J Ind Microbiol Biotechnol (2004) 31: 353–361
DOI 10.1007/s10295-004-0152-7

ORIGINAL PAPER

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Removal of carbonaceous and nitrogenous pollutants from a synthetic wastewater using a membrane-coupled bioreactor

Received: 12 December 2003 / Accepted: 7 April 2004 / Published online: 28 July 2004

Abstract Two modified Ludzack-Ettinger (MLE)-type membrane-coupled bioreactors (MBRs) were investigated in this study for the purpose of removing both nitrogenous and carbonaceous pollutants from a synthetic wastewater. During the first MBR experiment, removal efficiencies were high (>90%) for chemical oxygen demand (COD) and ammonia, but total nitrogenous pollutant removal efficiency was poor (~25%). Bacterial community analysis of ammonia oxidizing bacteria (AOB) by a nested PCR-DGGE approach detected two *Nitrosomonas*-like populations and one *Nitrosospira*-like population. During the initial portion of the second MBR experiment, COD and ammonia removal efficiencies were similar to the first MBR experiment until the COD of the influent wastewater was increased to provide additional electron donors to support denitrification. Total nitrogen removal efficiencies eventually exceeded 90%, with a hydraulic residence time (HRT) of 24 h and a recirculation ratio of 8. When the HRT of the MBR experiment was decreased to 12 h, however, ammonia removal efficiency was adversely affected. A subsequent increase in the HRT to 18 h helped improve removal efficiencies for both ammonia (>85%) and total nitrogenous compounds (~70%). Our research demonstrates that MBRs can be effectively designed to remove both carbonaceous and nitrogenous pollutants. The ability of the microbial community to switch between anoxic (denitrifying) and oxic (nitrifying) conditions, however, represents a critical process constraint for the application of MLE-type MBR systems, such that little benefit is gained compared to conventional designs.

Keywords Ammonia · Denitrification · Membrane-coupled bioreactor · Nitrification · PCR-DGGE

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Introduction

Municipal and industrial wastewater contains inorganic nutrients (nitrogen and phosphorus) that stimulate the growth of photosynthetic microorganisms, causing increased turbidity and decreased dissolved oxygen concentrations. Although a considerable amount of research over the last several decades has focused on removing phosphorus from wastewater, there is renewed interest in total nitrogen removal from wastewater because of recent reports linking coastal eutrophication with inland sources of nitrogenous pollution [11, 16, 31]. New and more efficient wastewater treatment biotechnologies for nitrogenous pollutant removal are therefore needed to maintain surface water quality.

Complete biological nitrogen removal from wastewater poses a significant problem. Nitrification requires aerobic conditions so that autotrophic nitrifying bacteria can oxidize ammonia to nitrite and then to nitrate. Denitrification requires anoxic conditions so that nitrate can be reduced first to nitrite and then to nitrogen gas. The challenge from the wastewater treatment perspective is that heterotrophic metabolism proceeds under the same conditions needed for nitrification, thereby eliminating the organic compounds necessary to serve as electron donors during denitrification. Three different approaches have been used to solve this problem. First, an exogenous carbon source can be added following nitrification to a denitrification reactor (typically methanol); this approach, however, is often cost prohibitive and leads to increased organic levels in the treated effluent [36]. Second, internal carbon storage during rapid heterotrophic growth in a sequencing batch reactor has been used to promote denitrification [2]. Finally, untreated wastewater is first sent to an anaerobic reactor, which receives recirculated water from a downstream aerobic reactor containing nitrate [4].

Membrane-coupled bioreactors (MBRs) are a relatively new biotechnology for wastewater treatment that offer some unique advantages compared to conventional

bioreactors. MBRs utilize a membrane to achieve up to 100% biomass separation from the treated effluent. The retention of these cells within the MBR leads to substantially higher concentrations of biocatalyst, resulting in a net increase in the amount of wastewater that can be treated per reactor volume compared to conventional batch, fed-batch, or continuous-flow bioreactors [19, 24]. This improvement in biomass retention is critical because previous research has demonstrated that treatment efficiency by conventional bioreactors is limited by biomass separation instead of process microbiology [13]. MBRs are particularly useful for the treatment of high-strength industrial wastewaters that would otherwise require very large and expensive bioreactors to meet regulatory requirements [9, 41].

Previous research has demonstrated that MBRs can accommodate slow-growing bacteria [25], although it remains unclear whether such bacteria are useful for the purpose of biological wastewater treatment. The goal of the research described herein was to apply MBRs to the removal of both carbonaceous and nitrogenous pollutants from a synthetic wastewater. We hypothesized that MBRs would better support high concentrations of slow-growing nitrifying bacteria and thus lead to better treatment performance. The two different reactor configurations tested in this work were similar to modified Ludzack-Ettinger (MLE) designs [39]. The reactors were used to examine the effects of the hydraulic residence time (HRT) and the recirculation rate from the nitrification reactor(s) to the denitrification reactor on total nitrogen removal efficiency.

