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Bacterial community analysis of marine recirculating aquaculture system bioreactors for complete nitrogen removal established from a commercial inoculum

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Abstract

An experimental recirculating aquaculture system was constructed under ambient seawater conditions to compare microbial community diversity of nitrifying and denitrifying biofilters that were derived from a commercial inoculum used for aquarium applications. Next generation sequencing revealed distinct and diverse microbial communities in samples analyzed from the commercial inoculant and the denitrification and nitrification biofilters. In all samples, communities were represented by a few dominant operational taxonomic units (OTUs). Bacteria having the capacity to carry out ammonia and nitrite oxidation were more abundant in the nitrification biofilter. Similarly, the proportion of the bacterial taxa known to carry out heterotrophic and autotrophic denitrification and participate in sulfur cycling were found in the denitrification bioreactor, and likely originated from the ambient environmental water source. Our

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Authors contributions

Marina Brailo: Conceptualization, Methodology, Writing - Original draft preparation.
Harold J. Schreier: Conceptualization, Methodology, Writing - Reviewing and Editing.
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Declarations of interest

None.

results indicated that environmental seawater can be a favorable enhancement to the bacterial consortium of recirculating aquaculture systems biofilters.

Keywords

Microbial community; Denitrification; Illumina MiSeq sequencing; Nitrification; Recirculating aquaculture system

1. Introduction

Development of environmentally sustainable farming of marine and freshwater species using recirculating aquaculture systems (RAS) requires a complete understanding of the biological components involved in water treatment. These components integrate biofilters comprised of microbial communities whose structure, dynamics, and activities are responsible for system efficiency. In comparison with other physicochemical factors that affect fish survival, such as dissolved oxygen, temperature, salinity, and pH, which can be easily monitored and controlled, RAS biofilters are more complicated because their performance critically relies on the interactions of microbial communities in dynamic environments (Ruan et al., 2015). Molecular tools not only have allowed for evaluating microbial diversity but also have contributed insight into their activities and interactions (Schreier et al., 2010). These include the application of 16S rRNA gene sequencing that provide a broad representation of biofilter communities in different RAS compartments. These analyses should yield information about spatial and temporal relationships within and between compartments and help identify critical contributors to a particular process or for evaluating the development of biofilter communities in start-up systems.

Better understanding of bacterial community structures is certain to provide novel RAS biofilter arrangements as well as insight into new processes and tools to enhance and monitor these systems. Characterization of the microbial communities in biofilters in RAS is of interest not only because it increases our understanding of the system's microbial ecology, but also because it may provide the basis for managing such communities (Kumar et al., 2013; Michaud et al., 2014; Rurangwa and Verdegem, 2015), enhancing their function and reliability (Huang et al., 2016).

The use of fixed film bioreactors is the most common choice for both nitrification and denitrification processes in RAS technology (Pedersen et al., 2015) Management of biological filters begins with the acclimation process, which must be inoculated with the appropriate nitrifying bacteria. Bacterial biomass and metabolism must then be elevated to levels required for removal of the ammonia produced by the cultured fish (Wheaton et al., 1994). Pond sediment or uncontaminated soils can serve as natural sources of the desired bacteria (DeLong and Losordo, 2012). Using water or active media from an already operating system will accelerate this process. Alternatively, stocking the system with low densities of small fish and providing reduced levels of feed will provide a limited ammonia concentration that will establish the filter slowly without harming the fish (Van Gorder, 2000). After the filter achieves full acclimation, the remainder of the fish can be added and feed levels increased (Van Gorder, 2000). The most commonly used, and rapid method of

biofilter pre-acclimation, is through seeding the biofilter with a commercial source of nitrifying bacteria followed by the addition of appropriate concentrations of ammonia and nitrite.

The aim of this study was to compare the composition of a commercial biofilter inoculant to microbial communities established on nitrification and denitrification RAS biofilters that were established using the inoculant and developed with influent from an ambient environmental marine source. This study revealed that in addition to the community provided by the commercial inoculum, a large proportion of bacteria that participate in nitrogen and sulfur cycling originated from environmental seawater.

2. Materials and methods

2.1. Marine recirculating aquaculture system design

A RAS of the University of Dubrovnik's Business and Innovation Centre for Mariculture, Croatia was used for this study and a schematic is shown in Fig. 1. The round conical bottom main tank (breeding pool) was composed of high density polyethylene with a diameter of 1.90 m, average height 1.02 m, and cone slope of 18°. Total tank volume was 2.89 m^3, although a volume of 1 m^3 was used throughout the experiment. Solid waste from the system was removed by a granular mechanical filter (PolyGeyser Bead Filter DF3; Aquaculture Systems Technologies LLC, Jefferson, Louisiana, USA). After filtration and UV sterilization (SMART High-Output UV Sterilizer, Emperor Aquatics, Inc., Pottstown, Pennsylvania, USA) seawater flowed to biofiltration components, which consisted of aerobic nitrification and anaerobic denitrification sections. For the nitrification process a rotating biological contactor was used (RBC 1400; Fresh Culture Systems, Breinigsville, Pennsylvania, USA). Anaerobic biological filtration was carried out in two cylindrical containers filled with mesh stockings that were used for mussel production as a medium for the settlement and growth of denitrification bacteria. System temperature was maintained at 24 °C by an air conditioner (Toshiba RAS-18SKH, Toshiba Corporation, Japan) located in the room.

The RAS was filled with local seawater with a salinity of 30 ppt, filtered through a 5 μm cartridge. Biofilters were inoculated using a commercially obtainable mixture of bacteria for aquaria and biological filtration processes were initially stimulated by the addition of nitrogen, and carbon to ensure the optimal environment for nitrification and denitrification (Brailo, 2016). Oxygen was provided by an air stream that was used to rotate the RBC and ammonia and carbon was provided by the daily addition of NH_4Cl and $NaHCO_3$ to the unit. The supply of nitrate and carbon was provided by the addition of$NaNO_3$ and wort, a raw material for beer production (Pivovara Medvedgrad d.o.o., Zagreb, Croatia), directly to the denitrification filters providing a COD/ N ratio of 2.5/1 (van van Rijn and Barak, 1998).

