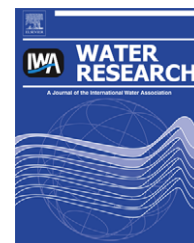


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Total nitrogen removal in a hybrid, membrane-aerated activated sludge process

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ABSTRACT

The hybrid (suspended and attached growth) membrane biofilm process (HMBP) is a novel method to achieve total nitrogen removal from wastewater. Air-filled hollow-fiber membranes are incorporated into an activated sludge tank, and a nitrifying biofilm develops on the membranes, producing nitrite and nitrate. By suppressing bulk aeration, the bulk liquid becomes anoxic, and the nitrate/nitrite can be reduced with influent BOD. The key feature that distinguishes the HMBP from other membrane-aerated processes is that it is hybrid; heterotrophic bacteria are kept mainly in suspension by maintaining low bulk liquid BOD concentrations. We investigated the HMBP's performance under a variety of BOD and ammonium loadings, and determined the dominant mechanisms of nitrogen removal. Suspended solids increased with the BOD loadings, maintaining low bulk liquid BOD concentrations. As a result, nitrification rates were insensitive to the BOD loadings, remaining at $1 \text{ gN m}^{-2} \text{ day}^{-1}$ for BOD loadings ranging from 4 to $17 \text{ gBOD m}^{-2} \text{ day}^{-1}$. Nitrification rates decreased during short-term spikes in bulk liquid BOD concentrations. Shortcut nitrogen removal was confirmed using microsensor measurements, showing that nitrite was the dominant form of oxidized nitrogen produced by the biofilm. Fluorescence *in situ* hybridization (FISH) showed that ammonia oxidizing bacteria (AOB) were dominant throughout the biofilm, while nitrite oxidizing bacteria (NOB) were only present in the deeper regions of the biofilm, where the oxygen concentration was above 2 mg/L . Denitrification occurred mainly in the suspended phase, instead of in the biofilm, decreasing the potential for biofouling. When influent BOD concentrations were sufficiently high, full denitrification occurred, with total nitrogen (TN) removal approaching 100%. These results suggest that the process is well-suited for achieving concurrent BOD and TN removal in activated sludge.

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1. Introduction

The removal of total nitrogen (TN), i.e., ammonium, nitrate, and nitrite, is an increasingly important goal for municipal and industrial wastewater treatment plants. Practical and cost-effective technologies are needed, especially for older plants with limited space for expansion. TN removal typically

is achieved through successive nitrification and denitrification. For nitrification, slow-growing nitrifying bacteria must be retained. Retention typically is achieved by operating at high solid retention times (SRTs), which may not be feasible for older activated sludge plants. Another approach is to add fixed or suspended biofilm attachment surfaces, which is the basis for the integrated fixed-film activated sludge (IFAS)

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process. IFAS can be readily retrofitted into conventional activated sludge plants for increased nitrification (Sen et al., 2000), but provides limited denitrification.

For denitrification, anoxic conditions and an electron donor are required. Common processes for combined nitrification and denitrification include the modified Ludzack–Ettinger process, the UCT process, and the Bardenpho process (Rittmann and McCarty, 2001). All these rely on predenitrification, where a portion of the nitrified effluent is recycled to an upstream, anoxic zone where influent BOD serves as an electron donor. Disadvantages include the need for high rates of water recirculation, loss of BOD to oxygen recirculated from aerobic zones, the need for long SRTs, and limited TN removals of up to 75–80% (Tchobanoglous et al., 2001).

Another approach to TN removal is through membrane-aerated bioreactors (MABRs), which use oxygen-supplying, biofilm-supporting membranes in a non-aerated, well-mixed tank. Oxygen is supplied at the base of the biofilm, producing aerobic conditions deep in the biofilm and anoxic conditions in the outer biofilm. Nitrifying bacteria grow in the deep, aerobic portions of the biofilm where BOD concentrations are low, and heterotrophic denitrifying bacteria grow in the outer, anoxic portions where they use nitrate or nitrite as electron acceptors. MABRs allow BOD removal, nitrification, and denitrification to take place concurrently in a single biofilm with little to no suspended growth in the system (Timberlake et al., 1988), and has been shown to be a promising technology for municipal wastewaters (Hibiya et al., 2003; Satoh et al., 2004; Semmens et al., 2003; Yamagiwa et al., 1994) and industrial wastewaters (Terada et al., 2003). Advantages of the MABR process include passive aeration, providing energy savings of up to 70% (Semmens, 2005); reduced tank volume; elimination of internal water recycle; and maximized use of influent BOD for denitrification.

A potential added benefit of membrane aeration is shortcut TN removal, which has been suggested for MABR systems (Hibiya et al., 2003; Terada et al., 2003, 2006). Shortcut TN removal is the reduction of nitrite rather than nitrate during denitrification, which can reduce carbon requirements by up to 40%. Nitrite accumulation is based on selection of ammonia oxidizing bacteria (AOB) over nitrite oxidizing bacteria (NOB), thus limiting the amount of nitrite oxidized to nitrate. Conditions that favor AOB over NOB include high temperature (Hellinga et al., 1998), high free ammonia (FA) (Anthonisen et al., 1976), high pH (Villaverde et al., 1997), and low dissolved oxygen (DO) (Munich et al., 1996). Inhibition of NOB via low DO may occur when membrane-aerated biofilms operate in an anoxic environment, where the outer biofilm regions have low or zero oxygen concentrations. AOB can outcompete NOB at lower oxygen concentrations, as they have a lower half saturation coefficient (K_o) value for oxygen (Schramm et al., 1998). In a nitrifying MABR, microsensor studies indicated that nitrite was the main product of nitrification (Schramm et al., 2000). MABRs achieving TN removal were shown to have low levels of nitrate in the biofilm (Hibiya et al., 2003), and an oxygen mass balance indicated that the majority of oxygen transferred to a MABR was used for ammonia oxidation, with little oxygen remaining for nitrite oxidation (Terada et al., 2003).

A major challenge with membrane aeration for wastewater treatment is biofouling. In MABRs used for TN removal, the

biofilm can include 60% heterotrophic biomass (Cole et al., 2004; LaPara et al., 2006), resulting in thick biofilms, with mass transfer limitations (Semmens et al., 2003). The effect is exacerbated at higher BOD:N loading ratios, significantly decreasing nitrification rates due to increased competition between heterotrophic and nitrifying bacteria in the biofilm (Walter et al., 2005).

Hybrid treatment systems, where both suspended and attached growth are utilized, can reduce biofouling by minimizing heterotrophic attachment while maintaining a nitrifying biofilm. At biofilm depths where the BOD is below the growth threshold concentration, S_{min} , cells can no longer grow. Therefore, if the bulk liquid BOD is close to S_{min} , then the heterotrophic biofilm thickness should be minimal. This was shown in a biofilm airlift suspension (BAS) reactors, where suspended heterotrophic growth increased and attached growth decreased with increasing SRT (Tijhuis et al., 1994).

