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Competition between heterotrophic and autotrophic nitrifiers for ammonia in chemostat cultures

E.W.J. van Niel, P.A.M. Arts, B.J. Wesselink, L.A. Robertson and J.G. Kuenen

Kluyver Laboratory for Biotechnology, Delft University of Technology, Delft, The Netherlands

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1. SUMMARY

Mixed cultures of a heterotrophic nitrifier/aerobic denitrifier, *Thiosphaera pantotropha*, and an autotrophic nitrifier, *Nitrosomonas europaea*, were grown in chemostats under dual ammonia- and acetate limitation. Because of simultaneous nitrification and denitrification by *T. pantotropha*, the activity of the cultures was evaluated from nitrogen balances as complete as possible. Under most conditions studied, no interaction took place between the two bacteria. Only above a critical *C/N* ratio of 10.4, *T. pantotropha* was able to outcompete *N. europaea* for ammonia (dilution rate = 0.04 h^{-1}). At dissolved oxygen concentrations below 10 μM, the autotroph became oxygen-limited and the heterotroph dominated in the culture. Moreover, when the dilution rate was increased to 0.065 h^{-1}, *N. europaea* could not maintain itself successfully in the chemostat, even when the *C/N* ratio was as low as 2.2. Nitrification by *T. pantotropha* was equivalent to that of *N. europaea* when the cell ratio of heterotrophs/autotrophs was 250. The relevance of these observations to the nitrogen cycle in natural environments is discussed.

2. INTRODUCTION

Ammonia can be oxidized to nitrite by two types of bacteria. The best known are the autotrophic nitrifiers, of which *Nitrosomonas europaea* is the most extensively studied. The other type consists of a group of heterotrophic species (for an extensive list see [1] and [2]), including many denitrifiers [3,4].

There are many differences between autotrophic and heterotrophic nitrifiers. Ammonia is the sole energy source for growth for the autotrophic ammonia oxidizers. All studies done thus far on heterotrophic nitrification indicate that these organisms do not gain energy from the ammonia oxidation (e.g., Refs. 5–7). It has now

Correspondence to: E.W.J. van Niel, Department of Microbiology, Agricultural University, H. van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands.

been recognised that heterotrophic nitrification rates cannot be evaluated from nitrite or nitrate accumulation measurements, as simultaneous denitrification may take place [4,8,9]. Published rates for heterotrophic nitrification (generally 10^4–10^5 times lower than autotrophic nitrification rates) are therefore probably often underestimates. Calculation of heterotrophic nitrification rates based on ammonia disappearance has revealed that the rates are actually only about 10^2–10^3 times lower per unit biomass [8]. Thus, in some situations where heterotrophs greatly outnumber autotrophs (e.g., in sludge or soil [10]), the total nitrification rates due to the two types of nitrifiers may be comparable. It has been proposed that heterotrophic rather than autotrophic nitrification could play a significant role under certain conditions, such as low or high pH [2] and at low oxygen concentrations [11]. Attempts to estimate the numbers of autotrophic nitrifiers present in samples have generally given lower figures than expected. Several reasons for this discrepancy have been suggested, including anomalies in the Most Probable Number (MPN) or other counting methods, or the occurrence of microbial interactions [2]. Another possibility is that the original nitrification figures were partially based on nitrite production by heterotrophs. Castignetti and Gunner [12] showed that sequential nitrification of ammonia to nitrate by a coculture containing a heterotrophic nitrifier isolated from soil and *Nitrobacter agilis* was possible.

This paper reports on the coexistence of a heterotrophic nitrifier/aerobic denitrifier, *Thiosphaera pantotropha*, and an autotrophic nitrifier, *Nitrosomonas europaea* in continuous culture under different environmental conditions, including changes in the dissolved oxygen and the C/N ratio. A comparison of the nitrification rates due to the two types of nitrifier will also be made.

