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Sequentially aerated membrane biofilm reactors for autotrophic nitrogen removal: microbial community composition and dynamics

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Summary
Membrane-aerated biofilm reactors performing autotrophic nitrogen removal can be successfully applied to treat concentrated nitrogen streams. However, their process performance is seriously hampered by the growth of nitrite oxidizing bacteria (NOB). In this work we document how sequential aeration can bring the rapid and long-term suppression of NOB and the onset of the activity of anaerobic ammonium oxidizing bacteria (AnAOB). Real-time quantitative polymerase chain reaction analyses confirmed that such shift in performance was mirrored by a change in population densities, with a very drastic reduction of the NOB Nitrospira and Nitrobacter and a 10-fold increase in AnAOB numbers. The study of biofilm sections with relevant 16S rRNA fluorescent probes revealed strongly stratified biofilm structures fostering aerobic ammonium oxidizing bacteria (AOB) in biofilm areas close to the membrane surface (rich in oxygen) and AnAOB in regions neighbouring the liquid phase. Both communities were separated by a transition region potentially populated by denitrifying heterotrophic bacteria. AOB and AnAOB bacterial groups were more abundant and diverse than NOB, and dominated by the r-strategists Nitrosomonas europaea and Ca. Brocadia anamoxidans, respectively. Taken together, the present work presents tools to better engineer, monitor and control the microbial communities that support robust, sustainable and efficient nitrogen removal.

Introduction
The discovery of anaerobic ammonium oxidizing bacteria (AnAOB, aka anammox bacteria) two decades ago has launched a new phase in wastewater biotechnology. Many reactor concepts have been developed to take advantage of this functional group for the treatment of nitrogen (N)-rich waste streams. AnAOB can grow symbiotically with aerobic ammonium oxidizing bacteria (AOB) in biofilms with redox gradients, allowing the conversion of equimolar mixtures of ammonium (NH4+) and nitrite (NO2−) to nitrogen gas (N2) without the addition of organic carbon (Terada et al., 2011). In membrane-aerated biofilm reactors (MABRs) such redox gradient conditions can establish in a counter-diffusion mode, with oxygen (O2) entering the biofilm through the membrane–biofilm interface and NH4+ diffusing from the liquid phase into the biofilm at its surface. We have recently shown that this biofilm reactor configuration can effectively support autotrophic N removal from synthetic waste streams at a lower energy, spatial, and environmental footprint than is feasible by conventional (i.e. based on co-diffusion) biofilm technologies (Pellicer-Nàcher et al., 2010; Gilmore et al., 2013).

\[ \text{NO}_2^- \text{ is a central intermediate in autotrophic N conversions: it is the product of AOB and is a necessary substrate for AnAOB. Therefore, metabolically active nitrite oxidizing bacteria (NOB) are undesirable during autotrophic N removal, as the oxidation of } \text{NO}_2^- \text{ to nitrate (NO}_3^- \text{), catalysed by NOB, would hamper AnAOB activity. Several strategies have been explored to suppress or control NOB growth in suspended growth or co-diffusion biofilm systems such as operation at elevated pH, low dissolved} \]

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oxygen (DO) concentration, or higher temperatures (Van Hulle et al., 2010). However, none of these procedures has proven efficient to suppress NOB activity in MABRs (Wang et al., 2009; Terada et al., 2010). In large part, this difficulty in outcompeting NOB may be due to the localization of NOB, this microbial guild, in MABR biofilms. NOB, when present, grow in the aerobic regions of the biofilm (inner biofilm regions due to O₂ diffusion across the biofilm base) where DO and NO₂⁻ concentrations are highest, and any changes applied to the liquid phase will have minimal effects. New approaches such as careful inoculum selection or implementation of cyclic aeration patterns have been explored and proven successful to moderate NOB activity in MABRs (Pellicer-Nàcher et al., 2010; Terada et al., 2010).

While the above studies have demonstrated the feasibility and identified suitable operational conditions for MABRs targeting autotrophic N removal (Pellicer-Nàcher et al., 2010; Gilmore et al., 2013), direct inspection of the microbial community structure and composition in the resulting MABR biofilms has been limited. Such inspection is not only necessary to assess the robustness of the process through the study of the diversity of the established microbial community, but also to deepen the understanding about how biofilms can be engineered for a certain operational purpose by applying selected operational strategies. In addition, information from direct biofilm inspection can be used to support or modify biofilm process models, where community compositions are easily predicted but rarely verified (Terada et al., 2007; Lackner et al., 2008).

