RESEARCH ARTICLE

Ammonia-oxidizing archaea and nitrite-oxidizing nitrospiras in the biofilter of a shrimp recirculating aquaculture system

Monisha N. Brown[1], Aurelio Briones[2], James Diana[3] & Lutgarde Raskin[1]

[1]Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI, USA; [2]Plant, Soil & Entomological Sciences Department, University of Idaho, Moscow, ID, USA; and [3]School of Natural Resources and the Environment, University of Michigan, Ann Arbor, MI, USA

Correspondence: Lutgarde Raskin, Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI 48109, USA. Tel.: +1 734 647 6920; fax: +1 734 763 2275; e-mail: raskin@umich.edu

Received 10 February 2012; revised 5 July 2012; accepted 5 July 2012.
Final version published online 9 August 2012.

DOI: 10.1111/j.1574-6941.2012.01448.x

Editor: Cindy Nakatsu

Keywords
ammonia oxidation; nitrite oxidation; *Nitrospira*; *Nitrosopumilus maritimus*; shrimp aquaculture; recirculating aquaculture system.

Abstract

This study analysed the nitrifier community in the biofilter of a zero discharge, recirculating aquaculture system (RAS) for the production of marine shrimp in a low density (low ammonium production) system. The ammonia-oxidizing populations were examined by targeting 16S rRNA and *amoA* genes of ammonia-oxidizing bacteria (AOB) and archaea (AOA). The nitrite-oxidizing bacteria (NOB) were investigated by targeting the 16S rRNA gene. Archaeal *amoA* genes were more abundant in all compartments of the RAS than bacterial *amoA* genes. Analysis of bacterial and archaeal *amoA* gene sequences revealed that most ammonia oxidizers were related to *Nitrosomonas marina* and *Nitrosopumilus maritimus*. The NOB detected were related to *Nitrospira marina* and *Nitrospira moscoviensis*, and *Nitrospira marina*-type NOB were more abundant than *N. moscoviensis*-type NOB. Water quality and biofilm attachment media played a role in the competitiveness of AOA over AOB and *Nitrospira marina*-over *N. moscoviensis*-type NOB.

Introduction

Recirculating aquaculture systems (RAS) for the production of marine shrimp are a potentially sustainable alternative to traditional aquaculture systems because RAS reduce water requirements and limit the concentration of nutrients discharged to receiving waters. RAS require biological filters to oxidize toxic ammonia and nitrite, and aeration of the water to remove carbon dioxide and increase oxygen concentrations (Ebeling, 2000). Nitrifying biofilters keep ammonia and nitrite concentrations below toxic levels. For the white shrimp, *Litopenaeus vannamei* (Boone), ammonia toxicity levels range from 2.44 to 3.95 mg L^{-1} of total ammonia nitrogen (Lin & Chen, 2001), while nitrite toxicity levels vary between 6.1 and 25.7 mg L^{-1} of nitrite-nitrogen (Lin & Chen, 2003).

Nitrification is a two-step process in which ammonia is oxidized to nitrite by ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA) and nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). The sensitivity of AOB and NOB to a wide variety of environmental factors is well known, so much, so that nitrification has been regarded as the 'Achilles heel' of wastewater treatment (Daims *et al.*, 2006). In recirculating aquaculture settings, the challenges associated with accumulation of ammonia and nitrite are similar to those in the wastewater treatment field and also include problems with low dissolved oxygen (DO) levels, pH outside the optimal range for nitrifying microorganisms (7.5–8.6) and accumulation of trace amounts of toxic sulphides (Joye & Hollibaugh, 1995; Masser *et al.*, 1999; Ling & Chen, 2005).

Less is known about the sensitivity of AOA to environmental parameters. Archaeal ammonia monooxygenase subunit A (*amoA*) gene has been found to be ubiquitous in the environment, including in marine waters, biofilters of aquaria, coral reefs, estuaries, wastewater treatment plants, hot springs, sediments and soils (Prosser & Nicol, 2008; Erguder *et al.*, 2009; You *et al.*, 2009). In most studies in which the abundances of archaeal and bacterial *amoA* gene copies were investigated, the archaeal *amoA* outnumbered the bacterial *amoA* gene copies (Erguder *et al.*, 2009). The factors that influence this distribution are still unclear, but Erguder *et al.* (2009) proposed that AOA might be important ammonia oxidizers in low

nutrient, low pH and sulphide containing environments. Furthermore, Martens-Habbena *et al.* (2009) have shown that AOA have adapted to survive at low ammonia concentrations, for example, 0.2 μM, concentrations at which AOB cannot grow.