Materials and methods

Membrane-coupled bioreactors

Two different MBR configurations were designed to remove carbonaceous and nitrogenous pollutants from a synthetic wastewater (Fig. 1). The first MBR setup consisted of three reactors operated in series. Reactor fluid flowed from the first reactor (300 ml; not aerated) into the second reactor (700 ml; aerated) and then into the third reactor (300 ml; not aerated). Reactor fluid was removed from the third reactor, passed through a 0.2 μm pore size polysulfone microfilter membrane cartridge (surface area $= 0.011\ m^2$; A/G Technology, Needham, Mass.), and returned to the second reactor. This fluid exchange between the second and third reactors was sufficiently rapid such that they were considered fully intermixed. Reactor fluid was also slowly pumped from the third to first reactor (Fig. 1a). The second MBR experiment consisted of two reactors operated in series. Reactor contents flowed from the first reactor (300 ml; not aerated) to the second reactor (300 ml; aerated). Fluid from the second reactor was removed and rapidly passed through the membrane cartridge and returned to the second reactor. Reactor fluid was also

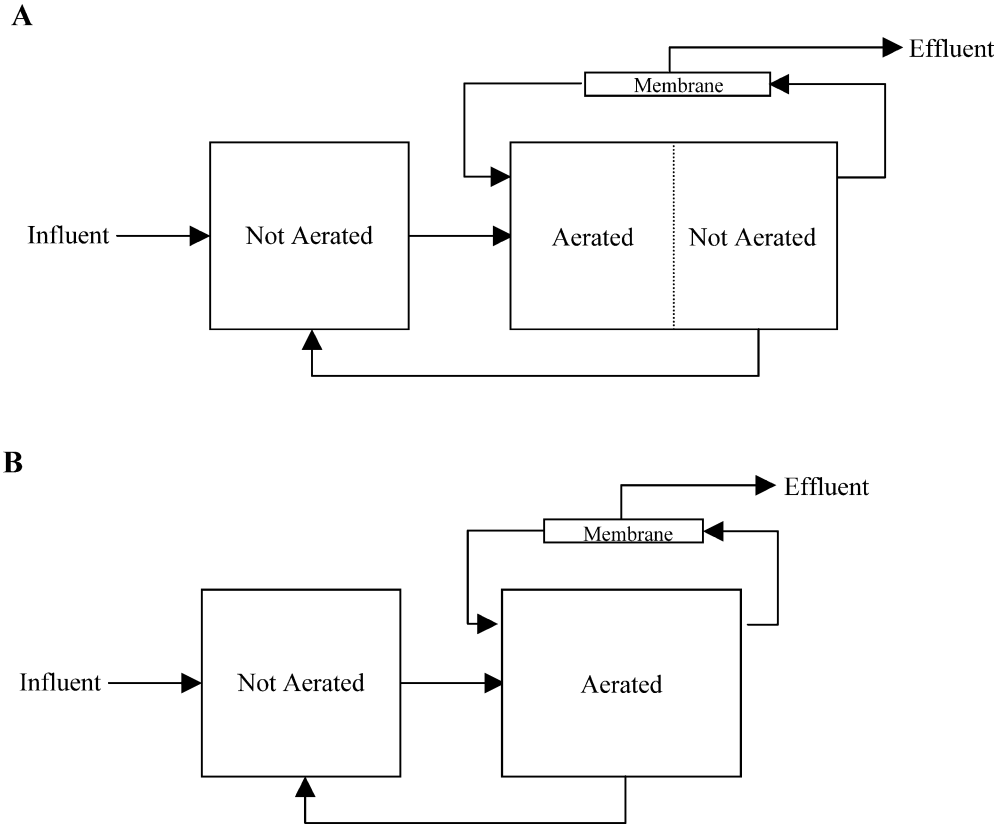

Fig. 1a, b Schematic diagrams of modified Ludzack-Ettinger (MLE)-type designs used during the first (**a**) and second (**b**) membrane-coupled bioreactor (MBR) experiments. The *dotted line* indicates that these two physically separate reactors were completely intermixed

slowly pumped from the second reactor to the first reactor (Fig. 1b). Membrane filtrate was removed during each MBR experiment at a specific rate to maintain a constant total volume of reactor fluid. All reactors were placed on magnetic stir plates and mixed rigorously. Specific reactors were aerated by passing 0.2–0.3 l min^{-1} ambient air through the reactor.

Sterile synthetic wastewater was pumped into the first reactor of each MBR experiment at specified rates using a peristaltic pump (Masterflex variable-speed console drive pump; Cole-Parmer, Vernon Hills, Ill.). The synthetic wastewater contained the following constituents per liter of deionized water: 150 mg gelatin, 70 mg starch, 10 mg yeast extract, 10 mg casamino acid, 50 mg ammonium sulfate (as nitrogen), 25 mg sodium phosphate, 30 mg potassium phosphate, 60 μg calcium chloride, and 0.1 ml SL7 trace mineral solution [8]. Reactor pH was controlled by including 100–1,000 mg l^{-1} sodium bicarbonate with the feed medium. During the second MBR experiment, the influent starch concentration was increased to 350 mg l^{-1} to increase the amount of carbonaceous pollution in the feed medium. The concentration of total nitrogenous pollutants (ammonia + organic nitrogen) was 70 mg l^{-1}.

