Diversity and dynamics of ammonia-oxidizing bacterial communities in a sponge-based trickling filter treating effluent from a UASB reactor


ABSTRACT

Changes in ammonia-oxidizing bacterial (AOB) population dynamics were examined in a new sponge-based trickling filter (TF) post-upflow anaerobic sludge blanket (UASB) reactor by denaturing gradient gel electrophoresis (DGGE), and these changes were linked to relevant components influencing nitrification (chemical oxygen demand (COD), nitrogen (N)). The sponge-based packing media caused strong concentration gradients along the TF, providing an ecological selection of AOB within the system. The organic loading rate (OLR) affected the population dynamics, and under higher OLR or low ammonium-nitrogen (NH$_4^+$-N) concentrations some AOB bands disappeared, but maintaining the overall community function for NH$_4^+$-N removal. The dominant bands present in the upper portions of the TF were closely related to *Nitrosomonas europaea* and distantly affiliated to *Nitrosomonas eutropha*, and thus were adapted to higher NH$_4^+$-N and organic matter concentrations. In the lower portions of the TF, the dominant bands were related to *Nitrosomonas oligotropha*, commonly found in environments with low levels of NH$_4^+$-N. From a technology point of view, changes in AOB structure at OLR around 0.40–0.60 kgCOD m$^{-3}$ d$^{-1}$ did not affect TF performance for NH$_4^+$-N removal, but AOB diversity may have been correlated with the noticeable stability of the sponge-based TF for NH$_4^+$-N removal at low OLR. This study is relevant because molecular biology was used to observe important features of a bioreactor, considering realistic operational conditions applied to UASB/sponge-based TF systems.

Key words | ammonia-oxidizing bacteria (AOB), domestic wastewater, nitrification, polymerase chain reaction-DGGE, post-treatment, UASB/TF system

INTRODUCTION

Trickling filter (TF) is a successful alternative for the post-treatment of anaerobic effluents in developing countries, presenting good efficiency, very low energy consumption, and operational simplicity (Mergaert et al. 1992; Chernicharo 2006). However, ammonium-nitrogen (NH$_4^+$-N) removal in TF post-upflow anaerobic sludge blanket (UASB) reactors and its microbiological aspects need to be better understood. For nitrifying trickling filters (NTFs), Rowan et al. (2003) demonstrated that ammonia-oxidizing bacteria (AOB) community structure was selected in a non-random manner along the system, presenting greater diversity with depth. All the sequences identified were grouped within the genus *Nitrosomonas*. A similar trend was observed by Persson et al. (2002) regarding AOB genus, but they did not find the expected differences in hybridization pattern with depth in the NTF. Whether or not downward substrate-loading patterns within TFs influences AOB diversity, the influence of local conditions on AOB distribution still lacks investigation. In this sense, the packing media and the operational conditions, as well as the form of a multistage treatment process can define the pattern of AOB communities in TFs, and this is probably the case for TFs treating effluents from UASB reactors.

In a simpler flowsheet of TFs post-UASB reactors filled with rock or plastic media the establishment of nitrifying bacteria within the reactor is still challenging (Almeida et al. 2009), even at low organic loading rates (OLRs). It suggests that detachment and growth rates are not in balance for adequate ammonia removal rates.
One of the main limitations related to TF technology is the sludge residence time (SRT) control, a key point in achieving stable nitrification. To overcome SRT control in TF, polyurethane sponges can be applied for microorganism immobilization (Chu & Wang 2011). The mechanical strength of sponges and its potential for biomass retention can avoid high detachment rates, increasing the capability of sustaining slow-growers. A remarkable example using sponges as a medium is downflow hanging sponge (DHS) system post-UASB reactors. In DHS systems, 100 d SRT and sufficient availability of dissolved oxygen within the interior space of sponges are reported, providing an amicable environment for nitrifiers (Araki et al. 1999; Tandukar et al. 2006).