There are four validly described genera of NOB: *Nitrobacter*, *Nitrospina*, *Nitrococcus, Nitrospira*, as well as a newly communicated NOB species, '*Candidatus* Nitrotoga arctica' (Bartosch *et al.*, 1999; Alawi *et al.*, 2007). *Nitrospira* spp. have been found to be the main nitrite oxidizers in wastewater treatment plants (Juretschko *et al.*, 1998; Burrell *et al.*, 1999; Daims *et al.*, 2001, 2006) and the biofilters of a marine RAS (Keuter *et al.*, 2011) because they are better scavengers for nitrite and oxygen than *Nitrobacter* spp. (Schramm *et al.*, 1999; Koops & Pommerening-Roser, 2001). *Nitrospira* spp. have also been found in a hot spring (Lebedeva *et al.*, 2011), a marine sponge (Off *et al.*, 2010), and various soils (Bartosch *et al.*, 2002). An analysis of the publicly available sequences of the *Nitrospirae* phylum (Daims *et al.*, 2001) revealed that the genus *Nitrospira* consists of at least four distinct sublineages.

Nitrification is important to aquaculture in general, but particularly to zero discharge RAS, in which water quality (specifically ammonia and nitrite concentrations) is maintained by biofiltration.

The success of these systems depends on stable and reliable performance of its biofilters. The long-term goal of this work is to understand the nitrogen cycling processes in zero discharge RAS. In the present study, we examined the nitrifier community in the biofilter of a marine, zero discharge, indoor, recirculating shrimp maturation system, a type of RAS that was operated for the growth and reproduction of *L. vannamei*. We evaluated the identities and abundances of nitrifying bacteria and archaea and found AOA and nitrospiras to be the dominant nitrifiers. Few studies have characterized both AOA and nitrite oxidizers in marine systems that allow for process control. In addition to their industrial importance, such systems may serve as important model systems to improve our understanding of the ecophysiology of AOA and NOB.

Materials and methods

Sample collection

Samples were collected from an indoor, zero discharge, marine RAS shrimp farm in Okemos, MI (Supporting Information, Fig. S1). At the time of sampling, the system had been run continuously for 3 years with minimal water exchange and stable production, although not at intensive levels (> 100 shrimp m^{-2} culture area; Fast & Lester, 1992). Because the facility is located away from the coast, artificial seawater was prepared from a commercial salt solution to fill the system. Water quality in the culture tank was measured on site as follows: ammonium, nitrite and nitrate concentrations were measured using colorimetric assays using Hach kits, DO and temperature were measured with a YSI model 55 DO meter (Yellow Springs, OH), salinity was measured using a YSI model 30 salinity meter and pH was determined with a Mettler-Toledo SevenGo portable pH meter (Schwerzenbach, Switzerland). All samples for biomass analysis were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI), and three replicate samples were collected from each location. Samples were collected using sterile equipment from four locations: the culture tank, the bioballs compartment, the oyster shell compartment and sludge from the basin beneath the filter tower. All samples were stored on ice during transport to the laboratory and processed within 24 h.

DNA extraction

DNA from two replicate samples collected from the four RAS compartments was extracted using the following procedures: for water samples, each replicate consisted of DNA extracted from the pellet obtained after centrifuging 250 mL tank water at 3220 ***g*** for 30 min. For the bioball compartment, each replicate consisted of DNA extracted from the biofilm stripped from two bioballs. Biofilm (average of 260 mg fresh weight) was stripped by immersing each bioball in phosphate-buffered saline [130 mM NaCl, 10 mM sodium phosphate buffer (pH 7.2)] and manually brushing the surface with an endocervical brush while sonicating for two minutes using a sonicator bath (L&R, Kearny, NJ). Each sludge sample (average of 560 mg fresh weight) was obtained by centrifuging 1 mL of the sample at 5000 ***g*** for 10 min. The oyster shells (average of 260 mg fresh weight) were placed directly into bead-beating tubes used for DNA extraction. DNA was extracted from all biomass samples using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's protocol. Extracted DNA in each sample was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