Here, we present the first exhaustive characterization study of the structure and composition of microbial biofilms that support autotrophic N removal in MABRs (Pellicer-Nàcher et al., 2010). We are especially keen to verify whether the imposed redox stratification results in the predicted ecological stratification of the involved functional groups, whether suppression of NOB and stimulation of AnAOB activity by sequential aeration is mirrored by the abundances of NOB and AnAOB, whether operational and reactor conditions have resulted in a more or less diverse set of functional guilds, and whether heterotrophic bacteria (HB) coexist in this autotroph-dominated community. Hence, we used a complementary set of molecular and microscopic tools to identify, quantify and assess the microbial diversity and structure of biofilms in a long-term operated MABR run under O₂ limitation and treating a synthetic NH₄⁺ rich influent.

Results and discussion

Microbial dynamics during reactor operation

From inoculation to month 13, the reactor was operated in continuous aeration mode. The O₂ to NH₄⁺ loading ratio was controlled at the optimal value for complete NH₄⁺ conversion to N₂ via the nitrification-anammox pathway (Terada et al., 2007). Notwithstanding the imposed O₂ limited operation, most of the NH₄⁺ was simply converted to NO₃⁻, resulting in limited N removal (Fig. 1A). From month 13 onward, O₂ was supplied in cycles of aeration/non-aeration, dramatically affecting reactor performance. Initially, strong NO₂⁻ accumulation was observed, followed by an increase in AnAOB activity and nearly maximal N removal (70%). In addition, at this point, minimal nitrous oxide (N₂O) emissions were observed (Pellicer-Nàcher et al., 2010).

Imposition of the sequential aeration regime caused clear shifts in the microbial community, as suggested by the results obtained from real-time quantitative polymerase chain reactions (qPCR) using relevant primers (Fig. 1B): the abundance of Nitrobacter spp. decreased by an order of magnitude. Nitrobacter spp. are considered r-strategist NOB (Schramm, 2003), whose presence is correlated with poor nitrification efficiencies (Terada et al., 2010). Both 16S rRNA gene (Fig. 1B) and nuxA (Fig. 1; in the Supporting Information, Fig. S1) targeted NOB quantifications were consistent. The gene copy numbers of Nitrobacter spp. increased by the end of the experiment (still under sequential aeration), but they did not negatively affect reactor performance (Fig. 1A). The abundance of the Nitrospira spp. was also severely affected by the onset of the sequential aeration: it decreased and dropped to the quantification limit until the end of the experiment. These data suggest that the
Nitrosospira NOB, despite their lower abundance vis-à-vis Nitrobacter NOB, were responsible for the conversion of NO$_2^-$ to NO$_3^-$ during the antecedent continuous aeration phase. This NOB genus is known to thrive in environments with low NO$_2^-$ concentrations (K-strategist, Schramm, 2003). The overall reduction in NOB abundance was mirrored by a significant increase in AnAOB numbers, as reflected by 16S rRNA gene (Fig. 1B) and hzo-targeted quantifications (Fig. S1). The exact reasons explaining the observed suppression of the NOB upon onset of the sequential aeration regime are unknown, but we hypothesize that AOB are less affected by the feast/famine conditions associated with cyclic aeration (Geets et al., 2006) and display a higher affinity for O$_2$ at low concentrations than NOB (Blackburne et al., 2008), which would allow AOB to outcompete NOB for the transiently available limiting O$_2$. AOB are known to release hydroxylamine and NO during O$_2$ transients, compounds which may have also inhibited NOB (Schmidt et al., 2003; Noophan et al., 2004; Kostera et al., 2008). The increased NO$_2^-$ availability (due to NOB suppression), and the postulated transiently available NO may also have enhanced NO$_2^-$ uptake by AnAOB upon the onset of cyclic aeration (Jetten et al., 2009; Kartal et al., 2010). Although NO$_2^-$ concentrations (in the bulk phase) attained values as high as 200 mg-N l$^{-1}$, they did not prevent the stimulation of AnAOB activity, in contrast with earlier studies that reported NO$_2^-$ values as low as 28 mg-N l$^{-1}$ to negatively affect their activity (van der Graaf et al., 1996).

