

CULTIVATING ECOSYSTEMS: MICROBIAL COMMUNITIES IN RECIRCULATING
AQUACULTURE SYSTEMS

by

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ABSTRACT

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Under the Supervision of Professor Ryan J. Newton

Intensive cultivation of fish is necessary to meet future global market demands. Recirculating aquaculture systems (RAS) enable dense growth of fish, while occupying less space than traditional aquaculture farms. However, RAS often experience complications and high fish mortalities due to disease and improper waste management. In properly functioning systems, the microorganisms associated with fish (gut, scales) as well as those found in the system environment (water, component surfaces) remove waste and maintain fish health by discouraging growth of opportunistic pathogens. Previous RAS microbiome studies are small in scope, utilize coarse methods, and contain limited long-term spatial or temporal data. With advances in computation, microbial ecology, and RAS technology it is possible to test the relationship between RAS operational management practices and microbial community composition. Using the RAS at the UW-Milwaukee School of Freshwater Sciences, I used massively parallel DNA sequencing platforms, cutting-edge fluorescent microscopy, and classical molecular and microbiological methods to rigorously examine microbial community structures. Results from this dissertation advance our knowledge of aquaculture by analyzing RAS microbiota throughout the system over time; evaluate waste removal function, and track system condition correlations to pathogen blooms. These analyses will provide insight as to how environmental changes during rearing cycles affect system function and fish health. To

investigate the connection of waste componentry failure to pathogen blooms, this dissertation uses *Flavobacterium columnare* as a model organism, since *F. columnare* infects fish across a myriad of freshwater systems. Genome sequencing of pathogenic *F. columnare* strains gives insight into the metabolic connections between fish waste and persistence of opportunistic pathogens. By better understanding the role of the microbiome in RAS, we can improve fish health, optimize waste removal, and increase yields and profits for aquaculturalists.

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I would like to dedicate this work to the memory of Robert Biersack, as well as to my parents, family, friends, and partner Caroline. Without their love and support this work would not have been possible.

1 Introduction

1.1 Background

The original practice of aquaculture dates back approximately 4000 years ago in ancient China, where the first text was written about pond-based cultivation of Koi (*Cyprinus carpio*) (Rabanal, 1988). The practice of cultivating aquatic species in ponds remained largely unchanged until the development of flow-through and raceway cultivation methods. All of these cultivation methods for aquatic species are subject to large external costs (i.e. water use, system footprint). These systems are also susceptible to environmental perturbations similar to those found in ponds or lakes, for example diel oxygen dynamics (Timmons and Ebeling, 2013). In the 1970's, indoor high-density cultivation of aquatic species was explored using recirculating aquacultural system (RAS) technology (Timmons and Ebeling, 2013). These systems borrowed technology from the wastewater and drinking water treatment industries, to allow high-density cultivation of fish or shellfish with minimal water use. Contemporaneously, experiments were conducted using supplementary plants to scavenge toxic nitrogenous compounds from these systems (Lewis et al., 1978; Naegel, 1977; Sneed et al., 1975). These hybrid aquaculture-hydroponic systems are often called aquaponic systems, a portmanteau of aquaculture and hydroponics. In the 21st Century, we are only beginning to explore the potential of RAS or aquaponic systems.

1.2 Literature Review

Increased research and development in integrated aquaculture may help meet global fisheries demand, while reducing commercial overfishing (Diana et al., 2013). However, the United States lags behind the world in aquacultural technologies and production. In

2013 the United States imported edible fisheries products at a \$12.42 billion deficit, even though overall fisheries productivity in the US increased (NOAA, 2013). Only 11.6% of United States aquaculture farms that participated in the 2013 USDA Agricultural Census were utilizing recirculating aquaculture, while the majority of aquaculture products were from pond and raceway systems (Vilsack and Reilly, 2013). The design and componentry required by recirculating aquaculture systems (RAS) relies on a consortium of microorganisms and mechanical devices to process the fish waste. The various parts of the system allow for minimal wastewater discharge from RAS, while reducing environmental variance (Tucker and Hargreaves, 2008). In 2050 it is projected that aquaculture will service the majority of product demand (Diana et al., 2013), therefore research to better facilitate RAS implementation should play a vital roll in this.

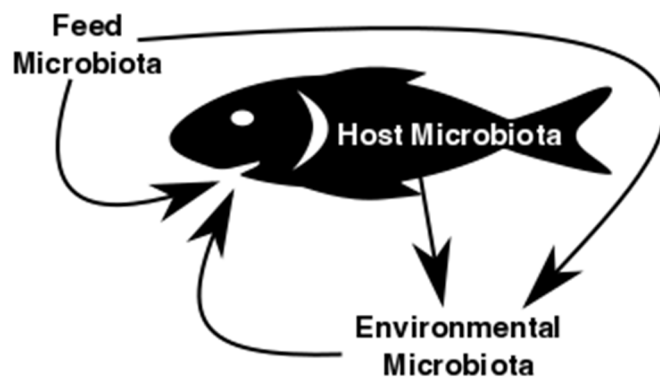


Figure 1.1 Diagram of Fish Interaction with RAS Microbiota (Modified from De Schryver and Vadstein, 2014)

Despite the farmed fish frequently interacting with three classes of microbiota (Figure 1.1), applied microbial ecology is underutilized in RAS (De Schryver and Vadstein, 2014). Whole bacterial and archaeal community analyses of aquaculture systems are lacking, but the application of these studies to aquaculture management are broad (De Schryver and Vadstein, 2014). Gut microbiome research is a steadily growing field of research in aquaculture. For example, the gut microbiomes of traditionally

cultivated Rainbow Trout were surveyed using fluorescence in situ hybridization and 16S Sanger sequences (Huber et al., 2004). While in another study, wild Stickleback gut microbiomes were sequenced from ten sites near Vancouver, British Columbia, but the study made little mention of taxonomic assignments or the relative abundances of taxa (Smith et al., 2015). Recently it was demonstrated that probiotic manipulation of Atlantic Salmon (*Salmo salar*) microbiomes had a positive effect on host health after prophylactic antibiotic exposure. In another *S. salar* study, the results suggest a fish meal free diet correlated to changes in gut microbiome composition (Schmidt et al., 2016). While in that same study, the feed change had no effect on the nitrifying microorganisms inside the RAS's biological filters (biofilters).

Nitrogen inputs into RAS consist of undigested feed and the catabolic byproducts of fish protein metabolism (Timmons and Ebeling, 2013). This is quite different than the nitrogen cycle redox observed in lakes and oceans, where nitrogen is input from the atmospheric nitrogen gas fixation (Wetzel, 1983). Commonly, nitrogen budgets in RAS are calculated relative to feed rate and protein content (Timmons and Ebeling, 2013). This is often modeled using the following equation where production of total ammonia nitrogen is relative to: the feeding rate (F) in kg fish feed/day, the protein concentration of feed (PC), and t is the time period of ammonia production in days (Timmons and Ebeling, 2013). The equation for estimating TAN production is as follows (Timmons and Ebeling, 2013):

$$\text{Production of TAN} = \frac{F \times PC \times 0.092}{t}$$

The constant 0.092 is derived from protein catalysis by the fish reared. Within the constant calculation, the authors cited 90% of the byproducts from fish protein catabolism are ammonia (Timmons and Ebeling, 2013). Since ammonia is an inherently

toxic compound to fish, nitrifying biofiltration is critical to the success of RAS (Timmons and Ebeling, 2013). Ammonia oxidation is considered the rate limiting step of nitrification in the environment and biofiltration (Kowalchuk and Stephen, 2001). Currently, aquacultural nitrification rates are based on easily cultivated organisms, with *Nitrosomonas spp.* performing ammonia oxidation, and *Nitrobacter spp.* carrying out nitrite oxidation (Ebeling et al., 2006; Timmons and Ebeling, 2013). The estimation of ammonia production and assumption of rates based upon easily cultured microorganisms may lead to inefficient process design, since non-canonical nitrifiers persist in intensive aquaculture and aquaria (Bagchi et al., 2014; Brown et al., 2013; Kruse et al., 2013; Sauder et al., 2011).

The mitigation of pathogens is another challenge faced by aquaculture system managers and operators. Various bacteria, viruses, fungi, and parasites may have detrimental health consequences for fish in RAS (AFS-FHS, 2014). It is beyond the scope of any dissertation to broadly focus on pathogens in aquaculture, therefore *Flavobacterium columnare* will be used as a model opportunistic pathogen, since *F. columnare* infects most freshwater fish (Lafrentz et al., 2012). Suboptimal rearing conditions, such as low dissolved oxygen, high ammonia levels, elevated nitrite levels, or overstocking are often associated with outbreaks of *F. columnare* in aquaculture (Lafrentz et al., 2012). Little is known about the ecological niche of *F. columnare*, though it has been shown to cause yearly die-offs in wild fish (McBride, 2014). Currently there are limited molecular methods to detect *F. columnare* before lesions become visible on the tank stock (Panangala et al., 2007). With few published genomes, many incidences of infection around the world, and little insight into the ecological role of *F. columnare*, this organism was used as a model for both developing detection methodologies and

applying microbial ecology to RAS (McBride, 2014; De Schryver and Vadstein, 2014; Tekedar et al., 2012).

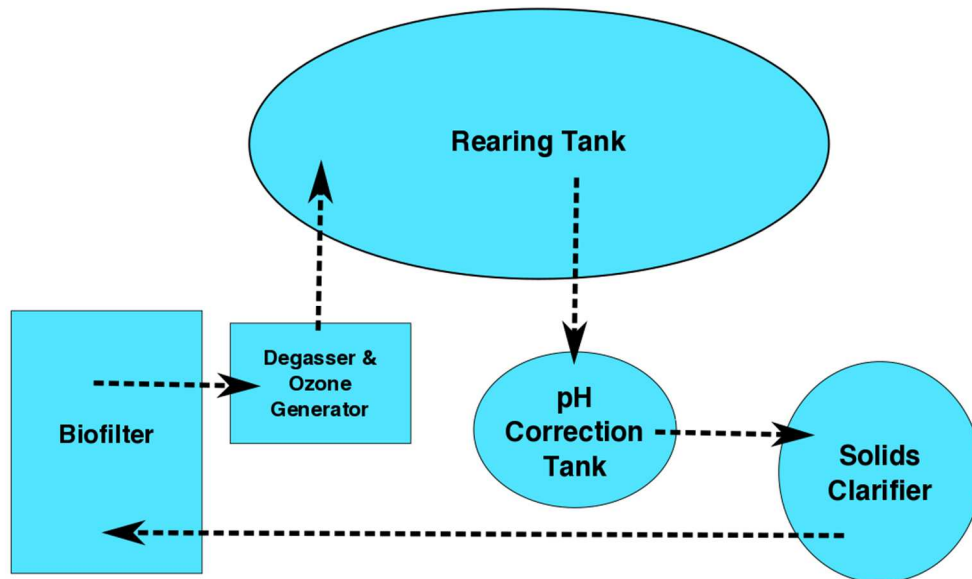


Figure 1.2 Illustration of UWM RAS Layout and Flow Path

This dissertation utilizes the UWM School of Freshwater Sciences RAS as a model system (Figure 1.2). RAS consist of a rearing tank coupled to devices designed to maintain optimal water conditions. Buffer tanks may be used to maintain optimum system pH and amend nutrient deficiencies in multi-trophic systems. Waste management components that remediate solids and nitrogenous waste are the most critical and misunderstood devices of RAS (Badiola et al., 2012; Martins et al., 2010). Solid wastes are composed of undigested feed and feces, which, results in suboptimal water chemistry when collected outside specific componentry. The UWM SFS RAS uses a modified bead filter for solid waste capture and settling, these require frequent backwashing to remove the build up of solids (Pfeiffer and Malone, 2006). Large scale commercial systems, such as those found at Bell Aquaculture (IN, USA), or the Cold Water Institute (WV, USA), utilize drum filters and settling tanks appropriated from wastewater treatment technology. Many systems utilizing solids drum filtration often lack redundant componentry, and

often when drum filters fail, the resulting low water quality becomes difficult to manage leading to large crop losses and increased incidence of disease (Bell Aquaculture, *Personal Communication*). Placement of the solid waste clarifier before the biofilter reduces influent total carbon loads, and should limit unchecked heterotrophic competition within the biofilm microbial consortia (Michaud et al., 2006, 2013).

Biofilter technology is broadly applicable to water treatment across all scales of aquaculture, whether the systems are monoculture, or multi-trophic. Aquaneering Technologies manufactured the UWM SFS RAS biofilter, and in 1999 the system went online. The biofilter vessel is 108 inches tall, with a diameter of 72 inches. The biofilter sand matrix has a height of approximately 68 inches, and is comprised of hydraulically fluidized Wedron 510 silica sand.

Before biofilter effluent returns to the rearing tank, and after re-oxygenating the water, aquacultural engineers employ devices to remediate populations of microbes and dissolved organic matter (DOM). Two common methods of microbial and DOM remediation are, ozone generation and UV irradiation. Ozone is very chemically unstable, which presents difficulties when engineers attempt to design ozone generators applicable to both marine and freshwater systems (Gonçalves and Gagnon, 2011). In most ozone systems, oxidation-reduction potential (ORP) measurements are used to regulate ozone generation, however in seawater, residual ORP stays in the system at potentially dangerous levels ($>500\text{mV}$) (Gonçalves and Gagnon, 2011; Tango and Gagnon, 2003). More importantly, proper ozonation requires *a priori* knowledge about the microbial community to optimize output abundance of microbes and viruses (Summerfelt, 2003; Timmons and Ebeling, 2013). The alternative, UV irradiation has both high initial and continuous operating costs. Additionally UV components have a large impact on the total

microbial community, including the reduction of potential probiotic organisms from the system (Summerfelt, 2003; Timmons and Ebeling, 2013). The UWM SFS RAS utilizes an ozone generator below the degasser tower before degasser effluent is redistributed to the rearing tank (Figure 1.2). Both UV and ozone generators directly impact the microbiome of RAS, and add selective pressure to the microbial community unique to these engineered environments. This dissertation will focus on three levels of inquiry, ecological context, an in depth analysis of RAS process microbial communities, and developing new methods to study fish pathogens. Specifically the topics researched are: comparing the UWM SFS RAS to itself and other systems in the Upper Midwest, characterizing the biological filter and enriching nitrifiers from the filter substrate, and using genomics to create a transgenic strain of *F. columnare* to study infection in larval and adult fish.

2 UW-Milwaukee Recirculating Aquaculture System

2.1 Microenvironments within a Recirculating Aquaculture System and Biogeography Across Recirculating Aquaculture/Aquaponic System Components and Facilities

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Abstract

Flow-through and pond aquaculture system microbiome management practices mitigate fish disease and stress. However, recirculating aquaculture system (RAS) operational success depends directly on system microbial community composition. Each component

environment is engineered to a specific microbial niche for waste management, as the water continually flowing through the system must be processed before returning into the rearing tank. In this study, we compared waste management component microbiomes (rearing tank water, pH correction tank, solid waste clarifier, biofilter, and degassing tower) within a commercial scale freshwater RAS, by high-throughput 16S rRNA gene sequencing. We found a planktonic bacterial assemblage circulates continuously through the system, but distinct microbial communities assemble in the nitrifying biofilter and solids clarifier, reflecting their intended engineered processes. To assess consistency among freshwater RAS microbiomes, we compared the microbial community composition among six aquaculture and aquaponic farms. Community assemblages reflected site and source water relationships, but some sequence variants classified to *Flavobacterium*, *Cetobacterium*, and the family *Sphingomonadaceae*, were common across all facilities. The presence of a hydroponic subsystem was a major community determinant. Nitrifying guilds of ammonia-oxidizing archaea and *Nitrospira* were also consistent across systems. The findings of this study suggest core taxa exist across RAS, independent of system design; but system design appears to inform the individual aquatic microbiome assemblages.

Introduction

Aquaculture is the cultivation of fisheries products for human use or consumption. Early system designs consisted of ponds, pens, and continuous water flow-through setups for cultivating finfish or other products. Now practices also include highly engineered recirculating aquaculture systems (RAS). These systems are constructed to optimize water use often achieving a 90-99% reduction in water consumption compared to more

conventional methods (Timmons and Ebeling, 2013). Nevertheless, recirculating water results in decreased water quality primarily through the accumulation of fish waste and uneaten food (Olsen et al., 2008). RAS typically manage these water quality concerns using engineered components that capture and remove solid and nitrogen waste products (Badiola et al., 2012; Verdegem et al., 2006). In the 1970's, aquacultural engineers supplemented nitrifying biofilters with plants for secondary treatment of nitrogenous waste (Lewis et al., 1978; Naegel, 1977; Sneed et al., 1975). Today such systems are commonly called aquaponic systems, a portmanteau of aquaculture and hydroponics. RAS offer a potentially long-term sustainable means to offset declining capture fisheries productivity (Barange et al., 2014). RAS success rates must grow in concert with increased demand for global fisheries products (FAO, 2014). Conversely, aquaponic systems are only often profitable when operated primarily around a plant centered production schedule (Love et al., 2015a) and therefore do not offer the same benefits for offsetting declining capture fisheries. Both RAS and aquaponic systems have the ability to reduce the distance to market (Love et al., 2015a; Martins et al., 2010). Soilless systems can also have lower energy and water footprints compared to traditional soil agriculture (Barbosa et al., 2015). Additionally, aquaponic systems introduce a grow season unbound by climate, and offer an ease of pest management not seen in traditional agricultural practices (Fox et al., 2012).

Aquaculture and aquaponic systems depend on a diverse consortium of microorganisms to carry out waste removal. Microbes also likely re-mineralize nutrients to support plant growth in aquaponic systems (Goddek et al., 2016). However, it is important to consider that fish are very sensitive to their external microbiome (De Schryver and Vadstein, 2014), and in both aquaculture and aquaponic systems the

individual component microbiomes are connected and can influence fish and/or plant health (Bartelme et al., 2018; De Schryver and Vadstein, 2014). For example, tank water microbiota composition was correlated with improved larval fish survival in RAS (Attramadal et al., 2014) and large taxonomic overlap was observed between the fish gut microbiota and the rhizosphere of plant roots (Hacquard et al., 2015). These results suggest microbial community assembly in one component could influence host health in a separate component. Also, the engineered nature of RAS may alter typical relationships between hosts and their microbiome, as significant taxonomic differences in gut microbiome composition between farm raised and wild fish have been noted (Dehler et al., 2016).

Despite the multifaceted importance of microorganisms to RAS success, RAS microbiomes are poorly defined. It is not well understood whether one RAS component influences the community assembly in separate components, how engineered conditions select for RAS microbiomes, or whether community assemblages are consistent across many facilities. The connection between fish and plant production microbial communities is also minimally defined. Ultimately, deciphering the role the microbiome has in both fish and plant growth may increase system yields.

This study investigated microbiome compositional correlation within a RAS's components over a short time-course, and among seven geographically separate freshwater RAS. To compare bacterial communities within a system, we examined the RAS at the University of Wisconsin – Milwaukee (UWM) School of Freshwater Sciences (SFS), which is equivalent to a medium scale commercial system. The microbial communities in this system were then compared to two other RAS (Marinette and Bell Aquaculture), three aquaponic systems (Marinette, PortFish, and the Urban Farm

Project), and recirculating freshwater aquaria from Discovery World, Milwaukee, WI. The systems were divided categorically by RAS and aquaponic system, source water, system scale (small, medium, or large), and species reared. From these comparisons, we sought to identify microbes that are common across systems and those that distinguish system component communities. Additionally, we investigated the influences of plant presence (i.e. aquaponic system) and source water on bacterial community composition. Since nitrifying guilds are critical to both RAS and aquaponic system success, we examined nitrifier assemblages in detail across all systems studied.

Materials & Methods

Sample Collection, Processing, & DNA Extraction

We collected samples from the UWM SFS RAS components (rearing tank, pH tank, solids clarifier, biofilter, and degasser) over a period of 7 months. A generalized diagram of components sampled across all systems is shown in Figure 2.1 All samples were collected using autoclaved 500mL plastic bottles. All water samples were filtered onto 0.22 μm filters (47 mm mixed cellulose esters, EMD Millipore, Darmstadt, Germany) and frozen at -80°C until further processing. The filtered volume for each sample is listed in Supplementary Table 1

(https://www.dropbox.com/s/g9lni7p2lb4s8u0/Supplementary_Table1.xlsx?dl=0). If applicable, biofilter pore water samples were collected and decanted from the biofilter solid medium substrate, and ~1 gram (wet weight) of remaining substrate was frozen at -80°C until extracted. Sample sites, available operator data, and weights or volumes of samples extracted are found in Supplementary Table 1

(https://www.dropbox.com/s/g9lni7p2lb4s8u0/Supplementary_Table1.xlsx?dl=0).

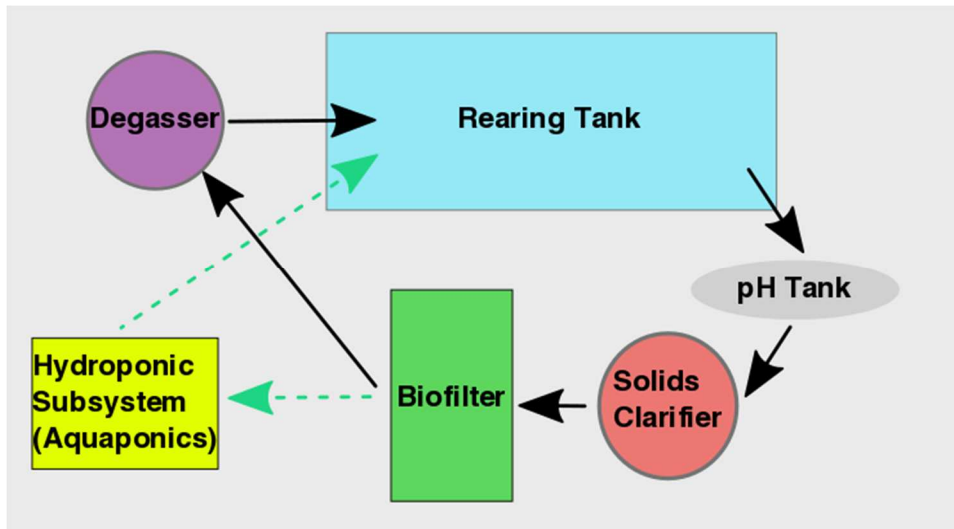


Figure 2.1 - Generalized System Diagram of All Components Sampled

Prior to DNA extraction, sample filters were removed from the freezer and macerated with a sterilized spatula. DNA was then extracted using the MP Bio FastDNA® SPIN Kit for Soil (MP Bio, Solon, OH, USA) according to the manufacturer's instructions except that each sample underwent 2 min of bead beating with the MP Bio FastDNA® SPIN kit's included beads using a Mini-BeadBeater-16 at the units' fixed speed (Biospec Products, Inc., Bartlesville, OK, USA). Initial quality of extracts was assessed using a NanoDrop® Lite (Thermo Fisher Scientific Inc., Waltham, MA, USA).

High-throughput Sequencing Reactions

Two different illumina platforms were utilized for massively parallel paired-end sequencing of bacterial 16S rRNA gene amplicons. For the within-system component comparison, we targeted the V6 region of the 16S rRNA gene (Eren et al., 2013)). We used 5-20 ng of the UWM SFS RAS component DNA extracts in a reaction consisting of 4 units Invitrogen Platinum HiFi *Taq* polymerase, 2 mM MgSO₄, 0.2 mM Invitrogen dNTPs, and 0.2 μM combined primers (Table 1) at a volume of 100 μl. These master mix reactions were split in triplicate, amplified with PCR, cleaned, etc. as described in (Eren

et al., 2013). Barcoded amplicon libraries were generated and sequenced on an illumina HiSeq at the Marine Biological Laboratory (MBL) in Woods Hole, MA. For the cross-site comparisons, the V4-V5 region of the 16S rRNA gene was targeted. Each sample was PCR amplified in triplicate using three separate Eppendorf Mastercycler Pro thermocyclers (Eppendorf, Mt Laurel, NJ, USA) with previously published primers (Table 2.1) purchased from IDT (Integrated DNA Technologies, Coralville, IA, USA). Prior to library preparation, all PCR products were cleaned using Ampure Beads (Beckman Coulter, Inc., Brea, CA, USA) and the resultant DNA quality and concentration of all samples was checked using the BroadRange Qubit 2.0 Spectrophotometric assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). MiSeq sequencing was carried out at either the Great Lakes Genomic Center (Milwaukee, WI, USA) or at MBL (Woods Hole, MA, USA).

Table 2.1 High-Throughput Sequencing Primer Sets

Gene Target	Forward Primer(s) (5'-3')	Reverse Primer(s) (5'-3')	Component Surveyed	Sample Site(s)	Citation
Bacterial 16S rRNA gene V6 region	CTAACCGANGAACCTYACC, CNACGCGAAGAACCTTANC, CAACGCGMARAACCTTACC, ATACGCGARGAACCTTACC	CGACRRCCATGCANCACCT	All	UWM RAS	Eren, et al. 2013
Bacterial 16S rRNA gene V4-V5 region	CCAGCAGCYGCGGTAAN	CCGTCAATTCNTTTRAGT, CCGTCAATTTCTTTGAGT, CCGTCTATTCCTTTGANT	All	All	Nelson, et al. 2014
Archaeal 16S rRNA gene V4-V5 region	GCCTAAAGCATCCGTAGC□, GCCTAAARCGTYCGTAGC, GTCTAAAGGGTCYGTAGC, GCTTAAAGNGTYCGTAGC, GTCTAAARCGYYCGTAGC	CCGGCGTTGANTCCAATT	Biofilters	All Biofilter Samples	Topçuoğ lu, et al. 2016
Betaproteobacterial <i>amoA</i>	GGGGHTTYTACTGGTGGT	CCCCTCKGSAAAGCCTTCTTC	Biofilters	All Biofilter Samples	Christman, et al. 2011; Rothauwe, et al. 1997
Comammox <i>amoA</i>	GGAYTTYTGGNTNGATTGGA	WRKTNNGACCACCASKACCA	Biofilters	All Biofilter Samples	Modified from Fowler, et al. 2018
<i>Nitrospira nxrB</i>	TACATGTGGTGGAAACA	CGGTTCTGGTCRATCA	Biofilters	All Biofilter Samples	Pester, et al. 2013

Bacterial rRNA Gene Sequence Data Processing

All bacterial 16S rRNA gene sequences were trimmed of their respective primers using the Great Lakes Genomic Center GNU parallel implementation of CutAdapt (Martin, 2011). After primer trimming, reads were merged with PEAR (Zhang et al., 2014), and the PEAR output was converted from FASTQ format to FASTA using the FASTX Toolkit. The V6 and V4-V5 16S rRNA gene datasets were decomposed into representative MED nodes (equivalent to OTU's/Amplicon Sequence Variants [ASV's]) with default settings except that the respective minimum substantive abundance cutoffs were set to 330 and 398, respectively. Chimera checking was carried out against the SILVA gold reference database with the implementations of Chimera Slayer and Uchime in mothur (Schloss et al., 2009). Chimeric node sequences were removed from the FASTA and absolute abundance tables generated by MED before taxonomy or statistical calculation. Taxonomy was assigned to non-chimeric MED nodes using the SILVA 128 SSU database and SINA online (Pruesse et al., 2012). FASTA files of representative nodes exceeding the SINA sequence number limit were split using the Great Lakes Genomic Center's SplitFA program. MED nodes not matching known bacterial taxonomies were removed from the MED node absolute counts table and eliminated from downstream statistical analyses.

Within System Diversity Calculations and Statistical Tests

Alpha- and beta-diversity comparisons were used to test influences on component bacterial community composition pertaining to the RAS environment and resultant environmental influences on bacterial taxonomic abundance. Alpha-diversity was

calculated using the natural logarithm base Shannon-Weaver Index (H') from the vegan R package diversity function (Oksanen et al., 2015). Pielou's evenness (J) was derived manually in R according to the vegan manual, where $J = H'/\log(S)$ and S was calculated using the vegan function specnumber on the relative abundance table of MED nodes (Oksanen et al., 2015). Kruskal-Wallis rank sum was then utilized to hypothesis test the influence of sample type (planktonic, sludge, and biofilm) on alpha diversity.

The chosen beta-diversity metric, Bray-Curtis dissimilarity, was calculated using the vegdist function from vegan across the UWM SFS RAS V6 16S rRNA gene dataset (Oksanen et al., 2015). ADONIS was then used to test the hypothesis that Bray-Curtis dissimilarity would reflect association with each component as its own environment with significantly different relative taxonomic abundances (Oksanen et al., 2015). The ADONIS function was run with 999 permutations, where Bray-Curtis was the dependent variable and component association, the independent variable. Component association was defined as a sample originating from the interstitial water, biofilm sand, solid sludge, or effluent, of a particular RAS component (rearing tank, pH tank, solids clarifier, biofilter, and degasser).

Cross-System Analyses, Ordination, and Shared Taxonomic Calculations

Samples were collected from six aquaculture and aquaponic facilities to generate the V4-V5 16S rRNA gene data used in this cross-system comparison (Supplemental Table 1 https://www.dropbox.com/s/g9lni7p21b4s8u0/Supplementary_Table1.xlsx?dl=0). The system component classes were extended to include hydroponic subsystem samples from aquaponic facilities, and conditioning water for calculating facility diversity metrics. The V4-V5 MED node table input into vegan's metaMDS function, using $k = 5$

dimensions and Bray-Curtis Dissimilarity between samples to calculate nMDS (Oksanen et al., 2015).

Nitrification Marker Gene Amplification, Multiplex Reaction, and Analysis

A multiplex MiSeq assay was constructed targeting nitrification marker genes: *amoA* from *Betaproteobacteria*, *amoA* of complete ammonia-oxidizing *Nitrospira*, *nxB* from *Nitrospira*, and the V4-V5 region of the Archaea 16S rRNA gene was used to detect ammonia-oxidizing archaea (Table 2.1). Only samples associated with biological filtration were used as templates for the multiplex assay. Briefly, copies of the primers from Table 1 were ordered with illumina TruSeq adapter sequences from IDT (Integrated DNA Technologies, Coralville, IA, USA). Each 20 μ l reaction consisted of Kapa Biosystems HiFi 2x master mix 10 μ l, 200 nM final concentration of forward and reverse primer, with 10-100 ng of sample gDNA. PCR products were amplified in triplicate across three separate Eppendorf Mastercycler Pro thermocyclers at the Great Lakes Genomics Center. Triplicate products were pooled by gene target, and cleaned with Ampure XP beads according to manufacturers instructions. Concentrations of template were quantified with QuantIT PicoGreen (ThermoFisher) before pooling the 4-amplicon types (Supplemental Table 2 https://www.dropbox.com/s/sjh bivsi367g8t7/Supplemental_Table2.xlsx?dl=0). After quantification amplicons from the four PCR assays were pooled in a volume of 40 μ l at an approximate concentration of 1.8 ng/ μ l (Betaproteobacterial *amoA*, *Nitrospira nxB*, & Archaea 16S V4-V5), while 0.9 ng/ μ l of Comammox *amoA* products were used to account for the shorter product length. After pooling, each well was barcoded by sample using Nextera Adapter sequences.

Amplicons were demultiplexed off the MiSeq by their Nextera tags, then merged and further demultiplexed by target genes using mothur (Schloss et al., 2009). Sequences were decomposed into unique ASV's using MED (Eren et al., 2015b). To further denoise the amplicon data, 95% clustering of MED node ASV's was done within mothur for: Betaproteobacterial *amoA*, *Nitrospira nxrB*, and complete-ammonia oxidizing *Nitrospira* (comammox) *amoA*. After representative sequences were denoised, taxonomy was assigned via two methods. Archaea V4-V5 16S rRNA gene identity was assigned using SINA and version 128 of the SILVA database (Pruesse et al., 2012). Any unknown sequences were removed before further analysis. For betaproteobacterial *amoA*, comammox *amoA*, and *Nitrospira nxrB*, reads were aligned to the ARB databases from Bartelme *et al.*, 2017. Sequences falling outside (i.e. basal to) the known marker gene diversity in phylogenetic reconstructions were compared against the NCBI nucleotide database using blastn on default settings. Those nitrification marker sequences that matched a small portion of a known corresponding gene were assumed to be chimeras and removed from downstream analyses.