For systems with BOD and ammonium, heterotrophic attachment can be minimized with little impact on nitrifying biofilms, as nitrifying bacteria have a much lower μ value than heterotrophic bacteria, and therefore require a much larger SRT to approach their S_{min} . Application of a hybrid system with suspended heterotrophs and attached nitrifiers by maintaining low bulk BOD concentrations has been shown in BAS reactors (van Benthum et al., 1997) and in the IFAS process (Randall and Sen, 1996; Sen et al., 2000; Sriwiriyarat and Randall, 2005). The attachment media incorporated into the activated sludge process in IFAS systems allow nitrification at reduced bulk liquid SRTs (bSRTs) while limiting heterotrophic attachment by maintaining a suspended heterotrophic population. This reduced bSRT allows for a much smaller reactor footprint, and also allows for nitrification to be achieved in wastewater treatment facilities designed only for BOD and TSS removal. In such plants, the higher bSRTs needed to maintain a suspended nitrifying population typically are not obtainable without major capital improvements. The limitation of the IFAS process is that oxygen is supplied in the bulk liquid, producing aerobic conditions in the outer biofilm and bulk liquid. Little BOD diffuses to the deep portions of the biofilm, where anoxic conditions exist, and therefore limited denitrification is achieved in IFAS.

The hybrid membrane biofilm process (HMBP) is the first attempt to use membrane aeration in a hybrid configuration for TN removal (Downing and Nerenberg, 2007). Hollow-fiber membranes are placed in a completely mixed activated sludge tank, providing oxygen to nitrifying biofilms on the outside of the membranes. Bulk liquid aeration is suppressed, allowing nitrite and nitrate produced by the nitrifying biofilm to be reduced by suspended denitrifying bacteria, using influent BOD as an electron donor. The bSRT can be as low as 2–3 days, which is sufficient to lower the bulk liquid BOD concentrations to near the S_{min} value, limiting heterotrophic growth in the biofilm. Thus, the HMBP effectively accomplishes nitrification and denitrification in a single tank, maximizes the use of influent BOD for denitrification, and improves nitrification performance by preventing thick biofilms.

Previous research showed that the HMBP concurrently removes BOD, nitrate, and nitrite, and that nitrifying bacteria were predominant in the biofilm, while heterotrophic bacteria

were mainly in the bulk liquid (Downing and Nerenberg, 2007). In this study, we (a) examine whether the HMBP is less sensitive to BOD loadings, and achieves less heterotrophic attachment, than the MABR; (b) examine the mechanisms of TN removal by measuring biofilm microgradients; and (c) use microbial ecology studies to reconcile biofilm structure and function.

2. Materials and methods

2.1. Reactor configuration

The HMBP reactor was similar to that described previously (Downing and Nerenberg, 2007). A bank of hollow-fiber membranes was inserted into an open rectangular tank (Fig. 1). The fibers were collected into bundles of 32 and potted at each end into 3-mm ID plastic tee fittings, resulting in 12.5 cm of exposed fiber length. The fibers were made of microporous polyethylene with a dense, polyurethane core (HFM200TL, Mitsubishi Rayon, Japan), and with a 280- μm OD. The total membrane surface area was 1900 cm^2 and the total tank volume was 6 L, resulting in a membrane specific surface area of 32 m^{-1} . Mixing was provided by a centrally located axial mixer with a 9-cm mixing blade, operated at 150 rpm. Given the high mixing rate, the system approximated a completely mixed tank reactor, where the bulk liquid concentrations are equal to the effluent concentrations. The effluent was directed to a 2-L conical settling tank, with a solid loading rate of 1.3 $\text{kg m}^{-2} \text{h}^{-1}$. The settler included a scraper arm, which was set to rotate intermittently for 1 min every 5 min. Solids were recycled to the rectangular tank from the bottom of the settler.

The influent flow rate was either 7.8 or 15.7 L day^{-1} and was achieved with a peristaltic pump (Masterflex pump, L/S 16 tubing), resulting in hydraulic retention times (HRTs) of 9.2 or 18 h, respectively. The solids' recirculation flow was 75% of the influent flow rate, and was supplied by a peristaltic pump

(Masterflex pump, L/S 17 tubing). The bulk liquid SRT (bSRT), as defined below, was maintained at approximately 2.5 days.

In previous studies, it was found that re-aeration via diffusion over the tank's liquid surface was significant (Downing and Nerenberg, 2007). This would be much less significant in a larger system, which would have a much lower surface-to-volume ratio. Therefore, the bulk liquid was continuously sparged with nitrogen gas to limit oxygen transfer from the water surface to the bulk liquid.

The assumption that the bench-scale reactor was completely mixed was verified using a tracer study. The HRT was tested with the membrane bank in place, but prior to inoculation. The aeration tank was filled with distilled water amended with 100 mg/L chloride. An influent flow of distilled water without chloride was fed to the aeration tank at a flow rate of 7.8 L day^{-1} . The decrease in chloride concentration with time was measured using ion chromatography (IC2500 with AS11/AG11 column, Dionex Corp, Sunnyvale CA, USA), and the data were fitted to the equation for a completely mixed tank (Droste, 1997). The measured HRT in the system was 9 h. The calculated HRT for this flow rate and active tank volume was 9.2 h; therefore, it was assumed that adequate mixing was achieved in the system.

2.2. Analytical methods

Ammonium ($\text{NH}_4^+\text{-N}$) was measured using a colorimetric method (Hach Company, Loveland, CO). Bulk liquid dissolved oxygen (DO) concentration was measured using a DO probe (YSI Model No. 55/25 FT). Nitrate ($\text{NO}_3^-\text{-N}$), nitrite ($\text{NO}_2^-\text{-N}$), and acetate were analyzed by ion chromatography (IC2500 with AS11/AG11 column, Dionex Corp, Sunnyvale CA, USA). Acetate was the BOD source, and BOD was calculated from the measured acetate concentration. Suspended solids were measured according to Standard Methods in Water and Wastewater (Rand et al., 1978).

