Nitrification, denitrification and growth in artificial *Thiosphaera pantotropha* biofilms as measured with a combined microsensor for oxygen and nitrous oxide

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Abstract

Cells of the aerobic denitrifier and heterotrophic nitrifier *Thiosphaera pantotropha* and of the traditional denitrifier *Paracoccus denitrificans* were immobilized in a 1.5 mm thick agar layer (biofilm) and submersed in liquid medium. A combined microsensor for O_2 and N_2O was used to record microprofiles of these two species in biofilms where the reduction of N_2O was inhibited by acetylene. Nitrification in *T. pantotropha* was not affected by the addition of acetylene and by using a diffusion-reaction model to simulate the N_2O profiles it was possible to calculate depth profiles of both nitrification and denitrification. The validity of the calculations when both nitrification and denitrification were operating in concert was confirmed by performing identical calculations on data obtained for a *P. denitrificans* biofilm. At high NO_3^- concentrations, part of the NO_3^- reduced by *T. pantotropha* biofilms was reduced only to NO_2^- and N_2O production thus did not reflect total NO_3^- reduction. When NO_2^- and no NO_3^- was present in the water above the biofilm N_2O production was recorded in the anoxic zone directly below the oxic zone. Nitrous oxide production was never detected in the oxic zone of the biofilms, although aerobic denitrification was described for the original isolate of this bacterium. The growth rate of *T. pantotropha* in the oxic region of the biofilms was estimated to be 0.42 h^{-1} which is slightly higher than rates previously obtained in liquid culture. In the *T. pantotropha* biofilms nitrification was calculated to account for more than 50% of the O_2 consumption whereas this process only consumed about 10% of the O_2 in liquid culture.

Keywords: *Thiosphaera pantotropha*; Heterotrophic nitrification; Denitrification; Growth; Biofilm; Immobilized cells

1. Introduction

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The demonstration of simultaneous nitrification and dissimulatory NO_3^- reduction in *Thiosphaera pantotropha* [1,2] has attracted much attention. The

organism is one of the most metabolically versatile bacteria known and its ability to perform aerobic denitrification may be seen as an adaptation to a rapidly fluctuating oxic/anoxic environment. Following the initial demonstration of aerobic denitrification in *T. pantotropha*, the process has also been demonstrated in other bacteria (*Alcaligenes faecalis* and *Pseudomonas denitrificans*, [3]). Significant rates of aerobic denitrification have, however, not been demonstrated in natural environments, possibly because organisms capable of performing this process are outcompeted. The study of denitrification in natural environments has, however, been complicated by the lack of a method that allows determination of in situ O_2 concentrations at the exact site where denitrification occurs. The development of a combined microsensor for O_2 and N_2O [4] has enabled us to analyze denitrification in relation to O_2 distribution in stratified microbial communities. However, application of this technique to biofilms [5–7], freshwater sediments [8] and marine sediments [9] has not revealed denitrification at more than 10–20 μM O_2.

The fact that autotrophic nitrification is inhibited by acetylene (C_2H_2) [10,11] has limited the work done with the O_2/N_2O microsensor on natural communities to focus on denitrification [4–8]. However, heterotrophic nitrification has been shown to be insensitive to C_2H_2 [12] and since *T. pantotropha* is a heterotrophic nitrifier it is possible to study nitrification in acetylene-inhibited cells of this organism.

Earlier work with *T. pantotropha* has mostly been carried out in liquid culture. However, most organisms in natural microbial ecosystems live and grow in gradients [13]. To approximate the natural environment as much as possible, the present study was done with cells of *T. pantotropha* immobilized in a thin agar layer on a glass plate in which supply of nutrients and removal of metabolic products was limited by diffusion. The growth conditions therefore simulate those occurring in natural biofilms and the term 'biofilm' will therefore be used to designate this experimental system.

The microsensor technique in combination with an organism insensitive to acetylene allowed us to study both denitrification, oxygen consumption, nitrification, and growth in an experimental setup simulating the natural gradient environment. The results obtained in experiments with *T. pantotropha* were compared to results obtained with *Paracoccus denitrificans* under exactly the same conditions. The latter organism is a classical denitrifier incapable of either nitrification or aerobic denitrification [2].

