $\text{NH}_4^+$ , and would respond to the increased amount of available  $\text{NH}_3$  [27]. Similarly, the increase in  $^{14,14}\text{N}_2$  and  $^{14,14}\text{N}_2\text{O}$  production after the addition of  $^{15}\text{N}$ -labelled compounds can be attributed to the increase in the overall concentration of the N-compound concerned, stimulating activity.

Growth of cultures on thiosulphate has previously been shown to reduce heterotrophic nitrification [28]. The effect of thiosulphate on gas production by these cultures was therefore tested in order to confirm that the labelled  $\text{N}_2$  and  $\text{N}_2\text{O}$  were biologically rather than chemically produced. Adding  $10 \text{ mM}$  thiosulphate to a culture grown on acetate and actively

producing labelled gas did not inhibit gas production, indicating that thiosulphate did not act as a chemical inhibitor. However, when a culture was changed to medium supplemented with  $10 \text{ mM}$  thiosulphate, and a new steady state allowed to develop, the total production of labelled gas was half that of the original culture (Table 3, lines 3 and 7), even though the dissolved oxygen had fallen because of the increase in the oxygen demand of the culture.

*T. pantotropha* had previously been shown to preferentially nitrify  $\text{NH}_2\text{OH}$  when provided with a mixture of  $\text{NH}_4^+$  and  $\text{NH}_2\text{OH}$ . This reaction appeared to be a suitable additional confirmation that the labelled gases were biologically rather than chemically produced. If the reaction had been chemical, as the addition of  $^{15}\text{NH}_2\text{OH}$  to a culture growing on  $^{14}\text{NH}_4^+$  should primarily result in gases with mixed labels. However, the addition of  $5 \text{ mM } ^{15}\text{NH}_2\text{OH}$  to a culture grown to steady-state at a dissolved oxygen concentration of 60% air saturation and with  $\text{NH}_4^+$  as the sole N-source resulted in the immediate production of  $^{15,15}\text{N}_2$  (approximately  $1.7 \text{ nmol N min}^{-1} \text{ mg dry weight}^{-1}$ ) and  $^{15,15}\text{N}_2\text{O}$  ( $2.25 \text{ nmol min}^{-1} \text{ mg dry weight}^{-1}$ ). Mixed label gases were not produced. After about an hour, not only did the production of  $^{15,15}\text{N}_2$  and  $^{15,15}\text{N}_2\text{O}$  fall off, but the 44 signal also began to decrease sharply. As it fell well below the background levels, this was assumed to be a decrease in  $\text{CO}_2$  production rather than  $^{14,14}\text{N}_2\text{O}$ . The dissolved oxygen concentration also increased. When  $10 \text{ mM } ^{15}\text{NO}_2^-$  was then added, gas production fell even more sharply, rather than increasing, and the  $\text{O}_2$  concentration in the off-gas began to increase, indicating both that the culture was dying, and that the possibility of chemical reactions could be discounted.

### 3.3. Expression of denitrifying capacity in aerobic cultures

In order to examine the denitrifying capacity of aerobically grown cells under the sudden onset of anaerobiosis, culture samples from the aerobic, nitrate-containing chemostat cultures were sparged with  $\text{N}_2$  (see Materials and methods). Nitrate disappeared immediately after the addition of acetate (Fig. 3), confirming previous observations that denitrifying enzymes were expressed constitutively. The initial

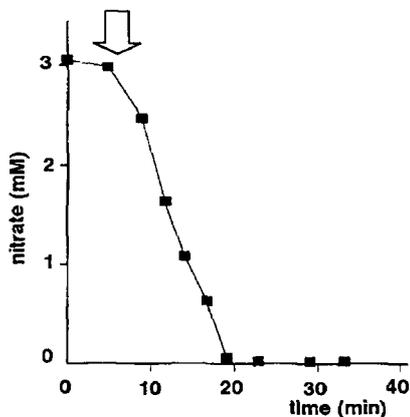


Fig. 3. Denitrification by *T. pantotropha* under anaerobic conditions (pH 8.2,  $T = 37^{\circ}\text{C}$ ). Cells were pre-grown in an aerobic acetate-limited continuous culture (pH 8.2,  $T = 37^{\circ}\text{C}$ ). The arrow indicates addition of 5 mM acetate.

nitrate reduction rate was  $0.6 \mu\text{mol NO}_3^- \text{min}^{-1} (\text{mg dry weight})^{-1}$ , which is comparable to previously measured rates (H. Nanninga, L.A. Robertson and J.G. Kuenen, unpublished data). In anaerobic, steady-state chemostat cultures, denitrification rates were similar to those previously observed (Table 1).