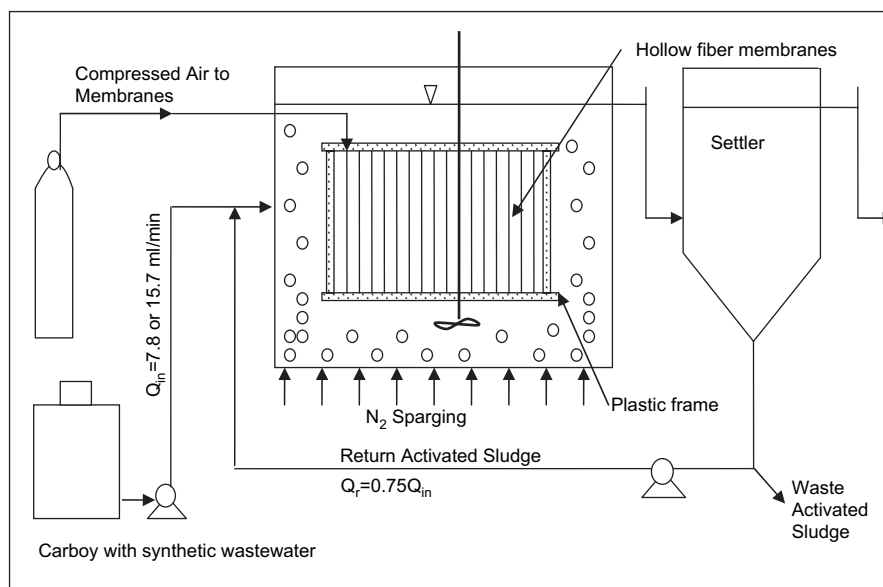


Fig. 1 – Schematic of the bench-scale HMBP. Nitrogen sparging of the bulk liquid was performed to maintain an anoxic bulk liquid.

The bSRT was calculated as the total suspended solids in the activated sludge tank divided by the solids lost in the effluent and lost through wasting (Rittmann and McCarty, 2001). The membrane-attached solids were not included in the total suspended solids in the tank. However, detached biomass from the biofilm system contributed to the effluent and wasted solids. This should not have significantly impacted the actual bSRT of the system, due to the low solid production by the nitrifying biofilm. For example, in a similar HMBP reactor under similar influent conditions, effluent solids produced by the nitrifying biofilm were less than 2 mg/L (Downing and Nerenberg, 2007).

2.3. Synthetic wastewater medium

A synthetic wastewater was prepared from distilled water amended with 1.386 g Na₂HPO₄, 0.849 g KH₂PO₄, 0.05 g MgSO₄·7H₂O per liter, as well as a trace mineral solution (Nerenberg et al., 2002). (NH₄)₂SO₄ was added to achieve either 20 or 12 mg/L NH₄⁺-N. Various BOD concentrations were achieved via acetate addition, where BOD was calculated by multiplying the acetate concentration by 1.085 mg BOD/mg acetate. The medium was kept anoxic by sparging with nitrogen and maintaining a positive nitrogen headspace in the medium bottle. The medium was maintained anoxic to prevent growth of heterotrophs and nitrifiers in the feed container. Influent pH was approximately 7.0 and the temperature was approximately 22 °C.

2.4. HMBP startup

Initially, the reactor was operated without hollow-fiber membranes, only an aeration tank and settler. Bulk liquid aeration was supplied with 41-cm porous tubing (Elite silicon tubing, Rolf C. Hagen, Inc, Montreal, Canada). The tank was inoculated with 2 mL of nitrifying activated sludge from a municipal wastewater treatment plant (Mishawaka, IN, USA). The influent consisted of 100 mg/L BOD and 20 mgN/L ammonium. Once BOD was consistently reduced and a consistent suspended solid concentration was observed, the membrane bank was inserted into the tank. Bulk liquid aeration was continued, with a DO of around 6 mg/L, until nitrification occurred. After observing consistent nitrification, bulk liquid aeration was discontinued.

2.5. Influent concentrations and loadings

A variety of loading conditions were used to determine the impact of the influent BOD:N surface loading ratio and the impact of bulk BOD concentration on nitrification (Table 1). Reactor startup lasted from day 1 to 30, after which the loading tests were initiated. At day 127, the membrane surface area was decreased to increase the surface loading. Over the course of the HMBP operation, effluent BOD increased occasionally, typically due to excess solid wasting or solid loss through the settling tank. These BOD spikes were used to assess the short-term impact of bulk liquid BOD concentrations on the nitrification rates.

Nitrogen assimilation was calculated based on the BOD consumed and the bSRT of the system (Rittmann and McCarty,

2001). Assimilation by the biofilm was assumed to be negligible compared to assimilation by suspended heterotrophs, and cells were assumed to contain 12.4% nitrogen by weight. Denitrification was calculated as the difference between ammonium removed and the sum of nitrate and nitrite formed plus nitrogen assimilated.

2.6. Microsensor measurements

The gradients of oxygen, ammonia, nitrate, and nitrite within the hollow-fiber membrane biofilm were measured in situ using microsensors. Clark-type oxygen microelectrodes with a 10-μm tip diameter (Ox10, Unisense, Århus, Denmark) were used to measure oxygen gradients (Revsbech and Jørgensen, 1986). Liquid ion exchange (LIX) microelectrodes were fabricated to measure ammonium (De Beer and van den Heuvel, 1988), nitrate (De Beer and Sweerts, 1989), and nitrite gradients (De Beer et al., 1997). Nitrate and ammonia electrodes had a tip diameter of 3–5 μm, and the nitrite electrodes had a tip diameter of 10–15 μm. The spatial resolution of microelectrodes is approximately twice the tip diameter (Geiseke and De Beer, 2004); therefore, measurements were taken at spatial intervals of 30 μm through the biofilm. A micromanipulator was used to move the microsensors (MM33-2, Unisense A/S, Århus, Denmark). For each measurement, gradients were measured at five locations on each of three different membrane bundles in situ. A single membrane was identified on each bundle, and the five gradient measurements were conducted at different positions along the length of the membrane.

Fluxes out of the biofilm were calculated to assess the nitrite and nitrate production rates (Geiseke and De Beer, 2004). The flux of the average profiles was used for calculations. Flux, J (g m⁻² day⁻¹), between two points can be calculated using Fick's first law

$$J_{ab} = -D_{\text{eff}} \frac{C_a - C_b}{X_a - X_b} \quad (1)$$

where J_{ab} is the mass flux between points a and b , D_{eff} is the effective diffusion coefficient (m² day⁻¹), C_a and C_b are the concentrations at points a and b (g m⁻³), and X_a and X_b are the depths of points a and b (m). The D_{eff} values were assumed to be 0.8 of the diffusion coefficient (D) for each constituent. The values used for D were 1.48×10^{-4} , 1.4×10^{-4} , and 1.4×10^{-4} m² day⁻¹ for ammonium, nitrate, and nitrite, respectively (Shanahan and Semmens, 2004). For calculating production rates in the biofilm, the flux through the diffusion boundary layer was used. The thickness of the diffusion boundary layer, δ_b , replaced $X_a - X_b$ in Eq. (1). C_a and C_b were the concentrations at the outer edge of the biofilm and in the bulk liquid, respectively. The diffusion boundary layer thickness was determined from the Sherwood number, Sh , using the following equation (Thibodeaux, 1979)

$$Sh = \frac{r_o}{\delta_b} \quad (2)$$

The radius of the biofilm, r_o (m), is the distance from the center of the membrane to the outer edge of the biofilm. The Sherwood number was found using an empirical relationship for turbulent flow (Reynolds number, Re , less than 1×10^5) (Thibodeaux, 1979)