Following a similar perspective, a new sponge-based packing media called Rotosponge is currently in development. In comparison with a plastic-based TF, the results indicate that Rotosponge significantly improves nitrification in the system (Almeida et al. 2015). Despite the attractive results using sponge-based packing media, the AOB population dynamics and its relationship with operational and local environmental conditions have not been elucidated, with little information about relevant communities related to N-transformations. The aim of this study was to contribute to a comprehensive investigation on AOB community developed in a sponge-based TF post-UASB reactor treating domestic wastewater. Polymerase chain reaction (PCR)-denaturating gradient gel electrophoresis (DGGE) and sequencing were used to investigate the diversity and the dynamics of AOB community, and its relationship with OLR, NH₄-N and organic matter concentrations.

**MATERIALS AND METHODS**

**Experimental setup and operational conditions**

A UASB reactor followed by a sponge-based TF (TF-Rotosponge) were monitored over 350 d with the physical and operating characteristics shown in Table 1. The domestic wastewater (raw sewage after screening and grit removal) was from the Arrudas wastewater treatment plant (Belo Horizonte – Brazil). The TF were naturally ventilated biotowers with four compartments filled with Rotosponge packing media (Figure 1(a)). Rotosponge, a sponge-based packing media, is constituted of sponge sheets attached to the surface of corrugated plastic sheets (Figure 1(b)). We defined three operational periods for the TF. During the first, second, and third periods the OLRs (average values) were 0.45 (P₁), 0.55 (P₂) and 0.55 (P₃) kgCOD m⁻³ d⁻¹, respectively. In the third period, OLR was significantly lower than in the first and second periods, and no significant changes between periods 1 and 2 were observed (Kruskal–Wallis test, α = 5%). Because our focus was on NH₄ removal in the TFs while operating the UASB-TF without secondary settlers, we applied a relatively low OLR. The operational conditions applied to the TF are on the same range to real operations, when nitrification is desired.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>UASB reactor</th>
<th>TF-Rotosponge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packing media/reactor volume ratio</td>
<td>m³ m⁻³_reactor</td>
<td>–</td>
<td>0.49</td>
</tr>
<tr>
<td>Reactor height</td>
<td>m</td>
<td>4.8</td>
<td>4.1</td>
</tr>
<tr>
<td>OLR</td>
<td>kgCOD m⁻³ d⁻¹</td>
<td>1.2</td>
<td>0.30–0.60</td>
</tr>
<tr>
<td>Hydraulic loading rate</td>
<td>m³ m⁻² d⁻¹</td>
<td>–</td>
<td>10–12</td>
</tr>
<tr>
<td>Chemical oxygen demand (COD): N</td>
<td>ratio (applied to TF)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRTb d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydraulic residence time (HRT)c</td>
<td>h</td>
<td>9.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*COD:N at each period (P₁; P₂; P₃).
*Estimated from the overall detached biomass to the amount of biomass within the sponges.
*Estimated using a radioactive tracer method.

During the operational period, we sampled from the raw sewage, UASB and TF effluent for physical–chemical analyses, as described in Almeida et al. (2015). The profiles for soluble COD, NH₄-N, and alkalinity were composed with grab samples taken during 3 h.

**System monitoring and sampling**

**Liquid-phase sampling**

During the operational period, we sampled from the raw sewage, UASB and TF effluent for physical–chemical analyses, as described in Almeida et al. (2015). The profiles for soluble COD, NH₄-N, and alkalinity were composed with grab samples taken during 3 h.

**Biomass sampling**

Representative pieces of polyurethane foam were sampled during the periods P₁, P₂ and P₃ at four different depths along the TF: 0–102 cm (RS₁), 102–204 cm (RS₂), 204–306 cm (RS₃), and 306–408 cm (RS₄). The biomass was extracted by squeezing and washing the sponges several times with phosphate buffer saline solution (0.13 M sodium chloride, 0.7 mM disodium hydrogen phosphate, 0.3 mM monosodium phosphate; pH 7.2). The resulting
suspension was centrifuged at 4,000 rpm for 15 min. The pellet was recovered and stored at –20 °C for subsequent DNA extraction. Because the system was kept stable at each period, we understand that our sampling characterizes the AOB communities at each OLR range.

