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Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system

Yossi Tal^a, Joy E.M. Watts^a, Susan B. Schreier^a,
Kevin R. Sowers^a, Harold J. Schreier^{a,b,*}

^aCenter of Marine Biotechnology, University of Maryland Biotechnology Institute,
701 E. Pratt St., Baltimore, MD 21202, USA

^bDepartment of Biological Sciences, University of Maryland Baltimore County,
1000 Hilltop Circle, Baltimore, MD 21250, USA

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Abstract

The microbial consortium of a moving bed bioreactor (MBB) connected to a marine recirculating aquaculture system was examined by denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA gene fragments. Both ammonia and nitrite oxidizers, *Nitrosomonas cryotolerans* and *Nitrospira marina*, respectively, were found associated with the marine system as well as a number of heterotrophic bacteria, including *Pseudomonas* sp. and *Sphingomonas* sp. In addition, two *Planctomycetes* sp. were detected in the system suggesting the capability for anaerobic ammonia oxidation (anammox). The potential for carrying out different nitrogen transformation processes—nitrification, denitrification and anammox—by the bead consortium in both low and high organic load MBBs was measured by short-term batch incubation. Beads with a high organic load exhibited a lower nitrification rate (25 mg NH₃-N/m²/h) than low organic load beads (31.5 mg NH₃-N/m²/h) as well as the ability to carry out denitrification and anammox processes. The potential of using MBBs to induce different nitrogen transformation processes was evaluated, and it was found that this type of bioreactor has the capa-

* Corresponding author. Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 E. Pratt St., Baltimore, MD 21202, USA. Tel.: +1-410-234-8874; fax: +1-410-234-8896.

E-mail address: schreier@umbi.umd.edu (H.J. Schreier).

bility to serve as a platform for mediating desired anoxic processes such as denitrification and anammox.

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1. Introduction

Optimizing the parameters for biological nitrogen removal filters in recirculated aquaculture systems is an important research area in modern aquaculture. Despite their importance, there is a dearth of information about the identity and ecology of the microorganisms involved in catalyzing nitrogen (N) removal in these systems (van Rijn, 1996). Most studies on N-removing bacteria in recirculated aquaculture filters or wastewater treatment plants have focused on the nitrifying consortia belonging to the β - and α -subdivisions of the *Proteobacteria*, i.e. *Nitrosomonas* sp., *Nitrobacter* sp., and *Nitrospira* sp., that occupy aerobic biofiltration units (Juretschko et al., 1998; Princic et al., 1998; Kloop et al., 2000). For many years, the general assumption has been that ammonia- and nitrite-oxidizing species were identical in marine and freshwater environments. Recent innovations in microbial ecology techniques, however, have shown that this is not the case. Using oligonucleotide probes to examine nitrifying bacterial populations associated with freshwater and marine aquaria, Hovanec and Delong (1996) found that bacteria responsible for ammonia oxidation, *Nitrosomonas europaea*, appear to be present at high levels in seawater aquaria and at very low levels in freshwater aquaria. Other studies demonstrated that the important nitrite-oxidizing bacteria in fresh and marine environments belong to *Nitrospira* sp. and not to *Nitrobacter* sp. as was previously thought (Burrell et al., 1998; Daims et al., 2000). Thus, while a small number of studies have begun to examine biological filtration systems used in aquaculture, almost no information is available about the specific members that compose the bacterial consortia in nitrifying filters and the role that they play in N-removal processes. For instance, the filters often contain anaerobic regions and the involvement of anaerobic ammonia oxidizing (anammox) bacteria (Strous et al., 1999) in contributing to ammonia removal has not been examined. The wide range of physical and chemical conditions present in these biofilters as well as the various nutrients that are available for bacterial consumption makes it likely that many key microorganisms have not been reported.

One of the main problems in exploring microbial diversity in complex environments, such as those associated with biological filters, is the fact that many of the important microorganisms cannot be cultivated by traditional methods (Amann et al., 1995). The availability of molecular tools, such as those used to analyze 16S ribosomal DNA (rDNA) sequences, have made it possible to explore slow growing or uncultivated bacterial species in different environments (Heal et al., 1998; O'Donnell and Gorres, 1999). The goal of the present study was to use a molecular biological approach to survey the bacterial community of a moving bed bioreactor (MBB) that is a component of a marine recirculated aquaculture system. It was expected that this approach would provide

information about the bacterial diversity in these filters and provide some insight into the roles for different bacterial species, both aerobic and anaerobic, in the inorganic nitrogen removal process. Such information could be used to enhance the efficiency of these biofilters by optimizing operating conditions (i.e. oxygen levels, water flow, temperature, nutrient load) to induce a desirable process that is necessary for closing the nitrogen cycle in the system and releasing the fixed nitrogen back to the atmosphere. By amplifying bacterial DNA extracted from MBBs, we were able to link members of the microbial consortia with different nitrogen processes. Short-term batch incubation of beads from MBBs was conducted to examine inorganic nitrogen transformation processes, (i.e., nitrification, denitrification, and anammox) that can potentially be carried out by the microbial consortium in the MBBs.

