Aquaculture 306 (2010) 177–184

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Biodiversity of N-cycle bacteria in nitrogen removing moving bed biofilters for freshwater recirculating aquaculture systems

Maartje A.H.J. van Kessel [a,b], Harry R. Harhangi [a], Katinka van de Pas-Schoonen [a], Jack van de Vossenberg [c], Gert Flik [b], Mike S.M. Jetten [a], Peter H.M. Klaren [b], Huub J.M. Op den Camp [a,*]

[a] Department of Microbiology, IWWR, Radboud University Nijmegen, Heyendaalseweg 135, NL-6525 AJ Nijmegen, the Netherlands
[b] Department of Organismal Animal Physiology, IWWR, Radboud University Nijmegen, Heyendaalseweg 135, NL-6525 AJ Nijmegen, the Netherlands
[c] KWR Watercycle Research Institute, Groningenhaven 7, NL-3433 PE Nieuwegein, The Netherlands

ARTICLE INFO

Article history:
Received 12 January 2010
Received in revised form 19 May 2010
Accepted 19 May 2010

Keywords:
N-cycle bacteria
Moving bed biofilm reactor
Freshwater aquaculture system
Nitrification
Denitrification
Anammox

ABSTRACT

Maintenance of optimal water quality and removal of nitrogen compounds pose challenges to aquaculture worldwide. Presence and activity of different bacteria involved in nitrogen cycling in the biofilm of a moving bed biofilm reactor (MBBR) connected to a Koi carp tank were investigated experimentally. For this MBBR system, a nitrogen removing rate of 3.5 g nitrogen per day was found in activity assays, whereas the predicted nitrogen removal rate was calculated to be 58 g nitrogen per day. The presence of ammonium-oxidizing and nitrite-oxidizing bacteria in the biofilm was demonstrated by situ hybridization (FISH) analysis. A large number of planctomycetes were shown to be present in the biofilm of the reactor, but they did not hybridize to oligonucleotide probes of known genera of anammox bacteria. Molecular 16S rRNA gene analysis of the MBBR biofilm revealed a novel group of planctomycete cells was present in this biofilter, which may explain the difference in experimentally found and calculated nitrogen removal of the system. The novel 16S rRNA gene sequences showed a low similarity to described anammox bacteria. This and other studies may implicate that the anammox reaction can be incorporated in biofilter systems for aquacultural freshwater systems, which makes it possible to remove nitrogen compounds from these systems in a relatively inexpensive and sustainable manner.

1. Introduction

In the Netherlands, fish aquaculture is carried out in closed, recirculating systems. A major problem in these systems is the maintenance of a constant and optimal water quality. Elimination of waste is generally managed through mechanical filtration to remove solids, and biofiltration for the conversion of toxic nitrogen metabolites into less toxic forms (Crab et al., 2007). The identity of the microorganisms responsible for this conversion of nitrogen metabolites is largely unknown (Tal et al., 2003). Given the low ammonium and nitrite concentrations in these systems, K-specialists (bacteria with high substrate affinity) such as *Nitrosospira* and *Nitrospira* may be likely candidates for aerobic ammonium and nitrite oxidation. Further, the recently discovered ammonium-oxidizing crenarchaea (Könneke et al., 2005) could play a role. Nitrate accumulation in aquaculture systems can be prevented by a daily replacement of a volume of pond water (van Rijn, 1996; Hargreaves, 1998), but this is expensive and inefficient. Furthermore, strict environmental legislation on nitrate levels in the discharge water (van Rijn, 1996) prompts the aquaculture industry to invest in more efficient nitrogen removal systems. This should result in the removal of nitrate in aquaculture waste water, for example by denitrification, by some considered to be the best candidate to remove nitrate from aquaculture systems. Denitrification is carried out by facultative anaerobic bacteria that utilize either organic (heterotrophic denitrification) or inorganic (autotrophic denitrification) compounds as electron sources to reduce nitrate to form nitrogen gas (N_2). Difficulties associated with the denitrification process, such as release of nitrous oxide as well as the requirement for an external electron source, have thus far prevented its application in full-scale commercial recirculation systems.

Another attractive option for completing the nitrogen cycle in aquaculture systems is the integration of the anaerobic ammonium-oxidation (anammox) process in these systems. As an important pathway of the nitrogen cycle, anammox allows ammonium to be oxidized by nitrite under anoxic conditions yielding molecular nitrogen. The importance of this process in the global nitrogen cycle is shown by the estimation that anammox bacteria are responsible for 30%–50% of the global present-day marine nitrogen loss (Brandes et al., 2007; Lam et al., 2009). The application of anammox is attractive for the complete nitrogen removal from aquaculture systems since it is economically advantageous; it requires at least 50% less oxygen compared with the conventional nitrification–denitrification process (Jetten et al., 2001). Furthermore, no additional (in)organic electron donor is required for this autotrophic nitrogen removal cycle in which ammonium is oxidized via hydrazine (N_2H_4) as an intermediate

* Corresponding author. Tel.: +31 24 3652657; fax: +31 24 3652830.
E-mail address: h.opdencamp@science.ru.nl (H.J.M. Op den Camp).

