Evaluating heterotrophic growth in a nitrifying biofilm reactor using fluorescence *in situ* hybridization and mathematical modeling

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Abstract The objective of this study was to evaluate the significance of heterotrophic growth in nitrifying biofilm reactors fed only with ammonium as an energy source. The diversity, abundance and spatial distribution of nitrifying bacteria were studied using a combination of molecular tools and mathematical modeling, in two biofilm reactors operated with different hydraulic retention times. The composition and distribution of nitrifying consortia in biofilms were quantified by fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes combined with confocal laser scanning microscopy (CLSM) and digital image analysis. Autotrophic and heterotrophic biofilm fractions determined by FISH were compared to the output from a multispecies model that incorporates soluble microbial products (SMP) production/consumption. In reactor R1 (short retention time) nearly 100% of the total bacteria could be identified as either ammonia- or nitrite-oxidizing bacteria by quantitative FISH analyses, while in reactor R2 (long retention time) the identification rate was only 73%, with the rest probably consisting of heterotrophs. Mathematical simulations were performed to evaluate the influence of the hydraulic retention time (HRT), biofilm thickness, and substrate utilization associated SMP production on the growth of heterotrophic bacteria. The model predicts that low HRTs resulted in a lower availability of SMPs leading to purely autotrophic biofilms. These model predictions are consistent with experimental observations. At HRTs that are about an order of magnitude larger than the reciprocal of the net maximum growth rate the majority of the active biomass will grow suspended in the bulk phase rather than in the biofilm.
Keywords Nitrification; biofilm; fluorescence *in situ* hybridization; mathematical modeling

Introduction

The so-called nitrification process, that is the catalytic oxidation of ammonia to nitrite and then to nitrate, is carried out by chemolithoautotrophic ammonia and nitrite oxidizing bacteria, which are therefore summarized as nitrifying microorganisms. Nitrifying microorganisms reduce inorganic carbon to form cell mass and excrete organic carbon (soluble microbial products, SMP) into the medium that supports heterotrophic growth. Coexistence of nitrifiers and heterotrophs has been found in autotrophic nitrifying suspended cultures (Rittmann *et al.*, 1994) and biofilms (Kindaichi *et al.*, 2004) grown without an external organic carbon source. This feature of nitrifying microorganisms has already been considered in mathematical models used to describe the competition for oxygen and space between autotrophs and heterotrophs (Furumai and Rittmann, 1994, and Lu *et al.*,

2001). In this work, heterotrophic growth in a nitrifying biofilm reactor was evaluated using fluorescence *in-situ* hybridization (FISH) and mathematical modeling.

Materials and methods

Experimental set-up and routine analyses

Two laboratory-scale circulating bed biofilm reactors of 1.2 l each, described in detail elsewhere (Nogueira *et al.*, 2002), were employed for this study. The operating conditions are presented in Table 1. The temperature was maintained at 30 °C and the pH was controlled at 7.5 with NaOH (1 M). High density polyethylene granulate with a particle size of 1 mm and a density of 731 kg/m^3 was used as support material for biofilm growth. Ammonium, nitrite and nitrate ions were determined photometrically (LCK, Dr. Lange). Biofilm mass was measured gravimetrically according to APHA (1995).

FISH and confocal laser scanning microscopy

In situ hybridization of cells in the biofilm was performed with fluorescently labeled rRNA-targeted oligonucleotide probes according to the method of Manz *et al.* (1992). First the samples were hybridized with a EUB338 probe set (EUB338, EUB338-II, EUB338-III) designed to target almost all bacteria (Daims *et al.*, 2001). Then, within this domain, the beta and gamma-subclasses of *Proteobacteria* were labeled with the respective group specific probes Bet42a and Gam42a (Manz *et al.*, 1992). Within the beta-subclasses, in turn, the ammonia-oxidizing bacteria (AOB) were detected using the following probes: (i) Nso1225 and Nso190, which are specific for all ammonia-oxidizers in the beta subclass *Proteobacteria* (Mobarry *et al.*, 1996); (ii) Neu, which is specific for halophilic and halotolerant *Nitrosomonas* spp. and *Nitrosococcus mobilis* (Wagner *et al.*, 1995); and (iii) NmV, specific for the *Nitrosococcus mobilis* lineage (Juretschko *et al.*, 1998). The following probes were used to detect nitrite-oxidizing bacteria (NOB): (i) Nit3, which is complementary to a sequence region of all *Nitrobacter* species (Wagner *et al.*, 1996); (ii) Ntspa712, specific for most members of the phylum *Nitrospira* (Daims *et al.*, 2001); and (iii) Ntspa662, specific for the genus *Nitrospira* (Daims *et al.*, 2001). For quantification of the different probe-targeted bacteria, simultaneous hybridizations were performed with Cy3 labeled specific probes and the Cy5 labeled bacterial probe set. For each confocal image, the relative cellular area was determined, defined as the ratio of the area of those cells labeled by the specific probe versus the area of all bacteria stained by the EUB338 probe set. Fluorescence signals were recorded with an LSM 510 confocal laser scanning microscope (Zeiss, Germany) equipped with two HeNe lasers (543 nm and 633 nm) for detection of Cy3 or Cy5.