The ability of *T. pantotropha* to denitrify aerobically has been correlated with the presence of a periplasmic, oxygen-insensitive nitrate reductase [9]. Anaerobic denitrification was attributed to the activity of a membrane-bound nitrate reductase on the cytoplasmic side of the membrane, similar to that reported for *Pa. denitrificans*. To investigate whether the low rates of aerobic denitrification observed during the present study were due to the absence or low activity of this enzyme, its activity was assayed in aerobically grown cells. High activities were found ( $0.1 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ ). This value is in the same order of magnitude as reported by Bell et al. [9]. Nitrate reduction by aerobically grown cells was not sensitive to azide (10 mM), confirming the presence of the periplasmic enzyme.

#### 4. Discussion

The experimental results presented in this paper do not confirm the very high rates of aerobic denitrification reported previously for chemostat cultures of

*T. pantotropha* [7]. The results of the experiments based on the chemical analysis of dissolved nitrogen compounds and biomass when nitrate was included in the medium confirm those of Thomsen et al. [11]. With the more sensitive  $^{15}\text{N}$ -mass spectrometry, however, a low level of aerobic denitrification was measured at all dissolved oxygen concentrations. The denitrification rates based on mass spectrometry of off-gases from cultures grown at 50% air saturation and given  $^{15}\text{NO}_2^-$  (Fig. 1, Table 3) were only about 5% of the rate to be expected from an equivalent anaerobic culture (equivalent to 40 and  $760 \mu\text{mol h}^{-1} \text{l}^{-1}$ , respectively). The original denitrification rates for similar cultures growing on  $\text{NO}_3^-$  were about 55% of the anaerobic rate. The heterotrophic nitrification (and concomitant aerobic denitrification) rates revealed by both the chemical analyses (Table 1) and the mass spectrometry (Figs. 1 and 2, Tables 2 and 3) were more significant. However, these rates were still only about 30% of the rates originally reported for similar cultures (8 and  $28 \text{ nmol NH}_4^+ \text{oxidized min}^{-1} \text{mg biomass}^{-1}$ , respectively). The cultures were clearly responding to the dissolved oxygen, both the fall-off of  $\text{N}_2$  and  $\text{N}_2\text{O}$  production as the oxygen level increased (Table 3), and in the increase in heterotrophic nitrification as the dissolved oxygen concentration approached limiting levels (Table 2). Indeed, one of the similarities between the behaviour of these and the earlier cultures is the fact that the continuous cultures grown with  $\text{NH}_4^+$  as the sole source of N gave lower biomass yields (Table 2) than those supplied with both  $\text{NH}_4^+$  and  $\text{NO}_3^-$  (Table 3).

A comparison of the results from the chemical balances and the mass spectrometry clearly shows that a careful statistical analysis of experimental data is required before conclusions on the magnitude of the 'gap' in the nitrogen balances can be drawn. Even then, propagation of experimental errors may easily obscure (or enhance) very low biological activities. Both the denitrification of  $\text{NO}_2^-$  at 50% air saturation (equivalent to  $0.7 \text{ mM NO}_2^-$ ) and the nitrification/denitrification of  $\text{NH}_4^+$  at 95% air saturation (equivalent to  $0.75 \text{ mM NH}_4^+$ ) readily disappear into the cumulative error of the chemical determinations (Tables 1 and 2). However, given the fact that the errors are absolute, rather than relative, it is extremely unlikely that this explains the previously

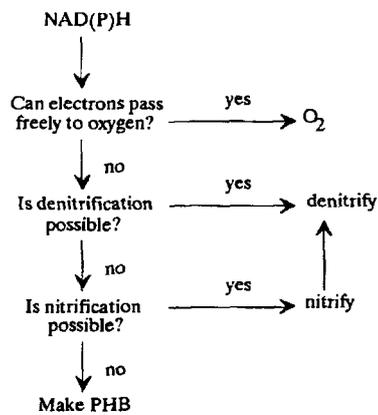


Fig. 4. A flow chart to show, diagrammatically, the working hypothesis to explain the control of heterotrophic nitrification and aerobic denitrification in *T. pantotropha* (originally published in [7]).

described disappearance of up to 17 mM of nitrate in aerobic chemostat cultures [7].