DNA extraction, PCR and DGGE

DNA was extracted from 0.5 g of frozen biomass sample according to Egli et al. (2005). DNA samples were diluted 100 times for amplification reactions in order to reach a concentration range of 25–50 ng/μL. All samples showed this concentration range except for sample RS4 (P1 and P3), which showed 10 ng/μL. As some variation in DNA concentration between the samples might have occurred, the interpretation of the results was focused on the predominant bands of each sample. Molecular fingerprinting of AOB community was conducted via PCR-DGGE using the primer set amoA1FGC and amoA2R targeting the NH₄⁺ monooxygenase subunit A of AOB (amplifying 533bp), as described in Rotthauwe et al. (1997). DGGE was performed twice at 60 °C in 0.5× TAE (Tris base, acetic acid, and ethylenediaminetraacetic acid pH 8.0) at 75 V for 16.5 h on a DCode system (Bio-Rad, USA) on a 6% polyacrylamide gel with 45–60% (M/V) gradient of urea formamide denaturant. Gels were stained with ethidium bromide and visualized in ultraviolet transillumination. Specific gel bands were excised and sequenced. The amoA gene partial sequences were aligned with those of known species of AOB. The phylogenetic distance tree was inferred with MEGA 4 software package using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method (Tamura et al. 2007).

RESULTS AND DISCUSSION

Molecular ecology of the AOB community

Figure 2(a) shows the DGGE profiling of AOB for the sponge-based TF, considering the operational periods investigated. Five different AOB bands were observed during TF operation (Bands AOB1, 2, 3, 4 and 5 in Figure 2, rendered on a phylogenetic tree in Figure 3). Two sequences, closely related to Nitrosomonas europaea (AOB1), and with limited sequence affiliation to Nitrosomonas eutropha (AOB2), were predominant at the upper compartments of the TF (RS₁ and RS₂), when the OLR ranged under higher values (0.45–0.55 kgCOD m⁻³ d⁻¹). Thus, those AOB populations were well adapted to the presence of higher NH₄⁺-N and soluble COD concentrations (10–25 mgN L⁻¹; 80–100 mgCOD L⁻¹), suggesting the ability to coexist with heterotrophs at the upper portions of the TF. The sequence AOB3 followed a similar trend, but reduced its intensity from the period P₁ to P₃ (Figure 2(a)). This AOB sequence did not show affiliation to the known sequences used (Figure 3). The sequences AOB4 and AOB5 were related to Nitrosomonas oligotropha.
being more intense from the samples taken at lower compartments (RS₃ and RS₄), but also visible at RS₂ when lower OLR was observed (period P₃).

These findings suggest successional changes of AOB communities within TF-Rotosponge with the system being colonized by different AOB species along the reactor. DGGE profiling was considerably altered from RS₂ down the reactor, indicating the change in downward substrate-loading patterns (Figure 2(b)) may be an important factor for such spatial organization. The simultaneous observation of DGGE, COD and NH₄⁺-N profiles (Figure 2(a) and (b)) can support this assertion. The predominance of the sequences related to *N. europaea* and *N. eutropha* at higher NH₄⁺-N concentrations, and sequences related to *N. oligotropha* at lower NH₄⁺-N concentrations (close to a limiting NH₄⁺-N concentration) suggest that the former could be considered *r*-strategists and the last, *K*-strategists, as suggested in previous studies (Gujer 2010). In fact, *N. europaea* strains have been found in eutrophic environments rich in NH₄⁺-N, while *N. oligotropha* predominate in environments with low NH₄⁺-N concentrations (Limpiyakorn et al. 2005; Lydmark et al. 2006).
During periods $P_1$ and $P_2$ the average OLR varied from 0.45 to 0.55 kgCOD m$^{-3}$ d$^{-1}$, but NH$_4^+$-N removals were around 80%, with no statistical differences in performance (Almeida et al. 2013). However, the AOB community structure was affected by OLR (Figure 2(a)). This could be explained by a functional redundancy, whereas different AOB populations were capable of carrying out similar biochemical functions. In period $P_2$, most of bands disappeared with the predominance of sequences related to N. europaea (AOB1), and with limited affiliation to N. eutropha (AOB2). It reinforces the hypothesis that the high substrate environment was suitable of harboring the related AOB species. Considering the related OLR range (0.45–0.55 kgCOD m$^{-3}$ d$^{-1}$), the results suggested that diversity was not a factor for overall NH$_4^+$-N removal. From a technology point of view, such changes in AOB structure and diversity did not affected TF performance for NH$_4^+$-N removal.