2. Materials and methods

2.1. Aquaculture facilities and filter setup

Nitrifying MBBs connected to recirculated marine systems in the Center of Marine Biotechnology's Aquaculture Research Center were used for these studies. A 5-m³ circular fiberglass tank containing gilthead seabream, *Sparus aurata*, was operated at a density of 80 kg/m³ and feeding rate of 6 kg/day. The tank was connected to a 2-m³ MBB filled with 1 m³ of polyethylene beads having a specific surface area of 500 m²/m³ (4.86 cm²/bead) (Water Management Technologies, Baton Rouge, LA, USA). A flow rate of 10 m³/h was set to enable two exchanges of water per hour through the filter. After four months of operation, 3000 beads from this high organic load MBB were transferred to a small experimental salt water system having a filter volume of 5 l and tank volume of 150 l. The system was operated without any organic load under aerobic conditions with water retention times similar to the original system. The MBB of this latter system was the source for low organic load beads; ammonium chloride was added daily to maintain a constant ammonia–nitrogen (both NH₃-N and NH₄⁺-N) concentration of 5 mg/l.

2.2. Batch experiments

Batch experiments were performed using 200 beads removed from high or low organic load filter systems. Beads were incubated in 250-ml flasks under a range of conditions. For nitrification activity, incubations were performed under aerobic conditions with the addition of 3 mg NH₃-N/l. Denitrification activity was examined by incubating filters under anaerobic conditions with 130 mg nitrate–nitrogen (NO₃-N)/l with and without 10 mM sodium acetate. Anammox activity was induced under anaerobic conditions with 7 mg NH₃-N/l and 7 mg nitrite–nitrogen (NO₂-N)/l without additional carbon source. For denitrification and anammox conditions, anaerobiosis was created by flushing the system with an atmosphere of nitrogen. All incubations were repeated at least three times at different time intervals in a synthetic sea water medium (Atkinson and Bingman, 1996) with a final salinity of 20 ppt at 26 °C.

2.3. Chemical and physical analyses

Oxygen and temperature measurements were done using a YSI (model 57) temperature/oxygen probe (Yellow Springs Instruments, USA). Oxygen uptake was measured by incubating beads in seawater saturated with oxygen and measuring oxygen depletion rates with the oxygen probe. Salinity was monitored with a refractometer (model S-10E, Atago, Japan). Total ammonia–nitrogen (TAN; NH_3 and NH_4^+) was determined by the hypochlorite oxidation reaction as described by Scheiner (1976), nitrite was determined by reaction with sulfanilamide according to Strickland and Parsons (1968), and nitrate was measured spectrophotometrically according to Clesceri et al. (1989). To prevent salt interference, nitrate analyses were conducted on water samples that were diluted up to 1000-fold with deionized water.

2.4. Bead dry weight

The organic matter load carried by beads was inferred by measurements of bead dry weight. Ten beads were removed from filters and dried at 65 °C to a constant mass ± 0.0001 g. The net weight of organic matter carried by beads was calculated by subtracting the average weight of 10 unused beads from the average weight of 10 beads taken from the filters.

2.5. DNA extraction and polymerase chain reaction (PCR) amplification

Biofilm from two to four MBB beads was stripped by vigorous vortexing with 0.25 g of silica (0.1 mm; Biospec Products, Bartlesville, OK) in 2 ml of extraction buffer (0.15 M NaCl, 0.1 M Na_2EDTA , pH 8.0). After centrifugation at $14,000 \times g$, 10 min, 4 °C, the pelleted material was resuspended in 300 μl of lysozyme solution (15 mg lysozyme/ml, 0.15 M NaCl, 0.1 Na_2EDTA , pH 8.0) and incubated at 37 °C for 1 h with mixing by inversion every 15 min. After cooling on ice, 300 μl SDS buffer (0.5 M Tris–HCl, 0.1 M NaCl, pH 8.0, 4% sodium dodecylsulfate) was added and the sample was incubated for 10 min and then placed at 55 °C for 10 min. This process was repeated three times. Genomic DNA was then extracted and purified from lysates by three sequential phenol–chloroform extractions (Sambrook et al., 1989) followed by precipitation with isopropanol. DNA pellets were washed with 70% ethanol and resuspended in sterile TE (50 mM Tris–HCl, 1 mM Na_2EDTA , pH 8.0) and stored in –20 °C.

PCR amplification of 16S rDNA genes from extracted DNA was done using universal bacterial-specific 16S rRNA primer 1055f (*Escherichia coli* position 1055 to 1070; 5'-TGGCTGTCGTCAGCT-3') and GC clamp primer 1392r (*E. coli* position 1392 to 1406; 5'-CGCCCCGCCGCCCCGCGCCCGCCGCCCCGCCCCGCCCCACGGGCGGTGTGTAC-3') (Ferris et al., 1996). Reaction mixtures (50 μl) contained 5 μl of $10 \times$ PCR buffer, 4 μl dNTPs (25 mM), 3 μl MgCl_2 (25 mM), 0.1 μl *Taq* polymerase (250 U) (Applied Biosystems, California), 2 μl DNA template (10–100 ng), and 1 μl of each primer (100–200 ng each). Reaction cycle parameters included an initial denaturation step of 5 min at 95 °C followed by seven cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 62, 60, 59, 58, 57, 56, 55 °C, elongation for 30 s at 72 °C, followed by 30 cycles of

94 °C, annealing for 30 s at 54 °C, elongation 30 s at 72 °C, with a final extension step of 5 min at 72 °C (Watts et al., 2001). PCR products were checked for size and yield on a 0.8% (w/v) TAE (20 mM Tris–HCl, 10 mM sodium acetate, 0.5 mM Na₂EDTA, pH 8.0) agarose gel.

