Nitrifying Community Analysis in a Single Submerged Attached-Growth Bioreactor for Treatment of High-Ammonia Waste Stream

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ABSTRACT: This study investigated the nitrifying community structure in a single-stage submerged attached-growth bioreactor (SAGB) that successfully achieved stable nitrogen removal over nitrite of a high-strength ammonia wastewater. The reactor was operated with intermittent aeration and external carbon addition (methanol). With influent ammonia and total Kjeldahl nitrogen ranging from 537 to 968 mg/L and 643 to 1510 mg/L, respectively, 85% nitrogen removal was obtained, and effluent was dominated by nitrite ($NO_2/NOx > 0.95$). Nitrifying community analysis using fluorescence in situ hybridization (FISH), with a hierarchical set of probes targeting known ammonia-oxidizing bacteria (AOB) within beta-proteobacteria, showed that the AOB community of the biofilter consists almost entirely of members of the *Nitrosomonas europaea/eutropha* and the *Nitrosococcus mobilis* lineages. Image analysis of FISH pictures was used to quantify the identified AOB, and it was estimated that *Nitrosomonas europaea/eutropha*-like AOB accounted for 4.3% of the total volume of the biofilm, while *Nitrosococcus mobilis*-like AOB made up 1.2%; these numbers summed up to a total AOB fraction of 5.5% of the total volume on the biofilm. Nitrite-oxidizing bacteria (NOB) were not detectable in the biofilm samples with probes for either *Nitrospira* sp. or *Nitrobacter* sp., which indicated that NOB were either absent from the biofilters or present in numbers below the detection limit of FISH (<0.1% of the total biofilm). Nitrite oxidizers were likely outcompeted from the system because of the free ammonia inhibition and the possibility that the aeration period (from intermittent aeration) was not sufficiently long for the NOB to be released from the competition for oxygen with heterotrophs and AOB. The nitrogen removal via nitrite in an SAGB reactor described in this study is applicable for high-ammonia-strength wastewater treatment, such as centrate or industrial wastes. *Water Environ. Res., 79* (2007).

KEYWORDS: nitrogen removal, sidestream treatment, nitrifying community structure, ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, fixed-film.

Introduction

Many publicly owned treatment works (POTWs) are facing increasingly stringent nutrient limits. The solutions associated with stringent nutrient limits are quite complex; not only do they require

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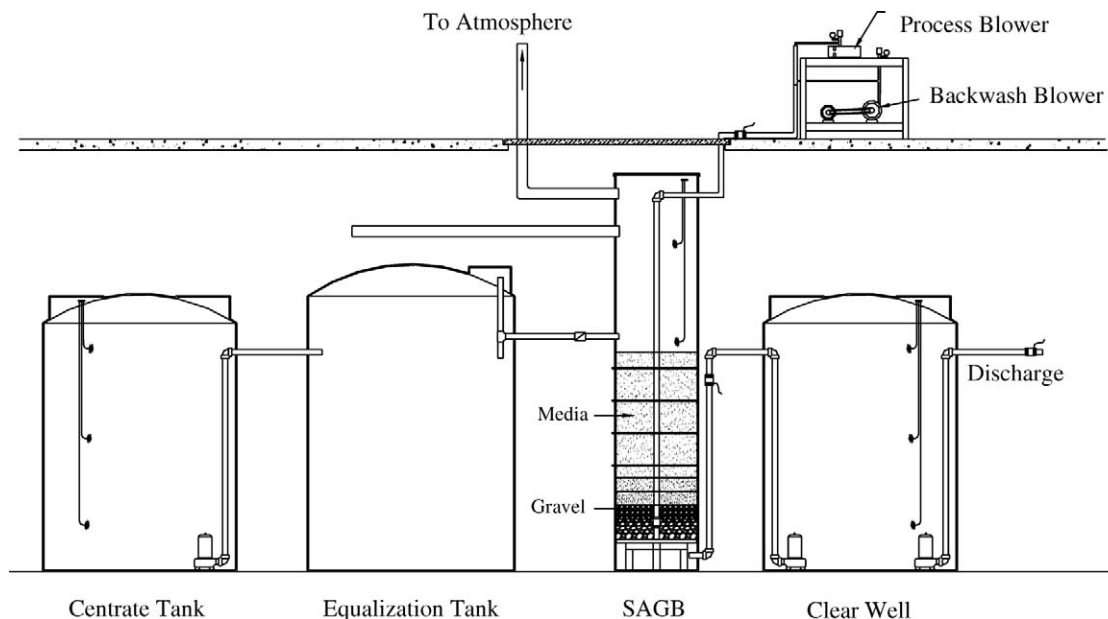
more sophisticated and reliable nutrient removal processes, but also a more comprehensive and integrated management of all treatment components. One important component of the process is the recycled sidestream, especially if anaerobic digestion is being applied. The digester sludge centrate/filtrate typically contributes 0.3 to 1.5% of the influent flow, but up to 10 to 30% of the total nitrogen load to the plant. Typically, the centrate is returned back to the head end of the process or to the secondary process. Recycle of centrate/filtrate negatively affects the nutrient-removal process stability and the effluent limits compliance for the following reasons:

(1) The recycled nitrogen load increases the demand of treatment capacity, in terms of reactor and aeration;
(2) The often-practiced intermittent operation of solids handling processes causes fluctuation in the influent nitrogen load, which can lead to bleeding through of ammonium-nitrogen (NH_4-N) in the effluent and sometimes biological system upset;
(3) The centrate/filtrate has a high NH_4-N concentration (600 to 1200 mg/L) and low biodegradable chemical oxygen demand (50 to 200 mg/L), thereby negatively affecting the denitrification efficiency, by reducing the carbon-to-nitrogen (C/N) ratio; and
(4) The increased NH_4-N load consumes influent alkalinity during the nitrification process and can cause alkalinity deficiency, which will negatively affect nitrification.