In the period $P_3$, the OLR was significantly lower (0.50–0.40 kgCOD m$^{-3}$ d$^{-1}$), and NH$_4^+$-N removal (around 95%) was more stable (Almeida et al. 2013). Changes in the banding patterns were observed (Figure 2(a)) related to lowering COD and N concentrations along the system, suggesting a deterministic selection. At the lower OLR range ($P_3$), the AOB diversity may be correlated with the noticeable stability of the reactor for NH$_4^+$-N removal, as shown in Almeida et al. (2013). At some extent, our results reinforce the notion presented by Daims et al. (2001), and supported by Rowan et al. (2003), that the level of AOB diversity can influence process stability.

The trend of spatial segregation of AOB was observed by Rowan et al. (2003) in a nitrifying TF. These authors discussed that the TFs in which AOB diversity was greater are more stable reactors in comparison with biological aerated filters. For such cases, N concentrations along the TF seemed to be a very important factor, as NTFs were designed to treat effluents from a high rate activated sludge (AS) process with lower concentrations of readily biodegradable organic matter. In our system (a combined organic matter removal and nitrification TF post-UASB), the variation of COD and NH$_4^+$-N concentrations during the periods and along the TF were clearly related to spatial organization and diversity of AOB. A similar tendency of NH$_4^+$-N and COD gradients, and ecological selection could not be observed in a plastic-based TF operated at the same conditions (data not shown). Thus, the sponge-based medium caused strong concentration gradients along the TF, providing a significant ecological selection of AOB within the system.

Higher HRT and SRT are important factors influencing soluble COD and NH$_4^+$-N profiles along the sponge-based TFs. Using a radioactive tracer method, we found 120 min HRT for TF-Rotosponge (Table 1), whereas in a full-scale nitrifying TF with vertical plastic medium a HRT around 15 min was observed (Wik 2003). Similar results have been obtained for plastic and rock TFs (18 and 12–22 min HRT, respectively) in small-scale systems (Almeida et al. 2013; Vieira 2013). The smaller differences in terms of spatial organization and diversity of AOB within the plastic-based TF post-UASB could be related to the lower HRT and SRT.

The SRT within the sponge-based TF (80–120 d), was four to five times higher than the SRT generally adopted for AS systems, even though extended aeration is considered. In combination with HRT, higher SRT probably contributes to the selection of microorganisms by location in the system, as the detachment rates seem to be very low (Almeida et al. 2013). In some cases (e.g. low OLR), it may contribute to spatial segregation of different species carrying out similar biochemical functions. From a technology point of view, the larger HRT and SRT provided by sponge-based packing media can increase the process stability for NH$_4^+$ removal rates.

The spatial segregation and diversity along filter beds at low organic loadings has been previously identified (Persson et al. 2002; Rowan et al. 2005; Montràs et al. 2008). However, our results indicate a different pattern for predominant species in comparison with the NTF post-AS process mentioned in Rowan et al. (2003). Thus, the form of a multistage treatment process probably defined the pattern of AOB communities. This hypothesis needs to be further investigated.