In order to avoid operating within the error range of our illumina MiSeq sequencer, remaining ASV's of the four genes with absolute abundances <10 were assumed to be noise, lane drift, or chimeras. These abundances were converted to zero within R before further analysis. Non-amplification of a gene in a sample was also assumed to be equivalent to an absolute abundance of zero. After the data tables were cleaned in R, binary Jaccard dissimilarities were calculated for each gene using vegan (Oksanen et al., 2015). To test correlations between the binary dissimilarity matrices, Spearman's ρ Mantel tests were conducted for 999 iterations for all possible pairwise combinations of the four Jaccard dissimilarities.

Results & Discussion

RAS Microenvironments Harbor Distinct Microbial Communities

Table 2.2 Mean Alpha-Diversity (Shannon-Weaver) & Evenness (Pielou's) within UWM RAS by Site & Sample category

Site	$H' \pm StDev$	$J \pm StDev$	n	Sample Category
Biofilter Effluent	4.22±0.32	0.68±0.05	4	Planktonic
Biofilter Sand	4.66±0.20	0.75±0.04	6	Biofilm
Biofilter Water	4.26±0.74	0.68±0.12	4	Planktonic
Clarifier	4.27±0.21	0.68±0.03	6	Sludge
Clarifier Effluent	4.38±0.35	0.70±0.06	4	Planktonic
Degasser	4.20±0.31	0.67±0.05	5	Planktonic
pH Buffer Tank	3.54±1.41	0.57±0.22	3	Planktonic
Rearing Tank	4.13±0.13	0.66±0.02	4	Planktonic

Two ecological diversity metrics (alpha- and beta-diversity) were calculated to evaluate the existence of RAS system component microenvironments and their effects on microbial community composition. Mean values of Shannon-Weaver index and Pielou's evenness were nearly equivalent across the different system components of the UWM RAS (Table 2.2). It was determined that sample class (planktonic water, clarifier sludge, or biofilter biofilm) significantly influenced Shannon-Weaver indices and, subsequently Pielou's evenness (Table 2.2; $\chi^2=8.092$, $df = 2$, $p = 0.01749$). The ozonation incorporated into the UWM RAS is a possible explanation for the differences in alpha diversity, since a previous study saw ozonation change taxonomic abundances in RAS biofilms (Wietz et al., 2009). The study of shifts in planktonic microbial communities due to ozonation warrants further study. These results also support the hypothesis that planktonic microbial assemblages significantly differ from biofilm communities formed in sludge digestion or within the biofilter (Blancheton et al., 2013). Interestingly, rearing tank Shannon-Weaver index ($H'=4.13 \pm 0.13$) was approximately 1.6x greater than a previous study of rearing

tank alpha diversity assessed by DGGE ($H'=2.6\pm 0.09$), while maintaining similar levels of Pielou's evenness, where, respectively, $J=0.66\pm 0.02$ and $J=0.64\pm 0.09$ (Attramadal et al., 2014). In Attramadal et al., the authors postulate that the RAS system selects for K strategist bacterial assemblages, resulting in increased larval fish survival compared to a flow through system. However, given the Attramadal et al., 2014 samples, and low Shannon diversity relative to our results using the illumina HiSeq, it is difficult to associate K strategist niche occupation as explaining their overall results. Our higher alpha diversity values indicate massively parallel sequencing may better capture co-occurring populations in recirculating aquaculture systems than molecular finger printing methods such as DGGE.

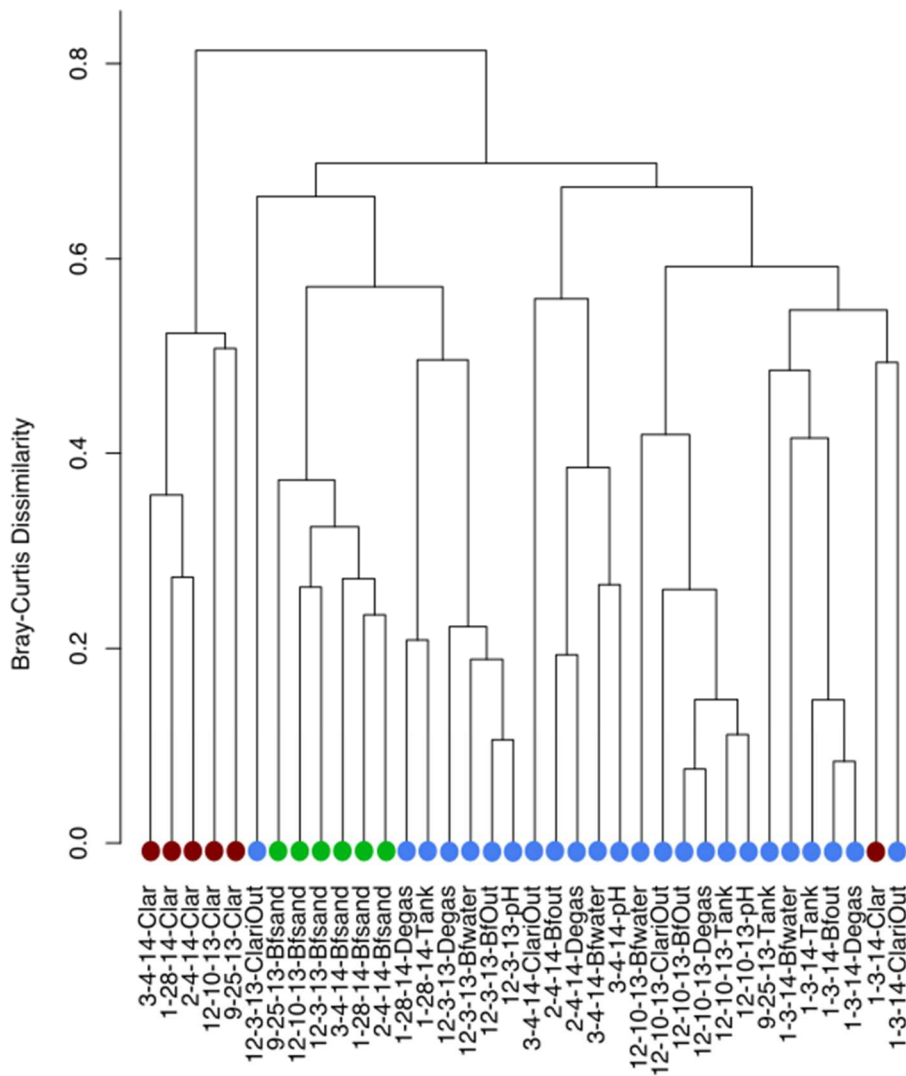


Figure 2.2 Dendrogram of Bray-Curtis Dissimilarity Between UWM RAS Components. Maroon nodes indicate sludge samples, green nodes indicate biofilter biofilm samples, and blue nodes indicate all planktonic samples.

Beta diversity was used to test whether each component was a unique microenvironment within the UWM SFS RAS. Bray-Curtis dissimilarity results, like the alpha-diversity measures, cluster approximately by sample class (Figure 2.2), with 27.6% of the beta-diversity (Bray-Curtis) explained by component association alone (ADONIS, $df = 4$, $p = 0.001$). This clustering pattern and relatively high variance residuals indicates there is not a complete separation between the microenvironments and is indicative of interactions between our defined independent variable (component association) and other system parameters. Most discordant environment linkages resulted from samples

classified as sludge that are chronologically linked to non-sludge samples and a division in linkage patterns between water samples and the sand biofilm samples (Figure 2.2). Since all RAS components are connected by water flow, any single sampling period could reflect a relatively high release of microbes from one component (e.g. tank, biofilter, or digester) into the others. This action would act to homogenize the community composition across components and explain some of our observed patterns. Additionally, changes in operator conditions during a rearing cycle can influence RAS microbial communities (Bartelme et al., 2017). These temporally punctuated whole system changes also may be acting to homogenize briefly communities across system components.

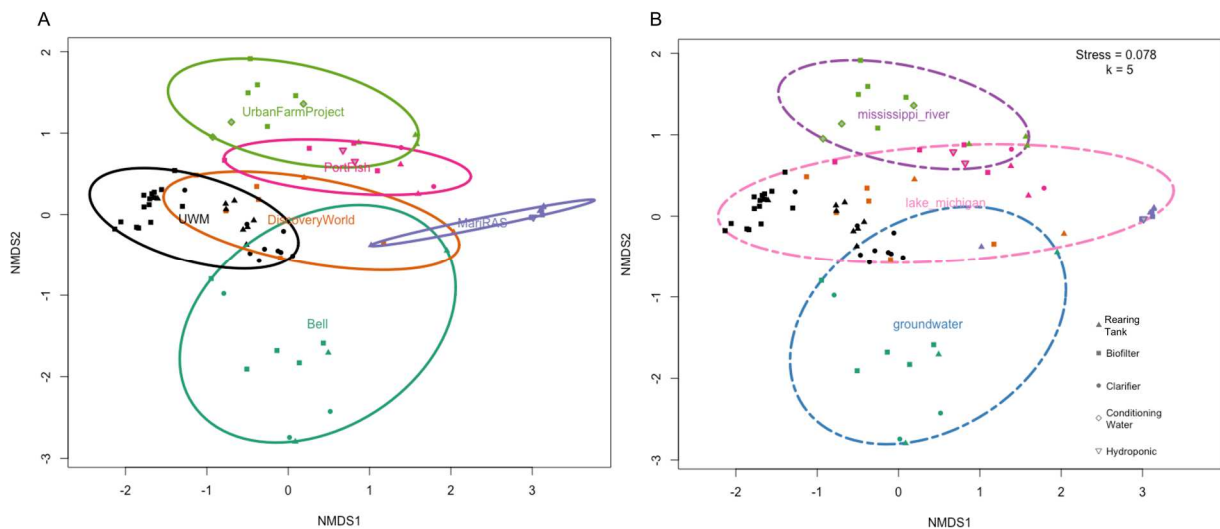


Figure 2.3 Cross-System Bray-Curtis Dissimilarity nMDS. Panel A – by Site, Panel B – by Source Water

Cross-System Comparison

To assess taxonomic differences in system component microbiomes, community composition comparisons were made in ordinate space. We found both system site (i.e. individual facility) (envfit, vegan; R^2 0.6491, $p=0.001$) and water source (envfit, vegan; R^2 0.2179, $p=0.001$) correlated with beta diversity (nMDS from Bray-Curtis dissimilarity with $k=5$ dimensions and stress equal to 0.078; Figure 2.3). System site, as the

dominating factor related to community composition, indicates the conditions in each facility dictate strongly the resultant community assemblages. Although the facilities all operate differently and have unique community compositions, it was clear source water shapes the outcome of RAS/aquaponic system microbiomes. This is particularly apparent when one considers how closely related the system microbiomes are within the Milwaukee Water Works distribution area. While beta diversity correlated less strongly with categorical factors such as: system scale (envfit, vegan; R^2 0.2016, $p=0.001$), component type (envfit, vegan; R^2 0.1935, $p=0.001$), and aquaponics system (binary TRUE/FALSE; envfit, vegan; R^2 0.1510, $p=0.001$), these factors appear to also inform microbiome composition. Since all facilities, except Discovery World, rear Yellow Perch (*Perca flavescens*), our system comparison results also reflect previously reported tank effects (Schmidt et al., 2016) on the scale of system operations and source water.

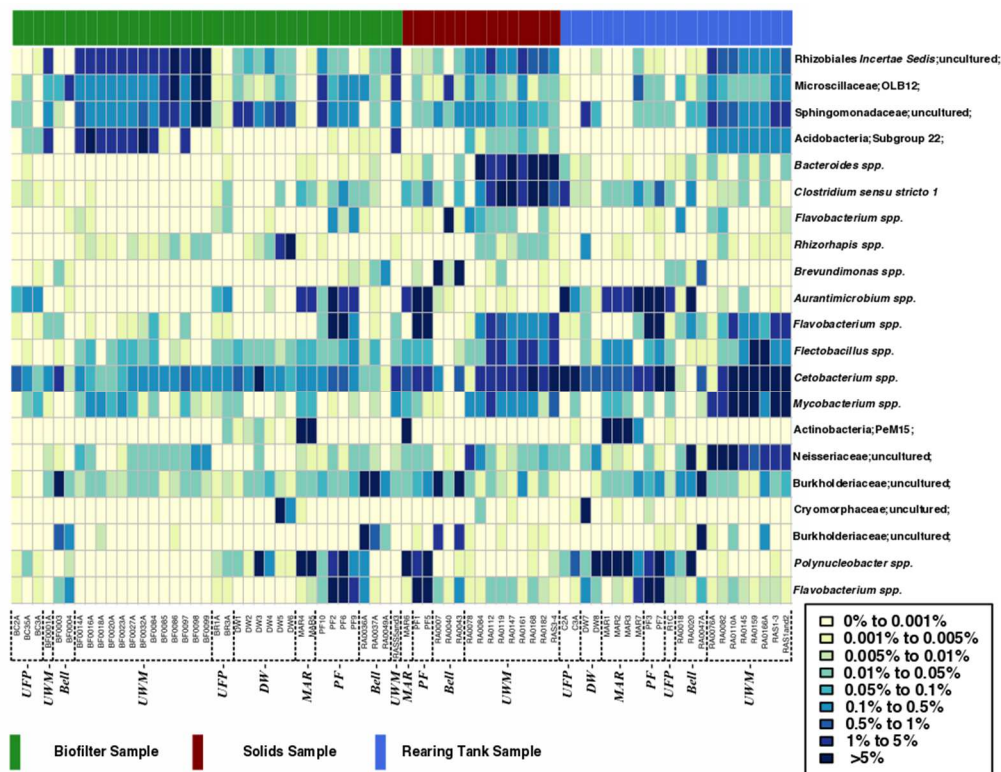


Figure 2.4 Heatmap of Top 10 Taxa Across Biofilters (Green columns), Rearing Tanks (Blue columns), and Solids Clarifiers (Maroon columns).

In contrast to the significant microbial community differences among facilities, some taxa (represented by unique ASVs) were abundant in all samples (Figure 2.4), showing that some taxa were maintained across all of the systems investigated. For example, a high abundance sequence was identified as a potential *Cetobacterium* spp (>1-5%, in all but 5 of the rearing tank and solids clarifier samples). *Cetobacterium* taxa are present in freshwater fish intestinal tracts (Tsuchiya et al., 2008) and, in one study, occupied >75% of the fish fecal microbiome (Schmautz et al., 2017). Furthermore, two different sequences of an unclassified *Flavobacterium* spp. were respectively represented in either the solids and rearing tank samples, while a third *Flavobacterium* spp. sequence was present in both. *Polynucleobacter* spp. and *Aurantimicrobium* spp. were most abundant in the aquaponic system samples. In the case of the Marinette facility, one tank was converted from an aquaponic system to a RAS, and the aquaponic taxa were maintained at the same abundance after conversion. This suggests the potential to maintain microbiome composition even in response to changes in overall system design. As such, this highlights the need to better understand the processes that drive and define the onset of microbial community in RAS and aquaponics, as subtle differences in microbial assemblages may possibly impart significantly different health, production, and operations outcomes beyond what is traditionally known. A number of species associating with the order *Rhizobiales* were present in high abundance across RAS rearing tanks and biofilters, but could not be taxonomically classified deeper than the order level. A *Sphingomonadaceae* sequence (closest cultured relative *Sphingorhabdus* sp. WM51; BLASTN: 100% query coverage, e=0.0, 100% identity) was present at relative abundances >0.001% in all but 12 of the 74 samples. These results were consistent with what was reported previously in the UWM SFS RAS (Bartelme et al., 2017).

Interestingly, these sphingomonads could be a boon for aquaculturalists as some actively and cooperatively degrade geosmin (Hoefel et al., 2006), which produces off flavors in fish (Houle et al., 2011). Additionally, when grown in co-culture with a species of *Pseudomonas*, a sphingomonad was also shown to degrade 2-methylisoborneol (MEB) (Eaton, 2012). MEB is another off flavor producing compound in aquaculture systems (Klausen et al., 2005). It is likely that the high abundance taxa present across components represent a planktonic community that continuously recirculates through a given system.

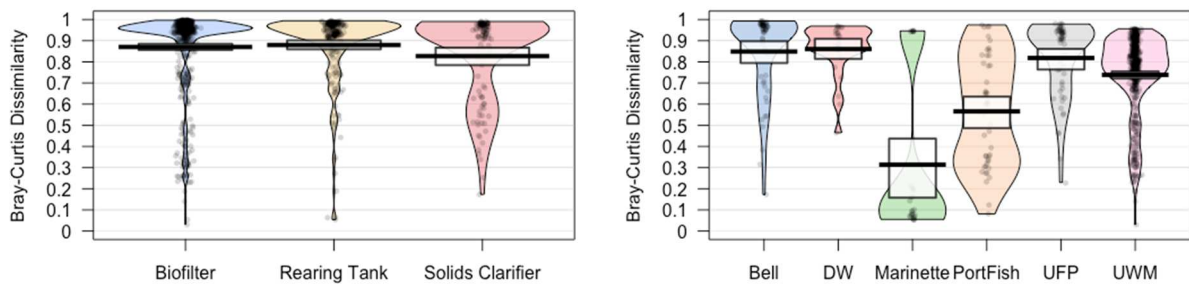


Figure 2.5 Distributions of Bray-Curtis Dissimilarity by Component and Site

Most research on solids clarification in RAS and aquaponics focuses on a reduction of dissolved organic matter (Wold et al., 2014) and a capture of solids to maintain nitrification rates in the biological filter (Michaud et al., 2006, 2013). According to recent reviews, solids management is critical to controlling populations of heterotrophic bacteria, some of whom may be opportunistic pathogens (Blancheton et al., 2013; Rurangwa and Verdegem, 2015). Our data suggest that these opportunistic pathogens, many of which are *Flavobacterium* spp. may proliferate in the solids capture systems (Figure 2.4). The heterotrophic bacterial communities and nitrifying guilds recovered from the biological filters studied resemble those found previously (Bartelme et al., 2017; Sugita et al., 2005). Examples of top taxonomic assignments shared with these previous biofilter studies are: uncultured *Acidobacteria*, uncultured *Rhizobiales*, as well as

Flavobacterium spp. Compared to the rearing tank and solids clarifier samples, the biofilter heterotrophic community appears to have the least number of taxa shared across systems (Green columns; Figure 2.4). The filter substrate or operational conditions may account for the dissimilarity in biofilter taxa recovered. The distinct system biogeography is probably most apparent in the bacterial V4-V5 biofilter communities, and is also reflected in the mean Bray-Curtis dissimilarity for biological filters (Figure 2.5). Though the bacterial communities within and across systems are very dissimilar, they seem to share the most common taxa in the solids clarifiers and rearing tanks. Whereas, at the core of each biofilter the nitrifying guilds appear quite conserved, the heterotrophic community surrounding appears more informed by factors such as system source water.

Nitrifier Guilds Across Biological Filters

Despite biogeographical differences in bacterial community composition, certain nitrifying consortia were consistent across the systems surveyed. All sites had ASV's of ammonia-oxidizing archaea (AOA) (based on 16S rRNA gene sequence) and *Nitrospira* (based on *nxB* gene sequence). The occurrence patterns of the AOA and *Nitrospira* genotypes were correlated across sites (Spearman's rho Mantel Test: $\rho = 0.5212$, $p = 0.001$). Previously we noted that AOA and *Nitrospira* genotype abundance patterns in a RAS biological filter were correlated across a fish rearing cycle (Bartelme et al., 2017). AOA are favored over AOB due to the low *in situ* concentrations of ammonia in RAS (Hatzenpichler, 2012). Since ammonia oligotrophy is a design constraint in RAS and aquaponic systems – this makes sense.

Based on current kinetic experiments (Kits et al., 2017), complete ammonia-oxidizing *Nitrospira* (comammox) species should be competitive with AOA in RAS and

aquaponic systems. We saw potential for this niche competition as some facility biofilters contained both AOA and comammox, where the binary beta diversity of the amplicons was significantly correlated (Spearman's rho Mantel Test: $\rho=0.3871$, $p=0.001$). Although comammox were previously found in RAS (Bartelme et al., 2017; van Kessel et al., 2015), we found their appearance in this study's surveyed samples to be informed by system water source. Predominantly, the comammox *amoA* amplicons were recovered from samples inside the Lake Michigan water basin (there were three outlier samples: 2 with groundwater fed system water and 1 from the Mississippi River). It is also possible, given how recently discovered comammox were, that the primers developed at the time of this writing do not fully capture the diversity of comammox *amoA*.

In this study, we found a diverse number of comammox *Nitrospira amoA* genotypes in systems with source water from the Lake Michigan area (PortFish, UWM RAS, and Discovery World; Marinette did not). The Urban Farm Project samples lacked comammox, and comammox was detectable in only three of the five samples from Bell Aquaculture. This association may originate in the drinking water treatment plant (DWTP), as DWTP's often contain comammox in rapid sand filters (Fowler et al., 2018; Palomo et al., 2016; Pinto et al., 2015) and have been enriched from point of use (Wang et al., 2017). These results indicate a potential for ammonia-oxidizer seeding based upon system source water. Unsurprisingly, in samples containing comammox *amoA* amplicons, we found them to correlate with *nxB* genotype occurrence patterns (Spearman's rho Mantel Test: $\rho=0.4411$, $p=0.001$).

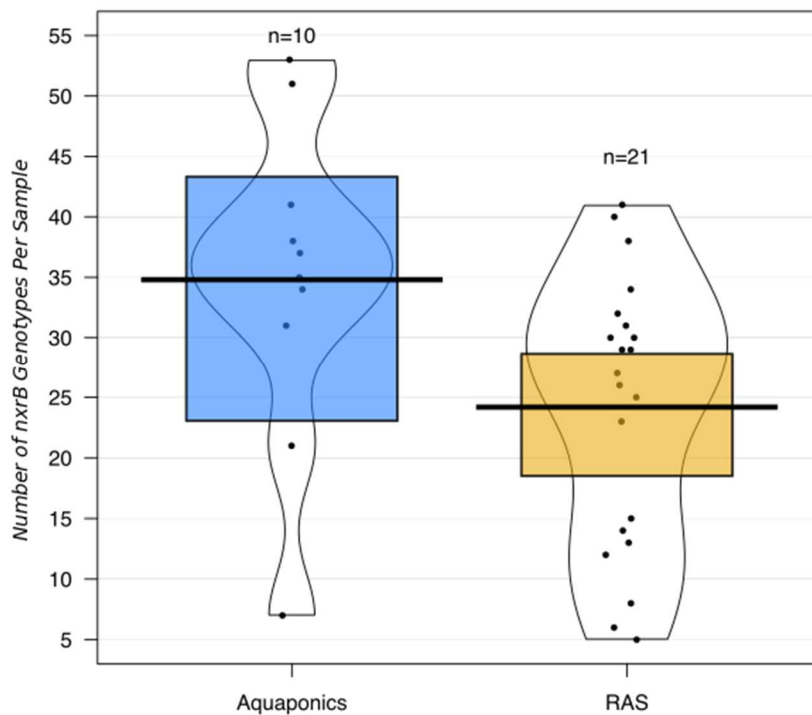


Figure 2.6 Distribution of Unique *nxrB* Genotypes in Aquaponic Systems & RAS

When examining *nxrB* genotypes, as a proxy for nitrite-oxidation potential, aquaponic facilities harbored significantly more genotypes of *Nitrospira nxrB* than RAS (Figure 2.6; Kruskal Wallis Rank Sum $\chi^2= 5.2134$, $df=1$, $p=0.02241$). Some of the differences could be accounted for by the presence of *nxrB* genotypes from comammox, since *nxrB* does not often distinguish comammox from NOB *Nitrospira* (Daims et al., 2016). To the best of our knowledge we believe this is the first such comparison of the recovery of *Nitrospira nxrB* genotypes from aquaponic and RAS facilities. It is unknown whether or not this is a consequence from increased trophic levels in an aquaponic system, driven by source water, or simply a stochastic event. Regardless, the mechanism of this selection merits further study, since these results suggest the potential for nitrogen cycle niche partitioning in aquaponic systems and may thusly increase system wide nitrifier diversity.

Conclusions

Despite differences in operations and beta-diversity all facilities retained some common (i.e. core) taxa associated with each of the major components (Rearing Tank, Solids Clarifier, and Biofilter). Although dominant taxa occur across all systems, it is apparent that source water and components shape overall community composition. While, within each facility, each component appears to drive overall community structure, but due to water recycling these component communities may be more similar on short time-scales. The results of this study offer some support for the decoupled aquaponic system model (Goddek et al., 2016), since the beta diversity within a single system, and across systems is coupled to component class. By decoupling components, in RAS or aquaponics, we can avoid unwittingly designing a system's "Achilles Heel". One of the systems surveyed had continual issues with solids clarifier failure, which in turn led to a suppression of nitrification, spike in nitrite levels, and subsequent die off of fish due to *Flavobacterium columnare* outbreaks. Since each component could, based on the results of this study, be considered its own microenvironment, decoupling components would allow aquaculturalists and aquaponics practitioners a greater level of system control.

The nitrogen cycle marker work in this study presented a novel schema for surveying nitrification using massively parallel sequencing technologies. After conducting our survey of nitrogen cycle amplicon markers, it is apparent that the AOA+NOB nitrifying guild seems to be the most common across freshwater aquaria and RAS (Bagchi et al., 2014; Bartelme et al., 2017; Sauder et al., 2011). It is still unclear as to why comammox *Nitrospira* appear in these systems; however, the most oligotrophic systems that contained fluidized sand filters harbored comammox. Rapid sand filters

(RSF) used in drinking water treatment have been shown to harbor comammox (Palomo et al., 2016; Pinto et al., 2015), and have even been found in RSF's processing groundwater (Fowler et al., 2018). But the commonality across DWTP's RSF and freshwater RAS biofilters merit further study as the trends we saw reflected those previously found in DWTP's, but are inconclusive. It is also worth noting that, although the aquaculture practitioners from our survey were knowledgeable about nitrification as a system process, many believed that *Nitrosomonas* and *Nitrobacter* species were the sole nitrifying taxa present. The results from this study and others (Hovanec and DeLong, 1996) indicate this is not the nitrification schema we see in operational RAS, and it is our recommendation that aquacultural organizations incorporate new nitrogen cycle findings into stakeholder outreach plans to better inform system operators when they select starter cultures or substrate for a biological filter. Furthermore, our work suggests, like these reviews (Bartelme et al., 2018; Munguia-Fragozo et al., 2015; De Schryver and Vadstein, 2014), more “-omics” studies would benefit both aquaculture and aquaponic system development.

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3 The Nitrogen Cycle in Recirculating Aquaculture

3.1 The Importance of Nitrogen Management in RAS

In recirculating aquaculture systems, nitrogen management is critical to operational success. The total ammonia nitrogen production (P_{TAN}) is estimated by the following equation: $P_{TAN} = \frac{F*PC*0.092}{t}$, where F is the feeding rate in kg/day, PC is the protein concentration of the feed, and t is a time period in days (Timmons and Ebeling, 2013). The 0.092 constant accounts for fish protein catabolism producing an approximate ratio of 90% ammonia to 10% urea (Timmons and Ebeling, 2013). Unionized ammonia is toxic to fish, while at a neutral pH, the concentration of unionized ammonia is minimal (~0.7%), as pH increases the concentration goes up exponentially (Timmons and Ebeling, 2013). Nitrite, which is produced as a nitrification intermediate, is also toxic to fish. Nitrite will react with the heme of fish hemoglobin, changing the oxidative state of iron, thereby inducing methemoglobinemia and oxidative stress in the fish (Timmons and Ebeling, 2013). The toxicity of either nitrogen species is fish dependent, but independent of aquatic species reared, all systems rely on guilds of nitrogen cycling organisms to facilitate waste remediation. Since, nitrate accumulation in freshwater RAS is assumed to be benign due to its low toxicity (Lee et al., 2000). For example, Atlantic Salmon (*Salmo salar*), during the post-smolt transition from freshwater to saltwater, there were no adverse reproductive or physiological effects at nitrate levels of ~100mg/L (Davidson et al., 2017; Good et al., 2017). Largely, denitrification processes have been utilized in aquaculture systems to mitigate nitrate levels in waste discharge (Klas et al., 2006), where the processes closely resemble a traditional waste water treatment plant. There is little information on the effects of nitrate in *Perca flavescens* reared in RAS. The experiments in this dissertation focus on aerobic nitrification in the UW-Milwaukee RAS and investigated chemolithoautotrophic nitrifier community assemblies.

Nitrification in the UWM SFS RAS was interrogated in 3 separate ways, apart from the amplicon survey in Chapter 2 of this dissertation. First, an in-depth characterization of nitrifier and heterotrophic bacterial genotypes were assessed in the biological filter over time (Bartelme, *et al.* *Frontiers in Microbiology*, 2017). The Archaeal community was also investigated and consisted of multiple 16S rRNA genotypes associated with ammonia-oxidizing archaea. Second, scaled down biological filters were constructed to manipulate nitrifier inocula in the laboratory and measure the response of nitrifiers from a period of system dormancy. In these experiments, the dormant nitrifying consortia were challenged with a commercially available aquarium biofilter starter culture. Last, a complete ammonia-oxidizing *Nitrospira* (comammox) species was enriched from the biological filter within the UWM SFS RAS, analyzed by shotgun metagenomic sequencing, and characterized versus other known comammox assemblies.

3.2 Freshwater Recirculating Aquaculture System Operations Drive Biofilter Bacterial Community Shifts Around a Stable Nitrifying Consortium of Ammonia-oxidizing *Archaea* and Comammox *Nitrospira*

Modified from the published form in Frontiers in Microbiology, Please See the Published Work for Supplemental Materials (doi: 10.3389/FMICB.2017.00101)

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Running title: Recirculating aquaculture biofilter microorganisms

Keywords: recirculating aquaculture system, biofilter, nitrifiers, ammonia-oxidizing
archaea, comammox, microbial communities, Nitrospira

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Abstract: Recirculating aquaculture systems (RAS) are unique engineered ecosystems that minimize environmental perturbation by reducing nutrient pollution discharge. RAS typically employ a biofilter to control ammonia levels produced as a byproduct of fish protein catabolism. *Nitrosomonas* (ammonia-oxidizing), *Nitrospira* and *Nitrobacter* (nitrite-oxidizing) species are thought to be the primary nitrifiers present in RAS biofilters. We explored this assertion by characterizing the biofilter bacterial and archaeal community of a commercial scale freshwater RAS that has been in operation for >15 years. We found the biofilter community harbored a diverse array of bacterial taxa (>1000 genus-level taxon assignments) dominated by *Chitinophagaceae* (~12%) and *Acidobacteria* (~9%). The bacterial community exhibited significant composition shifts with changes in biofilter depth and in conjunction with operational changes across a fish rearing cycle. *Archaea* also were abundant, and were comprised solely of a low diversity assemblage of *Thaumarchaeota* (>95%), thought to be ammonia-oxidizing archaea (AOA) from the presence of AOA ammonia monooxygenase genes. *Nitrosomonas* were present at all depths and time points. However, their abundance was >3 orders of magnitude less than AOA and exhibited significant depth-time variability not observed for AOA. Phylogenetic analysis of the nitrite oxidoreductase beta subunit (*nxrB*) gene indicated two distinct *Nitrospira* populations were present, while *Nitrobacter* were not detected. Subsequent identification of *Nitrospira* ammonia monooxygenase alpha subunit genes in conjunction with the phylogenetic placement and quantification of the *nxrB* genotypes suggests complete ammonia-oxidizing (comammox) and nitrite-oxidizing *Nitrospira* populations co-exist with relatively equivalent and stable abundances in this system. It appears RAS biofilters harbor complex microbial communities whose

composition can be affected directly by typical system operations while supporting multiple ammonia oxidation lifestyles within the nitrifying consortium.

