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 O₂ and N₂O was used to record microprofiles of these two species in biofilms where the reduction of N₂O 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 N₂O 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 NO₂⁻ concentrations, part of the NO₂⁻ reduced by *T. pantotropha* biofilms was reduced only to NO₃⁻ and N₂O production thus did not reflect total NO₂⁻ reduction. When NO₂⁻ and no NO₃⁻ was present in the water above the biofilm N₂O production was recorded in the anoxic zone directly below the oxic zone. Nitrous oxide production was never detected in theoxic 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 O₂ consumption whereas this process only consumed about 10% of the O₂ in liquid culture.

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

1. Introduction

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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$_2$O [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$_2$H$_2$) [10,11] has limited the work done with the O$_2$/N$_2$O microsensor on natural communities to focus on denitrification [4–8]. However, heterotrophic nitrification has been shown to be insensitive to C$_2$H$_2$ [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. *Thiobacillus 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, NH₄⁺ and NO₃⁻ (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 NO₃⁻ 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 O₂ and N₂O 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 O₂ and N₂O was linear with a detection limit of 1 μM for N₂O and 0.1 μM for O₂. The signal from the microsensor was higher in stirred than in stagnant medium for identical concentrations. This difference was 10% for O₂ and less than 1% for N₂O. The O₂ 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 O₂ solubility; [16]). The other calibration point was the signal for zero O₂ deep in the biofilm. Nitrous oxide calibration was done by dilution of N₂O saturated water to concentrations close to those encountered in the biofilms. Tabulated values for N₂O solubility were used [17].

The biofilms were incubated with C₂H₂, which blocked N₂O reduction [18,19], and allowed denitrification to be quantified as N₂ production. To maintain a partial pressure of 10 kPa C₂H₂, the medium was continuously sparged with a mixture of 90% air and 10% C₂H₂. 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 O₂ and N₂O 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 N₂O 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]:

$$\frac{\delta C(x,t)}{\delta t} = D(x) \frac{\delta^2 C(x,t)}{\delta x^2} + \left[ \frac{\delta D(x)}{\delta x} + \frac{D(x)}{\phi(x)} \frac{\delta \phi(x)}{\delta x} \right] \frac{\delta C(x,t)}{\delta x} + P(x,t) - R(x,t)$$

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 N₂O concentration in the overlying water was required as the upper boundary condition. The lower boundary condition required a N₂O concentration of zero at the lowest point for which the simulation was run. As the N₂O 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$_3^-$ profile using the NO$_3^-$ concentration in the medium as the upper boundary condition and assuming that the NO$_3^-$ consumption equalled N$_2$O production (in N equivalents).

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

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

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 $D(NO_3^-) = 2.13 \times 10^{-5}$ 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 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%.

![Fig. 1. Average specific O$_2$ uptake rate ($\square$) and penetration depth of O$_2$ (○) 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).](image)

<table>
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<th>Table 1</th>
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<tr>
<td>Rates of oxygen consumption, nitrification, and denitrification in <em>Thiophaera pantotropha</em> and <em>Paracoccus denitrificans</em> biofilms at various NO$_3^-$ concentrations in the medium above the biofilms.</td>
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<tr>
<th>Experiment</th>
<th>NO$_3^-$ in medium (μM)</th>
<th>O$_2$ consumption (nmol O$_2$ cm$^{-2}$ s$^{-1}$)</th>
<th>Nitrification (nmol N cm$^{-2}$ s$^{-1}$)</th>
<th>Denitrification (nmol N cm$^{-2}$ s$^{-1}$)</th>
<th>O$_2$ consumed by nitrification (O$_2$) consumed total</th>
<th>Denitrification (O$_2$ consumption)</th>
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<tr>
<td><em>T. pantotropha</em> (Fig. 3A)</td>
<td>5</td>
<td>0.99</td>
<td>0.34</td>
<td>0.17</td>
<td>0.51</td>
<td>0.17</td>
</tr>
<tr>
<td><em>T. pantotropha</em> (Fig. 3B)</td>
<td>165</td>
<td>0.43</td>
<td>0.14</td>
<td>0.10</td>
<td>0.48</td>
<td>0.23</td>
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<tr>
<td><em>T. pantotropha</em> (Fig. 3C)</td>
<td>497</td>
<td>0.98</td>
<td>0.64</td>
<td>0.55</td>
<td>0.98</td>
<td>0.56</td>
</tr>
<tr>
<td><em>P. denitrificans</em> (Fig. 4)</td>
<td>147</td>
<td>0.27</td>
<td>–</td>
<td>0.07</td>
<td>–</td>
<td>0.26</td>
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The individual experiments are shown in Figs. 3 and 4 as indicated. The medium contained 10 mM acetate and 5.6 mM NH$_4^+$. |
3.2. Growth of immobilized cells