A possible explanation for the change in behaviour of *T. pantotropha* cultures is that physiological changes have occurred since its isolation nearly 15 years ago. It was postulated that the high rates of aerobic denitrification observed with *T. pantotropha* were due to a rate-limiting step in electron transport to  $O_2$ , as outlined in Fig. 4 [7]. Parallel respiration of  $O_2$  and denitrification would permit faster regeneration of  $NAD^+$ . Any adaptation that relieved this rate-limiting step would diminish the degree of reduction of the cytochrome chain, removing the need for high aerobic denitrification rates and permitting higher growth rates. Indeed, the growth rates of batch cultures of currently available strains [12,29] were not stimulated by the presence of  $NO_3^-$  (about  $0.45\ h^{-1}$ ), but were also much higher than the rates previously reported ( $0.28\ h^{-1}$  with only  $NH_4^+$  present,  $0.34$  with both  $NH_4^+$  and  $NO_3^-$  [6]). Moreover, currently available *T. pantotropha* cultures do not produce the mesosome-like structures and large amounts of poly- $\beta$  hydroxybutyrate previously observed in cultures grown in the presence of hydroxylamine, or under  $O_2$ -limitation [30]. Different degrees of adaptation would give different efficiencies for electron transport to  $O_2$ , and thus varying levels of heterotrophic nitrification and/or aerobic denitrification. Further support for this hypothesis has been gained from experiments using *T. pan-*

*totropha* cultures with deliberate mutations blocking electron flow to oxygen via cytochrome  $aa_3$  (M. Hazelaar, T. de Boer, L.A. Robertson and J.G. Kuenen, unpublished data). Similar adaptive changes in other bacteria (e.g. loss of pathogenicity after prolonged culture on synthetic media) are well-known. Indeed, genetic instability of *T. pantotropha* was proposed to be the reason for the low aerobic denitrification rates observed by van Niel [12]. Aerobic denitrification was routinely measured in this and other laboratories over a number of years immediately following the original isolation of *T. pantotropha* (see, for example, [6–10]). This makes it extremely unlikely that the results of the earlier studies were based on the use of a variant strain [11].

It has also been suggested [11] that the reported high rates of aerobic denitrification [7] might have been due to the formation of biofilms, which could easily lead to oxygen-limited micro-environments. *T. pantotropha* does readily form biofilms in acetate-limited cultures at high growth rates [31,32] and under nitrogen limitation. However, significant biofilm formation was not observed during the earlier work on aerobic denitrification (which had been carried out at a range of growth rates well below the observed values for  $\mu_{max}$ ). Indeed, if the high aerobic denitrification rates had been due to anoxic micro-sites, over 50% of the biomass present in the chemostat cultures should have been inaccessible to oxygen. Thus, the presence or absence of biofilm formation cannot explain discrepancy between the older and current results. As the cultures described here were grown at relatively low growth rates, maintained for only one or two weeks, substrate-limited, and carefully inspected for biofilm growth, the possibility that the low rates of aerobic denitrification observed during the mass-spectrometry experiments were due to the occurrence of anaerobic micro-environments can be excluded.

Thus far, it has not been possible to identify the reasons for the changes in the behaviour of *T. pantotropha*. It is possible that it is due to an, as yet, unidentified change in the growth conditions. However, cultures have been grown in a number of different types of vessel (including chemostats of different shapes), but the Kluver flasks used for batch cultures are the same. Other possibilities, including the sources of demineralized water and

chemicals, the metal and type of tubing used for the continuous cultures, and the stirrer speed have all been checked, but these experiments have so far not identified a cause for the discrepancy. It seems possible that a physiological change, as discussed above, has occurred. We therefore advise others working with *T. pantotropha* to periodically check its actual aerobic denitrifying and heterotrophic nitrifying activities if they are critical factors in their studies.

Finally, the low rates of aerobic denitrification now observed with the 'model organism' *T. pantotropha* in axenic, well-stirred cultures by no means imply that aerobic denitrification is only a marginal ecological phenomenon: high aerobic denitrification rates, assayed both by chemical nitrogen determinations and mass spectrometry have been demonstrated in a variety of Gram-negative bacteria [33–41]. Even at its current low level of activity, *T. pantotropha* has retained the constitutive nature of its denitrifying system (Fig. 3), a property that may give it a considerable advantage in some ecological niches. Furthermore, the phenomenon of combined heterotrophic nitrification with aerobic denitrification has clearly been confirmed as a principle process in the conversion of ammonia in one single organism.

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