Performance and microbial ecology of the hybrid membrane biofilm process for concurrent nitrification and denitrification of wastewater

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Abstract We report on a novel process for total nitrogen (TN) removal, the hybrid membrane biofilm process (HMBP). The HMBP uses air-supplying hollow-fibre membranes inside an activated sludge tank, with suppressed aeration, to allow concurrent nitrification and denitrification. We hypothesised that a nitrifying biofilm would form on the membranes, and that the low bulk-liquid BOD concentrations would encourage heterotrophic denitrifying bacteria to grow in suspension. A nitrifying biofilm was initially established by supplying an influent ammonia concentration of 20 mgN/L. Subsequently, 120 mg/L acetate was added to the influent as BOD. With a bulk-liquid SRT of only 5 days, nitrification rates were 0.85 gN/m^2 per day and the TN removal reached 75%. The biofilm thickness was approximately 500 μm. We used DGGE to obtain a microbial community fingerprint of suspended and attached growth, and prepared a clone library. The DGGE results, along with the clone library and operating data, suggest that nitrifying bacteria were primarily attached to the membranes, while heterotrophic bacteria were predominant in the bulk liquid. Our results demonstrate that the HMBP is effective for TN removal, achieving high levels of nitrification with a low bulk-liquid SRT and concurrently denitrifying with BOD as the sole electron donor.
Keywords Biofilm; denitrification; hybrid reactor; membrane; nitrification

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Introduction

Total nitrogen (TN) is an increasing concern for municipal wastewater, as it can lead to nutrient-induced eutrophication of receiving waters. This research reports on a novel process for TN removal, the hybrid membrane biofilm process (HMBP). The HMBP integrates air-supplying hollow-fibre membranes into a conventional completely mixed activated sludge system. An aerobic nitrifying biofilm develops on the membranes, exporting nitrate or nitrite to the bulk liquid. By suppressing aeration in the activated sludge tank, denitrifying heterotrophs can reduce nitrate and nitrite using BOD as an electron donor, effectively achieving TN removal in a single tank (Figure 1a). Since the nitrifying bacteria are attached, nitrification can occur with short bulk-liquid solids retention times (bSRTs). A significant benefit is that the HMBP may be retrofitted into existing treatment plants. This is especially attractive for older plants unable to achieve the long bSRTs required for nitrification. Using short bSRTs reduces loadings to the settling tanks and reduces the suspended solids concentration in the aeration tank, which limits filamentous growth.

The HMBP builds on two existing processes: the integrated fixed film activated sludge (IFAS) process (Sen *et al.*, 2000) and membrane aerated bioreactor (MABR) process (Pankhania *et al.*, 1999; Semmens *et al.*, 2003) (Figure 1b and 1c, respectively). Similar to the IFAS, the HMBP relies on attached-growth nitrification and suspended-growth BOD oxidation. Completely mixed conditions provide low bulk-liquid BOD concentrations that minimise heterotrophic growth on the attachment surface (Tijhuis *et al.*, 1994; van Benthum *et al.*, 1997). Unlike IFAS, the HMBP membranes supply

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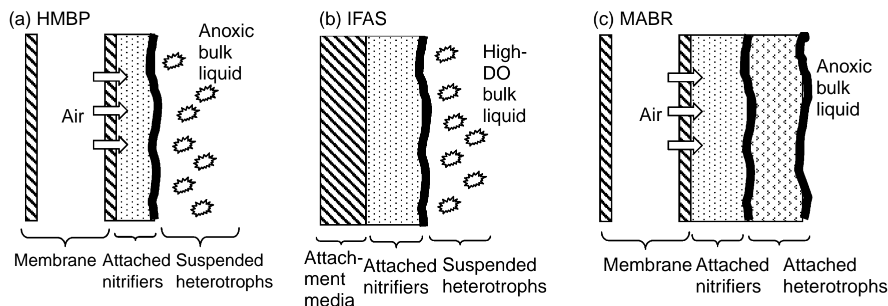