PCR amplification

Duplicate PCRs for each sample replicate were run for each primer set. The primers 8F (Lane, 1991) and 1387R (Marchesi *et al.*, 1998) were used to amplify the bacterial 16S rRNA gene (Briones *et al.*, 2007). The archaeal 16S rRNA gene was amplified with the primer set 109f/934b

(Grosskopf *et al.*, 1998). PCRs were 50 μL and each reaction contained 5 μL of 10 × buffer, 200 μM of each dNTP, 2 mM $MgCl_2$, 0.2 μM of each primer, 1.25 units of *Taq* Polymerase (ExTaq DNA polymerase; Takara Bio, Clontech Laboratories Inc., Madison, WI) and 1 μL template.

Archaeal *amoA* gene fragments were amplified using primers Arch-amoAF (Francis *et al.*, 2005) and Arch-amoARmod (5′-TTWGACCARGCGGCCATCCA-3′; this work). The PCR mixture was as described above. Thermal cycling consisted of initial denaturation of 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min; final extension was at 72 °C for 19 min. Bacterial *amoA* gene fragments were amplified using primers amoA-1F/amoA-2R (Rotthauwe *et al.*, 1997). The PCR mixture was as described above. Thermal cycling consisted of initial denaturation of 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51.5 °C for 30 s and extension at 72 °C for 30 s; final extension was at 72 °C for 10 min. All PCR results were confirmed with agarose gel electrophoresis.

Cloning, sequencing and phylogenetic analysis

Triplicate PCRs were first pooled and purified using the QIAquick® PCR Purification Kit (Qiagen, Germantown, MD) and then the appropriate band was gel extracted as follows except for the archaeal 16S rRNA gene product. The bacterial 16S rRNA (1396 bp), archaeal *amoA* (645 bp) and bacterial *amoA* (491 bp) genes' PCR products were run on 0.8%, 2% and 2%, respectively, agarose gels. The desired bands were excised and purified using the MinElute Gel Extraction Kit (Qiagen). The amplified archaeal 16S rRNA genes were purified using the UltraClean® PCR Clean-Up Kit (MO BIO Laboratories, San Diego, CA). The PCR products for archaeal 16S rRNA, archaeal *amoA* and bacterial *amoA* genes were pooled before cloning to create each respective clone library for the RAS system. All purified PCR products were cloned using a TOPO TA cloning kit (Invitrogen Corp., San Diego, CA) according to the manufacturer's protocol. All sequencing was carried out at the Genome Sequencing Center at Washington University, School of Medicine, except for the AOB *amoA* gene sequencing, which was performed by Agencourt Bioscience Corporation (Beverly, MA).

The archaeal and bacterial 16S rRNA gene sequences were aligned with the NAST alignment tool (DeSantis *et al.*, 2006a) available at the Greengenes website (www.greengenes.lbl.gov). The aligned sequences were chimera-checked using the BELLEROPHON version 3 tool (DeSantis *et al.*, 2006b) at the Greengenes website. The aligned, nonchimera sequences were classified using the classification tool at the Greengenes website. The archaeal and bacterial *amoA* and NOB 16S rRNA gene sequences were aligned using MEGA version 4 (Tamura *et al.*, 2007). From the alignment, phylogenetic analyses were conducted in MEGA version 4 (Tamura *et al.*, 2007).

Sequences obtained in this study have been deposited in GenBank under the following Accession Numbers: HM345608–HM345611 (archaeal *amoA* clones), HM345612–HM345622 (bacterial *amoA* clones) and HM345623–HM345625 (*Nitrospira* spp. clones).

Quantitative PCR

Quantitative PCR (qPCR) was used to quantify the AOA and AOB *amoA* gene abundance as well as *Nitrospira* 16S rRNA gene abundance. All sample and standard reactions were carried out in triplicate using SYBR green chemistry on a Mastercycler ep realplex (Eppendorf North America, Hauppauge, NY) qPCR machine. Standard curves were generated for all experiments from plasmids containing cloned *amoA* or 16S rRNA gene PCR amplicons previously sequenced to verify identity. Samples were diluted to contain 10 ng μL^{-1} DNA. All qPCR assays were carried out in 25 μL reactions consisting of 1 μL template DNA, 100 nM (archaeal *amoA*) or 300 nM of each primer (bacterial *amoA* and *Nitrospira* 16S rRNA genes) and 12.5 μL 2 × Quantitect MasterMix (Quantitect; Qiagen). The specificity of amplification for all qPCR assays was verified via generation of melting curves and agarose gel electrophoresis.

