Project Title: Metagenomic Analysis of Microbial Populations in Aquaponic Systems [Termination Report]
Key Words: Aquaponics, metagenomics, bacteria, nitrogen cycle
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Reason for Termination: Project objectives completed.

Project Objectives:

1. Collect microbial samples from multiple sites within an aquaponics system and from various systems across a geographic range and extract bacterial DNA using commercially-available kits.
2. Submit DNA samples to the UW-Madison Biotechnology Center for PCR amplification, Illumina next-generation sequencing, and bioinformatics analyses to compare microbial populations among sampling sites within a system, among systems at a single production site, and among production sites in the North-Central Region (NCR).
3. Develop a fact sheet explaining the importance of microbial populations in an aquaponics system, including the risk that certain organisms can cause (e.g., plant, fish, and human pathogens), develop a recorded presentation for dissemination using the NCRAC website, and produce oral presentations and a peer-reviewed manuscript.

Project Summary

Aquaponic food production is dependent upon the microbial populations present in the system.
Microbes play a role in nitrogen conversion from animal waste to plant fertilizer and mineralization of organics in the system, but may also cause fish, plant, or human diseases. Although the importance of microbes is recognized, little is known about the specific types of microbes found in an aquaponics system or where those microbes are found. Without this knowledge, it is difficult to manage those populations to best enhance fish and plant growth while reducing the risk of plant, fish, and human pathogens in the system. This study used a metagenomic approach to studying microbial populations in aquaponics systems. Microbial 16S RNA was sequenced using Illumina next generation sequencing and microbes were identified to taxonomic levels. Analyses were performed to determine the relative abundance and richness of taxa at each site, compare among different sites within a system, compare replicate systems, and to compare the microbial diversity between the two facilities.

Technical Summary and Analysis

Objective 1.—The University of Wisconsin-Stevens Point Aquaponics Innovation Center (UWSP-AIC) houses six replicate commercially-available systems (Nelson and Pade, Inc.). Each system contained two 416-L fish tanks that flowed by gravity into 416-L settling tanks, then to a mineralization tank containing plastic netting to provide surface area for microbial colonization. Water flowed to a degassing tank, then to four 4'W×6'L×1'D raft tanks. Water was then pumped back to the fish tanks. The fish stocked into the tanks were hybrid walleye at low, medium, or high density, and the plants were butterhead lettuce, romaine lettuce, kale, and pak choi.

To sample microbial populations in these systems, samplers were placed into the system on 28 October 2016. For microbial populations that grow on tank wall surfaces, four 7.5cm × 7.5cm pieces of tank wall material were suspended in each system. Two samplers were placed in the fish tanks, and two were placed in the raft tanks. To sample the microbial populations that colonize the netting of the mineralization tank, eight layers of 30cm × 30cm netting material were attached to a 30cm × 30cm PVC frame and placed into the mineralization tank of each system. To sample the microbial populations that colonize the underside of the foam rafts, 7.5cm× 7.5cm pieces of foam material were placed into raft tanks. Two foam samplers were placed into each system. The samples were undisturbed in the systems for 80 days before being removed 19 January 2017. Water and tank detritus samples were also collected 19 January 2017.

Accumulation of material on each tank wall and foam sampler was scraped from the samplers using a sterile cell lifter and placed into sterile 2ml microcentrifuge tubes containing 500µl sterile deionized water. From each netting sampler, a 7.5cm × 7.5cm portion of the eight-layer sampler was cut using sterile scissors and placed into a sterile 50ml conical-bottom centrifuge tube with 25ml of sterile deionized water. Water samples (25ml) were taken from the mineralization tank and raft tanks using a 25ml serological pipet and placed into a sterile 50ml conical-bottom centrifuge tube. Detritus samples were taken near the inflow pipe of the raft tank using a 25ml pipet. The tip of the pipet was placed into the detritus and the pipet was filled to the 25ml mark. The detritus sample was placed into a sterile 50ml conical-bottom centrifuge tube. Root cuttings were taken from random plants in the system using sterilized forceps and scissors and placed into sterile 2ml microcentrifuge tubes. All samples were stored on ice or refrigerated until DNA extraction.

