pH and inoculum effects on microbial communities in bench-scale aquaponics systems

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Microbiology and Biotechnology

Department of Biological Sciences

University of Alberta

Abstract

The world is facing a number of serious problems of which urban population growth, climate change, decrease of arable land, constrained freshwater supplies and food security are among the most important. Aquaponics, as a commercially viable food production system that combines the production of fish (aquaculture) and plants (hydroponics) into a soilless recirculating water system, could contribute to addressing these problems. In aquaponics, some groups of microorganisms (e.g. nitrifiers and heterotrophs) clean up fish waste and leftover fish food by mineralizing it into a constant supply of nutrients to support plant growth. However, reconciling water quality parameters for optimum operation of the different aquaponics compartments has challenged the development of economically feasible aquaponics systems. Managing pH is a common concern among aquaponics growers as fish, plants, and microbial activity must be equally supported. Due to the higher solubility and availability of essential plant nutrients at lower pH values, operating aquaponics systems at a slightly acidic pH can result in higher plant yield. In contrast, nitrification (the microbial conversion of highly toxic ammonia released by fish to nitrate) is limited at pH values below 7.5 and can result in enhanced nitrous oxide emissions.

The first aim of this thesis (Chapter 2) was to adapt a microbial inoculum for a recirculating aquaponics system from an operational pH of 7.6 to 5.6 (desirable for plants), compare nitrification activity and production of N_2O, and describe changes in the adapted versus un-adapted communities. Four adaptation strategies were tested; our results indicated that a gradual reduction from pH 7.6 to 5.6, along with a gradual reduction followed by a gradual return of available ammonium, was the best strategy resulting in retention of 81% nitrification activity at pH 5.6 compared to pH 7.6. 16S rRNA gene amplicon sequencing and qPCR

enumeration of nitrification-related genes showed that the composition of pH 5.6 adapted microbial communities from all four adaptation strategies was similar to one another and distinct from those operating at pH 7.6, with enrichment of complete ammonia oxidizer (comammox) clade B bacteria over ammonia-oxidizing bacteria and Thaumarchaeota. N_2O production of the pH 5.6 adapted microbial communities was below detection in all adaptation experiments, likely due to the increased proportion of comammox bacteria. Aquaponics biofilters enriched with comammox bacteria and adapted to function at pH 5.6 can be a desirable inoculum for freshwater recirculating aquaponics systems to retain nitrification activity and improve crop yields.

In Chapter 3, the effects of pH and microbial inoculum on nitrification activity and dynamics of microbial communities from biofilters, plant roots, and fish intestines/gills in lab-scale aquaponics systems were examined. Although the initial ammonium concentrations were significantly lower in systems with comammox-enriched inocula than in systems with unenriched inocula, the systems stabilized to an ammonium removal rate of 72-87% over the course of operation, with slightly higher rates of ammonium removal in systems with unenriched inocula. *Nitrosomonas* and *Nitrosospira* were dominant ammonia-oxidizers in systems operating at pH 7.6-7.8, whereas *Nitrospira* (comammox) and plant growth-promoting microbes were more abundant in systems operating at pH 5.8 - 6. This study demonstrated functional stability and redundancy of aquaponics microbiota as a function of pH, although enrichment of comammox nitrifiers operating at a reduced pH led to consistently higher plant biomass with a small decrease in ammonium removal efficiency.

In Chapter 4, metagenomes extracted from lettuce roots and biofilters in two aquaponics mini-systems operating at pH 6 and pH 7.6 were examined using PacBio® sequencing

technology. Taxonomy analysis of HiFi reads using DIAMOND-MEGAN tools indicated that pH is a determinant of the composition of *Nitrospira* (comammox and nitrite-oxidizing bacteria) communities. Moreover, *Nitrosospira* (ammonia-oxidizing bacteria) and *Nitrosocosmicus* (ammonia-oxidizing archaea) species populated the systems at pH 7.6 and pH 6, respectively, with differing sensitivity to pH.

Annotating the metagenome reads against the SEED protein database revealed potentially higher siderophore production in the system operating at pH 7.6 than pH 6, indicating better iron solubility, even though plant growth measured in Chapter 3 was more robust at pH 6. In addition, sequence assembly and contig binning using MetaBat2 and SemiBin2 tools resulted in five high quality MAGs from the biofilter metagenomics.

Together, this thesis show that slightly acidic pH can increase crop production with no fish mortality. Different nitrifiers groups could be active in nitrification in aquaponics systems with different pH values. Comammox *Nitrospira* are the most efficient nitrifiers that can benefit systems operating at low pH. This study showed that despite differences in the abundance of particular phyla, the core microbiota tended to converge, regardless of operating pH and source of inoculum. Nevertheless, some microbial activities, such as siderophore production were influenced by changes in pH.

Preface

This thesis is an original work done by Peyman Derik Vand at the Department of Biological Sciences, University of Alberta, under supervision of Dr. Lisa Y. Stein. Thesis is written according to the guideline provided by the Faculty of Graduate Studies and Research, University of Alberta.

Chapter 1 of this thesis comprises a literature review to accompany the following data-based chapters.

Chapter 2 of this thesis has been published as Derikvand, P., Sauter, B., & Stein, L. Y. (2021). Development of an aquaponics microbial inoculum for efficient nitrification at acidic pH. *Applied Microbiology and Biotechnology*, *105*(18), 7009-7021. Peyman Derikvand designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Brittany Sauter assisted in data acquisition and analysis and provided input to the experiment design. Dr. Lisa Y. Stein provided funding for the work, input on the experimental design and data analysis, and edited the manuscript.

Chapter 3 of this thesis has been submitted as Derikvand, P., Sauter, B., Keddie, A., & Stein, L. Y. (2023). Inoculum and pH effects on nitrification and microbial community dynamics in aquaponics systems. submitted to *iScience*. Peyman Derikvand, Dr. Andrew Keddie and Dr. Lisa Y. Stein conceptualized and designed the study. Peyman Derikvand, Brittany Sauter, Dr. Andrew Keddie and Dr. Lisa Y. Stein designed the methodology. Peyman Derikvand and Brittany Sauter monitored the systems, collected samples and interpreted the data. Peyman Derikvand curated the data and wrote the original draft. Peyman Derikvand and Dr. Lisa Y. Stein reviewed and edited the manuscript. Dr. Lisa Y. Stein provided funding for the work. Dr. Lisa Y. Stein and Dr. Andrew Keddie supervised the study.

Chapter 4 of this thesis will be submitted for publication as Derikvand, P., & Stein, L. Y. (2023). Tentative title: Effects of pH on microbial activity and metagenomic diversity in a lab-scale aquaponics system. As primary author, I was responsible for the conceptualization, designing, performing experiments, bioinformatic analysis and will write the manuscript. Dr. Lisa Y. Stein was the supervisor and contributed in all stages of conceptualization, design, results analysis, manuscript composition, and advice.

Chapter 5 of this thesis presents overarching conclusions derived from this thesis and poses future directions for optimizing the function of aquaponics systems by understanding the dyanmics of microbial populations.

To my parents,

For their inspiration and support in my life and being my first teachers who taught me to believe in hard work and encouraged me to go on every adventure, especially this one

To my sisters and brother,

For being my best friends forever.

Acknowledgements

I would like to thank my esteemed supervisor, Dr. Lisa Stein for her invaluable supervision, continuous support, and advice during my PhD degree. I will never forget her kind and friendly manner. Without her valuable guidance and suggestions, it would have been impossible to complete my study. Her enthusiasm for research allowed me to accomplish more than I could have imagined. It was an enjoyable journey spending four years of research with her.

I would also like to thank Dr. Andrew Keddie, my supervisory committee member, for his valuable suggestions and providing me with facilities for conducting the experiments, and for his critical help in preparation for my candidacy examination and thesis defense. I appreciate him for investing his time in writing reference letters for me and reviewing my manuscripts and providing me with his expertise advice.

My sincere thanks to Dr. George Owttrim, Dr. Keith Tierney And Dr. Sylvie Quideau to be in my candidacy examination committee, help me in preparation for my candidacy examination, and providing me with their valuable feedback on my thesis.

I am grateful to Dr. Marc Strous to be a part of my final defense examination committee and investing his time in evaluating my thesis.

My gratitude extends to my lab mates, Hem Sharma, Dr. Marina Lazic, Shibashis Das, Brittany Sauter, Mariah Hermary, Philip Sun, Sujani Gomez, Cerrise Weiblen, and Elizabeth Arenas Diaz for a cherished time spent together in the lab, and in social settings.

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List of abbreviations

AMO: ammonia monooxygenase

ANOVA: analysis of variance

AOA: ammonia oxidizing archaea

AOB: ammonia oxidizing bacteria

ASV: amplicon sequence variant

Comammox: complete ammonia oxidizer

GC: gas chromatography

HAO: hydroxylamine dehydrogenase

HiFi: high fidelity

DNA: deoxyribonucleic acid

NCBI: National Centre for Biotechnology

NMDS: Nonmetric multidimensional scaling

NOB: nitrite oxidizing bacteria

NXR: nitrite oxidoreductase

PacBio: Pacific Biosciences

PCR: polymerase chain reaction

PERMANOVA: permutational analyses of variance

PGPM: plant growth-promoting microbes

qPCR: quantitative polymerase chain reaction

RNA: ribonucleic acid

Chapter 1: Literature review

1.1 Rationale for aquaponics

The increasing demand for water and food by an increasing human population have challenged the sustainability of traditional food production models, which often have high energy costs and large waste discharges. There is thus a need for efficiently engineered agricultural systems to supplement or replace traditional land and water intensive food production methods to obtain high crop yields with less environmental cost. Technological advancements have positively impacted the world in terms of food production. The chemical fertilizers and pesticides that sprung up after World War II resulted in a significant growth of crop yields. However, rapid growth of artificial fertilizer production, driven by the growing world population and shifting toward a more protein-rich diet, has severely impacted the environment. The enormous increase of global nitrogen input in agriculture, estimated at 114.6 Mt in 2024 (IFA, 2019), has altered the balance of nitrogen at a global scale. Due to low nitrogen use efficiency, approximately 60% of reactive nitrogen forms (e.g., ammonia and nitrate) escape from agriculture soils via leaching and runoff, resulting in eutrophication of freshwater and estuarine ecosystems, nitrate contamination of groundwater, and greenhouse gas emission (Zhang et al., 2015).