At the beginning of the experiments the penetration depth of O₂ was up to 700 μm but decreased to only 20 μm after 12–15 h (Fig. 1). The decrease in O₂ penetration was due to increased O₂ 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 O₂ cm⁻³ s⁻¹) was obtained by dividing the O₂ consumption rate by the thickness of the O₂ consuming layer, assuming zero order kinetics. The 2 substrates, acetate and NH₄⁺, giving rise to both growth and O₂ consumption, were in excess throughout the experiment. The ratio of acetate to O₂ and NH₄⁺ to O₂ was ca. 40 and 25 respectively in the water outside the biofilm. Since both acetate,
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 \textit{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 \textit{T. pantotropha} was incubated with 20 mM NO$_3^-$ and 10 kPa C$_2$H$_2$. 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$_2$O profile was linear there was no production or consumption of N$_2$O. However, in the region of the biofilm where the N$_2$O profile is non-linear N$_2$O 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$_2^-$ instead of NO$_3^-$ the N$_2$O 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$_2^-$ increased the N$_2$O flux out of the biofilm from 2.11 to 3.14 nmol N cm$^{-2}$ s$^{-1}$.

3.4. Denitrification in \textit{T. pantotropha} biofilms

Denitrification was also measured in \textit{T. pantotropha} biofilms under varying NO$_3^-$ concentrations. The experiment presented in Fig. 3A was conducted with 5 \( \mu \)M NO$_3^-$ in the medium above the biofilm. Even at this extremely low NO$_3^-$ concentration there was a 50 \( \mu \)m thick denitrification zone exhibiting an activity of 35 nmol N cm$^{-2}$ s$^{-1}$. Denitrification rates at higher NO$_3^-$ concentrations are shown in Fig. 3B (165 \( \mu \)M) and Fig. 3C (497 \( \mu \)M). Total rates of denitrification are given in Table 1. The relationship between NO$_3^-$ concentration and denitrification rates can not be compared directly in 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$_3^-$ concentrations (Table 1). The upper boundary of the denitrification zone was found immediately below the oxic zone at all three NO$_3^-$ concentrations (Fig. 3).

3.5. Denitrification and oxygen respiration in \textit{P. denitrificans} biofilms

At the time when the O$_2$ and N$_2$O profiles in Fig. 4 were measured in the \textit{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 NH₄⁺ was added to suppress NO₂⁻ assimilation. Simulation of NO₂⁻ profiles was therefore performed using the NO₂⁻ concentration in the medium above the biofilm as the upper boundary condition and using N₂O production rates (in N-equivalents) as NO₂⁻ consumption rates. In the P. denitrificans biofilms NO₂⁻ was calculated to penetrate to just below the lower edge of the denitrifying zone. A perfect match between the simulated NO₂⁻ penetration and the analyzed lower edge of the denitrification zone was obtained when a value of 144 μM, instead of the measured NO₂⁻ concentration in the medium (147 μM), was incorporated in the model. When using the NO₂⁻ concentration in the overlying water as the upper boundary condition for the T. pantotropha biofilms, however, the simulated NO₂⁻ concentration profile decreased to zero well above the lower limit of the denitrification zone. In order to make the modelled NO₂⁻ profile reach the bottom of this zone it was necessary to assume that NO₂⁻ was also produced in the biofilm. Nitrification in T. pantotropha requires O₂ [25] and hence this process was assumed to take place only in the oxic zone of the biofilms. It was also assumed that NH₄⁺ was only oxidized as far as NO₂⁻ and that it proceeded at the same rate throughout this zone. Nitrification rates in the model were adjusted until NO₂⁻ 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 O₂ uptake only allowed determination of growth rate with O₂ 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 Q₁₀ 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 N₂O profiles it was assumed that the diffusion coefficient for N₂O was the same in the biofilm as in the overlying water; a similar assumption was also made for NO₂⁻ when calculating NO₂⁻ 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 $\text{N}_2\text{O}$ in the presence of $\text{C}_2\text{H}_2$. This has been verified in other studies using liquid cultures [27,28].