The University of Minnesota aquaponics facility houses six replicate systems representative of "homemade" systems in which the foam rafts are in the same tank as the fish. Water is circulated through a 208-L gravel filter bed. Tank wall and foam raft surfaces were sampled in duplicate from each system using sterile swab samplers. Water samples (50ml) were taken in duplicate from the fish/raft tank and water exiting the gravel filter using sterile 50ml conical-bottom centrifuge tubes. Root cuttings were taken in duplicate using a sterile blade and placed into Whirl-Pak bags. Duplicate samples of gravel bed medium (approximately 20 pieces of gravel) were collected from each system and placed into 50ml conical-bottom centrifuge tubes. Pump filter material (approximately 2.5cm × 2.5cm × 10cm) was collected in duplicate from each system and placed into sterile 50ml conical-bottom centrifuge tubes. All samples were stored on ice and sent to UW-Stevens Point for DNA extraction.

Due to the different substrates and methods of sample collection, the samples required different methods of preparation for DNA extraction. Water samples from WI and MN were prepared by centrifuging 25ml of the sample at 10,000 × g for 5min. Pellets were resuspended in 250µl sterile deionized (DI) water. Tank wall and foam swabs from MN were resuspended in 250µl sterile DI water by pressing and turning the swab against the wall of the microcentrifuge tube. Tank wall and foam scrapings from WI were centrifuged at 10,000 × g for 5min, and pellets were resuspended in 250µl sterile DI water. Twenty-five grams of each MN gravel sample was added to 25ml of sterile DI water vortexed vigorously to dislodge material from the gravel. The gravel pieces were aseptically removed, and the suspensions were centrifuged at 10,000 × g for 5min. The pellet was resuspended in 250µl sterile DI water. The MN pump filter material was placed into 25ml of sterile DI water and vortexed vigorously to dislodge material from the filter. The material was removed, and the suspension was centrifuged at 10,000 × g for 5min, and the pellet was resuspended in 250µl sterile DI water. The MN root samples were placed into 25ml of sterile DI water and vortexed vigorously. Samples were centrifuged at 10,000 × g for 5min, and the pellet was resuspended in 250µl sterile DI water. The suspension was transferred to a new microcentrifuge tube to reduce plant root material in the sample. The WI root cutting samples were suspended in 1ml sterile DI water and vortexed vigorously. The suspension was centrifuged at 10,000 × g for 5min, and the pellet was resuspended in 250µl sterile DI water. The suspension was transferred to new microcentrifuge tube to reduce plant root material in the sample. The WI netting samples were vortexed vigorously to dislodge material from the plastic. The netting was aseptically removed, and the suspension was centrifuged at 10,000 × g for 5min. The pellet was resuspended in 250µl sterile DI water. The WI detritus samples were vortexed vigorously to suspend the detritus. A 250µl sample of the resuspended material was removed for DNA extraction protocols.

DNA extraction was performed on 250µl of each sample by using the DNeasy PowerSoil Kit (Qiagen, Inc., Germantown, MD) per the manufacturer's protocol. DNA concentrations were determined fluorometrically using the Qubit dsDNA BR Assay Kit (Thermo-Fisher Scientific, Waltham, MA) per manufacturer's instructions for 10µl of each sample. Samples that had a concentration below the detectable limit (< 0.20ng/µl) were recorded as zero.

The six WI systems were stocked with high, medium, or low densities of hybrid walleye at two systems per stocking density. Fish stocking density had no significant effect on the DNA concentrations extracted from samples throughout the system. The six MN systems have red wiggler worms in the gravel biofilter of three systems and no worms in the other three systems. The DNA concentrations purified from samples were significantly lower in the pump filter medium ($P = 0.002$, Mann-Whitney Test) and gravel samples ($P = 0.002$, Mann-Whitney Test) in the systems containing worms (Fig. 1). This correlated with the observance of smaller pellets after sample centrifugation, although pellet weights were not measured. This suggests that the presence of the worms is reducing

the amount of organic material buildup in the gravel filters and on the pump filter.

