In-situ characterization of microbial community in an A/O submerged membrane bioreactor with nitrogen removal

A. Sofia, W.-T. Liu, S.L. Ong and W.J. Ng*

Centre for Water Research, Department of Civil Engineering, National University of Singapore, Blk E1A, #07-03, 1 Engineering Drive 2, Singapore, 117576, Singapore

* Corresponding author

Abstract The bacterial community involved in removing nitrogen from sewage and their preferred DO environment within an anoxic/oxic membrane bioreactor (A/O MBR) was investigated. A continuously operated laboratory-scale A/O MBR was maintained for 360 d. At a sludge age of 150 d and a C/N ratio of 3.5, the system was capable of removing 88% of the influent nitrogen from raw wastewater through typical nitrogen removal transformations (i.e. aerobic ammonia oxidation and anoxic nitrate reduction). Characterization of the A/O MBR bacterial community was carried out using fluorescence in situ hybridization (FISH) techniques. FISH results further showed that *Nitrosospira* spp. and *Nitrospira* spp. were the predominant groups of ammonia and nitrite oxidizing group, respectively. They constituted up to 11% and 6% of eubacteria at DO below 0.05 mg/l (low DO), respectively, and about 14% and 9% of eubacteria at DO between 2–5 mg/l (sufficient DO), respectively, indicating preference of nitrifiers for a higher DO environment. Generally low counts of the genus *Paracoccus* were detected while negative results were observed for *Paracoccus denitrificans, Alcaligenes* spp, and *Pseudomonas stutzeri* under the low and sufficient DO environments. The overall results indicate that *Nitrosospira* spp., *Nitrospira* spp. and members of *Paracoccus* spp. can be metabolically functional in nitrogen removal in the laboratory-scale A/O MBR system.

Keywords Denitrification; fluorescent in situ hybridization (FISH); membrane bioreactor; nitrification

Introduction

Biological nitrogen removal from wastewater is an important process as it reduces water quality problems such as eutrophication. Dissolved nitrogen removal in wastewater is a two step process comprising the oxidation of ammonia to nitrate (nitrification) followed by the conversion of nitrate to nitrogen gas (denitrification). In the natural environment, the nitrification process is carried out by autotrophic ammonia oxidizing bacteria (e.g. *Nitrosomonas* spp. and *Nitrosospira* spp.) and nitrite oxidizing bacteria (e.g. *Nitrobacter* spp. and *Nitrospira* spp.). Except for *Nitrospira* spp. which was only recently identified (Daims *et al*., 2001), the ammonia and nitrite oxidizing bacterial groups have been relatively well studied in activated sludge systems (Wagner *et al*., 1996, 1998). These bacterial groups are phylogenetically related autotrophic aerobes with relatively long cell doubling times. They are also sensitive to substrate and oxygen levels, alkalinity change, and availability of electron acceptors resulting in many treatment plants failing to maintain good nitrification. Unlike nitrifying bacteria, the ability to denitrify is widespread among bacteria of mostly phylogenetically unrelated heterotrophs (Stewart, 1988; Zumft, 1992) and this has led to uncertainty in estimating the extent to which each of these denitrifying bacteria contributes to nitrate and nitrite reduction under anoxic conditions. According to Otlanabo (1993) various species of the genera *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium, Hyphobacterium, Pseudomonas*, and *Vibrio* are responsible for denitrification in soil. Some findings in identification of denitrifiers in nitrogen removal showed that *Paracoccus* spp. made up an average of 3.5% of total cell counts in a denitrifying fluidized bed reactor (Neef *et al*., 1996), while other studies reported isolation

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of *Alcaligenes* spp. from anoxic reactors (Etchebehere *et al.*, 2001) and *Pseudomonas stutzeri* from sludge (Takaya *et al.*, 2003). The recent developments of molecular biology in wastewater treatment have resulted in the application of fluorescent labeled 16S and 23S rRNA targeted probes for single-cell identification of bacteria samples drawn from activated sludge (Wagner *et al.*, 1995). Studies on nitrifying and denitrifying communities in biofilms had, conventionally, been based on cultivation dependent approaches (Okabe *et al.*, 1996) and are thus limited to the cultivable part of the microbial population. Application of fluorescence in-situ hybridization (FISH) has shown that cultivation-based methods often fail to represent the true microbial community structure (Wagner *et al.*, 1998). Application of the FISH technique to study the microbial community in the activated sludge process revealed the dominance of a beta-subclass among the five members of *Proteobacteria* (Manz *et al.*, 1994; Snaidr *et al.*, 1997). Although the microbial community in activated sludge processes has been extensively studied, attempts at identifying the nitrifying and denitrifying bacteria and their correlation to nitrogen removal in the membrane reactor (MBR) has been more limited. This study aimed to use the FISH technique to identify the nitrifying and possible denitrifying species, and to investigate their structure, location, and their quantification with respect to eubacteria in two different dissolved oxygen (DO) environments and hence their possible influence on nitrogen removal in an anoxic-oxic membrane bioreactor (A/O MBR).