2.6. DGGE analysis

Analysis of PCR products by DGGE was performed as described by Muyzer et al. (1993) and modified by Watts et al. (2001) using the D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA). Products from triplicate PCR reactions were combined and applied directly to 10% (w/v) polyacrylamide gels that contained a 40–60% gradient of denaturants (100 ml of 100% denaturant contained 42 g urea, 40 ml deionized formamide, 25 ml of 40% acrylamide/bis solution [37.5:1], 1 ml of 50 × TAE buffer) and electrophoresis was performed for 5 h at 60 °C in TAE at a constant voltage of 200 V. After electrophoresis, nucleic acids were stained with SYBR-Green II (1:10000 dilution; Molecular Bio-Probes, Eugene, OR) and the staining pattern was immediately recorded using a fluoroi-mager (Molecular Dynamics, Sunnyvale, CA). Isolation of DNA fragments was achieved by first staining gels with ethidium bromide (0.5 µg/ml) and visualizing using a UV transilluminator. Gel sections containing stained fragments were excised and slices were placed in TE buffer and incubated at 4 °C overnight. DNA obtained in this manner was used as template for the PCR reaction, as described above.

2.7. Cloning, sequencing, and database analysis

PCR-amplified DNA fragments obtained from DGGE gel slices were introduced into a pCR2A vector and transformed into *E. coli* using the TOPO TA cloning kit (Invitrogen, California) as described by the manufacturer. The presence of cloned inserts was verified by PCR amplification and sequence analysis was done by the Center of Marine Biotechnology's BioAnalytical Services Lab according to the method of Ferris et al. (1996) using an ABI 373 automated sequencer (PE Applied Biosystems, Foster City, CA). DNA sequences were analyzed using BLASTN, search of nucleic acid databases (Altschul et al., 1990).

3. Results

3.1. MBB bead biofilm formation and characteristics

To examine the characteristics of the microbial community from an MBB in a closed recirculated mariculture system, the biofilm from MBB beads established in a high organic load filter was compared to beads that were conditioned to a low organic load system (see Materials and methods). Initially, fresh beads were placed into the high organic load filter established in the marine recirculated system to allow for the formation of a biofilm. Bead samples were removed over the course of a 30-day period and dry organic net weight was determined. As expected, a progressive increase in dry organic weight was detected for

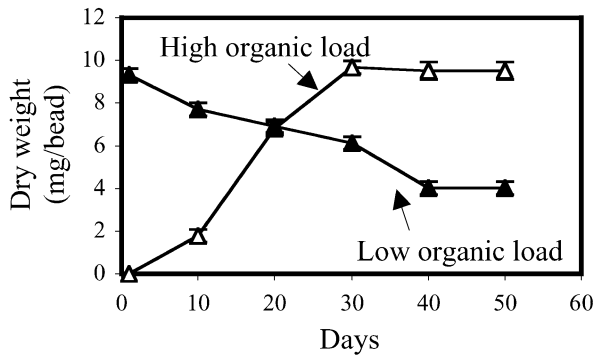


Fig. 1. Dry organic weight of beads. Dry weights for beads in high (Δ) and low (\blacktriangle) organic load filters during a time-course of incubation was determined as described in Materials and methods.

these beads, which reached a peak value of approximately 9.6 mg/bead after 30 days (Fig. 1). A portion of these beads were then transferred to a low organic load filter (see Materials and methods) and a decrease in the dry organic net weight was observed, which continued to a minimum and steady value of approximately 4 mg/bead after 40 days of incubation (Fig. 1). As can be seen in a photograph of representative beads from both filter systems (Fig. 2), the biofilm attached to the beads from the high load filter was thick and dark brown in color compared to the thinner and lighter yellow color observed for beads from