Technology-based or near-limit-of-technology nutrient removal processes are already being proposed in a number of regions, including Long Island Sound and the Chesapeake Bay. Typically, 3.0 mg/L total nitrogen and less than 0.1 mg/L total phosphorus are considered as technology limits for secondary wastewater treatment plants. At these very low levels, it becomes more and more difficult for the POTWs to consistently meet effluent limits. To eliminate sporadic inconsistencies and upsets, management and separate treatment of recycle centrate/filtrate becomes crucial for the plant to meet compliance. Therefore, efficient, reliable, and cost-effective treatment technologies for nitrogen removal in dewatering centrate/filtrate are needed.

There are a number of emerging technologies for removing nitrogen from ammonia-rich return streams, including physical/chemical treatment processes and biological processes. Physical/chemical treatment technologies include ammonia stripping with steam or hot air; ion-exchange, such as the ammonia-recovery process; and the magnesium-ammonium-phosphate (struvite)

Figure 1—Schematic of SAGB pilot plant.

precipitation process. A number of biological centrate treatment processes have been developed, including enhanced nitrification/denitrification with bioaugmentation, such as In-Nitri (Inexpensive Nitrification, M2T Technologies, State College, Pennsylvania) and bioaugmentation batch enhanced processes; partial nitrification and denitrification over nitrite, such as Single reactor High activity Ammonia Removal Over Nitrite (SHARON); and deammonification processes, such as anaerobic ammonium oxidation (ANAMMOX). Compared with physical and chemical treatment, biological treatment is more cost-effective and more compatible with existing plant facilities and operation. Although successful cases have been reported for these novel biological processes, there is still a lack of extensive full-scale experiences, thus limiting their design and application.

Recently, single-stage nitrogen removal in fixed-film systems has been proposed to treat ammonia-rich wastewater (Helmer et al., 2001; Pedros et al., 2006). A novel single-unit, single-zone submerged-attached growth bioreactor (SAGB) was proposed for nitrogen removal in centrate (Onnis-Hayden, 2006; Onnis-Hayden et al., 2006; Pedros et al. 2006). A SAGB pilot plant at the Massachusetts Water Resources Authority (MWRA) Deer Island Wastewater Treatment Plant (Winthrop, Massachusetts) was operated to treat the centrate generated from dewatering the sludge from the anaerobic digestion process. The first stage of the research demonstrated the successful nitrogen removal over nitrite with the SAGB for centrate treatment and its performance stability over 10 months. The objective of this study was to assess the nitrifying community structure within the SAGB reactor and gain fundamental knowledge of the biological nitrification/denitrification process, to assist mechanistic model development and optimize the system for best performance.

Methodology

Submerged Attached-Growth Bioreactor Reactor and Pilot Plant. A SAGB pilot plant at the MWRA Deer Island Wastewater Treatment Plant, shown in Figure 1, was operated from March 2005 to January 2006, to treat the centrate generated from dewatering the sludge from the anaerobic digestion process at the main wastewater treatment plant. This SAGB has been previously applied successfully for on-site wastewater treatments (Pedros et al., 2004). The system consisted of one centrate storage tank, one sand filter (SAGB), one equalization tank, and one clear well (Figure 1). The SAGB was 0.762 m (2.5 ft) in diameter and had a media depth of 1.22 m (4 ft.). The sand media, with a 3.0-mm nominal diameter, results in a media porosity of approximately 40%. The media specific surface area is 820 m^2/m^3. The wastewater flows from the equalization tank by gravity into the SAGB. The flow through the SAGB alternated between downflow (forward flow) and upflow (reverse flow) modes. The upflow was accomplished by pumping from the clear well back up through the SAGB. To achieve the desired aerobic and anoxic conditions within the biofilm, process air was supplied intermittently in the reactor (5 minutes on and 10 minutes off). Supplemental alkalinity was added during the initial startup period, when no external carbon was added. After several weeks of operation, methanol was added as the supplemental electron donor for denitrification, and the addition of supplemental alkalinity was then terminated because of the subsequent alkalinity recovery from the increased denitrification. The filter was backwashed daily. The temperature and pH in the SAGB were maintained between 17 and 24°C and 7 and 8.2, respectively. More detailed descriptions of the pilot unit and its performance can be found in previous publications (Onnis-Hayden, 2006; Onnis-Hayden et al., 2006; Pedros et al., 2006).

Nitrifying Community Analysis and Quantification. To identify and quantify ammonia- and nitrite-oxidizing bacteria (AOB and NOB, respectively) and to test for the presence of anaerobic ammonia-oxidizing bacteria (ANAMMOX) in the SAGB, fluorescence in situ hybridization (FISH) in combination with a polymerase chain reaction (PCR)-based assessment of 16S rRNA genes was used.

Probe Selection. Nitrifying bacteria have been described from six different phylogenetic lines—AOB of the beta and gamma subclasses of Proteobacteria (β- and γ-AOB, respectively) and NOB of the genera *Nitrobacter*, *Nitrococcus*, *Nitrospira*, and

Table 1—Oligonucleotide primers and probes used in this study and their respective target groups.