Figure 1 (a) Biofilm in the HMBP, where heterotrophic attachment is limited by maintaining a high suspended solids concentration and low bulk BOD; (b) biofilm in an IFAS with both nitrifying and heterotrophic bacteria present in the biofilm and oxygen supplied *via* the bulk liquid; (c) biofilm in an MABR with both heterotrophic and nitrifying biomass, leading to biofouling and mass transfer limitations

oxygen, allowing a nitrifying biofilm to co-exist with an anoxic, denitrifying bulk liquid. The HMBP is similar to MABRs, which also use membranes to deliver oxygen to biofilms. MABRs can remove BOD, nitrify and denitrify municipal wastewaters (Okabe *et al.*, 1996; Hibiya *et al.*, 2003; Semmens *et al.*, 2003; Satoh *et al.*, 2004) and industrial wastewaters (Terada *et al.*, 2003). Previous studies with MABR systems show that nitrifying bacteria compose the inner, dense portions of the biofilm, while heterotrophic bacteria dominate in the outer, porous portions of the biofilm (Hibiya *et al.*, 2003; Cole *et al.*, 2004). This stratification creates a suitable environment for TN removal. A key difference between the HMBP and MABRs is that the latter relies exclusively on membrane-attached growth, leading to very thick biofilms, mass transfer limitations and biofouling (Semmens *et al.*, 2003). In contrast, the HMBP is a hybrid process, where BOD oxidation is expected to occur in the suspended phase. Thus, HMBP biofilm thicknesses should be small and biofouling and mass transfer problems minimised.

Our research examined the ability of the HMBP to achieve TN removal in an activated sludge configuration, and also gained insights into the process's microbial ecology. A bench-scale system was tested under typical domestic wastewater conditions. The bSRT was maintained low enough to wash out suspended nitrifying biomass. After achieving sustained TN removal, biofilm and suspended biomass were collected for analysis. Denaturing gradient gel electrophoresis (DGGE) and clone library construction were used to determine (1) if nitrifying bacteria were only present in the biofilm and (2) if heterotrophic growth was mainly in the suspended phase.

Methods

Synthetic wastewater

A synthetic wastewater was prepared from distilled water amended with 1.386 g Na_2HPO_4, 0.849 g KH_2PO_4, 0.05 g $MgSO_47H_2O$ per litre, as well as a trace mineral solution (Nerenberg *et al.*, 2002). $(NH_4)_2SO_4$ was added to achieve either 20 or 10 mg/L NH_3-N. Various BOD concentrations were achieved *via* acetate addition. BOD is calculated by multiplying the acetate concentration by 64 mg BOD/59 mg acetate.

Analytical methods

NO_3^--N, NO_2^--N, and acetate were analysed by ion chromatography (IC2500 with AS11/AG11 column, Dionex Corp, Sunnyvale, CA, USA). The eluant was sodium hydroxide. NH_3-N was measured using a colorimetric method (High Range, Hach, Loveland, CO, USA). pH was monitored using a glass electrode pH meter. Bulk liquid

dissolved oxygen (DO) concentration was measured using a DO probe (YSI Model No. 55/25 FT). Suspended solids were measured according to *Standard Methods in Water and Wastewater* (Rand *et al.*, 1978).

HMBP reactor

The bench-scale HMBP reactor was designed to simulate an activated sludge system (Figure 2). An open rectangular chamber contained a bank of hollow fibre membranes. Owing to the anticipated high suspended solids in the bulk liquid, the membrane bank was designed so that solids would readily pass through it, reducing the potential for clogging and biofouling. Spacing between individual membranes was 1 mm from the edge of the membrane to the edge of the membrane. Spacing between rows of membranes was approximately 1 cm. The membranes were a composite, microporous polyethylene with a dense, polyurethane core (HFM200TL, Mitsubishi Rayon, Japan). The membrane outside diameter was 280 μm, and the total membrane surface area was 1,300 cm^2.