2.2. DNA Isolation, amplification, and sequencing

Bacterial samples were taken from both biofiltration compartments for DNA extraction three months after the biofiltration system was consistently removing nitrogen at a rate of 40 mgL^{-1} per day (Brailo, 2016). Sampling involved the scraping of material from each of the two

denitrification filters, which were combined and treated as one. For comparison, DNA was also extracted from the initial commercial inoculum. Samples were stored overnight at 4 °C, and DNA extraction was done using the DNA Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions, which included a lysozyme treatment at 37 °C for 30 min. Purified DNA was suspended in TE buffer (10 mM Tris-HCl, pH 8, and 0.1 mM EDTA) and stored at − 20 °C until further analysis. DNA concentration (mg/mL) and purity (A260/280) was measured using a Biophotometer (Eppendorf, Germany) and subsequent purification to remove any residual proteins was done using the Wizard® DNA Clean-Up System (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions.

The 16S metagenome sequencing libraries were prepared according to the manufacturer's instructions (Illumina, San Diego, CA). Briefly, the V3-V4 region of the 16S rRNA gene was amplified using the primer pair evaluated previously (Caporaso et al., 2010): forward primer, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and reverse primer, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′, using the 2×KAPA HiFi HotStart ReadyMix (Sigma-Aldrich, St. Louis, MO) with the following polymerase chain reaction (PCR) program parameters: initial denaturation step of 3 min. at 95 °C followed by 25 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, elongation for 30 s at 72 °C, followed by a final elongation for 5 min at 72 °*C. index* PCR was performed using the Nextera XT Index Kit according to the manufacturer's instructions (Illumina, San Diego, CA). PCR products were purified using AMPure XP beads (Beckman Coulter, Brea, CA), pooled and sequenced using the Illumina MiSeq platform (Illumina, Inc. San Diego, California, USA) (250 bp paired-end reads).

2.3. Community analysis

Raw reads were preprocessed using CLC workbench v8.0 (Qiagen). Reads were quality trimmed (qual. Limit =0.05; ambiguous nucleotide max. =2; min. Sequence length = 100 bp) prior to merging read pairs (mismatch cost = 2; gap cost = 3; max. Unaligned = 0; min. Score = 8). Community analysis was performed using QIIME (DeSantis et al., 2006). Reads were binned into operational taxonomic units (OTUs) at 97% identity using the open reference OTU picking method with the Greengenes (Greengenes 13_8) reference database (minimum OTU cluster size; $n = 2$). Evaluation of abundance of species richness in bacterial samples was carried out by Chao1 index calculated using the formula $S_p = S_o + (a^2/2b)$ where S_p is the estimated number of species, S_o is the observed number of species, a is the number of singleton species and b is the number of doubleton species (Chao, 1984). Species abundance was determined using Simpson's evenness index by the formula $E = 1/[\Sigma(n/N)^2 \times S]$ where E is the Simpson evenness index, n is the total number of individuals of a certain species, N is the total number of individuals of all species and S is the number of species (Simpson, 1949).

To better visualize trends in community composition across samples, OTU networks were generated in QIIME using the make_OTU_network.py script. To reduce the number of OTU

nodes in the final network, open reference OTU picking was repeated (as described above) with an increased minimum OTU cluster size (n = 10). The resulting network table was visualized in Cytoscape (v3.6) using an edge-weighted spring embedded layout (Shannon et al., 2003). Sequences used for these analyses were deposited in GenBank accessions nos. SAMN08624706 (commercial innoculum), SAMN08624707 (nitrification biofilter), and SAMN08624708 (denitrification biofilter).

2.4. Predictive functional profiling of microbial communities

Predictive functional profiling was performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013). All samples were normalized to the sample with lowest number of reads prior to OTU picking. OTUs were picked using the closed reference method against gg_13_8 (min. OTU cluster size = 2; OTU similarity = 0.94). The resulting BIOM table was normalized for 16S copy number prior to predicting functions for metagenomes and were visualized in Rstudio (v 0.98.1083) using the heatmap.2 function of the gplots package. For this analysis, KEGG orthologs (KO) were recorded. KOs were collapsed into pathways (L1–L3) using the *categorize _by_ function.py* script. OTUs that contributed particular functions were determined using the *metagenome_contributions.py* script. Analysis was limited to KEGG orthologs K10944 (*AmoA*), K03385 (*NrfA*), K00366 (*NirA*), and K00376 (*NosZ*), representing key enzymes in nitrification, nitrite reduction to ammonium, assimilatory nitrite reduction, and denitrification, respectively.

3. Results

OTUs identified in the commercial inoculant (CI), nitrification biofilter (RBC), and denitrification biofilter (DBR) and their abundance are shown in Table 1, with the highest observed OTU richness obtained in the nitrification reactor and lowest from the commercial inoculant. Values for Chao1 index were equal to the number of observed OTUs in all samples (Table 1), suggesting that the microbial community was well sampled. In addition, the Simpson's evenness indices were relatively low, with the highest value recorded in the CI, most likely reflecting the specificity of the biofilter environments for particular microbial lineages.

Bacterial community members identified in samples of CI, RBC, and DBR biofilters representing greater than or equal to 1% of the total reads are shown in Table 2 and Fig. 2. In general, communities from the CI and both biofilters were highly represented by a few dominant OTUs with the most abundant phylum being *Proteobacteria*, ranging from approx. 21% in the CI to 58% in the RBC and 78% in the DBRs. Nitrifying bacteria of the *Nitrospirae* phylum accounted for 17.9% in the CI and 3.1% in the RBC but were not identified in the DBR. The next important bacterial phylum was *Bacteroidetes* with nearly 14% in the commercial mixture, 21% in the RBC, and 9% in the denitrifying population. *Planctomycetes* were abundant in the CI and RBC samples, with little to none identified in the DBR denitrifying population. Similarly, representatives of the *Chlorobi* phylum, which were abundant in the commercial inoculum, were greatly reduced in the nitrifying and denitrifying communities. An abundant OTU that is classified in the nitrate-reducing

Caldithrix phylum was found in the CI sample but not detected in the biofilters. All samples contained, on average, 11 phyla that were present with <1% and some unassigned sequences.