2. Materials and methods

2.1. Immobilization of bacteria

Artificial biofilms were prepared from a homogeneous suspension of bacterial cells in 0.9% (w/v) agar (Difco Bacto Agar). The suspension was poured into an 'O'-ring (inside diameter of 23 mm and a thickness of 1.5 mm) which was attached to a glass plate with silicone grease. By pouring the melted agar into this 'O'-ring a circular 1.5 mm thick biofilm was formed. The agar was dissolved in medium without trace metals (see below) by boiling and subsequently cooled to 40° C before addition of trace metals and bacteria. Vigorous mixing for 1 min. on a Vortex mixer ensured homogeneous distribution of the bacteria in the agar. *Thiosphaera pantotropha* was obtained as frozen culture (LMD 82.5 Delft Culture Collection) which before freezing had been grown in a chemostat on NO_2^- and acetate while the oxygen concentration was maintained at 70% of air saturation. Before use the cells were thawed and suspended in medium to an optical density of approximately 1.9 (430 nm, 1 cm lightpath). The bacterial suspension (2–5 ml) was centrifuged to form a pellet which subsequently was resuspended in 2 ml of the agar/medium mixture and poured into the 'O'-ring. *Paracoccus denitrificans* (LMD 22.21 Delft Culture Collection) cells used in our experiments were taken from a chemostat culture growing aerobically on acetate. Two ml of this culture, with an optical density of 1.4 (430 nm, 1 cm lightpath), was centrifuged to form a pellet, resuspended in 2 ml agar/medium mixture, and poured into the 'O'-ring. Initial cell density in the biofilms was calculated assuming that an optical density of 1.9 in suspended culture corresponded to a cell density of 1×10^8 cells per ml. Cell density at the beginning of the experiment was calculated to be in the order of 1 cell per 3×10^{-12} l. The average distance between cells initially was hence approximately 15 μm.

2.2. Incubation and media

The glass plate with 3–4 'O'-rings containing the agar immobilized cells was submersed in *T. pantotropha* medium [14] which was contained in a 2 l aquarium. A pump circulated the medium through a glass tube helix submersed in a constant-temperature water bath, to maintain a temperature of 30° C in the aquarium. The pumping also created flow along the biofilm surface.

Medium was replaced every 3–4 h, or more frequently if significant suspended cell growth had occurred. Initial concentrations of acetate, NH_4^+ and NO_3^- (when added) were 10, 5.6 and 20 mm respectively. The concentrations of these species were not measured because high initial concentrations and frequent replacement of medium ensured little depletion. In experiments with low concentrations of NO_2^- it was crucial to know its actual concentration, so frequent measurements were performed using the Griess-Romijn reagent [15].

2.3. Microsensor measurements

Profiles of O_2 and N_2O were measured in the submersed biofilms using a combined microsensor for these 2 compounds. The microsensor was constructed according to the principles of Revsbech et al. [4] with modifications as described by Dalsgaard and Revsbech [7]. Because of the short lifetime of this type of sensor 2 different sensors were used in this study. Both had an outer tip diameter of 20 μm. The response to both O_2 and N_2O was linear with a detection limit of 1 μM for N_2O and 0.1 μM for O_2. The signal from the microsensor was higher in stirred than in stagnant medium for identical concentrations. This difference was 10% for O_2 and less than 1% for N_2O. The O_2 part of the electrode was calibrated using a 2-point calibration curve with one point being the concentration in the stirred medium above the biofilm (using tabulated values of O_2 solubility; [16]). The other calibration point was the signal for zero O_2 deep in the biofilm. Nitrous oxide calibration was done by dilution of N_2O saturated water to concentrations close to those encountered in the biofilms. Tabulated values for N_2O solubility were used [17].

The biofilms were incubated with C_2H_2, which blocked N_2O reduction [18,19], and allowed denitrification to be quantified as N_2O production. To maintain a partial pressure of 10 kPa C_2H_2, the medium was continuously sparged with a mixture of 90% air and 10% C_2H_2. The microsensor was mounted on a motor-driven micromanipulator and moved through the biofilms in increments as small as 10 μm, while the signals for O_2 and N_2O were collected by a computer equipped with an analogue-digital converter. All the profiles presented here represent steady-state conditions, which was confirmed by measuring consecutive profiles until at least 2 sets were identical.

2.4. Calculations

Denitrification rates were determined from N_2O concentration profiles using the diffusion-reaction model of Revsbech et al. [20]. The model is based on an extended version of Fick's second law of diffusion [21]:

$$\frac{\delta C(x,t)}{\delta t}$$

$$= D(x)\frac{\delta^2 C(x,t)}{\delta x^2}$$

$$+ \left[\frac{\delta D(x)}{\delta x} + \frac{D(x)}{\phi(x)}\frac{\delta \phi(x)}{\delta x} \right] \frac{\delta C(x,t)}{\delta x}$$

$$+ P(x,t) - R(x,t) \qquad (1)$$

where C represents concentration, D diffusion coefficient, t time, x depth, ϕ porosity, P production and R consumption. This equation was solved numerically on a computer [20] assuming that there was no consumption ($R(x,t) = 0$). A constant N_2O concentration in the overlying water was required as the upper boundary condition. The lower boundary condition required a N_2O concentration of zero at the lowest point for which the simulation was run. As the N_2O concentrations in the biofilms were above zero, and the glass plate was an impermeable boundary, it was necessary to mathematically simulate a glass plate. This was achieved by running the model with extremely low values of diffusion coefficients and porosity below the active zone enabling the simulated concentration to decrease to zero with no

flux towards the glass plate. Nitrous oxide profiles were modelled by estimating production rates, running the simulation to steady-state, and then comparing the simulated profile to the measured one. Based on the observed differences between the two profiles a new and better estimate of production rates was calculated and the simulation repeated. This procedure was repeated until a good agreement between measured and simulated profiles was obtained.