MBRs were inoculated with 1 ml cells from 100 ml enrichment cultures grown on the synthetic wastewater for 24 h. These enrichment cultures had been inoculated with 1 ml cryopreserved activated sludge (15% v/v in glycerol) collected from the aeration tanks of the Metropolitan Wastewater Treatment Plant (St. Paul, Minn.). A nitrifying enrichment culture was also maintained throughout both experiments. The composition of the growth medium for this enrichment culture was same as the synthetic wastewater, except that it did not contain any organic compounds. The enrichment culture was continuously aerated. Biomass was allowed to settle periodically and the supernatant was replaced by fresh feed medium.

The HRT and recirculation ratio (RR) were controlled during each MBR experiment by modifying the rate at which synthetic wastewater was fed to the denitrification reactor, and by controlling the rate at which reactor fluid was pumped from the nitrification reactor to the denitrification reactor, respectively. The HRT was calculated by dividing the total volume of all reactors used in each MBR experiment by the flow rate of synthetic wastewater into the denitrification reactor. The RR was calculated by dividing the recycle flow rate from the nitrification reactor to the denitrification reactor by the flow rate of synthetic wastewater into the denitrification reactor. HRT and RR values for each experiment are included in Table 1.

Table 1 Summary of reactor operating conditions and influent wastewater concentrations that were varied during the membrane-coupled bioreactor (MBR) experiments. *HRT* Hydraulic residence time, *RR* recirculation ratio, *COD* chemical oxygen demand

Days	Reactor configuration		Wastewater characteristics (mg l^{-1})	
	HRT (h)	RR	COD	Carbohydrate
First MBR				
1–77	12	10	250	70
77–91	12	12	250	70
91–105	12	8	250	70
105–120	12	14	250	70
Second MBR				
1–15	12	8	250	70
15–26	12	6	250	70
26–39	12	11	250	70
39–80	24	8	250	70
80–94	24	8	500	350
94–108	12	8	500	350
108–122	18	8	500	350
122–136	18	4	500	350

Analytical methods

Biomass concentrations were measured as optical density (OD_{600}) and particulate protein. Particulate protein was measured using the Lowry method [27] using bovine serum albumin (BSA) as a protein standard. Chemical oxygen demand (COD) was quantified using low-range accu-Test vials (Bioscience, Bethlehem, Pa.) with potassium hydrogen phthalate as a standard. Soluble protein was quantified using a modified Lowry method using BSA as a protein standard [14]. Soluble carbohydrate concentrations were measured using the anthrone method using glucose as a carbohydrate standard [15]. Total kjeldahl nitrogen (ammonia + organic nitrogen) was determined using a Hach Digesdahl Digestion Apparatus (Hach, Loveland, Colo.) following the manufacturer's instructions. Ammonia concentrations were measured using a modified Nessler Method using ammonium sulfate as a standard [12]. Nitrite and nitrate concentrations were measured using an ion chromatograph (761 Compact IC, Metrohm, Herisau, Switzerland) equipped with an anion column (Metrosep A Supp 5–150) using sodium nitrite and sodium nitrate as standards. The concentrations of all nitrogen-containing compounds are reported as nitrogen. Results reported herein are the arithmetic mean of triplicate analyses.

Community analysis

Biomass samples (1 ml each) were collected from the reactor, centrifuged, and resuspended in 1 ml lysis buffer (120 mM sodium phosphate, 5% sodium dodecyl sulfate, pH 8.0). Cells were lysed by performing three consecutive freeze-thaw cycles and a 90 min incubation at 70°C. Genomic DNA was then extracted using a Fast DNA Spin Kit (Qbiogene; Vista, Calif.) as per the manufacturer's instructions.

Partial 16S rRNA genes were amplified by polymerase chain reaction (PCR) using a PTC 100 thermal cycler (MJ Research; Watertown, Mass.). An initial

PCR amplified a 465 bp fragment of the 16S rRNA gene biased towards the clade of known nitrifying bacteria from the Betaproteobacteria using primers CTO189f (5′-GRA AAG YAG GGG ATC G-3′) and CTO654r (5′-CTA GCY TTG TAC TTT CAA ACG C −3′) [22]. The PCR protocol included a 5 min initial denaturation at 94°C, 35 cycles of 92°C for 1 min, 57°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 5 min. PCR products were then diluted 10^4- to 10^6-fold and used as template for PCR of the V3 region of these 16S rRNA genes using primers PRBA338F (5′-ACT CCT ACG GGA GGC AGC AG-3′) [23] and PRUN518R (5′-ATT ACC GCG GCT GCT GG-3′) [28] with a GC-clamp [28] attached to the forward primer. The PCR protocol included a 5 min initial denaturation at 94°C, 30 cycles of 92°C for 45 s, 55°C for 45 s, and 72°C for 45 s, followed by a final extension at 72°C for 10 min. The first and second reaction mixtures (volume = 50 µl) contained 1×PCR buffer with $MgCl_2$ (Promega; Madison, Wis.), 4 nmol deoxynucleoside triphosphates, 25 pmol of forward and reverse primers, and 1.25 U *Taq* DNA polymerase (Promega).