Introduction

The development of aquacultural technology allows societies to reduce dependency on capture fisheries and offset the effects of declining fish numbers (Barange et al., 2014). Aquaculture production now accounts for nearly 50% of fish produced for consumption, and estimates indicate a 5-fold increase in production will be required in the next two decades to meet societal protein demands (FAO, 2014). However, expanding production will increase the environmental impact of aquaculture facilities and raises important concerns regarding the sustainability of aquaculture practices.

Recirculating aquaculture systems (RAS) have been developed to overcome pollution concerns and stocking capacity limits of conventional terrestrial aquaculture facilities (Chen et al., 2006a; Martins et al., 2010). RAS offer several advantages over traditional flow-through systems including: 90-99% reduced water consumption (Badiola et al., 2012; Verdegem et al., 2006), more efficient waste management (Piedrahita, 2003), and potential for implementation at locations that decrease distance to market (Martins et al., 2010). RAS components are similar to those used in wastewater treatment, including solids capture and removal of nitrogenous waste from excess animal waste and undigested feed. The advancement of RAS technology and advantages over flow-through systems has led to increasing RAS use, especially among countries that place high value on minimizing environmental impacts (Badiola et al., 2012) and in urban areas where space is limiting (Klinger and Naylor, 2012).

Nitrifying biofilters are a critical component of most RAS and an important determinant of operational success. These biofilters are also cited as the biggest hurdle

for RAS start-up and the most difficult component to manage once the RAS is in operation (Badiola et al., 2012). RAS biofilters act to remove nitrogenous waste byproducts generated by fish protein catabolism and oxidation processes. Ammonia and nitrite are of most concern to freshwater aquaculturalists, with the toxic dose of both nitrogen species depending on pH and the aquatic organism being reared (Lewis and Morris, 1986; Randall and Tsui, 2002). In RAS process engineering, designers typically cite the principle nitrifying taxa as *Nitrosomonas* spp. (ammonia-oxidizers) and *Nitrobacter* spp. (nitrite-oxidizers) (Kuhn et al., 2010) and model system capacity from these organisms' physiologies (cite Ebeling & Timmons book). It is now clear *Nitrosomonas* and *Nitrobacter* are typically absent or in low abundance in freshwater nitrifying biofilters (Hovanec and DeLong, 1996) while *Nitrospira* spp. are common (Hovanec et al., 1998). More recent studies of freshwater aquaculture biofilters have expanded the nitrifying taxa present in these systems to include ammonia-oxidizing archaea (AOA), a variety of *Nitrospira* spp., and *Nitrotoga* (Bagchi et al., 2014; Hüpeden et al., 2016; Sauder et al., 2011). Further studies are needed to understand whether other nitrifying consortia co-inhabit RAS biofilters with *Nitrosomonas* and *Nitrobacter* spp., or if diverse assemblages of nitrifying organisms are characteristic of high-functioning systems. A more refined understanding of RAS biofilter nitrifying consortia physiology would inform system design optimization and could alter parameters that are now considered design constraints.

The non-nitrifying component of RAS biofilter communities also impact biofilter function. Heterotrophic biofilm overgrowth can limit oxygen availability to the autotrophic nitrifying community resulting in reduced ammonia-oxidation rates (Okabe et al., 1995). Conversely, optimal heterotrophic biofilm formation protects the slower-

growing autotrophs from biofilm shear stress and recycles autotrophic biomass (Kindaichi et al., 2004). Previous studies have suggested the diversity of non-nitrifying microorganisms in RAS biofilters could be large and sometimes contain opportunistic pathogens and other commercially detrimental organisms (Schreier et al., 2010). However, most of these studies used low-coverage characterization methods (e.g. DGGE, clone libraries) to describe the taxa present, so the extent of this diversity and similarity among systems is relatively unknown. Recently, the bacterial community of a set of seawater RAS biofilters run with different salinity and temperature combinations was characterized with massively parallel sequencing technology (Lee, *et. al.*, 2016). This study provided the first deeper examination of a RAS biofilter microbial community, and revealed a highly diverse bacterial community that shifted in response to environmental conditions but more consistent nitrifying assemblage typically dominated by *Nitrospira*-classified microorganisms.

In this study, we aimed to deeply characterize the bacterial and archaeal community structure of a commercial-scale freshwater RAS raising *Perca flavescens* (Yellow perch) employing a fluidized sand biofilter that has been in operation for more than 15 years. We hypothesized that the biofilter sand biofilm community would exhibit temporal variability linked to environmental changes associated with the animal rearing process and a diverse nitrifying assemblage. To address these questions, we used massively parallel sequencing to characterize the bacterial and archaeal biofilter community across depth and time gradients. We also identified and phylogenetically classified nitrification marker genes for the ammonia monooxygenase alpha subunit (*amoA*; Rotthauwe *et al.*, 1997; Pester *et al.*, 2012; van Kessel et al., 2015) and nitrite oxidoreductase alpha (*nxrA*; Poly *et al.*, 2008; Wertz *et al.*, 2008) and beta (*nxrB*; Pester

et al., 2013) subunits present in the biofilter, and then tracked their abundance with biofilter depth and over the course of a fish rearing cycle.

Materials and Methods

UWM Biofilter Description

All samples were collected from the University of Wisconsin-Milwaukee Great Lakes Aquaculture Facility RAS biofilter (UWM biofilter). Measured from the base, the biofilter stands ~2.74 meters tall, with a diameter of ~1.83 meters. The water level within the biofilter is approximately ~2.64 meters from the base, with the fluidized sand filter matrix extending to a height of ~1.73 meters from the base. The biofilter is filled with Wedron 510 silica sand, which is fluidized to ~200% starting sand volume by the use of 19 schedule 40 PVC probes, each with a diameter of 3.175 cm. The probes receive influent from the solid waste clarifier, which upwells through the filter matrix. Samples for this study were taken at three depths within the fluidized sand biofilter, defined as surface (~1.32-1.42 m from biofilter base), middle (~0.81-0.91 m from biofilter base), and bottom (~0.15- 0.30 m, from biofilter base). Depictions of the UWM biofilter and sample sites are shown in Figure 3.1. The maximum flow rate of the biofilter influent is 757 liters per minute, which gives a hydraulic residence time of ~9.52 minutes. Typical system water quality parameters are as follows (mean \pm standard deviation): pH 7.01 ± 0.09 , oxidation-reduction potential 540 ± 50 (mV), water temperature 21.7 ± 0.9 ($^{\circ}$ C), and biofilter effluent dissolved oxygen (DO) 8.20 ± 0.18 mg/L. The biofilter is designed to operate maximally at 10 kg feed per day, which is based on the predicted ammonia production by fish protein catabolism at this feeding rate (Timmons & Ebeling, 2013).

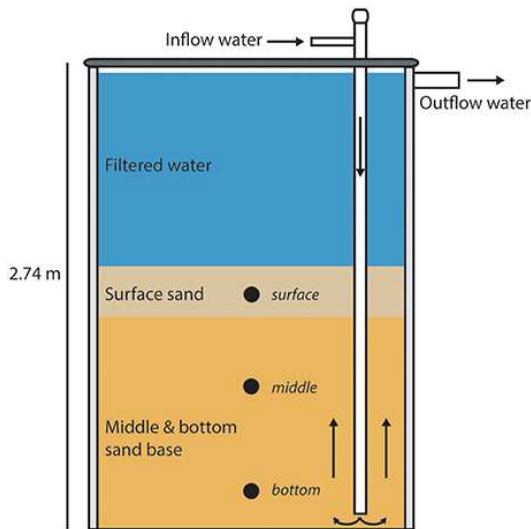


Figure 3.1 Illustration of UWM Biofilter Cross-Section

Sample Collection, Processing, & DNA Extraction

Samples from the top of the biofilter matrix were collected in autoclaved 500 mL polypropylene bottles. Two samples from the surface of the biofilter were collected during the final two months of one Yellow perch rearing cycle and then immediately before the initiation of a new rearing cycle in the system. After stocking the system with fish, samples were collected approximately every week through the first half of the new rearing cycle (the strains of Yellow perch present during this study need ~9 months to grow to market size). Following collection, water from the biofilter matrix samples was decanted into a second sterile 500 mL bottle for further processing. Then, approximately one gram wet weight sand was removed from the sample bottle and frozen at -80 degrees Celsius for storage prior to DNA extraction. Water samples were filtered onto 0.22 μ m filters (47 mm mixed cellulose esters, EMD Millipore, Darmstadt, Germany), frozen at -80°C, and macerated with a sterilized spatula prior to DNA extraction. To separately address the spatial distribution of bacterial taxa, depth samples were taken from the filter matrix by using 50 mL syringes with attached weighted Tygon tubing (3.2mm ID, 6.4mm OD) (Saint-Gobain S.A., La Défense, Courbevoie, France). Samples were binned into

categories by approximate distance from the filter base as surface, middle and bottom. Tubing was sterilized with 10% bleach and rinsed 3X with sterile deionized water between sample collections. DNA was extracted separately from biofilter sand and water samples (~1 g wet weight and 100 mL respectively) using the MP Bio FastDNA® SPIN Kit for Soil (MP Bio, Solon, OH, USA) according to the manufacturer's instructions except that each sample underwent two minutes of bead beating with the MP Bio FastDNA® SPIN kit's included beads at the Mini-BeadBeater-16's only operational speed (Biospec Products, Inc., Bartlesville, OK, USA). DNA quality and concentration was checked using a NanoDrop™ Lite (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sample details and associated environmental data and molecular analyses are listed in Table S1.

Ammonia and Nitrite Measurements

For both the time series and depth profiles, a Seal Analytical AA3 Autoanalyzer (Seal Analytical Inc., Mequon, WI, USA) was used to quantify ammonia and nitrite, using the manufacturer's supplied phenol and sulfanilamide protocols on two separate channels. To quantify only nitrite, the cadmium reduction column was not incorporated into the Auto Analyzer. RAS operators recorded all other chemical parameters from submerged probes measuring temperature, pH, and oxidation-reduction potential. Per the laboratory standard operating procedure, RAS operators used Hach colorimetric kits to measure rearing tank concentrations of ammonia and nitrite.

16S rRNA Gene Sequencing

To maximize read depth for a temporal study of the biofilter surface communities, we used the illumina HiSeq platform and targeted the V6 region of the 16S rRNA gene for *Archaea* and *Bacteria* separately. In total, we obtained community data from fifteen

dates for the temporal analysis. To interrogate changes in the spatial distribution of taxa across depth in the biofilter and obtain increased taxonomic resolution, we used 16S rRNA gene V4-V5 region sequencing on an illumina MiSeq. We obtained samples from three depths n=5 for the surface, n=5 for the middle, and n=4 for the bottom. Sample metadata are listed in Table S1. Extracted DNA samples were sent to the Josephine Bay Paul Center at the Marine Biological Laboratory (V6 *Archaea* and V6 *Bacteria*; V4-V5 samples from 12/8/2014 & 2/18/2015) and the Great Lakes Genomic Center (V4-V5 samples from 11/18/2014, 12/2/2014, 12/18/2014) for massively parallel 16S rRNA gene sequencing using previously published bacterial (Eren et al., 2013) and archaeal (Meyer et al., 2013) V6 illumina HiSeq and bacterial V4-V5 illumina MiSeq chemistries (Huse et al., 2014b; Nelson et al., 2014). Reaction conditions and primers for all illumina runs are detailed in the aforementioned citations, and may be accessed at:

<https://vampls.mbl.edu/resources/primers.php#illumina>. Sequence run processing and quality control for the V6 dataset are described in Fisher *et al.*, 2015, while CutAdapt was used to trim the V4-V5 data of low quality nucleotides (phred score < 20) and primers (Fisher et al., 2015; Martin, 2011). Trimmed reads were merged using Illumina-Utils as described previously (Newton et al., 2015). Minimum entropy decomposition (MED) was implemented on each dataset to group sequences (MED nodes = operational taxonomic units, OTUs) for among sample community composition and diversity analysis (Eren et al., 2015b). MED uses information uncertainty calculated via Shannon entropy at all nucleotide positions of an alignment to split sequences into sequence-similar groups (Eren et al., 2015b). The sequence datasets were decomposed with the following minimum substantive abundance settings: bacterial V6, 377; archaeal V6, 123; bacterial V4-V5, 21. The minimum substantive threshold sets the abundance threshold for MED

node (i.e. OTU) inclusion in the final dataset. Minimum substantive abundances were calculated by dividing the sum total number of 16S rRNA gene sequences per dataset by 50,000 as suggested in the MED best practices (sequence counts are listed in Table S2). The algorithm Global Alignment for Sequence Taxonomy (GAST) was used to assign taxonomy to sequence reads (Huse et al., 2008), and the website Visualization and Analysis of Microbial Population Structures (VAMPS) (Huse et al., 2014a), was used for data visualization.

Table 3.1 Primer Sets Used to Survey UWM Biofilter

Citation
(Christman et al., 2011; Rothauwe et al., 1997)
(Christman et al., 2011)
(Pester et al., 2012; Touma et al., 2008)
Fwd (Luesken et al., 2011) & Rev This Study
Fwd (Poly et al., 2008) & Rev (Wertz et al., 2008)
(Pester et al., 2013)
Modified from (Gantner et al., 2011)
(Øvreås and Torsvik, 1998)
This Study
This Study
This Study
This Study
This Study
This Study

Gene Target	Assay Type	Forward Primer	Reverse Primer	Primer Conc.	Thermocycler Temperature Programs
amoA	Qiagen Endpoint	1F 5'-GGG GHT TYT ACT GGT GGT-3'	2R 5'-CCC CTC KGS AAA GCC TTC TTC-3'	300 nM	1x 95°C 5:00 min; 30x 95°C 0:30 min, 53°C 0:30 min, 72°C 0:30 min; 1x 72°C 7:00 min
amoA	Qiagen Endpoint	3F 5'-GGT GAG TGG GYT AAC MG-3'	4R 5'-GCT AGC CAC TTT CTG-3'	300 nM	1x 95°C 5:00 min; 30x 95°C 0:30 min, 48°C 0:30 min, 72°C 0:30 min; 1x 72°C 7:00 min
amoA	Qiagen Endpoint	19F 5'-ATG GTC TGG YTW AGA CG-3'	616R 5'-GCC ATC CAB CKR TAN GTC CA-3'	300 nM	1x 95°C 5:00 min; 30x 95°C 0:30 min, 50°C 0:30 min, 72°C 0:30 min; 1x 72°C 7:00 min
amoA	Qiagen Endpoint	pmoA-189b-F 5'-GGNGACTGGGACTTYTGG-3'	Com_amoA_1_R 5'-CGAGATCATGGTGTGTGAC-3'	300 nM	1x 95°C 10:00 min; 35x 95°C 0:40 min, 56°C 0:40 min, 72°C 0:15 min; 1x 72°C 7:00 min
nxrA	Qiagen Endpoint	FInxrA 5'-CAGACCGACGTGCGAAG-3'	R2nxrA 5'-TCCACAAGGAACGGAGGTC-3'	300 nM	1x 94°C 5:00 min; 35x 94°C 0:30 min, 55°C 0:45 min, 72°C 1:00 min; 1x 72°C 10:00 min
nxrB	Qiagen Endpoint	nxrB169F 5'-TACATGTGGTGAACA-3'	nxrB638R 5'-CGG TTC TGG TCR ATC A-3'	300 nM	1x 95°C, 5:00 min; 35x 95°C 0:40 min, 50°C 0:40 min, 72°C 1:30 min; 1x 72°C 10:00 min
16s	SYBR Green	958F-m 5'-AAT TGG ANT CAA CGC CRG-3'	1000R-m 5'-GGC CAT GYA CYW CCT CTC-3'	500 nM	1x 95°C 10:00 min; 40x 95°C 0:15 min, 58°C 0:15 min, 72°C 0:15 min
16s	SYBR Green	Eub337F 5'-ACT CCT ACG GGA GGC AGC AG-3'	Eub513R 5'-ATT ACC GCG GCT GCT GG-3'	100 nM	1x 95°C 10:00 min; 40x 95°C 0:15 min, 53°C 0:15 min, 72°C 0:15 min
amoA	EvaGreen	Arch-amoAF 5'-CTG ACT GGG CGT GGA CAT CA-3'	Arch-amoAR 5'-CCC AAT GCA AAC CAT GCA CC-3'	200 nM	1x 95°C 2:00 min; 40x 95°C 0:05 min, 62°C 0:45 min
amoA	EvaGreen	Beta-amoA-m1-F 5'-TCG AAC AAG GTT CAC TCC GTA C-3'	Beta-amoA-m2-R 5'-ACA AAC GCT GAG AAG AAC GC-3'	200 nM	1x 95°C 2:00 min; 40x 95°C 0:05 min, 61°C 0:45 min
amoA	EvaGreen	Beta-amoA-O2-F 5'-ATT TGG ACC GAC CCA CTT ACC-3'	Beta-amoA-O2-R 5'-TAT GAC CAC CAA ACG TAC GC-3'	200 nM	1x 95°C 2:00 min; 40x 95°C 0:05 min, 60°C 0:45 min
nxrB	EvaGreen	NitrospiraG1-a-F 5'-TAT GGG GTG TTC GAA GGG ATG-3'	NitrospiraG1-a-R 5'-ATG TTC ACG AAG CGC CAT TC-3'	200 nM	1x 95°C 2:00 min; 40x 95°C 0:05 min, 67°C 0:45 min
nxrB	EvaGreen	NitrospiraG2-a-F 5'-ACG TCA AAA TCA CGC AGC TG-3'	NitrospiraG2-a-R 5'-CGG CAT CGA AAA TGG TCA TCC-3'	200 nM	1x 95°C 2:00 min; 40x 95°C 0:05 min, 65°C 0:45 min
amoA	EvaGreen	UWM_comammox_amoA_F1 5'-CGGACTACATGGGCTTTC-3'	UWM_comammox_amoA_R1 5'-GAGCCACTTCGATCATCC-3'	200 nM	1x 95°C 2:00 min; 40x 95°C 0:05 min, 59°C 0:45 min

Target Organisms	Betaproteobacteria AOB	Gammaproteobacteria AOB	Ammonia Oxidizing Archaea	Comammox amoA	Nitrobacter spp.	Non-Nitrobacter NOB	Archaea	Bacteria	UWM AOA - Total	UWM Nitroso - 1	UWM Nitroso - 2	Nitrospira nxrB uwm-1	Nitrospira nxrB uwm-2	Comammox Nitrospira amoA
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Comammox amoA PCR

To target comammox *Nitrospira amoA* for PCR and subsequent cloning and sequencing, *amoA* nucleotide sequences from van Kessel et al, 2015 and Daims et al., 2015 were aligned using MUSCLE (Edgar, 2004). The alignment was imported into EMBOSS to generate an *amoA* consensus sequence (Rice et al., 2000). Primer sequences were identified from the consensus using Primer3Plus (Untergasser et al., 2012), and the candidates along with the methane monooxygenase subunit A (*pmoA*) primers suggested by van Kessel et al., 2015, were evaluated against the consensus sequence in SeqMan Pro (DNAStar), using MUSCLE (Edgar, 2004). The *pmoA* forward primer (Luesken et al., 2011) and candidate primer COM_amoA_1R (this study; Table 3.1) offered the best combination of read length and specificity, and subsequently were used to amplify *amoA* genes from our samples.

Clone Library Construction and Phylogenetic Analysis

Multiple endpoint PCR approaches were used to investigate the nitrifying community composition of the RAS fluidized sand biofilter for *amoA* (*Gammaproteobacteria*, *Betaproteobacteria*, *Archaea*, and comammox *Nitrospira*), *nxA* (*Nitrobacter* spp.), and *nxB* (non-*Nitrobacter* NOB). The primer sets and reaction conditions used are listed in Table 3.1. All endpoint PCR reactions were carried out at a volume of 25 µl: 12.5 µl 2x Qiagen PCR master mix (Qiagen, Hilden, Germany), 1.5 µl appropriate primer mix (F&R), 0.5 µl bovine serum albumin (BSA), 0.75 µl 50 mM MgCl₂, and 1 µl DNA extract.

DNA samples of biofilter water and sand from four different rearing cycle time-points were used to construct clone libraries of archaeal *amoA* and *Nitrospira* sp. *nxB*. One sample from the center of the sand biofilter was used to construct clone libraries for betaproteobacterial *amoA* and comammox *amoA*. The center biofilter sample was chosen as it produced well-defined amplicons suitable for cloning target *amoA* genes. All PCR reactions for clone libraries were constructed using a TOPO PCR 2.1 TA cloning kit plasmid (Invitrogen, Life Technologies, Carlsbad, CA). Libraries were sequenced on an ABI 3730 Sanger-Sequencer with M13 Forward primers. Vector plasmid sequence contamination was removed using DNASTar (Lasergene Software, Madison, WI).

Cloned sequences of *Betaproteobacteria amoA*, *Archaea amoA* and *Nitrospira* sp. *nxB* from this study were added to ARB alignment databases from previous studies (Abell et al., 2012; Pester et al., 2012, 2013). Neighbor-Joining Jukes-Cantor (NJ-JC) corrected trees were created in ARB (Ludwig et al., 2004). Comammox *amoA* sequences from this study were aligned with those from van Kessel et al., 2015, Pinto et al., 2015, and Daims et al., 2015 using MUSCLE and imported into a new ARB database where the alignment was heuristically corrected before calculating an initial NJ-JC tree. A Maximum-Likelihood (ML) phylogenetic tree was calculated using RAxML on the Cipres Science Gateway (Miller et al., 2010; Stamatakis, 2014). Bayesian inference (BI) of phylogeny was carried out with instances of MrBayes on the Cipres Gateway, with the NJ-JC tree from ARB incorporated into a tree block within the input nexus file to reduce calculation time (Miller et al., 2010; Ronquist et al., 2012), using a significant posterior probability of <0.01. Consensus majority trees based on all three phylogenetic methods (NJ-JC, ML, and BI) were constructed using ARB's consensus tree algorithm (Ludwig et

al., 2004). Consensus trees were visualized with the Interactive Tree of Life (Letunic and Bork, 2011).

qPCR Assays for Target Marker Genes

Quantitative PCR assays were designed to differentiate two *Nitrospira nxrB* genotypes and two *Nitrosomonas amoA* genotypes in our system. Potential qPCR primer sequences were identified using Primer3Plus (Untergasser et al., 2012) on MUSCLE (Edgar, 2004) generated alignments in DNASTar (Lasergene Software, Madison, WI). Primer concentrations and annealing temperatures were optimized for specificity to each reaction target. Primers were checked using Primer-BLAST on NCBI to ensure the assays matched their target genes. The newly designed primers were tested for between genotype cross-reactivity using the non-target genotype sequence in both endpoint and real time PCR dilution series. After optimization, all assays amplified only the target genotype. Due to high sequence similarity between the two archaeal *amoA* genotypes (>90% identity) in our system, a single qPCR assay to target both genotypes was developed using the steps described above. The two closely related sequence types were pooled in equimolar amounts for reaction standards. A comammox *amoA* qPCR primer set was developed using the same methods as the other assays presented in this study. All assay conditions are listed in Table 3.1. All qPCR assays were run on an Applied Biosystems StepOne Plus thermocycler (Applied Biosystems, Foster City, CA). Cloned target genes were used to generate standard curves from 1.5×10^6 to 15 copies per reaction. All reactions were carried out in triplicate, with melt curve and endpoint confirmation of assays (qPCR standard curve parameters and efficiency are listed in Table S3).

Statistics and Data Analysis

Taxonomy-based data were visualized with heatmaps constructed in the R statistical language (R Core Team, 2014), by implementing functions from the libraries *gplots*, *Heatplus* from Bioconductor Lite, *VEGAN*, and *RColorBrewer*. MED nodes were used in all sample diversity metrics. The *EnvFit* function in the *VEGAN* (Oksanen et al., 2015) R package was used to test the relationship between RAS observational data and changes in the biofilter bacterial community composition. Pearson's correlations were calculated using the *Hmisc* package in R (Harrell, Jr. and Dupont, 2015) to test whether 16S rRNA, *amoA*, and *nxrB* gene copies correlated over time. Kruskal-Wallis rank sum tests were performed in the R base statistics package (R Core Team, 2014) to test whether the populations of the aforementioned genes were stratified by depth. The *ADONIS* function from *VEGAN* was used on the V4-V5 depth dataset to test the significance of the observed Bray-Curtis dissimilarity as a function of depth categorical factors, with *strata=NULL* since the same biofilter was sampled multiple times.

Biomass Model

To determine whether the observed ammonia removal could provide the energy needed to support the number of potential ammonia-oxidizing microorganisms (AOM) in the biofilter as quantified via qPCR, we modeled steady-state biomass concentration from measured ammonia oxidation with the following equation:

$$X_{AO} = \frac{\theta_x}{\theta} \left[\frac{Y_{ao}}{1 + \theta_{AO} \theta_x} \right] * \Delta S_{NH3}$$

X_{AO} is defined as the biomass concentration of ammonia oxidizers in milligrams per liter in previous models (Mußmann et al., 2011), however, in this study we converted to cells per wet gram of sand by identifying the mean grams of sand per liter water in the biofilter. θ_x is the mean cell residence time (MCRT) in days and was unknown for the

system. Θ is the hydraulic retention time in days, which, is ~ 9.52 min, or 0.0066 days in this system. Y_{AO} is the growth yield of ammonia oxidizers, and b_{AO} is the endogenous respiration constant of ammonia oxidizers, which were estimated as 0.34 kg volatile suspended solids (VSS)/kg NH_4^+-N and $0.15 d^{-1}$ from (Mußmann et al., 2011). ΔS_{NH_3} is the change in substrate ammonia concentration between influent and effluent in mg/L. To calculate X_{AO} , or biomass concentration, we used the mean cell diameter (0.96 μm) for *Candidatus Nitrosocosmicus franklandus* (Lehtovirta-Morley et al., 2016) to calculate the biovolume of a single cell, and used the conversion factor of $310 fg \cdot C / \mu m^3$ (Mußmann et al., 2011) to relate biovolume to endogenous respiration. The modeled biomass concentration was plotted vs. a range of potential MCRT for a RAS fluidized sand filter (Summerfelt, Personal communication). The results of all *amoA* qPCR assays were combined to estimate total ammonia-oxidizing microorganism biomass in copy numbers per gram wet weight sand. Modeled biomass was then compared to our AOM qPCR assay results. A commented R-script for the model is available on GitHub (<https://github.com/rbartelme/BFprojectCode.git>).

NCBI Sequence Accession Numbers

Bacterial V6, V4-V5, and Archaeal V6 16S rRNA gene sequences generated in this study are available from the NCBI SRA (SRP076497; SRP076495; SRP076492). Partial gene sequences for *amoA* and *nxB* are available through NCBI Genbank and have accession numbers KX024777-KX024822.

Results

Biofilter Chemistry Results

RAS operations data was examined from the beginning of a Yellow perch rearing cycle until approximately six months afterward. The mean biofilter influent concentrations of ammonia and nitrite were, respectively, $9.02 \pm 4.76 \mu\text{M}$ and $1.69 \pm 1.46 \mu\text{M}$. Biofilter effluent ammonia concentrations ($3.84 \pm 7.32 \mu\text{M}$) remained within the toxicological constraints ($<60 \mu\text{M}$) of *Perca flavescens* reared in the system. On occasion, nitrite accumulated above the recommended threshold of $0.2 \mu\text{M}$ in both the rearing tank ($0.43 \pm 0.43 \mu\text{M}$) and biofilter effluent ($0.73 \pm 0.49 \mu\text{M}$). No major fish illnesses were reported during the RAS operational period. Environment and operations data are listed in Table S1.

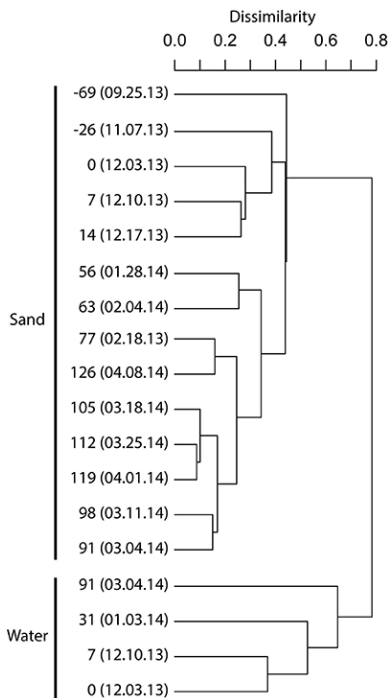


Figure 3.2 Dendrogram illustrating the bacterial community composition relationships among biofilter water samples

Bacterial and Archaeal Assemblages Within the Biofilter

The characterization of the RAS biofilter bacterial community revealed that both the sand-associated and water communities were diverse at a broad taxonomic level; 17 phyla averaged >0.1% in each of the biofilter sand and water bacterial communities (See Table S2 for complete sample taxonomic characterization). *Proteobacteria* (on average, 40% of biofilter sand community sequences and 40% of water sequences) and *Bacteroidetes* (18% in sand, 33% in water) dominated both water and sand bacterial communities. At family-level taxonomic classification, the biofilter sand-associated community was distinct from the water community. The greatest proportion of sequences in the sand samples were classified to the bacterial groups, *Chitinophagaceae* (mean relative abundance, 12%), *Acidobacteria* family unknown (9%), *Rhizobiales* family unknown (6%), *Nocardioideaceae* (4%), *Spartobacteria* family unknown (4%), and *Xanthomonadales* family unknown (4%), while the water samples were dominated by sequences classified to *Chitinophagaceae* (14%), *Cytophagaceae* (8%), *Neisseriaceae* (8%), and *Flavobacteriaceae* (7%). Using Minimum Entropy Decomposition (MED) to obtain highly discriminatory sequence binning, we identified 1261 nodes (OTUs) across the bacterial dataset. A MED-based bacterial community composition comparison (Figure 3.2) supported the patterns observed using broader taxonomic classification indicating that the biofilter sand-associated community was distinct from the assemblage present in the biofilter water.

In contrast to the large diversity in the bacterial community, we found the archaeal community to be dominated by a single taxonomic group, affiliated with the genus *Nitrososphaera*. This taxon made up >99.9% of the *Archaea*-classified sequences identified in the biofilter samples (Table S2). This taxon also was represented almost completely by a single sequence (>95% of *Archaea*-classified sequences) that was

identical to a number of database deposited *Thaumarchaeota* sequences, including the complete genome of *Candidatus Nitrosocosmicus oleophilus* (CP012850), along with clones from activated sludge, wastewater treatment, and freshwater aquaria (KR233006, KP027212, KJ810532-KJ810533).