The model was also used to calculate the NO_2^- profile using the NO_2^- concentration in the medium as the upper boundary condition and assuming that the NO_2^- consumption equalled N_2O production (in N equivalents).

Oxygen uptake was quantified as the flux J through the diffusive boundary layer using Fick's first law of diffusion:

$$J = \frac{\delta C(x)}{\delta x} D(x)\phi(x) \qquad (2)$$

As the agar gel contained only 0.9% w/v agar the porosity was assumed to be 1 and the diffusion coefficients were assumed to be the same as for pure water [22]. All experiments were done at 30° C and the following diffusion coefficients were used for calculations: $D(O_2) = 2.66 \times 10^{-5}$ cm^2 s^{-1}, $D(N_2O) = 2.69 \times 10^{-5}$ cm^2 s^{-1} [23] and $D(NO_2^-) = 2.13 \times 10^{-5}$ cm^2 s^{-1} [24].

3. Results

3.1. Oxygen uptake

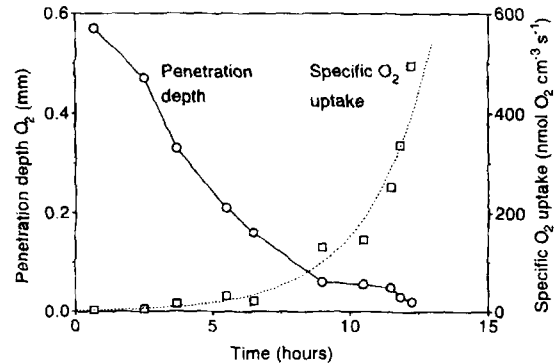Rates of O_2 uptake through the diffusive boundary layer (Eq. 2) are presented in Table 1 together

Fig. 1. Average specific O_2 uptake rate ($\square$) and penetration depth of O_2 ($\bigcirc$) in *T. pantotropha* biofilms grown with excess acetate (10 mM) and NH_4^+ (5.6 mM). Specific O_2 uptake rate was used as a relative measure of cell density to calculate a growth rate of 0.42 h^{-1}. The exponential growth curve was calculated from that growth rate (dotted line).

with other parameters determined simultaneously. The diffusive boundary layer was here defined as the layer above the biofilm in which the O_2 concentration decreased linearly. Calibration of the microsensor was done in turbulent medium above the biofilm, whereas the measurements used for calculations were made under stagnant conditions in the diffusive boundary layer and the biofilm. As the O_2 signal of the microsensor was 10% lower in stagnant than turbulent medium, the O_2 concentrations in the diffusive boundary layer and the biofilm were underestimated by 10%. To compensate for this oxygen concentrations in the diffusive boundary layer, used for calculating fluxes into the biofilm, were increased by 10%.

Table 1
Rates of oxygen consumption, nitrification, and denitrification in *Thiosphaera pantotropha* and *Paracoccus denitrificans* biofilms at various NO_3^- concentrations in the medium above the biofilms

Experiment	NO_3^- in medium (μM)	O_2 consumption (nmol O_2 cm^{-2} s^{-1})	Nitrification (nmol N cm^{-2} s^{-1})	Denitrification (nmol N cm^{-2} s^{-1})	O_2 consumed by nitrification (O_2 consumed total)	Denitrification (O_2 consumption)
T. pantotropha (Fig. 3A)	5	0.99	0.34	0.17	0.51	0.17
T. pantotropha (Fig. 3B)	165	0.43	0.14	0.10	0.48	0.23
T. pantotropha (Fig. 3C)	497	0.98	0.64	0.55	0.98	0.56
P. denitrificans (Fig. 4)	147	0.27	–	0.07	–	0.26

The individual experiments are shown in Figs. 3 and 4 as indicated. The medium contained 10 mM acetate and 5.6 mM NH_4^+.
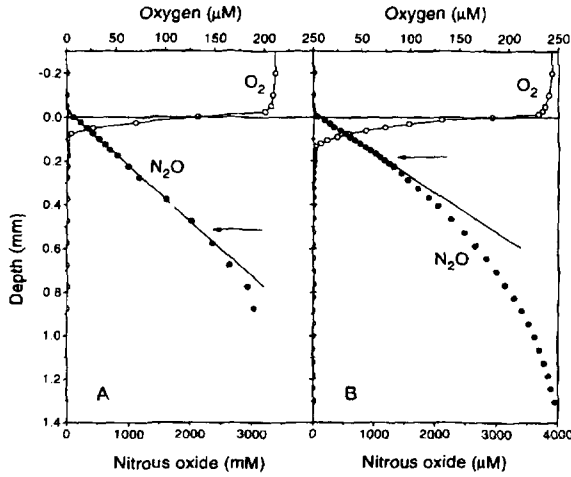
Fig. 2. Steady-state microprofiles of O_2 (O) and N_2O (●) in a C_2H_2 inhibited *T. pantotropha* biofilm. Incubation was done in medium containing 10 mM acetate, 5.6 mM NH_4^+ plus 20 mM NO_3^- (A) or 5 mM NO_2^- (B). Arrows indicate the upper boundary of the N_2O production zone. The medium/biofilm interface is at depth = 0.0.