Table 1 – Influent loading conditions

| Day | Loading condition | HRT (h) | Mem. area (cm ²) | Influent NH ₄ ⁺ (mgN/L) | NH ₄ ⁺ loading (gN m ⁻² day ⁻¹) | Influent BOD (mg/L) | BOD loading (gBOD m ⁻² day ⁻¹) | BOD:N surface loading ratio |
|---------|-------------------|---------|------------------------------|---|--|---------------------|---|-----------------------------|
| 30–50 | | 18 | 1900 | 20 | 0.83 | 140 | 5.8 | 7 |
| 51–75 | A | 9 | 1900 | 20 | 1.65 | 40 | 3.3 | 2 |
| 76–95 | A | 9 | 1900 | 20 | 1.65 | 80 | 6.6 | 4 |
| 96–126 | A | 9 | 1900 | 20 | 1.65 | 100 | 9.9 | 5 |
| 127–161 | B | 9 | 1400 | 20 | 2.24 | 40 | 4.5 | 2 |
| 162–175 | B | 9 | 1400 | 20 | 2.24 | 80 | 9.0 | 4 |
| 176–203 | B | 9 | 1400 | 20 | 2.24 | 100 | 12.4 | 5 |
| 204–240 | B | 9 | 1400 | 20 | 2.24 | 150 | 16.8 | 7.5 |
| 240–270 | C | 9 | 1400 | 12 | 1.46 | 150 | 16.8 | 12.5 |

$$Sh = 0.036Re^{1/2}Sc^{1/3} \quad (3)$$

Re was approximately 1×10^4 , and the Schmidt number, Sc, was calculated for each constituent using the following equation (Thibodeaux, 1979)

$$Sc = \frac{\nu}{D_{eff}} \quad (4)$$

where ν is the kinematic viscosity, ν is the same for each constituent, and D_{eff} was the same as used in Eq. (1).

The rates of denitrification in the biofilm and the bulk liquid were determined by mass balance, considering (1) the rate of denitrification in the biofilm is equal to the total flux of ammonia into the biofilm minus the flux of nitrite and nitrate out of the biofilm, and (2) the rate of denitrification in the bulk is equal to the rate of ammonium loss in the reactor, minus the outflow rate of nitrate/nitrite from the reactor, minus the rate of denitrification in the biofilm. Sensor measurements were taken on days 190, 240, and 270, when significant denitrification was occurring.

LIX sensors were calibrated initially and recalibrated frequently during measurements to minimize error. Calibrations were log-linear and nearly matched the Nernst equation for all measurements (Geiseke and De Beer, 2004). Oxygen sensors were calibrated initially and checked after completing measurements, as a negligible amount of drift occurred during measurements.

2.7. FISH

Molecular analysis of nitrifying biofilms allows insights into the stratification of AOB and NOB (Schramm et al., 1998, 1996, 2000), potentially revealing a basis for shortcut nitrogen

removal. The procedure used for maintaining the structure of the biofilm during FISH analysis was as described by Schramm et al. (1996). After microelectrode measurements, the target membrane was removed from the reactor, fixed in freshly prepared paraformaldehyde (4% in phosphate buffer solution (PBS)) at 4 °C for 1 h, and washed in PBS. Sectioning was performed with a cryostat (Leica CM1100) using OCT compound as an embedding agent. The membrane sample was embedded in OCT and stored at –80 °C for 24 h to allow full penetration of the OCT. Sectioning was performed laterally along the length of the membrane at –20 °C, with each section approximately 5 μm in thickness (Schramm et al., 2000). Each section was placed on a six-well slide (Erie Scientific). Biofilm sections were then dehydrated using sequential ethanol baths at 50%, 80%, and 100% concentrations, followed by drying at 46 °C.

Four oligonucleotide FISH probes were used for hybridization: Nso190, which targets the majority of AOB (Mobbary et al., 1996); NIT3, which targets *Nitrobacter* sp. (Wagner et al., 1996); NSR1156, which targets *Nitrospira* sp. (Schramm et al., 1998); and EUB338, which targets all bacteria (Amann et al., 1990). Probes, hybridization conditions, and washing conditions are summarized in (Table 2). Oligonucleotides were synthesized and fluorescently labeled with fluorochromes Cy3, Cy5, or FITC by Operon Biotechnologies, Inc. (Huntsville, Alabama, USA).

The hybridization procedure followed Manz et al. (1992). All hybridizations were completed at 46 °C for 90 min in hybridization buffer containing 0.9 M NaCl, formamide at the percentage shown in Table 2, 20 mM Tris–HCl (pH 7.4), and 0.01% SDS. The probe concentration was 5 ng/μL. Hybridization was followed by a stringent washing step at 48 °C for 15 min in a buffer containing 20 mM Tris–HCl (pH 7.4), NaCl

Table 2 – FISH probes used for ecology analysis in nitrifying membrane aerated biofilms

| Probe | Specificity | Probe sequence (5'–3') | Target site (rRNA positions) ^a | % FA ^b | NaCl (mM) ^c |
|---------|--|------------------------|---|-------------------|------------------------|
| EUB338 | Bacteria | GCTGCCTCCCGTAGGAGT | 16S (338–355) | 30–55 | 20–122 |
| Nso190 | Ammonia oxidizing β-subclass <i>Proteobacteria</i> | CGATCCCCTGCTTTTCTCC | 16S (190–208) | 55 | 20 |
| Nsr1156 | <i>Nitrospira</i> sp. | CCCCGTCTCCTGGGCAGT | 16S (1035–1048) | 30 | 56 |
| NIT3 | <i>Nitrobacter</i> sp. | CCTGTGCTCCATGCTCCG | 16S (1156–1173) | 40 | 122 |

a *Escherichia coli* numbering.

b Percentage of formamide in the hybridization buffer.

c Millimolar concentration of sodium chloride in washing buffer.

at the concentration listed in Table 2, and 0.01% SDS. The washing buffer was removed by rinsing the slides with distilled water. The slides were air dried in the dark, and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 ng/ μ L) for 10 min in the dark on ice, and rinsed again with distilled water. The slides were then mounted in Citifluor AF100 (anti-fading agent in phosphate buffered saline) and CFPVOH (aqueous solution of poly(vinyl alcohol)) (Citifluor, Ltd., London, UK) to avoid bleaching. Images were captured with an epifluorescence microscope (Nikon Eclipse 90i) and analyzed with MetaMorph software (MDS, Inc). For quantification, the area of a single, dispersed cell hybridized with each of the probes was first determined. Area of cell clusters in the biofilm was then assigned cell counts via the automated cell count function in MetaMorph. A grid of 10 μ m by 10 μ m squares was applied to each image, and cell counts in each grid square were made. These grids were then assigned distances from the membrane surface. Thresholding was completed manually and verified with manual cell counts of dispersed cells. A total of 10 images were analyzed for each probe at each condition. Sections of biofilm that hybridized with EUB 338, but not Nso1225, NIT3, or NSR1156, were assumed to be heterotrophic bacteria (Hibiya et al., 2003).