3. MATERIALS AND METHODS

3.1. Organisms

Thiosphaera pantotropha LMD 82.5 was originally isolated from a denitrifying, sulphide-oxidizing waste water treatment system [13]. A culture of *Nitrosomonas europaea* was kindly provided by Dr. J.I. Prosser, University of Aberdeen, UK. *N. europaea* was routinely maintained in batch cultures of the inorganic medium described for the chemostat. Phenol red (0.05 mg l^{-1}) was included as a pH indicator. Sodium carbonate (9% w/v) was used to adjust the pH to 8.0. The cultures were incubated at 30°C. Contamination by heterotrophs was monitored by plating onto tryptone and yeast extract agar with subsequent incubation at 30°C for 2–3 weeks.

3.2. Continuous cultures

Continuous cultures were grown in chemostats fitted with dissolved oxygen and pH control. The temperature was maintained at 30°C and the pH at 8.0 (titration with 7% Na_2CO_3 solution). The medium supplied to the chemostats contained (g l^{-1}): K_2HPO_4, 0.4; KH_2PO_4, 0.15; $(NH_4)_2SO_4$, 0.66; $MgSO_4 \cdot 7H_2O$, 0.4; and 2 ml of trace element solution [14]. Acetate was supplied in concentrations of 10, 12.5, 15, 17.5, 20, 29 and 35 mM, as indicated in the text. With the latter two acetate concentrations, 0.45 g l^{-1} $(NH_4)_2SO_4$ (6.8 mM ammonia) was used. The chemostats were wrapped in black paper to exclude light.

Neither organism excreted organic compounds in axenic cultures under these growth conditions as was confirmed by TOC analysis (detection level: 5 ppm carbon).

3.3. Oxygen uptake experiments

Oxygen uptake by whole cells was measured polarographically with a Clark-type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instruments, OH, USA) mounted in a thermostatically-controlled (30°C) vessel which is closed except for a small hole through which additions may be made. The system was calibrated using washed suspensions of pure cultures from the chemostat harvested during an appropriate steady-state. The cells were suspended in 50 mM potassium phosphate buffer at pH 8. The reaction was started by addition of acetate or ammonia. The acetate- and ammonia-dependent oxygen uptake rate was corrected for the endogenous respiration. The affinity constant for ammo-

nia of *N. europaea* was determined by measuring the rate of oxygen uptake at various concentrations of ammonia in washed cell suspensions of this organism. The K_s for oxygen was determined in the presence of excess ammonia.

3.4. Determination of the biomass and bacterial numbers

The biomass concentration of the pure cultures was calculated from the total organic carbon determination and the CHON analysis of washed cells. For *T. pantotropha* and *N. europaea*, CHON ratios of $CH_{1.81}O_{0.54}N_{0.25}$ and $CH_{1.82}O_{0.39}N_{0.21}$ were found, respectively. The cells were counted in a counting chamber by phase contrast microscopy. The size of the cells of the two species were determined from electron micrographs. The cell size and shape of the two species remained constant throughout the experiments. The cell sizes of *N. europaea* and *T. pantotropha* were 1.0 by 0.56 μm and 0.9 by 0.7 μm, respectively. The number of cells per mg biomass of *N. europaea* and *T. pantotropha* were about 0.99×10^{10} cells and 1.46×10^{10} cells, respectively. The yield of *N. europaea* on ammonia in chemostat cultures at a dilution rate of 0.04 h^{-1} was $1.1 \pm 0.14 \times 10^{10}$ cells mmol NH_3^{-1} at all air saturation levels above 5–10%. Below this value, the yield decreased.

Calibration curves were prepared of cell numbers versus maximum oxygen uptake rates with either acetate or ammonia as measured with the Biological Oxygen Monitor (BOM). Defined cell populations containing different, known ratios of *T. pantotropha* and *N. europaea* were used to confirm the validity of these curves (standard deviation was ca.5%). Acetate- and ammonia-dependent oxygen uptake rates for washed cells from the mixed cultures were then used to determine the numbers of heterotrophs and autotrophs, respectively. Cell numbers were calculated as the means of 6 to 12 determinations, using samples taken from the chemostat over a period of 3–6 volume changes during steady state. Further confirmation of the correlation between oxygen uptake and cell numbers was obtained using immuno-fluorescent staining, which was carried out using antibodies raised against *T. pantotropha* and *N. europaea* by the method described by Muyzer et al. [15].