Archaeal *amoA* gene copies were quantified using primers AOA-amoA-fm (5′-TTCTAYACTGACTGGGCYTGGACATC-3′) and AOA-amoA-rb (5′-AKGCCGTTTCTAGTGGGTCWGCTA-3′). These primers were modified from the primer set AOA-amoA-f/AOA-amoA-r (Coolen *et al.*, 2007) based on the archaeal *amoA* gene clone sequences obtained in this study. Linear response ($R^2 = 0.97$) was observed for plasmids containing archaeal *amoA* between 10^1 and 10^7 gene copies μL^{-1} template DNA; PCR efficiency was 1.78. The PCR conditions were as follows: 94 °C for 15 min, followed by 43 cycles consisting of 94 °C for 15 s, 58.5 °C for 30 s and 72 °C for 30 s. The one-point calibration method for absolute quantification, as described by Brankatschk *et al.* (2012), was used to calculate the gene abundance. The LINREGPCR program (v 2012.0) (Ruijter *et al.*, 2009) was used to calculate cycle threshold, C_T and PCR efficiency, E, values for samples and standards from amplification data. The mean E values for samples were 1.34 for tank water, 1.34 for bioballs, 1.29 for oyster shells and 1.37 for sludge. The mean E value for the standard used in the one-point calibration was 1.40.

Bacterial *amoA* was quantified using the primers amoA-1F/amoA-2R (Rotthauwe *et al.*, 1997). Linear response ($R^2 = 0.99$) was observed for plasmids containing bacterial *amoA* between 10^1 and 10^7 gene copies μL^{-1} template DNA and the PCR efficiency was 1.87. The PCR conditions were as follows: 95 °C for 15 min, followed by 45 cycles consisting of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. The standard curve method for absolute quantification was used to calculate gene abundance.

NOB 16S rRNA genes were quantified using the primer sets Ntspa4-821f/Ntspa4-1028r and Ntspa2-172f/Ntspa2-311r for Type IV nitrospiras (*Nitrospira marina* sublineage) and Type II nitrospiras (*Nitrospira moscoviensis* sublineage), respectively. The one-point calibration method for absolute quantification was used to calculate gene abundance as described for AOA *amoA*.

Ntspa4-821f (5′-GGGYACTAAGTGTCGGCGGT-3′) and Ntspa4-1028r (5′-RGSTCMTCMCCCTTTCAGGT-3′) were designed based on the sequences obtained in this study. Primer design and evaluation of specificity was performed using PRIMER3 software (Rozen & Skaletsky, 2000) implemented in the Primer-BLAST website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Optimization of annealing temperatures for each primer pair for real-time PCR was conducted using the gradient function of the Mastercycler ep realplex machine. Linear response ($R^2 = 0.99$) was observed for plasmids containing *Nitrospira marina* 16S rRNA gene between 5 and 1×10^6 gene copies μL^{-1} template DNA; PCR efficiency was 1.90. The PCR conditions were as follows: 95 °C for 15 min, followed by 44 cycles consisting of 94 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s. Mean *E* values for samples were 1.34 for bioballs and 1.38 for oyster shells. Mean *E* for standard used in one-point calibration was 1.37.

Ntspa2-172f/(5′-ATACCGCATACGRCTCCTGG-3′) and Ntspa2-311r (5′-GCTGATCGTCCTCTCAGACC-3′) were designed based on the sequences obtained in this study. Linear response ($R^2 = 0.99$) was observed for plasmids containing *N. moscoviensis* 16S rRNA gene between 5 and 1×10^6 gene copies μL^{-1} template DNA; PCR efficiency was 1.77. The PCR conditions were as follows: 95 °C for 15 min, followed by 44 cycles consisting of 94 °C for 15 s, 61.5 °C for 30 s and 72 °C for 30 s. The mean *E* values for samples were 1.25 for bioballs and 1.27 for oyster shells. The mean *E* value for standard used in the one-point calibration was 1.19.