Table 3.2 EnvFit - Eigenvector Correlations

Variable^{1,2}	Dim1	Dim2	R²	Pr(>r)
Days From Start	0.83616	0.54849	0.9425	0.002
Number of Fish	-0.83937	-0.54356	0.7719	0.024
Fish Mortalities	0	0	0	1
Culled Fish	0	0	0	1
System pH	-0.45394	0.89103	0.0342	0.911
Air Temperature	0.84354	0.53707	0.385	0.326
Water Temperature	0.75233	0.65879	0.6859	0.05
Conductivity	0.97036	-0.24168	0.8234	0.042
System Ammonia	0.65116	0.75894	0.5019	0.19
System Nitrite	0.82332	-0.56757	0.872	0.011
Biofilter PSI	0.47328	0.88091	0.7029	0.081
Biofilter Influent Ammonia	0.29711	0.95484	0.627	0.097
Biofilter Effluent Ammonia	-0.58214	0.81309	0.0333	0.949
Biofilter Influent Nitrite	0.68713	0.72653	0.6932	0.057
Biofilter Effluent Nitrite	0.78223	0.62299	0.8078	0.01
ORP	0.92752	-0.37378	0.8165	0.021
Feed Size	0.99105	-0.1335	0.8822	0.042
kg feed	0.7976	0.60319	0.4657	0.19
Percent Contribution ³	23.8	11.0	-	-

1. The V6 16S rRNA gene data were related to the system metadata in Table S1 using the VEGAN EnvFit function in R (1). Variables significantly influencing species composition are highlighted in grey.

2. Days From Start = Days following the start of a rearing cycle, Culled fish = the number of fish removed from the system up to the point of sampling, System pH = pH in the rearing tank, ORP = oxidation reduction potential, Biofilter PSI is the pressure within the biofilter manifold, in pounds per square inch.

3. Eigenvector percent contribution for the dimensions used in analysis

The initial biofilter community composition characterization revealed distinct communities between the biofilter sand and decanted biofilter water (Figure 3.2). Based on this data and that fluidized-bed biofilter nitrification occurs primarily in particle-attached biofilms (Schreier et al., 2010), we focused our further analyses on the biofilter

sand matrix. In the sand samples, we observed a significant change in bacterial community composition (MED nodes) over time (Table 3.2). The early portion of the study, which included a period while market sized Yellow perch were present in the system (sample -69 & -26), a fallow period following fish removal (sample 0), and time following re-stocking of mixed-age juvenile fish (sample 7 & 14), had a more variable bacterial community composition (Bray-Curtis mean similarity $65.2 \pm 6.5\%$) than the remaining samples (n=9) collected at time points after an adult feed source had been started ($20.0 \pm 6.4\%$, Figure 3.3). Several operational and measured physical and chemical parameters, including oxidation-reduction potential, feed size, conductivity, and biofilter effluent nitrite were correlated ($p < 0.05$) with the time-dependent changes in bacterial community composition (see Table 3.2 for environmental correlation results).

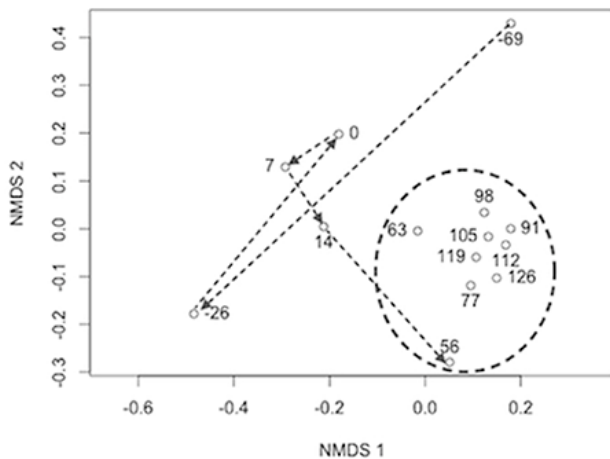


Figure 3.3 nMDS of Bray-Curtis Dissimilarity of Bacterial V6 Sequences at Biofilter Surface Processing Through Ordinate Space in Response to Perch Diet Change

Using a second sequence dataset (V4-V5 16S rRNA gene sequences), we examined the bacterial community composition associated with sand across a depth gradient (surface, middle, bottom). We found the bacterial communities in the top sand samples were distinct from those in the middle and bottom (ADONIS $R^2=0.74$, $p=0.001$; Figure 3.4). The *Planctomycetes* were a larger portion of the community in the surface

sand (on average 15.6% of surface sand vs. 9.6% of middle/bottom sand), whereas the middle and bottom layers harbored a greater proportion of *Chitinophagaceae* (7.4% in surface vs. 16.8% in middle/bottom) and *Sphingomonadaceae* (2.4% in surface vs. 7.9% in middle/bottom; Figure 3.4).

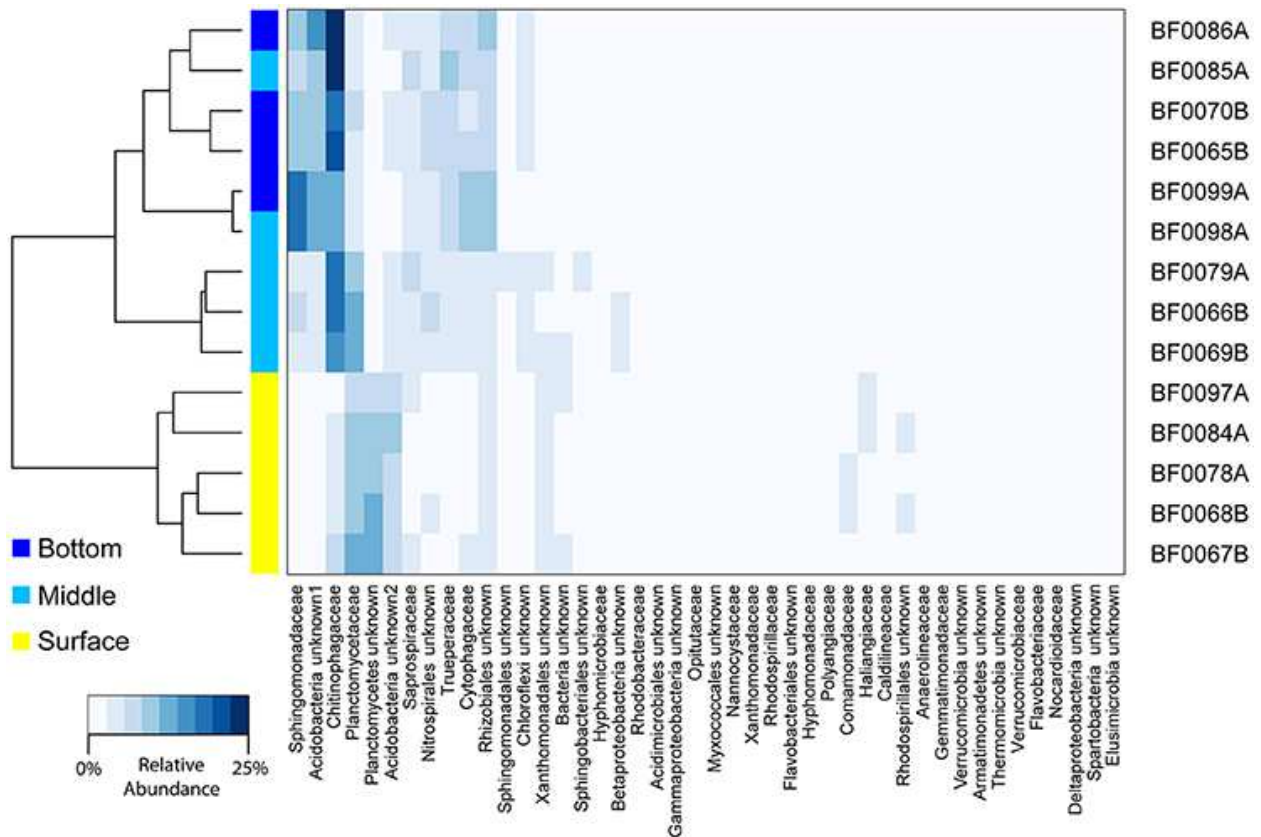


Figure 3.4 Depth Comparison of Bacterial Biofilter Community Composition

Nitrifying Community Composition and Phylogeny

The massively parallel 16S rRNA gene sequencing data indicated bacterial taxa not associated with nitrification comprised the majority (~92%) of the sand biofilter bacterial community. In contrast, >99.9% of the archaeal 16S rRNA gene sequences were classified to a single taxon associated with known ammonia-oxidizing archaea. Among the bacterial taxa, *Nitrosomonas* represented <1% of the total community across all samples and no *Nitrobacter* sequences were obtained. We also were unable to amplify

Nitrobacter nxrA genes (Figure S5) with a commonly used primer set (Poly et al., 2008; Wertz et al., 2008). In contrast, *Nitrospira* was fairly abundant, comprising 2-5% of the total bacterial community (Table S2).

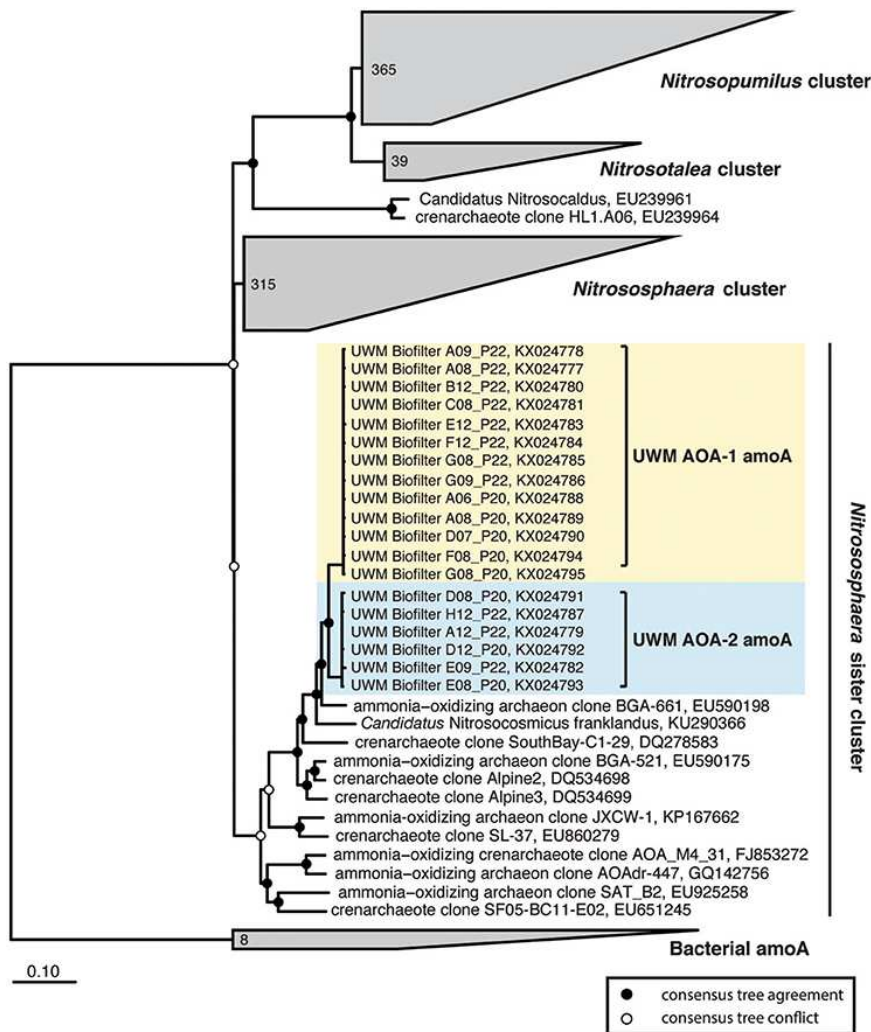


Figure 3.5 Ammonia-oxidizing Archaea Consensus Tree

In addition to the 16S rRNA gene community data, we amplified, cloned, and sequenced nitrifying marker genes representing the dominant nitrifying taxa in the UWM biofilter. The archaeal *amoA* sequences (KX024777-KX024795) clustered into two distinct genotypes, with an average nucleotide identity ranging from 97-99%. Both genotypes placed phylogenetically in the *Nitrososphaera* sister cluster (Figure 3.5), which includes the candidate genus, *Nitrosocosmicus* (Lehtovirta-Morley et al., 2016),

but the sequences were most closely related to the *amoA* genes from Archaeon G61 (97% nucleotide identity; KR233005). Sequenced amplicons for betaproteobacterial *amoA* (KX024803-KX024810) also revealed the presence of two AOB genotypes affiliated with *Nitrosomonas*. These *Nitrosomonas* genotypes were most closely related (99% identity) to environmental sequences obtained from freshwater aquaria and activated sludge (Figure 3.6).

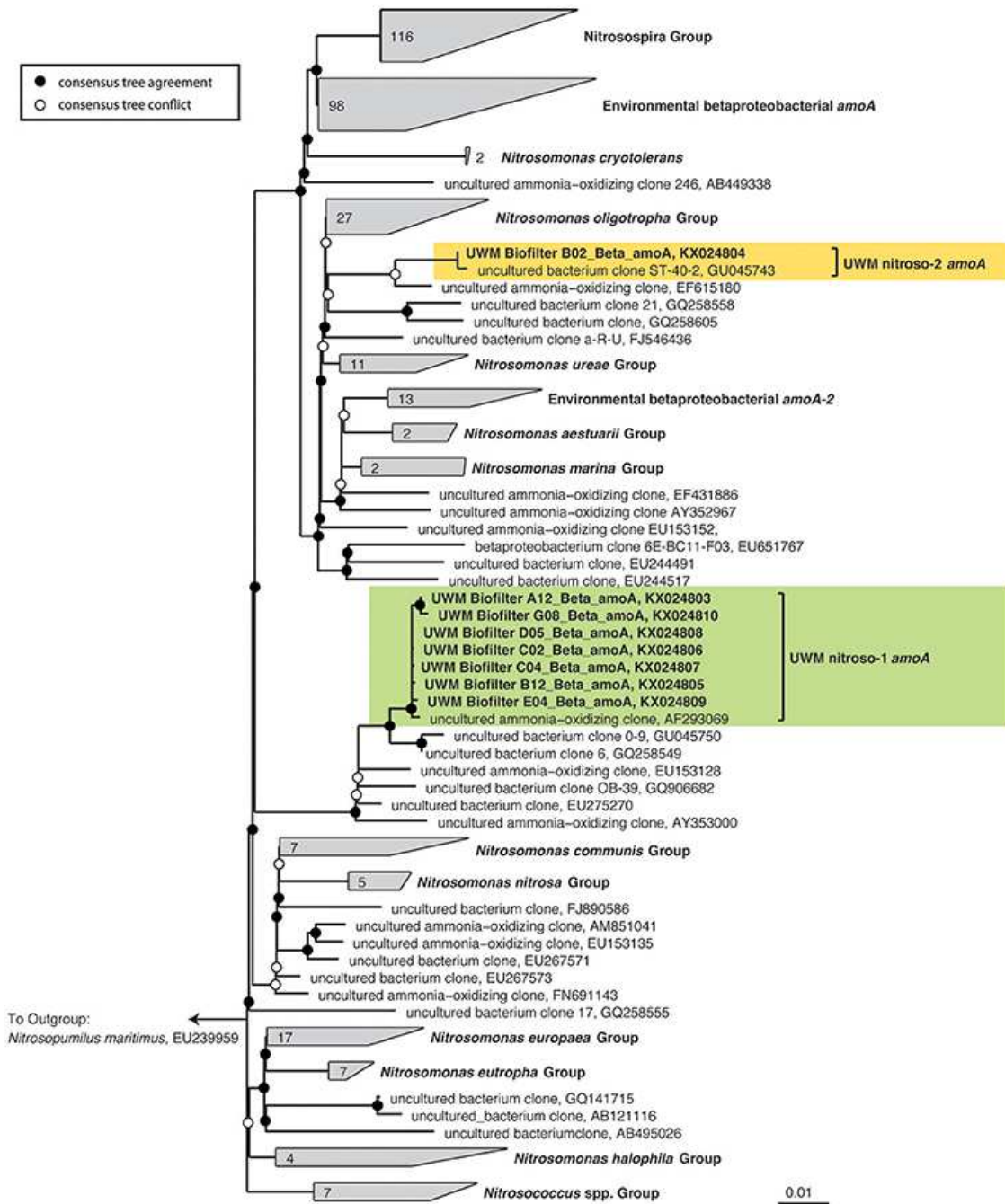


Figure 3.6 Ammonia-oxidizing Bacteria Consensus Tree

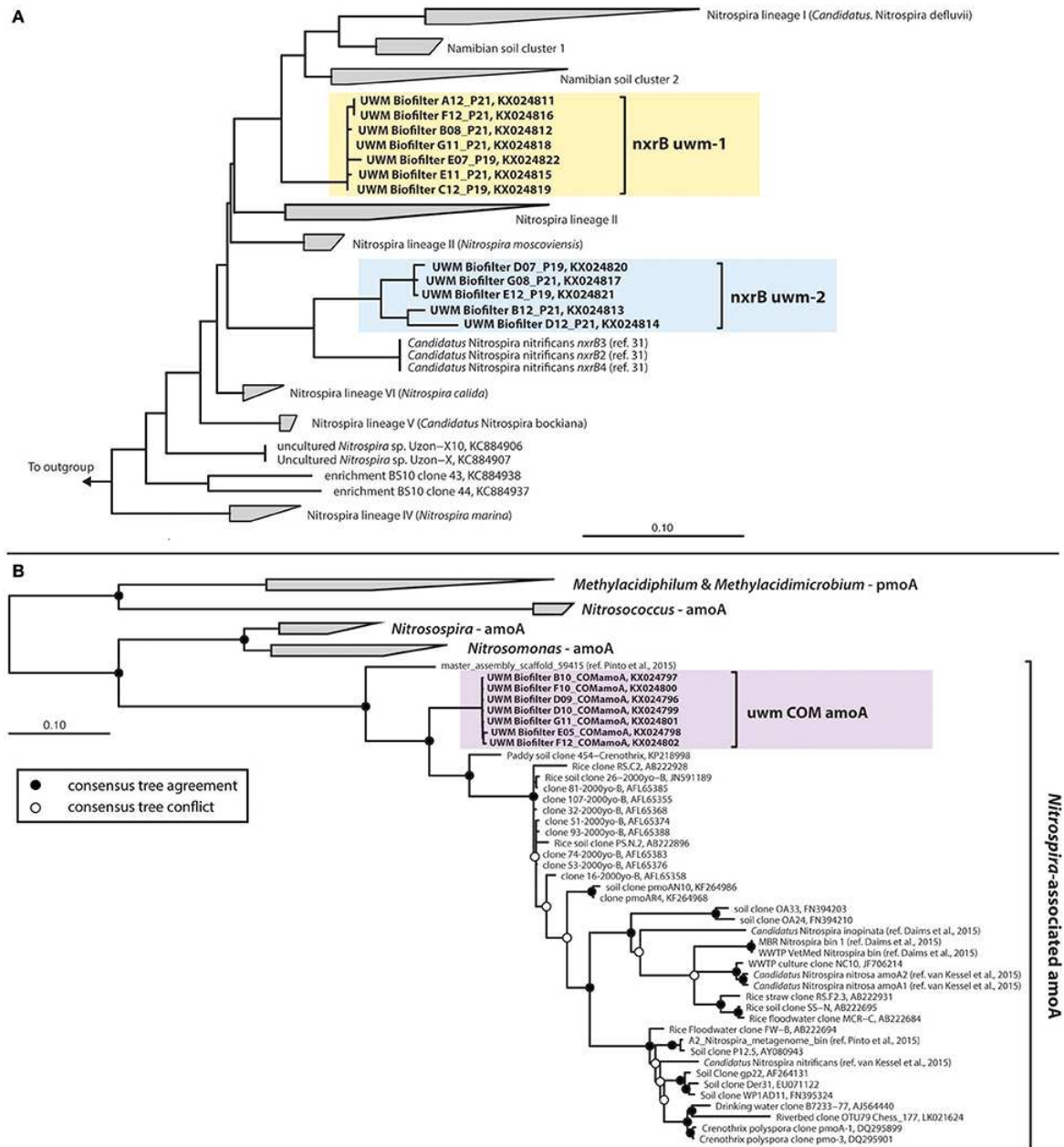


Figure 3.7 Consensus Trees for *Nitrospira*-like *nxrB* (A) and *amoA* (B) genes

The UWM biofilter sand also harbored two phylogenetically distinct and divergent clades of *nxrB* sequences (85-86% nucleotide identity between genotypes; KX024811-KX024822) affiliated with the genus *Nitrospira*. *Nitrospira nxrB* uwm-1 formed a clade distinct from cultivated *Nitrospira* spp. (~92% nucleotide identity to *Nitrospira bockiana*). *Nitrospira nxrB* uwm-2 clustered phylogenetically with *Nitrospira* spp., which have been implicated in complete nitrification (i.e. comammox) (Daims et al.,

2015; van Kessel et al., 2015) (Figure 3.7A). Because of the association of *Nitrospira nxrB* uwm-2 with comammox *nxrB* sequences, we further examined the biofilter for the presence of *Nitrospira*-like *amoA* genes. We subsequently amplified a single *Nitrospira*-like *amoA* out of the biofilter samples, and phylogenetic inference placed this *amoA* on a monophyletic branch with currently known *Nitrospira amoA* sequences, but in a distinct cluster (Figure 3.7B) with a drinking water metagenome contig (Pinto et al., 2015) and a “*Crenothrix pmoA/amoA*” Paddy Soil Clone (KP218998; (van Kessel et al., 2016)). A link to ARB databases containing these data may be found at https://github.com/rbartelme/ARB_dbs.

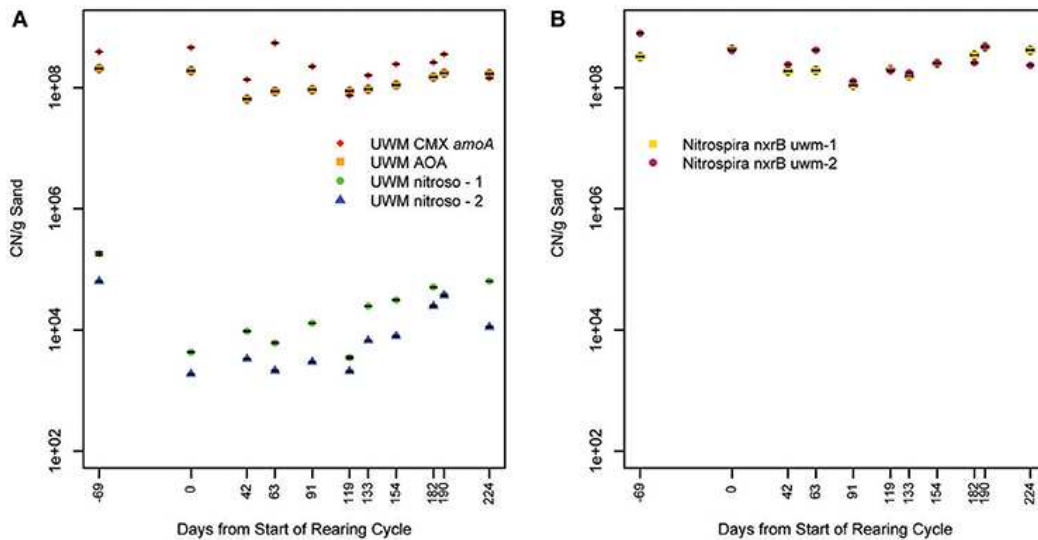


Figure 3.8 Nitrification Marker Gene Concentration Over Time (A) *amoA* (B) *nxrB*

Temporal and Spatial Quantification of Nitrification Marker Genes

We investigated the temporal and spatial stability of the nitrifying organisms in the UWM biofilter by developing qPCR assays specific to identified *amoA* and *nxrB* genes. Within the ammonia-oxidizing community, the AOA and comammox-*Nitrospira* (*amoA* assay) had space-time abundance patterns distinct from that of the *Nitrosomonas* genotypes. For example, the AOA and comammox-*Nitrospira* were numerically

dominant (range = 450-6500:1) to *Nitrosomonas* (combined UWM nitroso-1 & nitroso-2 genotypes) across all samples (Figure 3.8; Table 3). The AOA and comammox-*Nitrospira* also had more stable abundances over time (Coefficient of variation (CV) = 0.38 & 0.55 vs. 1.33 & 1.32 for nitroso-1 and nitroso-2; Figure 8), copy number concentrations that were less impacted by biofilter depth (Table 3), and comammox-*Nitrospira* were approximately 1.9x more abundant than AOA throughout the biofilter. Lastly, the two *Nitrosomonas amoA* genotypes exhibited a strong temporal abundance correlation (Pearson's R=0.90, pseudo p=0.0002) that was not shared with AOA or the comammox-*Nitrospira* (Pearson's R =0.65 & 0.69, and pseudo p=0.031 and 0.019, respectively).

Table 3.3 Nitrification Marker Gene Concentrations in Biofilter Sand

qPCR Assay ¹	Bottom (CN/g) ²	Middle (CN/g)	Surface (CN/g)	Significance ⁴
UWM AOA-Total (<i>amoA</i>) ³	2.1x10 ⁸ +/- 0.2x10 ⁸	2.6x10 ⁸ +/- 0.8x10 ⁸	1.0x10 ⁸ +/- 0.06x10 ⁸	$\chi^2=5.4$ & p=0.07
UWM Nitroso-1 (<i>amoA</i>)	4.6x10 ⁵ +/- 0.3x10 ⁵	3.6x10 ⁴ +/- 1.3x10 ⁴	4.5x10 ⁴ +/- 2.9x10 ⁴	$\chi^2=5.6$ & p=0.06
UWM Nitroso-2 (<i>amoA</i>)	2.0x10 ⁴ +/- 0.4x10 ⁴	4.0x10 ³ +/- 1.7x10 ³	3.5x10 ³ +/- 1.9x10 ³	$\chi^2=5.4$ & p=0.07
<i>Nitrospira nxrB</i> uwm-1	5.8x10 ⁸ +/- 1.0x10 ⁸	7.4x10 ⁸ +/- 3.9x10 ⁸	4.6x10 ⁸ +/- 1.3x10 ⁸	$\chi^2=2.3$ & p=0.32
<i>Nitrospira nxrB</i> uwm-2	4.9x10 ⁸ +/- 1.8x10 ⁸	4.6x10 ⁸ +/- 2.1x10 ⁸	4.2x10 ⁸ +/- 1.4x10 ⁸	$\chi^2=0.35$ & p=0.84
<i>Comammox (amoA)</i>	3.5x10 ⁸ +/- 0.7x10 ⁸	3.9x10 ⁸ +/- 1.0x10 ⁸	2.5x10 ⁸ +/- 0.9x10 ⁸	$\chi^2=1.7$ & p=0.43

1. Mean and standard deviation are listed.
2. Bottom, middle, and surface depth categories are defined as: surface (~1.32-1.42 m from biofilter base), middle (~0.81-0.91 m from biofilter base), and bottom (~0.15- 0.30 m, from biofilter base).
3. For *nxB*, n=4, and for *amoA* n=3. Corresponding samples are listed in Table S1.
4. χ^2 and P-values from Kruskal-Wallis Rank Sum assessment of depth as a significant factor in nitrification marker gene distribution.

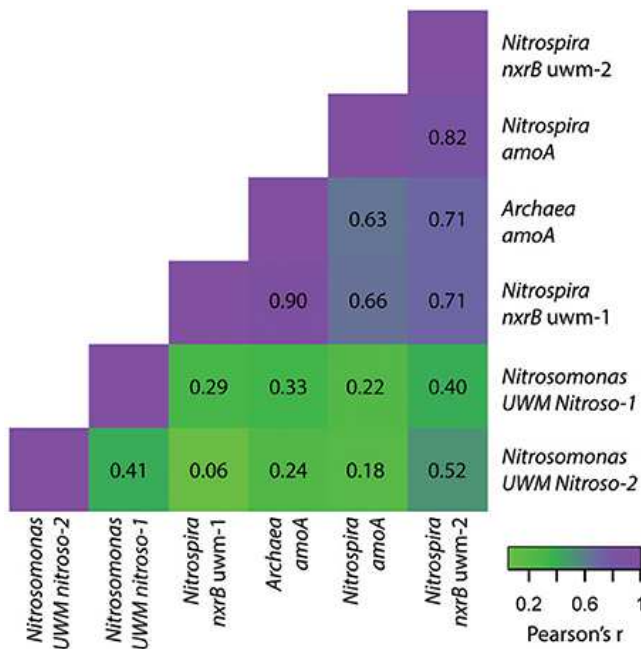


Figure 3.9 Heatmap of Abundance Pattern Correlations for Nitrifier Genotypes

Within the nitrite-oxidizing community, the abundance of both *Nitrospira* genotypes (*nrxB* uwm-1 & uwm-2) was in the range of 10^8 CN/g sand, and each exhibited temporal and spatial (depth) abundance stability (Table 3; Figure 3.8). The two genotypes also exhibited abundance co-variance across all samples (Pearson's $R=0.71$, pseudo $p=0.0002$). Despite these abundance pattern similarities, the two genotypes had differential associations with other nitrifying taxa marker genes. Genotype uwm-1, which is phylogenetically associated with strict nitrite-oxidizers, had strong abundance co-variation with the AOA *amoA* (Pearson's $R=0.90$, pseudo $p\leq 0.0001$), while genotype uwm-2 (phylogenetically associated with comammox-*Nitrospira*) had a stronger relationship to the *Nitrospira amoA* (Pearson's $R=0.82$, pseudo $p\leq 0.0001$; Figure 3.9).

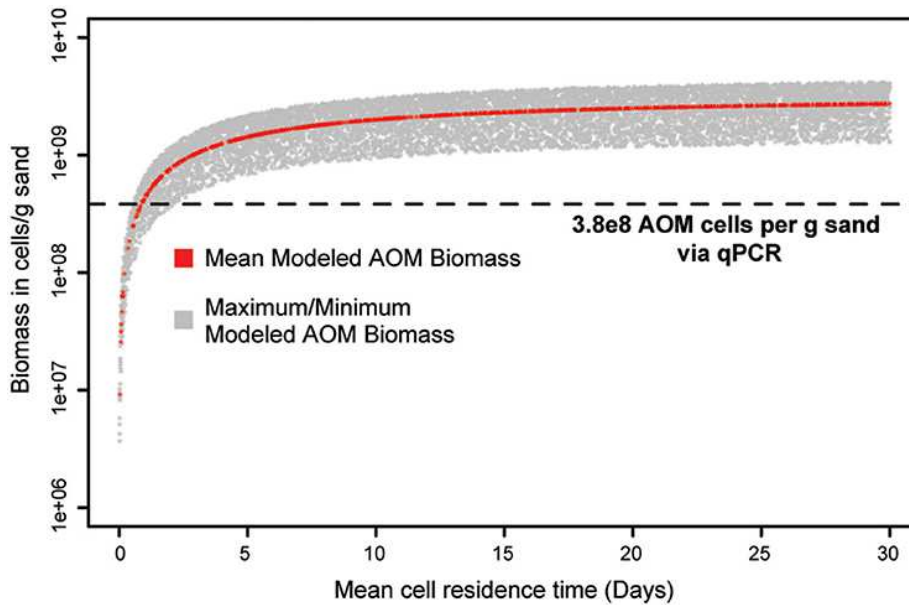


Figure 3.10 Model Output of Ammonia-oxidizer Cell Concentration as a Function of Biofilter Mean Cell Residence Time (MCRT)

Ammonia-oxidizing Microorganism Biomass Model

The estimated cell densities for ammonia oxidizers in the biofilter were modeled as a function of mean cell residence time (MCRT). Since the biofilter MCRT was unknown, a range of values (1-30 days) was used in the model. The model suggests the combined estimated ammonia oxidizer cell densities (*Nitrosomonas* + AOA + commamox-*Nitrospira*) could be supported by the ammonia oxidation observed, and in fact over-estimated these densities. For example, the model indicates ammonia oxidizer biomass reaches near maximum by a mean cell residence time (MCRT) of 20 days (Figure 3.10). At this 20-day MCRT, the model indicates the ammonia removal rate measured could support ~6.2X more cells than we observed (Figure 3.10).