In addition, population growth has tripled the demand for aquaculture production, from 34 Mt in 1997 to 112 Mt in 2017 (Naylor et al., 2021), bringing several adverse effects to the environment. Aquaculture effluent contains large amounts of suspended solids, dissolved organic material, and nitrogenous and phosphorus compounds, which requires appropriate treatment prior to being discharged into receiving water bodies (Piedrahita, 2003). The nutrient-rich

effluent can be used as a source of fertilizer for soilless plant cultivation through combining recirculating aquaculture and hydroponics into aquaponics (Fig. 1.1). The aquaponics system is ideal for reducing water usage, fertilizer input, and waste discharge to the environment as compared to traditional agriculture or to hydroponics and aquaculture acting as separated systems.

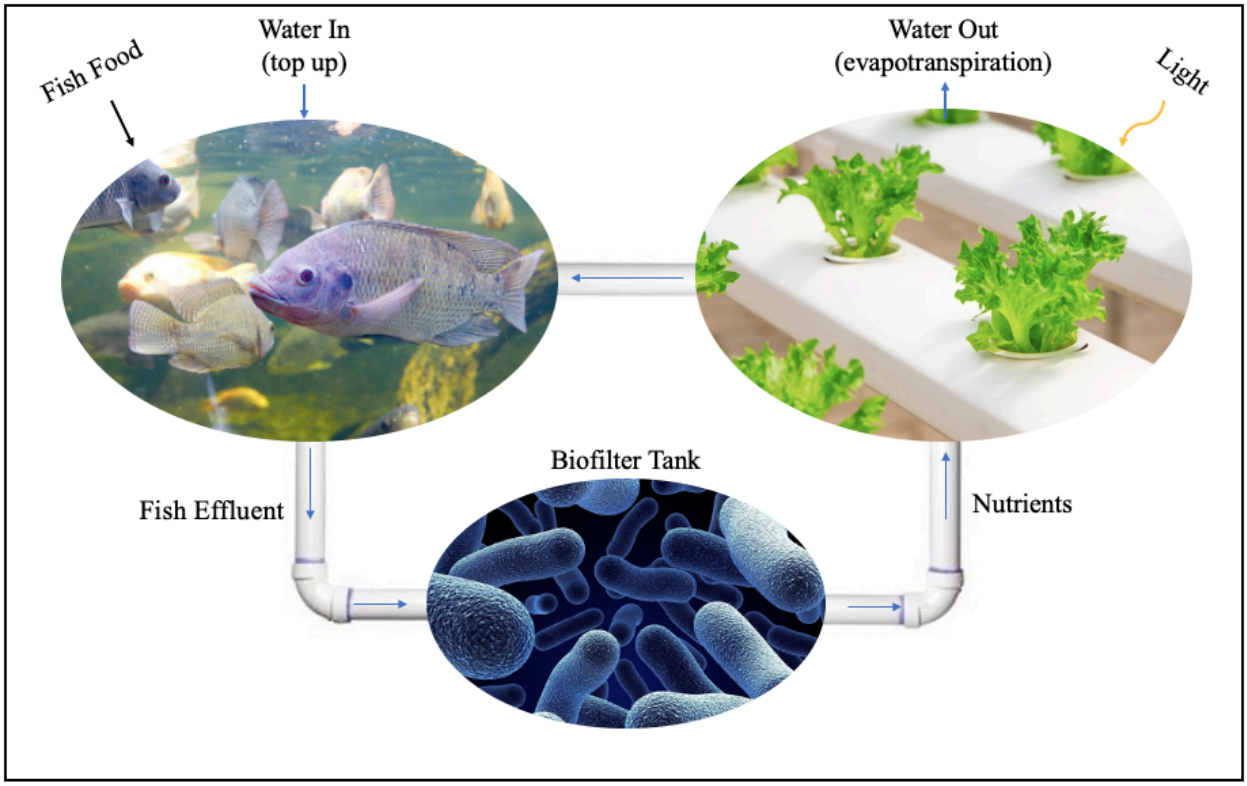

Figure 1.1 An overview of coupled aquaponic system. Fish food and light are the only energy inputs in this system. Microorganisms degrade and mineralize fish waste into nutrients for plant growth. Plants absorb elements from water and purified water circulates back to the fish tank.

Replacement of a portion of traditional agriculture with aquaponics could be a turning point in human history, providing a promising system for healthy food production, while decreasing environmental impact on the planet. Furthermore, aquaponics would enable providing fresh protein and vegetables in space stations and long distance space travels. Fertilized eggs of

herbivorous fish like tilapia have been suggested to be taken to space and then grown on board (Rezende et al., 2020). Plant and fish waste products can be recycled into efficient fish feed for further protein production in aquaponics. The viability of these methods needs to be studied in detail if we are to rely on a radically different system for providing an essential organic food resource.

1.2 Hydroponics

Hydroponics is a technique of soilless plant cultivation without the use of mechanical supports such as perlite, gravel, and rockwool. In this technique, plant roots are immersed in a mineral-based nutrient solution. Most of the commercial scale hydroponic systems have been designed to control the water level, nutrients, and photoperiod automatically (Sharma et al., 2018). This cultivation method is rapidly growing to sustainably feed the world's growing population, especially in urban areas where access to land is limited, and in harsh arid environments where both arable land and water are scarce. Efficient management of resources makes hydroponics suited for cultivating a variety of crops including leafy vegetables, tomatoes, cucumbers, peppers, strawberries, and medicinal plants (Sardare et al., 2013).

Hydroponics circumvents the limitations of soil-based agriculture, such as the presence of disease-causing microbes and nematodes, unsuitable soil reactions, and poor drainage, and provides many advantages including higher crop yield, low water consumption, and reduced need for herbicides and pesticides (Romeo et al., 2018). Critical levels of seventeen elements, categorized as macro- and micro-nutrients, that are needed for cultivating common crop plants in hydroponics systems have been determined. Potassium nitrate, potassium phosphate, magnesium sulfate, iron chelate, and calcium nitrate are common soluble salts added to hydroponics as

sources of mineral nutrients. The frequency and amount of mineral salts added to hydroponics systems depends on the plant type, irrigation system, and the size of the container (Mugundhan et al., 2011). However, along with significant start-up cost as well as demanding labor and management, the expense and resources to maintain a high concentration of nutrients in hydroponics water systems has challenged crop production and output (Khan, 2018).

1.3 Aquaculture

Fish is a highly beneficial food source for human nutrition and provides a good source of protein, unsaturated fatty acids, minerals, and vitamins. Aquaculture has been the fastest expanding sector of protein production during the past decades, and the world's population growth has tripled the demand for aquaculture production from 34 Mt in 1997 to 112 Mt in 2017 (Naylor et al., 2021). Regarding an estimated population of nine billion people in 2050 (FAO, 2012), development of efficient and environmentally friendly aquaculture systems with minimum water usage is imperative to meet future world food needs.

In aquaculture, approximately 95% of feed is consumed by farmed fish, around 5% remains in the water. Of the consumed feed, around 30-40% is conserved into fish biomass, based on the feeding and fish type, and 60-70% is released into water in the form of feces and urine (Robaina et al., 2019). Furthermore, ammonia resulting from digestion of proteins is released through the gills (Khakyzadeh et al., 2015). Large amounts of suspended solids, dissolved organic matter, and nitrogenous and phosphorus compounds released into aquaculture effluent requires appropriate treatment prior to being discharged into receiving water bodies (Piedrahita, 2003). Recirculating aquaculture systems (RAS) have been designed with the aim of lowering water consumption. In RAS systems, waste reduction and water treatment are achieved

within a recirculating loop. RAS water treatment is accomplished by incorporation of sludge digestion and nitrification as well as denitrification (Van Rijn, 2013). An alternative biological ammonia removal method considered for RAS is anaerobic ammonium oxidation (anammox) in the anoxic segment of water treatment facilities. The anammox process eliminates nitrogen by combining ammonium oxidation and nitrite reduction to produce nitrogen gas (Strous et al., 2006).

According to Rakocy et al (2003), fish waste and leftover fish food provide most of the nutrients required by plants if the ratio between fish feeding and plant growth is optimally sustained. Therefore, instead of denitrification/anammox removal of nitrogen, RAS can be combined with hydroponics into aquaponics, thus reducing water usage, fertilizer inputs, and waste discharge to the environment.

1.4 Aquaponics Overview

Aquaponics, the portmanteau word of aquaculture and hydroponics, is a term that was coined in the 1970s (Lewis and Wehr 1976; Naegel 1977), but the progenitor of nearly all modern aquaponics is thought to be the work performed by, and the systems produced by, James Rakocy and his team at the University of the Virgin Islands (UVI) starting in the early 1980s. The design of systems for aquaponics farming can vary from simple mini-systems containing a single fish tank with plants floating in water above the fish to more elaborate industrial systems with entire warehouses converted to vertical agriculture units. Regardless of the complexity of systems, the aim of aquaponics design is almost always to farm plants and seafood for profit. Combining recirculating aquaculture and hydroponics for commercially viable food production is supported by a biological water filtration tank (microbial biofilter) (Junge, *et al*., 2017). Fish

are raised in a separate tank which release ammonia-rich waste from protein digestion into the water. Water from the fish tank is pumped into a biofilter tank. In the biofilter, microorganisms clean up fish sludge (i.e. feces and uneaten fish food) by mineralizing it into a constant supply of nutrients to support plant growth (Fig. 1.1). Plant roots filter the water for fish by absorbing excess elements in the water. The only energy inputs to most aquaponics systems are fish food and light which distinguishes them from hydroponics systems in which the essential plant nutrients come from manufactured fertilizers. Oxygen required to support fish survival and microbial metabolism enters the system via an air pump.