Denaturing gradient gel electrophoresis (DGGE) was performed using a D-Code apparatus (Bio-Rad; Hercules, Calif.). Approximately equal amounts of PCR products were loaded onto 8% (w/v) polyacrylamide gels (37.5:1, acrylamide: bisacrylamide) in 0.5×TAE buffer [34] using a denaturing gradient ranging from 25 to 50% (100% denaturant contains 7 M urea, 40% v/v formamide in 0.5×TAE buffer). Electrophoresis was performed at 60°C, initially at 20 V (15 min) and then at 200 V (180 min). The gel was stained with SYBR Green I (Molecular Probes; Eugene, Ore.; diluted 1:5,000 in 0.5×TAE buffer), viewed on a UV transilluminator, and photographed with a CCD camera (BioChemi System; UVP; Upland, Calif.). The contrast and brightness of the photographs were adjusted using Adobe PhotoShop v 6.0.

Specific PCR-DGGE bands were manually excised from the gel, suspended in 20 µl sterile water, and incubated overnight at room temperature. PCR-DGGE was repeated using these samples as template until a single band remained in each lane. A final PCR step was performed without the GC clamp attached to the forward primer. PCR products were then purified using a Geneclean II Kit (QBiogene) and nucleotide sequences were determined fully in both directions for each PCR-DGGE band using PRBA338F and PRUN518R as sequencing primers. Sequencing was performed at the Advanced Genetic Analysis Center at the University of Minnesota using an ABI 3100 Genetic Analyzer (Applied Biosystems; Foster City, Calif.). Reported nucleotide sequences do not include the original PCR primer sequence. Reference nucleotide sequences were obtained from the GenBank database [6]. The nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers AY428951–AY428955.

Results

First MBR experiment

Following the initiation of the first MBR experiment, anoxic conditions developed within 24 h in the first (not aerated) reactor and were sustained throughout the rest of the experiment (data not shown). The second (aerated) and third (not aerated) reactors maintained dissolved oxygen concentrations >2 mg l^{-1}. Biomass concentrations increased quasi-linearly (23 mg protein l^{-1} day^{-1}; $r^2 = 0.8$) for the first 37 days (Fig. 2a), at which time about 70% of the biomass was accidentally lost from the system. Following this accidental biomass loss, biomass again increased linearly (30 mg protein l^{-1} day^{-1}; $r^2 = 0.89$). After day 63, biomass was manually removed to maintain a constant biomass level ($OD_{600} \approx 2.5$; mean cell protein = 1,100 mg l^{-1}). Effluent COD concentrations decreased to <35 mg l^{-1} within

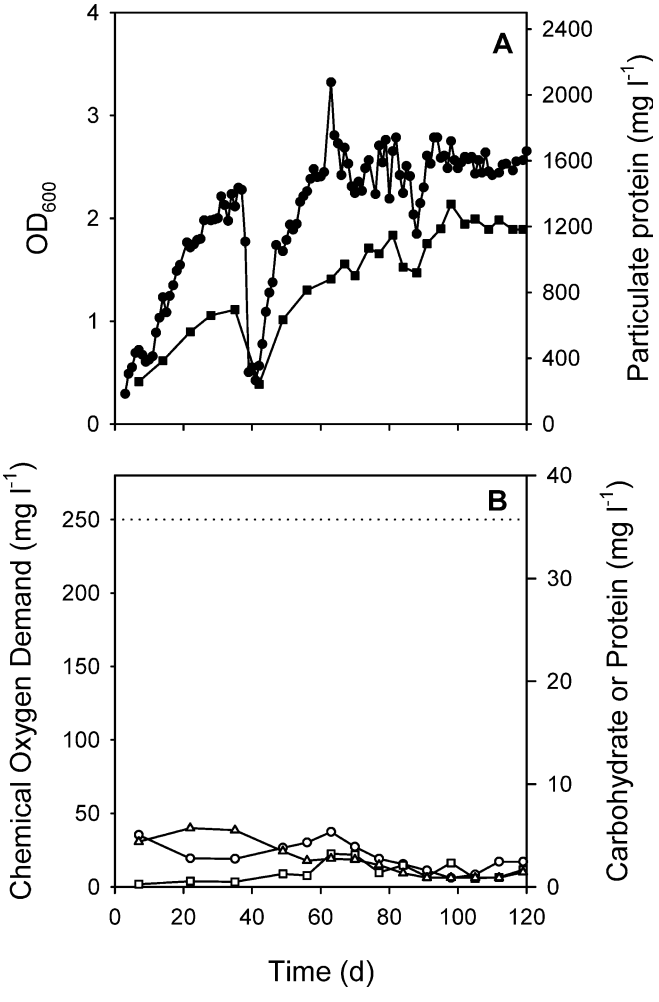

Fig. 2a, b Accumulation of biomass (**a**) and effluent water quality (**b**) during the first MBR experiment. Biomass was quantified as OD_{600} (*filled circles*) and particulate protein (*filled squares*). Effluent water quality was quantified as COD (*open circles*), carbohydrate (*open squares*), and protein (*open triangles*). The *dotted line* indicates influent COD concentration

the first 7 days and remained low throughout the rest of the experiment (Fig. 2b). Effluent soluble carbohydrate and protein concentrations were continuously < 5 mg l^{-1} (Fig. 2b).