**Community structure of AOB within the compartments of the TF-Rotosponge**

In order to capture important features related to the influence of local conditions on AOB distribution, changes in AOB community structure were examined considering NH$_4^+$-N and soluble COD profiles. Table 2 shows NH$_4^+$-N and COD concentrations and removals along the TF for the operational periods.

**Community structure of AOB within the compartment 1 (RS$_1$)**

From period $P_1$ to $P_2$ (higher OLR) NH$_4^+$-N removal decreased with the increase in COD concentration, but the community structure remained the same (Figure 2(a)).
Table 2 | NH₄⁺-N and COD concentrations and removals along the TF for the investigated periods

<table>
<thead>
<tr>
<th>Sampling point</th>
<th>NH₄⁺-N (mgN L⁻¹)</th>
<th>NH₄⁺-N removal (%)</th>
<th>Soluble COD (mgCOD L⁻¹)</th>
<th>COD removal rate (gCOD m⁻³ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P₁</td>
<td>P₂</td>
<td>P₃</td>
<td>P₁</td>
</tr>
<tr>
<td>UASB₁ (inlet)</td>
<td>32.9</td>
<td>45.6</td>
<td>25.7</td>
<td>63.3</td>
</tr>
<tr>
<td>RS₂ (1.02 m)</td>
<td>25.0</td>
<td>42.7</td>
<td>20.9</td>
<td>23.9</td>
</tr>
<tr>
<td>RS₃ (2.04 m)</td>
<td>16.0</td>
<td>31.4</td>
<td>4.2</td>
<td>31.4</td>
</tr>
<tr>
<td>RS₄ (3.06 m)</td>
<td>17.2</td>
<td>31.4</td>
<td>8.5</td>
<td>6.8</td>
</tr>
<tr>
<td>RS₅ (4.08 m)</td>
<td>6.6</td>
<td>6.6</td>
<td>1.4</td>
<td>52.7</td>
</tr>
</tbody>
</table>

*NH₄⁺-N and alkalinity concentrations for UASB effluent are the influent concentrations of the TF-Rotosponge. NH₄⁺-N and alkalinity concentrations for RS₁ effluent are the influent concentrations for RS₂ and so on.

| Note: The boldface (significantly different values) and grey-shaded entries support the discussion in the text. |

Probably, the increase of the OLR locally affected AOB activity in RS₁. In period P₂ (lower OLR), the AOB community differed, and only the sequence related to *N. europaea* (AOB1) was dominant. The AOB community within the upper part of the TF (RS₁) had changed, but maintained the overall community function for NH₄⁺-N removal.

Community structure of AOB within the compartments RS₂ and RS₃

The higher COD removal rates at RS₁ were reflected in the AOB community (Figure 2(a)), with the appearance of different AOB populations (related to *N. oligotropha*). Low COD (~10–35 mgCOD L⁻¹) and N (~5–15 mgN L⁻¹) concentrations down the TF (Table 2) were probably factors selecting AOB species more adapted to the related conditions, reinforcing the ability of these AOB species to scavenge low NH₄⁺-N concentrations.

Within the compartments RS₂ and RS₃, as well as in RS₁, the changes in COD and N over time resulted in changes of AOB community structure. The AOB populations involved in NH₄⁺-N removal at low OLR were clearly different when compared with AOB community submitted to higher organic loadings. Even in lower portions (e.g. RS₃) OLR probably was a factor.