Results and discussion

Samples were collected from an indoor, zero discharge, marine RAS shrimp farm in Okemos, MI. At the time of sampling (May 17, 2007), the system had been run continuously for 3 years with minimal water exchange and stable production, although not at intensive levels. Because the facility is located away from the coast, artificial seawater was prepared from a commercial salt solution to fill the system. The RAS relies on biofiltration in a multi-stage, nitrifying trickling filter that contains multiple types of biofilm attachment media, including plastic bioballs, plastic corrugated block and crushed oyster shells (Fig. S1). The bioballs (polyethylene; Aquatic Eco-System Inc., Apopka, FL) had a diameter of 3.5 cm with a specific surface area of 525 $m^2\ m^{-3}$. The crushed oyster shells are used as a supplement for chicken feed and were obtained from an animal feed store. Stocking densities for this maturation system are 6–9 shrimp m^{-2} of tank area (personal communication with farmer), which is considerably less than typical stocking densities of ultra-intensive production systems (> 100 shrimp m^{-2} of tank area; Fast, 1991). In this system, water from the culture tank is pumped and filtered by gravity through the different biofilm attachment media. Beneath the filter tower is a basin to collect water and settled particles (sludge) before the water is pumped back into the culture tank.

To analyse the composition of the microbial community in this RAS, we constructed 16S rRNA gene clone libraries of *Archaea* (Fig. S2), *Bacteria* (Table S1) and *Planctomycetes* (data not shown). Bacterial clone libraries were generated using biomass collected from each sampling location in the RAS (tank water, bioballs, oyster shells and sludge; Fig. S1). However, the archaeal and *Planctomycetes* libraries were generated using pooled PCR products obtained from DNA extracted separately from biomass samples obtained from each of the four sampling locations. Analysis of the archaeal 16S rRNA gene clone library (Fig. S2) revealed that 17% of clones were *Nitrosopumilus* type, a group related to AOA (Könneke *et al.*, 2005). Analysis of the bacterial clone libraries failed to detect any representatives of the proteobacterial AOB and NOB (Table S1). However, NOB from the genus *Nitrospira* were detected (Table S1). No sequences closely related to known anaerobic ammonium-oxidizing bacteria were detected in the *Planctomycetes* clone library (data not shown).

To confirm the presence of AOA and to determine their distribution within the RAS, a PCR assay was used to detect archaeal *amoA* genes in biomass samples collected from tank water, bioballs, oyster shells and sludge (data not shown). The initial PCR result indicated the presence of AOA *amoA* genes and revealed the highest abundance in the samples obtained from oyster shells (data not shown). These results were confirmed by a qPCR assay targeting archaeal *amoA* (Fig. 1). The abundance of archaeal *amoA* in the biomass attached to oyster

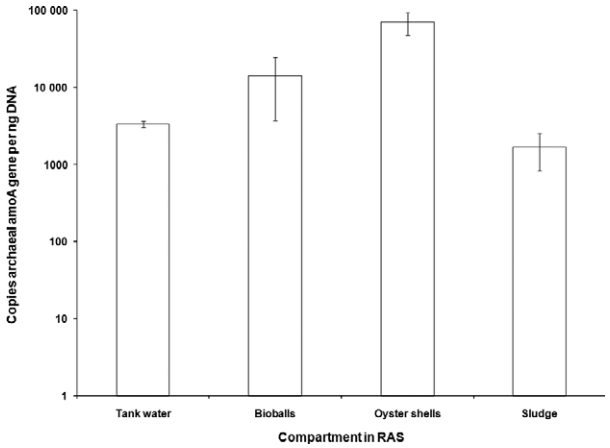

Fig. 1. Archaeal *amoA* gene abundance in four different RAS samples (Fig. S1), as measured by qPCR. Error bars show standard deviation.