Effluent ammonia concentrations were > 55 mg l^{-1} for the first 28 days, while the pH of all three reactors was between 8.0 and 8.5 (Fig. 3a). At this time, the reactor was re-inoculated with biomass from a nitrifying enrichment culture and the pH was manually reduced by decreasing the pH of the feed medium. Ammonia concentrations in the effluent began to decline by day 35. Once nitrification began, pH decreased to < 7; at this time, the pH of the synthetic wastewater was re-adjusted to sustain pH levels of approximately 7.5 in the reactors. Ammonia concentrations in the effluent were typically < 5 mg l^{-1} from day 50 until the end of the experiment.

Nitrite and nitrate were not detectable in the effluent for the first 35 days (Fig. 3b), after which effluent nitrite concentrations started to increase. Effluent nitrite concentrations peaked on day 63 ($\sim$22 mg l^{-1}); nitrite levels

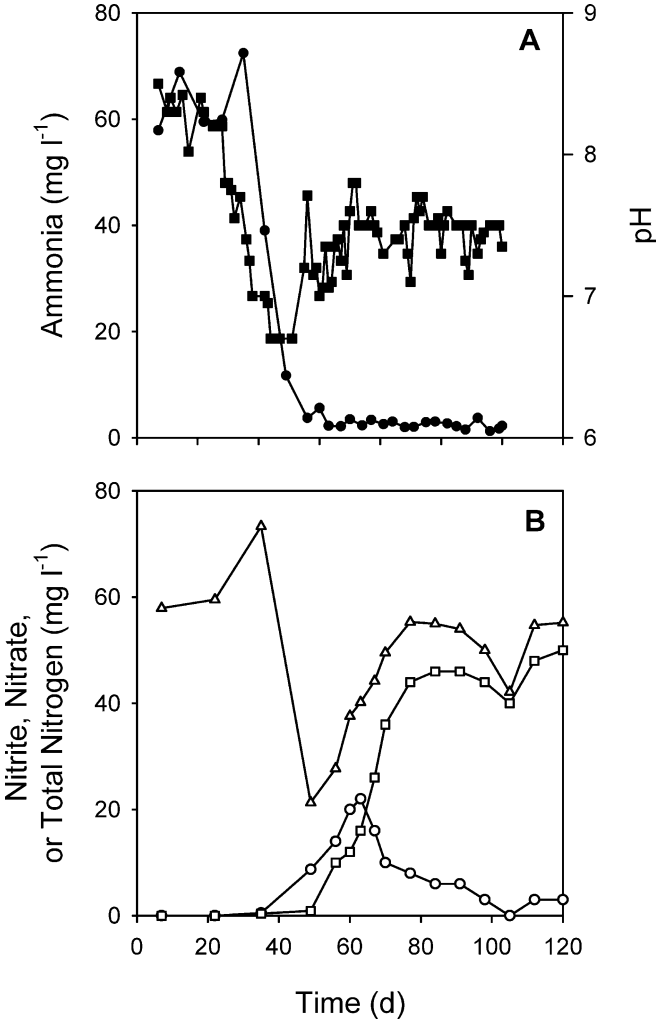

Fig. 3 a Effluent ammonia concentrations (*filled circles*) and reactor pH (*filled squares*), and **b** effluent concentrations of nitrite (*open circles*), nitrate (*open squares*), and total nitrogeneous pollutants (*open triangles*) during the first MBR experiment

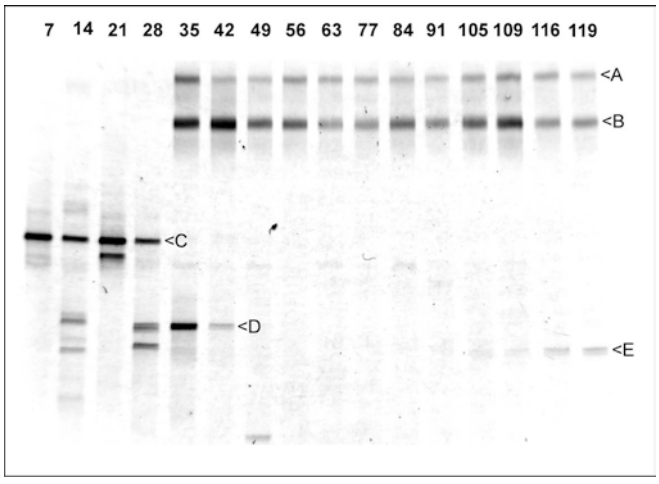

Fig. 4 Denaturing gradient gel electrophoresis (DGGE) of nested PCR-amplified 16S rRNA gene fragments. The nested PCR was biased towards the ammonia oxidizing bacteria (AOB) within the Betaproteobacteria. Lane numbers indicate the time (days) at which community fingerprints were determined. Specified bands were excised and sequenced (see Table 2)

then decreased to approximately 5 mg l^{-1} by day 85, and then remained relatively constant for the remainder of the experiment. Effluent nitrate concentrations also began to increase on day 35, reaching a relatively constant concentration of $\sim$50 mg l^{-1} after 70 days. Once nitrate concentrations stabilized, the total nitrogen removal efficiency was $\sim$25%. During the last 8 weeks of this experiment, the RR of reactor fluid from the nitrification reactors to the denitrification reactor was varied between 8 and 14 to test its impact on denitrification. Total nitrogen removal efficiency, however, was not substantially affected.

