Study of nitrifying bacteria diversity in biofilms using a molecular approach

Regina Nogueira*, Ulrike Purkhold, Luís F. Melo and Michael Wagner

Centro de Engenharia Biomédica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

*Correspondence to: regina@deb.uminho.pt

Summary

The purpose of this work was to investigate the microbial community structure of nitrifying biofilms. Biofilm samples were collected from two reactors operated with distinct retention times. The composition and spatial distribution of nitrifying consortia in biofilms was quantified by fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes combined with confocal laser scanning microscopy (CLSM), and digital image analysis. High-resolution analyses of ammonia-oxidizer diversity in both reactors were performed using the gene that encodes the catalytic subunit of the ammonia-monoxygenase enzyme (amoA) as a marker.

At least two populations of beta-subclass ammonia-oxidizing bacteria (AOB) were detected in both reactors. As demonstrated by oligonucleotide probing and comparative amoA sequence analysis, one of these populations was closely related to the model organism Nitrosomonas europaea, while the other population surprisingly showed no close relationship with recognized ammonia-oxidizers. Nitrospira-like bacteria was the dominant nitrite-oxidizing bacteria (NOB) in the biofilm reactors studied. According to our results biofilms formed in the two studied reactors with distinct retention time were similar in their microbial diversity and spatial distribution of AOB and NOB populations. Differences occurred, however, in the relative abundance of AOB, which was higher in the reactor operated with shorter retention time. Despite of the fact that the environmental conditions within the reactors represented a common situation, the bacterial diversity was surprisingly diverse.

Keywords: Nitrification; Biofilm; Fluorescence in situ hybridization; Confocal laser scanning microscopy
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. In view of their biological systematics, cultured ammonia-oxidizing bacteria (AOB) comprise two monophyletic groups within the Proteobacteria (Koops et al., 2003): Nitrosococcus oceanus and N. halophilus belong to the gamma subclass of the class Proteobacteria, while the members of the genera Nitrosomonas, Nitrosospira, Nitrosovibrio and Nitrosolobus constitute a closely related assemblage within the beta subclass of Proteobacteria. Cultured nitrite-oxidizing bacteria (NOB) have been assigned to the four recognized genera Nitrobacter, Nitrospina, Nitrococcus and Nitrospira. While the genus Nitrobacter is a member of the alpha subclass of Proteobacteria, the genera Nitrospina and Nitrococcus belong to the delta and gamma subclasses of Proteobacteria, respectively. The remaining genus, Nitrospira is a member of the Nitrospira phylum of the domain Bacteria.

Biofilms are ubiquitous in nature and can be defined as surface growth of microbial cells embedded in extracellular polymeric substances (Davey and O'Toole, 2000). They are of great practical importance for technologies such as water and wastewater treatment. Methods to analyze the relevant populations in biofilms must meet at least two requirements: firstly to reveal a comprehensive picture of the community structure, i.e. of the identity and abundance of its members, and secondly to detect their spatial arrangements inside the biofilm. The past decade has witnessed the development of several new techniques to elucidate the microbial community structure of biofilms. Examples include: fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy (CLSM). The combination of these two techniques allows the detection, localization and quantification of microorganisms directly in the biofilm (Schramm et al., 1999; Daims et al., 2001).

Molecular fine scale diversity analysis of ammonia-oxidizing populations in various environments has extensively been made using the gene that encodes the catalytic subunit of the ammonia-mono-oxygenase enzyme (amoA) as a phylogenetic marker (Purkhold et al., 2000). This molecular tool can give a more detailed picture of AOB
diversity than FISH analysis since the number of available rRNA oligonucleotide probes is still limited. However, quantitative information on the community composition and structure is lost.

In wastewater treatment systems, the susceptibility of nitrifying bacteria for environmental disturbances makes nitrification the unstable step. Thus, in order to ensure a stable performance of the treatment processes, it is necessary to investigate their microbial community structure. Nitrifying bacteria are slow-growing microorganisms and recalcitrant to cultivation attempts (Koops et al., 2003). Therefore, only after the development of FISH nitrifying bacteria could be monitored in natural and engineered systems almost under real time conditions.