3.5. Analytical techniques

Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer), or as total organic carbon with a TOCA master 915-B analyser (sensitivity level at 5 ppm carbon). Nitrite was determined using the Griess-Romijn reagent [16]. Hydroxylamine was determined colorimetrically by means of the method described by Frear and Burrell [17]. Ammonia was determined colourimetrically by means of the method described by Fawcett and Scott [18]. Protein was measured spectrophotometrically, by means of the micro-biuret method [19]. The total organic carbon of washed cells was determined with a TOCA master 915-B analyser. For the qualitative

Table 1

Comparison between the population composition of a defined mixed culture determined using oxygen uptake rates in the biological oxygen monitor and fluorescent antibodies

O_2 μM	C/N mol mol^{-1}	Biological oxygen monitor		Fluorescent antibodies	
		T. pantotropha (%)	*N. europaea* (%)	*T. pantotropha* (%)	*N. europaea* (%)
118	2.8	97.1 ± 0.4	2.9 ± 0.4	96.9 ± 0.9	3.0 ± 0.7 (8)
118	3.7	98.0 ± 0.4	2.0 ± 0.4	97.9 ± 0.6	2.1 ± 0.6 (4)
10	1.9	96.8 ± 0.9	3.2 ± 0.9	96.4 ± 1.3	3.6 ± 1.3 (8)

The number of total count preparations is given in parentheses. The cultures had been grown in the chemostat with a dilution rate of 0.04 h^{-1} and varying oxygen concentrations and/or different C/N ratios. The medium contained growth-limiting acetate- and ammonia concentrations. Acetate served as the carbon and energy source for *T. pantotropha* and ammonia as the energy source for *N. europaea*. Ammonia also served as the nitrogen source for both organisms.

determination of nitrous oxide the Clark-type oxygen electrode was used under anaerobic conditions [20].

Ammonia and ammonium will both be present at the pH values used in these experiments. For the sake of convenience, the term 'ammonia' will be used throughout to indicate both the protonated and unprotonated forms. Because of the very low ammonia concentrations in the culture, ammonia loss from stripping can be discounted.

The C/N ratio is expressed as mol carbon (of acetate) per mol nitrogen (of ammonia) in the influent.

4. RESULTS

4.1. Confirmation of the population estimates by means of fluorescent antibodies (FA)

Table 1 shows the results of determining the numbers of *N. europaea* with fluorescent antibodies and from its oxygen uptake rates obtained with samples from mixed cultures grown at steady state under three different sets of conditions. In all three cases, there was good agreement between the two counting methods. The greatest error was found in the FA counting method because the cells were not homogeneously distributed on the glass surface.

4.2. Growth characteristics of T. pantotropha and N. europaea in mixed chemostat culture

The bacteria were cultivated separately in continuous culture at a dilution rate of 0.04 h^{-1}. *T. pantotropha* was grown with acetate as the sole limiting substrate. *N. europaea* was grown under ammonia limitation. Wall growth was insignificant during almost all experiments (see below). When both cultures had reached steady state, *T. pantotropha* was added to the *N. europaea* culture. This procedure was used for most experiments because of the large volume of the *N. europaea* culture (which had a much lower cell density) that would have been necessary to add to the *T. pantotropha* culture in order to achieve the same cell ratio. In addition, autotrophic cultures were slower to develop and stabilize. However, for verification, one mixed culture was started by

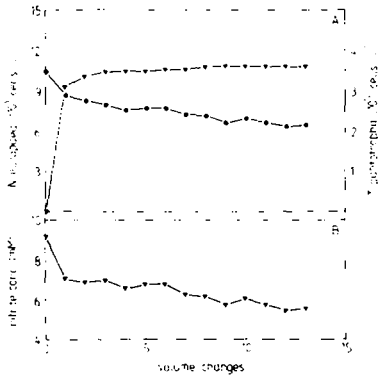

Fig. 1. Development of a co-culture at a dilution rate of 0.04 h^{-1}; a dissolved oxygen concentration of 118 μM and a C/N ratio of 4. At the start a steady-state culture of *N. europaea* was inoculated with *T. pantotropha*. (A) ● = *N. europaea* (10^{10} cells l^{-1}); ▼ = *T. pantotropha* (10^{12} cells l^{-1}). (B) ▼ = nitrite (mM).