The density of denitrifying bacteria was approximated by quantifying the abundance of the nirK and nirS genes; nirK and nirS encode NO$_3^-$ reductase enzymes in both heterotrophic denitrifying bacteria (nirS and nirK) or nitrifiers (nirK, Braker et al., 1998; Cassiotti and Ward, 2001; Heylen et al., 2006). Onset of cyclic aeration caused a significant decrease in nirK abundance, while nirS remained relatively constant. Because AOB abundance remained fairly constant, this trend suggests a shift in the heterotrophic denitrifying guild, becoming more nirS abundant as AnAOB density and activity increased. The decrease in NOB, also known to contain nirK in their genome (Schreiber et al., 2012), could have contributed as well to the observed drop. Prior work revealed that N$_2$O emissions from our and other MABRs were very low when AnAOB activity was high ($<0.015\%$ and $<0.001\%$ N-N$_2$O/ N-load during the aerated and non-aerated phase of an aeration cycle, respectively, Pellicer-Nàcher et al., 2010), but can be very high when AnAOB activity is low (2–11% N-N$_2$O/ N-load; Gilmore et al., 2013). Because N$_2$O emissions are, in part, caused by NO$_2^-$ reductase (Zumft, 1997), it will be interesting to verify whether the dramatic drop in nirK abundance directly correlates with reduction in N$_2$O emissions.

Microbial community composition and architecture

After 23 months of operation (630 days), the developed biofilm exhibited a very pronounced radial stratification of the microbial community (Fig. 2A). The biofilm region adjacent to the hollow fibre membrane was clearly dominated by AOB, as indicated by the simultaneous signal from EUB — all bacteria — and AOB targeting probes (Table 1, combination 1). The thickness of the observed AOB layer ranged from 110 to 170 μm across all analysed samples, comparable to the O$_2$ penetration measured with microsensors during reactor operation here and in other nitritating MABRs (Fig. S2, Terada et al., 2010). Moreover, since the DO concentration in the bulk liquid was close to the detection limit of the DO probe used (Pellicer-Nàcher et al., 2010), it could be concluded that the AOB group consumed most of the O$_2$ transferred from the membrane, and mediated the partial conversion of the NH$_4^+$ diffusing from the bulk liquid to NO$_2^-$ High magnification micrographs in this area revealed rod-shaped cells in very compact strata around the hollow fibre membranes (Fig. 2D). The average biofilm porosities calculated here were 0.36 ± 0.07, half of what is considered normal in co-diffusion biofilm systems (Zhang and Bishop, 1994). This packed and ordered structure of AOB is very different from the cauliflower-shaped clusters observed in other biofilm systems (Okabe and Kamagata, 2010), which may be caused from the pressure of upper biofilm strata onto the biofilm base, high growth velocities or the high competition for O$_2$ and space in this region. Isolated cauliflower-shaped clusters could only be seen in biofilm regions distant from the membrane where DO concentrations were lower.

The use of probes with higher phylogenetic resolution (Table 1, combinations 2 and 3, Fig. 2B and C), indicated that halophilic and halotolerant Nitrosomonas spp. were the dominant AOB in the system. Halophilic and halotolerant Nitrosomonas spp. are known to outcompete other AOB species in environments with high substrate availability (Okabe and Kamagata, 2010). The high NH$_4^+$ concentrations expected across the whole biofilm thickness (bulk concentrations ranged from 100 to 400 mg-N l$^{-1}$) may hamper the ability of Nitrosospira spp. to thrive even in biofilm areas with lower DO concentrations, as previously reported (Schramm et al., 2000). Our observation is in agreement with the work by Terada et al. (2010), who noted that MABR biofilms populated by Nitrosomonas spp. rather than Nitrosospira spp. supported higher NO$_2^-$ production. Halophilic and halotolerant Nitrosomonas spp. are typically the most abundant AOB in co-diffusion biofilms performing autotrophic N removal (Sliékers et al., 2003; Vázquez-Padín et al., 2010; Liu et al., 2012).