1.5 Variation in aquaponics designs

Based on the hydroponics compartment, aquaponics systems are primarily designed in three different types: Nutrient Film Technique (NFT), Media Bed, and Deep-Water Culture (DWC). The NTF design uses horizontal pipes, usually polyvinyl chloride (PVC), to create a channel with a shallow stream of water carrying dissolved nutrients to the plant compartment (Fig. 1.2a). Because of their space efficiency and lower labor costs, NFT systems are widely used in design of commercial aquaponics systems. Crops in NFT networks can also be grown on vertical shelves, which increases crop yields per unit area and provides easy access to and harvesting of plants (Al-Tawaha et al., 2018). This method is best suited for leafy greens like lettuce as it provides a continuous supply of water, oxygen, and nutrients. However, this design is not suited for large fruiting plants like tomatoes and cucumbers, as there is a higher risk of disease spread, root masses may clog the channel, and the weight of the plants may not be supported. In addition, in NFT systems the plant roots are exposed to the air, which can leave them vulnerable to temperature fluctuations (Lennard and Leonard., 2006).

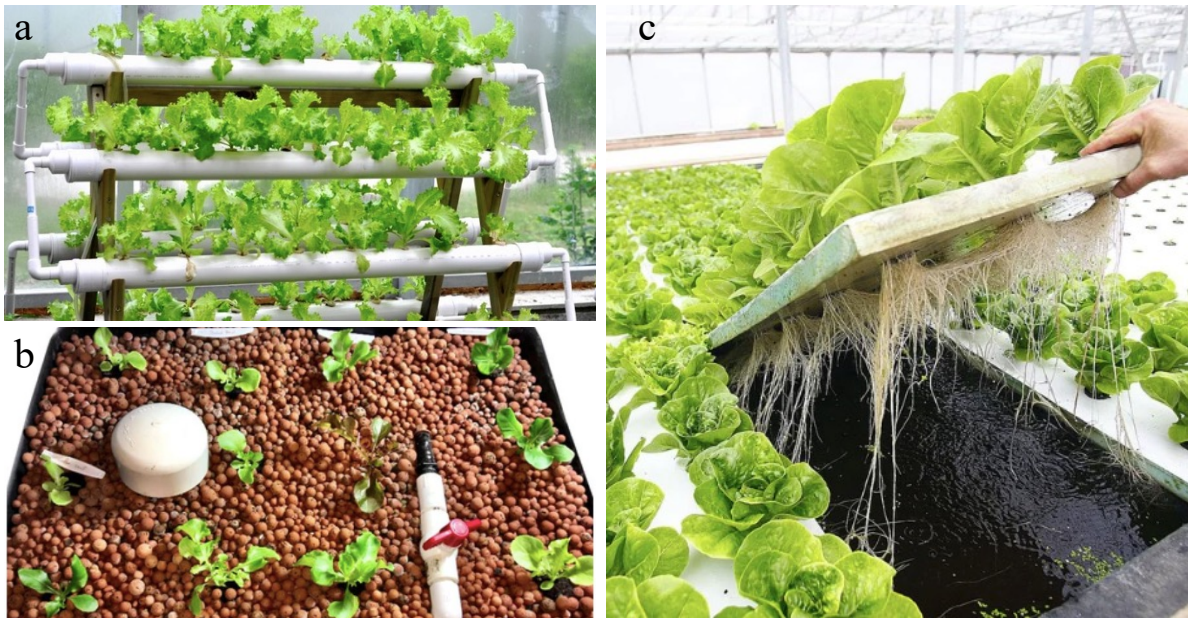

Figure 1.2 Three types of aquaponics system design: a) Nutrient Film Technique (NFT) is a channel of horizontal pipes with a shallow stream of water coming from fish tank passing through pipes network, b) media bed system in which the flood and drain of nutrient rich water in the rock bed provides the plants the nutrients to grow, and c) Deep Water Culture (DWC) with plant roots suspending directly into aerated and nutrient-rich water coming from fish tank (https://gogreenaquaponics.com/blogs/news/what-is-a-raft-based-aquaponics-system https://nipgroup.com/aeroponic-aquaponic-hydroponic-vertical-farm/ https://rgjaquaponics.weebly.com/media-bed-technique.html)

Unlike NFT, media bed systems use rock media such as gravel or expanded clay (hydroton) to support the roots of plants (Fig. 1.2b). The flooding and drainage of nutrient rich water in the rock bed provides nutrients and oxygen to the plants to support their optimal growth. In addition to acting as a mechanical support for plants, the gravel in the media bed also provides a high surface area for microorganisms to create biofilm which acts as a biofilter to capture and breakdown wastes (Goddek et al., 2019). The media bed system is simple to construct at small scales, which has made this aquaponics design the best for beginners and is widely used for personal gardening. Because the media bed provides a mechanical support for plants similar to

soil, large root masses from fruit-bearing and flowering plants can be farmed in these systems. However, media bed aquaponics systems are hard to scale up into commercial systems since the design of the hydroponic component is not space efficient at scale and requires more input of labor (Lennard and Leonard., 2006).

DWC, also known as the raft system, uses floating rafts to support plants with plant roots suspended directly into the aerated, nutrient rich, water coming from the fish tank (Fig. 1.2c). In these aquaponics systems, the biofilter tank must be built into the design as there is no solid media (like media bed systems) or enough surface area (like NFT) to capture and process the solid wastes. Therefore, more advanced aquaculture techniques and system design is necessary for DWC systems which increases the upfront costs. DWC aquaponics is the most common design for commercial production as it is more scalable and stable as compared to the other two system types. A higher volume of water in the system reduces drastic fluctuations of nutrients and temperature (Goddek et al., 2019).

1.6 Microorganisms in aquaponics systems

1.6.1 Nitrifying microorganisms

In aqueous media, ammonia nitrogen can be found in two chemical forms: a non-ionized form (NH_3) that is highly toxic to aquatic organisms and an ionized form (NH_4^+) that has lower toxicity to aquatic organisms. Together they form the total ammonia nitrogen (TAN), wherein the ratio between the two forms is mainly controlled by pH, and to a lesser extent by temperature and salinity of water. Ammonia in aquaponics is the product of protein digestion by the fish, released into water mainly through their gills, and accumulates to toxic concentrations if left untreated. Elevated ammonia concentration causes convulsions, coma, and death in fish and

other aquatic animals through displacing K^+ and depolarizing neurons which leads to the activation of NMDA type glutamate receptors and influx of excessive Ca^+, followed by cell death in the central nervous system (Randall and Tsui, 2002). Concentrations >1mg NH_3/L has shown toxicity to carp species (Abbas, 2006).

Ammonia can be oxidized in biofilters by a communities of microorganisms called nitrifiers. These organisms are aerobic chemolithotrophs that obtain their energy from the oxidation of inorganic nitrogen compounds (ammonia and nitrite) and grow very slowly. Therefore, these organisms can be outcompeted by heterotrophic bacteria if organic carbon, mostly present in biosolids suspended in the water, is allowed to accumulate in the system. Solid forms (sand grains, stones, plastic elements, etc.) are used as substrates to provide surface area for bacterial attachment and biofilm formation, which retains nitrifying bacteria in the system (Goddek et al., 2019).

The nitrification process is facilitated by ammonia oxidizing bacteria (AOB) and archaea (AOA) and nitrite oxidizing bacteria (NOB) (Stein, 2019). In this process, AOB (such as *Nitrosomonas* and *Nitrosospira* genera) and AOA (such as *Nitrososphaera*) catabolize ammonia to nitrite, which serves as the substrate for NOB (such as *Nitrobacter* and *Nitrospira*) to produce nitrate (Fig. 1.3). For more than a century, nitrification was thought to be a two-step process with metabolic labor divided between ammonia oxidizers and nitrite oxidizers. However, oligotrophic bacteria capable of complete oxidation of ammonia to nitrate were postulated to exist in the environment (Costa et al., 2006), and ten years later, complete ammonia oxidizing *Nitrospira* (comammox *Nitrospira*) were discovered from biofilm samples (Daims et al., 2015; Van Kessel et al., 2015).