Suspended biomass was also analyzed using FISH. The same hybridization procedures, probes, and analysis were carried out for suspended samples, except that homogenized suspended samples spread over a slide were used, rather than using a sectioned portion of biofilm.

3. Results

3.1. Reactor performance

The reactor performance was monitored for 270 days, during which the influent concentrations and membrane area were varied as shown in Table 1. Results are shown in Fig. 2. The nitrification rate was between 1 and 1.2 $\text{gN m}^{-2} \text{day}^{-1}$ over the majority of the 270 day operation. The goal of the performance study was to determine the applicable nitrification rates under varying loading conditions, not to optimize TN removal, and only partial nitrification was achieved during most of the testing period. TN removal generally increased with increasing influent BOD, and the majority of the oxidized ammonium was typically denitrified. Effluent BOD was typically below the detection limit of 0.2 mg/L, except for occasional spikes of up to 2.5 mg/L. These spikes occurred most frequently during the last tests, which had the highest BOD loadings. Effluent BOD spikes correlated with decreased suspended solid concentrations, which resulted from excess wasting or poor settling. Effluent nitrite initially dominated over nitrate, but nitrate became dominant over time. Increasing nitrate accumulation over time was previously observed in a nitrifying MABR, possibly from NOB gradually establishing themselves in the deeper portions of the biofilm (Terada et al., 2006). For the HMBP, it is also possible that nitrite was preferentially reduced in the bulk liquid, as discussed below.

The maximum denitrification rate occurred during the last loading condition, where the influent BOD was high and the influent ammonium low. Suspended solids ranged from

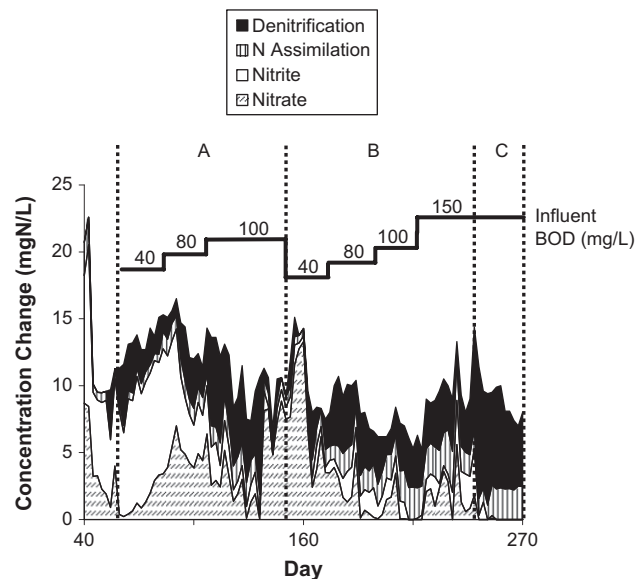


Fig. 2 – Fate of ammonium in the HMBP. The total area is the ammonium oxidized in the HMBP, which can be accounted for by the production of nitrate (cross-hatched) or nitrite (white); nitrogen assimilation (vertical line hatched); or denitrification (black). Changes in nitrogen loadings are indicated by vertical lines, and influent BOD concentrations are denoted for the different time periods.

50 mg/L to 120 mg/L, and were within 15% of the theoretically estimated concentrations (Rittmann and McCarty, 2001). The bSRT ranged from 2 to 3 days.

Nitrification rates were not affected by the loading condition, as long as the effluent BOD concentration was below the detection limit (Fig. 3). Nitrification rates decreased during brief spikes in effluent BOD, probably due to increased competition between heterotrophs and nitrifiers in the biofilm. This may be explained by greater BOD penetration into the biofilm at higher bulk BOD concentrations, allowing heterotrophic

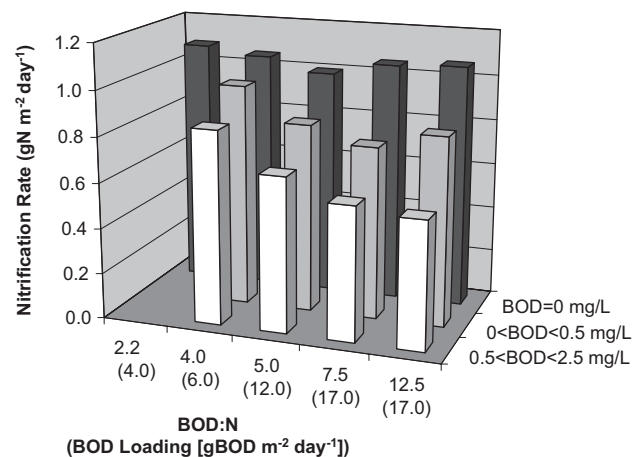


Fig. 3 – Impact of effluent BOD concentration of average nitrification rates at varying influent BOD:N ratios. BOD concentrations indicated on the right axis are for the bulk liquid.

bacteria to reduce oxygen levels. Although the BOD spikes were all below 2.5 mg/L, the BOD source was acetate, which is readily degradable and consequently can stimulate growth at very low concentrations. A typical municipal wastewater contains more complex BOD, and significant effects on nitrification would not be expected at these low concentrations.

3.2. Microgradient studies

Microsensor measurements of ammonium, nitrite, nitrate, and oxygen were made *in situ*, and were used to quantify the biofilm activity at BOD:N ratios of 5, 7.5, and 12.5 (Fig. 4). The average biofilm thickness was $120 \mu\text{m} \pm 25 \mu\text{m}$, and did not change appreciably over the course of the experiments. Based on light microscopy observations, the biofilm covered the membranes with a continuous biofilm with relatively consistent thickness. To allow averaging, gradients were plotted in terms of relative biofilm thickness, i.e., the depth of sample relative to the total biofilm thickness.

As seen in Fig. 3, oxygen concentrations were around 3–4 mg/L at the membrane surface, dropping to nearly zero at the outer edge of the biofilm. Ammonium concentrations decreased from the outer edge of the biofilm towards the membrane surface. At the membrane surface, nitrite and nitrate were present at similar concentrations. As oxygen concentration decreased through the biofilm, nitrite became dominant over nitrate, indicating reduced nitrite oxidation. Profiles suggest that some oxygen was exported to the bulk liquid, along with nitrate and nitrite. The loss of BOD to oxygen exported from the biofilm may have limited denitrification during the tests at lower BOD loadings.

The nitrite production fluxes were 3–4 times greater than those for nitrate for BOD:N ratios of 5 and 7.5 (Fig. 5). For the BOD:N ratio of 12.5, the nitrate production flux was zero. The nitrification fluxes were higher than those determined using influent and effluent ammonium measurements. This suggests that not all of the membrane surface area in the reactor was actively nitrifying, which may be caused by bundling of some membranes, where diffusion limitation would occur for those fibers deep within a bundle. The nitrification flux also decreased under the last loading condition, possibly due to the higher bulk BOD on the day the microsensor measurements were carried out.