The filtered seawater used to establish the RAS appeared to be a source for many of the major OTUs and bacterial lineages identified in the RBC and DBR (Fig. 2). Of the 1262 OTUs identified in all samples, only 42% originated from the CI. In addition, the RBC and DBR shared more OTUs (181) than either biofilter did with the CI (52 with RBC and 23 with DBR). The majority of CI OTUs did not flourish in the RAS as only approximately 23% were detectable in either or both biofilters at the time of sampling. While it is presumed that most of the OTUs not identified in the CI originate from the filtered seawater, we recognize that the RAS is an open system and subject to contaminating bacteria from the environment.

In many cases, bacterial members that were likely to play a role in nitrogen transformation activities were only found in the CI exclusively, CI and RBC, or DBR exclusively but not all three, as might be expected, since community structure is influenced by differences in biofilter environment. For instance, OTUs with high sequence similarity to the nitrite-oxidizing genus *Nitrospira*, which dominated the CI (at 17.7%), were not detected in communities of either biofilter. However, other *Nitrospiraceae* OTUs (3.1% in RBC), which were present in the CI at <1%, were enriched by the biofilter environment and could account for nitrite oxidation in this filter. The nitrate- and nitrite-reducing *Candidatus Solibacter* OTUs were not identified in the RAS even though they represented 1.5% of the CI. The genus *Planctomyces*, a group of bacteria ubiquitous in the marine environment was represented in all three samples, with the greatest abundance in the RBC (8.3%) compared to the CI (1.7%) and least in the DBRs (0.1%). Only the *Saprospiracaea* OTUs were identified in all three communities at frequencies >1%, with highest frequencies in the CI and RBC samples. Members of this family are important in the digestion of complex organic compounds and the utilization of wort likely contributed to their enrichment in the biofilters. Finally, there were several bacterial groups that were found in the RBC and/or DBR that were not identified in the CI, including *Pseudoaltermonadacaea*, and the genera *Vibrio* and *Arcobacter*, which was heavily enriched in the DBR (26.4, 16.9%, and 5.3% respectively) and are likely contributors to denitrification in this biofilter.

A predictive functional profile of the CI and each biofilter was determined based on their microbial community profiles, focusing on nitrogen cycling pathways (Fig. 3). Our analysis indicated that the type and number of pathways were not evenly distributed across the three microbial communities. While each community possessed functionally distinct, predicted profiles, hierarchical cluster analysis showed that the CI and RBC population were most similar. Unlike the DBR, both the RBC and CI were predicted to possess the full suite of genes necessary for nitrification. However, the predicted abundance of ammonia monooxygenase (*amoABC*) was much higher in the RBC, which is likely due to the enrichment conditions found in this biofilter. All three communities possessed membrane bound nitrate reductase (*nar*) in addition to other reductases necessary for denitrification and anaerobic nitrate respiration. In contrast to denitrification, only the DBR community appeared well-suited for dissimilatory nitrate reduction as it was predicted to have relatively

high abundance of all periplasmic (*nap*) nitrate reductase genes. All three communities possessed genes for assimilatory nitrate reduction through both NADH (*nasAB*) and ferrodoxin/ flavodoxin (*narB*/*nirA*) dependent pathways. However, within each of these pathways, the relative abundance of nitrate and nitrite reductases varied greatly. None of the communities appeared well-suited for anaerobic ammonia oxidation (anammox) as many essential genes were missing from the predicted metagenome, which is consistent with the absence of anammox *Planctomycetes* OTUs in the community analysis.

The type and number of microorganisms contributing to each of the major nitrogen cycling pathways was highly variable (Fig. 4). For this analysis, a representative gene was selected from each pathway and the relative contributions of major taxonomic groups was recorded. The removal of nitrogen from the system by denitrification was carried out by distinct communities in each of the reactors. For the RBC this process appears to be carried out almost exclusively by members of the families *Flavobacteriaceae* and *Saprospiraceae* (phylum *Bacteroidetes*). The potential denitrifying community in the DBR was more diverse and consisted of several families of *Bacteroidetes*, *Proteobacteria*, and *Chloroflexi*. Both *nosZ*-containing denitrifying communities differed from the initial starting inoculum, which was comprised primarily of members of the *Bacteroidetes* and *Chloroflexi*, as well as several uncharacterized species. Similar to denitrification, distinct communities in the RBC and DBR were responsible for assimilatory and dissimilatory nitrate reduction, which were predicted based on the presence of *nirA* and *nfrA* genes, respectively. In the RBC, these processes were carried out primarily by members of the *Planctomycetes*, while in the DBR these were *Proteobacteria*-mediated processes. In both the biofilters as well as the CI, *amoA*-directed nitrification was predicted to be carried out exclusively by members of the family *Nitrosomonadaceae.*

4. Discussion

A phylogenetic analysis of the nitrifying and denitrifying microbial populations of a simulated RAS was compared to the population of the starting commercial inoculum. Identical values were calculated for Chao1 and observed OTUs indicating that microbial communities were well-sampled and increased sequencing effort would unlikely significantly improve the observed biodiversity. The community in the RBC was richest in species and the CI community was the poorest. Differences in species richness of the three populations can be explained by the fact that the CI was added to the RAS, which had been filled with filtered seawater from the surrounding area. It is likely that additional bacterial species from the environment settled in compartments possessing favorable growth conditions. This is consistent with the finding that, regardless of start-up period, pioneer colonizing bacteria originating from the same body of water responded to their environment and were forced to occupy their particular ecologic niche (Michaud, 2007).