Materials and methods

Experimental set up and operational conditions used in this study

A schematic diagram of the laboratory-scale A/O system is given in Figure 1. The reactor consisted of anoxic and oxic zones, each about 10 and 9 L, respectively. The membrane used in this study was a flat-sheet membrane (Kubota Co.) with 0.4 μm pore size and a total filtration area of 0.1 m^2. The flux was kept at 0.48 m/d with intermittent suction (8 mins on/2 mins off). The system was acclimated for three months before the experiments were conducted with an HRT and SRT of 11 h and 150 d, respectively. About 40 L/d of screened raw sewage was fed continuously to the anoxic zone using a peristaltic pump. The recycle line, pumping from the aerobic to the anoxic zone, was set at a recycle ratio of 3:1 with respect to the influent flow rate. Due to the relatively low organic carbon content in the raw sewage, it was decided to supplement the feed with sodium acetate to maintain a C/N ratio of 3.5.

In-situ hybridization

FISH of all biomass samples was performed according to the protocol described by Amann *et al.* (1995). The following 16S rRNA-targeted oligonucleotide probes were used: one for all hitherto sequenced bacteria (EUB338, Amann *et al.*, 1990; EUB338-II, EUB338-III,

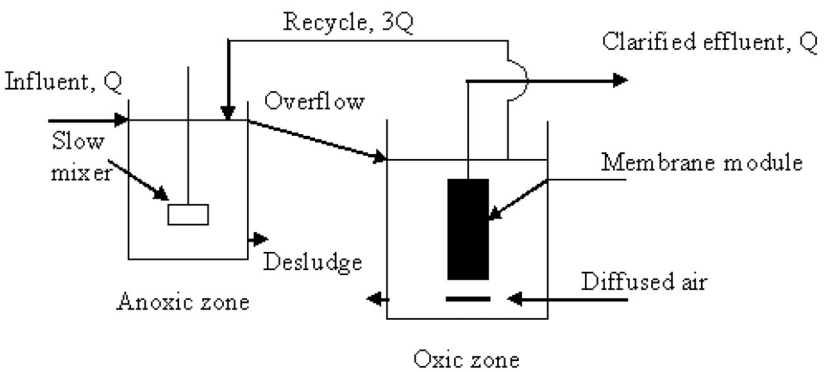

Figure 1 Schematic of laboratory-scale A/O submerged MBR

Daims *et al*., 1999); a control probe complementary to EUB338 (NONEUB, Wallner *et al*., 1993); probes specific for the alpha, beta and gamma *Proteobacteria* (ALF1b, BET42a and GAM42a, respectively, Manz *et al*., 1992); a probe specific for the *Actinobacteria* subclass within the *Firmicutes* family (HGC69a, Roller *et al*., 1994); a probe specific for the *Cytophaga-Flavobacterium* subclass (CF319a, Manz *et al*., 1996); a probe specific for ammonia oxidizing beta *Proteobacteria* (NSO1225, Mobarry *et al*., 1996); a probe specific for *Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosococcus mobilis* and *Nitrosomonas C56* (NSM156, Mobarry *et al*., 1996); a specific probe for most *Nitrosospira* spp. (NSV443, Mobarry *et al*., 1996); a probe specific for most members of the phylum *Nitrospirae* (NTSPA712, Daims *et al*., 2001); a probe competitor for NTSPA712 (CNTSPA712, Daims *et al*., 2001); a probe specific for *Nitrobacter* spp. (NIT3, Wagner *et al*., 1996); probes specific for the genus *Paracoccus* (PAR651, PAR1244, PAR1457, Neef *et al*., 1996), a probe specific for *Paracoccus denitrificans* (PDV198, Neef *et al*., 1996); a probe for *Pseudomonas stutzeri* (PST997, Amman *et al*., 1996); and a probe specific for *Bordetella* spp. and *Alcaligenes* spp. (ALBO34a, Stoffels *et al*., 1998). Except for NONEUB and CNTSPA712, probes were synthesized and labeled with either Cy3 or Cy5 (MWG-Biotech AG, Germany). Percentage area quantification of probe-conferred fluorescence was done using the Metamorph software with epifluoresence microscopy (Olympus BX51), while processing of the final image was done using a confocal laser scanning microscope/ CLSM (Zeiss 5 PASCAL).