Fig. 6 shows the NO_x (nitrite plus nitrate) production rate, the amount of denitrification occurring in the bulk liquid, and the amount of denitrification occurring in the biofilm. The bulk liquid BOD was below 0.2 mg/L for measurements at BOD:N loadings of 5 and 7.5; however, the bulk liquid BOD was 1.2 mg/L for the BOD:N loading of 12.5. For BOD:N loadings of 5 and 7.5, denitrification occurred exclusively in the bulk liquid. However, at the BOD:N loading of 12.5, there was decreased NO_x production and a significant amount of denitrification in the biofilm. Biofilm denitrification probably occurred because the BOD concentrations in the biofilm were high enough to support heterotrophic growth.

3.3. FISH studies

FISH was used to study the distribution of AOB, NOB, and heterotrophic bacteria in the suspended and biofilm phases,

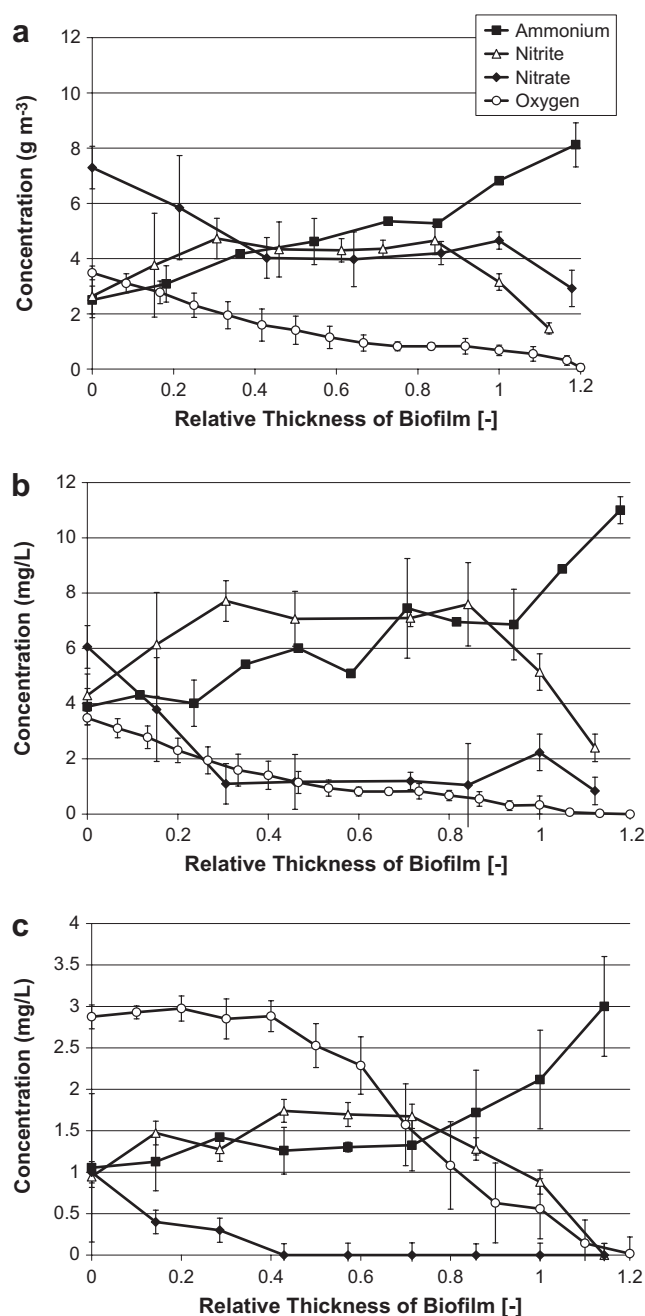


Fig. 4 – Average microsensor measurements at BOD:N loadings of (a) 5:1, (b) 7.5:1, and (c) 12.5:1. Fluxes into and out of the biofilm were used to calculate rates of nitrification, nitrite production, and nitrate production by the biofilm. The data is shown over a normalized biofilm thickness, with 0 as the membrane surface and 1 as the biofilm surface. The biofilm thickness was approximately $120 \mu\text{m}$ for all conditions. Error bars are the standard deviation of 15 profiles. Note the different vertical scale in (c).

following each set of microsensor tests (Fig. 7). The large standard deviation associated with the cell densities is due to the tendency of the NOB to form clusters within the biofilm. Total biofilm area per sample was typically $10,000 \mu\text{m}^2$, and when NOB were present in a sample, they were in dense

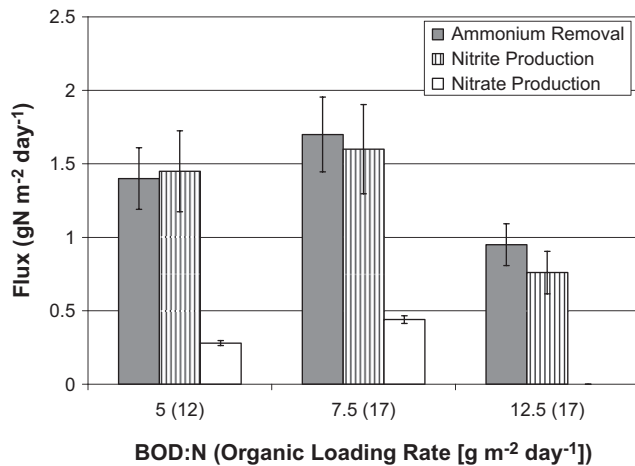


Fig. 5 – Rates of nitrification (solid), nitrite production (vertical lines), and nitrate production (white) by the biofilm in the HMBP at varying loading conditions, as calculated from biofilm microgradients.

clusters, usually about 900 μm^2 in area. Some samples contained multiple clusters, while others had only one or no clusters present.

With a BOD:N ratio of 5, AOB were dominant throughout the biofilm, while NOB were only present near the membrane–biofilm interface (Fig. 7a). At BOD:N ratios of 7.5 and 12.5, the overall AOB population density decreased and the non-nitrifying population increased (Fig. 7b, c), although the AOB population density was similar at the membrane–biofilm interface under all the loading conditions. At the BOD:N ratio of 12.5, BOD spikes were more frequent, possibly accounting for the greater presence of putative heterotrophic bacteria in the biofilm.