Simpson’s evenness indices for all samples were relatively low indicating the populations were strongly influenced by a few dominant species. This is supported by the fact that only approx. 17% of the OTUs were shared by all samples, which is similar to previous findings (Ruan et al., 2015). However, denitrifying and nitrifying bacteria coexisted in all biofilter samples, which agrees with findings of others (Okabe et al., 2002; Preena et al., 2017).

Proteobacteria and *Bacteroidetes* were most numerous in samples of the nitrifying and denitrifying biofilters, finding that agrees with those made by Ruan et al. (2015) and Huang et al. (2016) for marine RAS biofilters. Even though present in large amounts in the CI, the percentage of bacteria from the *Nitrospirae* phylum were significantly reduced in the RBC; the relatively low abundance of these bacteria in RAS biofilters has been reported previously (Ruan et al., 2015; Huang et al., 2016). The *Planctomycetes* were numerous in the CI and RBC and they have been found in different freshwater (Sugita et al., 2005; van Kessel et al., 2010) and marine (Tal et al., 2003; Michaud, 2007; Interdonato, 2012; Ruan et al., 2015; Huang et al., 2016) nitrification filters, marine RAS (Cytryn et al., 2003) and sludge of wastewater treatment plants (Shu et al., 2015).

The relative abundance of bacteria at the class level differed significantly among the samples in this system; the variation of the microbial composition is expected considering the functions of the starting inoculum and the two biofiltration units. Observed communities in the nitrification and denitrification reactors are consistent with the findings of Huang et al., (2016) for marine biofilters where *Alphaproteobacteria*, *Flavobacteria*, and *Gammaproteobacteria* comprised 35–75% of the observed OTUs and the *Nitrospira* comprised approximately 1–18%. Also, the prevalence of *Gammaproteobacteria* in denitrification biofilters is consistent with the finding of Michaud et al. (2014) who showed that an increased C/N ratio resulted in reduced bacterial diversity and selected for *Gammaproteobacteria*. The specialization of bacteria in the denitrification population compared to the CI and the RBC is also evident from the abundance of bacteria supporting this process, which was suggested by their predicted gene contributions (Figs. 3 and 4).

As expected, one of the most numerous taxons of the CI was the genus *Nitrospira*. Found in freshwater and marine aquaculture filters, these bacteria are responsible for oxidizing nitrite to nitrate (Hagopian and Riley, 1998; Schreier et al., 2010; Blancheton et al., 2013). As the genus *Nitrospira* was not found in the biological filters of the marine RAS, it is likely that those detected in the CI were freshwater species that were not capable of adapting to the increased salinity (30 ppt) of the system. It is noted, however, that the small proportion of undetermined *Nitrospiraceae* members identified in the CI that increased in the RBC were likely halotolerant species. The relatively low prevalence of these nitrifying bacteria in the RBC is consistent with findings for marine biofilters (Ruan et al., 2015; Huang et al., 2016). Conditions in the RBC were also favorable for proliferation of members of the family *Nitrosomonadaceae*, which is plausible considering their ammonia-oxidizing activity and the fact that they are regularly found in freshwater and marine aquaculture filters (Hagopian and Riley, 1998; Schreier et al., 2010; Ruan et al., 2015; Huang et al., 2016*). Nitrosomonads* increased from a very small occurrence of the CI to 11.4% of the RBC population and are likely the major ammonia oxidizing clade based on the predicted metagenomic analyses.

Bacteria of the *Phyllobacteriaceae* family and genera *Acinetobacter* and *Halorhodospira* were low abundance clades in the CI that significantly increased in the RBC. Representatives of the family *Phyllobacteriaceae* are a group of aerobic bacteria that include some rhizobia species capable of using various forms of nitrogen for growth (Mergaert and Swings, 2005) and have been identified in a marine filter (Michaud, 2007). Members of *Acinetobacter* genus have showed both heterotrophic nitrification and aerobic denitrification

ability (Yang et al., 2015). The anaerobic conditions of the DBRs were not suitable for these bacteria but the environment in the RBC promoted their growth. The abundance of nitrate and oxygen enabled aerobic denitrification, while the heterotrophic nitrification was stimulated by the presence of ammonia and compounds that probably originated from the seawater used for filling the RAS. *Acenitobacter* have also been identified from the Mediterranean (Cavallo et al., 2009), sea bass reared in northern Adriatic Sea (Čož-Rakovac et al., 2002), coke plant wastewater (Liu et al., 2015), freshwater (Itoi et al., 2007; Schneider et al., 2007; Schreier et al., 2010) and marine aquaculture systems (Michaud, 2007). Representatives of the genus *Halorhodospira* perform oxidation of sulfides (Imhoff and Süling, 1996) and their relatives have been found in the wastewater treatment systems (Shu et al., 2015) and in marine RAS (Interdonato, 2012).

Members of the order *Flavobacteriales* were a minor group in the CI that were enriched in both biofiltration compartments. Some of these bacteria are strictly aerobic organisms and others are facultative anaerobic chemoorganotrophs. Most *Flavobacteriales* grow on substrates with organic compounds as carbon and nitrogen source, while some use inorganic nitrogen, and many require NaCl or sea water for growth (Bernardet, 2011). It is conceivable that the RBC dominating *Flavobacteriales* were aerobic bacteria that use inorganic nitrogen, while the ones inhabiting denitrification reactors were anaerobes able to use organic compounds from the wort. This group of bacteria has been found in aeration basin (Preut, 2014) and anaerobic sludge (Shu et al., 2015) of different full-scale wastewater treatment plants as well as the Eastern Mediterranean water column (Techtmann et al., 2015).