Chemical analysis of samples

Determination of NO_3-N and NO_2-N concentrations was by ion chromatography (DIONEX QIC Analyser) while NH_4-N was measured with the autophenate method. DO concentrations were automatically monitored and recorded by YSI DO meters.

Results and discussion

Reactor performance

The laboratory-scale A/O MBR was operated for 360 days. Despite variations in feed concentration due to fluctuations in raw wastewater quality, the sludge concentration was maintained at around 7–9 g/l. DO concentration in the anoxic zone was maintained at below 0.05 mg/l while it was between 2–5 mg/l in the oxic zone. To more easily differentiate between the two DO environments, the anoxic and oxic zones were defined as low and sufficient DO environments, respectively. The mean influent total nitrogen concentration was 42 mg/l. This was made up of ammonium nitrogen (about 86%) while nitrite and nitrate nitrogens were almost negligible. Dissolved oxygen rich liquor recycled from the oxic zone did not seem to inhibit the nitrate reduction process and nitrogen removal performance was stable throughout the study. At the long sludge age of 150 d and HRT of 11 h, it was observed that the system had a TN removal efficiency of about 88%. Effluent nitrogen, comprising mostly of nitrate nitrogen, was consistently about 5 mg/l. Considering the zero effluent suspended solids and a relatively stable sludge concentration, nitrogen loss due to assimilation can be expected to be marginal and thus the influent nitrogen would primarily be removed by ammonia oxidation and subsequent anoxic nitrate reduction.

Identification of bacterial communities

FISH analysis was performed to characterize the overall phylogenetic composition and diversity of the microbial community in the A/O MBR. The oligonucleotide probes applied covered five rRNA targeted groups: ALF1b, BET42a, GAM42a specific to the alpha-, beta-, and gamma-subclass of *Proteobacteria*, respectively; HGC69a for gram positive bacterium with a high G+C DNA content; and CF319a for *Cytophaga-Flavobacterium*

group. These were used with eubacterial probes (EUB338, EUB338-II, EUB338-III) and a complementary to probe EUB338 (NON338). The results are depicted in Table 1. Generally, members of *Proteobacteria* were 20–30% higher in samples from the 2–5 mg/l DO (sufficient DO) environment compared to those from the below 0.05 mg/l DO (low DO) environment. The A/O bacterial community was dominated by members of the beta-subclass *Proteobacteria*. The next dominant subclass was the gamma-subclass *Proteobacteria,* while members of the alpha-subclass *Proteobacteria*, *Cytophaga-Flavobacterium* group and Gram positive high G+C bacteria did not constitute numerically dominant groups, with less than 4% targeted for each group. The predominance of beta *Proteobacteria* is in general conformance to studies reported on many activated sludge processes (Manz *et al*., 1994; Snaidr *et al*., 1997) and the aerobic MBR (Luxmy *et al*., 2000; Witzig *et al*., 2002). Despite the predominance of beta-subclass *Proteobacteria*, the total detectable microbial population within the A/O MBR was relatively low, with 52–62% of total active bacteria remaining undetected. This relatively high percentage of no detection was similarly reported by Luxmy *et al*. (2000) for an infinite cell residence time MBR. This would suggest long SRTs had caused a shift in the microbial population from the *Proteobacteria*.