In this work, we investigated the microbial community structure of nitrifying biofilms formed in biofilm reactors with distinct hydraulic retention times (i.e. the average residence time of the liquid flowing through) using molecular tools, fluorescence microscopy and digital image analyses. The specific objectives were: (i) to study the composition of nitrifying biofilms using a comprehensive set of rRNA-targeted oligonucleotide probes for FISH, (ii) to carry out a fine-scale diversity analyses of ammonia-oxidizers, using the gene that encodes the catalytic subunit of the ammonia-monooxygenase enzyme (amoA) as a marker and iii) to determine the spatial distribution and abundance of nitrifying bacteria in the biofilm using a combination of FISH and CSLM techniques.

Materials and Methods

Experimental set-up

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 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. The operating conditions are presented in Table 1.
Table 1. Operating conditions and performance of reactors R1 and R2 at steady-state *

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Reactors →</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydraulic retention time, $HRT / h$</td>
<td></td>
<td>1.0</td>
<td>6.1</td>
</tr>
<tr>
<td>ammonium-nitrogen loading rate, $B_{NH_4}^+ / g \cdot L^{-1} \cdot d^{-1}$</td>
<td></td>
<td>0.93</td>
<td>1.02</td>
</tr>
<tr>
<td>influent concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ammonium-nitrogen, $CB_{NH_4}^+ / mg \cdot L^{-1}$</td>
<td></td>
<td>39</td>
<td>250</td>
</tr>
<tr>
<td>effluent concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ammonium-nitrogen, $C_{NH_4}^- / mg \cdot L^{-1}$</td>
<td></td>
<td>1.8</td>
<td>0.4</td>
</tr>
<tr>
<td>nitrite-nitrogen, $C_{NO_2}^- / mg \cdot L^{-1}$</td>
<td></td>
<td>1.9</td>
<td>0.6</td>
</tr>
<tr>
<td>nitrate-nitrogen, $C_{NO_3}^- / mg \cdot L^{-1}$</td>
<td></td>
<td>35</td>
<td>249</td>
</tr>
<tr>
<td>biofilm mass concentration, $C_{RX} / mg \cdot L^{-1}$</td>
<td></td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>suspended biomass concentration, $C_{RX} / cell \cdot mL^{-1}$</td>
<td></td>
<td>$1.3 \times 10^6$</td>
<td>$5 \times 10^6$</td>
</tr>
</tbody>
</table>

* For all parameters average values are given; retention time is defined as reactor volume divided by effluent flow rate.

**Fluorescence in situ hybridization**

Biofilm particles sampled from both reactors were immediately fixed in 4% paraformaldehyde as described previously (Amann et al., 1990). *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). *In situ* characterization of microbial populations followed a top to bottom approach: First the samples were hybridized with a EUB338 probe set (EUB338, EUB338-II, EUB338-III) designed to target almost all bacteria (Daims et al., 1999). Then, within this domain, the beta and gamma-subclasses of Proteobacteria were labeled with the respective group specific probes Bet42a and Gam42a previously published by Manz et al. (1992). Within the beta-subclasses, in turn, the ammonia-oxidizing bacteria 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: (i) Nit3, which is complementary to a sequence region of all Nitrobract 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). The probe Non338, which is complementary to probe EUB338, was used to control the binding specificity of EUB338 in the FISH experiments. The whole particles with adhering biofilm were subjected to FISH. Simultaneous hybridization with several probes was performed in subsequent steps by first hybridizing with the probe of higher stringency.

Oligonucleotide probes labeled with the fluorescent sulfoindocyanine dyes Cy3 and Cy5 were obtained from Interactiva (Ulm, Germany).

**Microscopy**

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. Images were acquired in stacks consisting of horizontal (xy) sections with a vertical step interval of 5 μm. Each stack contained 6 sections. The objective used was a 63×/1.4 NA Plan Apochromat oil immersion objective.

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 (RCA) 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. Determination was carried out semi-automatically using the procedure described previously (Daims et al., 2001).

Biofilm thickness was determined for fresh, unfixed biofilm samples stained with a 0.25 g·L⁻¹ fluorescein isothiocyanate (FITC) solution for
3 h at room temperature, using CLSM optical sectioning in the sagittal (xz) direction. The objective used was a 40×/1.2 W corr C-Apochromat water immersion objective.

**Comparative sequence analyses of the amoA gene**

To supplement results from FISH, high-resolution analyses of ammonia-oxidizer diversity in both reactors were performed using the gene that encodes the catalytic subunit of the ammonia-mono-oxygenase enzyme (amoA) as a marker. Amplification, cloning, sequencing and phylogenetic analyses of the biofilm-derived amoA fragments were performed as described by Purkhold et al. (2000).