For all three conditions, the FISH results revealed a uniquely stratified biofilm. Near the membrane–biofilm

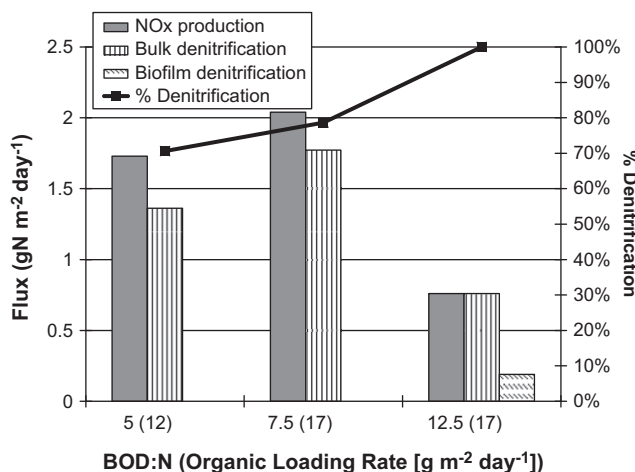


Fig. 6 – Total NO_x production by the biofilm (solid bar), denitrification occurring in the bulk liquid (bar with vertical lines), and denitrification occurring in the biofilm (white bar). The percent denitrification is indicated by the line with square markers. Results are calculated from microsensors gradients.

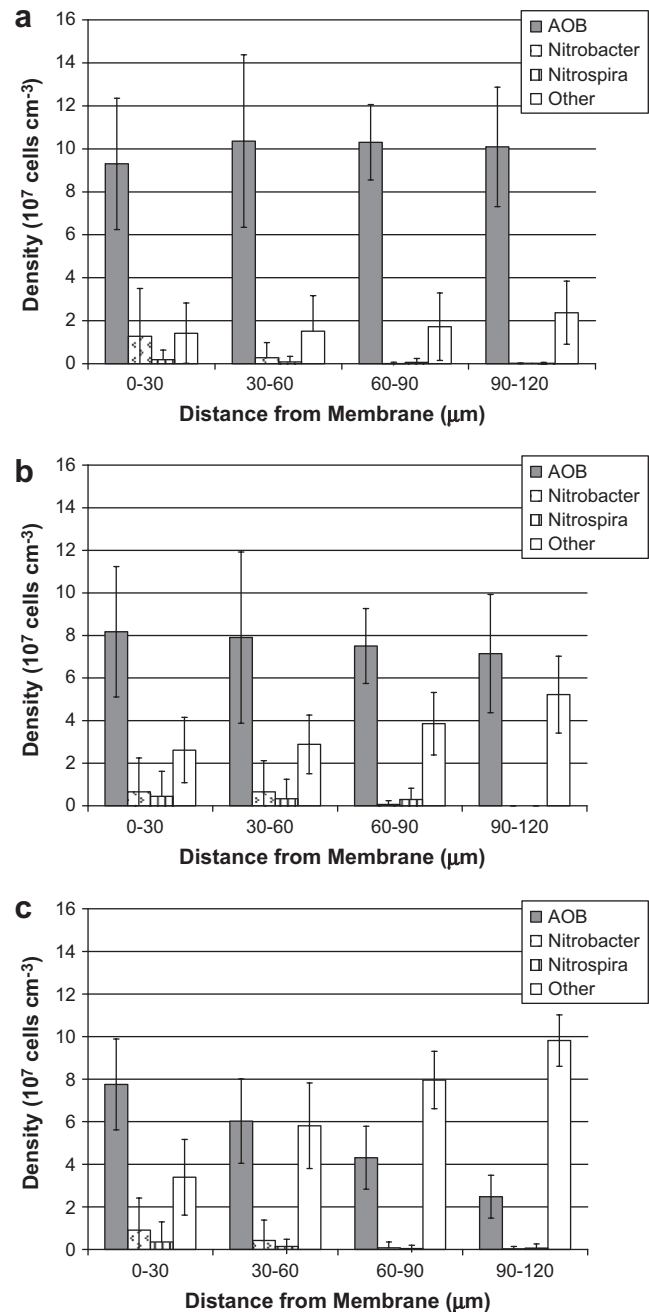


Fig. 7 – Distribution of AOB, *Nitrobacter* and *Nitrospira* (NOB), and other (putative heterotrophic) bacteria in the HMBP biofilm at BOD:N ratios of (a) 5, (b) 7.5, and (c) 12.5. Error bars are the standard deviation of 10 analyzed images. Effluent BOD concentrations during the 12.5 ratio spiked to above 0.5 mg/L on a more consistent basis than at previous loading conditions, possibly causing the increase in non-nitrifying bacteria in the biofilm.

interface, a mixture of AOB and NOB was observed. At a distance of approximately 60 μm from the membrane–biofilm interface, NOB populations decreased and AOB were present almost exclusively. The outer edge of the biofilm was composed almost exclusively of putative heterotrophic

bacteria, which increased in abundance with increasing BOD loading, but still were at much lower densities than reported in MABRs (Cole et al., 2004). The increased heterotrophic density and decrease in AOB density with BOD:N loading consistent with the decreased nitrification rate observed during the last operating period.

The NOB population density at the membrane–biofilm interface was approximately 10% of the density of the AOB population for all three conditions. At distances greater than 60 μm from the membrane surface, very few NOB were detected. This is the approximate distance where the oxygen concentration decreased to below 2 mg/L. It is also the distance where nitrite became the dominant form of oxidized nitrogen present in the biofilm. An oxygen concentration of 2 mg/L has previously been shown to limit nitrate production in a biofilm system (Chung et al., 2007). From these results, AOB appear to effectively be selected over NOB in the HMBP, accounting for the shortcut nitrogen removal.

4. Discussion

The average nitrification rate throughout the performance tests was $1.0 \pm 0.3 \text{ gN m}^{-2} \text{ day}^{-1}$, with a maximum nitrification rate of $1.9 \text{ gN m}^{-2} \text{ day}^{-1}$. The maximum occurred when the ammonia loading was $2.24 \text{ gN m}^{-2} \text{ day}^{-1}$ the influent BOD was 40 mg/L. Nitrification rates in the HMBP were similar to those observed in previous MABR studies at similar nitrogen loading rates (Table 3). However, unlike the MABR (Walter et al., 2005), the HMBP's nitrification rates were unaffected by the BOD loading. The overall rates of nitrification observed by Walter et al. (2005) were elevated due to their use of pure oxygen, resulting in maximum oxygen concentrations of approximately 20 mg/L, as compared to around 3 mg/L in the HMBP. Semmens et al. (2003) initially reported high nitrification rates at a BOD loading of $11.8 \text{ gBOD m}^{-2} \text{ day}^{-1}$.

However, the rates decreased over time due to increased heterotrophic biomass accumulation in the biofilm.

Nitrification rates in the HMBP and MABRs are lower than nitrification rates reported for membrane-aerated biofilms operated in an aerobic bulk liquid. Completely aerobic nitrifying MABRs achieved nitrification rates in the range of 6.6 (Brindle et al., 1998) to $8.0 \text{ gN m}^{-2} \text{ day}^{-1}$ (Yamagiwa et al., 2004). Although these nitrification rates are significantly higher, operation in an aerobic bulk liquid eliminates the ability to nitrify and denitrify in a single tank, limiting the use of influent BOD for denitrification and increasing the potential need for carbon augmentation for TN removal.