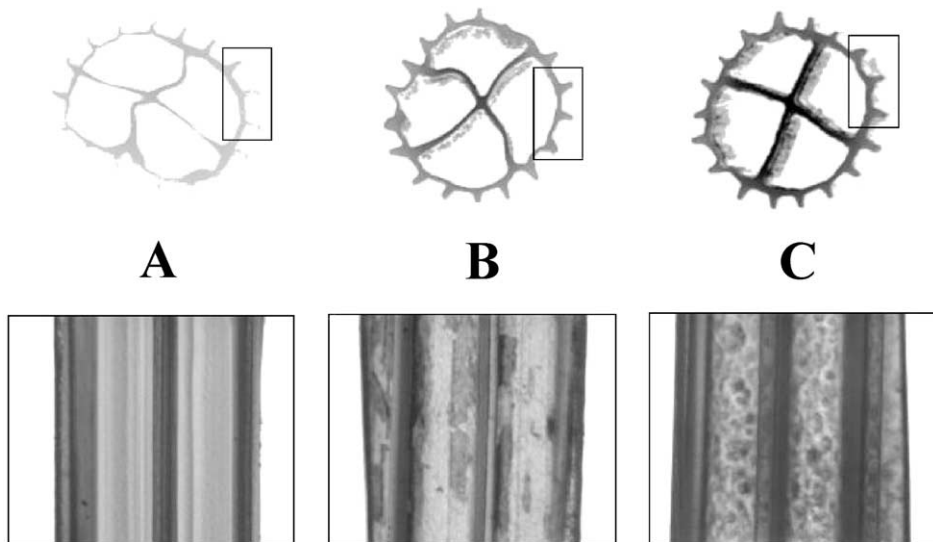


Fig. 2. Biofilm formation on beads from different organic load MBBs. Photograph of a representative bead from the low (B) and high (C) organic load systems are shown as well as an untreated bead (A). Magnification ($\times 3$) of a small section of the matrix (boxed area) is shown under each bead. The dark area between bead segments is due to biofilm formation.

the low load filter. The oxygen demand of beads from the different MBBs was determined and results are presented in Fig. 3. The initial oxygen consumption rate for the high organic load filters was $0.41 \mu\text{g oxygen/bead/min}$ compared to a value of $0.20 \mu\text{g oxygen/bead/min}$ for the low load beads. These rates are consistent with the bead dry weights and reflect the biomass differences between the high organic and low organic load systems.

3.2. Bacterial diversity

The diversity of the microbial consortium in biofilms of beads from both high and low organic load conditions was examined by PCR amplification of DNA extracted from biofilm material followed by DGGE analysis as described in Materials and methods. To examine dissimilarities between marine and freshwater bacterial consortia, a freshwater sample taken from the MBB of the freshwater system was also analyzed. The freshwater system configuration was identical to that described for the seawater system in Materials and methods. A typical result is shown in Fig. 4. As expected, band patterns from low (lane A) and high load (lane B) filter systems as well as the filter from the freshwater system (lane C) verified the presence of a high degree of diversity within the bacterial consortia of these systems. In general, the banding pattern for the marine systems were more similar to each other than either was to the freshwater system.

To identify the individual bacteria associated with these filters, DNA fragments were extracted from DGGE gels and were analyzed as described in Materials and methods. The highest similarity results (by BLAST analysis) of partial 16S rRNA sequences of 10 of these fragments (lane D and numbered arrows, Fig. 4) are summarized in Table 1. In the marine filters bacteria having significant sequence identity to the 16S rDNA of the nitrifier *Nitrosomonas cryotolerans* were identified. Nitrite oxidizers were found, represented by a clone having strong homology to *Nitrospira marina*. These nitrifiers (bands 1, 10) were detected in both high and low organic load beads of the marine system but not in the freshwater filter system. Similarly, PCR products having sequences that shared high similarities with a sulfite-oxidizing *Sulfitobacter* sp. (band 4) were identified

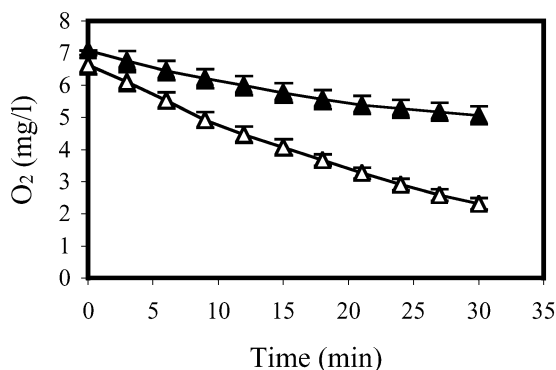


Fig. 3. Oxygen consumption pattern of filters. Oxygen levels for high (Δ) and low (\blacktriangle) organic load systems were measured as described in Materials and methods.

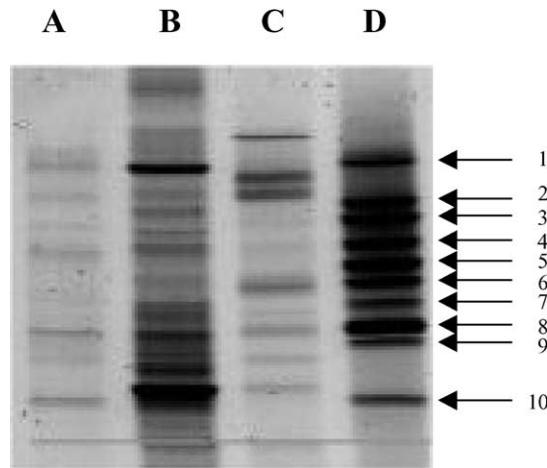


Fig. 4. DGGE analysis of PCR-amplified DNA extracted from filter biofilms. Samples from low (A) and high (B) organic load filters were compared to a freshwater filter sample (C). Also shown (D) is a collection of individual fragments that were isolated from these samples, cloned and characterized. Numbers and arrows correlate with the identification listed in Table 1.