inoculating a *T. pantotropha* culture with *N. europaea*. The steady-state values were similar to those obtained with steady-state cultures started by the standard mixing sequence (results not shown), indicating that the mixing procedure did not influence the outcome of the experiments.

A representative example of the development of a mixed population, from the time of mixing to steady state, at a dissolved oxygen concentration of 118 μM and a C/N ratio of 4, is given in Fig. 1. After inoculation of the *N. europaea* culture with *T. pantotropha*, the latter grew at its maximum growth rate, until the acetate became growth-limiting. The specific growth rate then decreased, eventually becoming equivalent to the dilution rate. Concomitantly with the increase of *T. pantotropha*, a steady decline in the *N. europaea* population was observed. Control experiments showed that each of the two species could grow well in the spent medium of the other (results not shown). Thus the most likely explanation for the drop in the *N. europaea* population is that *T. pantotropha* competed with the autotroph for the available ammonia. The decrease in nitrite is most likely due to reduced nitrification by *N. europaea*. Nitrogen balances (see below) showed that only part of this decrease in nitrite level was due to aerobic denitrification ($\approx 12\%$). The ammonia level remained at or around the

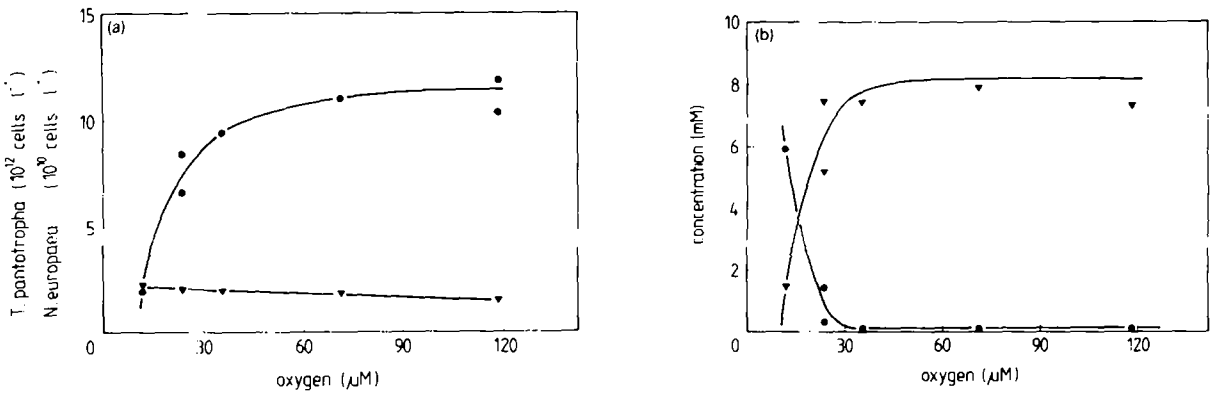

Fig. 2. Results of counting cells in mixed chemostat cultures in steady states at different dissolved oxygen concentrations at a dilution rate of 0.04 h^{-1} and a C/N ratio of 2. (a) ▼ = *T. pantotropha* (10^{12} cells l^{-1}); ● = *N. europaea* (10^{10} cells l^{-1}). (b) ▼ = nitrite (mM); ● = ammonia (mM).

detection limit of the assay (50 ± 30 μM), except in experiments where *N. europaea* washed out. The co-culture eventually reached steady state after at least 7 volume changes.