Probe	Target group	Sequence (3′-5′)	FA/NaCl[a]	Reference
NSO 1225	Betaproteobacterial AOB	CGCCATTGTATTACGTGTGA	35/80	Mobarry et al., 1996
NSV443	Most *Nitrosospira* spp. NOB	CCGTGACCGTTTCGTTCCG	30/112	Mobarry et al., 1996
NEU23a[b]	*N. europaea/eutropha/halophila*	CCCCTCTGCTGCACTCTA	40/56	Wagner et al., 1995
NSE1472	*N. europaea/eutropha*	ACCCCAGTCATGACCCCC	50/28	Juretschko et al., 1998
NmV	*Nitrosococcus mobilis*	TCCTCAGAGACTACGCGG	35/80	Pommerening-Röser et al., 1996
NmIV	*Nitrosomonas cryotolerans*-lineage	TCTCACCTCTCAGCGAGCT	35/80	Pommerening-Röser et al., 1996
NmII	*Nitrosomonas communis*-lineage	TTAAGACACGTTCCGATGTA	25/159	Pommerening-Röser et al., 1996
Nmo218	*N. oligotropha/urea*-lineage	CGGCCGCTCCAAAAGCAT	35/80	Gieseke et al., 2001
NIT3[b]	genus *Nitrobacter* NOB	CCTGTGCTCCATGCTCCG	40/56	Wagner et al., 1996
Ntspa712[b]	Phylum *Nitrospira* NOB	CGCCTTCGCCACCGGCCTTCC	50/28	Daims et al., 2001
Ntspa662[b]	Genus *Nitrospira* NOB	GGAATTCCGCGCTCCTCT	35/80	Daims et al., 2001
Pla46/Pla46F	Phylum *Planctomycetes*	GAC TTG CAT GCC TAA TCC	30/112	Neef et al., 1998
AMX368	All ANAMMOX bacteria	CCT TTC GGG CAT TGC GAA	15/338	Schmid et al., 2003
AMX368F	All ANAMMOX bacteria	TTC GCA ATG CCC GAA AGG	NA[c]	Schmid et al., 2003
AMX820/AMX820R	ANAMMOX genera *Brocadia*, *Kuenenia*	AAA ACC CCT CTA CTT AGT GCC C	40/56	Schmid et al., 2003
BS820	ANAMMOX genus *Scalindua*	TAA TTC CCT CTA CTT AGT GCC C	20/225	Kuypers et al., 2003
1390R	All bacteria	GAC GGG CGG TGT GTA CAA	NA[c]	Lane, 1991

[a] Percent formamide in the hybridization buffer and micromolar NaCl in the washing buffer, respectively.
[b] Used together with an equimolar amount of unlabeled competitor oligonucleotides, as indicated in the reference.
[c] NA = not applicable (only used as primer in PCR).

Nitrospina; however, only three major groups (AOB within Betaproteobacteria and NOB of the genera *Nitrospira* and *Nitrobacter*) are to be expected in wastewater treatment (Koops et al., 2003; Purkhold et al., 2000; Schramm, 2003). Therefore, probes specific for these groups were chosen, and additional probes were used to resolve the various lineages within the genus *Nitrosomonas*. Anaerobic ammonia-oxidizing (ANAMMOX) bacteria belong phylogenetically to a distinct branch of the Planctomycetes (Jetten et al., 2003). Therefore, one probe targeting all Planctomycetes and specific probes for ANAMMOX bacteria were used to screen for ANAMMOX; furthermore, the presence of ANAMMOX-like sequences was assessed by a semispecific PCR-approach. All probes and primers used in this study and their specificity, hybridization conditions, and references are listed in Table 1.

Fluorescence In Situ Hybridization. Samples (biofilm on sand grains) were fixed on-site with paraformaldehyde (PFA, 4%) for 1 hour, washed twice with phosphate-buffered saline (PBS), and stored in 50% ethanol in PBS at −20°C until analysis. The FISH was performed according to standard protocols (Manz et al., 1992; Pernthaler et al., 2001). The PFA-fixed samples were divided into small aliquots, and the biofilm was removed from its substratum by vigorous shaking and vortexing. Biofilm material was then immobilized on gelatin-coated glass slides and dehydrated in increasing concentrations (50, 80, 96%) of ethanol. Hybridization was performed, as previously described (Manz et al., 1992; Pernthaler et al., 2001), in moisture chambers at 46°C for 90 minutes. The hybridization buffer contained 0.9 M sodium chloride (NaCl), 20 mM Trishydrochloride (Tris-HCl) (pH 8.0), 0.01% sodium dodecyl sulfate (SDS), 5 ng/μl CY3-labeled oligonucleotide probe (http://www.biomers.net, see Table 1), and probe-specific concentrations of deionized formamide (Table 1). Hybridization was followed by a stringent washing step (15 minutes, 48°C) in 20 mM Tris-HCl (pH 8.0), 5 mM ethylenediamine tetraacetic acid (EDTA), 0.01% SDS, and probe-specific concentrations of NaCl (Table 1). Hybridized samples were investigated on an epifluorescence microscope (Zeiss Axiovert 200M, Carl Zeiss, Jena, Germany) equipped with appropriate fluorescence filter sets and an Apotome module (Carl Zeiss) for optical sectioning. The abundance of FISH-positive populations was quantified by image analysis using the freeware software Daime (Daims et al., 2006).

Polymerase Chain Reaction-Based Search for ANAMMOX. DNA was directly extracted from the frozen biofilm samples, by a combination of enzymatic lysis (Juretschko et al., 1998) and bead beating (DNA spin kit for soil, Qbiogene Inc., Heidelberg, Germany), a method that has proved successful in the laboratory for lysing ANAMMOX cells in freshwater and marine sediments. Specific PCR amplification of ANAMMOX-like 16S rRNA gene sequences was attempted with the primer pair MX368F/AMX820R; for semispecific amplification of ANAMMOX and Planctomycetes, primer pairs Pla46F/AMX820R and AMX368F/1390R, respectively, were used. The PCR products were checked on agarose gel and bands of the expected size excised, purified, and cloned into *Escherichia coli* JM109 using the pGEM-T vector (Promega, Mannheim, Germany). Clones were screened for inserts of the right length by PCR with vector primers M13F/M13R, and 30 insert-positive clones were randomly picked for commercial sequencing (http://www.macrogen.com). Sequences were run against the Genbank database to identify closest relatives using BLAST (http://www.ncbi.nih.gov).