3.2. Growth of immobilized cells

At the beginning of the experiments the penetration depth of O_2 was up to 700 μm but decreased to only 20 μm after 12–15 h (Fig. 1). The decrease in O_2 penetration was due to increased O_2 consumption as a result of cell growth. Growth occurred as colonies because of the fixed position of the cells. The distance between colonies (ca. 15 μm) was about the same as the tip diameter of the microsensor and any concentration differences on this scale could not be resolved. The average specific uptake rate (nmol O_2 cm^{-3} s^{-1}) was obtained by dividing the O_2 consumption rate by the thickness of the O_2 consuming layer, assuming zero order kinetics. The 2 substrates, acetate and NH_4^+ giving rise to both growth and O_2 consumption, were in excess throughout the experiment. The ratio of acetate to O_2 and NH_4^+ to O_2 was ca. 40 and 25 respectively in the water outside the biofilm. Since both acetate,
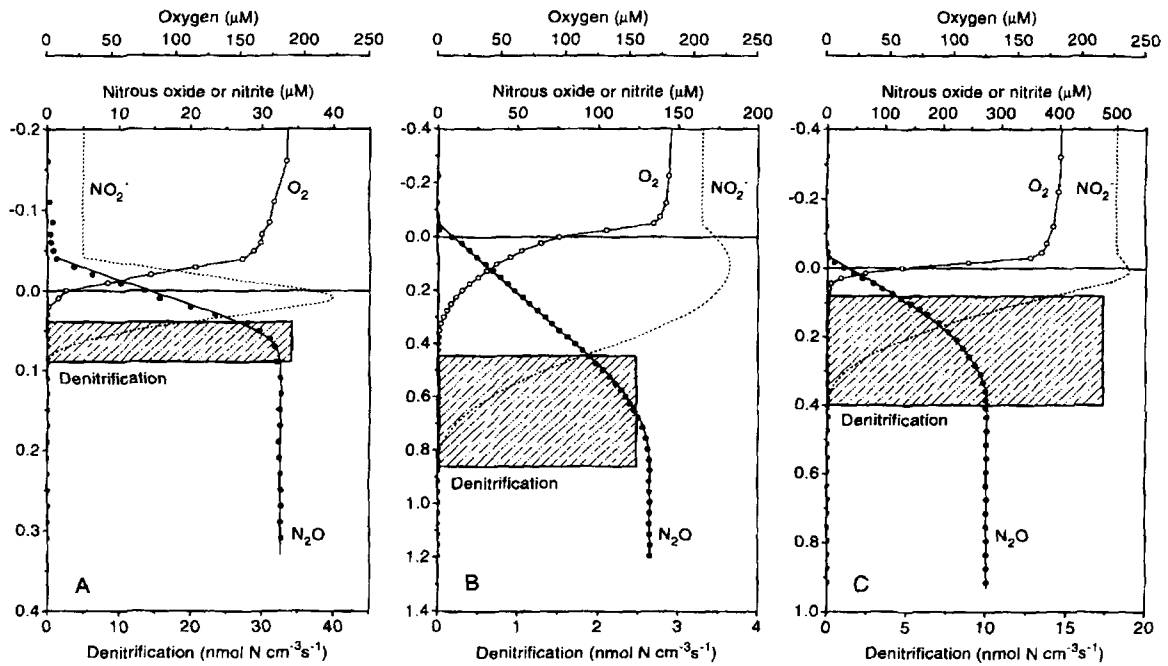

Fig. 3. Measured microprofiles of O_2 (O) and N_2O (●) and calculated profiles of NO_2^- (dotted line) and denitrification (bar) in a *T. pantotropha* biofilm. The line through the measured N_2O concentrations is the one simulated from the denitrification shown. The medium above the biofilm contained 10 mM acetate and 5.6 mM NH_4^+ plus 5 μM NO_2^- (A) or 165 μM NO_2^- (B) or 497 μM NO_2^- (C). Medium/biofilm interface is at depth = 0.0. Note the differences in scale.