The increased nitrification rate observed in the HMBP at higher loading rates is a result of the decreased heterotrophic competition in the biofilm. Significant biofilm thickness, causing fouling and mass transfer resistance, has been reported as a limitation of the MABR (Semmens et al., 2003), and the HMBP addresses this limitation by separating the heterotrophic and nitrifying growth between the suspended and attached phases, respectively. In MABR systems, biofilms were as thick as 3 mm (Cole et al., 2004), and under similar BOD and nitrogen loading conditions as tested for the HMBP, heterotrophs were shown to account for approximately 60% of a 2500 μm thick biofilm in an MABR (LaPara et al., 2006). High COD:N loading ratios have also been shown to impact nitrification and anaerobic ammonium oxidation in a modeled membrane-aerated biofilm (Lackner et al., 2008). Step-change perturbations with COD:N loading ratios of 0–1 and 1–5 resulted in decreasing AOB populations and nitrification rates, due to increased heterotrophic activity. When the COD:N loading ratio was 5, less than 10% of the biofilm consisted of AOB, whereas over 60% of the biofilm was heterotrophic biomass. In the HMBP, heterotrophs accounted for between 5 and 15% of a 120–150 μm thick biofilm with influent BOD:N ratios of 5, 7.5, and 12.5. The thin biofilm and limited heterotrophic growth in the biofilm of the HMBP is what leads to increased nitrification rates at

Table 3 – Performance from this study and previous studies on denitrifying MABRs

| Ammonium–N loading ($\text{gN m}^{-2} \text{ day}^{-1}$) | BOD loading ($\text{gBOD m}^{-2} \text{ day}^{-1}$) | Nitrification rate ($\text{g m}^{-2} \text{ day}^{-1}$) | Denitrification (%) | Reference |
|--|---|---|---------------------|-----------------------|
| 2 | 7 | 0.77 | >95 | Hibiya et al. (2003) |
| 0.7 | 3.3 | 0.63 | >90 | Semmens et al. (2003) |
| 1.9 | 8.3 | 1.71 | | |
| 2.6 | 11.9 | 2 | | |
| 2.5 | 0 | 1.8 | NA | Walter et al. (2005) |
| | 10.6 | 1.14 | | |
| | 18.6 | 0.68 | | |
| 5.19 | 78 | 2.6 | >99 | Jacome et al. (2006) |
| 13.3 | 53 | 0.9 | >99 | |
| 1.65 | 3.3 | 1.0 | 24 | This study |
| | 6.6 | | 29 | |
| | 9.9 | | 67 | |
| 2.24 | 4.5 | 1.0 | 10 | This study |
| | 9.0 | | 40 | |
| | 12.4 | | 70 | |
| | 16.8 | | 80 | |
| 1.46 | 16.8 | 1.0 | 99 | This study |

increased BOD loadings, and this is a direct result of the incorporation of suspended heterotrophic bacteria to limit attached heterotrophic growth.

Past researchers have indirectly inferred that nitrite is the main product of nitrification in membrane-aerated biofilms achieving TN removal, either through the lack of nitrate production (Hibiya et al., 2003) or through oxygen transfer mass balances (Terada et al., 2003). Nitrite accumulation also has previously been confirmed in nitrifying membrane-aerated biofilms that were not achieving TN removal (Schramm et al., 2000; Terada et al., 2006). In this study, we experimentally determined nitrite and nitrate production rates by a membrane-aerated biofilm achieving TN removal. As shown in Fig. 5, microsensor measurements indicated that nitrite was the main oxidized nitrogen species produced by the biofilm under all three loading conditions tested. This was not determinable from the bulk liquid data, as the nitrite to nitrate ratio did not typically match the flux of nitrite and nitrate from the biofilm due to denitrification in the bulk liquid.

In situ microsensor measurements also indicated that denitrification mainly occurred in the bulk liquid (Fig. 6). Some denitrification occurred in the biofilm under the highest BOD:N loading ratio (12.5). This resulted in the highest percent of TN removal, but the increased diffusion of BOD into the biofilm also resulted in decreased nitrification rates. A tradeoff exists between complete TN removal and decreased nitrification rates, and incomplete TN removal (i.e., some effluent nitrite and nitrate) and higher nitrification rates.

Microsensor measurements indicating that denitrification occurred mainly in the bulk liquid (Fig. 6), combined with the relatively low heterotrophic growth shown in the HMBP biofilm relative to an MABR biofilm (Fig. 7), confirm that heterotrophic activity was mainly in the bulk liquid of the HMBP. However, there was some heterotrophic biomass present in the aerobic zone of the biofilm at all three loading conditions examined. Nitrifying biofilms typically have some heterotrophic biomass, even if no influent BOD is present, due to the production of soluble microbial products (SMPs) (Nogueira et al., 2005). Modeling has been used to assess the contribution of SMPs to activity in a membrane-aerated biofilm (Lackner et al., 2008), and the heterotrophic biomass in this model was shown to comprise approximately 5% of the biofilm. Therefore, the small amount of heterotrophic growth deep in the biofilm may be the result of SMPs produced by the nitrifying microorganisms.

The results suggest that the HMBP may be an effective means for achieving TN removal from wastewater. It is potentially retrofittable into existing wastewater treatment plants, even those designed for short SRTs; it maximizes use of influent BOD for denitrification; and can achieve shortcut nitrogen removal. Future research should address potential challenges for its implementation, including determining cost-effective designs for scaleup (Syron and Casey, 2008), ensuring low bulk BOD concentrations to prevent low nitrification rates, ensuring adequate mixing when larger membrane banks are used, ensuring good settling properties for the suspended solids, and ensuring there is no excess BOD or oxidized nitrogen in the effluent due to imbalances in the influent concentrations.

5. Conclusions

The combination of bulk performance data, microsensors studies, and molecular analyses of microbial community structure provides insights into the functioning and capabilities of the HMBP process. Several important conclusions concerning the HMBP can be drawn.

- Nitrite is the main product of nitrification, resulting in shortcut nitrogen removal. This is explained by the significant presence of AOB observed throughout the biofilm during FISH tests, and minor presence of NOB, which were limited to regions where the DO was greater than 2 mg/L.
- Nitrification rates are insensitive to the BOD loadings; as the BOD loading increases, suspended solids increase, preventing changes in the bulk BOD concentration.
- At lower BOD:N ratios, incomplete denitrification occurs. This is probably due to oxygen diffusion from the biofilm to the bulk liquid. At higher BOD:N ratios, full denitrification occurs, with TN removals approaching 100%.
- Under low effluent BOD concentrations, denitrification occurs exclusively in the bulk liquid. Increased effluent BOD concentrations allow greater BOD penetration into the biofilm, resulting in greater heterotrophic presence and greater denitrification in the biofilm.

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