Results and Discussion

Nitrogen Removal in Submerged Attached-Growth Bioreactor. In this phase of the research, the objective was to oxidize the ammonia to nitrite and to denitrify from nitrite. The centrate was fed to the reactor at a flowrate of 0.19 m³/d (50 gpd). Influent NH_4-N and effluent NH_4-N, nitrite (NO_2^-), and nitrate
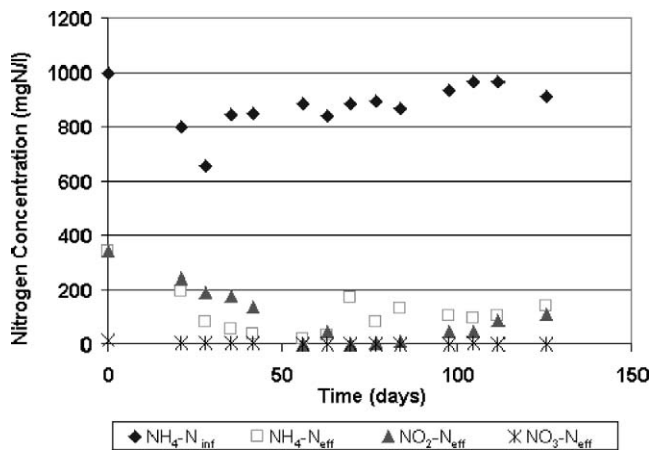
Figure 2—Nitrogen removal performance in the SAGB. Ammonia removal and nitrite/nitrate production in the SAGB during the pilot test.

(NO_3^-) during the pilot testing period are shown in Figure 2. The effluent consisted mainly of NH_4-N and NO_2^-N, and there was a marginal amount of NO_3^--N, indicating nitrogen removal over nitrite. The average influent NH_4-N and total Kjeldahl nitrogen (TKN) concentration was 880 mg/L and 1253 mg/L, respectively. The average NH_4-N/TKN ratio was 0.68. An overall total nitrogen removal of approximately 85% was achieved, with an average effluent total nitrogen concentration of 254 mg/L (118 mg/L of NH_4-N, 39 mg/L organic nitrogen, and 97 mg/L NO_2-N). The average nitrification efficiency (ammonia oxidation) was 91%. The total nitrogen removal rates ranged from 0.49 kg-N/m^3 · d at a loading

of 0.58 kg-N/m^3 · d to 1.19 kg-N/m^3 · d at a loading of 1.51 kg-N/m^3 · d. More detailed discussion of reactor performance was presented by others (Onnis-Hayden, 2006; Onnis-Hayden et al., 2006; Pedros et al., 2006).

Nitrifying Community Analysis. *Community Structure of Ammonia-Oxidizing Bacteria.* Ammonia-oxidizing bacteria were identified by a hierarchical set of probes (NSO1225, NEU23a, Nse1472, and NmV) as members of the *Nitrosomonas europaea/eutropha* and the *Nitrosococcus mobilis* lineages (see Figure 3). Figure 4 shows representative FISH images of identified specific AOBs. None of the other four probes (Nsv443, Nmo218, NmII, and NmIV) targeting beta-proteobacterial AOB yielded any positive results, indicating that the AOB community of the biofilter consists almost entirely of these two populations. Image analysis of FISH pictures was used to quantify the identified AOBs, and it was estimated that *Nitrosomonas europaea/eutropha*-like AOBs accounted for 4.3% of the total volume of the biofilm, while *Nitrosococcus mobilis*-like AOBs made up 1.2%; these numbers sum up to a total AOB fraction of 5.5% of the total volume on the biofilm retrieved from the SAGB reactor. Note that the FISH image analysis method actually quantifies the probe-specific area (or volume) within a sample, not cell numbers.

Because of the difference in enzyme affinities and reaction kinetics, activity and abundance distribution of nitrifiers in a system can be affected by many factors, including dissolved oxygen (DO) concentration; substrate concentration (i.e., ammonia, nitrite, and nitrate concentrations); competing substrate (i.e., carbon); and other environmental conditions, such as pH, temperature, and salinity. The two dominant AOB found in the SAGB reactor, namely *Nitrosomonas europaea/eutropha* and *Nitrosococcus mobilis*, were also found in other high ammonium/high salt environments (Jurestschko et al., 1998; Koops et al., 2003). Jurestschko et al. (1998)
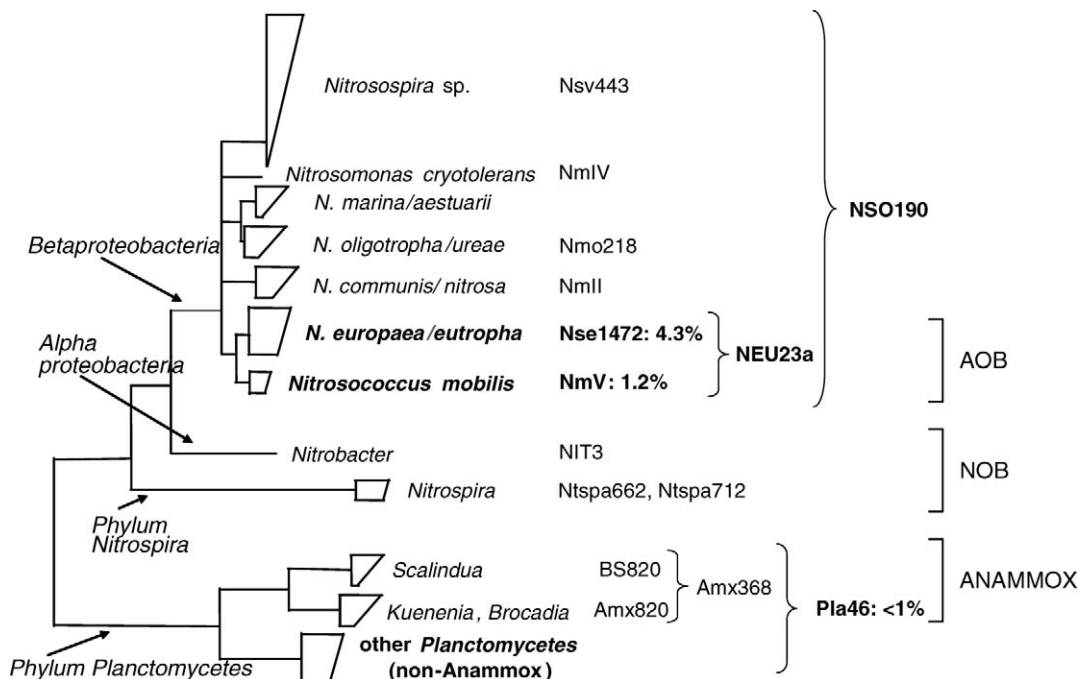