4.3. *Dissolved oxygen concentration*

Oxygen was required, both as a terminal electron acceptor for respiration and for the oxygenase reaction of ammonia to hydroxylamine [21,22]. As *T. pantotropha* has a much lower K_s for oxygen (1–2 μM [23]) than *N. europaea* (15–20 μM [24]), it would appear that competition between the two bacteria for oxygen might become a significant factor. This would imply that the heterotroph should have a competitive advantage at low dissolved oxygen concentrations. This hypothesis was tested with the mixed culture grown in a chemostat at a C/N ratio of 2.

In experiments carried out at dissolved oxygen concentrations ranging from 10–118 μM, cell numbers of the two bacteria were measured at steady state (Fig. 2a). It was found that the autotrophic population declined as the dissolved oxygen concentration decreased. There was a slight increase in the *T. pantotropha* population, even at a dissolved oxygen concentration of 10 μM. In Fig. 2b, the steady-state concentrations of nitrite and ammonia in the culture medium are presented as a function of oxygen concentration. It can be seen that ammonia started to accumu-

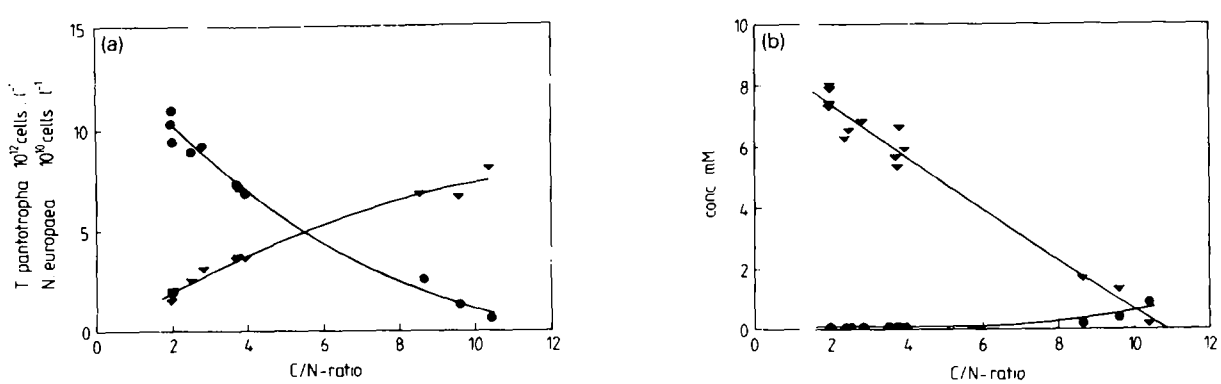

Fig. 3. Results of steady states at different C/N ratios at a dilution rate of 0.04 h^{-1} and a dissolved oxygen concentration of 118 μM. Symbols as described in Fig. 2.

late, and simultaneously the nitrite concentration decreased when the oxygen concentration decreased to 25 μM. This was due to the decline in the autotrophic population, indicating that under these growth conditions *N. europaea* was oxygen limited.

4.4. *C/N ratio*

Since the size of the *T. pantotropha* population was controlled by the acetate concentration, the effect of population size on the behaviour of the mixed cultures was investigated at different *C/N* ratios. This was achieved by altering the acetate concentration, while the ammonia concentration was generally kept constant. The exceptions were experiments at *C/N* ratios of 8.7–10.4 when the ammonia concentration in the feed was reduced from 10 to 6.8 mM. Data presented in Fig. 3a shows that increasing the *C/N* ratio resulted in an increase in *T. pantotropha* numbers. Nitrogen-balances (see below) showed that, at high *C/N* ratios, substantial quantities of nitrogen had disappeared from the culture, presumably by assimilation and combined nitrification and denitrification by *T. pantotropha*. Thus the concomitant decrease in *N. europaea* numbers was the result of an increase in the amount of ammonia assimilated and nitrified/denitrified by the heterotroph.

At a *C/N* ratio of 10.4, *N. europaea* had almost washed out from the chemostat, and 90% of the ammonia in the feed was being used by the heterotroph. Following the disappearance of the autotrophic nitrifier at higher *C/N* ratios the ammonia concentration increased slightly and low concentrations of nitrite were present in the spent medium (Fig. 3b). The reasons for this are not clear, but suggest that simple competition for ammonia is not the determining factor. Extrapolation of the line representing the number of *T. pantotropha* cells (Fig. 3a) indicated that this bacterium will become nitrogen-limited at a *C/N* ratio between 11 and 11.5.