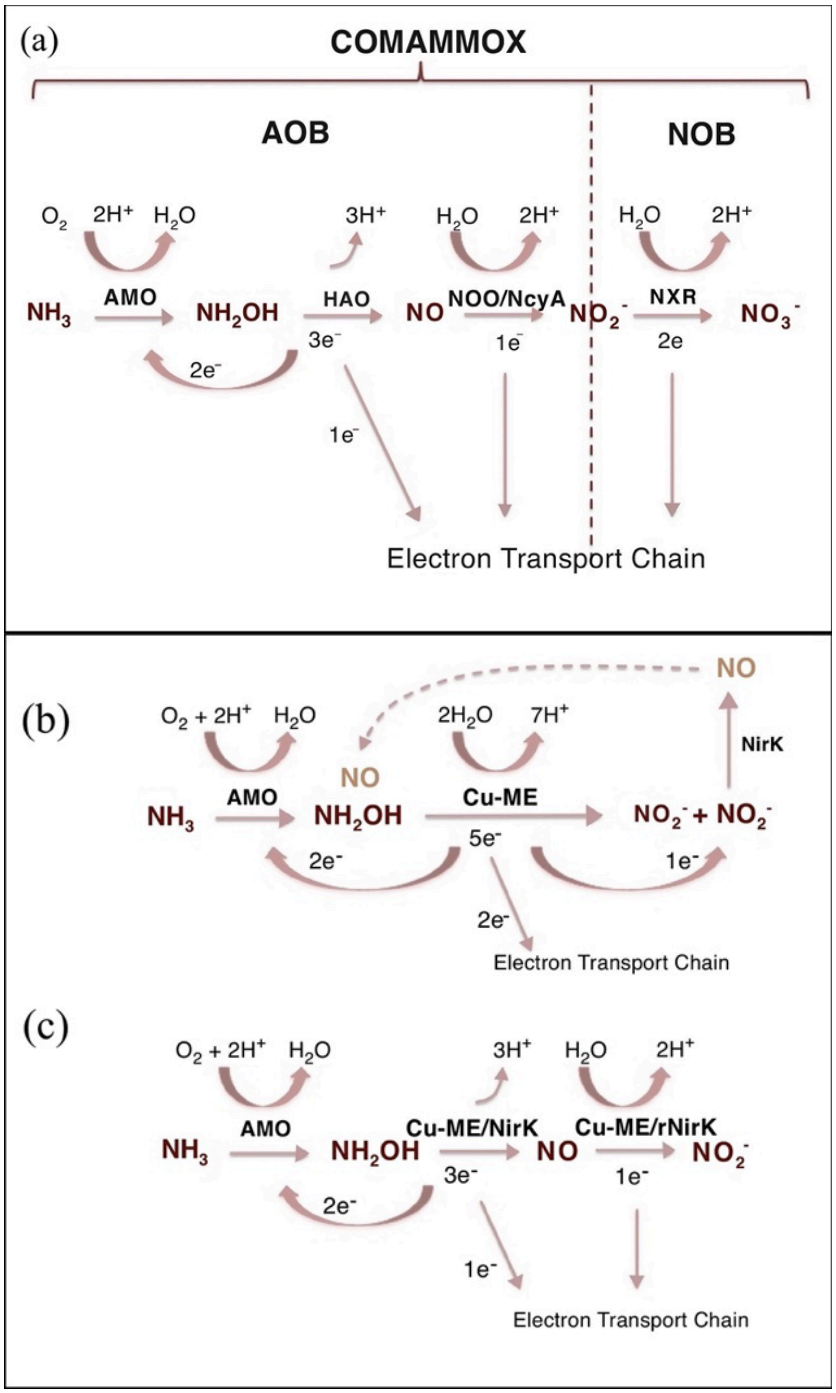

Figure 1.3 Pathways for energy conservation by a) ammonia oxidizing bacteria, nitrite oxidizing bacteria and comammox *Nitrospira*, b) and c) postulated pathways for energy conservation by ammonia oxidizing archaea. AMO: ammonia monooxygenase; HAO: hydroxylamine dehydrogenase; NOO: nitric oxide oxidoreductase; NXR: nitrite oxidoreductase; Nir: nitrite reductase; Cu-ME: copper-based metalloenzyme complex (Adapted with permission from Stein, 2019, Current Opinion in Chemical Biology, Copyright Clearance Center, 2023).

Nitrate (NO^{-}_{3}), the end product of nitrification, has significantly lower toxicity to fish, as compared to nitrite and ammonia. At very high concentrations (> 150-300 mg/L depending on the fish species), nitrate limits the capacity of oxygen-carrying molecules in fish blood, similar to the toxicity mechanism of nitrite. However, unlike nitrite, nitrate cannot easily penetrate through the fish gill membrane, and low toxicity of nitrate is mainly attributed to its low permeability (Wongkiew et al., 2017).

Nitrous oxide (N_2O), a potent greenhouse gas (~300-fold greater warming potential than CO_2 on a 100 year time scale) and a major cause of stratospheric ozone depletion, is also a byproduct of nitrification which is formed via both biotic and abiotic reactions (Fig. 1.4). Ammonia oxidation is one of the pathways to generate N_2O, and low pH and hypoxia in aquatic and coastal ecosystems have been shown to promote N_2O production from nitrification (Babbin et al., 2015; Stein and Klotz., 2016). AOB are the main source of nitrification-related N_2O emissions relative to AOA and comammox microorganisms. Under sub-oxic conditions, many AOB species reduce nitrite to nitric oxide (NO) and N_2O via a process termed "nitrifier denitrification" which is performed to maintain intracellular redox balance at low O_2 concentrations (Stein, 2019). Enzymatic generation of N_2O by AOB may also occur through anaerobic oxidation of hydroxylamine by the enzyme cytochrome P460 (CytL) (Caranto et al., 2016).

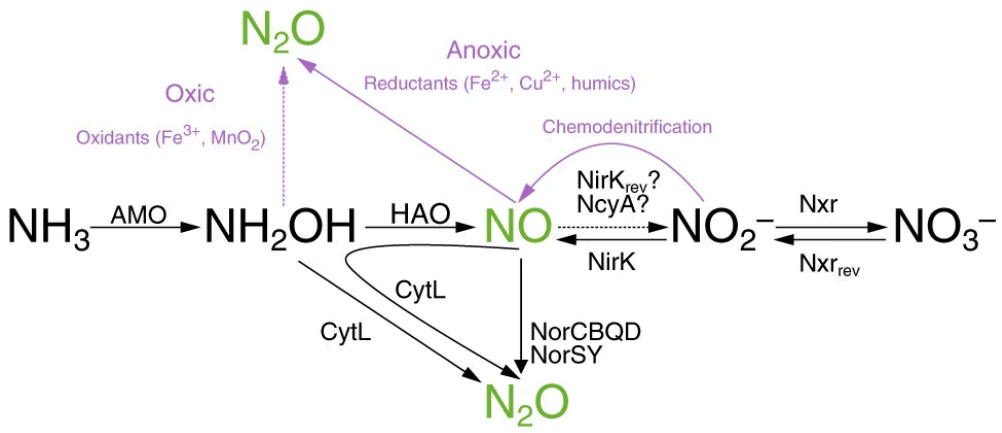

Figure 1.4 Biotic and abiotic production of N_2O in AOB. Black arrows indicate enzymatic pathways, and purple arrows indicate abiotic interaction leading to N_2O generation. Interaction of hydroxylamine, nitric oxide and nitrite with each other and with organic and inorganic substances, including humics, ferrous and ferric iron, Cu^{2+} and MnO_2, leads to formation of N_2O at both oxic and anoxic conditions (Adapted with permission from Kits et al., 2019, Nature Communications, Rights and Permissions, 2023).

AOA and comammox *Nitrospira* do not have the enzymatic machinery to perform nitrifier denitrification (Kozlowski et al., 2016; Kits et al., 2019). Nevertheless, abiotic reactions resulting in N_2O generation can occur through nitrification intermediates released by these organisms and also by AOB. Interaction of hydroxylamine, nitric oxide and nitrite with each other and with organic and inorganic substances, including humics, ferrous and ferric iron, Cu^{2+} and MnO_2, leads to formation of N_2O at both oxic and anoxic conditions (Stein, 2019).

1.6.2 Heterotrophic microorganisms

Heterotrophic microbial communities are important constituents of aquaponic systems. These microorganisms play significant roles in nutrient recycling and biodegradation of organic matter. Heterotrophic bacteria in aquaponics and RAS mainly originate from fish feces and are key organisms for sludge treatment and water quality. These microorganisms are actively involved in the biodegradation of organics and removal of biosolids in aquaponics; thus,

lowering the turbidity and improving water quality. Microbial degradation of organics in aquaculture mainly includes fine solids (particles < 30 μm) which cannot be removed by sieving (Goddek et al., 2019).

Another major role of heterotrophic bacteria in aquaponics could be phosphorous cycling. Different phosphatase producing strains of *Bacillus, Pseudomonas*, and *Enterobacter* enhance phosphorous availability to plants by mineralizing organic phosphorous (mainly phytates) into orthophosphates (da Silva Cerozi and Fitzsimmons, 2016). Phosphorus has a fundamental role in nucleic acid production and ATP formation and can induce rapid growth of buds and flowers and stimulate root development (Goddek et al., 2019). Strains of *Bacillus* and *Pseudomonas* can also ameliorate the deficiency of ferric ion non-availability by producing bacterial siderophores (e.g. organic iron-chelating compounds) (Goddek et al., 2019).

Despite their importance for efficient performance of aquaponics, control of heterotrophic bacteria can be a major difficulty in systems with an unbalanced C/N ratio. Since biosolids serve as a substrate for the growth of heterotrophic microorganisms, an increase in the concentration of organics in the water may eventually result in increased oxygen consumption and poor water filtration.

1.7 Effects of pH on aquaponics

pH represents one of the most important water quality compromises present in the aquaponics industry. pH controls a wide variety of equilibrium reactions and water solubility of chemicals in both hydroponics and aquaculture. The recommended pH range for hydroponic systems is between 4.5 and 6.0 whereas the RAS industry typically applies pH settings between 7.0 and 8.0 (Goddek et al., 2019). Water pH affects the solubility of ions, and at pH > 7, most of

the nutrient elements required for plant growth, such as $SO4^{2-}$, $PO4^{3-}$, Ca^{2+}, Fe^{2+}, and Mn^{2+}, precipitate into insoluble and unavailable salts (Goddek et al., 2019). Lowering the water pH in hydroponics to slightly acidic values increases root uptake of ions and enhances crop yield (Goddek et al., 2015). In contrast, acidic water is a major stressor in aquaculture and low pH values negatively affect the welfare of fish (Yavuzcan Yildiz et al., 2017). In freshwater fish, pH < 5.5 can increase the turnover of ion-transporting cells and mucus accumulation in gills, as well as decrease in plasma Na^{+} and Cl^{-} levels (Kwong et al., 2014). Mukherjee et al (2019) showed that acidic pH induces detrimental oxidative damage (including alteration in glutathione system and superoxide dismutase-catalase system) followed by associated neurotoxicity effects in carp species. However, freshwater fish species farmed in aquaponics systems can tolerate a pH range of 6.1-8, and growth and reproduction of freshwater fish is not limited as long as pH > 5.5 (Crane, 2006; Makori et al., 2017).