NH_4^+, and O_2 were transported into the biofilm by diffusion NH_4^+ and acetate were in excess throughout the oxic zone. The organisms were therefore not substrate limited and O_2 uptake per unit volume was assumed to be proportional to the number of bacteria and used as a relative measure of cell density in the oxic zone. Plotting average specific O_2 uptake versus time yielded an exponential curve (Fig. 1). Logarithmic transformation (base e) gave a straight line (not shown) with a slope of 0.42 h^{-1} ($r^2 = 0.79$) which was taken to be the growth rate of T. pantotropha in the oxic zone of these biofilms. The data presented in Fig. 1 are pooled from several experiments with 1-3 measurements in each biofilm.

3.3. Reduction of nitrate at high concentrations

A biofilm containing T. pantotropha was incubated with 20 mM NO_3^- and 10 kPa C_2H_2. Nitrous oxide production began immediately after start of the incubation and a steady-state concentration profile evolved (Fig. 2A). In the region of the biofilm where the steady-state N_2O profile was linear there was no production or consumption of N_2O. However, in the region of the biofilm where the N_2O profile is non-linear N_2O production took place. Nitrous oxide production hence occurred below a depth of 0.5 mm in Fig. 2A. When the biofilm was exposed to 5 mM NO_3^- instead of NO_3^- the N_2O production zone moved upwards and production occurred from the bottom of the oxic zone downwards (Fig. 2B). The shift from 20 mM NO_3^- to 5 mM NO_3^- increased the N_2O flux out of the biofilm from 2.11 to 3.14 nmol N cm^{-2} s^{-1}.

3.4. Denitrification in T. pantotropha biofilms

Denitrification was also measured in T. pantotropha biofilms under varying NO_2^- concentrations. The experiment presented in Fig. 3A was conducted with 5 μM NO_2^- in the medium above the biofilm. Even at this extremely low NO_2^- concentration there was a 50 μm thick denitrification zone exhibiting an activity of 35 nmol N cm^{-3} s^{-1}. Denitrification rates at higher NO_2^- concentrations are shown in Fig. 3B (165 μM) and Fig. 3C (497 μM). Total rates of denitrification are given in Table 1. The relationship between NO_2^- concentration and denitrification rates can not be compared directly in
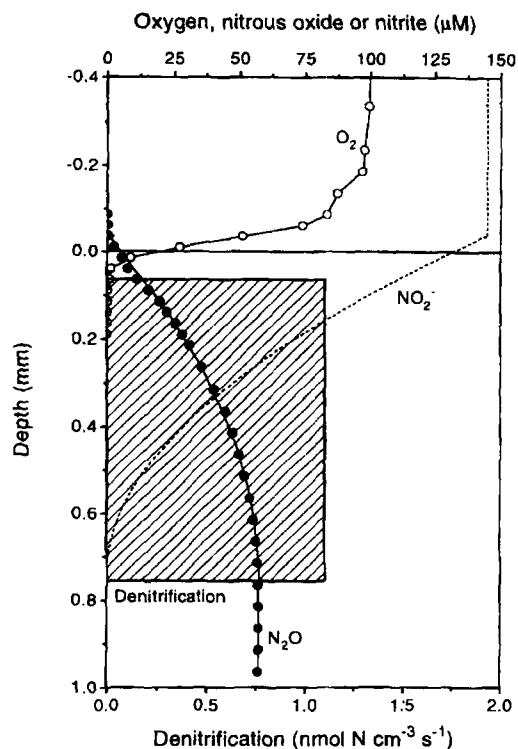

Fig. 4. Measured microprofiles of O_2 (O) and N_2O (●) and calculated profiles of NO_2^- (dotted line) and denitrification in a P. denitrificans biofilm. The line through the measured N_2O concentrations indicate the N_2O profile modelled from the denitrification activity shown. The biofilm was incubated in medium containing 10 mM acetate, 5.6 mM NH_4^+ and 147 μM NO_2^-. Medium/biofilm interface is at depth = 0.0.

these 3 experiments because of the differences in cell density and this is reflected in the differences in O_2 uptake rates (Table 1) and O_2 penetration (Fig. 3A-C). However, the ratio of denitrification rate to O_2 consumption was compared and as expected this increased with increasing NO_2^- concentrations (Table 1). The upper boundary of the denitrification zone was found immediately below the oxic zone at all three NO_2^- concentrations (Fig. 3).

3.5. Denitrification and oxygen respiration in P. denitrificans biofilms

At the time when the O_2 and N_2O profiles in Fig. 4 were measured in the P. denitrificans biofilm, the O_2 concentration in the medium had decreased due to suspended growth in the medium. This may have

decreased the acetate concentration, so to avoid acetate limitation in the biofilm acetate was added to increase the concentration by 20 mM. Denitrifying activity was then recorded from the oxic-anoxic interface to a depth of 0.75 mm.