The ammonia oxidizing bacteria (AOB) community belonging to the Betaproteobacteria was analyzed by nested PCR-DGGE of 16S rRNA gene fragments. Relatively simple community fingerprints (< 5 bands) were detected from day 7 to day 28 (Fig. 4). Following re-inoculation of the reactor with the nitrifying enrichment culture, two new bands appeared (bands A and B) and remained prominent for the rest of the experiment. Near the end of the experiment (day 105), a new band appeared (band E), which remained prominent for the rest of the experiment. Five of the prominent bands were excised and their nucleotide sequences were determined (Table 2). Two bands (A and B) were closely related to previously identified *Nitrosomonas* spp. ($> 98\%$ identical), while band E was related to a *Nitrosospira* sp. ($> 99\%$ identical). Bands C and D were closely related to known members of the Betaproteobacteria but did not group with known AOB.

Second MBR experiment

Following the initiation of the second MBR experiment, anoxic conditions developed within 24 h in the first (not

Table 2 The best phylogenetic match of the nucleotide sequences of prominent bands detected by nested PCR-DGGE during the first MBR experiment (Fig. 5). All sequences are 160 nucleotides in length and cluster with the Betaproteobacteria

PCR–DGGE band	Phylogenetic relationship	
	Most closely related sequence	Identity (%)
A	*Nitrosomonas* sp. R5c88 (AF386750)	100
B	*Nitrosomonas* sp. DYS317 (AF363292)	98.1
C	*Variovorax* sp. 5S2.A7 (AY043571)	99.4
D	Betaproteobacterium JDS4 (AY084085)	99.4
E	*Nitrosospira* sp. DNB_E1 (AY138531)	99.4

aerated) reactor, and were sustained throughout the remainder of the experiment (data not shown). The second (aerated) reactor continuously maintained dissolved oxygen concentrations >2 mg l^{-1}. Biomass increased quasi-linearly (27 mg protein l^{-1} day^{-1}; $r^2=0.8$) for the first 5 weeks of the experiment (Fig. 5a). On day 39, the HRT was increased from 12 to 24 h and the biomass level reached a plateau of $\sim$800 mg l^{-1} cell protein. On day 80, the starch content of the synthetic wastewater was increased 2.5-fold and the biomass again increased linearly (48 mg protein l^{-1} day^{-1}; $r^2=0.95$). Beginning on day 99, biomass was manually removed to maintain an approximately constant biomass concentration ($OD_{600}\approx4.5$; mean protein = 1,400 mg l^{-1}). The effluent concentrations of COD (<15 mg l^{-1}), carbohydrate (<1 mg l^{-1}), and protein (<1 mg l^{-1}) were low throughout the experiment (Fig. 5b).

Although reactor pH was consistently maintained between 7.5 and 8.0 from the initiation of the second MBR experiment, effluent ammonia concentrations were >50 mg l^{-1} for the first 28 days (Fig. 6a). On day 29, therefore, the aerated reactor was re-inoculated with biomass from the nitrifying enrichment culture. Effluent ammonia concentrations soon declined but still exceeded 25 mg l^{-1} on day 35 ($\sim$60% ammonia removal

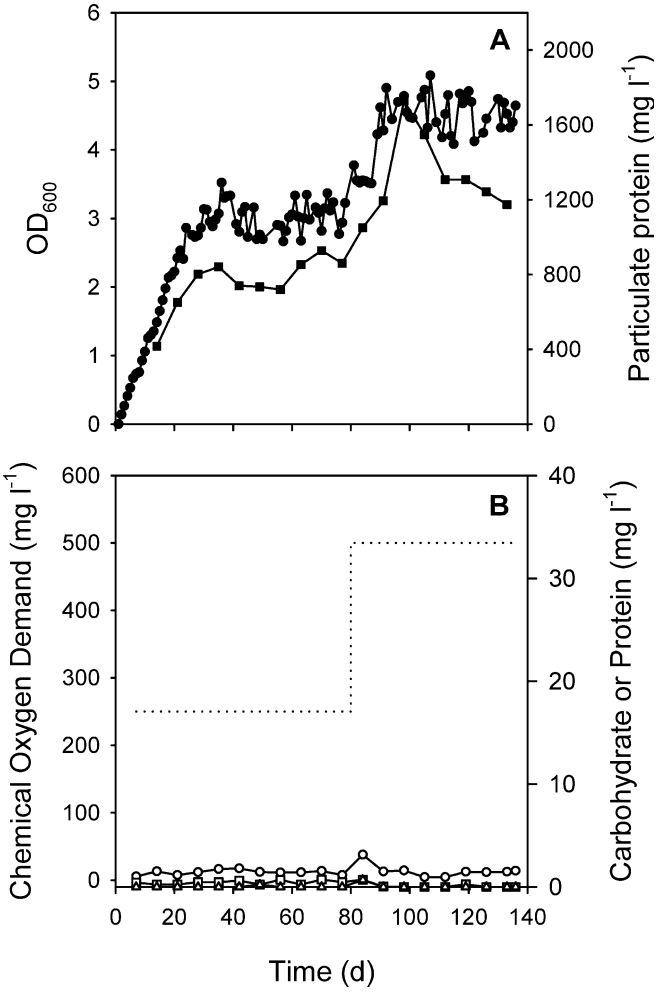

Fig. 5 **a** Accumulation of biomass and **b** effluent water quality during the second MBR experiment. Biomass was quantified as OD_{600} (*filled circles*) and particulate protein (*filled squares*). Effluent water quality was quantified as COD (*open circles*), carbohydrate (*open squares*), and protein (*open triangles*). The *dotted line* indicates influent COD concentration