Microcolonies of the N. oligotropha lineage (Table 1, probe combination 3, Fig. 1B) were occasionally observed and randomly distributed within aerobic biofilm regions.
Although *N. oligotropha* is expected in environments of low substrate availability, *N. oligotropha* and *N. europaea* can coexist in aerobic regions of nitrifying biofilms when operated under dynamic aeration conditions (Gieseke et al., 2001). In another MABR biofilm performing autotrophic N removal under continuous aeration *N. oligotropha* and halophilic and halotolerant *Nitrosomonas* spp. were also identified as the most abundant AOB (Gilmore et al., 2013).

In that study however, *N. oligotropha* signals were observed in anaerobic strata parallel to the membrane surface, suggesting cell maintenance without growth, as *N. oligotropha* is, among other AOB, able to maintain its ribosome content even under famine conditions (Gieseke et al., 2001).

AnAOD-positive signals were exclusively found close to the biofilm top, at least 300 μm away from the membrane.

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**Table 1.** Probe combinations used in the study (after Loy et al., 2007; Okabe and Kamagata, 2010). Probe sequences and hybridization conditions for each probe are available in Table S1.

| No. | Experimental Purpose | Fluorophore |
|-----|----------------------|-------------|
| 1.  | Verify spatial distribution of AOB and AnAOB | EUBmix, Nso190-Nmo218-Cluster 6a192<sup>a</sup>, Amx820 |
| 2.  | Examine abundance of *Nitrosospira* spp. vs halophilic and halotolerant *Nitrosomonas* spp. (AOB) | EUBmix, Nev443, NEU<sup>a</sup>, Nmo218-Cluster 6a192<sup>a</sup> |
| 3.  | Examine abundance of *N. oligotropha* vs halophilic and halotolerant *Nitrosomonas* spp. (AOB) | EUBmix, NEU<sup>a</sup>, Ntspa662<sup>a</sup> |
| 4.  | Examine abundance of *Candidatus* Kuenenia spp. vs *Candidatus* Brocadia spp. (AnAOB) | EUBmix, Kst157, Ban162 |
| 5.  | Examine abundance of *Nitrobacter* spp. vs *Nitrospira* spp. (NOB) | EUBmix, NIT3<sup>a</sup>, Ntspa662<sup>a</sup> |

<sup>a</sup> Probe requires a competitor

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Microbial study of MABR biofilms for autotrophic N removal

Microsensor measurements confirmed that DO was almost absent in this region (Fig. S2), while high NH$_4^+$ and NO$_2^-$ concentrations were expected, given their concentrations in the bulk liquid (Fig. 1A). AnAOB microcolonies were spearhead- or oval-shaped with round edges. High magnification micrographs (Fig. 3B) revealed doughnut-shaped cellular morphology, characteristic of AnAOB (rRNA distributes around the central anammoxome organelle, Kuenen, 2008).

Although it is rare to find multiple AnAOB species in bioreactors performing autotrophic N removal (Hu et al., 2010; Park et al., 2010a), both Ca. Brocadia anammoxidans (dark cyan) and Ca. Kuenenia stuttgartiensis (light orange) were observed here. However, Brocadia signals were clearly more abundant (Fig. 3A, Table 1, combination 4). These two AnAOB have different postulated growth preferences: Ca. Brocadia seems to thrive in environments with high substrate availability (r-strategist), while Ca. Kuenenia finds its niche in environments with nutrient scarcity (K-strategist) (van der Star et al., 2008). Microcolonies of both AnAOB lineages do not appear to be stratified within our biofilms. This observation might be due to the changing NH$_4^+$ and NO$_2^-$ concentrations during sequential aeration. In a continuously aerated MABR performing autotrophic N removal exclusively the Ca. Brocadia AnAOB lineage was observed (Gilmore et al., 2013).

Overall reactor mass balance calculations suggested that, although substantial N removal was observed (5.5 g-N m$^{-2}$ day$^{-1}$), approximately 30% of the NO$_3^-$ production was due to residual NOB activity (0.3 g-N m$^{-2}$ day$^{-1}$), while the remainder had been synthesized by AnAOB (0.7 g-N m$^{-2}$ day$^{-1}$, Pellicer-Nàcher et al., 2010). Here, NOB could still be detected in the biofilm (Table 1, combination 5, Fig. 4), even though they were clearly outnumbered by AOB in the aerobic biofilm regions. NOB in these biofilms were exposed to dual competitive pressure caused by fast growing AOB (consuming most O$_2$) and AnAOBs (consum-
Inert AOB HB

Fig. 5. Observed and predicted occurrence of metabolically active biofilm regions. Normalized biofilm thickness = 0 (membrane–biofilm interface).
A. Averaged relative activity profile with biofilm depth (n = 4). Standard deviations represented with error bars in each column.
B. Relative space occupancy profile for the considered bacterial communities calculated by mathematical modelling in a previous study describing heterotrophic activity in MABR biofilms for autotrophic N removal (Lackner et al., 2008).