It was observed that the percentage of minor groups of bacteria identified in the CI increased in the DBR, including bacteria of the *Hyphomicrobiaceae* family. These bacteria reduce nitrate under anaerobic conditions (Garrity et al., 2005) and have been found in a marine methanol-fed denitrification reactor (Labbe et al., 2003). In addition to heterotopic denitrification bacteria, representatives of bacteria were found that are capable of performing autotrophic (sulfide-dependent) denitrification or sulfide oxidation by nitrate. The most numerous group was the family *Rhodobacteraceae*, which have been identified in marine recirculation systems (Cytryn et al., 2005a, 2005b; Michaud, 2007; Schreier et al., 2010; Blancheton et al., 2013). Furthermore, bacteria of the order *Bacteroidales* were found as well, which are known to be present in marine nitrification aquaculture filters (Michaud, 2007; Interdonato, 2012), marine denitrification aquaculture filters (Cytryn et al., 2005a), and a sludge digestion basin of a zero-discharge mariculture system where sulfate reduction takes place (Cytryn et al., 2003).

The DBRs were populated by bacteria that were not detected in the CI suggesting that they originated from the environmental seawater used for filling the RAS. The most important of these were members of the *Gammaproteobacteria*, and especially order *Vibrionales*, which are common to marine and estuarine environments and the microbiome of marine animals, and *Vibrio* spp. were found to make up a significant proportion of the DBR population (16.8%). These bacteria have been found in both freshwater (Sugita et al., 1981) and marine filters (Diaz et al., 2012) as well as in sea bass reared in the northern Adriatic Sea (Čož-Rakovac et al., 2002) and they have been suggested to play a significant role in denitrification in marine RAS (Michaud, 2007; Interdonato, 2012). Bacteria of the order

Marinicellales identified in the DBR population have been isolated from the marine environment and have aerobic and chemoorganoheterotrophic metabolism (Romanenko et al., 2010). They have been found in an aeration basin of full-scale wastewater treatment plant (Preut, 2014) and biofilters of marine RAS (Ruan et al., 2015). Similarily, the DBR denitrification community included bacteria of the family *Colwelliaceae* and *Thalassospira* that were not represented in the CI. *Colwelliaceae* include marine aerobic and facultative anaerobic marine members that reduce nitrate to nitrite (Ivanova et al., 2004) and have been shown to inhabit industrial saline wastewater (Yoshie et al., 2004; Yoshie et al., 2006) and marine RAS denitrification systems (Interdonato, 2012; Martins et al., 2013). *Thalassospira* include members possessing aerobic or facultative anaerobic metabolism that use nitrate as electron acceptor (Wiese et al., 2009) and have been identified in denitrification bioreactor for saline water with poly butylene succinate as carbon source (Luo et al., 2013) and in the Mediterranean Sea (Hütz et al., 2011; Cortés-Lara et al., 2015). Finally, in addition to bacteria involved in nitrogen cycling, bacteria capable of sulfur metabolism were identified in the DBRs, including a representative of the genus *Arcobacter*, which oxidizes sulfide to sulfur (Vandamme et al., 2005). *Arcobacter* has been found in both freshwater (Schneider et al., 2007) and marine RAS (Welsh et al., 2011).

Potential metabolic capacities of each of the microbial communities was examined using a predictive metagenomics analysis. Based on the analysis, it appeared that most of the genes for the major nitrogen cycling pathways were present in each the communities. Except for those involved in nitrification, the number and types of microorganisms possessing these genes varied greatly between the CI and the two biofilters, which is consistent with large differences in overall microbial biodiversity. The removal of nitrogen via denitrification in the DBR appeared to be carried out by a mixed community of *Proteobacteria*, *Bacteroidetes*, and *Chloroflexi*, which are found in high abundance in this and other marine recirculating aquaculture systems (Cytryn et al., 2005b).

It should be noted, that the method of metagenonic analysis used here is only predictive and does not provide any information about gene expression or metabolic activity. Indeed, based on the large differences in community composition and environmental factors (available nitrogen/carbon, oxygen, etc.) in each of the biofilters and inoculum, we would expect to see large differences in gene expression and enzymatic activity. In addition, the quality of this analysis can be limited by the available sequences in the reference database as well as the quality of the genome annotations. In well sampled environments such as the human body and aquaculture systems the quality and accuracy of these analyses tends to be higher than rare or lesser sampled environmental communities.

5. Conclusions

Phylogenetic analysis of microbial populations in a recirculating aquaculture system by next generation sequencing showed that there was a significant number of bacteria that oxidize ammonia and nitrite in the nitrification biofilter, while in denitrification bioreactors there was a large proportion of bacteria that carry out heterotrophic and autotrophic denitrification, as well as those that participate in sulfur cycling. Comparing the stable microbial populations established on the biofilters for complete nitrogen removal with the

composition of the culture contained in the commercial inoculum, it can be concluded that environmental seawater used for filling the RAS served as an additional bacterial source, especially in denitrification reactors. Considering their role in nitrogen and sulfur cycling, this was a favorable enhancement to the commercial inoculum.