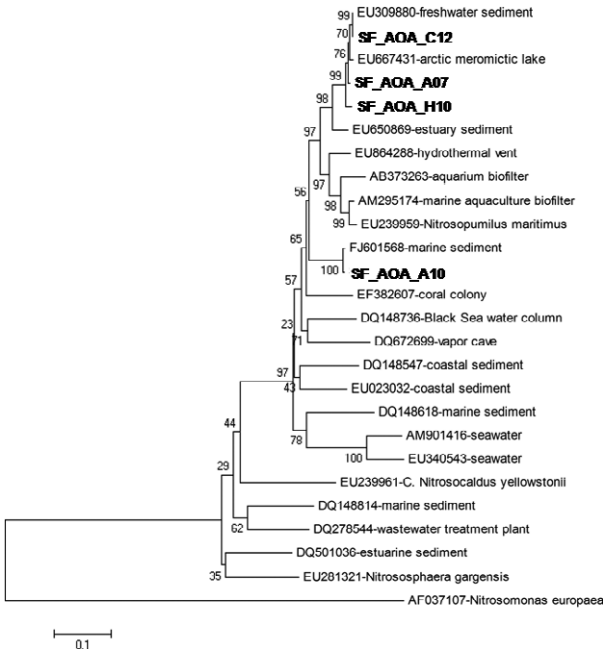

Fig. 2. Phylogenetic relationships of archaeal *amoA* gene. Sequences obtained in this study are indicated with the prefix 'SF_'. The tree was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the sequences analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site.

shells ($7.1 \times 10^4 \pm 2 \times 10^4$ copies archaeal *amoA* ng^{-1} DNA) was higher than the abundance of archaeal *amoA* associated with bioballs ($P < 0.5$, two-sample *t*-test) and an order of magnitude higher than the abundance associated with tank water ($P < 0.25$) and sludge ($P < 0.5$, Fig. 1). Sequence analysis of the archaeal *amoA* PCR products (Fig. 2) showed that most of the sequences amplified were related to the previously described *Nitrosopumilus* species (Könneke *et al.*, 2005) confirming our initial identification based on analysis of 16S rRNA genes. The sequences were obtained from two replicate samples pooled together to generate one clone library. A total of 24 clones related to *Nitrosopumilus* sequences were obtained, of which 16, five, two and one clones clustered within groups SF_AOA_A07, SF_AOA_A10, SF_AOA_C12 and SF_AOA_H10, respectively.

The bacterial 16S rRNA gene clone libraries did not detect the presence of AOB. However, using a PCR assay targeting the betaproteobacterial *amoA* gene, AOB were detected, but only in bioball samples (data not shown). These results were confirmed by a qPCR assay targeting bacterial *amoA* (Fig. 3). The abundance of bacterial *amoA* in the biomass attached to bioballs (83 ± 15 copies bacterial *amoA* ng^{-1} DNA) was approximately an order of magnitude higher than in the biomass associated with tank water, oyster shells and sludge (Fig. 3). Sequence analysis of bacterial *amoA* PCR products (Fig. 4) showed that 98.5% of the sequences amplified were related to obligately halophilic *Nitrosomonas marina*. One clone, designated SF_AOB_C09, was found to be closely related to *Nitrosomonas aestuarii*, which was isolated from brackish water and is closely related to *Nitrosomonas marina* (Purkhold *et al.*, 2000). In summary, the AOB population in the RAS was not abundant and consisted of AOB belonging to the *Nitrosomonas marina* cluster of betaproteobacterial AOB (Purkhold *et al.*, 2000).

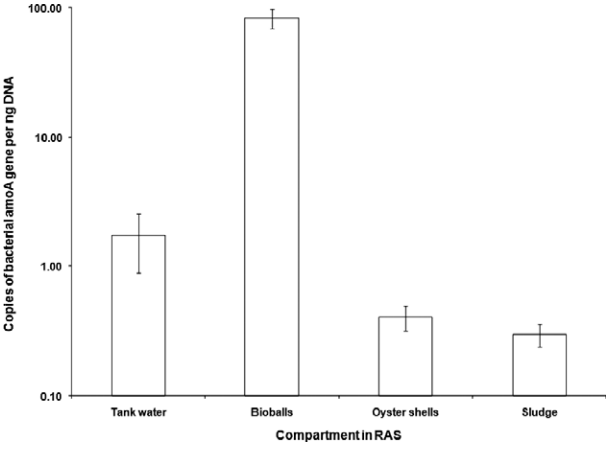

Fig. 3. Bacterial *amoA* gene abundance in four different RAS samples (Fig. S1), as measured by qPCR. Error bars show standard deviation.