Microbial community abundances
Quantitative image analysis of the presented FISH results revealed that AOB and AnAOB accounted for 53 ± 13% and 38 ± 6% of the biofilm, with 9 ± 8% of the EUB signals not accounted for by either AOB or AnAOB (Table 1, combination 1). A separate analysis indicated a highly variable NOB fraction constituting 11 ± 10% of all detected EUB signals (Table 1, combination 5). These AOB, NOB and AnAOB fractions are consistent with those observed by FISH analysis in co-diffusion biofilms performing autotrophic N removal (Liu et al., 2012).
estimated at about 5, 0.2 and 25% of the total community population (considering that each cell contained a single copy of the genes targeted by qPCR primers, Table 2). Differences in sampling procedure may have impacted considerably the AOB and NOB results obtained by qPCR. While the entire biofilm is probed and imaged during FISH analysis (both biofilm and membrane were cryosectioned), only the biomass that could be scraped off the membranes was quantified by qPCR. As nitrifiers were preferentially present adjacent to the aeration membrane, AOB and NOB underestimation by qPCR was likely. Additionally, DNA extraction methods are known to display different yields in different types of bacterial species, which may bias substantially later qPCR analyses (Juretschko et al., 2002; Pei et al., 2005). The targeted functional genes may be present in multiple copies per cell in the different biocatalysts studied, which could also lead to wrong estimates of the community fractions by qPCR calculated here (Pei et al., 2010). Finally, small differences could also be explained by the fact that, oligonucleotide probes (for FISH) and primers (for qPCR) do not necessary detect exactly the same phylogenetic clades (Hallin et al., 2005).

The presented results, however, seem to converge in the fact that AOB and AnAOB are the most abundant microbial guilds. Model calibration and process operation can greatly benefit from the dual detection approach presented here. While FISH and microelectrode results would give information on the position and relative abundances of the functional microbial communities (Schramm et al., 2000), qPCR performed on representative biomass samples would allow for routine observation measurements on the microbial dynamics of the system. These observations could alert about unwanted changes in the microbial community supporting the process and suggest the need of imposing process control to correct deviations from the desired set point (Park et al., 2010a).

**Table 2.** Relative abundance of functional microbial guilds after 630 days of operation assessed via different molecular techniques.

|        | Pyrosequencing | qPCR      | FISH      |
|--------|----------------|-----------|-----------|
| AOB    | 2.70 ± 0.87%   | 5.4 ± 0.2%| 53.7 ± 13.2%|
| NOB    | 0.57 ± 0.12%   | 0.2 ± 0.2%| 11.4 ± 10%  |
| AnOB   | 60.00 ± 3.42%  | 25.0 ± 12.1%| 38.2 ± 5.8% |

a. Taxonomy index of each functional guild was given in supplementary document (Table S1).

Diversity of community fractions assessed by deep sequencing

Microbial diversity of relevant functional guilds (AOB, NOB and AnAOB) was revealed by pyrosequencing the V3-V4 region of amplified community 16S rDNA. After implementation of quality control measures and denoising, 19,962 sequences were obtained from triplicate samples, which could be binned in 439 operational taxonomic units based on 97% phylogenetic similarity (OTU0.03). A total of 15, 2 and 6 OTUs0.03 could be assigned to AnAOB, NOB and AOB respectively (Fig. 6). All three sequence libraries shared 117 OTUs0.03 but were distinct due to abundant unshared genotypes (Fig. S3).