Discussion

Biofilter Microbial Community Composition

In this study, we generated data that deeply explored the microbial community composition for a production-scale freshwater RAS nitrifying biofilter, expanding our understanding of the complexity of these systems beyond previous reports (Blancheton et al., 2013; Sauder et al., 2011; Sugita et al., 2005). This deeper coverage gave us the

power to examine temporal and depth distributions for both total bacterial and archaeal communities and the potential nitrifying member consortia therein. In previous studies of freshwater RAS biofilters, *Actinobacteria*, *Gammaproteobacteria*, *Plantomycetes*, and *Sphingobacteria* were identified as dominant taxa, while at more refined taxonomic levels *Acinetobacteria*, *Cetobacterium*, *Comamonas*, *Flectobacillus*, *Flavobacterium* and *Hyphomicrobium* were common (Sugita et al., 2005). All of these genera were present and relatively abundant (>0.5% total community; genus level taxonomic breakdown in Table S2) in our biofilter sand samples, suggesting there may be selection pressures for heterotrophs that act universally across systems. Some researchers have hypothesized that each RAS biofilter should have a unique microbial community composition shaped by operational controls and components implemented in the RAS (Sugita & Sugata 2005; Blancheton 2013). In support of this idea, many of the most abundant bacterial genera in our system (e.g. *Kribbella*, *Niabella*, *Chitinophaga*, *Byssovorax*, *Hyphomicrobium*) had not been reported as abundant in other systems. While it is likely true that each microbial community assemblage will be unique among RAS biofilters, i.e. each biofilter has a unique “microbial fingerprint”, the low number of RAS biofilters with community composition information to date and the low sequencing depth within existing studies, prohibits making robust comparisons across systems and identifying underlying community composition trends that relate to system operations.

Different components of RAS are expected to have unique environmental selective pressures, and thus multiple distinct microbial communities should be present within a single RAS. Our community data indicates there are consistent and significant differences in the biofilter sand and water communities. These differences included community members that were ubiquitous in, but nearly exclusive to the water samples.

These taxa could be remnant members derived from previous components in the system (e.g. rearing tank, clarifier), but the high shear force in a fluidized sand bed may make for inconsistent passage of these inflow microorganisms. The water samples also had decreased representation of prominent sand-associated taxa, including most known nitrifiers, so studies sampling biofilter outflow water would not represent accurately the microbial assemblages associated with nitrification. These observations support previous observations to the same effect, further lending support to the idea that a transient planktonic microbial assemblage is constantly moving through RAS components while an independent community develops on the biofilter media (Blancheton et al., 2013).

Our time series indicates RAS biofilter bacterial community composition change correlates with environmental parameter shifts related to fish growth (i.e. number of fish, water temperature, conductivity, oxidation-reduction potential, and feed size). This result is consistent with the hypothesis that biofilter bacterial community variation follows feed and fish growth driven shifts in the C/N ratio (Michaud et al., 2006, 2013). The community variability is seemingly confined to the non-nitrifying members of the biofilter, as the dominant nitrifying organisms changed little in composition or abundance over time. Sampling different depths in the biofilter revealed distinct microbial communities in each sand stratum, suggesting a potential partitioning across physical and chemical gradients within the biofilter. In contrast to the observed temporal variation, these differences were present both in the heterotrophic assemblages, and in the abundance of nitrifiers. It appears this biofilter maintains a stable, but depth partitioned nitrifying community in the midst of a shifting bacterial community, whose composition is linked to variation in nutrient inputs, ultimately stemming from the output of fish growth.

Generally, the RAS biofilter heterotrophic microbial community is viewed only as competing with nitrifiers for resources, and system design guidelines recommend operations based on this premise (Okabe et al., 1995). However, this view may confine further development of biofilter technology, as it is becoming apparent that the heterotrophic community context can play a broader role in nitrification. Our data clearly indicates the heterotroph community varies substantially during “typical” fish rearing cycles. It is possible under some scenarios that these changes could impact nitrification. For example, certain heterotrophs are known to enhance nitrification rates in *Nitrosomonas* and *Nitrobacter* bioreactors (Sedlacek et al., 2016). It is unknown whether these interactions extend to other ammonia and nitrite-oxidizing taxa or other systems, but the interplay between heterotrophs and nitrifiers as a means to enhance nitrification rates in RAS should be investigated. Further data across systems and over longer periods in a single system are also needed to bound “normal” vs. stochastic system variability and identify key taxa or community assembly principles governing RAS.

Nitrifying Consortia

Prior to metagenomic studies, members of a few bacterial clades were believed to be responsible for ammonia oxidation. The isolation of the first ammonia-oxidizing archaeon, *Nitrosopumilus maritimus*, altered global nitrification models (Könneke et al., 2005). Ammonia-oxidizing archaea (AOA) are ubiquitous in both natural and engineered environments and are seemingly differentiated by niche from ammonia-oxidizing bacteria (AOB) based on ammonia concentration, where AOA outcompete AOB at relatively low concentrations (Hatzenpichler, 2012). This relationship appears to extend to freshwater biofilters, as it was shown recently that AOA dominate in freshwater aquaria biofilters

when ammonia concentrations are low (<30 μM ; (Pester et al., 2011)). Our data support these previous findings, as AOA were 6×10^5 times more abundant than both *Nitrosomonas* genotypes in the UWM biofilter, which maintains similarly low influent ammonia concentrations (mean = 9 μM). AOA showed little abundance variation with depth or over time (<3X change) while *Nitrosomonas* exhibited an order of magnitude greater abundance during later periods in the fish rearing cycle and deeper in the biofilter (Table 3). System ammonia is highest late in the rearing cycle (Table S1) and presumably deeper in the biofilter, which is nearest to the influent ports.

Although AOA were numerically dominant over AOB, a presumed third ammonia-oxidizer was also present in the biofilter sand matrix. Identification of *Nitrospira*-like *amoA* (Figure 3.7B) in the biofilter and the strong correlation between the abundance of the *Nitrospira nxrB uwm-2* gene and this *Nitrospira amoA*, suggests a complete ammonia-oxidizing *Nitrospira* spp. resides in the UWM biofilter. In fact, we found that the comammox *amoA* was the most abundant ammonia-oxidizing gene in the biofilter (on average 1.9X that of AOA *amoA*). Similar to the AOA, the comammox *Nitrospira* exhibited little abundance variation with depth or over time, which suggests the AOA and comammox *Nitrospira* stably co-exist throughout this system. The comammox reaction is predicted to be competitive in systems with limited substrate influx, and comammox *Nitrospira* have proven to be common in drinking water systems (Pinto et al., 2015). Part of the initial discovery of comammox included a comammox *Nitrospira* from a RAS (van Kessel et al., 2015), but in the anoxic portion of a trickling biofilter. Thus, RAS biofilters, which often have a municipal water source and relatively low nutrient influx may be a common reservoir of comammox *Nitrospira* colonization.

The physiology of the UWM RAS biofilter AOA cannot be interpreted from our dataset, but both the AOA genotypes cluster phylogenetically within the *Nitrososphaera* sister cluster, which is represented mainly by cloned *amoA* sequences from soil, sediment, and some AOA associated with freshwater aquaria. Recently an organism given the name *Candidatus Nitrosocosmicus franklandus* (Lehtovirta-Morley et al., 2016) was isolated from the *Nitrososphaera* sister cluster. *Ca. Nitrosocosmicus* spp. appear to be suited to tolerate higher concentrations of ammonia and nitrite than other AOA, and are capable of ureolytic growth (Lehtovirta-Morley et al., 2016), both of which could be beneficial traits in RAS environments. AOA, now have been detected in freshwater, brackish, and saline RAS that also span a variety of cultured species, ranging from finfish to crustaceans (Sakami et al., 2012; Sauder et al., 2011; Urakawa et al., 2008). Given the common AOA dominance over *Nitrosomonas* in RAS nitrifying biofilters, including in our study system, a greater understanding of AOA ecophysiology is needed to understand how system designs could be used to maximize AOA capabilities.

Although AOA appear widespread in RAS biofilters, the presence of AOA with comammox *Nitrospira* in our system suggests understanding AOA physiology may be only a part of understanding RAS biofilter nitrification. It is clear this environment generally favors the proliferation of organisms thought to be high affinity, low substrate specialists and can support a complex nitrifying consortium. However, further work is needed to understand how ammonia-oxidation partitions between the various ammonia-oxidizers competing for substrate and how system operations can take advantage of potentially flexible ammonia-oxidizer physiologies.

In our system, we did not detect *Nitrobacter*, whose physiological constraints are often used when calculating RAS biofiltration capacity. Instead we identified *Nitrospira*

as the dominant nitrite-oxidizing bacteria (NOB). *Nitrospira* are generally considered *K*-strategist NOB favoring oligotrophic environments, while *Nitrobacter* are *r*-strategist copiotrophs (Nowka et al., 2015). *Nitrospira* uwm-1 exhibited a strong abundance pattern correlation with AOA, had abundances roughly equal ($\sim 10^8$ *nxB* CN/g sand) to that of the AOA, and clustered phylogenetically with known nitrite-oxidizing *Nitrospira*. Together, this suggests *Nitrospira* uwm-1 is the primary strict nitrite-oxidizing bacterium in this biofilter. The dominance of *Nitrospira* in this system and several other RAS (Auffret et al., 2013; Brown et al., 2013; van Kessel et al., 2010; Kruse et al., 2013; Schreier et al., 2010) indicates there is a versatile metabolic network driving RAS biofilter nitrification. For example, nitrite-oxidizing *Nitrospira* spp. possess a diverse array of metabolic pathways, and have been shown experimentally to hydrolyze urea and cyanate to ammonia, thereby initiating nitrification through cross-feeding with AOA/AOB. This process is counter to the supposed role of nitrite oxidizers solely as converters of nitrite to nitrate (Daims et al., 2016). Whether or not *Nitrospira* in RAS move nitrogen pools through these alternate pathways is not yet known.

Given the diversity of nitrifiers and burgeoning understanding of nitrifier metabolic flexibility, it is possible that some of the identified ammonia-oxidizing organisms in our system were not carrying out ammonia oxidation, as this scenario has been observed in municipal wastewater treatment systems (Mußmann et al., 2011). Our model indicates the measured ammonia removal could support the predicted ammonia-oxidizer biomass, and in fact overestimated the number of ammonia oxidizing cells present. This overestimation could be the result of the model's reliance on biomass production from traditional AOM metabolisms, which many not represent accurately biomass production from ammonia oxidation for metabolically flexible ammonia-

oxidizers or comammox *Nitrospira* (Costa et al., 2006). Also, the cell volume used in the model is based on measurements of *Candidatus Nitrosocosmicus franklandus*, a relatively small microorganism; thus differences in cell size across ammonia-oxidizing taxa also may be contributing to the overestimation of biomass. In order to accurately predict ammonia consumption to biomass production ratios, which are used to used to constrain biofilter design, future models will need to account for the substrate kinetic differences between ammonia oxidizer metabolic pathways, differences in cell size among taxa, and include an updated understanding of cross-feeding between AOM and NOB (Daims et al., 2016; De Schryver and Vadstein, 2014).

This study builds upon the accumulating body of evidence that biofilter microbial communities in freshwater recirculating aquaculture systems are dynamic, diverse, and more distributed by resource availability than is often considered in the design process. Our results along with others (Brown et al., 2013; Sakami et al., 2012) indicate the microorganisms carrying out nitrification in RAS are different than those used traditionally to model RAS nitrifying capacity. This disconnect suggests there is potential to further fine-tune biofilter design to take advantage of these newly discovered physiologies and alter start-up procedures so that animal production objectives are matched to the nitrifying microorganisms most capable of meeting those demands. Incorporating this knowledge would provide opportunities to develop new system operations, such as operating at a lower pH (Hüpeden et al., 2016), and could move system optimization beyond that bound by current nitrification models. Yet, many unknowns remain, including how differences in system scale, water properties, and system initiation with subsequent founder effects influence biofilter community composition, stability, and ultimately performance. Further use of microbial ecological

theory in aquaculture has the potential to extend RAS capabilities, identify currently unrecognized interactions between microorganisms and system design, and facilitate replicable zero discharge systems (De Schryver and Vadstein, 2014).

3.3 Loss of Comammox *Nitrospira* Genotypic Diversity from Recirculating Aquaculture System Biofilter Inoculum After Fallow Period

Introduction

Recirculating aquaculture systems (RAS) are on-land systems for cultivating fisheries products. Highly engineered componentry in RAS allow for waste remediation while maintaining lower water footprint than traditional methods (Timmons and Ebeling, 2013). One such apparatus in RAS is the biological filter, or biofilter, which is used to reduce accumulated nitrogen waste. Biofilters oxidize, ammonia—a major catabolic byproduct of fish protein catabolism, to nitrate. The nitrification process is essential to reducing ammonia and nitrite concentrations in RAS, as both nitrogen species are toxic to fish (Timmons and Ebeling, 2013).

Initiation and continuous operation of biofilters remains difficult (Badiola et al., 2012). Standard recommendation for biofilter initiation relies on one or two approaches: seeding the filter with an ammonia source and/or progressively adding fish to the system to gradually increase the ammonia loading rate (DeLong and Losordo, 2012). DeLong and Losordo hypothesized new media is too slick to form biofilms during initiation (DeLong and Losordo, 2012). However, DeLong and Losordo's postulation seems simplified when one considers media turbulence and sheer stress (Chen et al., 2006a). Additionally, at all stages of operation, biofilters are sensitive to organic carbon fluxes (Michaud et al., 2006,

2013). Organic carbon loading leads to an increase in heterotrophic bacterial growth, reducing nitrification rates and process efficiency (Michaud et al., 2013).

The difficulty of initiating RAS biofilters is further confounded by nitrification rate kinetic calculations. Biofilter design often considers the Monod kinetics of *Nitrosomonas* and *Nitrobacter* (Chen et al., 2006a). However, ammonia-oxidizing archaea (AOA) and complete ammonia-oxidizing *Nitrospira* may also play roles in ammonia-oxidation in biofilters (Bagchi et al., 2014; Bartelme et al., 2017; Brown et al., 2013; Sakami et al., 2012; Sauder et al., 2011). This is problematic given the half saturation constants (K_m) of AOA, comammox, and nitrite oxidizing *Nitrospira* are 0.5-2 orders of magnitude lower than those of *Nitrosomonas* or *Nitrobacter* (Kits et al., 2017).

Biofilter microbiome studies have been critical to understanding both heterotrophic bacterial association and nitrifying community composition. To investigate community assembly dynamics in biofilters, we used volumetric portions of biofilter material from a previously studied RAS biofilter containing a consortia of comammox, AOA, *Nitrosomonas*, and nitrite-oxidizing *Nitrospira* (Bartelme et al., 2017). Knowing a priori what the biofilter community should consist of, we were interested in whether reducing the scale of the filters would retain the genotypes of nitrifiers we previously observed along with the accompany concomitant heterotrophic bacteria. Additionally, we were interested in how commercial biofilter starter cultures compare in initiation time to using an inoculum from an active biofilter. Furthermore, a competition dynamic was tested using both the commercially available starter culture and an inoculum of biofilter material from the UWM RAS biofilter. This study seeks to address the applicability of scaled nitrifying reactors to maintain nitrifier populations, so in the event of RAS filter failure an appropriate inoculum may be used to restart the biofilter.

Materials and Methods

Lab-scale Fluidized-bed Biological Filter Set-up

Six lab-scale fluidized sand biological filters were designed, constructed, and operated at the University of Wisconsin-Milwaukee's School of Freshwater Sciences (UWM SFS). The UWM SFS RAS facility biofilter characterized in Bartelme, *et al.* 2017 was used as a reference system. For UWM SFS biofilter dimensions see section 3.1 of Chapter 3, or Bartelme, *et al.* 2017. Hydraulic analysis was used to design our lab-scale filters and reservoir to proportions representative of a small RAS (Summerfelt & Cleasby, 1996).

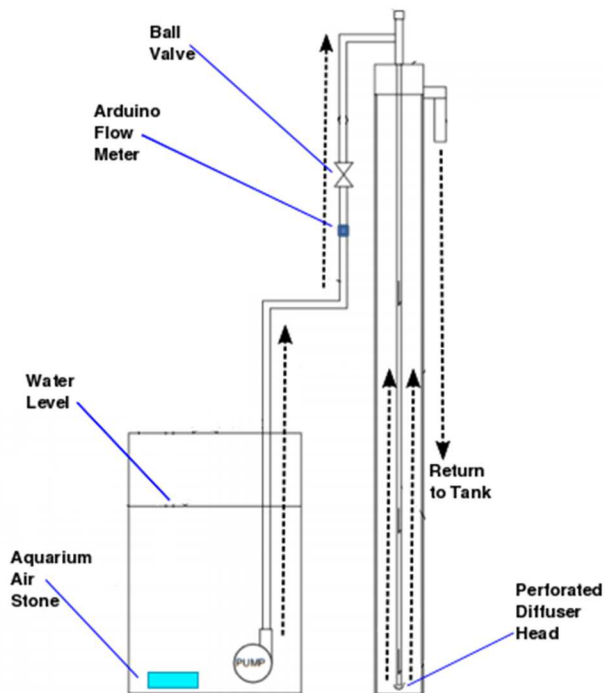


Figure 3.11 Lab-Scale Biofilter Diagram

The experimental system consisted of six separate 37 L biofilter vessels (Figure 3.11; dashed arrows represent direction of water flow), with water continuously pumped via a submersible pump. Each biofilter was connected to a 150 L tank representing the

rearing tank, where conditions were maintained by chemical addition (pH, ammonia, alkalinity) or physical control (pump, flow sensor, ball valve, check valve [not shown in Figure 3.11; upstream of ball valve], air stone). The list of materials used to construct the lab-scale biofilters may be found in Table 3.4. All six reactors were operated as fluidized-sand biofilters with the same silica filter substrate as the UWM SFS RAS (Wedron 510 100% silica sand). Each filter used 11.4 liters of silica sand as biofilter media. The tank water was re-circulated using a submersible pump with an upward flux of 7.08 gpm/ft² (0.47cm/s) resulting in ~100% volumetric expansion of the sand. The biofilters were operated at a flow rate of ~5.26 liters per minute and maintained a ~7.04 minute hydraulic retention time. Flow-rate was measured with an inline flow-meter (Adafruit, Product ID: 828) and an Arduino MEGA 2560 microcontroller (Adafruit Product ID: 191) implementing flow-rate code (https://github.com/rbartelme/LBF_code/). Lab-scale biofilter recycle rates were ~38 minutes, slightly faster than the UWM SFS RAS (~50 minutes).

Table 3.4 Lab-Scale Biofilter List of Materials

6" pipe x 6 ft	1
6" Diameter Pipe PVC 80" Length	1
6" Diameter Cap (Flat Bottom)	1
6" Diameter End Screw	1
6" Diameter Female Connection	1
3/4" Ball Valve Threaded Female Connection	1
3/4" Check Valve	1
3/4" Flow Control	1
3/4" 90 deg elbow	4
3/4" x 1/2" Male adaptor	1
1/2" Connection	1
1" Cap	1
1/2" x 1" Threaded Adaptor	1
1/2" Male Adaptor	1
1/2" Bulk Head	1
Flow Meter	1
Hose	2
Hose Clamps	4

Proof of Concept Experiments

After initial scaling of our laboratory biofilters, we conducted proof of concept experiments with varying inocula from the UWM SFS RAS biofilter. The three treatments were, by percent volume of silica sand, 100% UWM SFS RAS biofilter sand, 10% sand, un-inoculated sand. All treatments were run in duplicate biological replicate. Over the duration of these a continuous flow autoanalyzer, Seal AA3 (Seal Analytical, Mequon, Wisconsin, USA), was used to measure Ammonia and Nitrite concentrations, using manufacturer protocols G-102-93 and G-109-94 respectively. To only measure free nitrite concentrations, an in-line cadmium column was not used. A handheld probe, YSI 6-Series Sonde, was used to measure conductivity, pH, temperature, and dissolved oxygen at sampling times. Starting concentrations of 140 μM ammonium chloride and 140 μM sodium bicarbonate were respectively used as nitrification substrate and alkalinity.

Second Experiment Design and Sampling Regime

The results from the proof of concept experiments informed our decision to use a 10% (by volume sand) inoculum. In these experiments, treatments were again performed in biological duplicate with 10% UWM biofilter sand, 10% UWM biofilter sand plus starter culture, and fresh silica sand with the commercial nitrifying starter culture. Ammonia concentrations were measured using a Lamotte ammonia kit (Lamotte, Chesterpark, Maryland, USA) on ThermoFisher Scientific spectrophotometer, with a 1 cm path length cuvette. Nitrite was again measured using AA3 (Seal Analytical, Mequon, Wisconsin, USA) and the 6 series sonde was used to measure conductivity, pH, and dissolved oxygen.

Sequencing PCR and Illumina MiSeq Protocols

Bacteria V4-V5 16S rRNA gene PCR reactions were carried out in triplicate 33 μ l reactions, with Platinum HiFi taq polymerase as described previously (Huse et al., 2014b). These PCR reactions were pooled together, cleaned with AMPure XP beads (ThermoFisher, CITY, CA), and purified product concentrations quantified with Qubit (ThermoFisher, CITY, CA). Separate barcoding step was performed using Kapa Biosystems Hifi taq polymerase as described in the illumina MiSeq SOP. These sequences were clustered into amplicon sequence variants (ASV's) using minimum entropy decomposition with a minimum substantive abundance (-M) of 187 (Eren et al., 2015b).

A multiplexed illumina MiSeq assay surveyed the nitrification markers present in the biofilters across both experiments. Four gene targets were used in this assay, with primers modified to include illumina adapter sequences (Table 2.1). The V4-V5 region of the Archaea 16S rRNA gene, Betaproteobacterial *amoA*, and comammox *amoA* were used to identify genotypes of ammonia-oxidizing microorganisms (Table 2.1). There was no evidence of *Nitrobacter* spp. in the UWM RAS (Bartelme, et al. 2017); therefore the only *Nitrospira nxrB* nitrite-oxidation marker was used. Archaea V4-V5 16S rRNA genes were amplified in triplicate using previously described primers (Topçuoğlu et al., 2016), 2X Kapa Hifi Master Mix, in triplicate 20 μ l reactions, with 10-100ng template. The other three genes were amplified using the same reaction scheme. The triplicate reactions were pooled, and cleaned using AMPure beads according to manufacturers instructions. After pooling and clean-up, amplicons concentrations were quantified using QuantIT PicoGreen assay (ThermoFisher). Amplicon pooling prior to barcoding used template

concentrations of: 1.8 ng/μl Archaea 16S, Betaproteobacterial *amoA*, and *nxB*, while comammox *amoA* was added at 0.9 ng/μl to account for the shorter amplicon size.

Barcoding was performed according to the standard illumina MiSeq protocol.

Since the number of unique of amplicon sequence variants (ASV's) in Bartelme, *et al.* 2017 was low, fastq files were not only demultiplexed to their respective amplicon targets, but also underwent two rounds of sequence clustering. First, the multiplexed amplicon sequences were demultiplexed by sample name and primer set into their respective gene targets using mothur (Schloss et al., 2009). These fastq files were then processed using anvio 2.2's `anvi-script-reformat-fasta -simplify-names` before concatenating all samples into a single fasta file for each gene target. Initial clustering was conducted for each gene target using minimum entropy decomposition (MED) (Eren et al., 2015b). Resulting MED nodes (ASV's) were then clustered at 95% similarity in mothur to reduce noise. After 95% clustering, representative sequences were aligned to ARB databases from Bartelme, *et al.*, 2017. Sequences not falling into a previously defined clade of a particular gene were BLAST against the NCBI nucleotide database on default settings. All negative hits for gene targets were removed before downstream analysis and ASV's with an absolute abundance <100 were assumed to be effectively 0.

Statistical Analyses of Recovered Sequences

Statistical analyses were done in R using the VEGAN package (Oksanen et al., 2015). Overall similarity of Bacterial V4-V5 relative abundances across was determined using Bray-Curtis dissimilarity to construct a beta-diversity dendrogram. For each experiment's Bacterial V4-V5 dataset, nMDS were calculated using the vegan function `metaMDS` with `k=4` dimensions, `seed=444`, 999 permutations, and Bray-Curtis dissimilarity. Additionally, each nMDS was linked to the measured environmental

parameters using the vegan environmental fit function with `choices=1:4`, `na.rm=TRUE`, and `seed=444`. vegan's permutative ANOVA function, ADONIS, was used to determine the contribution of treatment effects to the recovered Bray-Curtis dissimilarity. Beta-dispersion was implemented to determine the likelihood two treatments share a homogenous beta-diversity pattern. If the beta-dispersion is not dissimilar, then one cannot reject the hypothesis the two communities share a beta-diversity centroid in ordinate space (Anderson, 2006; Anderson et al., 2006).

Results and Discussion

Hydraulic Analysis Results and Physiochemical Measurements

Conditions in experiment 1 were maintained at levels approximately those of the UWM RAS (water temperature $20.40 \pm 0.77^\circ\text{C}$, pH 7.5 ± 0.4 , dissolved oxygen 7.63 ± 3.53 mg/L, specific conductivity 1.45 ± 0.51 mS/cm). In the proof of concept experiments the 100% biofilter sand treatment removed all ammonia within 72 hours. The 10% sand condition filters oxidized ammonia at two different rates, one consumed all ammonia at the same rate as the 100% treatment, while the other took ~ 1.5 weeks. Whereas the null controls took ~ 1 month to consume all of the ammonia provided at the beginning of the experiment. In experiment 2 conditions were maintained similar to those of experiment 1, with the exception of a lower mean specific conductivity (water temperature $22.16 \pm 0.46^\circ\text{C}$, pH 7.17 ± 0.19 , dissolved oxygen 8.25 ± 1.48 mg/L, specific conductivity 0.10 ± 0.06 mS/cm).

Changes in Microbiome Composition by Treatment

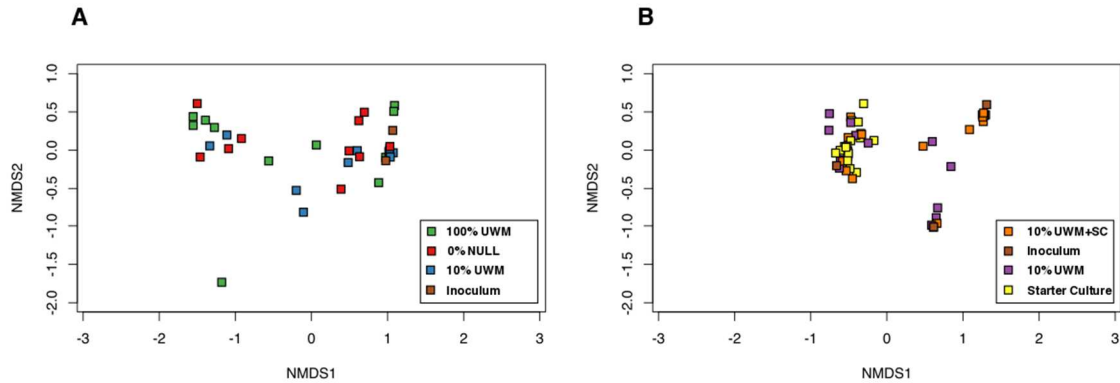


Figure 3.12 - nMDS plots of Lab-scale Biofilter Bacterial Amplicon Beta-diversity. Panel A represents Bray-Curtis Dissimilarity from the Proof of Concept Filters, where Green Squares indicate: 100% UWM RAS sand, Blue: 10% UWM RAS sand, Brown: inoculum samples, and Red: no inoculum control. Panel B represents the second experiment filters' Bray-Curtis Dissimilarity with the commercial starter culture competing against the UWM RAS sand. Orange: 10% UWM RAS sand + starter culture, Brown: inoculum samples, Yellow: Starter Culture + Null sand, and Purple: 10% UWM Sand.

When both experiments are considered as a single dataset, there is a significant treatment effect on recovered Bacterial V4-V5 beta-diversity (ADONIS of Bray-Curtis~treatment: $R^2 = 0.37428$, $P=0.001$). This is also apparent in the dendrogram pattern (Figure 3.13; node colors match nMDS plot keys). In experiment 2, it seems that the addition of a commercially available starter culture does not in fact help biofilter initiation. This is very apparent by the dendrogram branching (Figure 3.13) the sand+starter (yellow) clusters with a common root to the 0% NULL sand from experiment 1 (red). Moreover, the addition of the commercial starter culture to biofilters already containing 10% UWM sand reached equilibrium over the course of the second experiment (Figure 3.12B). There was no difference in beta-diversity between the 10% UWM treatment and the 10% UWM + Starter Culture treatment in experiment 2 (ADONIS: $R^2 = 0.0503$, $P=0.152$). It is also not possible to reject that the two 10% treatments from experiment 2 share a centroid (pairwise permutest of betadisper~treatment: $F=1.198$, $p=0.291$; Figure 3.14). The lack of significant difference of beta diversity and dispersion, may suggest that seeding with UWM biofilter material

leads to a priority effect governing community assembly (Fukami et al., 2010; Jiang and Patel, 2008). This is likely the first study to observe a priority effect while modeling RAS biofilter initiation.

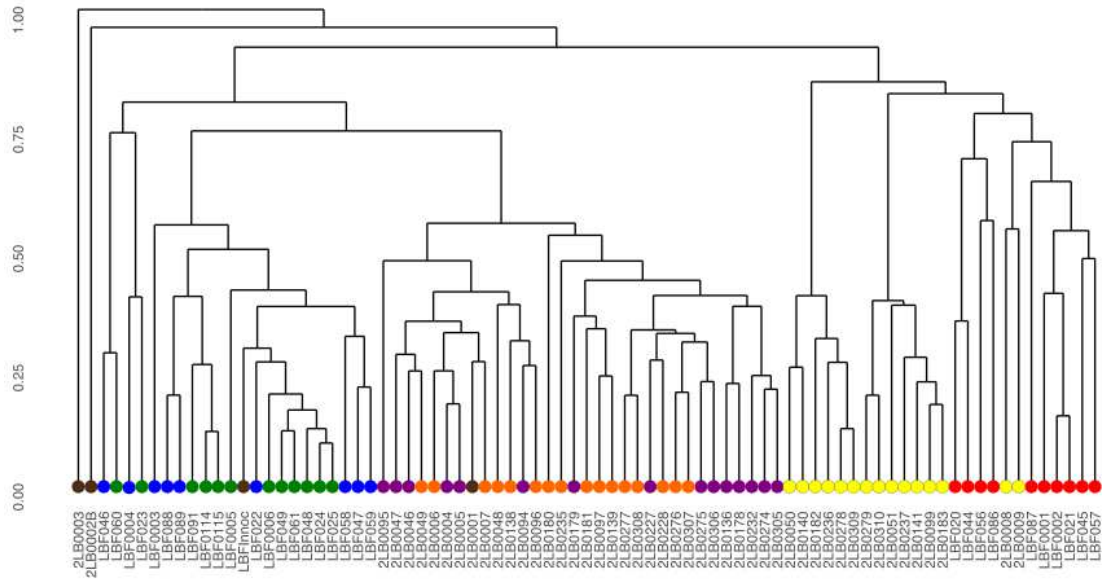


Figure 3.13 Bray-Curtis Dissimilarity + Average Linkage Dendrogram Across Lab-Scale Biofilter Experiments 1 and 2. Experiment 1 sample colors are as follows, Green: 100% UWM RAS sand, Blue: 10% UWM RAS sand, Brown: inoculum samples, and Red: no inoculum control Experiment 2 sample colors are as follows, Orange: 10% UWM RAS sand + starter culture, Brown: inoculum samples, Yellow: Starter Culture + Null sand, and Purple: 10% UWM Sand. The color codes also correspond to those used in Figure 3.12.