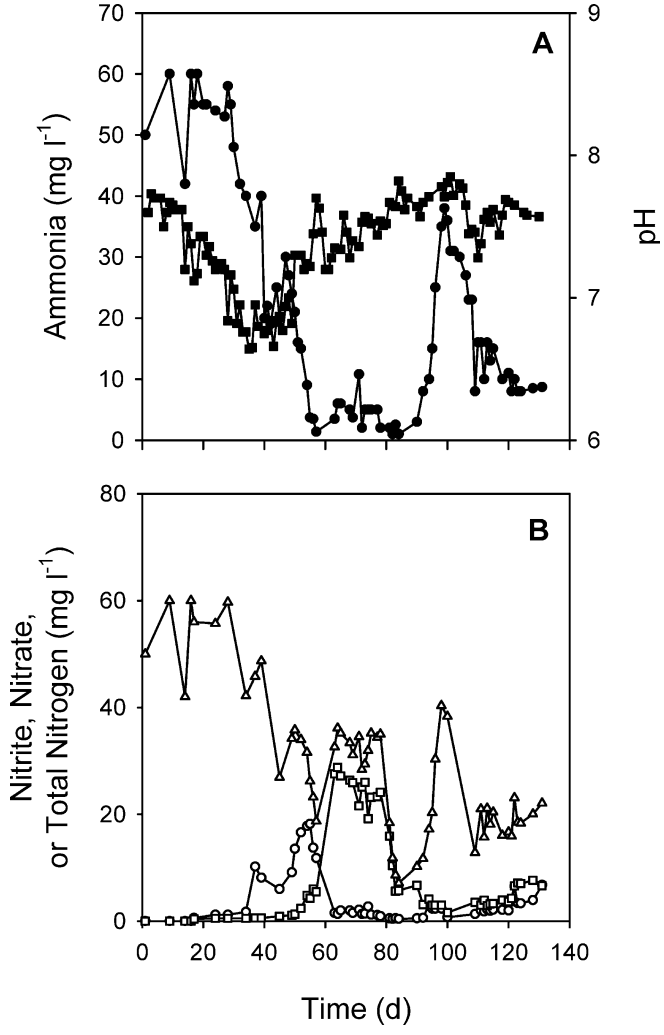

Fig. 6 **a** Effluent ammonia concentrations (*filled circles*) and reactor pH (*filled squares*), and **b** effluent concentrations of nitrite (*open circles*), nitrate (*open squares*), and total nitrogenous pollutants (*open triangles*) during the second MBR experiment

efficiency). On day 39, the HRT was increased to 24 h and the effluent ammonia concentrations decreased substantially (effluent $NH_3 < 5$ mg l^{-1}).

Nitrite and nitrate concentrations were low (< 1 mg l^{-1}) for the first 28 days of the experiment (Fig. 6b). After the effluent ammonia concentrations began to decrease, the effluent nitrite concentrations increased, reaching a maximum on day 56 ($\sim$15 mg l^{-1}) but then rapidly decreasing to < 2 mg l^{-1}. Effluent nitrate concentrations concomitantly increased and reached a peak concentration by day 64 ($\sim$30 mg l^{-1}). The total nitrogen removal on day 64 was $\sim$50%. Because this removal efficiency is low compared to previous studies on nitrogenous pollutant removal, denitrification efficiency was assumed to be limited by the availability of electron donor. Therefore, the starch content of the feed medium was increased on day 80. Effluent nitrate concentrations rapidly decreased to < 6 mg l^{-1}, corresponding to a total nitrogen removal efficiency of $> 90\%$.

Following this improvement in total nitrogen removal efficiency, the HRT was decreased to 12 h on day 94 and the effluent ammonia concentrations increased to > 35 mg l^{-1} (Fig. 6a). Although the effluent nitrite and nitrate concentrations remained low (< 5 mg l^{-1}), the total nitrogen removal efficiency declined to $< 50\%$ (Fig. 6b). The HRT was then increased to 18 h on day 108 and the effluent ammonia concentrations decreased rapidly to < 10 mg l^{-1} for the remainder of the experiment. The total nitrogen removal efficiency during this period was $> 70\%$, which was not substantially affected by decreasing the RR from 8 to 4.

Discussion

The goal of this research was to investigate the use of MBRs for the removal of both carbonaceous and nitrogenous pollutants from a synthetic wastewater. This research is of practical importance because MBRs are relatively new alternatives for the treatment of various industrial wastewaters [8, 18, 19, 37], but there is relatively little information in the technical literature regarding their ability to remove nitrogenous pollutants. MBRs have been particularly attractive treatment alternatives for situations where space is limited, such as the treatment of shipboard wastewater [7, 37].