in high and low load beads but not in freshwater beads, suggesting the presence of an anaerobic condition that might promote the reduction of sulfate to sulfite in the marine filters. Heterotrophs in both marine MBB samples were represented by clones having high similarities to *Pseudomonas* sp. (band 7) and two unclassified α -Proteobacteria (bands 2 and 6). In addition, a clone with very high sequence similarity to the heterotrophic *Aquaspirillum metamorphum* (band 3) was also identified in both marine filters. While *A. metamorphum* is a freshwater bacterium associated with shellfish, it is conceivable that the

Table 1
Characteristics of DNA fragments from DGGE gels

Band	Closest match ^a	Number of nucleotides compared	% Similarity with closest match	Accession number	Sample origin ^b
1	<i>Nitrosomonas cryotolerans</i>	327	99	AF272423	HL/LL
2	α -Proteobacterium	280	97	AB013442	HL/LL
3	<i>Aquaspirillum metamorphum</i>	244	100	Y18618.1	HL/LL
4	<i>Sulfitobacter</i> sp.	289	96	AF007254	HL/LL
5	<i>Planctomycete</i> sp.	347	95	AJ231182	HL
6	α -Proteobacterium	320	97	AB018689	HL/LL
7	<i>Pseudomonas cedrella</i>	353	100	AF064461	HL/LL
8	Uncultured bacterium	285	98	AF142794	HL/LL
9	<i>Planctomycete</i> sp.	337	91	AJ231171	HL
10	<i>Nitrospira marina</i>	279	91	X82559.1	HL/LL

^a Closest match was determined by aligning sequences to sequences in the database using the BLASTN program (see Materials and methods).

^b HL, high organic load filter; LL, low organic load filter.

bacterium present in the marine system is a salt-tolerant relative. Of interest was the presence of two PCR products having sequence similarities to two members of the *Planctomycetes* sp. (bands 5 and 9), which were only detected in the high organic load filters. Members of the *Planctomycetes* sp. have been found to be responsible for the process of anaerobic ammonia oxidation, also known as “anammox” (Strous et al., 1999). In many cases (e.g. band 8 and others not shown), sequences were found to be highly similar to 16S rRNA sequences that have been deposited in the sequence database for uncultured bacteria acquired from anoxic marine sediments (Bowman et al., 2000).

Since our DGGE analysis of the microbial consortia of the MBBs is based on PCR-derived products, we are not able to determine the relative abundance of the different bacterial species within these filters. However, the dominance of particular bacterial species may be estimated by their unique level of physiological activity. Therefore, we examined the high and low load filters for their ability to carry out processes related to the nitrogen cycle.

3.3. Inorganic nitrogen transformation processes

Batch incubations were performed to examine and measure the potential for carrying out different nitrogen transformation processes by the microbial consortia in the marine MBBs. Beads from both high and low organic load systems were incubated under nitrification, denitrification, and anammox conditions, and the rate of each process was determined.

3.3.1. Nitrification potential

Aerobic incubations of beads with ammonia as the sole nitrogen source demonstrated a maximum ammonia removal rate of 31.5 mg $\text{NH}_3\text{-N}/\text{m}^2/\text{h}$ for low organic load beads compared to 25 mg $\text{NH}_3\text{-N}/\text{m}^2/\text{h}$ rate of high organic load beads. A typical pattern for ammonia, nitrite and nitrate consumption/accumulation during these incubations can be seen in Fig. 5. Nitrate and nitrite concentrations increased as ammonia concentrations declined, a pattern that is consistent with the nitrification process proceeding to completion. A similar pattern was observed for both high and low organic load systems (data not shown). The relatively high oxygen consumption rate and low nitrification activity of the high load beads suggest an active heterotrophic population in the microbial consortia of the high load beads compared to the low load beads.

3.3.2. Denitrification potential

Beads from the high organic load filter demonstrated denitrification capabilities in the absence of carbon source (Fig. 6A). Calculating the denitrification rates from the results shown in Fig. 6A indicated that during 72 h of incubation nitrate reduction rates fluctuated from 1.7 mg $\text{NO}_3\text{-N}/\text{m}^2/\text{h}$ in the first 24 h, to a maximum of 10.8 mg $\text{NO}_3\text{-N}/\text{m}^2/\text{h}$ after the second 24-h interval. The same batch of beads was re-incubated with 130 mg $\text{NO}_3\text{-N}/\text{l}$ (Fig. 6B). During the second incubation, the denitrification rate stabilized at an average of 5 mg $\text{NO}_3\text{-N}/\text{m}^2/\text{h}$. After 72 h of incubation, the denitrification ability of the high load beads stopped completely.