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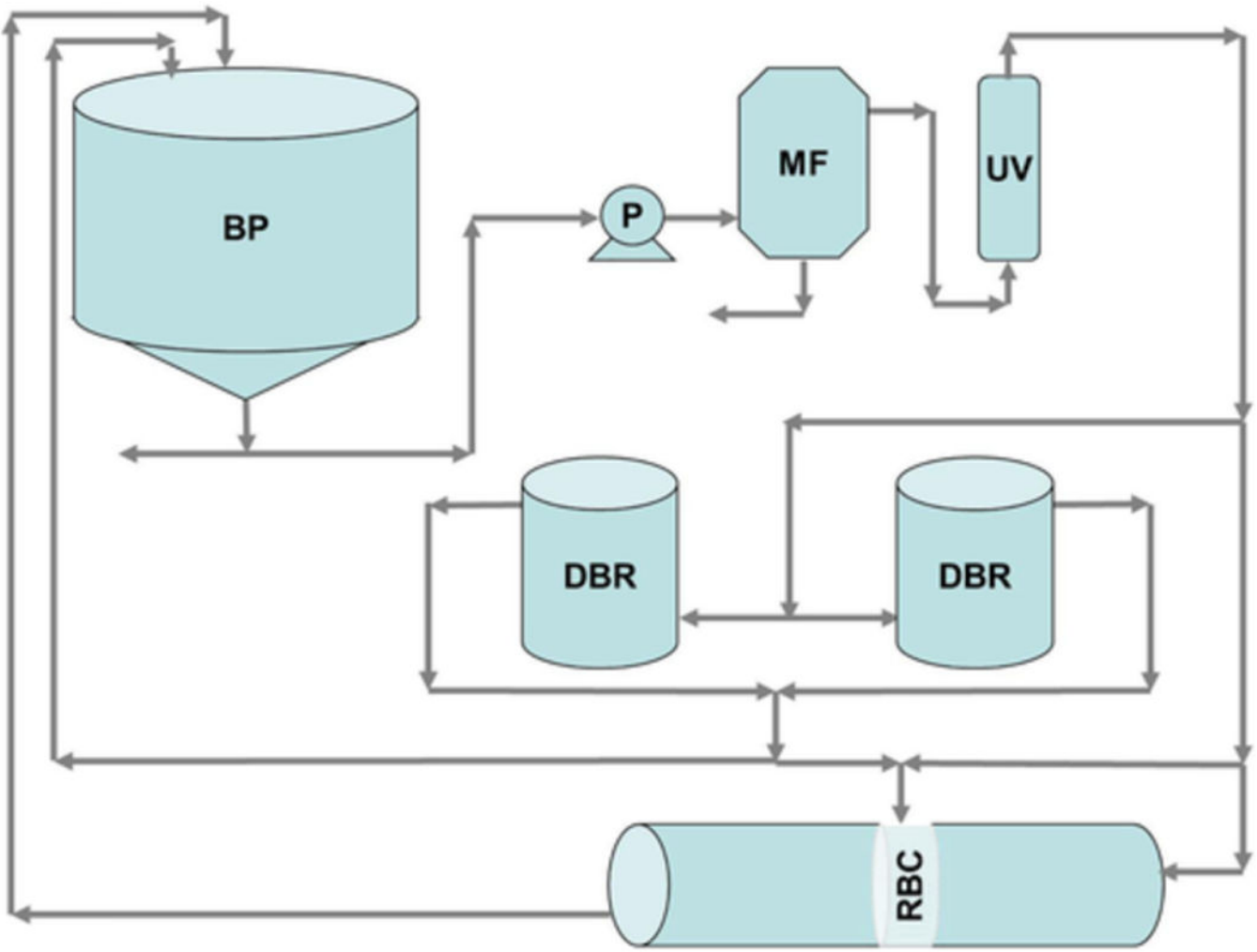

Figure 1.

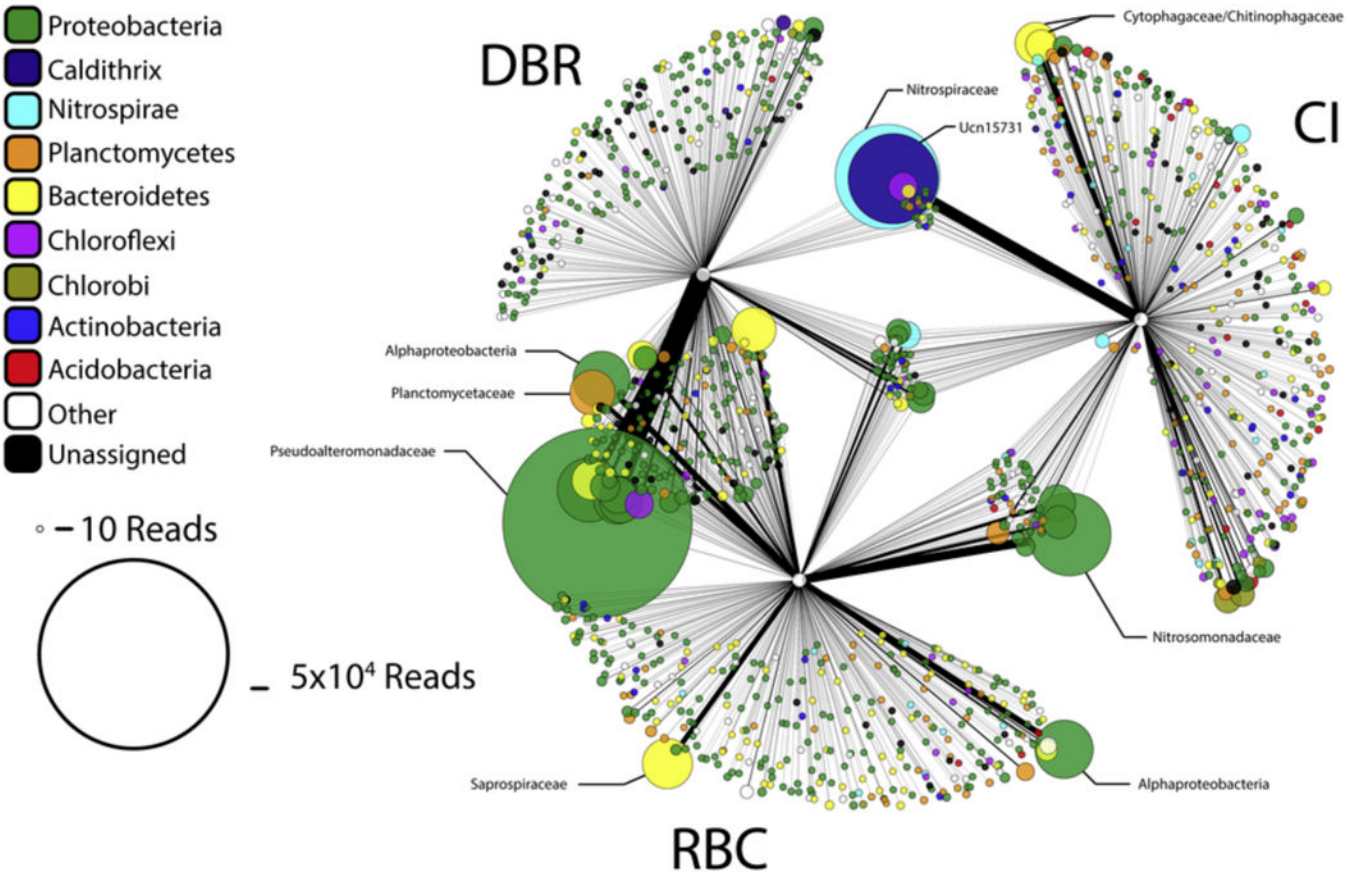

Figure 2.