Table 1 Operating conditions and performance of reactors R1 and R2 at steady-state[*]

↓ Parameters	Reactors →	R1	R2
Hydraulic retention time[†], *HRT* (d)		0.0175	0.13125
Influent C_{N-NH4+} (mg/l)		39	250
Effluent C_{N-NH4+} (mg/l)		1.8	0.4
Effluent C_{N-NO2-} (mg/l)		1.9	0.6
Effluent C_{N-NO3-} (mg/l)		35	249
Biofilm mass C_{BF} (mg/l)		2.7	2.7
Suspended biomass C_{bulk} (cell/ml)		1.3×10^6	5×10^6
Biofilm thickness (μm)		33	27

[*]For all parameters average values are given
[†]Retention time is defined as reactor liquid volume divided by effluent flow rate

Mathematical modeling

Mathematical modeling was used to evaluate mechanisms of competition between autotrophic and heterotrophic biomass within the biofilm and in the bulk phase of the biofilm reactor. Three particulate fractions were considered. The autotrophic biomass (X_A) is a combination of AOB and NOB. Organic substrate for the growth of the heterotrophic biomass (X_H) was derived solely from soluble microbial products (SMP) produced during autotrophic growth. Inert particulate matter (X_I) was the result of inactivation of X_A and X_H. Substrate utilization associated production of SMPs was modeled for autotrophic growth where $f_{UAP,A}$ ($g_{COD,SMP}/g_{COD,biomass}$) is the stoichiometric parameter linking the rate of SMP production to the growth of X_A. A one-dimensional biofilm structure was assumed with mass transport of soluble substrates (oxygen, ammonium, SMP) by diffusion and mass transport of particulate matter (X_A, X_H, X_I) by advection as a result of volume expansion due to growth. The rate of surface detachment was adjusted to result in different biofilm thicknesses. Detached biomass remained active and was suspended in the completely mixed bulk water. Basic model parameters were taken from Wanner and Reichert (1996). Simulations were performed using AQUASIM (Wanner and Morgenroth, 2004). All simulations were performed with a constant ammonia loading rate of $1.3\,g_N/d$, and result in similar conditions as in the experimental part of this study.

Results and discussion

Microbial community characterization

All AOB detected in this study belonged to beta-subclass *Proteobacteria*, of which at least two distinct populations were present in both reactors. One of these populations was closely related to the model organism *Nitrosomonas europaea*, while the other population showed no close relationship with any recognized ammonia-oxidizers. Nitrite oxidation was performed in both reactors exclusively by *Nitrospira*-like bacteria. Nitrifying populations detected by the different probes were quantified and compared on the basis of their cellular area. These analyses revealed that in both reactors R1 and R2 NOB (probe Ntspa662) made up about 50% of the total bacteria (EUB338 probe set). For AOB (probe Nso1225), however, the relative abundance was considerably smaller in R2 (long retention time) biofilm samples (23%) than in R1 (short retention time, 53%). Thus, while in reactor R1 nearly 100% of the total bacteria could be identified as either AOB or NOB, this rate was only 73% in reactor R2. The missing 27% are probably heterotrophic bacteria that grew on soluble microbial products (SMP) excreted by nitrifiers in the biofilm (Furumai and Rittmann, 1994). Experimental results of spatial distribution of nitrifiers in the biofilm (Figure 1) demonstrate that the nitrifying populations are uniformly distributed in reactor R2 biofilm. The AOB in reactor R1 displayed a small decrease in relative abundance towards the interior of the biofilm. These experimental results suggest that concentration gradients for soluble substrates into these relatively thin biofilms must not have been of significance.

### Simulation results compared with FISH analyses

Experimental results showed that the reactor operated with a larger HRT (at the same ammonium loading rate) had a larger heterotrophic biomass fraction. The purpose of the mathematical modeling was to evaluate: (a) under what conditions X_H can grow in the biofilm system; and (b) under what conditions X_H will mainly grow in suspension rather than in the biofilm.