The HMBP reactor was operated with an influent flow rate of 9.0 mL/min and a recycle sludge ratio of 1.0. The tank volume was 3.25 L, resulting in a hydraulic retention time (HRT) of 6 hours. A bSRT of 5 days was maintained. The HRT and bSRT were selected to mimic a conventional activated sludge system. The settler volume was 5 L, resulting in a combined HRT of 15 hours. The aeration tank was mixed with a 2-inch magnetic stir bar. The membranes were supplied with compressed air at 70 kPa. Suspended solids in the influent, reactor tank, effluent and settled sludge were measured to determine the volume of wasted sludge necessary to maintain the 5-day bSRT.

The hybrid reactor was operated under three loading conditions. The first included an influent of 20 mg/L NH_3-N and was used to establish a nitrifying biofilm. The second contained 20 mg/L NH_3-N and 120 mgBOD /L of acetate. The third contained 10 mg/L NH_3-N and 130 mgBOD /L acetate (Table 1).

DNA extraction and amplification

DNA was extracted from the biofilm and suspended biomass at day 50 (nitrifying biofilm) and day 99 (hybrid reactor). Suspended biomass was concentrated by centrifugation (Fisher Scientific) at 10,000 rpm for 5 minutes. Soil DNA extraction kits (Mo Bio Laboratories, Inc.) were used to extract DNA from 0.1 g of each biomass sample, following the manufacturer's instructions.

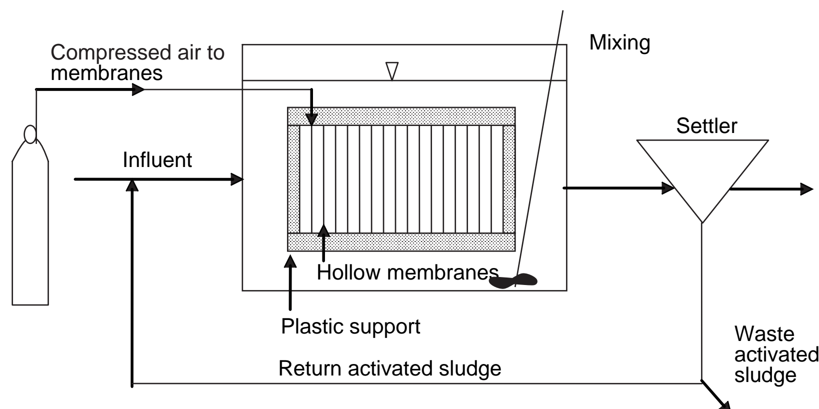

Figure 2 HMBP reactor configuration; an open tank replicates an activated sludge tank, while a settler and recycle line maintain a five-day SRT

Table 1 Loading conditions for HMBP

Loading condition	Time period (days)	NH_3-N (mgN/L)	BOD (mg/L)
1	0–50	20	0
2	51–74	20	120
3	75–99	10	130

PCR-DGGE

The 16S rRNA gene was amplified from the DNA extract by PCR using the 341F-GC/534R universal primer set (Muyzer *et al.*, 1993), resulting in a 193-bp fragment. A thermal cycler (Mastercycler epgradientS, Eppendorf) was used for the PCR with the following programme: 5 minutes at 94 °C; 40 cycles of 1 minute 94 °C, 1 minute 55 °C, 1 minute 72 °C, and 1 minute 72 °C; and then hold at 4 °C. DGGE was performed on a D-Code apparatus (Bio-Rad, Hercules, CA, USA). Ten microlitres of PCR amplicons were loaded onto an 8% (wt/vol) polyacrylamide gel (37.5:1 acrylamide-bisacrylamide) in 1X Tris-acetate-EDTA (TAE) buffer (Kowalchuk *et al.*, 2004) using a denaturing gradient ranging from 40 to 60%. Electrophoresis was performed at 60 °C, initially at 20 V (20 minutes), then at 60 V (840 minutes). Following electrophoresis, the gel was stained with ethidium bromide (Fisher Scientific) and visualised and photographed with a gel documentation system (Kodak, Model GP200).