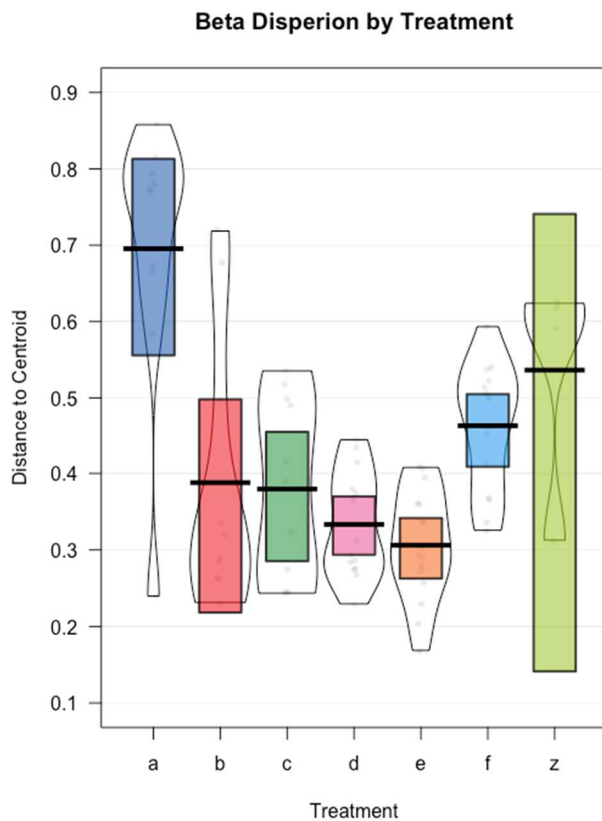


Figure 3.14 Distribution of Betadispersion Distance to Centroid by Treatment. a) 0% NULL b) 10% UWM c) 100% UWM d) 10% UWM-2 e) 10% UWM+Starter Culture f) Starter Culture+0% NULL z) Inoculum

Adding biofilter community to start the biofilters lowers the variability of change over time. This effect is independent of the experiment. When testing the dispersion of Bray-Curtis diversity around a centroid, there was no significant difference between the two experiments 10% UWM treatments (pairwise permutest of betadisper~treatment: $F=1.6692$, $p=0.225$; Figure 3.14) Therefore, the hypothesis that the two experiments' 10% inocula share similar dispersion patterns in ordinate space cannot be rejected, although overall there appears to be significant differences in community composition between experiments. The overall changes in dispersion (Figure 3.14) are most likely based upon the differences in starting conditions of the system, i.e. fish vs. no fish.

Varied Nitrification Genotype Recovery

Taking into account all experimental data, there is no correlation between AOA+nxrB presence/absence dissimilarities (Binary Jaccard). The null controls were too different to include in the analysis. When examining just the samples containing UWM SFS RAS biofilter material, the AOA+nxrB are strongly correlated (Mantel test, permutations = 999, spearman's rho = 0.455, p = 0.001). These results support previous work suggesting that AOA and *Nitrospira* dominate freshwater biofilters (Bagchi et al., 2014; Bartelme et al., 2017; Sauder et al., 2011). The comammox *amoA* sequences were difficult to recover from all biofilters with a UWM inoculum in the second set of experiments. However, all comammox *amoA* that were recovered (Figure 3.16) matched the clone sequences from Bartelme, *et al.* 2017. Betaproteobacterial *amoA* sequences were also difficult to acquire from the lab-scale biofilters, and those that were present more closely matched sequences from wastewater treatment plants than those found in Bartelme, *et al.* 2017.

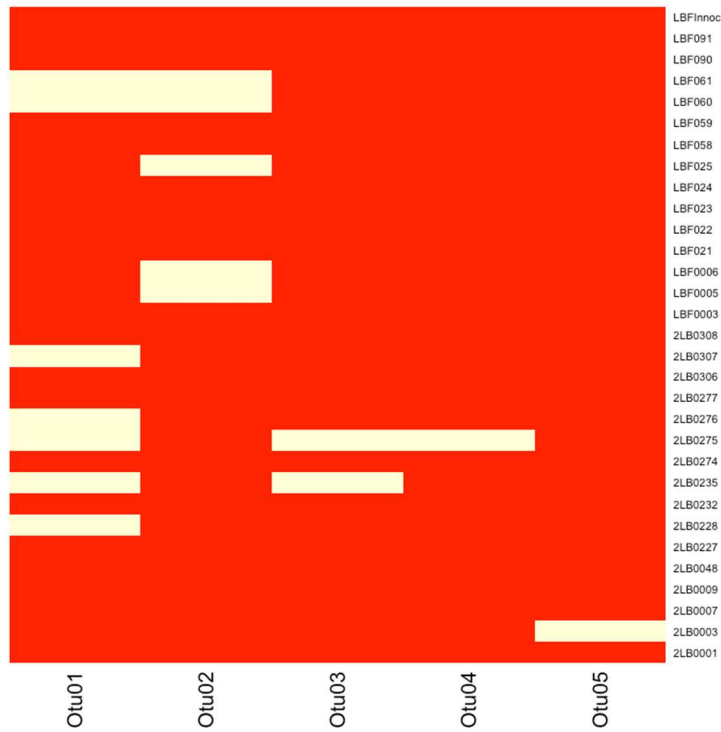


Figure 3.15 Betaproteobacterial *amoA* Presence-Absence Heatmap. Sample names are indicated on the y-axis labels, and operational taxonomic units (Otu's) are labeled on the x-axis. Red indicates the presence of an *amoA* genotype, while white indicates an absence of the genotype in that particular sample.

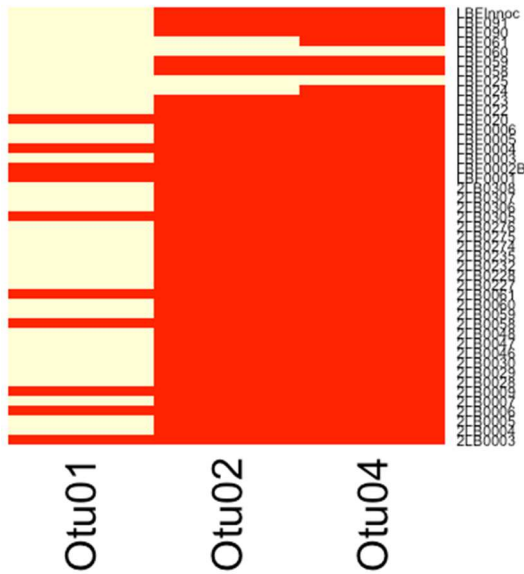


Figure 3.16 Comammox *amoA* Genotype Presence-Absence Heatmap. The y-axis labels denote the sample name, and the x-axis denotes the operational taxonomic unit (otu) of the *amoA* amplicon. Red indicates the presence of the otu sequence in a sample, while white indicates the absence of the otu.

Conclusions

In both the proof of concept experiments and the competition experiments, we see evidence of an inoculum priority effect on the trajectory of the lab scale biofilter community. In the proof of concept experiments we see a procession in time with the 10% inoculum filters resembling the 100% inoculum filters by the end of the experiment. And, although we saw populations of comammox *Nitrospira* disappear from the inoculum during the fallow period inoculation, we see the priority effect again in the competition experiment. The 10% inoculum community, and 10%+ starter culture reach an equilibrium and the dispersion of beta-diversity is also equivalent. This was also reflected in the ammonia-oxidation rate. These results indicate that if an idealized biofilter community is grown, in a bioreactor mimicking the turbulence and physiochemical constraints of design, the community may be maintained in some capacity. These results reflect the previous factors identified influencing nitrifier community assembly (Chen et al., 2006b). Furthermore, the standardized suggestions for biofilter initiation (DeLong and Losordo, 2012), may not be directly applicable to restarting filters after failure. This is particularly evident in how stochastic the 0% NULL community assembly was in experiment 1. Specifically applied to recirculating aquaculture, we recommend that facilities maintain a scaled bioreactor where 10% by volume of the original filter substrate may be withdrawn to restart the biofilter after a catastrophic failure. These recommendations are in line with the new decoupled aquaponic system proposed (Goddek et al., 2016), which could in turn be a system design worth exploring in RAS.

3.4 Enrichment of a Complete Ammonia-Oxidizing *Nitrospira* From the UW-Milwaukee Biofilter

Introduction

Canonical aerobic nitrification is facilitated as a two-step biogeochemical process facilitated by ammonia-oxidizing bacteria (AOB) or archaea (AOA), which oxidize ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) that oxidize nitrite to nitrate (Hatzenpichler, 2012; Klotz and Stein, 2008; Leininger et al., 2006; Sorokin et al., 2012). The classical two-step aerobic nitrification paradigm established by Winogradsky in the 19th Century was forever altered with the discovery of complete ammonia-oxidizing (comammox) bacteria (Bartelme et al., 2017; Costa et al., 2006; Daims et al., 2015; van Kessel et al., 2015; Kits et al., 2017; Pinto et al., 2015; Wang et al., 2017). The facilitation of nitrification by a single microorganism is predicted to come with certain caveats, such as a lower growth rate but a higher growth yield from ammonia-oxidation (Costa et al., 2006), but such attenuation would be advantageous in engineered applications, particularly those that rely on biofilm-based processes (Kreft, 2004). By catalyzing complete nitrification, COMAMMOX bacteria add a new dimension to aerobic nitrification, presenting both challenges and opportunities for managing nitrogen biotransformation in engineered systems and in biogeochemical modeling.

The greatest nitrogen removal energy cost associated with wastewater treatment plants is oxygen supply for nitrification and supplementation of electron donors for denitrification. Developing shortcut nitrogen removal processes for wastewater treatment such as utilizing anaerobic ammonia oxidation (ANAMMOX) by bacteria or heterotrophic denitrification downstream of ammonia-oxidizing bacteria can reduce aeration and electron donor requirements by 25-60% and 60-100%, respectively.

Complete ammonia-oxidizers have already been found in ANAMMOX reactors (van Kessel et al., 2015), so the future of applications of comammox bacteria represent a fundamental challenge to the theoretical underpinnings and operational benefits of shortcut nitrogen removal processes in WWTP.

These applications may be extended to other industries where nitrogen removal and/or management is important, such as recirculating aquaculture, or drinking water filtration systems (Bartelme et al., 2017; Pinto et al., 2015; Wang et al., 2017). Managing the impacts of complete ammonia oxidation to maximize the benefits in some systems while minimizing the deleterious impacts in others will require a detailed understanding of the ecology and physiology of COMAMMOX bacteria. To appropriately utilize comammox in engineered application and understand their ecology, it is necessary to combine studies of different environmental conditions, such as under oxic oligotrophic (Kits et al., 2017) or hypoxic conditions (van Kessel et al., 2015), with comparative genomic, evolutionary models, and bioreactor studies (Camejo et al., 2017; Palomo et al., 2016, 2017). What follows is a brief description of how the UWM comammox organism was enriched, sequenced via shotgun metagenomics, and placed in context with previous comammox assemblies.

Materials & Methods

UWM Comammox Nitrospira Enrichment

Enrichments were started from 3x500mL slurries of sand and water pooled together from a previously described recirculating aquaculture system's fluidized sand biofilter (Bartelme *et al.*, 2017). Mean *in situ* conditions of the biofilter water were 22.2±0.1°C, 8.2±0.2 mg/L O₂, specific conductivity of 0.33±0.0 mS/cm, and pH 7.92±0.01. Water was decanted from the pooled sand/water slurries, and 1%

weight/volume of sand was inoculated into NOB media with differing concentrations of nitrite (Nowka et al., 2015; Spieck et al., 2006). Originally, the enrichments targeting canonical *Nitrospira* were performed with two treatment conditions, however, comammox *Nitrospira* were abundant in both enrichments. One set of enrichments was grown with 100 μM NaNO_2 for 12 days, after which 200 μM NaNO_2 was used the duration of this enrichment. The second set of enrichments was incubated with 500 μM NaNO_2 . After an approximately 4 month enrichment period, ~ 160 mL of the 100/200 μM enrichment and ~ 35 mL of the 500 μM Enrichment were filtered onto 0.22 μm (47 mm mixed cellulose esters, EMD Millipore, Darmstadt, Germany), and frozen at -80°C prior to DNA extraction. Subsequently, the filters were sterilely macerated with a spatula, DNA was extracted using the MP Bio FastDNA® SPIN Kit for Soil (MP Bio, Solon, OH, USA) according to the manufacturer's instructions, with the exception of extending the bead beating step to two minutes. The DNA from the two enrichment conditions and two samples from Bartelme *et al.*, 2017 were sent to the Marine Biological Laboratory for shotgun metagenomic sequencing on an illumina NextSeq and use in downstream metagenome assemblies and analyses.

UWM Comammox MAG Sequencing, Assembly, Binning, ANVI'O processing, and Bin Extension

The extracted biofilter and enrichment DNA were sequenced on an illumina NextSeq 500 with 2x150 ungapped paired end reads. The UWM comammox bin was co-assembled from all UWM biofilter metagenome datasets in anvi'o (Eren et al., 2015a) using MegaHIT (Li et al., 2015a). The MegaHIT assembly was run with varying kmers from 21 to 99 selected in intervals of 5-mers, with a minimum assembly length of 1-2 kb.

Bowtie2 (Langmead and Salzberg, 2012) was used to map the reads to the assembled contigs, after which anvi'o was utilized for manual bin curation.

BLAST analyses of COMAMMOX *amoA* revealed that the *amoA* gene was truncated and therefore the metagenomic bin required refinement. The bin was enhanced *in silico* with PriceTI (Ruby et al., 2013) and the enrichment metagenome dataset with the most reads contributing to the comammox MAG (the 200 μ M NaNO₂ enrichment). *amoA* gene length was assessed before and after PriceTI enhancement with default Discontinuous MEGA-BLAST settings against a database of comammox clones from Bartelme, *et al.* 2017. PriceTI was run for fifteen cycles with a 600 bp target amplicon size, 98% identity, `-repmask 1 s 10 10 160 98` was employed to ignore all data constituting the first metagenome bin assembly, `-dbmax 160`, and `-lenf 600 0 -target 98 0`, and 30 processor cores. PriceTI reduced the number of contigs in the assembly by $\sim 1/3$; reducing the contigs count from 95 to 31 contigs.

Pangenomic Workflow:

Genomic content and open reading frames of complete ammonia-oxidizing *Nitrospira*, nitrite-oxidizing *Nitrospira*, and Betaproteobacterial ammonia-oxidizers were compared using the pangenomic workflow of anvi'o versions 2.2.2 through 4, (<http://merenlab.org/2016/11/08/pangenomics-v2/>). All open reading frame calls were made in anvi'o, and all protein annotation was done with anvi'o's blastp algorithm which matched translated ORF's to clusters of orthologous groups (COGS). Genomes were included from the aforementioned groups of bacteria if they were publicly available and were 80-100% complete by assessment with CheckM (Parks et al., 2015) and anvi'o (Table 3.5 values). The anvi'o analyses of genome completeness, size, GC content, and

redundancy are summarized in Table 3.5. A list of other genomes analyzed in this study may also be found in Table 3.5.

Table 3.5 Ammonia and Nitrite Oxidizing Bacterial Genomes

Genome Assembly	Genome Size (Mb)	Percent Complete	Percent Redundancy	GC%	Number of Genes	Average Gene Length	Genes per Kb	Environment	Citation
<i>Candidatus Nitrospira defluvii</i>	4.32	96.40	0.0	59.03%	4119	932	0.95	WWTP	Lücker <i>et al.</i> , 2010
<i>Candidatus Nitrospira inopinata</i>	3.30	96.40	1.4	59.23%	3191	921	0.97	Warm Pipe	Daims, <i>et al.</i> , 2015
<i>Candidatus Nitrospira nitrificans</i>	4.12	94.96	0.7	56.59%	4035	886	0.98	Aquaculture	van Kessel, <i>et al.</i> , 2015
<i>Candidatus Nitrospira nitrosa</i>	4.42	97.12	0.7	54.80%	4234	911	0.96	Aquaculture	van Kessel, <i>et al.</i> , 2015
<i>Nitrospira</i> CMX UWM	4.76	82.01	19.4	54.65%	4863	854	1.02	Aquaculture	Bartelme, <i>et al.</i> , 2017
<i>Nitrospira</i> SG bin1	4.42	94.24	2.2	56.08%	4480	861	1.01	DWTP	Wang <i>et al.</i> , 2017
<i>Nitrospira</i> SG bin2	3.66	97.12	1.4	56.77%	3796	849	1.04	DWTP	Wang <i>et al.</i> , 2017
<i>Nitrospira</i> ST bin4	2.94	94.96	2.2	57.02%	2983	865	1.02	DWTP	Wang <i>et al.</i> , 2017
<i>Nitrospira</i> ST bin5	4.01	96.40	0.7	58.02%	4016	894	1.00	DWTP	Wang <i>et al.</i> , 2017
<i>Nitrospira</i> sp CG24A	3.56	95.68	2.2	55.69%	3612	841	1.02	DWTP	Palomo <i>et al.</i> , 2017
<i>Nitrospira</i> sp CG24E	3.48	97.12	0.0	55.84%	3560	841	1.02	DWTP	Palomo <i>et al.</i> , 2017
<i>Nitrospira</i> sp CG24C	3.00	94.24	0.0	56.12%	2989	865	1.00	DWTP	Palomo <i>et al.</i> , 2017
<i>Nitrospira</i> sp CG24B	3.24	93.53	2.9	55.28%	3363	849	1.04	DWTP	Palomo <i>et al.</i> , 2017
<i>Nitrospira</i> sp CG24D	3.39	95.68	1.4	57.84%	3469	880	1.02	DWTP	Palomo <i>et al.</i> , 2017
<i>Nitrospira</i> comammox2 A2	4.18	94.96	8.6	58.13%	4697	781	1.12	DWTP	Pinto, <i>et al.</i> , 2015
<i>Nitrospira japonica</i>	4.08	97.12	1.4	58.96%	3894	942	0.95	WWTP	Ushiki <i>et al.</i> , 2013
<i>Nitrospira marina</i> NB295	4.69	94.24	0.0	50.04%	4129	941	0.88	Marine	Dupont, <i>et al.</i> , unpublished
<i>Nitrospira moscoviensis</i> strain NSP M 1	4.59	97.84	5.0	61.99%	4452	918	0.97	Warm Pipe	Spieck, <i>et al.</i> , 1998
<i>Nitrospira</i> sp Ga0074138	4.11	94.24	0.7	55.07%	4242	840	1.03	DWTP	Pinto, <i>et al.</i> , 2015
<i>Nitrospira</i> sp ND1	4.45	97.12	0.7	58.87%	4274	928	0.96	WWTP	Fujitani <i>et al.</i> , 2014
<i>Nitrospira</i> sp	3.75	91.37	1.4	60.36%	3747	913	1.00	WWTP	Speth <i>et al.</i> ,

OLB3 UZ03									2016
Nitrosomonas communis strain Nm2	4.07	97.84	1.4	44.73%	3697	870	0.91	Soil	Kozłowski <i>et al.</i> , 2016b
Nitrosomonas cryotolerans ATCC 49181	2.87	96.40	0.0	43.41%	2511	947	0.87	Marine	Rice <i>et al.</i> , 2017
Nitrosomonas europaea ATCC 19718	2.81	99.28	1.4	50.72%	2623	948	0.93	Multiple	Chain <i>et al.</i> , 2003
Nitrosomonas europaea isolate OLB2 UZ02	2.61	99.28	2.9	50.53%	2618	880	1.00	Multiple	Speth <i>et al.</i> , 2016
Nitrosomonas eutropha C91	2.66	97.84	1.4	48.49%	2551	909	0.96	WWTP	Stein <i>et al.</i> , 2007
Nitrosomonas sp AL212	3.18	97.84	0.0	44.84%	3009	914	0.95	WWTP	Yuichi <i>et al.</i> , 2011
Nitrosomonas sp Is79A3	3.78	97.84	1.4	45.44%	3492	931	0.92	Freshwater Sediment	Bollmann <i>et al.</i> , 2013
Nitrosomonas ureae strain Nm10	3.31	97.12	3.6	44.48%	3057	927	0.92	Soil	Kozłowski <i>et al.</i> , 2016a
Nitrospira briensis C 128	3.21	99.28	1.4	53.25%	2830	947	0.88	Soil	Rice <i>et al.</i> , 2016
Nitrospira multiformis ATCC 25196	3.18	99.28	1.4	53.94%	2889	949	0.91	Soil	Norton <i>et al.</i> , 2008
Nitrospira sp APG3	3.11	97.12	0.7	53.58%	2821	941	0.91	Freshwater Sediment	Garcia, <i>et al.</i> , 2013
Nitrospira sp NpAV	3.45	99.28	2.2	53.14%	3308	891	0.96	Soil	Norton <i>et al.</i> , 1996

Phylogenomic Analysis and Metabolic Pathway Prediction

To assess phylogenomic relationships between members of the genus *Nitrospira*, the *Nitrospira* core genome was identified in the anvi'o interactive view and exported using the anvi-export-pc-alignment function, skipping multiple gene calls, to generate an aligned concatenated amino acid fasta file. IQ-TREE (Nguyen *et al.*, 2014) was then used to construct a maximum-likelihood phylogenomic tree from the exported 190 aligned proteins. To place these comammox MAGs in context with Beta-AOB, open reading frames matching genes from a bacterial single copy gene hidden markov model (HMM; n=78) (Campbell *et al.*, 2013) were aligned for phylogenetic analysis. Individual phylogenetic trees were calculated using amino acid sequences with MrBayes 3.2

(Ronquist et al., 2012) on the CIPRES gateway (Miller et al., 2010). Members of Verrucomicrobia encoding a particulate methane monooxygenase, *Methylacidophilum fumariolicum* and *Methylacidophilum infernorum*, were used as outgroups in all AOB-comammox tree calculations. MrBayes tree quality was assessed with Tracer (Rambaut and Drummond, 2007) and RWTY (Warren et al., 2017). A species tree was then calculated from the individual HMM MrBayes gene trees using a Bayesian Coalescent model, BUCKy (Larget et al., 2010). To predict potential metabolic pathways, function, and completeness, individual anvi'o databases were constructed for all the genomes listed in Table 1. The ORF amino acid sequences from all AOB, comammox, and NOB *Nitrospira* were iteratively exported using a bash script within the anvi'o 4 virtual environment. These amino acid FASTA files were then annotated using GhostKOALA, which output a table of KEGG functions and metabolic pathway annotations. The GhostKOALA table was then translated into a heatmap of metabolic pathway completeness using Dr. Ben J. Tully's KEGGDecoder version 0.4 (<https://github.com/bjtully/BioData>).

Results & Discussion

Assembly Validation

The assembly of the UWM comammox MAG was validated using the anvi'o SOP, Sanger sequences from enrichment starting material, as well as NCBI BLAST against nitrification marker genes from previous studies (Daims et al. 2015, van Kessel, et al., 2015, Pinto et al., 2016, Bartelme et al., 2017). After enhancing the bin using PriceTI the UWM COMAMMOX MAG was ~85% complete according to the ANVI'O completeness algorithm. Bowtie2 was then used to remap reads to the UWM COMAMMOX MAG assembly. Redundant contigs were manually removed using

anvi'o's metagenome profile database visualization. Ultimately, the UWM MAG lacks the nitrification markers: *nxrA* and *amoB*.

Phylogenomics and Metabolic Features of the UWM Comammox MAG

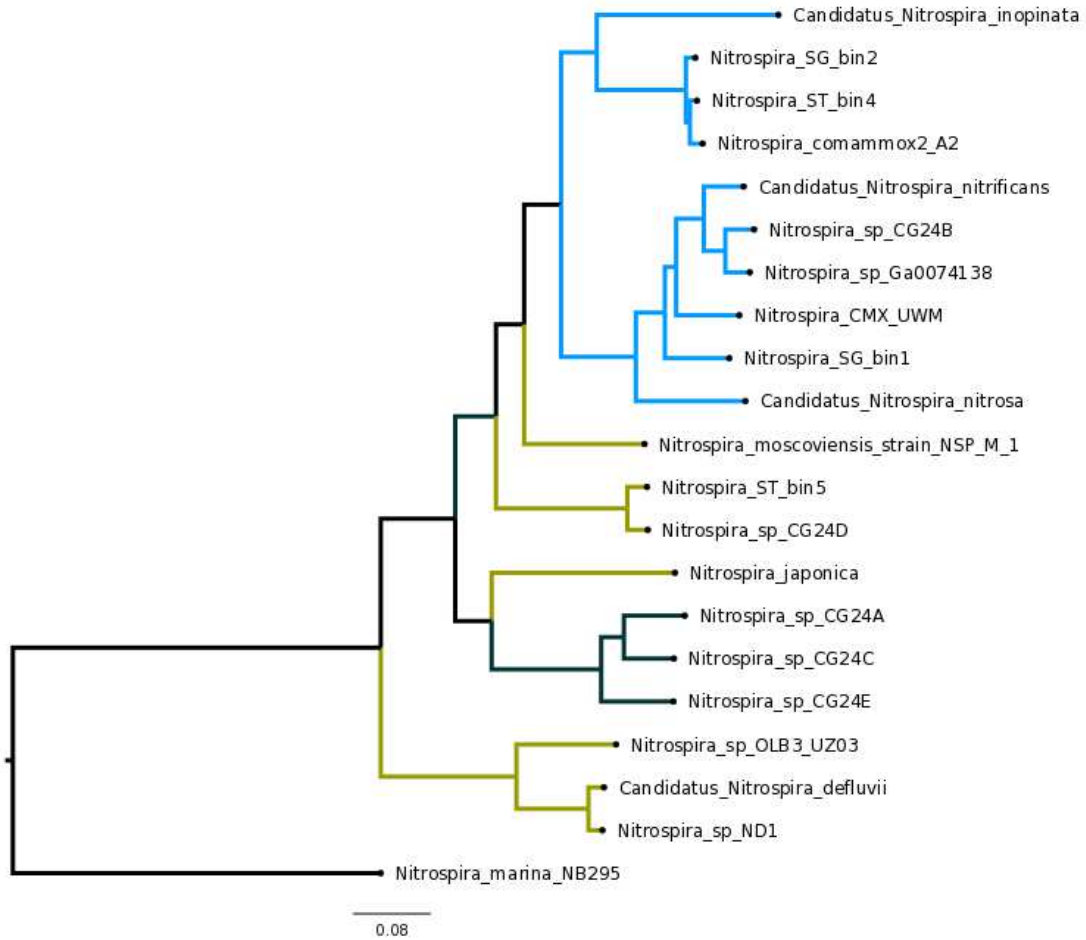


Figure 3.17 Concatenated *Nitrospira* Core Pangenome Maximum Likelihood Phylogenomic Tree

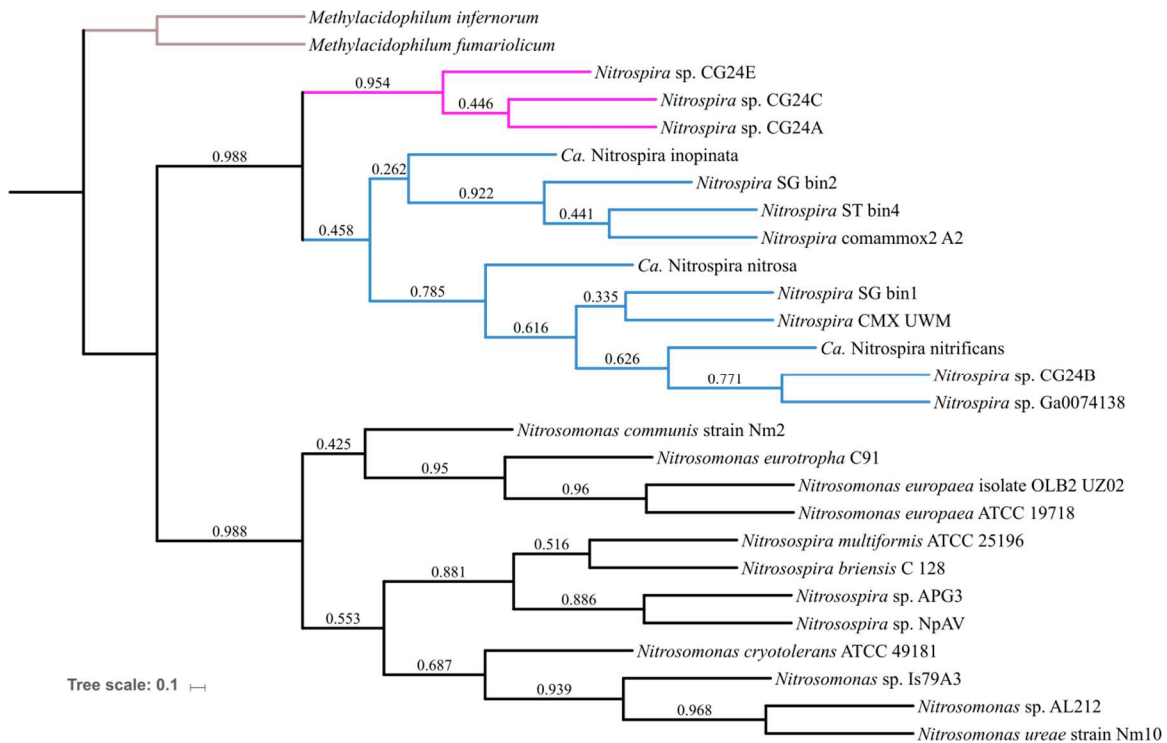


Figure 3.18 AOB & Comammox Bayesian Coalescent Species Tree

It is interesting that the UWM comammox MAG was assembled from a nitrite-oxidation enrichment. The coassembled MAG appears to be a Clade A comammox *Nitrospira* (Figures 3.17 & 3.18). This is the third Clade A comammox assembly from a recirculating aquaculture system, however, it is the first enrichment from the oxic portion of a biological filter. Interestingly, when we strictly compare the UWM comammox MAG to the other assemblies from aquaculture systems, *Ca. N. nitrosa* and *Ca. N. nitrificans*, one important metabolic pathway stands out. The UWM MAG encodes RuBisCo and a nearly complete Calvin-Benson-Bassham (CBB) cycle (Figure 3.19). Previous *Nitrosomonas europaea* work indicated the *cbb* operon is upregulated in response to low levels of CO₂, depending on cellular energy status (Wei et al., 2004). It is entirely possible that this same regulatory mechanism exists within the *in situ* UWM biofilter population of comammox. This may offer some explanation to the low diversity of comammox genotypes during the fallow period inoculum from Chapter 3, Section 3.2

compared to Section 3.1. However, the RuBisCo encoded by another *Nitrospira* was found to be a form IV molecule, and not involved in the CBB cycle due to a lack of key functional residues (Lücker et al., 2010). Regardless, little is known about commamox gene regulation mechanisms, so the gene regulation hypothesis and RuBisCo form IV function in commamox merit further study.