Beads from low load filters showed no denitrification ability during incubations without the addition of carbon source. When 10 mM sodium acetate was added to low organic load

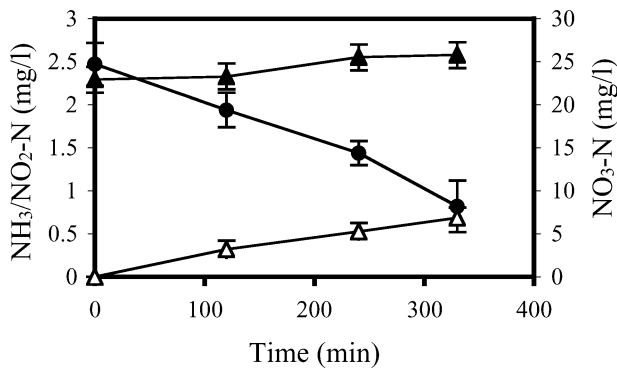


Fig. 5. Patterns for inorganic nitrogen consumption and accumulation during nitrification conditions. NH₃-N (●), NO₂-N (△) and NO₃-N (▲) levels were measured as described in Materials and methods.

beads, nitrate reduction was activated 24 h after the beginning of incubation (Fig. 6C). The maximum denitrification rate measured during these incubations was 10.7 mg NO₃-N/m²/h. The relatively high nitrite accumulation (28 mg NO₂-N/l) of these beads is indicative of a high activity of denitrifying bacteria, e.g. the *Pseudomonas* sp. detected in the filters, that are able to reduce nitrate only to nitrite (Tiedje, 1988). In a similar experiment, addition of

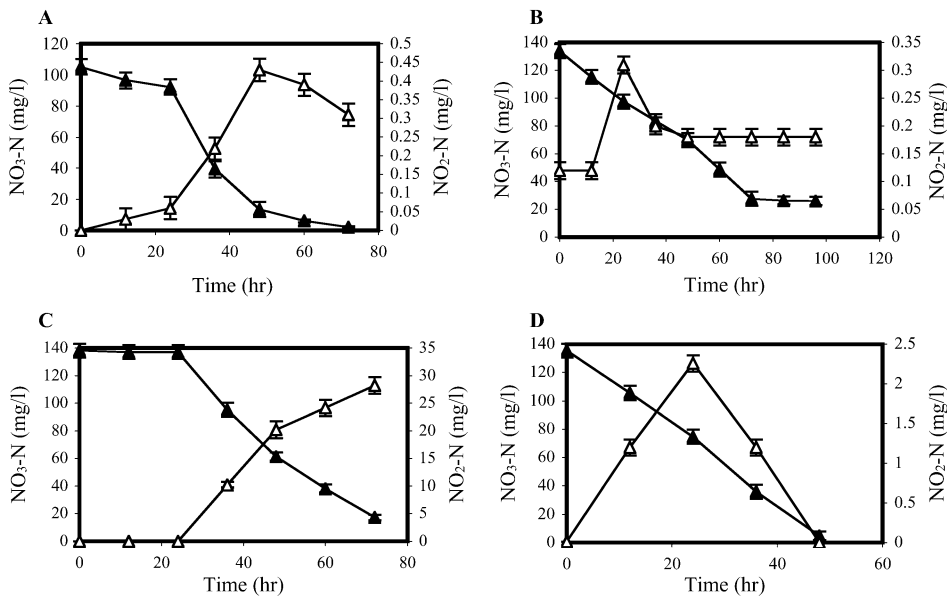


Fig. 6. Nitrate and nitrite concentration during incubation of MBBBs under denitrification conditions. Nitrate (▲) and nitrite (△) concentrations were determined as described in Materials and methods and incubations were done: (A) with high load beads in the absence of an external carbon source; (B) during a second incubation of high load beads without the addition of external carbon source; (C) with low load beads in the presence of 10 mM acetate as carbon source; and (D) with high load beads in the presence of 10 mM of acetate as carbon source.

a carbon source to the high load filter showed an increase in denitrification activity from the start of the incubation (Fig. 6D), which reached a maximum rate of 9.9 mg $\text{NO}_3\text{-N}/\text{m}^2/\text{h}$. The low nitrite accumulation (0.2–0.4 mg $\text{NO}_2\text{-N}/\text{l}$) during the incubation of the high load filter with or without acetate confirmed the existence of a denitrifying population as a component of the bacteria consortia of these beads.

3.3.3. Anaerobic ammonia oxidation potential

Detection of *Planctomycetes* sp. in the DGGE analysis suggested that the bacterial consortia of the MBBs have the potential of carrying out the anammox process. To determine whether anammox activity is occurring in the MBBs, batch incubations of beads from low and high organic load MBBs were performed under anammox conditions. For the three separate experiments, removal rates for ammonia and nitrite ranged from 1.5- to 3.6-fold higher under anammox conditions in beads taken from the high organic load filter compared to controls (Fig. 7A). The ammonia oxidation rate with nitrite as electron