3.6. Nitrite profiles

Nitrite consumption in both P. denitrificans and T. pantotropha biofilms (Figs. 3 and 4) was assumed to be only due to denitrification since 5.6 mM NH_4^+ was added to suppress NO_2^- assimilation. Simulation of NO_2^- profiles was therefore performed using the NO_2^- concentration in the medium above the biofilm as the upper boundary condition and using N_2O production rates (in N-equivalents) as NO_2^- consumption rates. In the P. denitrificans biofilms NO_2^- was calculated to penetrate to just below the lower edge of the denitrifying zone. A perfect match between the simulated NO_2^- penetration and the analyzed lower edge of the denitrification zone was obtained when a value of 144 μM, instead of the measured NO_2^- concentration in the medium (147 μM), was incorporated in the model. When using the NO_2^- concentration in the overlying water as the upper boundary condition for the T. pantotropha biofilms, however, the simulated NO_2^- concentration profile decreased to zero well above the lower limit of the denitrification zone. In order to make the modelled NO_2^- profile reach the bottom of this zone it was necessary to assume that NO_2^- was also produced in the biofilm. Nitrification in T. pantotropha requires O_2 [25] and hence this process was assumed to take place only in the oxic zone of the biofilms. It was also assumed that NH_4^+ was only oxidized as far as NO_2^- and that it proceeded at the same rate throughout this zone. Nitrification rates in the model were adjusted until NO_2^- was calculated to penetrate precisely to the bottom of the denitrifying zone. The rates obtained are presented in Table 1.

4. Discussion

4.1. Growth of T. pantotropha in biofilms

As the immobilized cells grew throughout the experiment, a true steady-state was never reached.

However, concentration profiles stabilized rapidly after each manipulation and reached an apparent short term steady-state. The generation time of this bacterium was ca. 1.65 h and the recording of a set of profiles lasted less than 15 min. The measured profiles therefore accurately reflect production and consumption in the biofilm and changed only slowly with time as a result of growth in the biofilm. The method of estimating growth rate from average specific O_2 uptake only allowed determination of growth rate with O_2 as electron acceptor. T. pantotropha has also been shown to grow under anoxic denitrifying conditions [1] and would therefore be able to grow in the anoxic part of the biofilm. That this in fact occurred can be elucidated from increasing specific denitrification rates with time data not shown). Quantification of the anaerobic growth rate from the increase in specific denitrification rates was not possible because the increased cell numbers in most of the anoxic zone were derived as a result of both aerobic and anaerobic growth.

The calculated aerobic growth rate of 0.42 h^{-1} was comparable to those reported by Hooijmans et al. [26] for T. pantotropha immobilized in spherical agarose beads (maximum specific growth rate of 0.52 h^{-1}) and those of Robertson and Kuenen [1] for suspended culture (maximum specific growth rate of 0.34 h^{-1}). The quoted growth rates were all measured at 37° C, which is the optimal temperature for this bacterium, whereas the cultures grown in this study were incubated at 30° C. The effect of temperature on growth rate for this bacterium in not precisely known but a Q_{10} of about 2 would seem appropriate. Conversion of the measured growth rate to 37° C would therefore give a higher value than found for cells immobilized in agarose beads. Hence the growth of immobilized T. pantotropha in gradients would appear to be faster than in liquid culture.

4.2. Calculation of nitrite profiles

When calculating denitrification activities from the N_2O profiles it was assumed that the diffusion coefficient for N_2O was the same in the biofilm as in the overlying water; a similar assumption was also made for NO_2^- when calculating NO_2^- profiles. It is possible, however, that the diffusion coefficients decreased during the experiment due to bacterial

growth. Another assumption made using the diffusion-reaction model was that there was no consumption of N_2O in the presence of C_2H_2. This has been verified in other studies using liquid cultures [27,28].

Diffusion of gasses is driven only by concentration gradients, whereas the diffusion of ions is also affected by electrostatic interactions. In a NO_2^- and acetate consuming biofilm the diffusion of these negatively charged species into the biofilm must be accompanied either by diffusion of positive charged species into or negatively charged species out of the biofilm. The rate at which these accompanying species diffuse will affect the diffusion coefficient for NO_2^- [29]. In the experiment with immobilized P. denitrificans the significance of this phenomenon could be investigated. This bacterium does not nitrify [2] and the NO_2^- profile that develops results from the interplay between diffusion and consumption of NO_2^- by denitrification. Based on the modelled denitrification activity, the NO_2^- profile was estimated and the NO_2^- concentration in the water was calculated to be 144 μM, whereas the measured NO_2^- concentration was 147 μM. This calculation was performed assuming that the ratio of diffusion coefficients for N_2O and NO_2^- was the same in the P. denitrificans biofilms as in the water and the close match between estimated and measured NO_2^- concentrations validates this assumption. There is no reason to assume different conditions with respect to diffusion in the T. pantotropha biofilms.