In addition, nitrification in aquaculture systems has been reported to be most efficient at a pH range of 7.5 – 9 (Hochheimer and Wheaton, 1998). Nitrification activity of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) decreases at pH values below 7.5, accompanied by enhanced nitrous oxide emission (Zou et al., 2016). NOB are typically more sensitive to acidic pH than AOB, and faster growth of AOB at lower pH values can result in the accumulation of toxic nitrite in water which causes detrimental effects on fish (Goddek et al., 2019).

Integrating RAS and hydroponics into aquaponics is, therefore, hampered by the challenges of pH. Optimally, an operational pH should be identified that matches requirements of plants, fish and microorganisms to achieve maximum nitrogen removal efficiency and production.

1.8 High throughput sequencing

Identification of the microbial composition of any habitat including animal intestines, soil, marine, aquatic, and industrial systems, to name a few, is referred to as microbiome analysis and is an essential activity in microbiology. Advances in high-throughput DNA sequencing have expanded our understanding of microbial diversity and ecology of different niches, which provides a basis for microbiome-based therapeutics and management of environmental and industrial systems. Obtaining a high resolution profile of microbial communities is necessary to accurately understand the impact of physicochemical factors on the functional capacity of microbiota regardless of the niche they occupy.

Microbial profiling using high-throughput sequencing technologies have rapidly evolved over the past decades, becoming increasingly fast, sensitive, and cost-efficient. Amplicon-based 16S rRNA gene sequencing is the most common method for studying community patterns of microorganisms. Next-generation sequencing (NGS) metabarcoding allows for an approximation of the relative abundances of different microbial taxa and to compare between groups of interest that cannot be easily cultured on selected media. This method also can help to investigate changes in the microbiomes over time, or between treatment groups. Various universal primers have been designed for profiling bacterial and archaeal communities, which target partial sequences in the hypervariable regions of the 16S rRNA gene (including V_1-V_2, V_1-V_3, V_2-V_4, V_3-V_4, and V_4) (Kameoka et al., 2021).

Nevertheless, studying microbiomes using amplicon sequencing has some limitations. For instance, because of high similarity between ribosomal genes of closely related organisms, in most cases the resolution of profiling is limited to the genus level. In addition, the primers used

for amplicon sequencing may introduce biases, as these primers typically bind regions that are not 100% conserved across all taxa (Gupta et al., 2019).

Shotgun metagenome sequencing (metagenomics) can provide unbiased profiling of microbial communities with higher taxonomic resolution. Unlike amplicon sequencing which targets specific gene regions, metagenomics predicts complete genomic sequences of all microorganisms in a sample, including bacteria, archaea, and viruses, and allows discovery of and linkages among unknown functional genes and insights into functional processes of specific ecosystems (Xie et al., 2020). Much of metagenomics studies are based on short-read sequencing using NGS technologies (e.g. Illumina sequencing). This method enables microbiologists to sequence thousands of organisms in parallel, with the ability to combine several samples in a single sequencing run. Nevertheless, short-read shotgun sequencing (<500 bp reads) rarely covers a gene of interest, or connects neighboring genes, and necessitates assembly before taxonomic and functional analysis. Short-read sequencing also makes *de novo* assembly of genomes a challenging and laborious task due to the presence of repetitive DNA regions and shared genomic regions among strains (Gehrig et al., 2022).

Third-generation sequencing technologies which provide longer sequencing reads can surmount many difficulties in gene alignment and metagenome assembly via spanning multiple genes and repetitive regions. Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio®) are the most popular third-generation platforms for long-read sequencing (~ 10 kb reads). ONT is a low cost and convenient technology but with high base error rate, which limits its utility in microbiome profiling. ONT sequencing is typically used in combination with Illumina shotgun sequencing to obtain complete metagenome assembled genomes (MAGs). On the other hand, the circular consensus sequencing (CCS) method developed by PacBio®

performs multiple passes of a circularized template molecule before a consensus sequence is reported, which generates high fidelity reads (HiFi reads) with low native error rate of ~0.1% (Portik et al., 2022). This technology has resulted in a significant increase in the average contig length and high numbers of complete circular bacterial genomes per dataset as compared to other methods (Feng et al., 2022).

Alternatively, bioinformatic workflows have been optimized for *in silico* analysis of long-read sequences. HiCanu (Nurk et al., 2020), metaFlye (Kolmogorov et al., 2020), and hifiasm-meta (Feng et al., 2022) are the pipelines developed for *de novo* assembly of long-reads. In a benchmarking study, Xiaowen Feng and colleagues (2022) evaluated the performance of these metagenome assemblers on PacBio® sequences of seven empirical datasets (including mock communities and real datasets) and indicated that hifiasm-meta workflow resulted in the highest number of complete circular bacterial genomes per dataset, consistently outperforming other assembler pipelines (Fig. 1.5a). In addition, Daniel Portik and colleagues (2022) performed a critical benchmarking study on PacBio® reads using eleven pipelines designed for taxonomic classification and functional profiling, and indicated that two long-read workflows, BugSeq and DIAMOND- MEGAN (Fig. 1.5b), performed the best and displayed high precision and recall, with accurate detection of all species down to 0.1% relative abundance level.

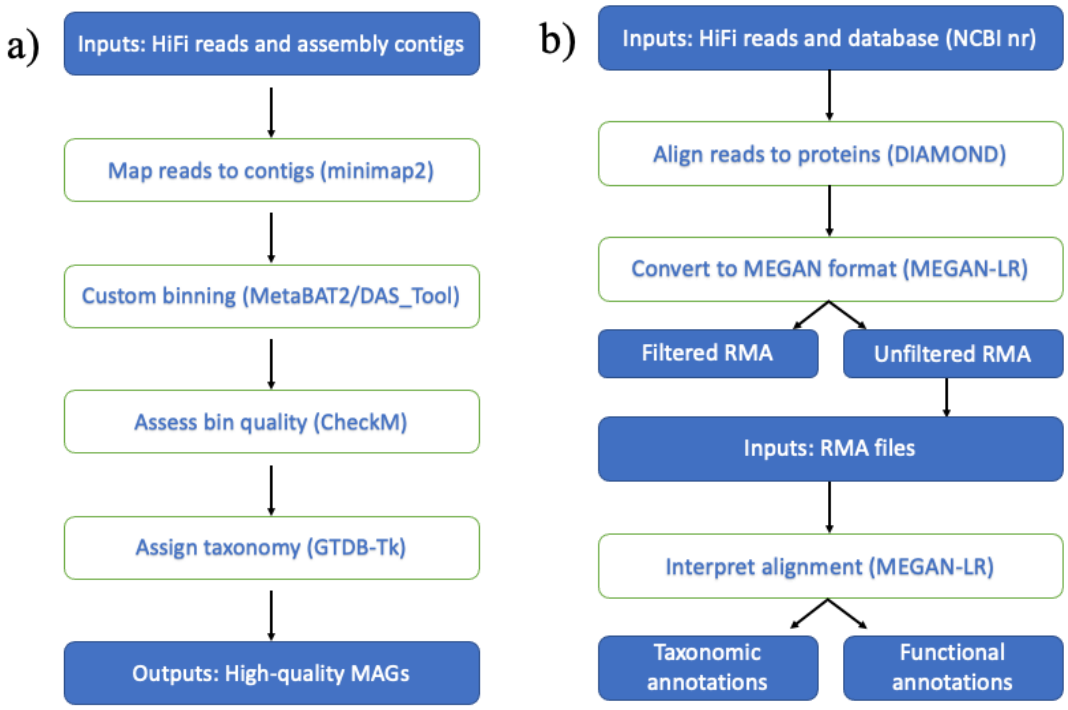

Figure 1.5 Workflows developed for analysis of HiFi PacBio® sequences: a) HiFi-MAG pipeline. In this circular-aware pipeline, minimap2 and MetaBAT2 are used for binning the MAGs, and DAS_Tool is used to merge the bins. CheckM tool is used to check the quality of MAGs (based of default criteria). GTDB-Tk is used to assign taxonomy to high quality MAGs, and b) taxonomic classification and functional profiling pipeline. Diamond is used to align the reads to NCBI-nr protein database, and Megan-LR (long read algorithm) is used for taxonomic and functional annotation of sequences. SAMtoRMA tool was used to convert SAM files to RMA format for Megan analysis.

As a result of these improvements, PacBio® CCS sequencing offers new opportunities for high resolution taxonomic classification, functional annotation, and metagenome assembly.

1.9 Objectives of thesis

Combining aquaculture and hydroponics into aquaponics is seen to be one of the key food production technologies which 'could change our lives' (Van Woensel, *et al*., 2015). However, reconciling water quality parameters for optimum operation of the different aquaponics compartments has challenged the sustainability and commercial efficiency of these systems.