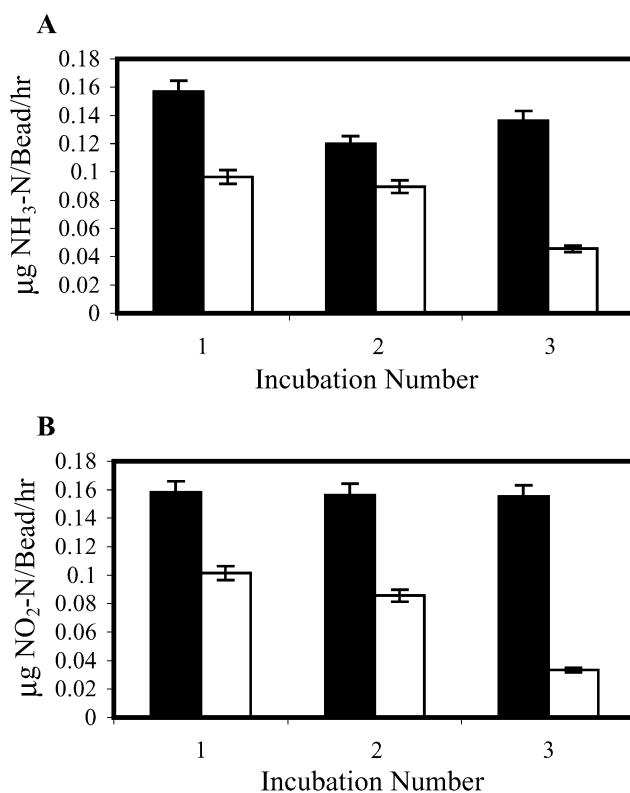


Fig. 7. Anammox potential for the high organic load filter. Ammonia (A) and nitrite (B) removal rates of high load beads were measured during incubations under anammox conditions as described in Materials and methods. For A, incubations were done in the presence of ammonia and nitrite (open boxes) or with ammonia alone (closed boxes); for B, incubations were done in the presence of ammonia and nitrite (open boxes) or with nitrite alone (closed boxes).

acceptor was between 0.29 and 0.33 mg NH₃-N/m²/h compared to values of 0.092 to 0.20 mg NH₃-N/m²/h during incubations without nitrite. Nitrite uptake values paralleled ammonia uptake values, ranging from 1.6- to 4.8-fold (Fig. 7B). With ammonia, nitrite removal rates averaged from 0.33 mg NO₂-N/m²/h compared to 0.067 to 0.2 mg NO₂-N/m²/h obtained from incubations without ammonia. The ability to measure ammonia and nitrite consumption under anammox conditions suggests that the bacterial consortia of the MBBs can carry out anammox activity.

4. Discussion

Nitrifying MBBs are considered to be a relatively inexpensive form for biofiltration in commercial recirculated aquaculture systems. Their low energy consumption and simple operation makes this filter format highly attractive. However, one of the drawbacks of MBBs is the accumulation of organic particles on and inside the beads. This phenomenon reduces the ability of oxygen to diffuse through biofilms that cover the beads and consequently reduces the nitrification performance of the filter. Microelectrode studies on trickling filter biofilms showed that the environment becomes totally anaerobic under a 100- μ m layer (Schramm et al., 1996) and no nitrification can take place.

In this study, ammonia removal rates were measured in high organic load beads that were 20% lower compared to low load beads. The latter consumed 50% less oxygen and their average dry weight was less than half of the high organic load beads. These results strongly suggest that these filters may be operational in the nitrifying mode, with a mechanical pre-filtration unit in place to lower the organic load of the treated water. A comparison of the nitrification rates of the marine MBBs to published nitrification rates in trickling filters and expandable granular biofilters (bead filter propeller washed) of freshwater systems is shown in Table 2. As can be seen, the maximum ammonia removal rates of the MBBs are equal or higher compared to the other filter systems. The significantly lower energy required to operate the MBB together with its high nitrification rates and simple operation makes this filter format very attractive to use for nitrification processes in a marine systems.

DGGE analysis showed that ammonia and nitrite oxidizers in low and high organic load beads were represented by *N. cryotolerans* and *N. marina*. These findings are consistent with other studies on nitrifying bacteria in a marine environment. Stephen et

Table 2
Ammonia removals rates of different filter configurations

Filter type	Ammonia removal rate (g TAN/m ² /day)	Ambient ammonia concentration (mg TAN/l)	Reference
Trickling filter	0.43	2	van Rijn and Rivera (1990)
Trickling filter	0.6–0.72	2–8	Bovendeur et al. (1990)
Bead filter (propeller washed)	0.28–0.55	1	Malone et al. (1993)
Moving bed bioreactor	0.59–0.75	3	This study

al. (1996) surveyed the ammonia oxidizing consortia in marine sediments located under fish cages and did not find any relationship between the nitrifying bacterial composition and the degree of organic pollution in the environment. Others studies showed that the dominant nitrite oxidizers in marine aquaria are *Nitrospira* sp. (Smith et al., 2001).