Figure 3—Schematic phylogeny of beta-proteobacterial AOB, NOB, and ANAMMOX targeted by the applied FISH probes. Probes that yielded positive results and the respective bacterial groups therefore detected in the bioreactor are indicated in bold.
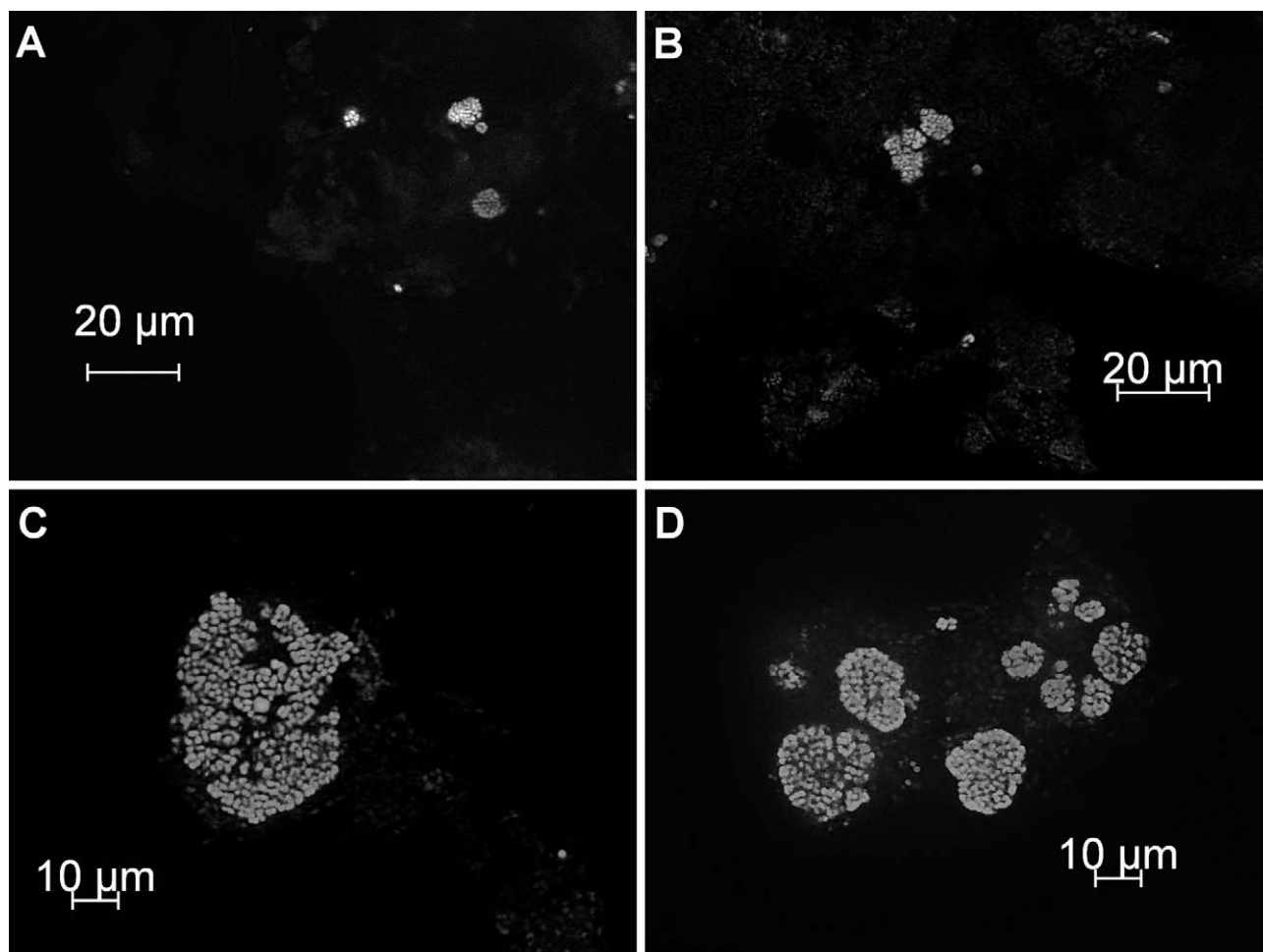
Figure 4—FISH images (A, B = overview, 630×; C, D = details of AOB cluster, 1000×) of AOB (lighter color) in the biofilm (darker background). A, C =: *Nitrosomonas europaea/eutropha*-like AOB, hybridized with probe Nse1472; and B, D = *Nitrosococcus mobilis*-like AOB, hybridized with probe NmV. Scale bars = 10 μm, except for panel C (5 μm).

investigated the nitrifying community at the Kraftisried wastewater treatment plant (Kraftisried, Germany), which receives wastewater with exceptionally high NH_4-N concentrations (up to 5000 mg/L), stemming from the decay of protein-rich material handled by the adjoining animal-waste-processing facility. The intermittent aeration allowed for more than 90% removal of the nitrogen compounds. The high influent ammonia and intermittent aeration that are common for both the Kraftisried plant and the SAGB in this study likely contributed to the selection of similar AOB. *N. mobilis*-like ammonia oxidizer was originally isolated from brackish water and had not been reported to contribute to nitrification in wastewater treatment, until the observation by Jurestschko et al. (1998). Okabe et al. (2002) also detected *Nitrosococcus mobilis*-like AOB in the autotrophic nitrifying biofilms with synthetic wastewater that contained high salt (4.8 mM Cl^-). *N. eutropha/europaea* were often found in nitrification systems and are known to be **r**-strategists, with low substrate affinity and higher growth rate (Gieseke et al., 2001; Okabe et al., 2002; Schramm, 2003). The high substrate (ammonia) concentration in the SAGB reactor likely favored the high-growth-rate AOB *N. eutropha/europaea*.