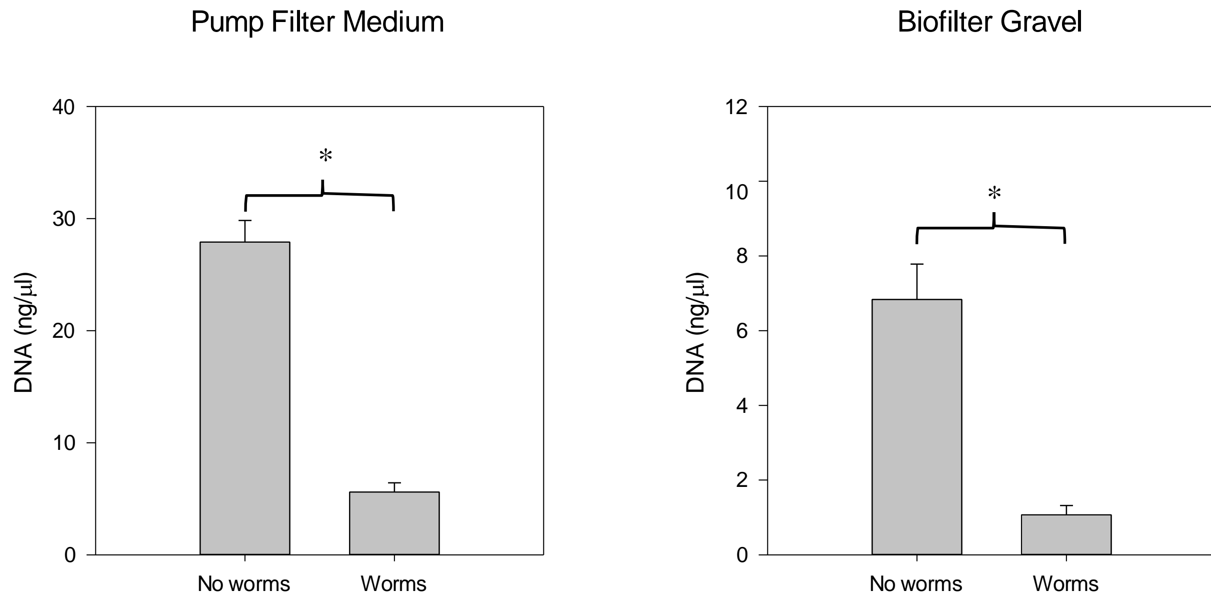

Figure 1. DNA concentrations of samples taken from pump filter medium and biofilter gravel used in the University of Minnesota aquaponics systems. Bars represent the mean DNA concentration (± standard error). Asterisks indicate a significant difference ($P < 0.05$) using a Mann-Whitney Rank Sum Test.

When comparing samples longitudinally in the system, there were significant differences in the concentration of DNA extracted from the samples in both the WI and MN systems (Tables 1 and 2). At both facilities, the water samples had relatively low DNA concentrations, suggesting fewer microbes present than on surfaces within the system. When comparing all DNA concentrations between the MN and WI facilities, the WI samples have a significantly higher value ($P < 0.001$) concentration. In pairwise comparisons, only the MN root cuttings have a higher average DNA concentration than the WI root cuttings. For samples of surfaces such as tank walls and foam, this difference is likely due to using samplers in the WI systems that were removed and scraped to acquire organic material compared to using a swab to sample a smaller area in the MN systems.

The amount of DNA extracted from samples may affect the ability to acquire quality DNA sequencing results and an accurate snapshot of the microbial community of the area sampled. In both the MN and WI systems, sampling 25mls of water resulted in low DNA concentrations, which may lead to microbial populations being underrepresented or unidentified in the water samples. Similarly, swabbing fixed surfaces within the MN systems resulted in lower DNA yields than surfaces from uniformly sized, removable samplers from which material was scraped and collected. The difference in DNA may be simply due to the MN systems having less area sampled, but the removable samples at the WI site also prevented material from being displaced from swabs by water in the system while surfaces were being sampled.

Table 1. Median DNA concentrations of samples taken from the WI aquaponics systems. Medians with the same letter are not significantly different ($P > 0.05$).

Sample	Amount Sampled	Median DNA Concentration (ng/µl)
Fish Tank Plastic Wall	$56.25cm^2$	75.000[a]
Mineralization Tank Water	25mls	4.570[b]
Mineralization Tank Netting	$56.25cm^2$	19.040[a]
Raft Tank Water	25mls	1.529[b]
Raft Tank Plastic	$56.25cm^2$	34.100[a]
Raft Tank Foam	$56.25cm^2$	18.510[ac]
Raft Tank Detritus	variable*	20.400[a]
Root Cuttings	variable†	4.010[bc]

*A 25ml serological pipet tip was placed into detritus, and a pipet-aid was filled to 25mls with detritus and water.
†Approximately 5cm of the lower portion of roots were cut.