A novel approach to operating a MBB system that results in complete nitrogen removal is dependent not only on the presence of nitrifying bacteria but also requires the induction of other nitrogen transformation processes through the action of heterotrophic bacteria on organic matter accumulated by the filters. This activity plays a role in producing an oxygen diffusion gradient that will create conditions favorable for denitrification and other anoxic processes (Schramm et al., 1996). Furthermore, aeration rates and water retention times can be modified to lower oxygen levels in the MBB system to promote these processes. Reports of denitrification taking place in aerobic nitrifying filters have been published (Wik, 1999). The limiting factor for sustaining localized anoxic conditions for long periods of time in these cases is usually the availability of an organic carbon source (Arbiv and van Rijn, 1995). In the present study, the accumulation of organic matter on the beads in MBBs can be used to some extent as a carbon source for denitrification. Using the information from Fig. 6A and B, we have calculated that the total amount of $\text{NO}_3\text{-N}$ that can be reduced by the heterotrophic bacteria in beads from high organic load MBBs, without additional carbon source, is as high as 0.34 mg $\text{NO}_3\text{-N}$ /bead. This suggests that each bead was “charged” with enough available carbon source to support reduction of 0.34 mg $\text{NO}_3\text{-N}$ at a maximum rate of 5.2 $\mu\text{g NO}_3\text{-N}/\text{bead}/\text{h}$ (0.45 mg $\text{NO}_3\text{-N}/\text{m}^2/\text{h}$). A growth system with a feed rate of 1 kg/day will produce approximately 30 g of $\text{NO}_3\text{-N}$ after complete nitrification (Timmons and Losordo, 1994). Incubation of approximately 250,000 beads for 24 h under denitrification conditions will reduce the entire nitrate to nitrogen gas. Therefore, this amount of beads has the potential of reducing 85 g of $\text{NO}_3\text{-N}$ before carbon source depletion.

Use of MBBs for denitrification can be accomplished by a configuration that includes either a sequence aerobic/anaerobic incubation of one MBB, or two bioreactors that will switch between aerobic and anaerobic conditions. Such a configuration has been tested for removing nitrogen waste in sewage treatment facilities (Pujol and Tarallo, 2000; Rusten et al., 2000). The limiting factor of using MBBs for nitrate controls in recirculated aquaculture systems is the regeneration rate of the carbon source that accumulates in the beads, which depends on the organic load of the treated water. In the present study, beads from the low load filter showed no detectable denitrification activity without the input of additional carbon source. This is despite the fact that DGGE analysis of the bacterial consortia in these beads revealed the presence of heterotrophic bacteria. The “recharging” rate of beads in MBBs and its dependence on the organic load of the treated water is currently being studied.

Another process that may be induced in MBBs under anoxic conditions is anammox, which involves that reduction of ammonia to nitrogen gas using nitrite as an electron acceptor (Strous et al., 1999). Broda (1977) predicted the existence of the anammox process almost 25 years ago based on thermodynamic calculations but the process was only recently confirmed by isolating chemolithoautotrophic bacteria capable of perform-

ing this unique process (Strous et al., 1999). Anammox bacteria were found to be a new autotrophic member of the order *Planctomycetes*, which belongs to one of the major distinct divisions of the Bacteria. *Planctomycetes* capable of anammox activity use nitrite in place of oxygen as the preferred electron acceptor and CO₂ as a sole carbon source. Since its discovery attention has been focused on the anammox process for its potential for decreasing oxygen utilization in wastewater treatment plants (Jetten et al., 2001). Two members of the *Planctomycetes* sp. were identified by DGGE analysis of the microbial consortia in the high organic load MBBs, The sequence identity between the partial 16S rDNA sequences of these *Planctomycetes* sp. to known anammox *Planctomycetes* sp. including *Brocadia anammoxidans* and *Kuenenia stuttgartiensis* was between 83% and 86%. These *Planctomycetes* were isolated from fresh wastewater treatment systems having very high concentration of ammonia, which would explain any variations from the *Planctomycetes* spp. found in the marine MBBs. Studies investigating the diversity of the anammox-capable *Planctomycetes* showed a range of similar but not identical bacteria that can carry out the anammox reaction (Schmid et al., 2000; Egli et al., 2001). This diversity may very well suggest the existence of marine anammox bacteria that are different from the anammox *Planctomycetes* sp. previously isolated.

The ammonia oxidation rates of high load beads under anammox conditions were two orders of magnitude lower compared to nitrification rates of the same beads. This low anammox activity may be due to a small population of the *Planctomycetes* sp. relative to the more abundant nitrifying bacteria. Unlike others studies where anammox bacteria was enriched by repeated incubation of samples with ammonia and nitrite (Egli et al., 2001), no attempt was made to enhance the anammox bacteria in our system. Moreover, the MBBs were operated under conditions favorable for nitrifying bacteria. The fact that no anammox activity could be measured in the low organic load filter may indicate a connection between biofilm structure and anammox activity. A more dominant anoxic zone favorable for anammox activity can be found in a thick biofilm structure such as the one that is produced in MBB beads that function under high organic loads compared to the low load beads. Our finding that an anammox activity is present in the MBB system has motivated us to examine the possibility of applying the anammox process as a component of water treatment in recirculated aquaculture systems.

5. Conclusion

Stricter environmental regulations on effluent discharge from aquaculture facilities are motivating this industry to reduce effluent volumes and nitrogen loads. Closing the nitrogen cycle within the treatment system is one way to accomplish this goal. In this study, we demonstrated that the microbial consortia present in MBBs has the potential to support different nitrogen transformation processes that enables closing of the nitrogen cycle and releasing nitrogen back to the atmosphere. The potential of using MBBs for complete nitrogen removal by combining nitrification with denitrification or anammox processes has been discussed and is yet to be applied in recirculated aquaculture systems.

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