4.5. *Growth rate*

T. pantotropha and *N. europaea* have very different maximum specific growth rates (0.35 [25] and 0.076 h^{-1} [24], respectively), and it seemed likely that growth rate could influence the outcome of the composition of the co-culture. At a dilution rate of 0.04 h^{-1}, both organisms could coexist at most *C/N* ratios used in this study. However, at a dilution rate of 0.065 h^{-1}, when *N. europaea* was growing at 77% of its maximum specific growth rate, it was unable to maintain itself in the co-culture at the high *C/N* ratio of 9.6, although ammonia was still present in sufficient amounts (Table 2).

As described previously, at *C/N* ratios greater than 10, *N. europaea* numbers declined dramatically. However, the cells never washed out completely because of continuous re-inoculation from a small amount of biofilm which formed above the level of the medium in the vessel at these high *C/N* ratios. Fluorescent antibodies showed

Table 2

Effect of dilution rate on the results from aerobic co-cultures grown at a dissolved oxygen concentration of 118 μM and different *C/N* ratios

Dilution rate (h^{-1})	*C/N* (mol mol^{-1})	Supernatant		*T. pantotropha* (10^{12} cells l^{-1})	*N. europaea* (10^{10} cells l^{-1})	Heterotrophic nitrification		Aerobic denitrification activity
		NO_2 (mM)	NH_3 (mM)			%	activity	
0.040	2.0	7.3	0.05	1.5	10.3	1	1.8	7.9
0.065	2.2	0.6	6.60	2.1	0.3	65	6.2	6.2
0.040	9.6	1.3	0.40	6.6	1.3	65	2.4	2.4
0.065	9.6	0.0	1.20	6.7	0.0	100	4.7	4.7

Heterotrophic nitrification is given as percentage of total nitrified ammonia and as activity (nmol NH_3 10^{10} cells $^{-1}$ min^{-1}); aerobic denitrification activity (nmol N 10^{10} cells $^{-1}$ min^{-1}). For relevant details see Table 1.

Table 3

Influence of dissolved oxygen concentration on the heterotrophic and autotrophic nitrification activities, and aerobic denitrification activity of the heterotroph, cell ratio and the amount of heterotrophic nitrification as a percentage of total ammonia nitrified (C/N = 2.0 mol mol^{-1}, dilution rate = 0.04 h^{-1})

Dissolved oxygen (μM)	nmol N 10^{10} cells^{-1} min^{-1}			*T. pantotropha* / *N. europaea* (cell ratio)	Heterotrophic nitrification (%)
	nitrification activity		aerobic denitrification		
	heterotroph	autotroph			
118	1.8	610	7.9	14	4
70	-	560	4.8	16	-
35	2.1	610	6.0	20	6
25	4.8	610	6.8	23	17
10	3.9	615	5.4	115	42

that both *T. pantotropha* and *N. europaea* were present in the biofilm.

4.6. *Heterotrophic versus autotrophic nitrification*

The amount of ammonia nitrified by *T. pantotropha* could be estimated from the N balance as follows:

$$[NH_4^+]_{hn} = [NH_4^+]_{in} - [NH_4^+]_{out} - [NH_4^+]_{an} - [NH_4^+]_{biom} \quad (1)$$

where hn is the ammonia nitrified heterotrophically; in, the ammonia in the feed; out, the ammonia in effluent; an, the ammonia nitrified autotrophically; and biom, the ammonia assimilated by the biomass.

The amount of ammonia nitrified by *N. europaea* could be calculated from the number of autotrophic cells in the culture divided by a growth yield factor (1.1 ± 0.14 10^{10} cells mmol NH_3^{-1}). Data in Table 3 show the nitrification rates of *T. pantotropha* and *N. europaea* at various air saturation levels. Heterotrophic nitrification became a significant part of total nitrification at dissolved oxygen concentrations below 25 μM. This was probably due to several factors including increased nitrification by *T. pantotropha* at low oxygen concentrations ($\leqslant 70$ μM) [6], and the availability of higher ammonia concentrations in the culture due to the washout of *N. europaea* (Fig. 2b).