PCR cloning and sequencing

The 16S rRNA gene was amplified a second time from the previously extracted DNA using the 341F-GC/907R primer set (Muyzer *et al.*, 1996). The above PCR programme was used for amplification. The resulting PCR product was cloned using TOPO TA cloning kits (Invitrogen Corporation, Carlsbad, CA, USA). Ligation and transformation reactions were performed according to the manufacturer's instructions. Twenty-four clones from each DNA sample were sequenced. Sequencing was performed by Sequetech Corporation (Mountain View, CA, USA). Nucleotide sequences were compared with known sequences in GenBank (Benson *et al.*, 2002) using the BLAST program (Altschul *et al.*, 1997).

Discussion and results

Hybrid reactor performance

The initial loading condition for the hybrid reactor, 20 mgN/L NH_3 and no BOD, was used to establish a nitrifying biofilm on the membranes. The reactor initially was fed from a 10 × concentrated medium stock and diluted with distilled water. However, fluctuating influent NH_3-N values were obtained. Non-concentrated feed began on day 24. From day 24 to day 50, the NH_3-N loading rate was 1.95 gN-m^{-2}-day^{-1}, while the nitrification rate was 1.65 gN-m^{-2}-day^{-1}. These results are consistent with MABR studies showing nitrification rates from 0.77 gN-m^{-2}-day^{-1} to 6.6 gN-m^{-2}-day^{-1}(Brindle and Stephenson, 1996). Effluent NH_3-N was consistently at or below 4.0 mgN/L, with the exception of day 41 (Figure 3). A spill occurred on day 40 after sampling, where some of the reactor contents were lost. This resulted in the increased effluent NH_3-N on day 41, followed by a quick return to the previous levels. No denitrification was observed. Bulk-liquid dissolved oxygen was approximately 1.0 mg/L, resulting from incomplete consumption of the oxygen from the membranes.

After achieving consistent nitrification, 120 mgBOD/L of acetate was added as a BOD source to promote denitrification. After only 24 hours, 99% BOD removal was achieved. However, the effluent NH_3-N increased to 10 mgN/L (Figure 3) and the apparent nitrification rate decreased to 0.85 gN-m^{-2}-day^{-1}. The lower nitrification rate was a result of

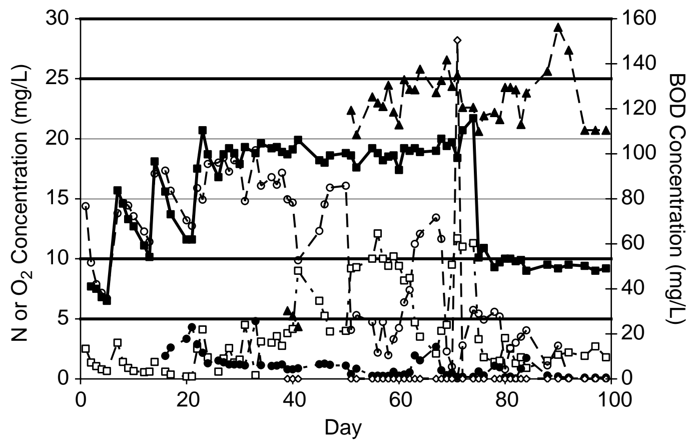

Figure 3 HMBP performance over a 99-day period. Full denitrification and 90% nitrification were achieved from day 85 to 99. Influent BOD (▲), effluent BOD (◇), influent ammonia (■), effluent ammonia (□), effluent nitrate (●), and bulk liquid DO (○)

the drop in bulk liquid DO to below 0.1 mg/L, which decreased nitrification activity in the outer portions of the biofilm. This was confirmed by increasing the bulk DO to 1.70 mg/L between days 61 and 67, which immediately decreased the effluent NH_3-N to below 4 mgN/L. The bulk liquid DO was increased by sparging the tank with compressed air. The effluent NO_3^--N was less than 5 mgN/L when the bulk DO was less than 0.3 mg/L, which indicated that approximately 5 mgN/L as NO_3^--N was reduced in the reactor.