Absence of Nitrite-Oxidizing Bacteria. Nitrite-oxidizing bacteria were not detectable in the biofilm samples, with probes for either *Nitrospira* sp. or *Nitrobacter* sp. (alpha proteobacteria), which indicates that NOB were either absent from the biofilter or present in numbers below the detection limit of FISH (<0.1% of the total biofilm). The absence of NOB is supported by the nitrite accumulation and lack of nitrite oxidation observed in the SAGB (Figure 2).

In most other studies of nitrifying biofilms or flocs using FISH, a close association of AOB and NOB has been observed (i.e., Mobarry et al., 1996; Schramm et al. 1998, 1999). For fixed-film systems, nitrification is often confined to a narrow zone (100 to 150 μm) at the biofilm surface, where oxygen and ammonia are available (Schramm et al., 1998). In the case of the sand-grain-attached biofilms of the SAGB, biofilm thickness is barely exceeding 50 to 100 μm, and thus the whole biofilm can be actively nitrifying under autotrophic and oxic conditions. However, the abundance and spatial distribution of nitrifiers are also affected by the mass-transfer rate and concentration gradient of oxygen and nitrogen species along the depth of a biofilm. Because of this, the carbon/nitrogen ratio also affects the abundance and activity of nitrifiers in the biofilm, as a result of the competition for oxygen between autotrophic nitrifiers and heterotrophic bacteria (Okabe et al., 2002; Satoh et al., 2000). The methanol addition applied to

the SAGB for enhancing denitrification produced a higher carbon/nitrogen ratio (2.7 g methanol/g N) than that required for partial denitrification via nitrite in the SAGB during the steady-state period of this study, which might lead to a relatively higher heterotrophic oxygen consumption rate at the biofilm surface. Thus, the competition for oxygen among heterotrophs, AOB, and NOB might contribute to the observed elimination of NOB from the reactor. Mechanisms for stable partial nitrification in the SAGB will be discussed in more detail below.

Absence of ANAMMOX Bacteria. Special efforts were made to detect ANAMMOX bacteria in the biofilm; although the phylum-specific probe Pla46 showed low numbers of Planctomycetes in the biofilm samples, none of the ANAMMOX-specific probes (AMX368, AMX820, or BS820) gave positive results (see Figure 3). This hybridization pattern indicates the presence of Planctomycetes other than the known ANAMMOX bacteria.

To possibly detect ANAMMOX bacteria not targeted under the stringent hybridization conditions by our specific probes or only occurring in numbers below the detection limit of FISH ($<0.01\%$ of all cells; because of the peculiar morphology of ANAMMOX cells, the detection limit for ANAMMOX is actually lower than for NOB), an additional PCR-based approach was initiated. A highly specific PCR reaction using primers AMX368F/AMX820R did not result in ANAMMOX-specific PCR products, while two semi-specific PCR reactions yielded products of the right size. However, none of the 30 sequences retrieved from these reactions were highly similar to known ANAMMOX bacteria or even close to the distinct ANAMMOX lineage. Instead, they were affiliated to other lineages within the Planctomycetes that contain environmental sequences and cultured heterotrophic representatives (i.e., *Isosphaera* and *Nostocoida*) with an aerobic, polymer-degrading metabolism. Several sequences even grouped outside the Planctomycetes in recently proposed candidate divisions or close to the Acidobacteria, Firmicutes, and Verrucomicrobia (data not shown). These results indicate the following; first, our PCR approach was broad enough to target a wide range of Planctomycetes and even sequences just outside the phylum Planctomycetes. Therefore, the detection of "novel" ANAMMOX sequences not targeted by our FISH probes should have been possible by PCR. Such sequences were, however, not detected. Second, Planctomycetes occurring in the system are not related to ANAMMOX, but most likely are involved in aerobic organic polymer degradation or fermentation. In conclusion, ANAMMOX is absent from the system; thus, anaerobic oxidation of ammonia with nitrite was not occurring in the reactor. The ANAMMOX activity requires partial nitrification (to produce nitrite), low dissolved oxygen (<0.6 to 0.8 mg/L), higher pH (>7.6), long solids retention time (SRT), and adequate NH_4-N/NO_2^- ratio. The oxygen-limited autotrophic nitrification and denitrification (OLAND) process (Kuai and Verstrate, 1998) in a biofilm reactor obtained ANAMMOX activity, with the dissolved oxygen concentration controlled between 0.6 and 0.8 mg/L. Although nitrite was produced and low dissolved oxygen conditions existed in the SAGB periodically, no ANAMMOX bacteria were detected. This suggests that ANAMMOX may require constantly low dissolved oxygen and cannot tolerate intermittent aeration; the presence and quality of organic carbon might be other regulating factors affecting the occurrence of ANAMMOX.

Denitrification Over Nitrite in Submerged Attached-Growth Bioreactor. The stable and high $NO_2/(NO_2 + NO_3)$ ratio (0.97 to 1.0) in the reactor effluent and the nondetectable NOB demonstrates that the operation conditions in the SAGB sequestered the nitrite oxidation and maintained stable nitrogen removal over nitrite. Biological nitrogen removal via the nitrite pathway is desirable, because the elimination of nitrite oxidation reduces the oxygen requirement by approximately 25% and decreases the external carbon source demand (for subsequent denitrification) by approximately 40%. In addition, it has been reported that denitrification rates with nitrite are 1.5 to 2 times greater than with nitrate (Abeling and Seyfried, 1992). Furthermore, less sludge is produced (0.8 to 0.9 versus 1 to 1.2 kg dry weight/kg-N, according to Mulder [2003]).

Strategies that have been investigated to obtain nitrite as the main product of nitrification include the following:

(1) Use of pure AOB (i.e., *Nitrosomonas* sp.) cultures,
(2) Inhibition of NOB via free-ammonia inhibition and/or low dissolved-oxygen-limiting conditions, and
(3) Selection of AOB with higher specific growth rates compared with NOB at higher temperatures ($>25°C$) and lower SRTs.