4.3. Nitrification in T. pantotropha

The measured denitrification rates in the T. pantotropha biofilms can only occur if a high nitrification rate occurred in the oxic zone of the biofilms. As the assumptions about diffusion coefficients used in the calculations of nitrification rates were verified in the experiment with P. denitrificans, we concluded that nitrification in this bacterium was not inhibited by C_2H_2. Robertson and Kuenen [25] found that the ammonium monooxygenase of T. pantotropha showed a number of similarities with that of autotrophic nitrifiers (light sensitivity, $Mg2^+$ requirement, NAD(P)H utilization) but also differences (e.g. inhibition by NH_2OH). The ammonium oxygenase of the autotrophic nitrifier Nitrosomonas europaea is inhibited by C_2H_2 [10,30] so the ob-

served insensitivity to C_2H_2 reported here is yet another difference. Insensitivity of nitrification to C_2H_2 has been previously reported for the heterotrophic nitrifying organisms Arthrobacter sp. [12] and Aspergillus flavus [31]. The present results thus support the general assertion that heterotrophic and autotrophic nitrification can be distinguished based on the difference in sensitivity to C_2H_2 [32].

The relative importance of nitrification to the total metabolism of the biofilm community can be estimated by comparing the O_2 uptake by nitrification to the total O_2 uptake. Oxidation of NH_4^+ to NO_2^- proceeds according to the following overall reaction:

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$$

Hence, the O_2 demand by nitrification is 1.5-times the nitrification rate. In continuous culture, oxidation of NH_4^+ by T. pantotropha principally yields NO_2^- [25], and we have assumed that this also occurred within the biofilms. Therefore in the biofilms oxygen consumption due to NH_4^+ oxidation was assumed to be 1.5 times the nitrification rate. On the basis of this assumption the O_2 uptake due to the calculated nitrification accounted for between 48% and 98% of the total O_2 uptake (Table 1). It should be noted that total O_2 consumption was calculated from measurements using a stirring sensitive microelectrode. Using a stirring insensitive O_2 microelectrode would have improved the accuracy.

Nitrification rates were estimated assuming that the nitrification was occurring at the same rate throughout the oxic zone of the biofilms. Therefore approximately 50% of the NO_2^- produced would have diffused out of the biofilm whilst the remainder would have diffused into the denitrification zone. A lower nitrification activity would therefore be required to generate the NO_2^- needed to account for the measured denitrification rate if nitrification occurred closer to the denitrification zone. Under these conditions the ratio of O_2 consumption by nitrification to total O_2 consumption would be lower. Studies with continuous cultures of T. pantotropha have shown that nitrification rates increased by 1/3 when the O_2 concentration was decreased from 25 to 5% of air saturation [2]. However, the O_2 uptake per unit volume of biofilm in Fig. 3B, estimated using Eq. 1 [20], was highest close to the surface and decreased with depth (not shown, but about 5-times higher in

the uppermost 0.1 mm as compared to the lowermost 0.1 mm). Those data do not support the hypothesis that nitrification was occurring at higher rates close to the denitrification zone and the ratios of O_2 consumption by nitrification to total O_2 consumption are in all probability not much lower than those calculated (Table 1).

T. pantotropha is considered to be a heterotrophic nitrifier [2,25,28,33], and heterotrophic nitrification, although still poorly understood, is thought to be an energy consuming process [32]. Using hydrazine ($N_2H_5^+$) as substrate for nitrification in T. pantotropha, Castignetti [34] concluded that this bacterium does not conserve energy when metabolizing this substrate. It is at present unclear how organisms benefit from performing heterotrophic nitrification. In the present study more than half of the oxygen consumption was calculated to be attributed to nitrification. Thus oxygen consumption by heterotrophic nitrification must have been higher than oxygen respiration. This is a rather high rate for a process for which the actual advantages for the organisms are largely unknown. However, these data demonstrate that in a gradient environment heterotrophic nitrification can be a very important process in the nitrogen turnover. More research dealing with the possible advantages/disadvantages for the organisms carrying out this process is needed.

In continuous cultures of T. pantotropha up to 10% of the total O_2 consumption was due to nitrification and for cells immobilized in spherical agarose beads this value was found to be 11–12% [26]. Nitrification in these immobilized cells was modelled from measured microprofiles of oxygen and calculated profiles of acetate under acetate limitation. The values found in this study are hence much higher (48–98%).

In the present study measurements of the relative importance of nitrification were performed at higher cell densities than in the study of Hooijmans et al. [26]. Oxygen penetration of less than 0.63 mm was never reached in that study whereas we found O_2 penetration as low as 0.02 mm. There is, however, currently no evidence for correlation between cell density and nitrification rates per cell.