Table 2. Median DNA concentrations of samples taken from the MN aquaponics systems. Medians with the same letter are not significantly different ($P > 0.05$).

Sample	Amount Sampled	Median DNA Concentration (ng/µl)
Biofilter Gravel	25g	2.996[ac]
Biofilter Water	25mls	0.000[b]
Tank Water	25mls	0.000[b]
Tank Wall	variable*	3.940[ac]
Foam	variable*	0.709[bc]
Root Cutttings	variable†	6.320[a]
Pump Filter Medium	variable††	15.250[a]

*Area swabbed was approximately $1\text{-}4cm^2$
†Approximately 15cm of the lower portion of roots were cut.
†† Approximately a 2.5cm × 2.5cm × 10cm piece of filter was cut.

Objective 2. —Purified DNA samples were submitted to the University of Wisconsin Biotechnology Center (http://www.biotech.wisc.edu/), which requested samples to be 20ng/µl or less. Samples that met that criterion were submitted undiluted, including those samples that had undetectable amounts of DNA. The UW Biotechnology Center performed PCR of the V3-V4 16S region, PCR cleanup, Illumina Mi-Seq next generation sequencing, and bioinformatic analyses using the Qiime pipeline (http://qiime.org/).

A total of 11,252,375 sequences were generated and analyzed. Of those, 10,998,062 (97.7%) were identified as prokaryotes (Bacteria or Archaea). There was a weak positive correlation between the concentration of DNA samples submitted and the number of reads generated from the sample (Fig. 2). Interestingly, samples with DNA concentrations too low to be detected (recorded as zero) frequently had greater than 40,000 DNA sequencing reads.