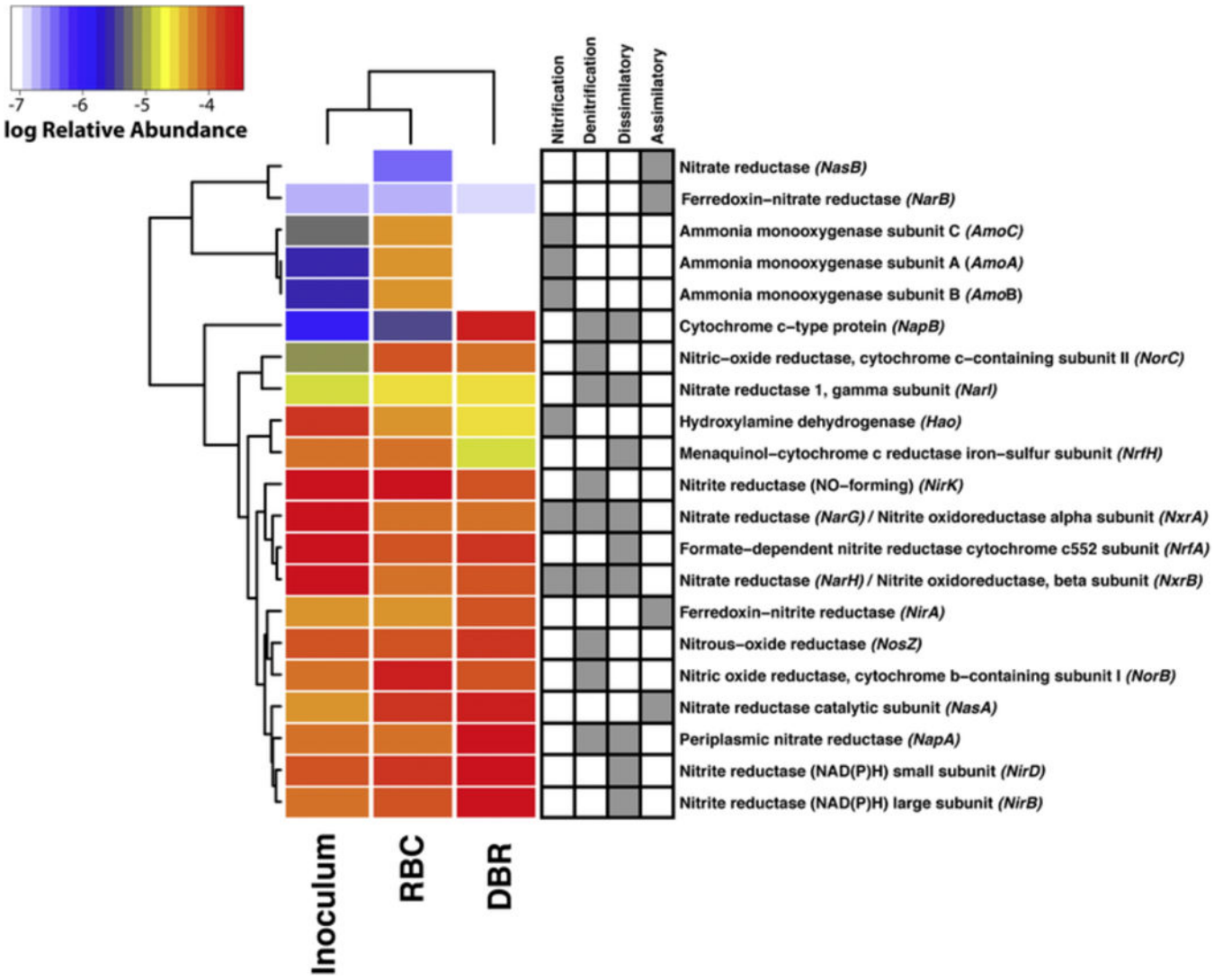

Figure 3.