In this study, COD removal efficiencies were high during both MBR experiments ($> 90\%$), independent of the various manipulations imposed with respect to HRT and RR. This is consistent with the results of previous studies investigating MBRs for wastewater treatment [20, 24, 38]. Nitrogenous pollutant removal, however, was more sensitive to HRT and RR. During the second MBR experiment, nitrogenous pollutant removal efficiency exceeded 90% once the influent COD concentration was artificially increased. This result is a substantial improvement compared to previous reports on MLE-type MBRs with respect to total nitrogenous pollutant removal efficiency [1, 35]. When the HRT was decreased in an attempt to optimize nitrogen removal per total reactor volume, however, there was a concomitant decrease in nitrogenous pollutant removal efficiency linked to incomplete nitrification. We conclude, therefore, that the ability of the microbial community to switch between anoxic (denitrifying) and aerobic (nitrifying) conditions represents a critical process constraint for the application of MLE-type bioreactor systems. Previous studies have observed a substantial lag period for denitrification [5, 26] during situations in which anoxic and aerobic conditions were alternated. Although not explicitly investigated herein, our results suggest that a similar lag is exhibited by nitrifying bacteria under these conditions.

From a practical perspective, our results suggest that MLE-type MBRs offer little benefit compared to conventional MLE bioreactors for nitrogenous pollutant removal. The critical parameter limiting nitrogenous pollutant removal efficiency appears to be the ability of the biomass to effectively alternate between anoxic and aerobic conditions. Because biomass retention does not limit treatment efficiency, as is normally the situation for COD removal by conventional aerobic treatment processes [13], biomass retention by gravitational separation is sufficient for optimum nitrogenous pollutant removal in MLE-type bioreactors. This conclusion is supported by previous reports of conventional MLE processes that routinely achieve $> 90\%$ nitrogenous pollutant removal efficiencies under similar operating conditions (i.e., HRT and RR) to those used here [39].

One of the hypotheses for the current research was that MBRs would accommodate slow-growing nitrifying bacteria better than conventional activated sludge processes. The results from the first MBR experiment demonstrate that MBRs can support high concentrations of ammonia- and nitrite-oxidizing bacteria, as demonstrated by the efficient removal of ammonia ($> 95\%$) without sustained accumulation of nitrite. High ammonia removal efficiencies have been reported by previous researchers examining MBRs for wastewater treatment [10, 32, 40]. The results from the second MBR experiment, however, demonstrate that nitrification efficiency in MBRs can be affected by HRT and RR. This suggests that caution should be exercised during the design of MBRs for complete nitrogenous pollutant removal to ensure that nitrification can still occur.

An implicit assumption of our research was that denitrification would rapidly proceed once nitrification was achieved. Stoichiometric calculations indicate that a COD:N ratio of at least 2.86:1 [3] is required for full denitrification, although previous researchers have reported that a ratio of 3.5–4.5:1 is actually necessary [29]. In our first MBR experiment, relatively poor total nitrogenous pollutant removal efficiency ($\sim$25%) was achieved while the COD:N ratio was 3:1. A similarly poor extent of total nitrogen removal was achieved during the second MBR experiment until the composition of synthetic wastewater was manipulated such that the COD:N was increased to 7:1.

Previous research has demonstrated that MBRs impose an increasingly stringent nutrient limitation on the heterotrophic bacterial community [17, 20, 25]; however, little has been known regarding the effects of MBR operation on the AOB community. Although two *Nitrosomonas*-like populations appeared soon after re-inoculation, the AOB community did not change until a *Nitrosospira*-like population appeared 70 days after re-inoculation. Previous researchers have demonstrated that most full-scale wastewater treatment facilities support *Nitrosomonas*-like populations but not the slower-growing *Nitrosospira* spp. [21, 33, 42]. This result provides additional support for our hypothesis that MBRs can support slowly growing bacterial populations.

The biases associated with application of PCR-based techniques for bacterial community analysis have been previously discussed in detail [43]. In this study, however, we intentionally biased the PCR towards the known AOB within the Betaproteobacteria. This nested-PCR-DGGE approach, combined with nucleotide sequence analysis, provided useful information on the presence or absence of AOB during the first MBR experiment. Prior to the onset of ammonia-oxidation, however, this technique detected bacterial populations that were clearly not AOB. This indicates that our nested PCR procedure was not completely specific for AOB. In addition to the detection of non-AOB populations, our nested PCR-DGGE approach could have also excluded relevant populations because the CTO primers [22] are not completely general to amplify all of the known AOB [30].

In conclusion, MLE-type MBRs can efficiently remove carbonaceous and nitrogenous pollutants from wastewater. MBRs, however, offer little advantage compared to conventional MLE bioreactors involving gravitational clarifiers because nitrogenous pollutant removal efficiency is limited by the ability of the bacterial community to rapidly transition between anoxic and aerobic conditions. Additional research is therefore needed to develop more efficient biotechnologies for the removal of nitrogenous pollutants from wastewater.

Acknowledgement This research was financially supported by the University of Minnesota Grant-in-Aid program.

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