The relative nitrification rates obtained during the experiments with different C/N ratios can be seen in Table 4. The amount of ammonia nitrified by *T. pantotropha* increased with its relative numbers in the culture, i.e., with the C/N

Table 4

Effect of C/N ratios on heterotrophic and autotrophic nitrification activities and on aerobic denitrification activity of the heterotroph, the cell ratio and the amount of heterotrophic nitrification as a percentage of total ammonia nitrified (dissolved oxygen concentration = 118 μM, dilution rate = 0.04 h^{-1})

C/N ratio (mol mol^{-1})	nmol N 10^{10} cells^{-1} min^{-1}			*T. pantotropha* / *N. europaea* (cell ratio)	Heterotrophic nitrification (%)
	nitrification activity		aerobic denitrification		
	heterotroph	autotroph			
2.0	1.8	610	7.9	14	4
2.4	4.6	580	6.9	29	19
2.8	2.0	610	4.8	34	10
3.7	2.6	610	3.4	49	18
8.7	2.2	600	2.9	264	47
9.6	2.4	670	2.4	508	65
10.4	2.2	645	2.6	1290	79

ratio. At a C/N ratio of 10.4, the heterotroph was responsible for up to 80% of the total nitrification. It should be noted that at C/N ratios higher than 8, the total nitrification (heterotrophic plus autotrophic) decreased to 25–40% of that at lower C/N ratios, because most of the ammonia (60–75%) was assimilated.

Higher heterotrophic nitrification rates per cell were recorded at a growth rate of 0.065 h^{-1} than at 0.04 h^{-1} (Table 2). This was due to the higher dilution rate, and was also found with pure cultures [6]. Furthermore, the higher NH_3 concentration and the presence of little or no NO_2^- in these cultures also permitted higher nitrification activities (see also Ref. 24). At a growth rate of 0.065 h^{-1}, nitrification was predominantly heterotrophic, even at a C/N ratio as low as 2.2, despite the fact that most of the NH_3 remained in the culture (Table 2). As discussed earlier, at this higher growth rate, and at a C/N ratio of 9.6, *N. europaea* washed out, and nitrification was only carried out by the heterotroph.

The specific nitrification activity ($6.2 \pm 0.3\ 10^{-8}$ nmol NH_3 min^{-1} $cell^{-1}$ or 517 ± 78 nmol NH_3 min^{-1} mg $biomass^{-1}$) of *N. europaea* did not vary very much under the different conditions. These data are consistent with those observed with pure cultures [24]. However, the specific nitrification activity of *T. pantotropha* varied more, and appeared to be influenced by the growth conditions. Higher heterotrophic nitrification activity per cell was observed at lower dissolved oxygen concentrations and, as already mentioned at higher growth rates, confirming previous results [6]. The overall mean heterotrophic nitrification rate was $2.5 \pm 0.9\ 10^{-10}$ nmol NH_3 min^{-1} $cell^{-1}$ (2.3 ± 0.5 nmol NH_3 min^{-1} mg $biomass^{-1}$) at a dissolved oxygen concentration of 118 μM. The standard deviation was high, because of the indirect way of calculating the amount of ammonia nitrified heterotrophically. This activity was comparable with the nitrification activity found in pure cultures of *T. pantotropha* in the presence of limiting ammonia concentrations and high nitrite concentrations [24]. It can be calculated that under these conditions, 250 times more *T. pantotropha* cells were required to oxidize the same amount of ammonia metabolized by the autotroph. This value is close to that found at a C/N ratio of 8.7 where, indeed, the heterotroph accounted for almost 50% of the total nitrification recorded (Table 4).