The amount of SMP produced due to autotrophic growth will determine the growth potential for heterotrophic biomass in the system. In Figure 2, the amount and location of heterotrophic biomass in the system is shown as a function of $f_{UAP,A}$ and HRT. It can be
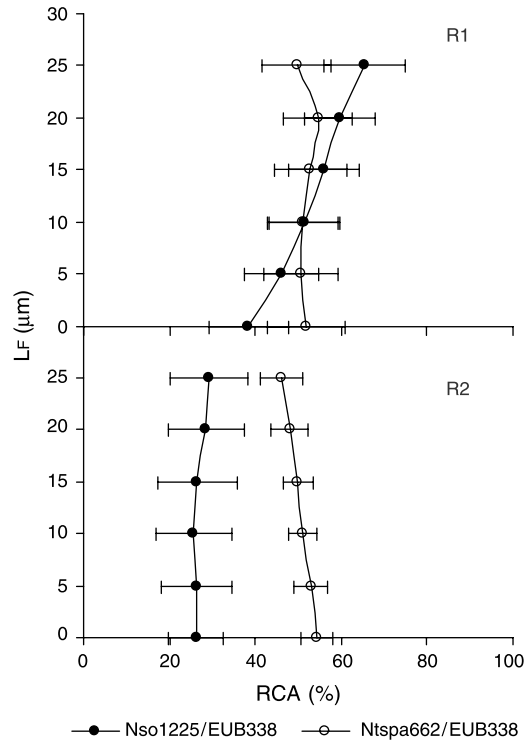
Figure 1 Averaged profiles of nitrifying bacteria over the thickness of the biofilm where RCA is the relative cellular area and L_F is the distance from the substratum. Data points are the average $\pm$ 95% confidence interval

seen that there is a lower limit for $f_{UAP,A}$ below which SMP production is insufficient to support heterotrophic growth. For lower HRTs, SMPs are washed out of the reactor faster and the total amount of ammonia converted decreases (data not shown). Comparing the different HRTs in Figure 2 shows that this minimum $f_{UAP,A}$ required to support heterotrophic growth increases for decreasing HRTs. For example for $f_{UAP,A} = 0.2\,g_{COD}/g_{COD}$ the heterotrophic biomass is negligible for a HRT of 0.0175 d while there is a significant amount of X_H for larger HRTs. Thus, a reduced availability of SMP in R1 could potentially explain the observed experimental results with a larger fraction of X_H in R2. However, mechanisms and kinetics of SMP production are not well understood and values for $f_{UAP,A}$ in the literature vary between 0.25 (Furumai and Rittmann, 1992) and $1.56\,g_{COD}/g_{COD}$ (Lu *et al.*, 2001). The extent of SMP production (i.e., $f_{UAP,A}$) is strongly correlated
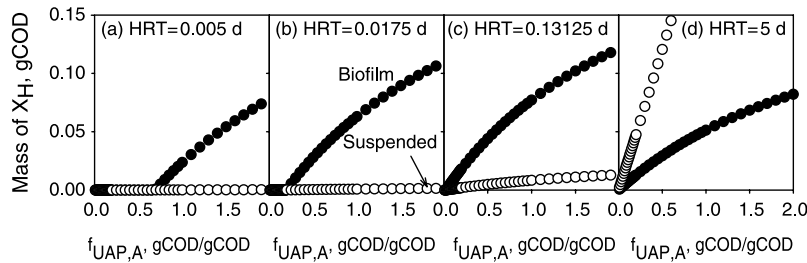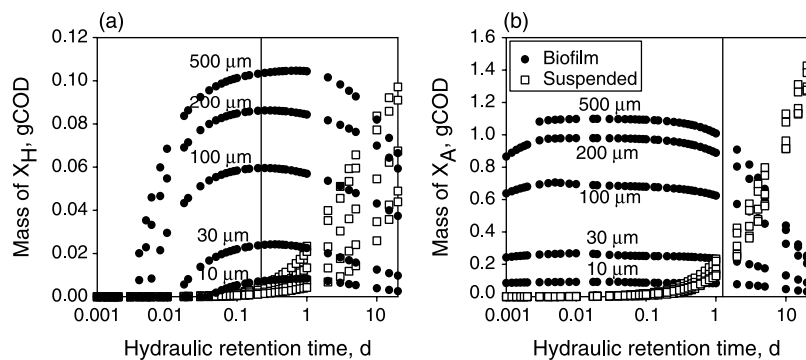

Figure 2 Amount of X_H in the biofilm (●) and suspended in the bulk phase (○) as a function of SMP production due to autotrophic growth ($f_{UAP,A}$) for different hydraulic retention times. HRTs used in the experimental part of this study were 0.0175 d (R1) and 0.131 d (R2) (simulations for a biofilm thickness of 30 μm)

with the amount of X_H in the system (Figure 2); however, the relationship between concentration of SMP in the effluent and the amount of X_H is very weak (data not shown). For the case of HRT = 0.0175 d the concentration of SMP in the effluent increased only from $1.3\,\text{mg}_{COD}/\text{l}$ to $1.4\,\text{mg}_{COD}/\text{l}$ for $f_{UAP,A}$ increasing from $0.2\,\text{g}_{COD}/\text{g}_{COD}$ to $2\,\text{g}_{COD}/\text{g}_{COD}$ – the majority of SMP produced inside the biofilm are directly used up and never reach the bulk phase. Thus, for actual biofilm systems it will be difficult to predict the existence of heterotrophic growth based on bulk phase measurements of the organic substrate.