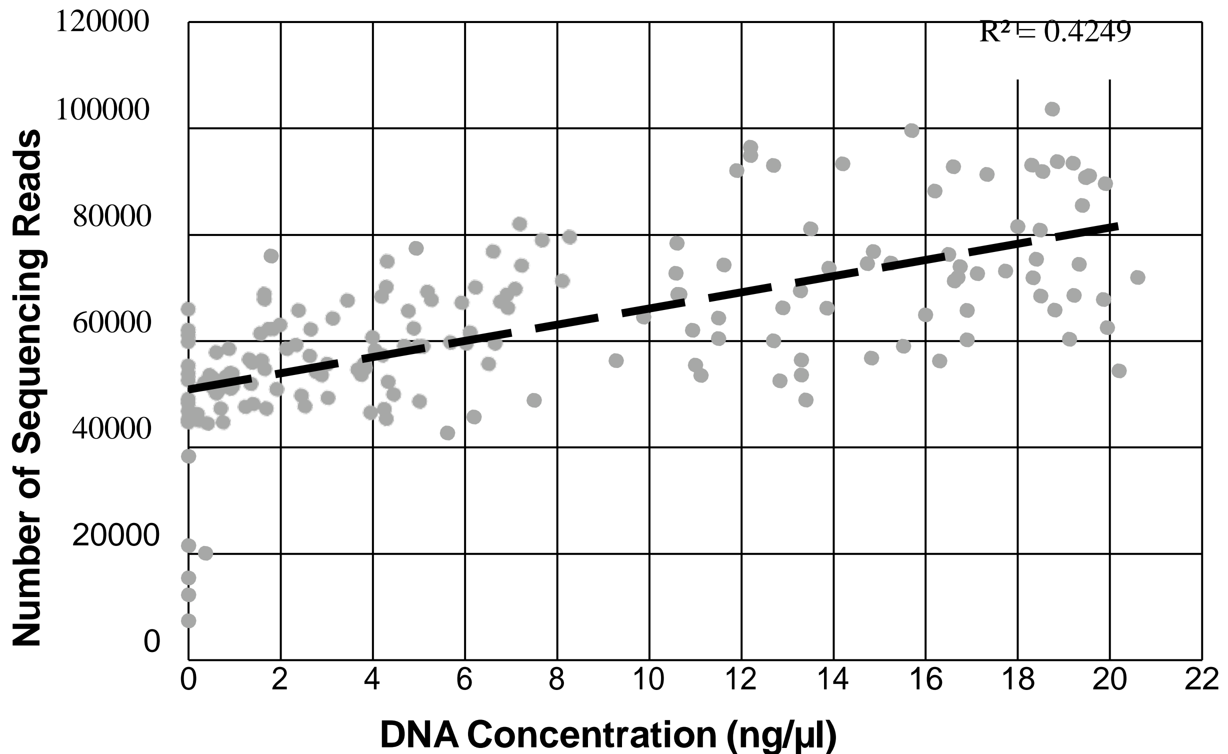

Figure 2. Scatter plot of the number of DNA sequences generated against the concentration of DNA sent for analysis. The data indicate a weak positive correlation between the concentration of DNA submitted for analyses and the number of sequences read during analysis.

The metagenomic analyses identified organisms from phyla to species, with 1,080 different operational taxonomic units (OTUs) at the species level. Only 10% of the species, however, were named (Table 3). As the taxonomic level approached species, the proportion of named groups decreased. Although these data indicate 1,080 species, it is possible there are more, because some sequences could not be resolved past taxonomic levels higher than genus and species. Because of the lack of species names for many of the organisms present, these analyses may be suboptimal for determining the abundance of pathogens, because pathogenic species share taxonomic groupings (genus, family, etc.) with non-pathogens.

It is unknown if different facilities will have different types of microbes in their systems. The two aquaponics facilities sampled differed in many ways, including design, fish species used, stocking densities, etc. Raup-Crick analyses were used to compare populations. Values 5 or less indicate significant dissimilarity, values 95 or greater indicate significantly similarity, and values from 6 to 94 indicate any population overlap is no different than random. At the phylum and class levels, the microbial populations are not significantly dissimilar (Table 4). From order to species, however, the Raup-Crick values indicated significant dissimilarity between communities of microbes MN and WI. Much of the endemic taxa at a facility, however, are rare taxa. Whether or not rare taxa should be included in the analyses is unknown, because we do not know if rare taxa perform significant functions in the system, e.g., nitrogen fixation, denitrification, iron reduction, sulfur reduction or oxidation, etc. In MN, 23% of the species identified are not present in WI, but those taxa make up only 0.3% of the microbial community sampled (Fig. 3A). Similarly, 16% of the species identified in WI systems were not present in MN systems, but that accounts for only 0.11% of the WI communities (Fig. 3B). Essentially, all species occurring at a frequency greater than 0.1% of the total population were present in both the MN and WI systems. This indicates that many rare taxa were sampled at each facility, but we do not know what role, if any, these taxa have in an aquaponics system. The analyses described below were performed with all taxa included, and many results show that the communities are significantly similar; removing rare taxa, however, was observed to result in conclusions that communities were different. Part of

determining the significance of the rare taxa is determining the functional roles of the bacterial populations present in the system and determining what proportion of microbes are performing that function. As such, it may be more beneficial to dispense with analyzing these communities based on taxonomic identification and analyze based on their functional identity. We are continuing to analyze the date to determine the appropriate handling of rare taxa.

Table 3. The number of taxonomic groups identified and the number of taxonomic groups named from metagenomic analyses of aquaponics systems.

Taxonomic level	Total	Taxonomic Name Assigned	Percent Name Assigned
Phylum	50	48	96
Class	165	132	80
Order	326	210	64
Family	539	255	47
Genus	936	390	42
Species	1080	108	10

Table 4. Count of taxa identified in Wisconsin and Minnesota aquaponics facilities. The numbers represent the counts of taxa in the entire study (Total), in Wisconsin only (WI), in Minnesota only (MN), and in both WI and MN (Both). The MN/WI overlap is the percentage of the total count of taxa identified in both WI and MN. The Raup-Crick value indicates significant dissimilarity (0) to significant similarity (1).