Community structure of AOB within the compartment 4 (RS₄)

We observed a specific case in RS₄, and changes in the AOB community structure were examined adding NH₄⁺-N removal rates and alkalinity consumption (Table 3). DGGE banding patterns at Period P₁ revealed the presence of two dominant populations related to *N. oligotropha* (AOB4 and AOB5 in Figure 2, and rendered in Figure 3) at low COD and NH₄⁺-N concentrations. However, the amount of PCR products loaded to the gel was the same for the following periods, and yet these bands were hardly seen in periods P₂ and P₃. This might indicate a lower proportion of these populations. NH₄⁺-N and calcium carbonate (CaCO₃) in the lower

Table 3 | NH₄⁺-N and carbonate alkalinity concentrations and consumption in the TF

<table>
<thead>
<tr>
<th>Sampling point</th>
<th>NH₄⁺-N concentration (mgN L⁻¹)</th>
<th>NH₄⁺-N removal rate (gNH₄⁺-N d⁻¹)</th>
<th>Alkalinity concentration (mgCaCO₃ L⁻¹)</th>
<th>Alkalinity consumption (gCaCO₃ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P₁</td>
<td>P₂</td>
<td>P₃</td>
<td>P₁</td>
</tr>
<tr>
<td>UASB (inlet)</td>
<td>32.9</td>
<td>45.6</td>
<td>25.7</td>
<td>245.8</td>
</tr>
<tr>
<td>RS₁ (1.02 m)</td>
<td>25.0</td>
<td>42.7</td>
<td>20.9</td>
<td>35.4</td>
</tr>
<tr>
<td>RS₂ (2.04 m)</td>
<td>17.2</td>
<td>31.4</td>
<td>8.5</td>
<td>35.4</td>
</tr>
<tr>
<td>RS₃ (3.06 m)</td>
<td>16.0</td>
<td>17.6</td>
<td>4.2</td>
<td>5.2</td>
</tr>
<tr>
<td>RS₄ (4.08 m)</td>
<td>7.6</td>
<td>6.6</td>
<td>1.4</td>
<td>38.0</td>
</tr>
</tbody>
</table>

*NH₄⁺-N and alkalinity concentrations for UASB effluent are the influent concentrations of the TF-Rotosponge. NH₄⁺-N and alkalinity concentrations for RS₁ effluent are the influent concentrations for RS₂ and so on.

| Note: The boldface (significantly different values) and grey-shaded entries support the discussion in the text. |
portion of the TF probably were limiting substrates, resulting in loss of AOB bands. The lower NH$_4$+N removal rate at RS$_4$ (period $P_3$) reinforces such perspective.

In general, we observed a consolidated trend in which at higher NH$_4$+N concentrations the bands closely related to N. europaea or with some sequence affiliation to N. eutropha prevailed. In this case, being such AOB species $r$-strategists, they were capable of fast growth over $K$-strategists at the upper part of the reactor and/or at higher NH$_4$+N concentrations. Within the lower compartments of the TF, AOB species related to N. oligotropha were able to scavenge lower NH$_4$+N concentrations, as those bands related to $r$-strategists reduced in intensity.

CONCLUSIONS

For the operational conditions imposed on the TF, the sponge-based packing media caused a strong concentration gradient along the system, providing an ecological selection of AOB communities. In this regard, higher HRT and SRT within the sponge-based TF were probably important factors, as we did not observe the same tendency of ecological selection in a TF filled with vertical plastic media operated under the same conditions. The dominant sequences present in the upper portions of the TF were closely related to N. europaea and more distantly affiliated to N. eutropha. In the lower portions of the TF, the dominant sequences were related to N. oligotropha. The form of a multistage treatment process (UASB/TF) may be defined in the pattern of AOB communities, but such a hypothesis needs to be further investigated.

The AOB communities changed over time, but maintained the overall function for NH$_4$+N removal. In this case, a high substrate environment tended to reduce the species richness of AOB within the TF, but with no statistical differences in performance for NH$_4$+N removal. From a technology point of view, it suggests that diversity was not a factor for overall NH$_4$+N removal, considering higher OLR range (0.45–0.55 kgCOD m$^{-3}$ d$^{-1}$). However, the AOB diversity may be correlated with the noticeable stability of the reactor for ammonium-N removal at the lower OLR range. This study is relevant because molecular biology was used to observe important features of a potential bioreactor, considering realistic operational conditions applied to UASB/sponge-based TF systems.

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