The realization of stable nitrite formation by selecting AOB is based on differences in growth rate, oxygen affinity, and/or inhibition characteristics between AOB and NOB.

Among these approaches, the SHARON process (Hellinga et al., 1998) is one of the most promising approaches to achieve stable nitrification/denitrification over nitrite, and several full-scale reactors are in operation. The SHARON reactor is operated at a high temperature (30 to 40°C), a neutral pH (approximately 7.0), and a short SRT (<1 to 2 days). Under these conditions, NOB grow slower than AOB, so that they are washed out from the reactor. The SHARON process is well suited for reducing the nitrogen load of streams with a high ammonium content (>500 mg NH_4^+-N/L), but it is has not been indicated for obtaining strict effluent standards. The SHARON reactor is typically operated as a continuous, completely mixed tank reactor (chemostat), and denitrification is applied with an external carbon source for pH control. Nitrification and denitrification either take place in a single reactor working with intermittent oxic and anoxic phases or in two separate reactors (an oxic one and an anoxic one), with recirculation between both.

In our study, stable nitrogen removal via nitrite was achieved in a single-stage fixed-film reactor at room temperature (20 to 25°C) and at pH 6.5 to 8.3 (mostly 7 to 8), which would not eliminate NOB by washing them out. A broad range of factors, such as dissolved oxygen, pH, temperature, free ammonia (NH_3), free nitrous acid (HNO_2), free hydroxylamine (NH_2OH), and SRT influence the nitrite accumulation and inhibition and/or elimination of NOB (Garrido et al., 1997; Yoo et al., 1999). Literature shows that the accumulation of nitrite frequently occurs in systems treating high-strength ammonia wastewater, and it has been linked to the presence of free ammonia (Anthonisen et al., 1976; Yun and Kim, 2003), dissolved oxygen limitation (Garrido et al., 1997; Kuai and Verstraete, 1998), and dissolved oxygen-to-ammonia ratio (Çeçen, 1996). Evaluation of potential effects of these factors on nitrite accumulation in the SAGB is discussed in the following section.

To determine the type and level of inhibition, the concentration of un-ionized or free ammonia and free nitrous acid was calculated (Anthonisen et al., 1976; Onnis-Hayden, 2006; Pedros et al., 2006). The concentration of free ammonia within the filter at different depths ranged from 0.29 to 36 mg-NH_3-N/L (Figure 5); the graph also shows the concentrations in the centrate and the anoxic tank. Inhibition of nitrite oxidation has been reported at concentrations of free ammonia of 0.1 to 1 mg/L (Çeçen, 1996; Turk and Mavinc, 1986), 5 mg/L (Turk and Mavinic, 1989), and 5 to 20 mg/L (Ford
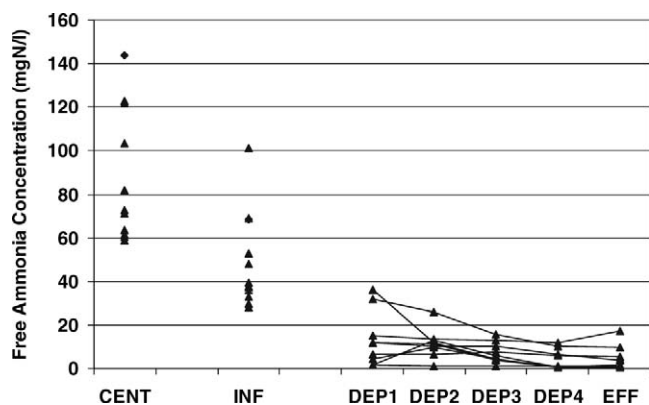
Figure 5—Calculated free ammonia concentrations in the feeding and equalization tanks, within different depth of the SAGB filter and in the effluent, during the testing period (CENT = centrate feed tank; INF = influent to the SAGB filter; DEP1,2,3,4 = different depth within the filter bed; and EFF = effluent).

Figure 6—Relation of nitrite production to the bulk ratio of dissolved oxygen concentration to ammonium concentration.

et al., 1980). Yun and Kim (2003) investigated the free ammonia inhibition on nitrite production. An influent ammonia loading ranging from 1.9 to 3.8 kg N/m³-d, the same level of nitrite accumulation as that observed in this study (NO_2/NO_x = 0.95), was demonstrated, regardless of the increasing aeration supply rates, indicating that free ammonia inhibition rather than dissolved oxygen limitation was the major cause for nitrite oxidation inhibition. However, further batch tests showed that, although nitrite oxidation was inhibited by high free ammonia concentration, NOB were still present, and nitrate production quickly resumed as soon as the ammonia levels dropped below a threshold level (Yun and Kim, 2003). Therefore, high free ammonia concentration could inhibit the nitrite oxidation, but would unlikely exclude the NOB from the system. Furthermore, adaptation of NOB to the free ammonia was observed by Turk and Mavinic (1986, 1987). In their study, nitrite buildup was achieved with intermittent contact to high free ammonia levels (5 mg NH_3-N/L) in the first cell of a four-cell system. However, nitrite buildup could not be sustained for a long term, because of the acclimation of the *Nitrobacter* sp. to free ammonia. Similar observations were made by many others (Ford et al., 1980; Wong-Chong and Loehr, 1978). In our study, nitrate production was not observed at various loading rates, and adaptation of NOB to a relatively high ammonia concentration did not occur in the SAGB during the 10-month period of operation, as indicated by the nondetectable nitrate in the effluent and absence of NOB in the sludge samples. This implies that factors other than high free ammonia level alone resulted in the inhibition of NOB activity from the SAGB.