Taxonomic level	Total	WI	MN	Both	MN/WI overlap	Raup-Crick
Phylum	50	46	49	45	90%	86
Class	165	142	155	132	81%	8
Order	326	275	295	244	75%	0
Family	539	449	483	393	73%	0
Genus	936	752	822	638	68%	0
Species	1080	864	938	722	67%	0

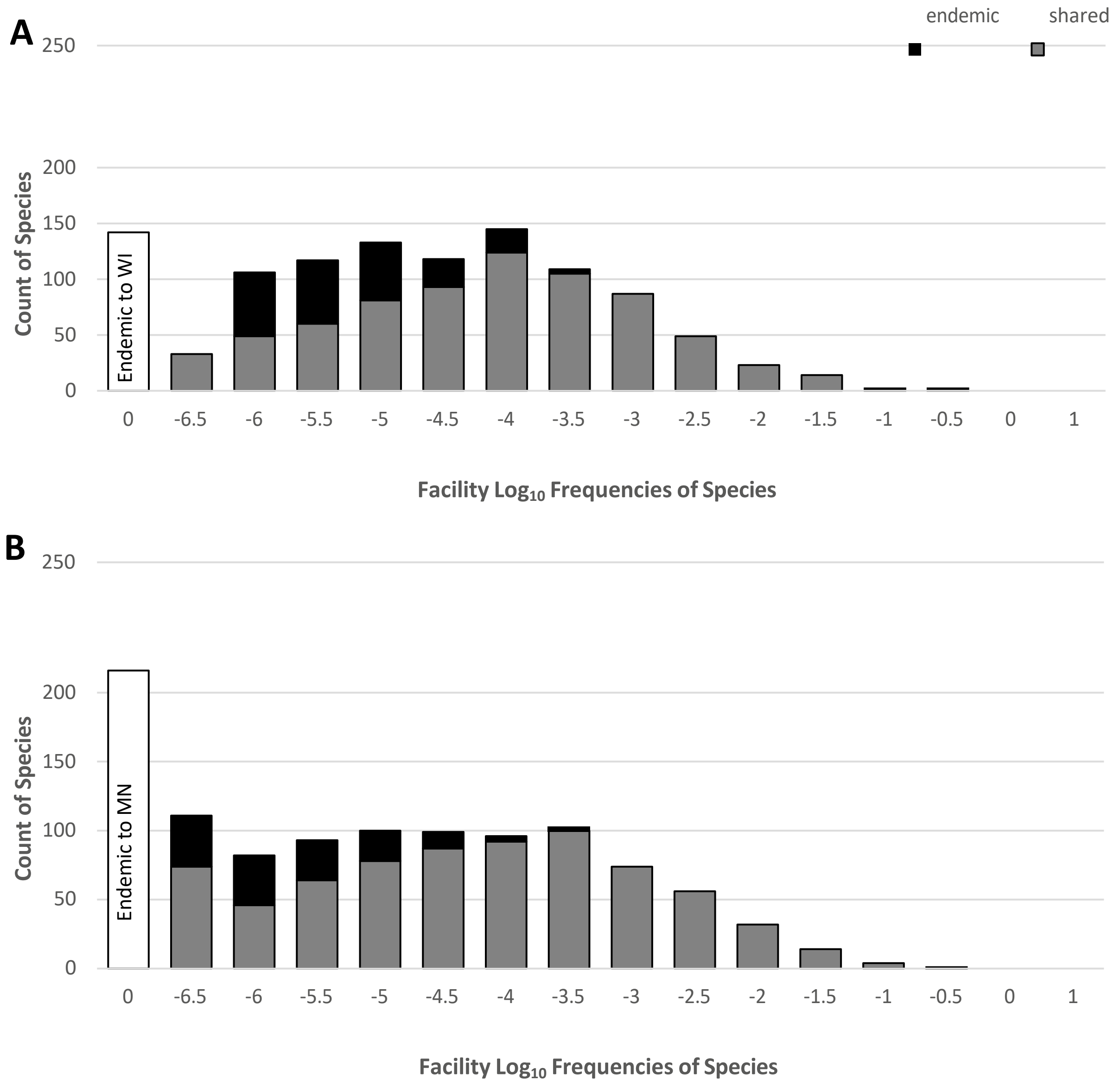

Figure 3. Frequency of species in MN (A) and WI (B) aquaponics systems. Bars represent the count of species occurring at a range of frequencies. Gray bars represent the taxa that are shared in the MN and WI systems, and black bars represent the taxa that are endemic to MN or WI. The white bar represents the number of taxa not present at that facility but present in the other.

Treatment effects on microbial populations

The WI aquaponics systems were stocked with varying levels of hybrid walleye, but there were no significantly dissimilar populations resulting from the different stocking densities. The MN aquaponics systems had red wiggler worms (*Eisenia fetida*) stocked into the gravel biofilters of three systems and three systems without worms.