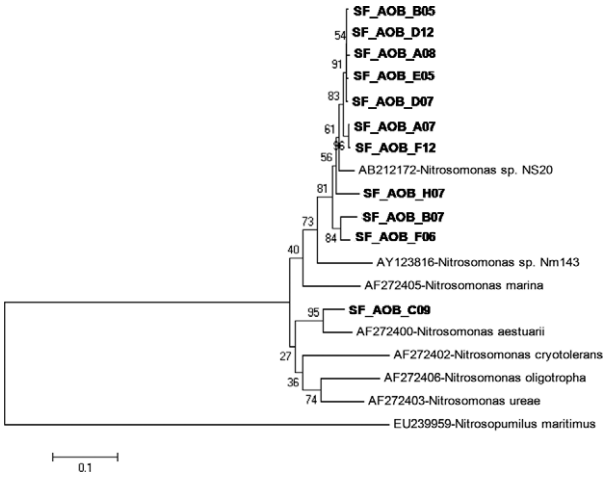

Fig. 4. Phylogenetic relationships obacterial *amoA* genes. Sequences obtained in this study are indicated with the prefix 'SF_'**.** The tree was created as described in the caption for Fig. 2.

The presence of a functional gene does not necessarily correlate to activity. However, the relatively low abundance of bacterial *amoA* genes in this marine RAS as compared to archaeal *amoA* genes suggests that AOB played a minor role in the function of the biofilter at the time of sampling.

NOB belonging to the phylum *Nitrospirae* were detected in the bacterial 16S rRNA gene clone libraries (Table S1) of the bioballs (14.3% of clones) and oyster shells (3.4% of clones). Phylogenetic analysis of these clones revealed that they belong to *Nitrospira* sublineage IV (*Nitrospira marina* sublineage) and sublineage II (*N. moscoviensis* sublineage) (Daims *et al.*, 2001) (Fig. 5). This is consistent with the results from other researchers who analysed the nitrifier community in freshwater aquarium (Hovanec *et al.*, 1998) and marine aquaculture (Foesel *et al.*, 2008; Keuter *et al.*, 2011) biofilters. A qPCR assay was used to quantify the abundance of NOB types in the RAS (Fig. 6). Both *Nitrospira marina* and

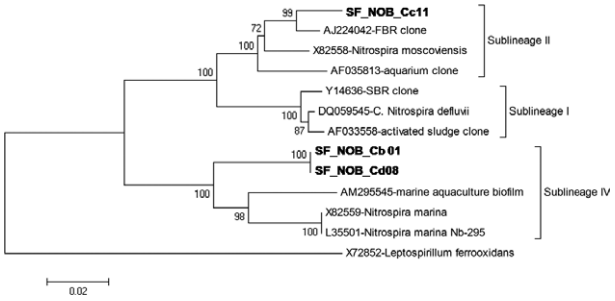

Fig. 5. Phylogenetic relationships of NOB 16S rRNA genes. The tree was created as described in the caption for Fig. 2.

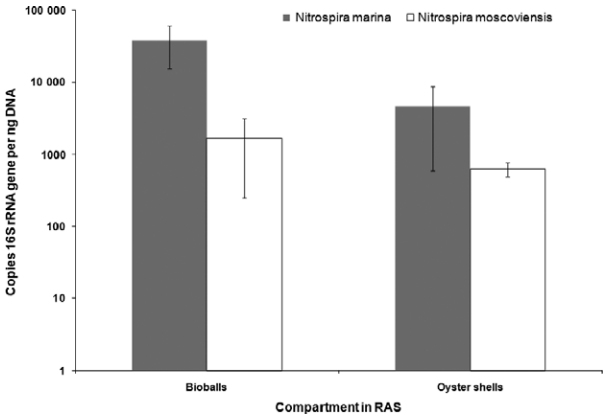

Fig. 6. NOB 16S rRNA gene abundance in two biofilter compartments (Fig. S1), as measured by qPCR. Error bars show standard deviation.

N. moscoviensis-like NOB were detected in the bioballs and oyster shell compartments of the biofilter. *Nitrospira marina*-like NOB ($3.8 \times 10^4 \pm 2.2 \times 10^4$ copies 16S rRNA gene ng^{-1} DNA) were an order of magnitude more abundant than *N. moscoviensis*-like NOB in the biomass obtained from the bioballs, while their abundances were similar in the oyster shell biomass.