Identification of nitrifers

The identification of specific groups in the nitrifying population was determined using five rRNA targeted probes: NSO1225 specific for ammonia oxidizing bacteria, NSM156 specific for *Nitrosomonas* spp., NSV443 specific for *Nitrosospira* spp., NIT3 specific for *Nitrobacter* spp. and NTSPA712 specific for *Nitrospira* spp. Biomass hybridization with NSV443, NSM156 and NSO1225 with EUB338 mix was conducted to identify the ammonia oxidizing bacteria. Likewise, detection of nitrite oxidizing bacteria was conducted with probe NIT3 and NTSPA712. In order to confirm the identification made with the NTSPA712 probe, comNTSPA712 which is a competitor probe to NTSPA712 was used. The results showed that NSO1225 constituted about 12–16% while NSV443 was about 11–14% of the total metabolically active bacterial cells (Table 1). The relatively similar readings provided by NSO1225 and NSV443 probes indicate *Nitrosospira* spp. represented the vast majority of ammonia oxidizing bacteria groups. No hybridization signal was observed with probe NSM156. For nitrite-oxidizing bacteria, hybridization signals were observed when the *Nitrospira*-specific probe NTSPA712 was used and this accounted for 6–9% of the total active cells. No detection of NIT3 was observed, indicating the dominance of *Nitrospira* spp. among the nitrite oxidizing bacterial groups. More signals of NSV443 and NTSPA712 were found in samples from the sufficient DO environment rather than the low DO environment, but in total, the numbers of probe-stained cells were lower compared to the numbers determined for ammonia oxidizing bacteria. Identification of

Table 1 Positive results on specific probe counts

Probe	Low DO[a] (%)[c]	Sufficient DO[b] (%)	Probe	Low DO (%)	Sufficient DO (%)
ALF1b	2.2	1.8	NSO1225	11.7	15.8
BET42a	17.2	21.9	NSV443	10.5	14.3
GAM42a	7.0	8.5	NTSPA712	6.0	8.5
CF319a	2.6	3.4	PAR1244	<1	–
HGC69a	3.1	3.7			

[a] DO below 0.05 mg/l

[b] DO between 2–5 mg/l

[c] Determined by simultaneous application of probes EUB338, EUB338-II, EUB338-III and NONEUB

Nitrosospira spp. and *Nitrospira* spp. lend support to the proposal that they might be responsible for ammonia and nitrite oxidation in the A/O MBR, respectively.

Identification of denitrifiers

Five rRNA oligonucleotide probes targeting specific commonly found denitrifying species: PAR651, PAR1244, PAR1457 specific for genus *Paracoccus*; PDV198 specific for *Paracoccus denitrificans*; PST997 specific to *Pseudomonas stutzeri*; and ALBO34a specific for *Alcaligenes* spp. were used. *In situ* hybridization with probes specific for the genus *Paracoccus* revealed that less than 1% of the total active cell counts in the low DO environment were assigned to PAR1244. No hybridization signals were noted when using probes PAR1244, PAR651 and PAR1457 on samples drawn from the sufficient DO environment. Negative results were also observed when the probes PDV198, PST997, and ALBO34a were used.

Analysis of the bacterial community structure and location

The community structure of the dominant beta-subclass *Proteobacteria* was characterized as rod-shaped, aggregated bacteria. As part of the beta-subclass, a similar structure was also observed in *Nitrosospira* spp. (Figure 2-a). Tight clusters of *Nitrosospira* spp. were found in the middle of aggregates with colony sizes of between 5.0 to 10.0 μm dia. These are twice as small compared to those reported for *Nitrosomonas* spp. clusters in an aerobic MBR (Luxmy *et al.*, 2000). Although the tendency to form tight clusters seems to be a typical characteristic among the ammonia oxidizing bacterial groups there are, however, differences among the different species. The reason for the smaller *Nitrosospira* spp. cluster is not yet known but may be due to its higher substrate affinity (Schramm *et al.*, 1998). The *Nitrospira* spp. also formed clusters (1.0 to 5.0 μm in size) and these were usually smaller than the *Nitrosospira* spp. clusters (Figure 2-b). The clusters of *Nitrosospira* spp. and *Nitrospira* spp. in the A/O MBR were usually irregular in shape like bunches of grapes or flowers and located in the middle of the flocs. Unlike nitrifying bacteria, the *Paracoccus* cells were normally found in small colonies made up of pairs of rod-shaped cells and located mostly on the outside of the flocs (Figures 2-c, 2-d). The occurrence of the denitrifiers and nitrifiers at different locations would suggest species selection resulting from differences in growth rates. Bacterial groups with the higher growth rate (denitrifiers) dominated