Sample site effects on populations

Comparisons of samples longitudinally through the WI aquaponics systems indicated significant similarities among all sample sites for all comparisons at the species level The MN systems are also similar when evaluating longitudinally. At the species level, all sample populations are significantly similar. This indicates highly uniform communities of microbes throughout the MN and WI systems.

Microbes involved in nitrogen cycling

Conversion of ammonia waste products of fish to nitrite and nitrate is essential for an aquaponics system to maintain the health status of fish and provide plants with usable forms of nitrogen. Microbes involved in various aspects of the nitrogen cycle were identified using metagenomic analyses

Ammonia oxidizing bacteria (AOB) convert ammonia into nitrite. Members of the family Nitrosomonadaceae are common AOB. Members of Nitrosomonadaceae were present in both the WI and MN systems (Table 5), making up 0.42% and 0.011% of the populations, respectively. Unlike the WI systems, MN systems also contained the archaeal genus *Nitrosopumilus*, which are ammonia-oxidizing archaea (AOA). This group made up only 0.0005% of the total population, however. *Nitrosopumilus* is in Family Cenarchaeaceae, and unidentified members of this family were present in the system (0.00074%), suggesting the possibility that additional AOA are present. While *Nitrosopumilus* is present in MN systems, the higher occurrence of Nitrosomonadaceae suggests it has a greater role in ammonia oxidation than AOA. Wisconsin systems had a greater percentage of Nitrosomonadaceae (P=0.004, Mann-Whitney Test) than MN systems, but the percentage of *Nitrosopumilus* was not different between the sites (P=0.397, Mann-Whitney Test). The reason for the difference between MN and WI systems is not known, but could be related to system design, management, setup, or some other extrinsic factor.

Microbes that perform anaerobic ammonia oxidation (anammox) were also present in both MN and WI systems. This metabolic process is strictly anaerobic and reacts ammonia with nitrite to produce nitrogen gas and water, thus decreasing the levels of both toxic forms of nitrogen for fish. The nitrogen, however, becomes unavailable to plants. Microbes performing anammox are classified in Candidate Order Brocadiales, which comprised 0.0001% and 0.053% of the microbial population in MN and WI, respectively. Wisconsin systems had a significantly greater percentage of Brocadiales than MN systems (P=0.002). The majority of Brocadiales appears to be in the raft tank detritus, which is likely to encourage anoxic conditions and the growth of anaerobic bacteria. Comparable material was not available to be sampled from the MN systems, and it is possible that isolated anaerobic environments in the MN systems or elsewhere in WI systems may contain higher levels of anaerobic bacteria, including Candidate Order Brocadiales.

Nitrite oxidizing bacteria (NOB) convert nitrite to nitrate. Historically, this process was attributed to organisms in Genus *Nitrobacter*. This genus, however, was not identified in any of the samples. The Genus *Nitrobacter* is in the Bradyrhizobiaceae family, which was identified in the system. Not all members of this family were identified to the genus level, so it is possible that *Nitrobacter* is present, but not identified. Unknown members of Bradyrhizobiaceae comprised 0.0232% and 0.0092% of the microbial population in MN and WI, respectively. Although *Nitrobacter* is commonly associated with important NOB microbes, the Genus *Nitrospira* is readily found in environmental samples and oxidizes nitrite. *Nitrospira* was identified in MN and WI systems. In MN systems, *Nitrospira* made up 0.33% of the microbial population, whereas in WI, it made up 3.1% of the microbial population. The lack of other NOB identified suggests that *Nitrospira* is the primary NOB present in both WI and MN systems. Wisconsin systems had significantly greater percentages of *Nitrospira* (P=0.014, Mann-Whitney Test). Again,the reason for the difference between MN and WI systems is not known, but could be related to system design, management, setup, or some other extrinsic factor.

Table 5. Percent of the microbial population comprised of microbes involved in nitrogen cycling.