0044-8486/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2010.05.019

(Jetten et al., 2009). Since their discovery in the late 1990s anammox bacteria, which belong to the order of the *Brocadiales* and are affiliated to the *Planctomycetales* (Jetten et al., in press), have been found in waste water treatment plants but also in natural ecosystems. Examples of the latter ecosystems are: the anoxic water column of the Black Sea (Kuypers et al., 2003), the Costa Rica shoreline (Dalsgaard et al., 2003), oceanic oxygen-minimum zones (Kuypers et al., 2005; Lam et al 2009), several marine sediments and estuaries (Thamdrup and Dalsgaard, 2002; Trimmer et al., 2003; Schmid et al., 2007) and in fresh water lakes (Schubert et al., 2006).

Since anammox and other bacteria of the nitrogen cycle are found in marine ecosystems and in wastewater treatment plants, these bacteria are most likely also present in (freshwater) aquaculture systems. Evidence for the presence of anammox bacteria in these systems has been obtained in a moving bed bioreactor connected to a marine recirculating aquaculture system (Tal et al., 2006; Lahav et al., 2009). Anammox bacteria in these systems are probably simultaneously active with nitrifying bacteria, which indeed has been shown in both natural and manmade environments (Lam et al 2007, 2009; Trimmer et al., 2005). This would mean that in aquaculture systems ammonium-oxidizing bacteria (AOB) or ammonium-oxidizing archaea (AOA) convert ammonium, excreted by the fish, under oxic conditions into nitrite. These nitrite and additional ammonium are then used by anammox bacteria in anoxic parts of the filter to form nitrogen gas, removing both nitrite and ammonium from the system without the need for denitrification (van Rijn et al., 2006). Here, we investigated the presence and activity of several functional groups of N-cycle bacteria in the biofilm of moving bed biofilters coupled to Koi carp tanks. Moreover, we compared anammox, nitrification and denitrification activities in these systems.

2. Materials and methods

2.1. Fish and system configuration

Eighty Koi carp (*Cyprinus carpio Koi*) with an average weight of 4 ± 0.5 kg were kept in a 25 m^3 tank. Fish were fed 1200 g commercially available food (*Cyprico spirulina*) per day, containing 37% protein, 6% fat and 1.2% phosphorus. Food contained two additional nitrogen sources: lysine (2.3%) and methionine (0.8%). Food was rationed in 3 equal portions during the day (at 10.00 h, 14.00 h and 18.00 h). System temperature and pH were maintained at 21 °C and 7.3, respectively, and a photoperiod of 12 L:12D was used. No growth of algae was observed by visual inspection. Water quality of the system was maintained by a Nexus 300 biofilter (Evolution aqua, UK) and a weekly water replacement of 10% of the total volume. A flow rate of 16 $m^3 \cdot h^{-1}$ was set to enable two exchanges of tank water per hour through the biofilter. The Nexus 300 biofilter has two compartments filled with Kaldnes K1 rings (1 cm diameter; Evolution aqua, UK) with a surface to volume ratio of 500 $m^2 \cdot m^{-3}$. Water is first filtered mechanically over an immobilized Kaldnes ring bed (40 L) to remove solids from the water. After this mechanical filtration, water is biofiltrated by a moving bed biofilm reactor (MBBR) process. In this compartment of the Nexus 300 biofilter (100 L) the Kaldnes rings, on which a microbial biofilm is formed, move freely through the biotank by air agitation. Due to this turbulence, substrate transport to the biofilm is optimal and a thin biofilm is maintained, which is important for optimal substrate penetration (Rusten et al., 2006). Furthermore, no nutrient or oxygen gradient is formed over the biofilter compartment which occurs in other types of filters, for example in trickling filters. Average concentrations over the past 350 days were 0.2, 0.05 and 12 mg of $N \cdot L^{-1}$ for ammonium, nitrite and nitrate respectively. For details on analyses see Section 2.2. Additional samples were taken from two similar systems (10 and 15 m^3), which had the same fish density and food supply (3.75 g/kg fish/day). During 24-h periods, water samples were taken at 30 min intervals from 9.00 h until 21.00 h, and then at 8.00 h of the next day. Sampling included four locations: in the tank, at the inlet of the biofilter, after mechanical filtration and after biofiltration.