Nitrite was also found to accumulate at low dissolved oxygen concentrations (Garrido et al., 1997; Kuai and Verstraete, 1998; Wiesmann, 1994) and at high-ammonia-loading conditions (Çeçen, 1996; Yun and Kim, 2003). In an investigation conducted by Çeçen (1996) relating to the treatment of fertilizer wastewaters in a submerged biofilm reactor, the author found that nitrite production rate increases generally with increased ammonia loading rate (kilograms nitrogen per cubic meter per day), the ratio of bulk dissolved oxygen to bulk ammonium (C_{DO}/C_{NH4-N}) has to be greater than 1 to achieve full nitrification, and nitrite accumulation occurred at a C_{DO}/C_{NH4-N} less than 1. Similar results were obtained by Yun

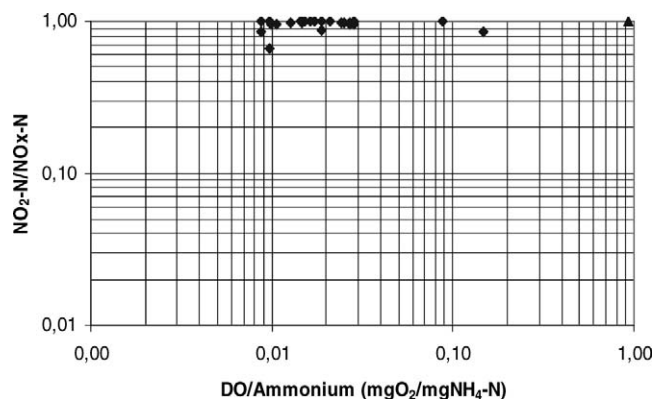and Kim (2003); indeed, a higher ammonia loading rate (>1.9 kg N/m³-d) led to nitrite buildup at bulk ammonium concentrations higher than 6 mg/L, with the dissolved oxygen concentration maintained at approximately 6 mg/L (C_{DO}/C_{NH4-N} <1). In our study, the ammonia loading rate ranged from 1.36 to 2.56 kgN/m³-d, and the fraction of NO_2 in NO_x produced versus the ratio C_{DO}/C_{NH4-N} in the bulk liquid (Figure 6) indicates that, for this study, the ratio of 0.01 to 0.12 resulted in almost 100% production of nitrite and very little nitrate. These results are consistent with observation by previous researchers (Çeçen, 1996; Yun and Kim, 2003). The inhibition of nitrite oxidation at lower dissolved oxygen concentration and/or high ammonia loading conditions is likely the result of the difference in their enzyme affinity to oxygen. Competition for oxygen exists between AOB, NOB, and heterotrophic bacteria at the oxic biofilm surface (Gieseke et al., 2001; Okabe et al., 2002; Satoh et al., 2000). For example, in a sequencing batch biofilm reactor for simultaneous phosphorus and nitrogen removal (Gieseke et al., 2001), nitrifiers coexisted and competed for oxygen with heterotrophs and phosphate-accumulating organisms (PAOs). Nitrification could only be maintained in the reactor with a sufficiently long aeration phase; at the end of this aeration phase, heterotrophs and PAOs became carbon-limited, allowing nitrification to resume. The NOB are considered to be more susceptible to oxygen limitation than AOB, because of their lower enzyme affinity than AOB, as indicated by their relatively lower oxygen half-saturation coefficients (K_s) than AOBs (Garrido et al., 1997; Kuai and Verstraete, 1998; Wiesmann, 1994). In our SAGB reactor, methanol was added at 2400 mg/L to enhance denitrification, leading to high heterotrophic oxygen uptake in the biofilm. During the aeration time, the dissolved oxygen concentration in the SAGB varied from 1.8 to 3.4 mg/L, whereas, without aeration, the dissolved oxygen concentration was typically below 0.5 mg/L. The aeration time was only 5 minutes, followed by 10 minutes without aeration, which was most likely not sufficient for NOB to compete with heterotrophs and AOB for oxygen. Preliminary results show that extended aeration times indeed result in nitrate production (data not shown), supporting our hypothesis that relatively low dissolved oxygen and insufficient aeration time length prohibited the proliferation of NOB in the SAGB, because of their relatively lower enzyme affinity to oxygen. Further investigation is needed to confirm and establish the correlation of dissolved oxygen level and aeration frequency and duration with NOB activity levels in the SAGB.

Conclusions

Efforts have been made to achieve nitrogen removal via nitrite because of its associated reductions of oxygen and external carbon source demands. Alternative systems exist, in which nitrite could be preferentially formed. These are immobilized pure ammonia-oxidizing cultures, fluidized beds, or fixed-film reactors that were operated at dissolved oxygen-limiting conditions or at high loading rates, to create ammonia inhibition conditions. However, most of these reported systems are laboratory-scale, and it is often difficult and problematic to control the aeration and dissolved oxygen in a fixed-film system, making it also difficult to control ammonia oxidation and nitrite accumulation. This study demonstrated that stable nitrogen removal over nitrite of a high-strength ammonia wastewater can be achieved by applying a single-stage SAGB (sand filter) with intermittent aeration and external carbon addition at a pilot-scale level. With influent ammonia and TKN ranging from 537 to 968 mg/L and 643 to 1510 mg/L, respectively, 85% nitrogen removal was obtained. The effluent was dominated with nitrite ($NO_2/NOx > 0.95$), and the effluent NH_4-N concentration averaged 117 m/L. Nitrifying community analysis showed that NOB were nondetectable in the reactor, and nitrogen was indeed removed via nitrite. The AOB were dominated by *Nitrosomonas europaea/eutropha* and the *Nitrosococcus mobilis* lineages, which may be associated with the high ammonia concentration and high reaction rates in the reactor. To our knowledge, this is the first report on a pilot-scale single-stage SAGB that successfully achieved stable nitrogen removal via nitrite. External carbon addition was needed, but no specific temperature, pH, and SRT control was required, as for other nitrite-production systems, such as the SHARON process. The NOB were likely inhibited and outcompeted from the systems, because of the free ammonia inhibition and the possibility that the duration of each aeration period was not sufficient for the dissolved oxygen-sensitive NOB to be released from the competition for oxygen with other heterotrophs and AOB. The nitrogen removal via nitrite in the SAGB described in this study is applicable for high-ammonia-strength-wastewater treatment, such as centrate or industrial wastes.

Credits

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