Sampling Site	NH_3 Oxidation Nitrosomonadaceae	NH_3 Oxidation *Nitrosopumilus*	Anammox Brocadiales	NO_2^- Oxidation *Nitrospira*
MN				
Biofilter Gravel	0.0424	0.0032	0	1.2026
Biofilter Water	0.0016	0	0	0.0516
Tank Water	0.0015	0	0	0.0453
Foam Raft	0.0013	0	0	0.0307
Tank Wall	0.0011	0	0	0.0251
Plant Roots	0.0104	0.0003	0.0003	0.5050
Pump Filter	0.0171	0	0.0005	0.4488
System mean	**0.0106**	**0.0005**	**0.0001**	**0.3299**
WI				
Fish Tank Wall	1.0616	0	0.0009	4.7397
Mineralization Tank Water	0.0214	0	0.0039	0.4212
Mineralization Tank Netting	1.1663	0	0.0011	4.4472
Raft Tank Water	0.0047	0	0.0007	0.0897
Raft Tank Wall	0.3711	0	0.0001	6.6135
Foam Raft	0.3371	0	0.0003	6.0694
Plant Roots	0.2223	0	0.0008	1.0216
Raft Tank Detritus	0.1703	0	0.4121	1.6142
System mean	**0.4194**	**0**	**0.0525**	**3.1271**

Objective 3. — Progress is being made toward completing a fact sheet outlining the importance of microbial populations in aquaponics systems. Topics will include different types of microbes necessary for the system, where they may be found in the population, and recommendations on sampling populations. Data generated from this project will be included. Once the fact sheet is completed, the recorded presentation will be produced using similar content and published to the NCRAC website. This project produce a large amount of data, and the analysis of the data is not complete for oral presentations or preparation of a manuscript. Analysis of the data will continue, and a manuscript and presentation is expected.

Principal Accomplishments

This project demonstrates the use of sampling techniques to study the microbial populations in aquaponics systems. Analyses of the microbial populations indicate that the various surfaces of the system are colonized by a diverse population of microbes, whereas the water exhibits fewer types of microbes. The surfaces, although made of different materials or found in different areas of a system, have similar microbial populations. This information is useful for future studies, because it indicates that not every variety or location of a surface needs to be sample. Although the organisms present in the MN and WI aquaponics facilities are similar, the proportion of specific taxa may be different, as evidenced by the proportions of microbes involved in nitrogen cycling. Those organisms are present in MN systems, but their proportions are greater in WI systems, suggesting that design or management of aquaponics systems can result in greater populations of beneficial organism. Finally, we have developed the computer coding programs to take the data generated from the Qiime pipeline and deconstruct the Qiime outputs to analyze the dataset in ways not done by Qiime. This will provide more streamlined analyses of metagenomic data in future projects. Much of the time spent on data analysis was related to developing the code to appropriately analyze the data in a meaningful way for aquaponics.

Impacts

This is the first study that has used metagenomics to study multiple locations within replicate aquaponics systems at different aquaponics facilities. The information collected from this project will be useful to future researchers interested in studying microbial populations in aquaponics systems. The use of samplers that can be put into systems and allowed to colonize will allow for more uniform sampling among sites within systems, different systems, and different facilities to provide more accurate comparisons between samples. Because the communities of microbes on various surfaces, i.e., plastic tank walls, foam, and netting, were similar, the sampling of all different types of surfaces may not be required. The reduction in sample sites within the system will allow for more repeated sampling over time to evaluate the stability of microbial communities in an aquaponics system.

The identification of the microbes nitrogen-related microbes, i.e., *Nitrosomonadaceae, Brocadiales*, and *Nitrospira*, suggest they are the organisms primarily involved in nitrogen waste conversion allows for development of management strategies to support their specific growth for optimal fish and plant health. The general identification of different types of microbes and the locations they inhabit within a system may be beneficial in developing strategies to prevent or reduce the possibility of harmful microbes, e.g., plant, fish, and human pathogens, from establishing themselves in a system.

Recommended future activities

This project provides a snapshot of two facilities. Future projects are needed to determine if the communities in a system are stable over time or if they fluctuate seasonally or in relation to facility management practices. As such, we recommend future support of studies that include the evaluation of microbial communities to determine if the frequency of populations of beneficial microbes, e.g., nitrogen-cycling organisms, can be increased for better plant growth. Additionally, although the MN and WI facilities demonstrated notable similarities between the types of microbes that make up a majority of the community, we cannot determine if this indicates an “aquaponics microbial fingerprint” or if the populations simply represent any indoors aquatic ecosystem or recirculating system. We suggest the sampling of a non-aquaponics system, such as a recirculating aquaculture system, a hydroponics system, or some other artificial aquatic environment as controls for microbial community studies in aquaponics.