Managing pH is a major difficulty in aquaponics systems. While nitrification, a key microbial process for maintaining water quality in aquaponics, is optimally active at a slightly alkaline pH (7.5 – 9.0), plants typically prefer a slightly acidic pH (5.5 – 6.5) for optimal growth. Most aquaponics facilities therefore target a pH between 6.8 and 7. However, crop yields are not reaching economically sustainable levels for most commercial aquaponics growers (Love, *et al*., 2014), which has stimulated increased research into plant growth optimization and nitrogen use efficiency in these systems (Junge, *et al*., 2017). In addition, operation of aquaponics systems at suboptimal pH for nitrification results in higher emission of nitrous oxide (Zou, *et al*., 2016). Lowering the pH of the aquaponics systems to a pH level for optimal plant growth would equal a higher yield and net increase in profits. Furthermore, if lowering the operating pH also lowers nitrous oxide production, that is a further reduction of negative environmental impacts brought about by aquaponics systems.

Towards achieving the goal of identifying an ideal operating pH in aquaponics systems to sustain nitrogen use efficiency, increase plant productivity, maintain fish health, and understand impacts of pH on microbiota, the objectives and chapter summaries in this thesis are as follows:

a) **Chapter 2**: adaptation of the microbial community from an aquaponics biofilter to an operating pH range that is optimal for plant growth without sacrificing microbial functionality,

b) **Chapter 3:** evaluating the effects of operating pH and microbial inoculum on ammonium removal rates and the spatial/temporal distribution of microbial communities within and between aquaponics compartments,

c) **Chapter 4:** performing an in-depth metagenomic analysis of microorganisms from aquaponics biofilters and plant roots operating at two pH levels to evaluate effects of pH on microbial nutrient cycling functions for sustaining water quality and system operation, and

d) **Chapter 5:** synthesizing the information gathered from the experimental chapters to make recommendations for how to best optimize nitrogen use efficiency and operation of aquaponics systems as a function of pH.

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Chapter 2: Development of an aquaponics microbial inoculum for efficient nitrification at acidic pH

2.1 Abstract

Maintaining an optimal pH that simultaneously supports plants, fish, and nitrifying microorganisms is a challenge in recirculating aquaponics systems as nitrification is optimal at a slightly alkaline pH and plant growth is optimal at a slightly acidic pH. Freshwater fish tolerate pH > 5.5. Our aim was to adapt a microbial inoculum for a recirculating aquaponics system from an operational pH of 7.6 to 5.6, compare nitrification activity and production of N_2O, and describe changes in the adapted versus unadapted microbial communities. Four adaptation strategies were tested; our results indicated that a gradual reduction from pH 7.6 to 5.6, along with a gradual reduction followed by a gradual return of available ammonium, was the best strategy resulting in retention of 81% nitrification activity at pH 5.6 compared to pH 7.6. 16S rRNA gene amplicon sequencing and qPCR enumeration of nitrification-related genes showed that the composition of pH 5.6 adapted microbial communities from all four adaptation strategies was similar to one another and distinct from those operating at pH 7.6, with enrichment of comammox clade B bacteria over ammonia-oxidizing bacteria and thaumarchaeota. N_2O production of the pH 5.6 adapted microbial communities was below detection in all adaptation experiments, likely due to the increased proportion of comammox bacteria. Aquaponics biofilters enriched with comammox bacteria and adapted to function at pH 5.6 can be a desirable inoculum for freshwater recirculating aquaponics systems to retain nitrification activity and improve crop yields.

2.2 Introduction

The increasing demand for water and food by an increasing global human population has challenged the sustainability of traditional food production models, which often have high energy costs and large waste discharges. There is thus a need for efficiently engineered agricultural systems to supplement or replace current food production methods to obtain high crop yields with less environmental cost. Aquaponics is a commercially viable food production system that combines the production of fish (aquaculture) and plants (hydroponics) into a soilless recirculating water system supported by a bio- logical water filtration tank (microbial filter) (Junge et al. 2017). In a recirculating aquaponics system, microorganisms clean up fish waste and leftover fish food by mineralizing it into a constant supply of nutrients to support plant growth. This system requires less water and has higher nitrogen and carbon use efficiency than traditional soil-based agriculture (Wongkiew et al. 2018).

Nitrification, the microbial conversion of ammonia to nitrate, is the primary microbial function that maintains water quality in recirculating aquaponics systems. While ammonia even at low concentrations affects the central nervous system of fish, eventually resulting in death, nitrate is less toxic and fish can tolerate high concentrations of up to 150–300 mg N/L (Yildiz et al. 2017). In normal aquaculture systems, ammonia-rich waste released through the fish excreta and gills accumulates in the water, which can potentially accumulate to toxic levels. However, in recirculating aquaponics, water from the fish tank is circulated through a microbial biofilter where ammonia- and nitrite-oxidizing microorganisms convert the ammonia to nitrate, which is then supplied to plants as their primary source of nitrogen before the water is circulated back to the fish. Nitrogen can also be lost from the aquaponics system through nitrification and denitrification activity in the form of nitrogen gas (N_2) or nitrous oxide (N_2O), a potent greenhouse gas (Stein

and Klotz 2016). Nitrogen loss in the form of N_2O in aquaponics is around 1.5–1.9%, depending on water quality, plant species, and microbial composition, which is slightly higher than that of conventional aquaculture (Hu et al. 2015, 2013b; Zou et al. 2016).

While combining aquaculture and hydroponics into aquaponics is considered by the European Parliamentary Research Service as one of the "ten technologies which could change our lives" (Van Woensel and Archer 2015), reconciling water quality parameters for optimum operation of the different aquaponics compartments has challenged the sustainability and commercial efficiency of these systems. Managing pH is difficult in aquaponics systems and needs to be balanced for different compartments: while nitrification is optimally active at a slightly alkaline pH (7.5–9.0), plants typically prefer a slightly acidic pH (5.5–6.5) for optimal growth. Freshwater fish species used in aquaponics systems, like tilapia, can tolerate a pH range of 6.1–8, and growth and reproduction of freshwater fish is not limited as long as pH > 5.5 (Crane 2006; Makori et al. 2017). Most aquaponics facilities therefore target a pH between 6.8 and 7. However, crop yields are not reaching economically sustainable levels for most commercial aquaponics growers (Love et al. 2014), which has stimulated increased research into plant growth optimization combined with nitrogen use efficiency in these systems (Junge et al. 2017). Suboptimal pH for microbial nitrogen transformation can also increase N_2O emission from the systems, which is mainly due to inhibition of complete denitrification activity (Zou et al. 2016), but can also arise from nitrification activity (Stein and Klotz 2016).

Lowering the pH of the aquaponics system to an optimal condition for plants and without affecting fish health would equal higher growth and yield along with a net increase in profits. Furthermore, if lowering the operating pH can also reduce N_2O production, this is a further net reduction in negative environmental impacts of aquaponics systems. A prior study (Day et al.

2021) showed that inoculating aquaponics with commercially derived microorganisms significantly increased plant growth when compared to transferring established bacteria from pre-existing aquaponics systems. Thus, the objective of this research was to develop a microbial inoculum that can be used to establish recirculating aquaponics systems for operation at a slightly acidic pH (5.6), determine the performance of this inoculum in terms of daily nitrification activity and production of N_2O, and describe the microbial community of the pH 5.6 adapted inoculum compared to the un-adapted community at pH 7.6.

2.3 Materials and methods

2.3.1 Adaptation of aquaponics biofilm carriers to laboratory medium

Thousands of microbial biofilm carriers suspended in water were collected directly from a commercial scale aquaponics microbial biofilter at NutraPonics Canada Inc. in Sherwood Park, Alberta, in Sept. 2019. The biofilm carriers were transported to the University of Alberta and stored at 4 °C in the original water from the commercial system. The microbial community of the biofilms and the original water were analyzed within 2 days of arrival as described below. To initiate the adaptation experiments, five biofilm carriers were placed into each of twenty 250-mL glass Wheaton bottles containing 100-mL mineral medium containing 0.4-mM KH_2PO_4, 0.2-mM $MgSO_4.7H_2O$, 1-mM $CaCl_2.2H_2O$, 1-mM KCl, 10-mM NaCl, trace solution 1 mL/L, 4.24 g/L HEPES buffer, 0.5-mM $(NH_4)_2SO_4$, and 2 g/100 mL zeolite (Hydor USA Inc.). Trace solution was composed of Na-EDTA 11.5 mM, $FeCl_2.4H_2O$ 10mM, $MnCl_2.2H_2O$ 0.5 mM, $NiCl_2.6H_2O$ 0.1 mM, $CoCl_2.6H_2O$ 0.1mM, $CuCl_2.2H_2O$ 0.1mM, $ZnCl_2$ 0.5 mM, $Na_2MoO_4.2H_2O$ 0.1mM, and H_3BO_3 1mM. Zeolite has been shown to improve water quality as well as increase the growth of plants in aquaponics systems. Zeolite acts as a filter media by

attracting and trapping ammonia, nitrate, and other ions. This process occurs through ion exchange and adsorption mechanisms (Rafiee and Saad 2006). Here, we studied the effect of adding zeolite during the adaptation of the biofilms. The bottles were incubated at 23 °C with shaking at 160 rpm. After each 24-h interval, the biofilm carriers were transferred to new bottles containing fresh mineral medium every day for 14 days prior to initiating the pH adaptation experiments.

For measuring dry biomass weight prior to adaptation and following the 40-day experiments, 20 biofilm carriers were placed in a vacuum chamber containing calcium sulfate (Drierite, Sigma-Aldrich) as a desiccant and incubated at room temperature for 24 h. After measuring the weight, the biofilm carriers were vortexed/washed with 1-M HCl several times to completely remove biofilm from the surface of carriers, and dried. The average dry biomass weight was calculated by subtracting the weight of the washed carriers from the weight of desiccated biofilm carriers prior to acid wash.