A remarkable diversity was detected within the AnAOB guild containing 15 OTUs0.03, while sampling depth may not yet have revealed the complete diversity. Sequences of both the *Kuenenia* and *Brocadia* lineages were detected. While species richness in both lineages was the same (8 each), *Brocadia* sequences were significantly more abundant (290:1), consistent with FISH observations. Sequences affiliated to the aerobic ammonium oxidizer *Nitrosomonas europaea* (509 seq.) and the NO3 oxidizer *Nitrobiater hamburgensis* (100 seq.) were the dominant nitrifying bacteria in the studied MABR biofilms (Fig. 6). While the diversity of AOB was relatively high, species different to *N. europaea* represented only minor fractions of the AOB guild (most having a single sequence for each OTU0.03, occurring in only one of the replicates, Fig. S4). Higher microbial diversity is normally reported in systems with dynamic operation conditions (Rowan et al., 2003).

Heterotrophic OTUs0.03 (41) were found in all samples despite the absence of organic carbon in the synthetic feed. The most abundant HB sequences were mainly assigned to the *Xanthomonadacea* (γ-Proteobacteria, 550 seq.) and *Clostridaceae* (Firmicutes, 356 seq.) families. Our results are in agreement with the findings of a study applying microautoradiography combined with FISH (MAR-FISH) to unravel the patterns in the cross-feeding of microbial products originating from nitrifier decay to HB (Okabe et al., 2005). In that study *Chloroflexi* and *Cytophaga-Flavobacterium* cluster cells were responsible for the degradation of slow biodegradable material (e.g. cells). Furthermore, members of the α- and γ-Proteobacteria (e.g. *Xanthomonadacea*, typical in low carbon environments) metabolized other low-molecular weight organic substrates. Members of the *Clostridaceae* family were not detected by their approach (gram positive bacterial require special fixation protocols), but, like *Xanthomonadacea*, they are also known to degrade complex organic substrates and use NO3 and NO2 as electron acceptor in anaerobic environments (Finkmann et al., 2000; Wüst et al., 2011). CARD FISH may further assist in the identification of the organisms present in this intermediate region.

The fractions of AOB, NOB and AnAOB were identified based on the number of sequences detected by pyrosequencing. The results were further compared to those obtained by qPCR and FISH (Table 2). The abundance results obtained by pyrosequencing confirm the
reduced NOB abundance with respect to AOB and AnAOB. The quantification of AOB and *Nitrospira* spp. was also affected with respect to qFISH. As previously indicated, such a divergence in results can arise by the difficulty in detaching the aerobic biofilm regions from the membrane or by a lower DNA extraction efficiency of the proposed treatment for these microbial species. The differences observed among PCR-based techniques could be related to the different amplification efficiency of universal primers (used in pyrosequencing) compared with family- and genus-specific primers (used in qPCR). Indeed, universal primers enhance the detection of highly abundant taxa but disfavour species represented by lower fractions (Gonzalez *et al.*, 2012).

**Conclusion**

Imposition of sequential aeration was successful in the rapid suppression of NOB activity and stimulating and maintaining AnAOB activity in the MABR, at very high effective O$_2$ loadings (4-14g-O$_2$ m$^{-2}$ day$^{-1}$). qPCR-based analysis revealed the following most remarkable shifts in the biofilm composition: a strong and moderate decrease in, respectively, the *nirK*, *Nitrospira* and *Nitrobacter* 16S rRNA gene abundance, and a strong increase in the AnAOB 16S rRNA gene abundance.

FISH analysis of the biofilms, after long-term sequential aeration, confirmed radial microbial stratification, with AOB at very high cellular densities in the O$_2$-rich areas close to the membrane surface, and AnAOB located close to the bulk liquid, separated by a transition region potentially harbouring denitrifying HB supported by decay products. Extant diversity assessed by using phylogenetically defined FISH probes revealed that halophilic and halotolerant *Nitrosomonas* spp., *Ca*. Brocadia anammoxidans and *Nitrospira* spp. were the most abundant lineages within the AOB, NOB and AnAOB guilds respectively. AOB and AnAOB constituted the most abundant community fractions, outnumbering NOB. Deep sequencing of the mature biofilm showed that the AOB guild was dominated by a single *N. europaea* OTU and confirmed a diverse AnAOB guild containing one most abundant *Brocadia* spp. OTU. Overall, our multipronged analysis confirmed that sequential aeration regimes can be used to successfully to engineer a microbial community to attain high-rate autotrophic N removal.
Experimental procedures