Diffusion of gases is driven only by concentration gradients, whereas the diffusion of ions is also affected by electrostatic interactions. In a $\text{NO}_2^-$ 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 $\text{NO}_2^-$ [29]. In the experiment with immobilized P. denitrificans, the significance of this phenomenon could be investigated. This bacterium does not nitify [2] and the $\text{NO}_2^-$ profile that develops results from the interplay between diffusion and consumption of $\text{NO}_2^-$ by denitrification. Based on the modelled denitrification activity, the $\text{NO}_2^-$ profile was estimated and the $\text{NO}_2^-$ concentration in the water was calculated to be $144 \mu\text{M}$, whereas the measured $\text{NO}_2^-$ concentration was $147 \mu\text{M}$. This calculation was performed assuming that the ratio of diffusion coefficients for $\text{N}_2\text{O}$ and $\text{NO}_2^-$ was the same in the P. denitrificans biofilms as in the water and the close match between estimated and measured $\text{NO}_2^-$ 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 $\text{C}_2\text{H}_2$. Robertson and Kuenen [25] found that the ammonium monooxygenase of T. pantotropha showed a number of similarities with that of autotrophic nitrifiers (light sensitivity, Mg$^{2+}$ requirement, NAD(P)H utilization) but also differences (e.g. inhibition by NH$_4^+$). The ammonium oxygenase of the autotrophic nitrifier Nitrosomonas europaea is inhibited by $\text{C}_2\text{H}_2$ [10,30] so the observed insensitivity to $\text{C}_2\text{H}_2$ reported here is yet another difference. Insensitivity of nitrification to $\text{C}_2\text{H}_2$ 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 $\text{C}_2\text{H}_2$ [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:

$$2\text{NH}_4^+ + 3\text{O}_2 \rightarrow 2\text{NO}_2^- + 2\text{H}_2\text{O} + 4\text{H}^+$$

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 O₂ consumption by nitrification to total O₂ 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 (N₂H₄) 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 O₂ 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 O₂ 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 NO₂⁻ concentrations rendered direct comparison of denitrification rates difficult. However, using O₂ 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 NO₂⁻ concentration (Table 1). A more direct comparison can be made between the rates in Fig. 3A and 3C since O₂ uptake rates (Table 1) and O₂ penetration depth were almost identical. The NO₂⁻ concentrations differed by a factor of 100, whereas the denitrification rates only differed by a factor of 3.2. Nitrification in theoxic zone of the biofilms, however, explains the relatively small effect of changing NO₂⁻ concentrations. At all applied concentrations of NO₂⁻, the *T. pantotropha* biofilms showed higher rates of nitrification than denitrification and therefore a net flux of NO₂⁻ out of the biofilms. This net flux was a result of 2 opposing fluxes; one being the flux of NO₂⁻ into the biofilm from the medium, which increased with increasing NO₂⁻ concentration. The other was the flux out of the biofilm of NO₂⁻ produced by nitrification. When the NO₂⁻ concentration in the medium was elevated the thickness of the denitrifying zone increased. The diffusion distance for NO₂⁻ from the nitrifying zone into the denitrifying zone hence increased and a relatively larger fraction of nitrified NO₂⁻ diffused out of the biofilm.

In *P. denitrificans* all NO₂⁻ 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 O₂ concentration in the medium was lower than when the profiles in the *T. pantotropha* biofilms were measured. This reduced the O₂ 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 NO₂⁻ concentrations.

4.5. Reduction of nitrate at high concentrations

Biofilms incubated with NO₃⁻ showed a rather large spatial separation between the zones of O₂
respiration and N₂O production (Fig. 2A). The apparently inactive zone in the uppermost anoxic layer disappeared when incubated with NO₂⁻, and N₂O production then took place immediately below theoxic zone (Fig. 2B). The fact that the organisms in this intermediary zone were capable of reducing added NO₃⁻ to N₂O suggests that they were metabolically functional also when NO₃⁻ was supplied, but under these conditions only reduced NO₃⁻ to NO₂⁻. This implies that N₂O production in the presence of C₂H₂ is not necessarily a measure of NO₃⁻ + NO₂⁻ reduction. In natural systems the NO₃⁻ concentration is invariably greater than that of NO₂⁻, and the possibility of erroneous NO₃⁻ reduction measurements using the C₂H₂ inhibition technique exists. However, denitrification measurements in natural biofilms and sediments with the O₂/N₂O microsensor has so far not revealed a separation of the O₂ 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 NO₃⁻ and NO₂⁻ reduction. In natural habitats with a complex microbial community a wide variety of regulatory mechanisms exist and reduction of both NO₂⁻ and NO₃⁻ 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 theoxic 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 O₂ and NO₂⁻ 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 C₂H₂ did not affect either O₂ respiration or denitrification, except for the expected inhibition of N₂O reductase under both aerobic and anaerobic conditions [27]. Similarly it was found that C₂H₂ inhibited aerobic denitrification just as it inhibited anaerobic denitrification [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 N₂O microsensor has never shown any denitrifying activity at O₂ 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.

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References