2.3.2 Adaptation of biofilm carriers from pH 7.6 to pH 5.6

The aim of this experiment was to adapt the microbial biomass attached to the biofilm carriers from pH 7.6 to 5.6 with minimum loss of nitrifying activity. This experiment was designed to study the effect of a gradual decrease in pH on the community composition and activity of nitrifiers, compared to sudden shift from pH 7.6 to 5.6. Unlike AOB and most NOB species (like *Nitrobacter*), *Nitrospira* are adapted to oligotrophic habitats (Kits et al. 2017); therefore, shifts in the substrate concentrations might influence the rearrangement of nitrifiers communities. The effect of reducing the ammonium concentration on the enrichment of higher affinity ammonia oxidizers (i.e., comammox *Nitrospira*) was investigated. Four variations of adaptation experiments (I–IV) were conducted over 40 days in which 5 biofilm carriers pre-

adapted for 14 days to mineral medium as described above were added to 250-mL glass Wheaton bottles containing 100-mL fresh mineral medium, with transfer to new bottles containing fresh medium every 24 h over the 40-day duration.

In all four experimental variations, the pH of the medium for the first 10 days was 7.6 with 1-mM ammonium to achieve a steady rate of nitrification activity prior to lowering the pH or changing the ammonium concentration (Table 2.1).

Table 2.1 Nitrification activity of microbial communities during adaptation from pH 7.6 to 5.6 in each of the four adaptation experiments (I–IV). In Experiment I, the pH of the medium was dropped to 5.6 on day 11 and subsequent transfers of the carriers were into medium at pH 5.6 for the remaining 30 days. In Experiment II–IV, the pH was gradually decreased from 7.6 to 5.6 from days 11 to 20 and kept at 5.6 for the remaining 20 days.

Day	Experiment I			Experiment II			Experiment III			Experiment IV		
	Initial pH	Initial NH^{4+}	NO_3^- (μM)	Initial pH	Initial NH_4^+	NO_3^- (μM)	Initial pH	Initial NH_4^+	NO_3^- (μM)	Initial pH	Initial NH_4^+	NO_3^- (μM)
1-10	7.6	1	296 ± 14.35	7.6	1	275 ± 17.11	7.6	1	282 ± 23.9	7.6	1	278 ± 19.7
11	5.6	1	287 ± 13.3	7.4	0.9	243 ± 21.9	7.4	1	287 ± 31.9	7.4	1	277 ± 15.2
12	5.6	1	25 ± 8.8	7.2	0.8	236 ± 10.4	7.2	1	243 ± 15.5	7.2	1	233 ± 8.9
13	5.6	1	19 ± 2.4	7	0.7	204 ± 9	7	1	246 ± 18.4	7	1	201 ± 22.5
14	5.6	1	13 ± 7.5	6.8	0.6	210 ± 17.3	6.8	1	276 ± 7.9	6.8	1	198 ± 18.6
15	5.6	1	19 ± 8	6.6	0.5	175 ± 23.1	6.6	1	268 ± 22.6	6.6	1	173 ± 9.4
16	5.6	1	19 ± 4.1	6.4	0.4	122 ± 9.3	6.4	1	179 ± 14.5	6.4	1	179 ± 16.1
17	5.6	1	28 ± 4.7	6.2	0.3	77 ± 13.2	6.2	1	177 ± 17.3	6.2	1	187 ± 11.3
18	5.6	1	12 ± 6.1	6	0.2	66 ± 5.8	6	1	193 ± 12.6	6	1	171 ± 20.7
19	5.6	1	38 ± 3.2	5.8	0.1	35 ± 6.9	5.8	1	155 ± 8	5.8	1	154 ± 17.3
20	5.6	1	43 ± 13.6	5.6	0.1	37 ± 12	5.6	1	128 ± 11.9	5.6	1	101 ± 19.3
21	5.6	1	30 ± 7.9	5.6	0.1	47 ± 9.9	5.6	1	103 ± 23.6	5.6	1	88 ± 9.8
22	5.6	1	42 ± 12.6	5.6	0.2	78 ± 11.4	5.6	1	109 ± 7.8	5.6	1	107 ± 16
23	5.6	1	58 ± 9.5	5.6	0.3	88 ± 11.9	5.6	1	111 ± 9.4	5.6	1	90 ± 7.3
24	5.6	1	54 ± 13.6	5.6	0.4	84 ± 4.6	5.6	1	104 ± 10.3	5.6	1	93 ± 14.2
25	5.6	1	67 ± 13.4	5.6	0.5	115 ± 15.5	5.6	1	120 ± 11.2	5.6	1	119 ± 11.5
26	5.6	1	69 ± 2.6	5.6	0.6	122 ± 12.6	5.6	1	122 ± 9.9	5.6	1	124 ± 16.7
27	5.6	1	63 ± 9.1	5.6	0.7	161 ± 9.4	5.6	1	152 ± 14.1	5.6	1	128 ± 11
28	5.6	1	71 ± 15.3	5.6	0.8	162 ± 22.1	5.6	1	157 ± 23.6	5.6	1	130 ± 15.2
29	5.6	1	80 ± 12.2	5.6	0.9	191 ± 14.7	5.6	1	155 ± 9.2	5.6	1	136 ± 17.6
30	5.6	1	81 ± 11.7	5.6	1	207 ± 18.3	5.6	1	179 ± 9.7	5.6	1	142 ± 9.2
31	5.6	1	86 ± 15.8	5.6	1	188 ± 10.8	5.6	1	189 ± 15.1	5.6	1	143 ± 24.1
32	5.6	1	94 ± 13.7	5.6	1	204 ± 7	5.6	1	191 ± 13	5.6	1	177 ±18.8
33	5.6	1	95 ± 18.1	5.6	1	201 ± 19.7	5.6	1	191 ± 22.5	5.6	1	179 ± 13.9
34	5.6	1	97 ± 14.4	5.6	1	202 ± 14.3	5.6	1	199 ± 20.8	5.6	1	175 ± 19.7
35	5.6	1	111 ± 19.7	5.6	1	221 ± 11.2	5.6	1	193 ±16.7	5.6	1	189 ± 10.6
36-40	5.6	1	110 ± 15.8	5.6	1	223 ± 19.5	5.6	1	201 ±17.9	5.6	1	186 ± 18.1

In Experiment I, the pH of the medium was dropped to 5.6 on day 11 and subsequent transfers of the carriers was into medium at pH 5.6 with 1 mM ammonium for the remaining 30

days. In Experiment II from days 11 to 20, the pH was gradually decreased from 7.6 to 5.6 by a daily increment of 0.2, while the initial ammonium concentration in the medium was decreased by a daily increment of 0.1 mM (Table 2.1). From days 20 to 40, the pH of the medium was kept at 5.6, but the initial ammonium concentration was gradually increased from 0.1 to 1 mM by a daily increment of 0.1 mM, and by day 30, the initial ammonium concentration was kept at 1 mM until the end of the experiment. Similar to Experiment II, for days 11 to 20 in Experiment III, the pH of the transfer medium was decreased daily from 7.6 to 5.6 by a daily increment of 0.2 but the initial ammonium concentration was maintained at 1 mM through- out (Table 2.1). Experiment IV was identical to Experiment III, but without the addition of zeolite to determine if this ion exchanger could be omitted and still maintain the same level of nitrification activity (Farhangi et al. 2013). Each adaptation experiment was initiated with 5 bottles containing 5 biofilm carriers each to ensure that there would be no nutrient limitation over each 24-h incubation. Two bottles were sacrificed for DNA extraction on days 10 and 20 (one bottle each), and a final bottle was sacrificed on day 40. Three bottles were used for daily triplicate measurements of nitrate and N_2O production throughout the 40 days of each experiment.

2.3.3 Measurement of nitrification activity and N_2O production

After each 24-h incubation period, the amount of N_2O in the gas headspace of the bottles was measured by GC-TCD (Shimadzu GC-8A; Hayesep Q column, Alltech). The biofilm carriers were removed and transferred to fresh medium while the spent medium was collected and centrifuged at 10,000 × g for 10 min to remove solid material. Total nitrite + nitrate was measured in the supernatant by colorimetric assay (Bollmann et al. 2011) using a 48-well microplate and microplate reader (Multiskan Spectrum, Thermo Fisher Scientific). To assess significant

changes in the nitrification activity of the microbial communities from the beginning to the end of the adaptation process, the mean ratio of nitrification activity (% ammonium conversion to nitrate per day) from days 36 to 40 was compared to that from days 1 to 5 using Kruskal- Wallis test and Dunn's multiple comparison test.

2.3.4 16S rRNA gene amplicon sequencing, ASV assignment, and statistical analysis

DNA was extracted from the biofilm carriers and surrounding water from the NutraPonics facility within 2 days of collection to determine the baseline com- position of the microbial communities. For the pH adaptation experiments, biofilm carriers were collected at three time points (days 10, 20, and 40) for DNA extraction. Water samples were filtered (0.2 micron) and filters were treated the same as biofilm carriers for DNA extraction. Five biofilm carriers from each sacrificed sample were added to a sterile 50-mL falcon tube containing 10-mL sterile Milli-Q water. Tubes containing biofilm carriers were vortexed vigorously for 5 min to detach the microbial biofilm from the carrier surfaces. Biofilm samples were centrifuged at 10,000 × g for 10 min and the resulting biomass pellets were used for genomic DNA extraction by FastDNA SPIN Kit for Soil (MP Biomedicals Inc.) along with positive and blank controls. Quality and quantity of the extracted DNA were measured using Nanodrop (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific) and a Qubit dsDNA HS Assay Kit, according to manufacturer's protocol (Invitrogen Qubit Fluorometer, Thermo Fisher Scientific).