Samples

Samples were obtained from a MABR performing stable N removal at 5.5 g-N m$^{-2}$ day$^{-1}$ via the nitritation-anammox pathway. This reactor housed 10 membrane bundles, each containing 128 30 cm long hollow-fibres (Model MHF3504, polyethylene/polyurethane, Mitsubishi Rayon, Tokyo, Japan). The reactor was inoculated in two steps with biomass from nitriﬁng (day −30) and AnAOB enrichment cultures (day 0), and was fed a synthetic NH$_4^+$-rich inﬂuent (100–500 mg-N l$^{-1}$), while air was passed through the ﬁbre lumens (2.5–40 kPa and 2.5–60 l min$^{-1}$). After 390 days of operation the reactor was aerated in sequential cycles comprising aerated and non-aerated periods (Pellicer-Nàcher et al., 2010). On days 90, 150, 390 and 480, biofilm samples were detached and removed from the reactor by inserting a Pasteur pipette through the installed sampling ports and aspiring biomass from several ﬁbre bundles at several heights. In these samples, bioﬁlm structure could not be preserved. More comprehensive and structurally intact biofilm samples were obtained sacrificially at the end of the reactor run (day 630).

FISH analysis

Bioﬁlm samples were collected after 630 days of operation by cutting one ﬁbre at three different locations along its length (lower, middle and upper). Specimens containing both bioﬁlm and membrane were ﬁxed in 4% paraformaldehyde, embedded in OCT compound (Sakura Finetek Europe, Zoeterwoude, the Netherlands), frozen at −21°C, cut in 20 μm-thick sections by using a microtome, and mounted on gelatine-coated slides. Sectioned samples were dehydrated and sequentially probed with Fluorescein (FLUO), Cy3- and Cy5- tagged 16S rRNA probes (Sigma Aldrich, St Louis, MO, USA, Table 1), following procedures described elsewhere (Terada et al., 2010). Bioﬁlm structure was further studied by staining the prepared sections with a general ﬂuorescent nucleic acid stain following manufacturer’s speciﬁcations (SYTO 60, Invitrogen, Carlsbad, CA, USA).

Hybridized and stained sections were inspected with a confocal laser-scanning microscope (CLSM, Leica TCS SP5, Leica Microsystems, Wetzlar, Germany) equipped with an Ar laser (488 nm), and two HeNe lasers (543 and 633 nm). Gain and pinhole settings were tuned for each registered image in order to allow unsaturated exposure values for all detected pixels. At least ﬁve micrographs obtained from specimens hybridized with probe combinations 1 and 5 (Table 1) were used to quantify the abundance of AOB, AnAOB and NOB using the image analysis software Daime (Daims et al., 2006). Images of SYTO stained bioﬁlms were analysed with Leica AS AF Lite (Leica) and ImagePro (MediaCybernetics, Des Moines, IA, USA) software to determine bioﬁlm porosities and probe intensity proﬁles along bioﬁlm depth.

DNA extraction

DNA was extracted from 0.5 g of bioﬁlm mass (dry weight) using the MP FastDNA™ SPIN Kit (MP Biomedicals, Solon, OH, USA) according to manufacturer’s instructions. The quality of the extracted genomic DNA was checked by measuring its 260/280 nm absorbance ratio with a NanoDrop (ThermoFisher Scientiﬁc, Waltham, MA, USA) and was later stored at −20°C in Tris-EDTA buffer until further processing.

High-resolution tag-based 16S rRNA sequence library

Bioﬁlm DNA sampled on day 630 was ampliﬁed with Phusion (Ftu) DNA Polymerase (ThermoFisher Scientiﬁc) and the 16S rDNA universal primers PRK341F (5′-C TTAYGGG RBGCACAG-3′) and PRK806R (5′-GG ACTACNGGG TATCTAAT-3′) (Yu et al., 2005) with 25 annealing and elongation cycles. Detailed description of the PCR ampliﬁcation conditions have been described before (Masoud et al., 2011; Sundberg et al., 2013). Ampliﬁed DNA was puriﬁed using the QiAquick PCR puriﬁcation kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s protocol. In a second 15-cycle PCR round, barcodes and tags were added. All 16S rDNA fragments comprising the V3 and V4 hypervariable regions were pyrosequenced using a 454 FLX Titanium sequencer (Roche, Penzberg, Germany). Detailed description of the pyrosequencing preparation process can be found elsewhere (Farnelid et al., 2011).