**Routine analyses**

Ammonium, nitrite and nitrate ions were determined photometrically (LCK, Dr. Lange). Biofilm solids were measured gravimetrically according to APHA (1995) using 0.22 μm membrane filters. Prior to this analysis, the biofilm was detached from the support material by treating it 120 s at 50 W with an ultrasonic homogenizer (Bandelin electronics D-1000, Berlin). The bacteria cell numbers in the effluent were counted with DAPI staining as described by Wagner et al. (1995).

**Results**

**General observations**

Table 1 summarizes the operating conditions and experimental results obtained at steady state operation. The reactor operated with a higher retention time (R2) presented lower effluent concentrations of ammonium and nitrite, though the ammonium load and biofilm mass concentration were identical in both reactors. This result might be explained by some nitrifying activity of the suspended biomass, the concentration of which was higher in reactor R2 than in R1 due to the longer retention time.
Diversity of nitrifying bacteria

The hybridization of biofilm samples from both reactors with a comprehensive probe set revealed the presence of at least two populations of ammonia-oxidizing bacteria (AOB) and one population of nitrite-oxidizing bacteria (NOB). The AOB in all biofilm samples could be labeled simultaneously with probes BET42a, Nso1225 and Nso190, but not with Gam42a. This result indicates that all occurring AOB were affiliated with the beta-subclass Proteobacteria. Among these, no ammonia-oxidizers of the Nitrosospira-cluster were detected. A fraction of the AOB was detectable with probe NEU specific for halophilic and halotolerant species of Nitrosomonas as well as Nitrosococcus mobilis. Since the latter was shown to be absent by probe NmV, the NEU-positive population of ammonia-oxidizers is most likely affiliated with the Nitrosomonas europaea/eutropha group (Wagner et al., 1998).

Comparative sequence analyses of amoA clones derived from the biofilm independently confirmed the presence of two different groups of ammonia-oxidizers. One amoA sequence cluster is closely related to Nitrosomonas europaea, most likely representing the NEU-positive ammonia-oxidizers, while the other amoA cluster is not closely related with any described ammonia-oxidizer reference strain and might correspond to the NEU-negative fraction of beta-subclass Proteobacteria AOB mentioned above.

In both reactors the only NOB detected were of the genus Nitrospira, as identified by hybridization with probes Ntspa712 and Ntspa662.

Abundance of nitrifying bacteria

In both reactors, ammonia and nitrite oxidizing populations formed microbial biofilm aggregates. Nitrifying populations detected by the different probes were quantified and compared on the basis of their cellular area (Table 2). 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.
Within the total AOB (probe Nso1225) the portion affiliated with the *Nitrosomonas europaea/eutropha* group (NEU probe) was similar in both reactors, 66% and 54%, for R1 and R2, respectively.

Table 2. Microbial community composition of reactors R1 and R2 biofilm, quantified as relative cellular area (RCA). Values are the average ± 95% confidence interval

<table>
<thead>
<tr>
<th>RCA % → AOB</th>
<th>Nso1225/EUB338</th>
<th>Neu/Nso1225</th>
<th>NOB</th>
<th>Ntspa662/EUB338</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>53 ± 4</td>
<td>66 ± 3</td>
<td>52 ± 4</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>23 ± 2</td>
<td>54 ± 4</td>
<td>50 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

*Spatial distribution of nitrifying bacteria*

In order to address the question of how nitrifiers are distributed in the biofilm, biofilm thickness and hydrated samples, stained with FITC, was measured by optical sectioning in the sagittal (xz) direction. The results revealed no significant difference in biofilm thickness between samples obtained from reactors R1 (33 μm ± 5 μm) and R2 (27 μm ± 3 μm). Figure 1 depicts the spatial distribution of nitrifiers in the biofilm, determined by optical sectioning in the xy direction. These experimental results demonstrate that the nitrifying populations are uniformly distributed in both reactor’s biofilms, except for the NEU-positive AOB in reactor R1, which displayed a small decrease in relative abundance towards the interior of the biofilm.