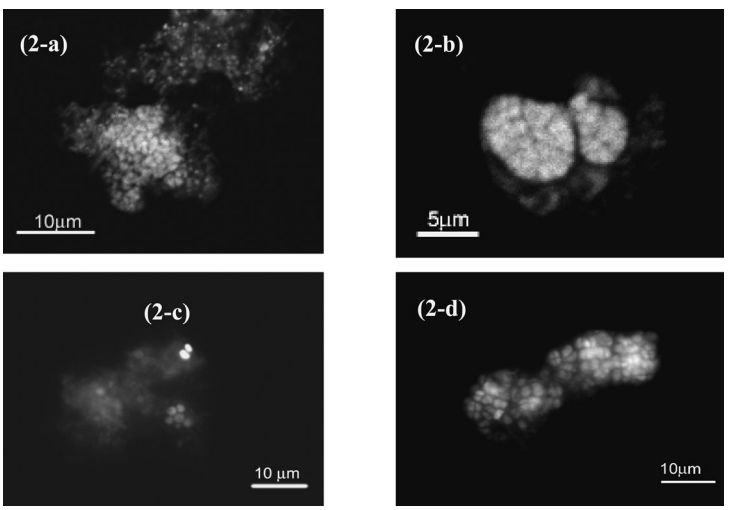

Figure 2 Clusters of *Nitrosospira* spp. (2-a), clusters of *Nitrospira* spp. (2-b), genus *Paracoccus* cells (2-c, 2-d)

the outer floc layers while those with the lower growth rate (nitrifiers) concentrated inside the flocs.

Influence of DO levels on spatial distribution of nitrifiers and denitrifiers

The concentration of DO affected the distribution of nitrifiers and denitrifiers in the MBR. Nitrifiers were found to predominate in the sufficient DO environment. As oxygen was removed as the limiting factor in the anoxic zone of the MBR, about a 36% and 40% increase in *Nitrosospira* spp. and *Nitrospira* spp. population, respectively, was observed in DO between 2–5 mg/l, indicating preference of nitrifiers for a higher DO environment. The former increase was accompanied by a decline in the *Paracoccus* population. The reduced *Paracoccus* population indicated the inhibitory effect DO had on its viability.

The ammonium and nitrite oxidation activity observed indicated the presence of active ammonia and nitrite oxidizing bacteria populations and *Nitrosospira* spp. and *Nitrospira* spp. were predominant in the system. The high population count observed showed *Nitrosospira* spp. and *Nitrospira* spp. were capable of surviving in the low DO environment. This was probably due to their higher affinity for oxygen or the utilization of alternative electron acceptors. Ammonia oxidizing bacteria were thought to possess lower K_m values for oxygen than nitrite oxidizing bacteria (Focht and Verstraete, 1977; Prosser, 1989). A low K_m value would enable ammonia oxidizing bacteria to better establish themselves in the low DO environment and this was in agreement with the higher numbers of ammonia oxidizing bacteria detected. The detection of *Nitrospira* spp. in this study indicates the diversity in nitrite oxidizing bacteria species and may possibly explain why *Nitrobacter* spp. may not be detected in large numbers in some nitrifying activated sludge systems (Wagner *et al*., 1996) and in an aerobic MBR (Witzig *et al*., 2002). The significant presence of *Nitrosospira* and *Nitrospira* spp. would therefore suggest these genera might be of greater importance in such systems than the more widely investigated *Nitrosomonas* spp. and *Nitrobacter* spp.

The genus *Paracoccus* had previously been reported as being responsible for the high denitrification activity observed in a denitrifying sand filter reactor (Neef *et al*., 1996). Although the genus was detected in the low DO environment, it was not so in the sufficient DO environment of this study. The presence of high dissolved oxygen had previously been reported to inhibit activity and synthesis of denitrifying enzymes (John, 1977). With the relatively low number of *Paracoccus* spp. detected and the negative results for *Alcaligenes* spp. and *Ps. stutzeri,* coupled with the relatively high denitrifying activity observed in this study, denitrification could not be attributed solely to the genus *Paracoccus*. The ability to denitrify is, however, widespread among bacteria of mostly phylogenetically unrelated heterotrophs (Stewart, 1988; Zumft, 1992). Hence, it is likely that there are many other possible bacterial species within the MBR culture, outside of the predominantly reported species, which are also capable of mediating the nitrate reduction process.

Conclusion and perspectives

The beta-subclass of *Proteobacteria* was found to be the predominant group within the MBR bacterial community for nitrification. The denitrifier species identified within the MBR system was the bacterial species from the genus *Paracoccus*. However, due to the relatively high denitrifying activity observed in this study, the former could not be attributed solely to *Paracoccus*. Further evaluations and identifications for other bacterial species outside of the predominantly reported species which are also capable of mediating the nitrate reduction process remain to be made.

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