4.4. Effect of nitrite concentration

Differences in densities of T. pantotropha in the biofilms when exposed to different NO_2^- concentra-

tions rendered direct comparison of denitrification rates difficult. However, using O_2 uptake as a relative measure of cell density, and normalizing the denitrification rates to this it is clearly evident that denitrification was stimulated by increased NO_2^- concentration (Table 1). A more direct comparison can be made between the rates in Fig. 3A and 3C since O_2 uptake rates (Table 1) and O_2 penetration depth were almost identical. The NO_2^- concentrations differed by a factor of 100, whereas the denitrification rates only differed by a factor of 3.2. Nitrification in the oxic zone of the biofilms, however, explains the relatively small effect of changing NO_2^- concentrations. At all applied concentrations of NO_2^-, the T. pantotropha biofilms showed higher rates of nitrification than denitrification and therefore a net flux of NO_2^- out of the biofilms. This net flux was a result of 2 opposing fluxes; one being the flux of NO_2^- into the biofilm from the medium, which increased with increasing NO_2^- concentration. The other was the flux out of the biofilm of NO_2^- produced by nitrification. When the NO_2^- concentration in the medium was elevated the thickness of the denitrifying zone increased. The diffusion distance for NO_2^- from the nitrifying zone into the denitrifying zone hence increased and a relatively larger fraction of nitrified NO_2^- diffused out of the biofilm.

In P. denitrificans all NO_2^- for denitrification originated from the overlying medium, and denitrification would therefore be expected to proceed at a lower rate than in the T. pantotropha biofilms. However, due to suspended growth, the O_2 concentration in the medium was lower than when the profiles in the T. pantotropha biofilms were measured. This reduced the O_2 uptake rate and stimulated the denitrification rate. The ratio of these rates (Table 1) was therefore higher than it would have been under the conditions at which the T. pantotropha biofilms were assayed. We concluded from these results that the cooccurrence of nitrification and denitrification in T. pantotropha biofilms greatly enhanced the latter process. Furthermore, it is evident that the relative stimulation of denitrification by nitrification was highest at the lowest NO_2^- concentrations.

4.5. Reduction of nitrate at high concentrations

Biofilms incubated with NO_3^- showed a rather large spatial separation between the zones of O_2

respiration and N_2O production (Fig. 2A). The apparently inactive zone in the uppermost anoxic layer disappeared when incubated with NO_2^-, and N_2O production then took place immediately below the oxic zone (Fig. 2B). The fact that the organisms in this intermediary zone were capable of reducing added NO_2^- to N_2O suggests that they were metabolically functional also when NO_3^- was supplied, but under these conditions only reduced NO_3^- to NO_2^-. This implies that N_2O production in the presence of C_2H_2 is not necessarily a measure of $NO_3^- + NO_2^-$ reduction. In natural systems the NO_3^- concentration is invariably greater than that of NO_2^-, and the possibility of erroneous NO_3^- reduction measurements using the C_2H_2 inhibition technique exists. However, denitrification measurements in natural biofilms and sediments with the O_2/N_2O microsensor has so far not revealed a separation of the O_2 respiration zone and the denitrification zone [5,6,8,7]. An explanation for this difference might be that in a pure culture biofilm all the cells possess the same regulatory mechanisms for the balance between NO_2^- and NO_3^- reduction. In natural habitats with a complex microbial community a wide variety of regulatory mechanisms exist and reduction of both NO_2^- and NO_3^- can therefore be expected to proceed at any ratio of the 2 species.

4.6. Denitrification in relation to oxygen

Denitrification in P. denitrificans biofilms only occurred below the oxic zone as expected. This was, however, also the case in T. pantotropha biofilms. Based on batch and continuous culture experiments this organism is considered to be able to respire O_2 and NO_2^- simultaneously and to be an aerobic denitrifier [1,2]. The discrepancy between the previous findings and those reported here can be explained by the fact that T. pantotropha has been found to lose the ability to perform aerobic denitrification after growth under culture conditions for extended periods of time [28,33].

For T. pantotropha it has been found that C_2H_2 did not affect either O_2 respiration or denitrification, except for the expected inhibition of N_2O reductase under both aerobic and anaerobic conditions [27]. Similarly it was found that C_2H_2 inhibited aerobic denitrification just as it inhibited anaerobic denitrifi-

cation [28]. Investigations using the acetylene inhibition technique on natural microbial communities indicate, however, that denitrifying activity is coupled with the occurrence of anoxic conditions (e.g., Christensen et al. [35]). Analysis of stratified microbial communities with the N_2O microsensor has never shown any denitrifying activity at O_2 concentrations above 10–15 μM [5–8]. The findings of aerobic denitrification in pure cultures do indicate, however, that special environments may be found where aerobic denitrification constitutes a significant sink for combined nitrogen.

In the present study it has been demonstrated how the occurrence of nitrification together with denitrification at close proximity within the same biofilm can greatly stimulate the latter process. Heterotrophic nitrification turned out to be a quantitative very important process in T. pantotropha growing in artificial biofilms. More work is certainly needed in elucidating the role of this process both on the physiological and ecological level.

Acknowledgements

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