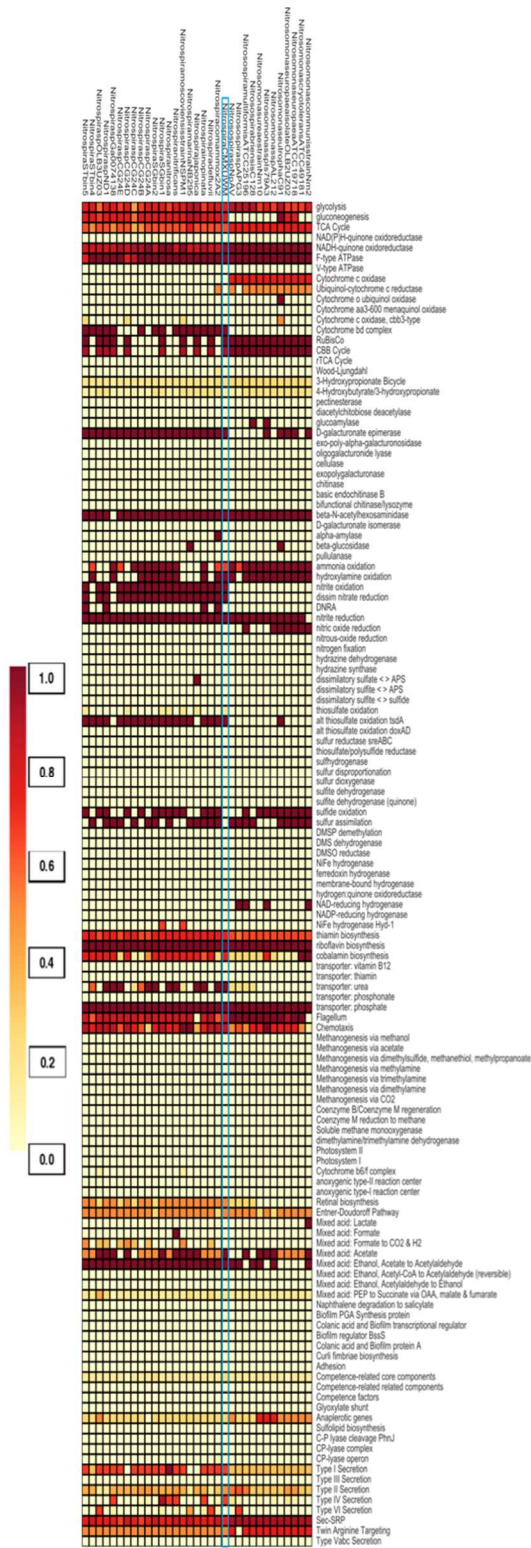


Figure 3.19 Heatmap of AOB and comammox Metabolic Pathway Completeness

4 *Flavobacterium columnare* – A Model Freshwater Fish

Pathogen

4.1 Review of *F. columnare* as an opportunistic fish pathogen in aquaculture

As of 2014, there are 51 fish diseases and pathologies recognized in the American Fisheries Society's (AFS) Blue Book of Fish Health. Bacteria cause approximately 30% of the diseases recognized by the AFS (AFS-FHS, 2014).

A gram-negative bacterial pathogen from the phylum Bacteroidetes, *Flavobacterium columnare*, is the causative agent of columnaris, or “fin rot”, in fish. In both wild ecosystems and intensive aquaculture systems, columnaris is a disease that infects a multitude of fish species causing deformation of gills and fins; and often results in death (Declercq et al., 2013; Durborow et al., 1998; Pulkkinen et al., 2010; Scott and Bollinger, 2014). Columnaris is of concern to aquaculturalists independent of system design and the persistence of these organisms majorly hinders crop yields. However, little is known about what causes this pathogen to bloom within aquaculture systems and even less is known about the ecological role *F. columnare* plays in the environment (McBride, 2014). Dead fish shed *F. columnare* at a high rate, after which, viable *F. columnare* cells persist for several months (Kunttu et al., 2009). To compound matters of microbial persistence, it is likely that *F. columnare* is not an obligate pathogen, and thus must have other means of surviving within an aquaculture system. Some Flavobacteria have been demonstrated to degrade dissolved organic matter (DOM), with an affinity for protein degradation (Cottrell and Kirchman, 2000). After cyanobacterial blooms die off in eutrophic lakes, the dead bloom enters the DOM cycle, where the recycling of proteins from the bloom detritus elicits an increased growth response within the Flavobacteria

community (Eiler and Bertilsson, 2007). It has been demonstrated previously that proteases, responsible for degrading the protein fraction of DOM, remain active after the death of bacterial populations (Kiersztyn et al., 2012).

Within closed loop agricultural systems, such as those found in recirculating aquaculture, undigested feed and feces likely become the primary source of DOM for organisms like *F. columnare*. It is unknown where opportunistic pathogens, like *F. columnare* reside in these systems, but they have been shown to persist for months (Kunttu et al., 2009). In Chapter 2 of this dissertation, there was evidence that *Flavobacterium* species are majority members of the planktonic community across multiple RAS. In *F. columnare*, we found the variable 6 region of the 16S rRNA gene to be a unique identifying sequence. Currently there are limited methods to specifically interrogate reservoirs of *F. columnare*, or observe population dynamics in response to elevated dissolved organic matter levels.

There are few methods that do not rely on traditional microbial culture methods to track the proliferation of *F. columnare* beyond an active outbreak. By relying on culture-based methods of detection, aquaculturalists are taking a reactive approach to pathogen management, rather than proactively managing their systems. Furthermore, these methods do not provide any means of tracking *F. columnare* back to the reservoir within an aquacultural system.

Strains of *F. columnare* are sub-classified into genomovars, which are analogous to “ecotypes” of other bacterial species. Genomovars are classified based upon 16S restriction fragment length polymorphism (RFLP) (Darwish and Ismaiel, 2005; Lafrentz et al., 2013; LaFrentz et al., 2018). Classification problems arise since, RFLP is highly variable and depends on agarose quality (Lafrentz et al., 2013). Recently 16S-RFLP was

combined with a comparative genomics approach (Kayansamruaj et al., 2017), where the authors noted that the RFLP assay failed to distinguish between genomovar II strains. In the same study, Kayansamruaj and colleagues stated the species *F. columnare* has an “open pangenome”, which is to say that as the number of *F. columnare* genomes sequenced increases there is an exponential increase in the number of genes called in each sequenced strain. Recently the term “genomovar” has been replaced with genetic group (LaFrentz et al., 2018). However, for the purpose of this dissertation the classic “genomovar” term will be used.

4.2 Strain selection for Transgenic *F. columnare* Experiments

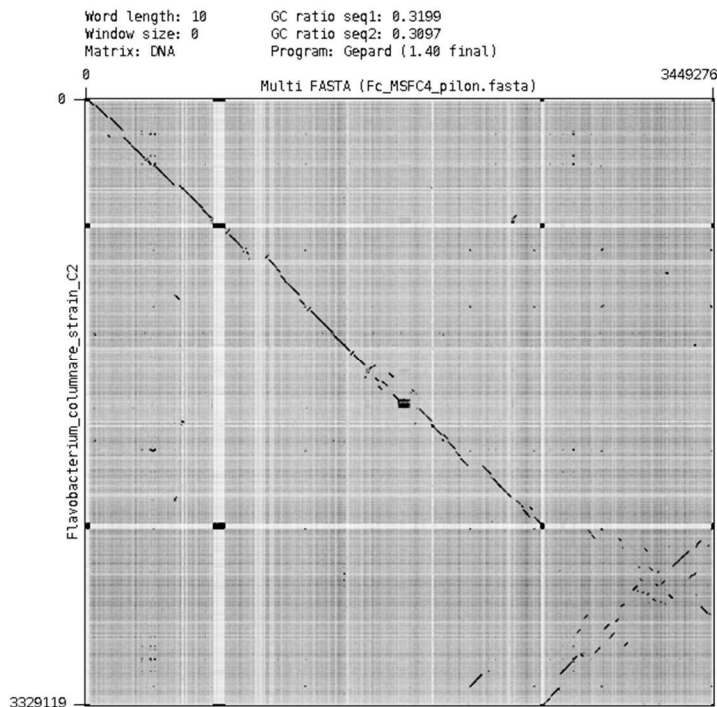


Figure 4.1 Dot Plot: Genome of *F. columnare* MS-FC-4 vs. C#2

As part of this dissertation, we sequenced two *F. columnare* genomes, a genomovar II strain C#2 (Appendix A) and a genomovar I strain MS-FC-4 (Appendix B). These two genomes share an average nucleotide identity of 91.18%, which is below the average nucleotide identity (ANI) threshold suggested for classifying bacteria to the species level

(Goris et al., 2007; Yoon et al., 2017). The two genomes share some amount of synteny (Figure 4.1). However, as mentioned in Appendix A, large chromosomal inversions occur within Flavobacteria as evidenced by the synteny fracturing in the lower right hand corner of Figure 4.1. Interestingly, despite syntenic differences, both C#2 and MS-FC-4 may be genetically manipulated (Appendices A & B, Li et al., 2015b). MS-FC-4 was chosen for further study, since it was isolated from rainbow trout (*Oncorhynchus mykiss*) (Evenhuis et al., 2016) and the preferred temperature range of trout (7-18°C) overlaps with that of yellow perch (17.5-25°C).

4.3 Construction of a Model GFP Transgenic *F. columnare* strain to Study Columnaris Infection

F. columnare MS-FC-4 was selected for its genetic malleability to develop a transgenic strain with green fluorescence protein (GFP). The integration of a functional GFP operon in MS-FC-4 would allow for rapid microscopy studies of infection localization. This transgenic strain may also develop a finer scale growth curve than those derived from turbidity or optical density, for use in growth substrate preference studies. To find an insertion site for the operon, it was determined that remnant bacteriophage regions of the genome would serve as the least metabolically disruptive site. Careful artificial operon construction would also ensure no frame-shift mutations were introduced into the genome. Conjugation between *E coli* and *F. columnare* MS-FC-4 was used to test the ability of MS-FC-4 to express GFP on a self-replicating plasmid according to previously published methods (Staroscik et al., 2008). The plasmid was selected for after conjugation using cefoxitin-resistance. MS-FC-4 colonies that grew on TYES media plus 10-µg/mL cefoxitin were tested for GFP expression using fluorescent microscopy (Figure 4.2).

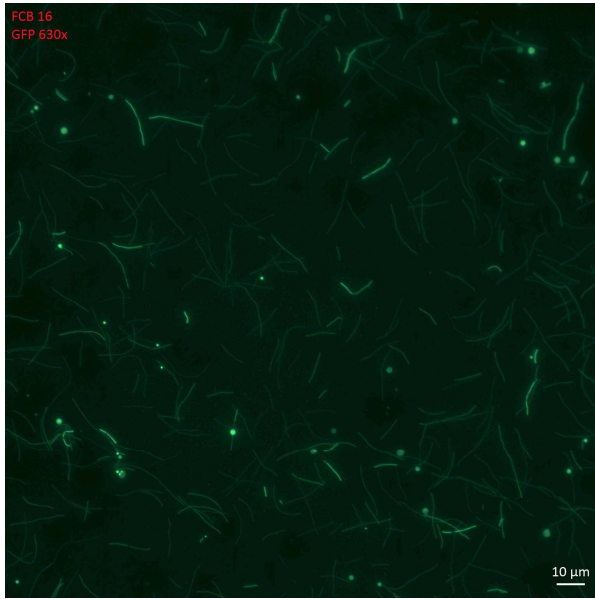


Figure 4.2 - *F. columnare* MS-FC-4 Expressing GFP from plasmid. 630X Magnification, GFP filter

After confirming the ability of MS-FC-4 to express GFP from a self replicating plasmid, the MS-FC-4 draft genome was analyzed for phage sites to insert the GFP operon using PHASTER (Arndt et al., 2016). After identifying the prophage region in the genome, primers were designed to amplify the upstream and downstream regions for double recombination of the GFP operon into the genome.

Table 4.1 Primer Sets Used to Construct Suicide Vector with Artificial *gfp* operon for Double Recombination in *F. columnare* MS-FC-4

Target	Forward (5'-3')	Reverse (5'-3')	Product Size (bp)
<i>F. johnsoniae ompA</i>	<u>Primer 2082:</u> CAGCTTTTCTTTTACTAAT TGCTCGGCAGCGCATACCA AAGAAC	<u>Primer 2083:</u> GCTAGGGATCCTTTTTTTA ATTACAATTTAGTTAATTA CAAGC	~208
<i>F. columnare</i> MS-FC-4 Upstream Prophage Region	<u>Primer 2080:</u> GCTAGGGTACCCATATTGG ATAGTTCAGTTAGGAAA	<u>Primer 2081:</u> GTTCTTTGGTATGCGCTGC CGAGCAATTAGTAAAAGA AAAAGCTG	~2675
Primers to clone <i>gfp</i> from pAS43	<u>Primer 2086:</u> GCTAGGGATCCATGAGTAA AGGAGAAGAACTTTTCAC	<u>Primer 2087:</u> GCTAGGTCGACCAGATCT ATTTGTATAGTTCATCCA	~1000
<i>F. columnare</i> MS-FC-4 Downstream Prophage Region	<u>Primer 2084:</u> GCTAGCTGCAGGAACGTTT TATTACTCTATAAAAACC	<u>Primer 2085:</u> GCTAGGCATGCCGAGAATT TGCCTTGATGATTTTATC	~2241

First, a 2475 bp region upstream of the identified prophage site was PCR amplified (primers 0280 & 2081, Table 4.1). This reaction was done using 30 ng of MS-FC-4 gDNA or 200 ng gDNA as template in 25 μ l volumes with final concentrations of: 1x Phusion Master Mix, 0.2 μ M F+R primer. The thermocycling conditions were as follows: 1x 30 sec 98°C, 25x 10 sec 98°C, 30 sec 58°C, 1 min 20 sec 72 °C, 1x 5 min 72°C. The PCR products from this reaction were excised from the gel and purified before downstream use. *ompA*, a strong promoter from *F. johnsoniae*, was amplified as described previously (Table 4.1, primers 2082 & 2083; Li et al., 2015b). Then the upstream region and *ompA* were fused into an artificial promoter using overlap PCR (Zhu et al., 2017). The complement of the reverse primer used to amplify the upstream prophage region was used as the forward primer in the overlap PCR and the *ompA* amplicon served as an equivalent to the reverse PCR primer. After confirming the success of the overlap PCR on an agarose gel (using primers 2080 & 2083, Table 4.1), the newly constructed promoter region was ligated into the restriction digested pBFc2 suicide vector plasmid. The *gfp* gene was then amplified from the plasmid used in the proof of concept experiments using previously published primers (primers 2086 & 2087, Table 4.1; Staroscik et al., 2008), the pBFc2 plasmid was restriction digested, and GFP was ligated into the suicide vector (now termed pBFc4, which is pBFc2+GFP). Finally, the region downstream of the prophage region was amplified with a different primer set under the same conditions as the upstream region. The suicide plasmid was restriction digested downstream of the artificially constructed *ompA+gfp* operon. The cleaned up PCR amplicon of the region downstream of the prophage (primers 2084 & 2085, Table 4.1) was then ligated into the plasmid before the conjugation and double recombination events. The double recombination methods used were identical to those from Li, *et al.*,

2015b (i.e. antibiotic selection, then sucrose sensitivity selection). Except, rather than simply removing the prophage; the *ompA+gfp* operon was inserted, in-frame, within the identified prophage region. After selecting the mutants from sucrose plates growth curves were generated from triplicate cultures of MS-FC-4+*gfp* and MS-FC-4 *wt*. There was no significant difference in growth rate between the *wt* and *gfp* transgenic strains.

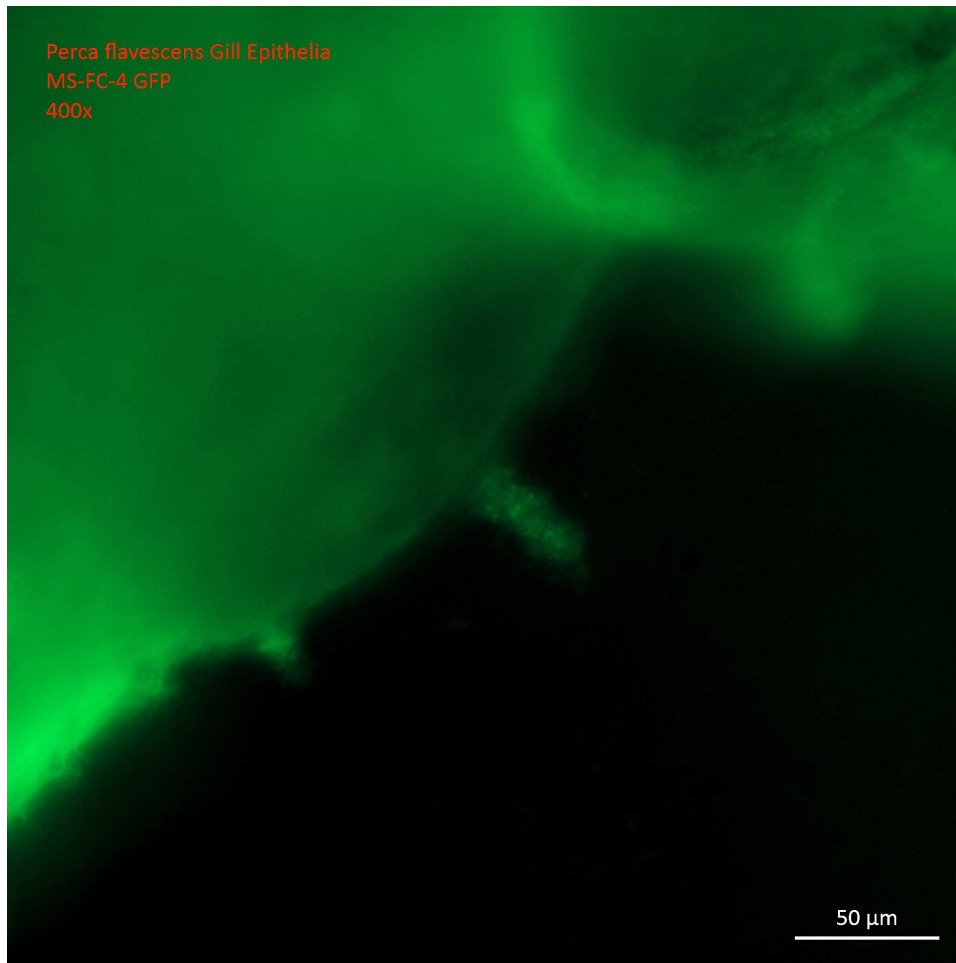


Figure 4.3 Columnar Aggregates of MS-FC-4+*gfp* on *Perca flavescens* Gill Epithelia, 400x magnification, GFP filter, wet mounted in DI water in ~2cm well glass slide.

Since MS-FC-4 *wt* is highly virulent (Appendix B), a small challenge study was conducted exposing zebrafish (n=6 per treatment) to MS-FC-4 *wt*, MS-FC-4+*gfp*, and a null control of TYES media. The virulence of the two strains in the zebrafish trial was identical when exposed to either 5×10^3 CFU/mL *wt* or *gfp* MS-FC-4, and all fish died at the same rate: 24 hours post-immersion 30% died, and 48 hours post-immersion there

were no surviving zebrafish. All fish in the TYES control group survived for 7 days. A small trial was also conducted to examine the localization of MS-FC-4+*gfp* in Yellow Perch, and the maintenance of GFP expression post-mortem. Use of the MS-FC-4+*gfp* strain allowed for the capture of *F. columnare*'s namesake columnar stacks of bacteria on the larval perch gills (Figure 4.3), as well as showing localization of the infection in the GI tract and head kidneys (Figure 4.4). The development of this GFP expressing strain will enable researchers to look at how gene deletion affects infection localization or infection severity. The results of these future experiments may be extended to generate attenuated vaccines against this common aquaculture opportunistic pathogen.

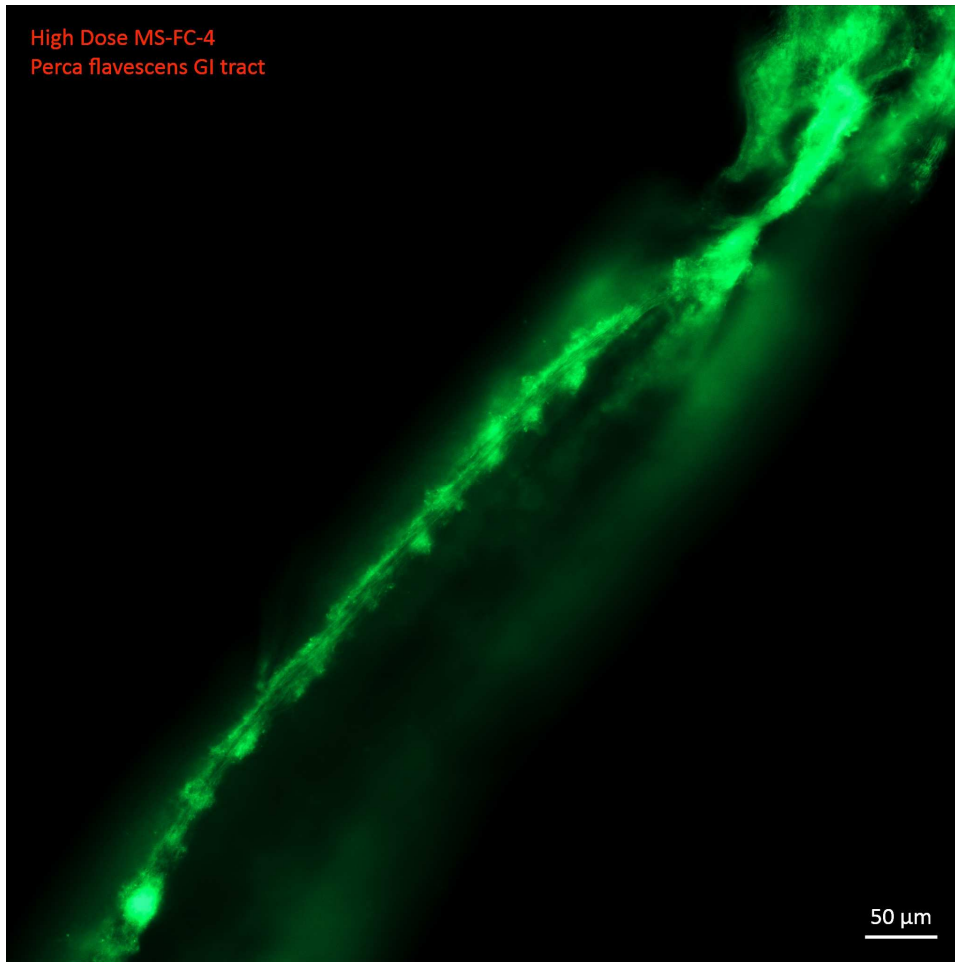


Figure 4.4 Juvenile *Perca flavescens* GI Tract after exposure to MS-FC-4+*gfp*. Post-mortem image taken at 400x magnification on an epifluorescent microscope with a *gfp* filter cube without fixative, mounted in DI water on a slide with a single ~2cm well.

4.4 The Cryptic Ecology of *F. columnare*

Little is known about the ecology of *F. columnare*, however, it is responsible for yearly larval fish die offs in natural ecosystems (Scott and Bollinger, 2014). During fish kills, *F. columnare* is able to maintain a saprophytic lifestyle (Kunttu et al., 2009), but loses its virulence after phage predation (Laanto et al., 2012). It is puzzling that only some strains conduct anaerobic denitrification while others do not (McBride, 2014). However, none of these metabolic features illustrate a clear ecological niche or role for *F. columnare* in natural ecosystems.

Nutrient availability from primary producer derived detritus may influence populations of *Flavobacterium* spp. For example, algal biomass was previously shown to have growth rate effects on heterotrophic bacterial populations in marine and freshwater environments (White et al., 1991). Furthermore, other members of the class *Flavobacteria* have been shown to be critical in algal-derived dissolved organic matter degradation in both marine and freshwater ecosystems (Eiler and Bertilsson, 2007; Mann et al., 2013). At an ecosystem level, it is possible *F. columnare* also participates in macromolecular degradation, but further experimentation is required. One hypothesis may be that the association with fish die offs in the spring is due to low algal substrate availability, particularly after the winter thaw during fish spawning season. In aquaculture systems, algal biomass is largely seen as a nuisance to fish production, and a potential source of fish kills (Tucker and Hargreaves, 2008). If infecting fish is a secondary ecological niche for *F. columnare*, given the persistence of *Flavobacterium* spp. in RAS outlined in Chapter 2, deciphering the ecological niche of *F. columnare* is a hypothesis worth exploring.

5 Future of Targeted Microbiological Approaches in Aquaculture

5.1 Beyond Basic Recirculating Aquaculture

RAS offers a viable means of combating declining fisheries (Barange et al., 2014), however it cannot address the availability of produce like aquaponic systems (Love et al., 2015a). RAS also lacks the trophic complexity of aquaponic systems without the addition of other salable crops, such as bivalves or crustaceans that act as nutrient sinks in multi-trophic aquaculture (Martins et al., 2010). Multi-trophic RAS may offer more salable products to practitioners who choose to combine cultivars, such as one study that combined white shrimp and Nile Tilapia cultivation (Muangkeow et al., 2007). However, in an educational setting, RAS does not offer the same benefits associated with the increased ecological complexity of aquaponic systems (Genello et al., 2015). In RAS, the ecosystem is largely unobservable to those without access to the latest methods in microbial ecology. Recent surveys of aquaponic systems suggest ecological gradients exist in the componentry, which is similar to the findings in previous chapters of this dissertation (Schmautz et al., 2017). These methods remain underutilized in both RAS and aquaponic systems (Munguia-Fragozo et al., 2015; De Schryver and Vadstein, 2014), which is perplexing given how the functionality of components in both classes of agricultural systems depends on their innate microbiota. If all agricultural systems that are dependent on their microbiota for process engineering were studied in as great of detail as wastewater or drinking water treatment, perhaps reproducibility of systems would be easier. What follows is a perspective on the applicability of microbial ecology to aquaponic systems for promoting plant growth. This work is meant to address, in hypothetical terms, the untapped potential of microorganisms to alleviate iron deficiency in aquaponic systems.

5.2 Stripping Away the Soil: Plant Growth Promoting Microbiology Opportunities in Aquaponics

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Stripping Away the Soil: Plant Growth Promoting Microbiology Opportunities in Aquaponics

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Keywords: Aquaponics, Plant growth promoting microorganisms, Recirculating aquaculture, Chlorosis, Rhizosphere, Microbiome

Running Title: Microbiology Research in Aquaponics

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Abstract

As the processes facilitated by plant growth promoting microorganisms (PGPMs) become better characterized, it is evident that PGPMs may be critical for successful sustainable agricultural practices. Microbes enrich plant growth through various mechanisms, such as enhancing resistance to disease and drought, producing beneficial molecules, and supplying nutrients and trace metals to the plant rhizosphere. Previous studies of PGPMs have focused primarily on soil-based crops. In contrast, aquaponics is a water-based agricultural system, in which production relies upon internal nutrient recycling to co-cultivate plants with fish. This arrangement has management benefits compared to soil-based agriculture, as system components may be designed to directly harness microbial processes that make nutrients bioavailable to plants in downstream components. However, aquaponic systems also present unique management challenges. Microbes may compete with plants for certain micronutrients, such as iron, which makes exogenous supplementation necessary, adding production cost and process complexity, and limiting profitability and system sustainability. Research on PGPMs in aquaponic systems currently lags behind traditional agricultural systems, however it is clear that certain parallels in nutrient use and plant-microbe interactions are retained from soil-based agricultural systems.

Aquaponics - Stripping Away the Soil

Aquaponics, the combined culture of fish and plants in recirculating water systems was pioneered in the 1970's (Lewis et al., 1978; Naegel, 1977; Sneed et al., 1975) as an environmentally sustainable agricultural method based on the concepts of minimal water use and minimal impact on environmental water quality compared to traditional agricultural methods (Blidariu and Grozea, 2011). In addition to producing salable crops, aquaponics is valued for its positive development of community and economic opportunity in urban areas (Goodman, 2011), and its wide-ranging educational benefits for students through the post-secondary level (Genello et al., 2015; Hart et al., 2014). Despite these benefits, microbial research supporting aquaponic crop production lags behind traditional agricultural systems. Here, we present the case that aquaponic systems provide a relatively untapped potential for research on plant-microbe interactions.

Aquaponics is a highly engineered agricultural system that uses fish effluent (which comprises both particulate waste solids and dissolved nutrients) from a recirculating aquaculture subsystem as nutrient medium to grow edible plants in attached hydroponic subsystems. In return, nutrient removal through plant absorption and growth, parallel to their associated microbiota, decreases dissolved solid and ionic concentrations, which, in turn, benefits fish production by improving overall water quality parameters, including the removal of toxic metabolites, such as ammonia and nitrite. Together, this circular and beneficial relationship between fish, plants, and microbes reduces water usage compared to traditional agriculture. Historically, aquaponics research was driven by the recirculating aquaculture community; so most technological advancement was focused on optimizing water quality for fish production purposes (Lewis et al., 1978; Naegel, 1977; Sneed et al., 1975). However, recent energy analyses and industry surveys of aquaponic systems concluded that profitability is greater when a plant-centric production approach is adopted (Love et al., 2015a, 2015b). Incorporation and testing of hydroponic method compatibility is therefore critical to integrating simultaneous fish and plant production. For example, using a hydroponic-centered production setup, Schmutz and colleagues found that the aquaculture-subsystem provided the necessary nutrients to produce cherry tomatoes with nutrient content that exceeded those available at local

markets (Schmautz et al., 2016). However, to improve plant growth in plant-centric aquaponic systems, micronutrient supplementation (e.g. iron, calcium, and potassium) is often required (Bittsanszky et al., 2016; Rakocy et al., 2004; Roosta, 2014). Additionally, chemical inputs are needed to counteract disease and other plant stressors (Nguyen et al., 2016), which adds long-term operational expenses (Love et al., 2015a). Thus, despite the benefits of these integrated agricultural systems, high capital expenses during system construction (Engle, 2015), and the abovementioned chemical supplementation make achieving economic sustainability a challenge. Continued research on micronutrient transport and transformation between aquaponic subsystems is needed to identify shortcomings and optimize engineering paradigms in aquaponic systems.

Plant growth promoting microorganisms (PGPMs) may be an effective alternative to chemical inputs for dealing with plant growth requirements and stressors in aquaponic systems. Plants recruit PGPMs from the surrounding environment to their rhizosphere using specific chemical signaling (DeVries and Wallenstein, 2017). For example, under phosphorus (P)-limiting conditions, the plant hormone strigalactone is released into the rhizosphere, where it serves as a signaling molecule to initiate associations with fungi (Akiyama et al., 2005). In soil-based environments PGPMs are known to enhance plant growth via a number of mechanisms, including: nitrogen fixation, organic matter mineralization, root growth promotion, protection against pathogens, and increasing the bioavailability of nutrients, including micronutrients such as iron (Cerozi and Fitzsimmons, 2016; Coleman-Derr and Tringe, 2014; Dias et al., 2014; Höflich et al., 1995; Khalifa et al., 2016; Loper et al., 2012; Loper and Henkels, 1997, 1999; Malusá et al., 2012; Marasco et al., 2012; Mendes et al., 2011; Pii et al., 2015; Rashid et al., 2012). In soilless environments, PGPM research is limited, but existing studies suggest PGPMs also play a significant role in plant growth and health (Cerozi and Fitzsimmons, 2016; Gravel et al., 2006; Nguyen et al., 2016; Sheridan et al., 2016; Villarroel et al., 2011).

Regardless of the agricultural system, root health is essential to the survival of plants; so one focus area for aquaponic PGPM research should be microbial root colonization. Arbuscular mycorrhizal fungi are well-documented plant growth promoting fungi that colonize plant roots. In traditional soil-based agriculture, arbuscular

mycorrhizal fungi promote phosphorus uptake and enhance biomass production (Govindasamy et al., 2011). Arbuscular mycorrhizal fungi also appear to be important for plant health in hydroponics. For example, in one hydroponic system study, arbuscular mycorrhizal fungi inhibited *Fusarium* spp. from inducing root rot in tomatoes grown under near-commercial conditions (Utkhede, 2006). While arbuscular mycorrhizal fungi are a commonly cited PGPM, many different microorganisms are thought to be PGPMs. One such group, rhizobia, were discovered in the 19th Century (Beijerinck, 1888), and now these diazotrophic bacteria are recognized as essential agents in promoting growth among crops such as legumes, rice, and wheat (Govindasamy et al., 2011; Ji et al., 2014; Majeed et al., 2015). Interestingly, iron siderophores facilitate the formation of rhizobium diazotrophic nodules (Barton et al., 1996; Brear et al., 2013), suggesting micronutrients may play a role in PGPM colonization in other agricultural systems, such as hydroponics/aquaponics. However, some PGPM benefits may come at an adaptive cost, such as increased sensitivity to insect herbivory (Barazani and Baldwin, 2013), but controlled environmental agriculture can account for invertebrate pest problems (Fox et al., 2012). Ultimately, research on PGPM in aquaponic systems may alleviate costly nutrient supplementation by properly integrating PGPM driven environmental processes into system design.