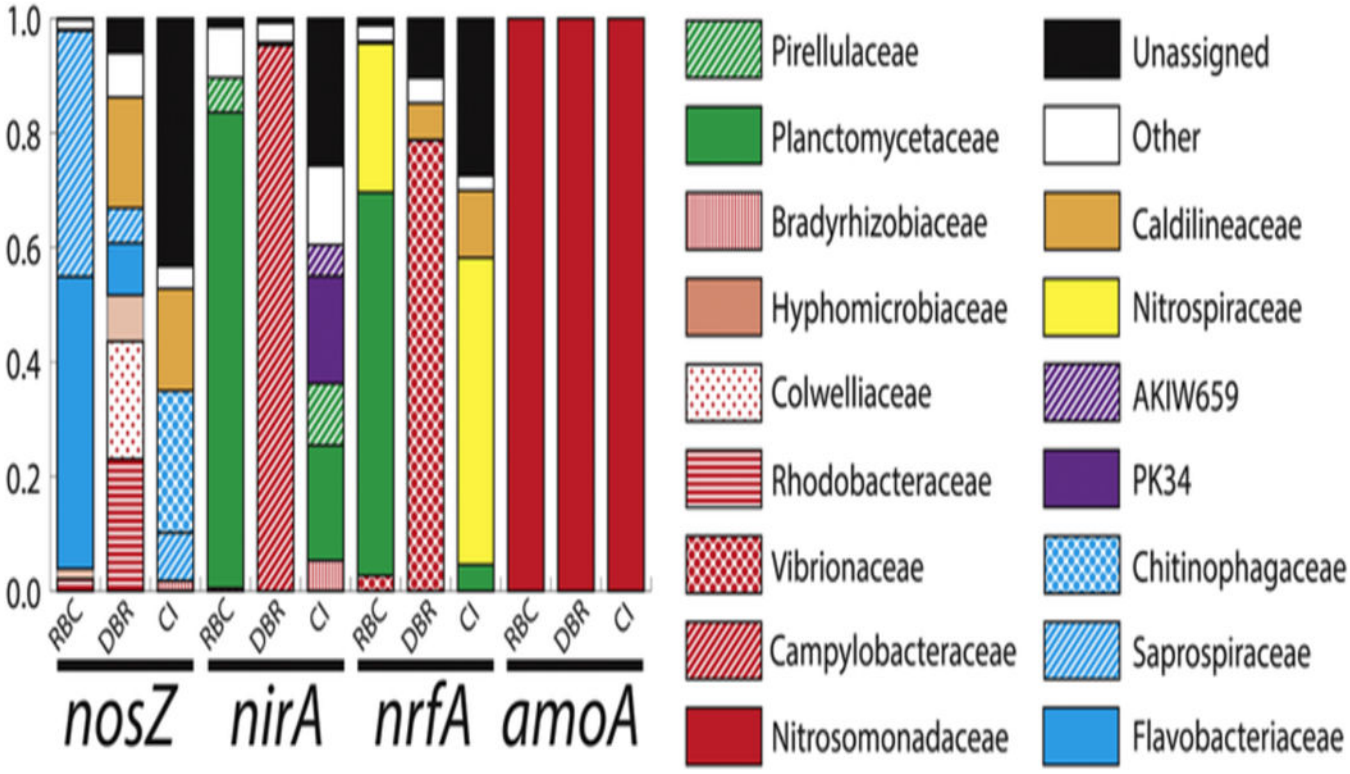

Figure 4.

Table 1

Sequence analysis parameters. See Methods for details.

Sample	Read count	Observed OTUs	Chao1	Simpson's evenness index
CI	4.2×10^5	467	467	0.048
RBC	6.5×10^5	688	688	0.036
DBR	7.4×10^5	533	533	0.021

Table 2

Proportions of bacterial community members found in samples of the commercial inoculum (CI), nitrification reactor (RBC) and denitrification reactors (DBR); NI, not identified. Entries in Family and Genus columns represent individual OTUs that account for >1% of total reads for at least one sample.

Phylum	Family	Genus	Sample proportion (%)		
Class			CI	RBC	DBR
Order					
Acidobacteria	*Solibacteraceae*	*Candidatus*	1.5	NI	NI
Solibacteres Solibacterales		*Solibacter*			
Bacteroidetes	*Saprospiraceae*		3.0	9.1	1.1
Bacteroidia					
Bacteroidales					
Cytophagia	*Cytophagaceae*		4.4	<1	NI
Cytophagales					
Flavobacteriia			<1	<1	4.8
Flavobacteriales	*Flavobacteriaceae*		<1	11.1	1.4
Sphingobacteriia			<1	<1	<1
Sphingobacteriales					
Bacteroidetes	*Chitinophagaceae*		5.5	0.3	NI
Chitinophagia					
Chitonophagales					
Chlamydiae			1.2	<1	NI
Chlamydiia					
Chlamydiales	*Parachlamydiaceae*		<1	1.5	
Caldithrix	*BA059*		NI	NI	1.1
Caldithrixae					
Caldithrixales					
Chlorobi			7.4	NI	NI
SJA-28					
Chloroflexi	*Caldilineaceae*		4.7	<1	3.1
Anaerolineae			<1	1.5	NI

Phylum	Family	Genus	Sample proportion (%)		
Class			**CI**	**RBC**	**DBR**
Order					
Caldilineales					
Gemmatimonadetes					
Gemm-1					
Nitrospirae	*Nitrospiraceae*		<1	3.1	NI
Nitrospira	*Nitrospiraceae*	*Nitrospira*	17.7	NI	NI
Nitrospirales					
Planctomycetes			2.7	NI	NI
OM190					
CL500–15					
Planctomycetia			1.4	0.1	NI
B97					
Pirellulales	*Pirellulaceae*		2.1	2.3	<1
Planctomycetales	*Planctomycetaceae*	*Planctomyces*	1.7	8.3	<1
Proteobacteria			2.8	18.8	<1
Alphaproteobacteria					
Kiloniellales			<1	4.8	NI
	Kiloniellaceae	*Thalassospira*	NI	NI	2.0
Rhizobiales			2.8	<1	<1
	Hyphomicrobiaceae		<1	1.3	1.3
	Phyllobacteriaceae		<1	2.8	<1
			<1	2.4	<1
Rhodobacterales	*Hyphomonadaceae*		2.0	<1	<1
		Hyphomonas	NI	1.6	NI
	Rhodobacteraceae		<1	1.2	7.4
Betaproteobacteria	*Nitrosomonadaceae*		<1	11.4	<1
Nitrosomonadales					
Epsilonproteobacteria	*Campylobacteraceae*	*Arcobacter*	NI	<1	5.3
Campylobacterales					

Phylum	Family	Genus	Sample proportion (%)		
Class			CI	RBC	DBR
Order					
Gammaproteobacteria			<1	<1	<1
	Colwelliaceae		NI	<1	3.2
Alteromonadales	*Pseudoalteromonadaceae*		NI	<1	7.2
			NI	<1	26.4
Vibrionales	*Vibrionaceae*	*Vibrio*	NI	<1	16.9
Chromatiales	*Ectothiorhodospiraceae*	*Halorhodospira*	<1	3.8	NI
Legionellales			5.4	<1	<1
Pseudomonadales	*Moraxellaceae*	*Acinetobacter*	<1	1.3	<1
[Marinicellales]	*[Marinicellaceae]*	*Marinicella*	NI	0.2	4.5
[Caldithrix]			11.8	NI	NI
Ucn15732					