In order better to match the HMBP's NH_3-N loading to its nitrification capacity under low bulk-liquid DO conditions, the influent NH_3-N concentration was reduced to 10 mgN/L, resulting in a 0.95-gN-m^{-2}-day^{-1} loading rate. Also, the influent acetate concentration was increased to 140 mgBOD/L to promote denitrification. These conditions were maintained from days 74 to 99. Nitrification continued with a rate of 0.85 gN-m^{-2}-day^{-1}, resulting in an effluent NH_3-N concentration of approximately 2 mgN/L. Acetate removal was 93% or greater. After 17 days of operation under these conditions, full denitrification was achieved while maintaining high levels of nitrification and BOD oxidation. TN removal efficiency was as high as 75%. The results of the third loading condition are shown in Figure 3. Higher TN removal efficiencies are likely to be attainable by increasing the membrane surface area or decreasing the NH_3-N loading. The bulk-liquid pH was 7 throughout all the experiments.

Hybrid reactor biomass

A critical goal of the HMBP is to achieve denitrification *via* suspended growth, not attached growth, in order to avoid thick biofilms. Suspended biomass was monitored to observe whether: (1) high concentrations of suspended biomass were produced; (2) the HMBP operated effectively in the presence of suspended biomass; and (3) denitrification was correlated to an increase in suspended biomass. From reactor startup to day 50, suspended biomass was very low, 0–2 mg/L. Following addition of acetate on day 51, suspended solids increased to between 150 and 250 mg/L. Denitrification increased with the rise in suspended biomass and full denitrification was achieved after 35 days. Nitrification was not significantly affected by the increase in suspended biomass. Biofilm thickness was approximately 500 μm, much lower than the 2–4 mm found in many MABRs (Terada *et al.*, 2003; Satoh *et al.*, 2004; LaPara *et al.*, 2006). The fact that denitrification was achieved while maintaining a relatively thin biofilm indicates that

(1) suspended heterotrophic biomass significantly contributed to denitrification and (2) maintaining low bulk BOD concentrations and high suspended biomass avoided excessive heterotrophic attachment.

DGGE

The reactor microbial ecology was studied using DGGE. Biomass was analysed from three samples: biofilm grown on ammonia, prior to the BOD addition (day 50); biofilm following BOD addition (day 99); and suspended growth following BOD addition (day 99) (Figure 4). The sample prior to BOD addition (lane 3) was dominated by putative nitrifying bacteria, as indicated by the four bands. The four bands present in lane 3 are identical to four dominant bands in lane 2, indicating that the nitrifying bacteria were the dominant species in the biofilm at day 99 (ammonia and BOD in influent). These four dominant bands are absent in the suspended biomass sample (lane 1). The suspended biomass lacks a significant number of nitrifying bacteria because the short bSRT and anoxic bulk liquid prevent their accumulation in suspended phase. Some heterotrophic bacteria appear to be present in the HMBP biofilm.