In Figure 3(a) the amount of X_H in the biofilm and in suspension are shown as a function of HRT for different biofilm thicknesses. It can be seen that the majority of X_H was located in the biofilm for HRTs smaller than 1 d. For increasing HRTs the rate of washout from the bulk phase decreases resulting in a corresponding increase of X_H growing in suspension and suspended X_H dominate the system for HRTs larger than 10 d. Van Benthum et al. (1997) had evaluated the location of X_H in a similar biofilm airlift suspension reactor and had observed that heterotrophic biofilms were mainly found for HRTs smaller than the reciprocal max growth rate of heterotrophic biomass. The HRT of 10 d where the transition between growth in the biofilm and in suspension occurred in our simulations is significantly larger than the reciprocal of the maximum specific growth rate of X_H (= 0.22 d). At HRTs between 1 d and 10 d heterotrophic biomass in the biofilm and in suspension coexist. For very low HRTs (< 0.001 d) the total amount of X_H decreases in the system. Suspended X_H are reduced due to increased rates of washout with decreasing HRTs. Decreased HRTs also result in an increased washout of SMP that are produced in the system. Modeling results show that the extent and location of X_H in the system are dependent on the HRT. In Figure 3(b) the mass of X_A in the biofilm and suspended in the bulk phase are shown. It can be seen that the mass of X_A in the biofilm is virtually independent of HRT for HRTs smaller than the reciprocal of the net maximum growth rate. For larger HRTs the amount of X_A growing in the bulk phase increases.

Biomass and substrate concentrations within the biofilm are shown in Figure 4. To illustrate a potential layering of biomass the case of a thick biofilm with a significant SMP production was simulated. Autotrophic biomass dominates at the surface of the biofilm while the concentrations of X_H increase towards the base of the biofilm. Such a biomass distribution is different from what has been reported in the literature (Wanner and Reichert, 1996; van Benthum et al., 1997) where heterotrophic biomass was expected to

Figure 3 The amount of heterotrophic biomass (a) and autotrophic biomass (b) in the biofilm (●) and suspended in the bulk phase (○) as a function of the HRT and biofilm thickness. The vertical line corresponds to the reciprocal maximum net growth rate where washout of suspended biomass would be expected for a completely mixed reactor without a biofilm (simulations with $f_{UAP,A} = 0.25\,\text{g}_{COD}/\text{g}_{COD}$)
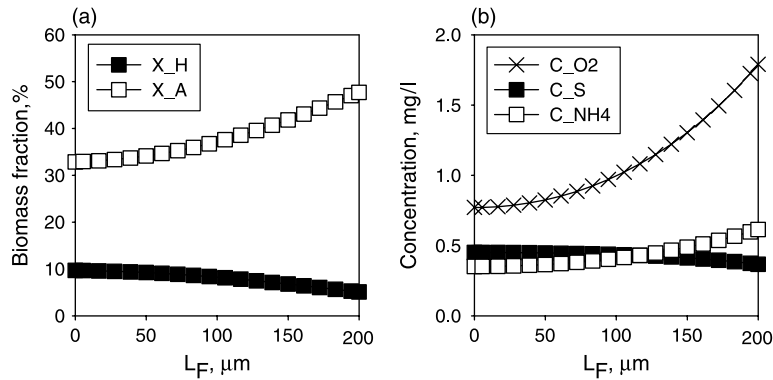
Figure 4 Biomass and substrate profiles over the thickness of the biofilm where L_F is the distance from the substratum (simulations for a biofilm thickness of 200 μm, HRT = 0.001 d, $f_{UAP,A}$ = 1.56 g_{COD}/g_{COD})

grow predominantly towards the surface of the biofilm. The difference in layering of X_A and X_H can be explained by the low growth rates of X_H that are limited by SMP production. Substrate concentrations are shown in Figure 4(b). The concentrations of SMP increase towards the base of the biofilm as SMP are being produced within the biofilm resulting in a flux of SMP towards the surface of the biofilm and into the bulk water.

Conclusions

In the reactor operated with short retention time, about 100% of the biofilm consisted of nitrifiers, as compared to only 73% in the long retention time reactor.

At low HRTs, less SMP are available for heterotrophic growth resulting in purely autotrophic biofilms. This model prediction is consistent with the experimental observations.

At HRTs that are about an order of magnitude larger than the reciprocal of the net maximum growth rate the majority of the active biomass will grow suspended in the bulk phase rather than in the biofilm.

Acknowledgements

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