4.7. Aerobic denitrification

Total denitrification was estimated from the nitrogen balance:

$$[N]_{den} = [NH_4^+]_{in} - [NH_4^+]_{out} - [NO_2^-]_{out} - [N_{biom}]_{out} \quad (2)$$

where den is the nitrite denitrified; in, the ammonia in the feed; out, the effluent; and biom, the biomass.

Total denitrification is thus the sum of the ammonia nitrified/denitrified by the heterotroph plus the nitrite produced by the autotroph and subsequently denitrified by the heterotroph. The denitrification rate did not appear to be strongly influenced by the dissolved oxygen concentration (Table 3). However, the increase of the C/N ratio resulted in a 2- to 3-fold decrease in the denitrification activity (Table 4). It was observed that with an increasing C/N ratio, the organic carbon content of the biomass in the culture increased by a greater factor than might be expected, while the percentage of the protein in the biomass decreased. It appeared that a higher C/N ratio resulted in an increase in the formation of storage products such as poly-β-hydroxybutyrate. The explanation for this might be that *T. pantotropha* behaves, physiologically, as if under ammonia limitation at higher C/N ratios, and is not well adjusted to the assimilation of mixed inorganic nitrogen compounds (i.e., ammonia and nitrite, see [24]).

5. DISCUSSION

This study has tested the hypothesis that heterotrophic nitrifiers could successfully compete with autotrophic nitrifiers for ammonia [8,26] under some growth conditions (e.g., high growth rates, high C/N ratios). The observations that *T. pantotropha* and *N. europaea* could coexist in mutual independence provided that the number

of cells of *T. pantotropha* were kept below a critical value, and that the heterotroph could, by simple numerical dominance, compete successfully with *N. europaea* for the available ammonia, tend to support this hypothesis. At high C/N ratios, more heterotrophic biomass was formed, and thus the outcome of the competition for ammonia was controlled by the availability of organic substrate.

Although specific nitrification rates greatly differed, *T. pantotropha* cultures could achieve total nitrification rates equivalent to those of *N. europaea* when the heterotrophs outnumbered the autotrophs by 250:1. Conditions which appear to favour heterotrophic nitrifiers include high C/N ratios, low oxygen concentrations and high growth rates. It seems likely that in environments which fulfil any of these conditions (e.g., in waste water treatment systems) heterotrophic nitrifiers may play a significant role in ammonia oxidation.

At a C/N ratio of 10.4, *T. pantotropha* dominated the mixed culture. Similar results were obtained by Verhagen and Laanbrock [27] in a co-culture of *N. europaea*, *Nitrobacter winogradskyi* and a heterotroph. They found critical C/N ratios of 12 and 10 at dilution rates of 0.004 h^{-1} and 0.01 h^{-1}, respectively.

The increase in nitrification by the heterotroph at dissolved oxygen concentrations below 15% air saturation (Table 3), compared to the small amounts of ammonia oxidized by the autotroph at low dissolved oxygen concentrations (< 25 μM), may indicate an ecological role for heterotrophic nitrification in situations where oxygen is low or limiting (Fig. 2a). A loss of nitrifying activity with decreasing dissolved oxygen was reported for *N. europaea* in co-culture with a *Nitrobacter* sp. [28], and with autotrophic ammonia oxidizers in wastewater [29]. The results may be explained by the relatively higher affinity constant for oxygen (10–15 μM) of *N. europaea* compared to that of the heterotroph (1–2 μM). However, although the heterotroph was responsible for up to 42% of the total nitrification at the lower oxygen concentrations, more than 50% of ammonia in the feed was not oxidized (Fig. 2b).

The results shown in Figs. 2 and 3 are in agreement with those predicted from a mathematical model which was primarily based on data from pure culture studies [24].

The implications of this work for research into natural nitrification systems are two-fold: (i) Rather than being inhibited by the presence of organic compounds, as has been proposed, autotrophic nitrifiers may simply be outgrown by heterotrophs, if suitable amounts of organic substrates are present. (ii) If autotrophic and heterotrophic nitrifiers compete for available ammonia, the C/N ratio may determine the contribution of the two populations to the total nitrifying activity.

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