2.2. Batch activity tests

Kaldnes K1 rings from the Nexus 300 biosystem were transferred to sterile 250 ml serum bottles (200 rings per bottle), which were then sealed with butyl rubber stoppers and degassed with argon. Approximately 1 mM ammonium was initially present in the samples. After shaking overnight at room temperature, soluble substrates were added to the bottles from 100 mM anoxic stock solutions. To measure anammox activity, ammonium and nitrite were added to final concentrations of 2 mM and 2.5 mM, respectively (Kartal et al., 2006). During activity tests, nitrite concentration was monitored using nitrite test strips (Merck) and added to a final concentration of 1 mM when the nitrite concentration was below the detection limit of the test strip (<2 mg$\cdot L^{-1}$). Samples were taken at 24-h intervals. Total ammonium nitrogen (TAN: NH_3 and NH_4^+) was determined using the orthophtaldialdehyde assay (Kartal et al., 2006). Nitrite was measured by the sulfanilamide reaction (Kartal et al., 2006). Nitrate was measured as described before (Haaijer et al., 2006). Hydroxylamine and hydrazine concentrations were determined spectrophotometrically using dimethylamino-benzylaldehyde and 8-hydroxyquinoline (Strous et al., 1998). All assays were performed in triplicate and had a variability of $\leq 10\%$.

2.3. DNA extraction, PCR amplification and sequencing

Genomic DNA isolated from the Kaldnes biofilm of the 10 m^3 tank was used as a template for PCR amplification of the 16S rRNA gene using primers specific for bacteria, archaea and planctomycetes (see below). Microbial DNA was extracted from the biofilter (11 rings per sample) by phenol extraction and ethanol precipitation. For PCR amplification 20 µl of 1:100 and 1:1000 dilutions of DNA was mixed with 25 µl of PCR master mix (Perfecta SYBR green Fastmix for PCR iQ; Quanta Biosciences, USA) and 2.5 µl of each primer (20 µM stock) was added. The primer sets used targeted bacterial 16S (616F: 5′-AGAGTTTGATYMTGGCTCAG-3′, 630R: 5′-CAKAAAGGAGGTGATCC-3′; Juretschko et al., 1998), archaeal 16S (Arch21: 5′-TTCCGGTTGATCCYGCCGG-3′, Arch915R: 5′-GTGCTCCCCCGCCAATTCC-3′; Baker et al., 2003), planctomycete 16S (pla46F: 5′-GGATTAGGCATGCAAGTC-3′, 1390R: 5′-GACGGGCGGTGTGTACAA-3′; Neef et al., 1998; Zheng et al., 1996), anammox 16S (pla46F: 5′-GGATTAGGCATGCAAGTC-3′, amx820R: 5′- GGATTAGGCATGCAAGTC-3′; Neef et al., 1998; Schmid et al., 2000), bacterial *amo*A (F: 5′-GGGGTTTCTACTGGTGGT-3′, R: 5′-CCCCTCKGSAAAGCCTTCTTC-3′; Rotthauwe et al., 1997) and archaeal *amo*A (F: 5′-STAATGGTCTGGCTTAGACG-3′, R: 5′-GCGGCCATCCATCTGTATGT-3′; Francis et al., 2005). PCR reactions were performed in a Biometra Tgradient thermocycler (Westburg, Leusden, NL) and started by an initial denaturation at 96 °C for 2 min followed by a 30-cycle amplification (96 °C for 1 min, 55 °C or 57 °C for 1 min, 72 °C for 1 min and 40 s) and a final extension for 10 min at 72 °C. PCR products were examined for size and yield using a 0.8% (wt/vol; Eurogentec Molecular biology grade Lot 88019D) agarose gel in TAE buffer (20 mM Tris–HCl, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 8.0). To construct clone libraries PCR products were ligated and cloned using the pGEMT-easy (Promega, Leiden, NL) or Topo TA (Invitrogen, Breda, NL) cloning kits following the instructions of the manufacturer. Plasmid-DNA was isolated with the Flexiprep kit (Amersham Biosciences). The screened clones (16S rRNA genes and amoA) were sequenced by an ABI Prism 3700 DNA Analyzer in combination with a BIG Dye Terminator kit (Applied Biosystems). Sequences were checked for chimera formation using the Check Chimera program of the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Sequences were submitted to the NCBI Basic Local Alignment Search Tool (BLAST) and the Ribosomal Database Project (RDP) to determine percentage similarity with other 16S rRNA genes. Alignments of the sequences together with representative 16S rRNA gene