Bioinformatic analyses

All raw 16S rDNA amplicons were processed and classiﬁed using the QIIME (http://qiime.org/index.html) software package (Caporaso et al., 2010b). Chimera checking and denoising were performed with the software AmpliconNoise (Quince et al. 2011). Retrieved sequences were clustered at 97% evolutionary similarity and aligned against the Greengenes reference set (DeSantis et al., 2006) using the Pynast algorithm (Caporaso et al., 2010a). Taxonomy index of each functional guild is given in Table S2. Phylogenetic trees were created in ARB using library sequences of interest together with selected representatives from a small subunit (SSU) reference library (SSU Ref. Nr. 111 Silva). Statistical calculations and phylogenetic comparisons were carried out using R software (R Development Core Team, 2012).

Quantitative PCR (qPCR)

Bioﬁlm DNA sampled and extracted throughout the whole operational period (days 0, 90, 150, 390, 480 and 630) was subject to qPCR to determine the numbers of AOB, NOB, AnAOB and denitriﬁying bacteria, based on appropriate 16S rRNA targets or functional genes (Table 3). Details on the procedure can be found elsewhere (Pellicer-Nàcher et al., 2010; Terada et al., 2010).

Microelectrode measurements

Oxygen Clark-type microsensors were constructed to measure DO concentrations within the bioﬁlm. Preparation, calibration and microsensor measurement were performed as previously described (Revsbech, 1989; Pellicer-Nàcher et al., 2010).

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Conflict of Interest

None declared.

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| Primers | Target Organism | Target molecule | Sequence (5′-3′) | Reference |
|---------|----------------|----------------|-----------------|-----------|
| ABI5f   | All Bacteria    | Eubacterial 16S rRNA gene | ATG GCT GTC GTG AGG T | Lane, 1991; Ferris *et al.*, 1996 |
| 1392r   |                | 16S RNA gene | ACG GCC GCT GTC TAC | |
| CTO189fa/b | β-proteobacterial AOBs | 16S RNA gene | GGA GAA AAG CAC GGG GTC ATC | Kowalchuk *et al.*, 1997; Hermansson and Lindgren, 2001 |
| CTO189fc |                | 16S RNA gene | GGA GAA AAG TAG GGC ATC | |
| RT1r    |                | 16S RNA gene | CGT CCT AGA CCA CCA ACT G | |
| FGPS872f | *Nitrobacter* NOB | 16S RNA gene | CTA AAA CTC AAA GGA ATT GA | Degrange and Bardin, 1995 |
| 1269r   |                | 16S RNA gene | TTT TTT GAG ATT TAG | |
| Nspra675f | *Nitrospira* NOB | 16S RNA gene | GCG GTG AAA TGG GTC GTG GAT | Graham *et al.*, 2007 |
| 746r    |                | 16S RNA gene | TCA GCG TCA GRW AGY TTC CAG AG | |
| F1370f1 | *Nitrobrother* | rnxA gene | CAG ACC GAC GTC GGC AAAG | Poly *et al.*, 2008; Wertz *et al.*, 2008 |
| F2843r2 | *Nitrococcus* NOB | | TCC ACA AAG AAC GGA AGG TC | |
| Amx809f | *AnAOB* | | GCC GTA AAG GAT GGG CAC T | Tsushima *et al.*, 2007 |
| Amx1066r | | | ATG GGC ACT MRG TAG AGG GGT TT | |
| hzoQf   | *AnAOB* | Hzo gene | CAT GGTCAA TTG AAA GRC CAC C | Park *et al.*, 2010b |
| hzoQr   | | | GCC ATC GAC ATA CCC ATA CTS | |
| cd3aF   | Denitrifying bacteria | nirS gene | AAC CYS AAG GAR ACS GG | Throbäck *et al.*, 2004 |
| R3cd    | | | GAS TTC GGR TGS GTC TTS AYG AA | |
| F1aCu   | Denitrifying bacteria | nirK gene | ATC ATG GT(C/G) CTG CCG CG | Hallin and Lindgren, 1999 |
| R3Cu    | | | GCC TCG ATC AG(A/G) TTG TGG TT | |
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