The water quality and biofilm attachment media likely play important roles in determining the relative levels of AOA vs. AOB and *Nitrospira marina*- vs. *N. moscoviensis*-type NOB. Low ammonia production rates are expected in the maturation system because of the relatively low shrimp stocking density (6–9 shrimp m^{-2} of tank area). The water quality data collected for a sample obtained from the culture tank (0.25 mg L^{-1} ammonium-N, nitrite-N below detection, 25 mg L^{-1} nitrate-N, 6.5 mg L^{-1} DO, 26.3 practical salinity units and pH 8.8) indeed indicated that ammonium and nitrite oxidation in the biofilter were effective in maintaining low levels of ammonium and nitrite in the culture tank, suggesting that AOA and *Nitrospira marina*-like NOB were most competitive at low substrate concentrations. Specifically, *Nitrosopumilus*-type AOA were more abundant than *Nitrosomonas marina*-type AOB at the time of sampling. Others have shown that AOA have adapted to survive under ammonia limited conditions where AOB cannot survive (Martens-Habbena *et al.*, 2009). The abundance of AOA may also be due to mixotrophic or heterotrophic growth of AOA (Prosser & Nicol, 2008). Similarly, *Nitrospira* spp. was the dominant NOB at the time of sampling, which was likely due to the low nitrite concentrations in the biofilter. Schramm *et al.* (1999) proposed that *Nitrospira*-like bacteria are *K*-strategists that grow efficiently at low nitrite and oxygen concentrations. The results obtained by a study characterizing NOB

in a nitrifying sequencing batch biofilm reactor with a nitrite concentration gradient ranging from zero to 50 mg nitrite-N L^{-1} confirmed this K/r-hypothesis (Daims *et al.*, 2001). A recent study examining the nitrifying populations of two municipal wastewater treatment plants (Whang *et al.*, 2009) detected the presence of both *Nitrospira marina*-like and *N. moscoviensis*-like bacteria. In the current study, *Nitrospira marina*-like NOB were significantly more abundant than *N. moscoviensis*-like NOB in the bioballs ($P < 0.01$, two-sample *t*-test) and oyster shells ($P < 0.05$), which was likely due to the advantage that *Nitrospira marina* has in halophilic environments. *Nitrospira marina* is obligately halophilic (Watson *et al.*, 1986), whereas *N. moscoviensis* has no salt requirement (Ehrich *et al.*, 1995). In addition to salinity, the metabolic versatility of *Nitrospira marina*-like NOB compared to *N. moscoviensis*-like NOB may also impact the abundance. Specifically, the accessible supplies of both organic and inorganic sources of energy in the RAS favour the growth of mixotrophs such as *Nitrospira marina* (Watson *et al.*, 1986), but not of *N. moscoviensis* (Ehrich *et al.*, 1995). To date, studies have not compared sublineage II to sublineage IV nitrite-oxidizing nitrospiras in any environment.

The role of the biofilm attachment media in determining the competitiveness of different ammonium and nitrite-oxidizing populations needs to be studied further. It is tempting to speculate that crushed oyster shells provide an environment conducive for AOA competiveness, possibly due to the release of alkalinity (carbonates) or trace elements, or due to low DO zones caused by the close packing and aggregation of the oyster shells. The low DO zones combined with the low ammonium levels expected at the bottom of the filter may explain the abundance of AOA in the oyster shells.

Conclusions

The ammonium- and nitrite-oxidizing populations of a mixed media trickling filter consisting of compartments with plastic bioballs and crushed oyster shells, which was operated to treat water from a marine RAS culturing white shrimp in a maturation system, were dominated by *Nitrosopumilus*-like AOA and *Nitrospira marina*-like bacteria (*Nitrospira* sublineage IV). The AOA were particularly abundant in the biofilter compartment containing oyster shells. The water quality (i.e. low ammonium and nitrite concentrations) and biofilm attachment media played a role in the competitiveness of AOA over AOB and *Nitrospira marina*- over *N. moscoviensis*-type NOB, but additional work is needed to elucidate their function and importance in determining the outcome of the competition.

Acknowledgements

We thank Russell Allen for allowing us to sample his facility. Funding was provided by the Graham Environmental Sustainability Institute at the University of Michigan and the US National Science Foundation (project CBET 0967546). M.N.B. was supported by a US National Science Foundation Graduate Research Fellowship.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Simplified schematic of a zero-exchange RAS for marine shrimp production in Okemos, MI.
Fig. S2. Phylum level affiliations of archaeal 16S rRNA genes that were PCR amplified, cloned, and sequenced using samples obtained from the RAS system.
Table S1. Phylum level affiliations of bacterial 16S rRNA genes that were PCR amplified, cloned, and sequenced for different RAS sampling locations (Fig. S1).

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