In order to study the spatial relationship between AOB and NOB dual hybridization of biofilm samples with both Nso1225 and Ntspa662 probes was carried out. It was found that both populations formed microbial aggregates in the vicinity of each other. The size of NOB aggregates was bigger than those of AOB.

CLSM pictures of biofilm particles labeled with the EUB338 probe set (total bacteria) revealed that only 20% of the polyethylene particles’ surface was colonized by bacteria. The particles had a relatively smooth surface and biofilm growth was mainly observed in cavities and ridges.
Figure 1. Averaged profiles of nitrifying bacteria: relative cellular area (RCA) as a function of biofilm depth (DB). Data points are the average ± 95% confidence interval.

Discussion

The microbial community structure of a biofilm encompasses the community's composition and spatial arrangement. In this work we studied the diversity, abundance and spatial distribution of nitrifying bacteria in two biofilm reactors operated with distinct retention times, using a combination of molecular tools and CLSM.

Composition of the microbial community

All AOB detected in this study belonged to beta-subclass Proteobacteria, of which at least two distinct populations were present in both reactors. As demonstrated by oligonucleotide probing and comparative amoA sequence analysis, one of these populations was
closely related to the model organism *Nitrosomonas europaesa*, while the other population surprisingly showed no close relationship with any recognized ammonia-oxidizers. Nitrite oxidation was performed in both reactors exclusively by *Nitrospira*-like bacteria confirming the recently recognized importance of these bacteria for nitrite oxidation in several environments (e.g. Juretschko et al., 1998; Schramm et al., 1999; Daims et al., 2001).

Our results show that the diversity of nitrifiers in biofilm reactors is not influenced by retention time, which is one of the most important parameters of reactor operation. The relative abundance of AOB in the biofilm was smaller in the reactor operated with longer retention time (R2). One possible explanation for this result is that the low ammonium-nitrogen concentration in R2 (possibly caused by the higher suspended biomass concentration) fell below the saturation constant for the genera *Nitrosomonas* (Koops et al., 2003), and thus limited the amount of AOB in the biofilm. Unlike the ammonia-oxidizers the relative abundance of nitrite-oxidizers was identical in both biofilm reactors. Although pure cultures of *Nitrospira*-like bacteria are missing as yet, Schramm et al. (1999) estimated their affinity constant in nitrifying aggregates for nitrite-nitrogen to be 0.14 mg · L⁻¹, using a combination of FISH and microsensor techniques. The nitrite-nitrogen concentration in both biofilm reactors was higher than this affinity constant, suggesting that *Nitrospira*-like bacteria were not limited by nitrite, which might explain the identical relative abundance.

Results of FISH demonstrated the co-existence of two ammonia-oxidizing populations in both biofilm reactors. However, close competition would be expected to lead to the exclusion of one of the populations. A possible hypothesis might be a symbiosis between the two populations, which necessarily kept them from outcompeting each other.

For the biofilm from reactor R1, 100 % of the total bacteria (detectable by the EUB338 probe set) could simultaneously be classified by at least one of the applied specific probes (Table 2). In reactor R2, however, this rate was only 73 %. Since it is well known (Rittmann et al., 1994) that exopolysaccharides excreted by nitrifiers can support heterotrophic growth, it is tempting to speculate that a presumably heterotrophic population might account for the missing 27 % (Nogueira et al., 2002).
What remains to be discussed is the spatial distribution of nitrifiers in the biofilm. Several studies reported that AOB and NOB occurred more or less spatially segregated, depending on the availability of substrates inside the biofilm and/or the respective kinetic parameters growth rate and affinity constant (Okabe et al., 1999; Schramm et al., 2000). In this study no significant stratification of AOB and NOB was observed in the biofilm matrix of both reactors. This result can be explained by the small thickness of both reactors' biofilms, which allows them to be fully penetrated by substrates.

Concluding remarks

– Under the conditions of our study the retention time does not affect the diversity of nitrifiers in the biofilm but influences the abundance of ammonia-oxidizing bacteria.

– In the comparably thin biofilms of the studied system no stratification of AOB and NOB occurs, while both form aggregates in close vicinity.

– Despite of the fact that the conditions within the reactors represent a well-studied environment, the bacterial diversity could not be completely described with the available tools.

– Molecular in situ investigations indicate that the species cultured so far in nitrifying environments represent a mere fraction of those really present. Isolation and characterization of new species seems to be one of the most important steps to advance on the characterization of such systems.

References