In addition to benefitting aquaponic crop production, PGPM research in soilless engineered environments has the potential to advance the fundamental understanding of rhizosphere microorganism associations. It is clear plants recruit PGPMs to their rhizosphere, but the mechanisms driving plant growth promoting rhizosphere interactions are difficult to disentangle from soil-based studies (DeVries and Wallenstein, 2017). Soil matrices are chemically complex and heterogeneous, exhibiting immensely diverse microbial communities. Additionally, given the large variability among soil and crop types, (DeVries and Wallenstein, 2017), rhizosphere recruitment of PGPMs in this environment remains mainly theoretical and often limited to a case-by-case basis. The complexity of the soil matrix also adds technological hurdles to studying PGPMs. The soil matrix often hinders nucleic acid extraction and, subsequently, sequence-based analyses of microorganisms, thus inhibiting the exploration of microbial community structure (Carini et al., 2016), genetic signatures pertaining to nutrient processing (Krsek

and Wellington, 1999; Martin-Laurent et al., 2001), and the identification of microbial guilds to utilize in rhizosphere engineering (Dessaux et al., 2016; Mueller and Sachs, 2015; Pii et al., 2015; Savka et al., 2013). In contrast to soil-based agricultural systems, aquaponic systems operate in highly monitored and controlled environments (e.g. pH, temperature, hydraulic retention time, nutrient concentrations, etc.), and lack the confounding variability and complexity of the soil-matrix. As a result, these systems represent, scalable, highly reproducible, and adjustable laboratories for PGPM research, where discoveries made in a research setting may be more directly transferred to an industrial or practical application.

The Challenges of Integrated System Design in Aquaponics

Aquaponic systems must balance the physiological requirements of both plant and fish in order to maintain their health. This balance makes even basic system design challenging, such as identifying plant and fish species that are compatible. The integration of distinct fish and plant subsystems also means operational change in any one component inherently impacts all other components, thereby creating a fairly high level of ecological complexity. For example, solid waste in aquaponic systems primarily consists of fish feed and feces, which, when decomposed, act as fertilizer for the hydroponic subsystem. Calculated fish feed rates relate to plant grow-bed size, but feed conversion and nutrient assimilation varies with feed protein type (i.e. plant-derived vs. teleost protein extracts) and plant crop-type (Hu et al., 2015; Medina et al., 2016; Rakocy et al., 2006; Timmons and Ebeling, 2013). Excess solid waste increases oxygen demand leading to hypoxic conditions in the rhizosphere, and may generate toxic concentrations of ammonia and nitrite (Danaher et al., 2013; Rakocy, 2012). Therefore, proper solids management is necessary to maintain the oxygen gradient around the plant roots allowing for colonization of PGPMs and preventing phyto-pathogen growth. However, in the roots of the hydroponic subsystem an appropriate level of solid waste re-mineralization is essential to supply micro- and macronutrients to the plants (Rakocy et al., 2012). Basic system design constraints influence overall microbial community structure and suggest microbial niche differentiation within system components, but the influence of environmental conditions has yet to be explored in aquaponic systems (Schmautz et al.,

2017). Food web interactions, including predation on bacteria and archaea and nutrient or energy transfer from microbial eukaryotic activity, such as that from micro-fungi, may also confound system designs in yet unknown ways. These food web interactions have had little consideration in system designs to date, but deserve thorough analysis as control points for microbial-plant interactions and in experiments aimed at optimizing aquaponic system technology.

Iron Limitation: A Case Study for PGPM Research in Aquaponic Plant Production

Commonly, iron is supplemented in the hydroponic subsystems of aquaponics configurations; however, little attention has been paid to exactly why this supplementation is required. Herein we review what is known about iron requirements in aquaponics and discuss possible iron supplementation strategies that do not require industrially produced chelated iron. Iron is an essential molecule for a multitude of metalloprotein structures (e.g. hemoglobin, chlorophyll, and cytochromes), and therefore, demand is high from all biological components of an aquaponic system. Fish assimilate low amounts of iron relative to terrestrial livestock (van Dijk et al., 1975), and often fish iron needs are met or frequently exceeded, with commercial feeds (Watanabe et al., 1997), so little attention is paid to this component of aquaculture operations. In contrast, although undigested fish feed contains excess iron, plant grow beds in aquaponics are often limited by bioavailable iron (Fe^{2+}). This nutrient deficiency is a known cause of chlorosis in the hydroponic subsystem crops, but may not be evident in a system until vegetable products have been raised for multiple generations (Rakocy et al., 2004). In the University of the Virgin Islands system, chelated iron is added at a concentration of 2 mg/L per day (Rakocy et al., 2004) to prevent chlorosis. One major factor driving iron deficiency is that soluble ferrous iron ($2+$) easily crosses the rhizoplane of the roots, but ferric iron ($3+$) is insoluble. Consequently, the competing chemical reactions driving Fe^{2+} to Fe^{3+} (i.e. the speciation of iron in natural water systems by hydroxyl radicals and ionic interactions) and pH dependency complicate the mass balance of iron in aquaponic systems (Rose and Waite, 2002; Waite, 2002). Biotic factors in aquaponic systems may also reduce available iron for plants. Endemic microbial communities scavenge iron for constructing metalloprotein centers (Andrews et al., 2003), but in aquaponics the extent of this iron demand remains unknown.

Limitation of biologically available iron is a relatively common phenomenon across environments containing photosynthetic organisms. One such environment is the ocean, where primary productivity depends on soluble iron, though iron remains sequestered in microbial amphiphilic siderophores (Boiteau et al., 2016). Published literature on the role of siderophores in soil-based agriculture point to both an enhancement of growth and a link to pathogenesis (Kloepper et al., 1980; Neilands and Leong, 1986). *Pseudomonas fluorescens* Pf-5 is one PGPM known to increase the bioavailability of iron through siderophore production in iron deficient soils (Loper and Henkels, 1997). Interestingly, some pseudomonads, like *Pseudomonas putida*, are able to scavenge iron from other siderophores under laboratory conditions and promote iron uptake in experimental cucumber seedlings (Loper and Henkels, 1997). The “siderophore theft” may be indicative of pseudomonad PGPM’s ability to remediate disease (Loper and Buyer, 1991; Loper and Henkels, 1997). Furthermore, genomic analyses of *Pseudomonas* spp. indicate a distinct ability to modulate the surrounding rhizosphere community through antifungal and bacteriocin production, in addition to siderophore production (Loper et al., 2012). In soil-based agriculture, other bacterial species such as, *Bacillus* and *Paenibacillus* spp. exhibit similar PGPM characteristics (Govindasamy et al., 2011). All of the aforementioned PGPM microbes found in soil studies may allow aquaponics practitioners to biologically remediate regularly occurring nutrient deficiencies. Since there is evidence PGPM ecophysiology work to overcome micronutrient deficiencies in engineered environments (Villarroel et al., 2011), there is ample opportunity to research their usefulness for aquaponic system design and management.

All nutrient rich hydroponic systems, including aquaponics, must balance the promotion of beneficial microorganisms while minimizing the growth and rapid spread of plant pathogens (Lee and Lee, 2015). Though the resiliency of aquaponic systems to phytopathogen infection requires experimental study, other groups of PGPM’s (such as *Bacillus* spp. and *Paenibacillus* spp.) may be linked to plant resilience (Govindasamy et al., 2011; Loper et al., 2012). In hydroponics, PGPM species have been identified from the bacterial genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Streptomyces*, *Gliocladium*, and *Trichoderma*; many of which produce siderophores (Lee and Lee, 2015). It also has

been demonstrated that siderophore production by a *Chryseobacterium* spp. alleviates iron starvation in tomato plants (Radzki et al., 2013). It is likely that other syntrophic or symbiotic relationships between plants and rhizoplane microbiomes exist, but as of now remain undiscovered or underutilized. As more PGPM's are discovered, operators may potentially integrate batch-culturing devices to facilitate the growth of PGPM's producing siderophores. Batch production could minimize industrially manufactured chelated iron input into the system, while aiding producers in ending the dispute over USDA organic certification for aquaponic and hydroponic produce (Birnbaum et al., 2016). At the 2017 National Organic Standards Board meeting no decision was made as to whether aquaponic or hydroponic crops could be certified organic under United States law.

Microbial iron use is a complicating factor in all environments with photosynthetic activity. In aquaponic systems, iron demand upstream of the hydroponic subsystem could induce a nutrient sink unless microbial micronutrient acquisition is considered in aquaponic system engineering. For example, an important design feature of aquaponics and recirculating aquaculture is solid waste decomposition, which induces anoxic or hypoxic conditions and facilitates methanogen growth when solid retention times (SRT) are greater than 10 days (Suhr et al., 2015). System iron deficits may be compounded if solids management component SRT allows for methanogenic growth, since many methanogens and methanotrophs require iron in metallo-protein complexes to catalyze reactions (Ettwig et al., 2016; Glass and Orphan, 2012; Speece, 1983). Additionally, iron is closely linked to the nitrogen cycle; a critical nutrient cycle to manage for both fish and plant growth (Glass, 2015; Klotz and Stein, 2008; Timmons and Ebeling, 2013). In some systems, such as the ocean or recirculating aquaculture system solids digesters, the link between iron and nitrogen is significant, as denitrification pathways found in heterotrophic microorganisms constitute one of the largest group of iron dependent metabolic pathways (Glass et al., 2015; van Rijn et al., 2006). Careful consideration of solids management and microbial nitrification is essential to iron management in aquaponics, if siderophore-producing PGPM are to successfully mitigate chlorosis.

The Microbial Future of Aquaponics

Integrating PGPMs into aquaponic system design has the potential to alleviate micronutrient fluctuations and phyto-pathogen blooms in the hydroponic subsystem of an aquaponic system. PGPM use in aquaponic process engineering may maintain optimal plant production with lower nutrient concentrations than those found in a typical commercial hydroponic system, thereby reducing the incidence of disease, and abiotic inhibition of plant nutrient uptake (Lee and Lee, 2015; Mill et al., 1996; Rakocy et al., 1997). These operational conditions also may be maintained in a hydroponic system, but without fish feed and feces serving as the basis of plant growth substrate, external costs and maintenance increase (Villarroel et al., 2011). Typically, the aquaponic system operator supplements iron, calcium, and potassium, but solid waste re-mineralization reduces supplementation cost compared to stand alone hydroponic systems (Bittsanszky et al., 2016; Rakocy et al., 2006). Raising Tilapia (*Oreochromis niloticus* L.) at low stocking densities was found to reduce nutrient costs incurred in hydroponic strawberry production, however, PGPM growth was not considered (Villarroel et al., 2011). These results and those from other studies (e.g. Schmautz et al., 2016), suggest that fish effluent could serve as primary growth media for hydroponic subsystems, but supplemental nutrients may be needed depending on the stocking density of the fish, plant crop grown, and presence of active PGPM.

Besides PGPM research, there are a number of additional areas where microbial-based research could benefit aquaponic production. For example, aquaponic practitioners must carefully balance the pH requirements of fish, nitrifying microorganisms, and plants by identifying a mean pH that facilitates biological growth throughout all components, even if it is not optimal for any one component. Typically this means aquaponic operation at a pH of 7.0, whereas plants grown hydroponically prefer a lower pH, from 5.5 to 6.5 (Rakocy et al., 2006). However, pH balancing does not follow a concrete rule for operation, as a review of aquaponic crop production (Tyson et al., 2011) suggests normal total crop yields may be obtained at pH levels above those recommended for traditional production. Recent research into nitrifying microorganisms suggests that certain species of nitrite-oxidizing bacteria become more competitive at lower pH levels (Hüpeden et al., 2016), presenting the opportunity for further research into operating aquaponic systems at a lower pH and allowing pH to be optimized for specific plant or

PGPM growth. There is also evidence of overlap between fish gut microbiomes and rhizosphere microbiomes (Hacquard et al., 2015), which could indicate microbial-based health benefits of fish-plant co-cultivation and an opportunity to use PGPM manipulation to benefit both plant and fish growth. Root-associated microorganisms are also known to influence plant phenology, such as flowering time (Pérez-Jaramillo et al., 2016; Wagner et al., 2014), and thus, in theory, could be used to manipulate desired plant biological characteristics in controlled settings such as those found in aquaponics. Finally, a recent model argues that a shift from continuous recirculation to decoupling aquaponic system components could lower incidences of nutrient supplementation and allow for PGPM growth planned into initial aquaponic system design (Goddek et al., 2016). Decoupling aquaponic components could free greater quantities of allochthonous iron and micronutrients from solid waste, while also sustaining crop growth during emergency or routine maintenance, but further research in this area and all those mentioned above is needed.

Despite the benefits of these integrated agricultural systems, achieving economic sustainability of aquaponic systems remains a challenge, primarily due to high capital expenses during system construction (Engle, 2015) and the need to reduce recurrent operational expenses (Love et al., 2015a). Microbial ecological theories governing nutrient cycling, host interactions, and community assembly underpin plant growth and health in aquaponic/hydroponic systems (Blancheton et al., 2013). Testing, understanding, and applying these theories to improve system design is crucial as global protein demands are becoming ever more reliant upon aquaculture/aquaponic systems, and many are turning to aquaponics to serve food deserts in cities and decrease agricultural water usage (Blidariu and Grozea, 2011; FAO, 2014; Goodman, 2011). We advocate that one “moon shot” research area for microbiologists should be enhancing sustainable agricultural systems, as this area presents opportunities to decentralize the food production system, simplifies agricultural logistics by decreasing distance to market, and enables more food safety and quality controls than traditional agriculture, while positively impacting local economies.

Author Contributions

RPB and JEB developed and outlined the initial concept for the perspective. RPB was the primary author and lead on writing and editing. BOO, JEB, and OJS-V contributed ideas and writing for particular sections relating to their expertise, and all were involved in writing and editing of manuscript drafts. RJN contributed to the initial outline and perspective focus, was involved in writing and editing of all manuscript stages, and was the primary source of funding for the project.

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5.3 Conclusions

The results from this dissertation synthesize multiple approaches to elucidating microbial communities in recirculating aquaculture. Information on RAS microbiomes is generally lacking (De Schryver and Vadstein, 2014), so it was necessary to compare the system at the UW-Milwaukee School of Freshwater Sciences (UWM SFS) to other systems in the Upper Midwest (Chapter 2). The results of this study indicated that each system appears to have its own microbiome structure. These differences in microbiome structure appeared to be significantly influenced by system source water. The biofilter microbiomes in Chapter 2 were different at a bacterial community level. However, when examining the nitrifiers present in all the biological filters surveyed, ammonia-oxidizing archaea (AOA) and nitrite-oxidizing *Nitrospira* were dominant taxa. The appearance of

Nitrosomonas and complete ammonia-oxidizing *Nitrospira* (comammox) seemed to be driven by biogeography and was again informed by source water.

Through the UWM SFS RAS biofilter survey in Chapter 3, it was found that the biofilter microbiome shifted in concert with feed regime changes over the Yellow Perch rearing cycle. It is also interesting that all three known freshwater ammonia oxidizing microorganisms were present (AOA, comammox, and *Nitrosomonas*). However, AOA and comammox dominated the biofilter over the rearing cycle, but all three organisms coexisted across a nutrient gradient within the filter. Furthermore, modeling ammonia-oxidation rate versus biomass as a function of solids residence time suggests that both AOA and comammox could occupy the ammonia-oxidation niche within the system. When scaled down to autotrophic bioreactor models of the UWM SFS RAS biofilter, a prominent priority effect was found for seeding the filters with sand from the RAS biofilter. The presence of Yellow Perch in the UWM RAS at the time of inoculum sampling had an effect on comammox abundance in the lab-scale filters. A strain of comammox from the UWM biofilter was enriched from biofilter sand during a Yellow Perch rearing cycle solely using nitrite. The enrichment was then sequenced using shotgun metagenomics, and the metagenomic-assembled genome (MAG) was placed into a phylogenomic context with other ammonia-oxidizers. Two other aquaculturally associated comammox are known (van Kessel et al., 2015). The UWM MAG shares most of the same metabolic pathways as these comammox, but contains nearly complete metabolic pathways for Ribulose-1,5-bisphosphate carboxylase/oxygenase synthesis and the reductive pentose phosphate cycle. These differences from the other two RAS comammox genomes may be indicative of an adaptation to fluctuating CO₂ levels in the aerobic biofilter, since the other *Nitrospira* were enriched from a RAS anaerobic digester.

Based on the results of Chapter 2, *Flavobacterium* spp. appear to persist in RAS, with no obvious connection to pathogen outbreaks in all systems studied. Based upon personal communications with the operators whose facilities were surveyed in Chapter 2, *F. columnare* infects opportunistically during times of physiological stress. For example, at one facility, the operators noted that as their solids management component failed, there was a cascading effect through the system. Dissolved organic matter fluxed into the biofilter, suppressing ammonia-oxidation, eventually causing a spike in nitrite concentration and an outbreak of *F. columnare*. The sequencing of *F. columnare* genomes in Appendices A and B may provide some insight into potential physiological adaptations these organisms have made to persist during normal RAS operations. The MS-FC-4+*gfp* strain represents a break through in pathogenesis studies in *F. columnare* allowing for rapid visualization of *F. columnare* without necessarily fixing the cells as one would when using other fluorescent microscopic techniques.

So in conclusion, the work in this dissertation reflects much of what De Schryver and Vadstein suggested in their 2014 review of ecological approaches to control aquaculture pathogens. In particular I conducted a system comparison study, detailed analysis of a biological filter at multiple spatial scales, shotgun metagenomics, and developed new ways to visualize an aquaculture pathogen. This work serves as a frame of reference for many more future hypotheses by addressing fundamental knowledge gaps in the microbial ecology of recirculating aquaculture. By no means is this work exhaustive, it is merely a representation of the potential microbial ecology has to change RAS operations by conducting research from the level of a single bacterial cell, to the larger picture of the unseen ecosystem cultivated within RAS.

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APPENDICES

Appendix A – Complete Genome Sequence of the Fish Pathogen

Flavobacterium columnare Strain C#2

Modified from the published form in Genome Announcements (doi:

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Abstract:

Flavobacterium columnare is a Gram-negative bacterial pathogen that causes columnaris disease of freshwater fish. *Flavobacterium columnare* strain C#2 was isolated from a diseased warm water fish and is typed as genomovar II. The genome consists of a single 3.33 Mb circular chromosome with 2,689 predicted coding genes.

Genome announcement:

Flavobacterium columnare is an important pathogen of freshwater fish (Darwish and Ismaiel, 2005; Lafrentz et al., 2013; Thomas-Jinu and Goodwin, 2004). Though *F. columnare* is known to result in large die-offs among wild and farmed fish, little is known regarding its virulence mechanisms or its ecology (McBride, 2014). Strains of *F. columnare* are classified into genomovars based on restriction digestion of the 16S rRNA gene and the 16S-23S rRNA internal transcribed spacer (Darwish and Ismaiel, 2005; Lafrentz et al., 2013), and *F. columnare* strain C#2 (originally referred to as *F. columnare* strain #2 (Staroscik et al., 2008; Thomas-Jinu and Goodwin, 2004)) is a genomovar II strain. The genome sequence of a genomovar I strain was previously reported (Tekedar et al., 2012). *F. columnare* strain C#2 was selected for genome sequencing because it is a virulent genomovar II strain that is amenable to genetic manipulations (Staroscik et al., 2008), allowing detailed study of its virulence mechanisms.

A single colony of strain C#2 was grown in 10 mL of modified Shieh Medium (Decostere et al., 1997) with shaking overnight at 25°C. Genomic DNA was extracted from 10 mL of the culture using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). 20 kb PacBio RSII libraries were constructed using size selection performed with AmPure beads in accordance with Pacific Biosciences library preparation protocol. Two PacBio SMRTcells were mag bead loaded with 0.01 and 0.015 nM concentrations of prepared library with Pacific Biosciences Sequencing Reagent 4.0, C4 sequencing chemistry, and P6 v2 polymerase. Sequencing produced 66,158 reads with an

N50 read length of 44,216 and mean read length of 20,496 representing a 296.7x coverage of the genome. Genome assembly was carried out using the PacBio PBcR HGAP 2.3.0 pipeline with default settings (Chin et al., 2013). The HGAP Pipeline has been shown to provide accurate microbial genome assemblies from PacBio sequence data (Liao et al., 2015). The assembly produced two contigs one containing 3,330,796 bases and the other of 8 Kb. The 8 kb contig was eliminated from the assembly because confidence scoring in the SMRT Portal assembly analysis was below the suggested quality cut-off value (mapQV<10). Comparison for genome synteny (Gepard (Krumsiek et al., 2007)) of the *F. columnare* strain C#2 genome to another genomovar II strain, 94-081 (CP013992) revealed three large syntenic regions split by a single chromosomal inversion and rearrangement. Similar chromosomal changes have been observed in other members of the genus *Flavobacterium* (Touchon et al., 2011). The polished assembly was trimmed using the check_circularity.pl script from SPRAI (Imai, 2015), and the resulting single circular contig was annotated using the Prokaryotic Genome Annotation Pipeline through NCBI (Tatusova et al., 2015).

The C#2 genome has a GC content of 30.97%, 13 rRNA operons, 93 tRNAs, 2 CRISPR arrays, and 93.4% of ORFs correspond to predicted coding genes. The genome was analyzed for secretion systems and potential secreted virulence factors (Chagnot et al., 2013; Sandkvist, 2001). *F. columnare* strain C#2 contains the core genes of the *Bacteroidetes*-specific type IX secretion system, including *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, *sprT*, *porU*, and *porV* (Abby et al., 2015). This secretion system may deliver virulence factors across the outer membrane. Genes encoding potential secreted virulence factors such as chondroitinases, proteases, and adhesins were also identified. The ability

of cells to move over surfaces by gliding motility may also be important in the disease process (Klesius et al., 2008). *F. columnare* strain C#2 exhibits gliding motility and the annotated genome contains all of the genes known to be required for this process (McBride and Zhu, 2013). The availability of complete genome sequence data and the ability to genetically manipulate *F. columnare* strain C#2 will enable experiments to reveal the critical virulence factors of this fish pathogen. These data also should facilitate further work toward construction of avirulent vaccine strains to prevent outbreaks of columnaris disease in aquaculture settings.

Nucleotide accession numbers. *Flavobacterium columnare* strain C#2 has been deposited in GenBank with the accession number CP015107. This paper describes the first version of the genome deposited.

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Appendix B – Draft Genome Sequence of the Fish Pathogen *Flavobacterium columnare* Strain MS-FC-4

Modified from the published form in Genome Announcements (doi: 10.1128/genomeA.00429-18)

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Flavobacterium columnare MS-FC-4 is a highly virulent genetic group 1 (formerly genomovar I) strain isolated from rainbow trout (*Oncorhynchus mykiss*). The draft genome consists of three contigs totaling 3,449,277 base pairs with 2,811 predicted open reading frames. *F. columnare* MS-FC-4 is a model strain for functional genomic analyses.

Flavobacterium columnare (Bernardet et al., 1996) is the etiologic agent of columnaris disease which affects cultured, wild, and ornamental fish. This pathogen is one of the most common species affecting farmed freshwater fish and causes major economic losses worldwide (Declercq et al., 2013; Wahli and Madsen, 2018). *F. columnare* isolates are genetically diverse and were classified historically into multiple genomovars (LaFrentz et al., 2017; Triyanto and Wakabayashi, 1999). Recently, the genomovars were described as four distinct genetic groups. Almost all isolates recovered from salmonids belong to genetic group 1 corresponding to the previous genomovar 1 (LaFrentz et al., 2018). *F. columnare* MS-FC-4 is a highly virulent strain that was isolated in 2013 from the head kidney of a diseased rainbow trout (*Oncorhynchus mykiss*) in Idaho, USA (Evenhuis et al., 2016). Strain MS-FC-4 has proven to be easily genetically manipulated. This manipulation capability, coupled with the available genome sequence, make it attractive for functional genomic studies of virulence.

F. columnare MS-FC-4 was cultured for 24 hours in tryptone yeast extract salt broth medium at 30°C and 150 rpm. Genomic DNA (gDNA) was isolated using a CTAB/phenol-chloroform/isoamyl alcohol protocol (Wilson, 2001) optimized for *F. columnare*.

For long read sequencing a gDNA library was prepared according to the standard Pacific Biosciences RSII large insert library protocol. Two flow cells (C4 Chemistry) were each loaded with 0.06 nM of a >10 kb BluePippin (Sage Science, Beverly, MA, USA) size selected SMRTbell gDNA library. One µg of MS-FC-4 gDNA was also sequenced on an Illumina HiSeq 2000 platform with 100 bp paired end reads. PacBio reads were assembled using Canu version 1.6 with options set as: genome size = 3.3 Mb, corrected error rate = 0.035, and corMaxEvidenceErate = 0.15 (Koren et al., 2017). Subread coverage of the genome was ~865x. The illumina HiSeq paired end reads were mapped to the PacBio assembly using Bowtie2 version 2.3.4 (Langmead and Salzberg,

2012) with the no-unaligned parameter to discard unaligned reads. HiSeq coverage of the contigs was ~360x. The output sam file was converted to a bam file using samtools version 1.4 (Li et al., 2009). Using the Canu assembly and the bam file as input, Pilon version 1.22 (Walker et al., 2014) corrected two insertions, seven deletions, and one substitution. However, the Pilon correction did not close the gaps in the input Canu PacBio assembly, resulting in three contigs of respectively 716,735; 23,378; and 2,709,164 bp length. The Pilon corrected MS-FC-4 genome assembly was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016).

The draft genome has a cumulative total size of 3,449,277 bp with 31.9% G+C content. This makes it the largest *F. columnare* genome sequenced to date. PGAP annotation showed that the *F. columnare* MS-FC-4 genome had 2,811 coding genes, 19 complete rRNA operons, 95 tRNAs, and two clustered regularly interspaced short palindromic repeat (CRISPR) arrays. Two incomplete prophage regions of 12.4 and 25.9 kbp were identified using PHASTER (Arndt et al., 2016). The two-way average nucleotide identities (ANIs) (Goris et al., 2007) between the MS-FC-4 genome sequence and those of strains ATCC 49512 (Tekedar et al., 2012), Pfl (Zhang Yulei, Nie Pin, 2016), and CSF 298-10 (Evenhuis et al., 2017) were >99%, confirming the *F. columnare* genetic group 1 (formerly genomovar I) classification (Evenhuis et al., 2016; LaFrentz et al., 2018).

The MS-FC-4 genome contains the core genes of the Bacteroidetes-specific type IX secretion system (T9SS) linked to gliding motility and virulence (Johnston et al., 2018; Li et al., 2017; McBride and Nakane, 2015; McBride and Zhu, 2013; Sato et al., 2010). The availability of the MS-FC-4 genome sequence should facilitate construction of targeted gene deletions to identify critical *F. columnare* virulence factors.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession PVLU00000000. The version described in this paper is version PVLU01000000.

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International Society for Microbial Ecology, 2018-Present

Invited Talks:

Bartelme RP. Escaping Biogeography: Consistent Nitrifying Consortia and Opportunistic Pathogen Presence in Recirculating Aquaculture System Microbiomes. Madison Microbiome Meeting. April 2018.

Bartelme RP. Cultivating Ecosystems: Microbial Communities in Recirculating Aquaculture. University of Vienna, Austria. April 2018.

Bartelme RP. Lessons from Graduate School, The Never Bored Game. WaTA Seminar Series. January 2018.

Bartelme RP. Scaling Ecology: Nitrifying Biofiltration from Recirculating Aquaculture Systems to Laboratory Models. Milwaukee Microbiology Society – American Society for Microbiology Chapter Meeting. December 2014.

Presentations:

*Bartelme RP, Smith MJ, Sepulveda-Vilet OJ, Newton RJ. System Scale and Source Water Influence Recirculating Aquaculture System Microbiomes. Great Lakes Genomic Center Retreat. March 2018. Milwaukee, WI, USA.

*Bartelme RP, Pinto AJ, Delmont TD, Eren AM, Newton RJ, Lückner S. Engineering Applications of Complete Ammonia-Oxidation: A Pangenomic and Evolutionary Context. North Central Branch Meeting, American Society for Microbiology. October 2017. De Pere, WI, USA.

*Bartelme RP, Pinto AJ, Delmont TD, Eren AM, Newton RJ, Lückner S. The Comammox Trade-Off: Loss of Metabolic Diversity for Thermodynamic Advantage. International Convention on Nitrification and Related Processes 5. July 2017. Vienna, Austria.

Bartelme RP and Newton RJ. Curious Co-occurrence: Ammonia Oxidizing Archaea, COMAMMOX *Nitrospira*, and *Nitrosomonas* spp. in a Recirculating Aquaculture Biofilter. International Society for Microbial Ecology 16th Meeting. August, 2016. Montreal, Quebec, Canada.

Bartelme RP, McLellan SL, Newton RJ. Nitrifiers in the Fluidized Sand Biofilter of A Recirculating Aquaculture System. International Convention on Nitrification and Related Processes 4. June 2015, Edmonton, Alberta, Canada.

*Bartelme RP, Garcia AL, Smith MS, Newton RJ, McLellan SL. Nitrifying Biofiltration in Recirculating Aquaculture Systems. International Fluorescence *in situ* Hybridization Workshop. February 2015. Department of Microbial Ecology, Vienna, Austria.

Bartelme RP, Binkowski FP, McLellan SL, Newton RJ. Community Comparison of Nitrifying Microorganisms Between Aquaculture and Aquaponic Systems. American Society for Microbiology General Meeting. May 2014. Boston, MA, USA.

*Denotes oral presentation

Teaching Experience:

- Guest Lecturer (2017) – University of Wisconsin – Milwaukee, Freshwater Science 512: Sequence Analysis.
- Guest Lecturer (2017) – University of Wisconsin – Milwaukee, Freshwater Science 564: Water Quality in Aquaculture
- Lecturer (2013-2015) - Growing Power, Inc., Milwaukee, WI: From the Ground Up Aquaponic Workshop Series

Stakeholder Involvement and Educational Outreach:

- Skype-A-Scientist: Science Communications for K-12 (2018-Present)

- Assistant - Science Bag: Friend or Foe? The Two Faces of Microbes. (2016) University of Wisconsin - Milwaukee, Milwaukee, WI
- Aquaculture Microbiology Consultant (2015) Will Radler - Private Goldfish Pond, Hales Corners, WI
- Preliminary Microbiology Analyst; *Flavobacterium columnare* Outbreak (2014) Great Lakes Aquaculture Center, University of Wisconsin-Milwaukee, Milwaukee, WI
- *Pro bono* Aquacultural Microbiology Consultant; *Flavobacterium columnare* Outbreak (2013) Bell Aquaculture, Albany, Indiana

Professional Society Memberships:

- American Society for Microbiology, Student Member (2014-Present)
- Aquacultural Engineering Society, Student Member (2014-Present)
- American Fisheries Society, Wisconsin Chapter, Student Member (2015-Present)

Workshop Participation:

- International Fluorescence *in situ* Hybridization Workshop Participant (2015)
- Introduction to R Workshop with Dr. Patrick Schloss (2014)
- Do-It-Yourself Microbial Genome Sequence Analysis Workshop, American Society for Microbiology General Meeting (2013)