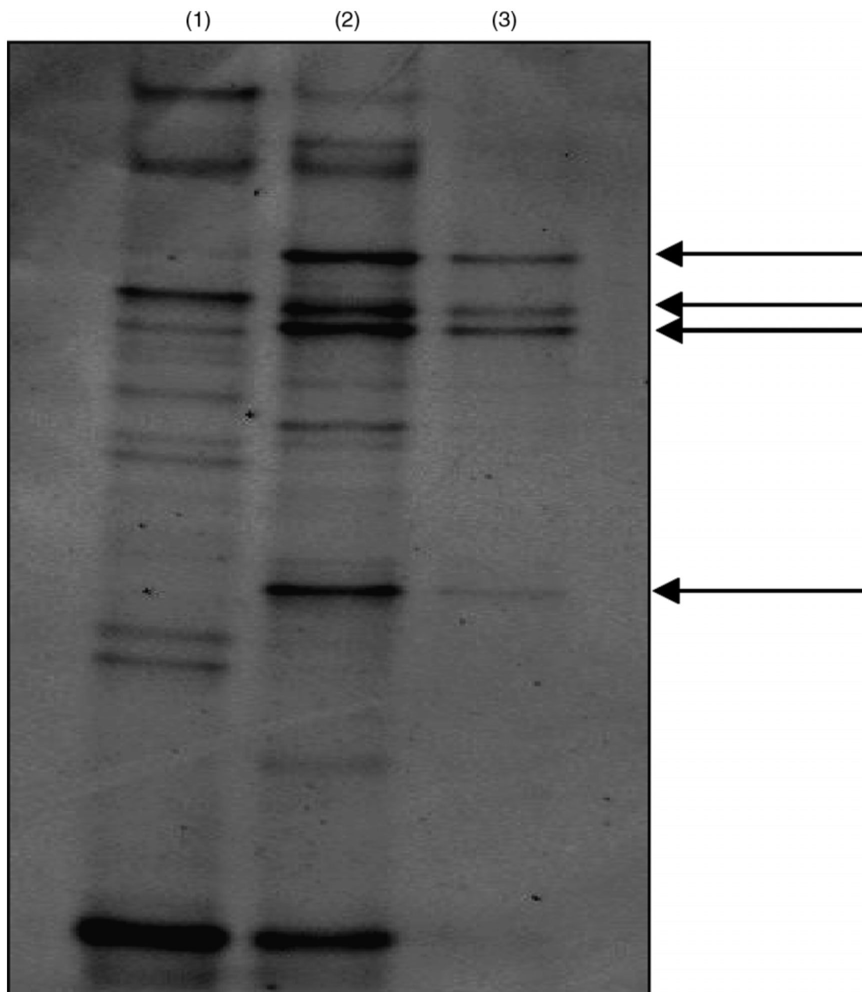

Figure 4 DGGE results for the three biomass samples: (1) suspended biomass from day 99; (2) biofilm when TN removal and BOD oxidation occurred (day 99); and (3) biofilm with no BOD source present (day 50). Arrows indicate dominant, putative nitrifying species

PCR cloning and sequencing

Cloning and sequencing results indicated a high abundance of *Nitrospira* spp. in biofilm samples from day 50 and day 99. Of the 24 clones from the biofilm on day 50, 14 were *Nitrospira* spp., one was *Nitrosomonas* spp., six were heterotrophic sequences and sequencing failed for three clones. On day 99, the biofilm samples produced nine *Nitrospira* spp sequences, ten heterotrophic sequences and the remaining five clones were not sequenced sucessfully. Lack of ammonia oxidising bacteria (AOB) sequences when using universal primer sets has been observed in other nitrifying systems (Schramm *et al.*, 1998) and may be due to a decreased DNA extraction efficiency with AOB *versus* NOB and heterotrophic bacteria. The suspended biomass from day 99 exhibited no nitrite oxidising bacteria (NOB) or AOB sequences, and the 20 successful clone sequences were all heterotrophs.

Conclusions

Our results suggest the HMBP can achieve high levels of nitrification with a low bSRT, while concurrently achieving denitrification without addition of an exogenous donor. Suspended heterotrophic biomass was maintained under anoxic conditions together with hollow fibre membranes supplying oxygen to a nitrifying biofilm. By maintaining suspended heterotrophic biomass, the biofilm thickness was limited. A maximum TN removal efficiency of 75% was achieved, with a nitrification efficiency of 80% and a BOD oxidation efficiency of 99%. A nitrification rate of $0.85\,\text{gN-m}^{-2}\text{-day}^{-1}$ was achieved by the attached biomass. DGGE and operating data suggest most heterotrophic bacteria were in the suspended phase. The HMBP can provide total nitrogen removal from municipal wastewater, and may be especially well-suited for upgrading existing activated sludge wastewater treatment plants not designed for nitrification or denitrification.

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