The extracted DNA was tested for the presence of enzymatic inhibitors via PCR amplification of 16S rRNA genes (515F and 806R primers). The V4 region of 16S rRNA genes, a control mock com- munity composed of DNA samples of seven different bacterial species in a defined ratio, and an extraction blank, was targeted for library preparation and sequencing by a

commercial sequencing provider (Microbiome Insights, Canada) using an Illumina MiSeq platform and the 250-bp paired-end kit (V2 500-cycle PE Chemistry; Illumina, USA). The unfiltered sequence data in this study was deposited in the NCBI Short Read Archive under accession number PRJNA716750. The paired-end fastq files were processed with DADA2 pipeline version 1.12 as an R script (in R 3.6.3). Filtering was performed with the FilterAndTrim function to keep the first 240 bases of the forward reads and 160 bases of the reverse reads. Error rates model learning [*(learnErrors)*] and ASV inference [*dada()*] were performed in R with the DADA2 default parameter. Chimeric sequences were identified and removed after sequence clustering [*removeBimera Denovo()* function and "consensus"].

Taxonomy was assigned to the resulting amplicon sequence variants (ASVs) using the Silva database (silva_nr_v138). Accuracy of the sequence variants inferred by DADA2 was evaluated by comparing to the accuracy of the control mock community. Sequences were rarified to the depth of the shallowest sample (16000 reads) using *rarefy_even_depth()* from phyloseq package. Matrices of dissimilarities for samples were calculated using the Bray-Curtis distance metric in vegan package. Patterns of similarity among samples were visualized with nonmetric multidimensional scaling (NMDS) in two dimensions using the metaMDS function in the R vegan package. Statistical support for differences between samples was calculated with the aid of PERMANOVA test. Alpha diversity of three sampling groups (samples obtained on days 10, 20, and 40) was assessed by Chao1, Simpson, and Shannon indices, and significant shifts in the richness and diversity across sample groups were detected using ANOVA and Tukey's HSD test.

2.3.5 Real-time quantitative PCR

To determine the abundances of ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and comammox *Nitrospira* during the adaptation experiments, the abundance of *amoA* genes in the DNA samples extracted at days 10, 20, and 40 was quantified on an optical real-time detection system (StepOnePlus, Applied Biosystems) with primer pairs *amoA* 1F/2R (Rotthauwe et al. 1997), CrenamoA-23F/616R (Tourna et al. 2008), and ComamoA F/R (Zhao et al. 2019), respectively. In addition, qPCR amplification of the *nxrB* gene with the primers *nxrB* 169F/638R (Pester et al. 2014) was used to separately quantify the abundance of total nitrite-oxidizing *Nitrospira* bacteria in the samples. Two PCR primer sets targeting either comammox *amoA* clade A or B were employed to determine the type of comammox *Nitrospira* present in the samples (Pjevac et al. 2017).

Previously described conditions (Pester et al. 2014; Zeng et al. 2011; Zhao et al. 2019) were used for qPCR and performed in triplicate. The reactions were performed in a 10-µL reaction mixture with 5 µL of 2X Mastermix, 2.5 µL of 3.2 µM F/R primers, and 2.5 µL of template. The 2X Mastermix contained Tris (pH 8.3), KCl, $MgCl_2$, Glycerol, Tween 20, DMSO, dNTPs, ROX as a normalizing dye, SYBR Green as the detection dye, and Taq polymerase. Melting curves and 1.5% agarose gel electrophoresis were performed after each qPCR amplification to verify single amplicons.

For standard curves, amplicons generated from each of the primer sets were cloned into pMiniT vectors and trans- formed into NEB 10-beta component *Escherichia coli* (NEB PCR cloning kit, New England BioLabs Inc.). Fifty micro- liters of transformed cultures were spread onto LB plates containing ampicillin (100 µg/mL). Amplicon-containing plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen, Germany), verified with 1% agarose gel

electrophoresis, and quantified by Qubit dsDNA HS Assay Kit according to the manufacturer's protocol (Thermo Fisher Scientific, Canada). The purity was also checked by Nanodrop. The cloned genes were verified using Sanger sequencing. qPCR standards were diluted in a tenfold series. PCR efficiencies of 90–98% and the correlation coefficients (R^2) of >0.98 were obtained.

2.4 Results

2.4.1 Nitrification activity of biofilms during adaptation experiments

Four variations of adaptation experiments were con- ducted over 40 days to adapt nitrifying biofilms grown on the surface of plastic biocarriers from pH 7.6 to 5.6. In all variations, the pH of the medium was kept at 7.6 for the first 10 days, and nitrate measurements showed a steady rate of nitrification activity during this period (Table 2.1). Twenty- eight to thirty percent of the total added ammonium (1 mM) was oxidized to nitrate over each 24-h period, indicating that the ammonium concentration was not limiting nitrification activity. The average dry biomass weight was calculated at 1.3 ± 0.1 mg per plastic biocarrier, for a total of ca. 6.5-mg biomass per incubation. The biomass did not change significantly over the 40-day time course of adaptation for any of the experiments.

In adaptation Experiment I, the pH was dropped from 7.6 to 5.6 at day 11 and nitrification activity decreased to 8.5% of the average activity level measured over days 1–10 (Table 2.1, Supp. Figure A-1). While keeping the pH of the transfer medium at 5.6 for the remaining 30 days, nitrification activity slowly increased, and reached a steady state of ca. 37% of the activity measured at pH 7.6 by day 36. In adaptation Experiment II, the transition from pH 7.6 to 5.6 was per- formed incrementally (0.2 units per day) from days 11 to 20 along with reducing the ammonium concentration to 0.1 mM in 0.1-mM increments to intentionally enrich

for higher affinity ammonia oxidizers, such as comammox bacteria (Kits et al. 2017). Even at the lowest ammonium concentration (0.1 mM), ca. 35–47% of the ammonium was oxidized to NO_3^- over each 24-h period. From days 21 to 30, the ammonium concentration was increased in 0.1-mM increments per day back to 1 mM while the pH was kept at 5.6. Nitrification activity from days 36 to 40 stabilized at 81% of the average activity measured at pH 7.6 from days 1 to 10 (Table 2.1, Supp. Figure A-1). In adaptation Experiments III and IV, the pH of the medium was also gradually decreased from 7.6 to 5.6 from days 11 to 20 in 0.2 unit increments, but the ammonium concentration was kept at 1 mM. By the end, nitrification activity in Experiments III and IV reached 71 and 67%, respectively, of the average activity measured at pH 7.6, and there was no significant difference between them (Dunn's test, $P > 0.05$) indicating that zeolite had no discernable effect on maintaining nitrification activity (Table 2.1).

In all four adaptation experiments, zero or negligible nitrite (NO_2^-) was measured over the 40-day duration. The highest nitrification activity at pH 5.6 was obtained in Experiment II (II > III > IV > I), where the ammonium con- centration was decreased to 0.1 mM over 10 days, and then increased back to 1 mM over the next 10 days. These results suggest that the gradual reduction followed by the gradual increase of ammonium during the adaptation process was more effective in retaining nitrification activity rather than keeping the supply of ammonium constant. Kruskal-Wallis test showed a significant difference of nitrification activities between all four adaptation experiments ($P < 0.05$). Dunn's multiple comparisons test indicated that nitrification activity at the end of Experiment II was significantly higher than that in Experiment I ($P < 0.05$), but there were no significant differences in end point activities between the other experiments.

2.4.2 N_2O emission during adaptation experiments

Over days 1–10, negligible N_2O was measured in the gas headspace of any incubation (Table 2.2, Supp. Figure A-1). However, in all four adaptation experiments, N_2O increased as the pH was reduced with the highest emission (325 nmol/mg biomass) measured in Experiment I on day 16 at pH 5.6. Experiment II showed the least overall N_2O emission; the highest level was measured at pH 7 (160 nmol/mg biomass). In Experiments III and IV, N_2O production reached its maximum between pH 6.6 and 6.2 (ca. 210 nmol/mg biomass). However, no N_2O was detected in any of the four adaptation experiments after day 20.

Table 2.2 Nitrous oxide (N_2O) production (nmol/mg biomass) of the microbial communities during adaptation from pH 7.6 to 5.6 in four adaptation experiments (I–IV). Limit of detection = 100 nmol/mg biomass. Dashes indicate no detection of N_2O.

	Experiment I	Experiment II	Experiment III	Experiment IV
Day	-	-	-	-
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	115 ± 26	103 ± 17
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	106 ± 44
9	-	-	-	-
10	-	-	105 ± 26	136 ± 31
11	152 ± 18	-	177 ± 30	157 ± 29
12	113 ± 32	135 ± 31	208 ± 36	154 ± 54
13	178 ± 20	160 ± 22	210 ± 16	158 ± 32
14	268 ± 17	132 ± 24	158 ± 25	145 ± 47
15	224 ± 42	133 ± 18	229 ± 20	210 ± 19
16	325 ± 28	102 ± 05	199 ± 42	212 ± 36
17	225 ± 25	-	188 ± 28	223 ± 39
18	-	-	170 ± 22	-
19	-	-	-	-
20–40	-	-	-	-

2.4.3 Shifts in microbial communities over the duration of the adaptation experiments

For investigating changes in the composition of microbial communities during the adaptation experiments, NMDS analysis based on Bray-Curtis distance was used to visualize the similarity of microbial communities (based on ASV analysis) on days 10, 20, and 40. Clear shifts in the composition of microbial communities were observed over time (PERMANOVA, $P < 0.05$) (Fig. 2.1). In addition, NMDS plots showed that at day 10, the distances among the four experiments were very close, indicating a high community similarity prior to lowering the pH and/or altering the ammonium concentration. However, upon changing the pH and/or ammonium concentration, the microbial communities diverged, with Experiment II showing greater variation than the other three experiments, likely due to the simultaneous change in both pH and ammonium concentration.

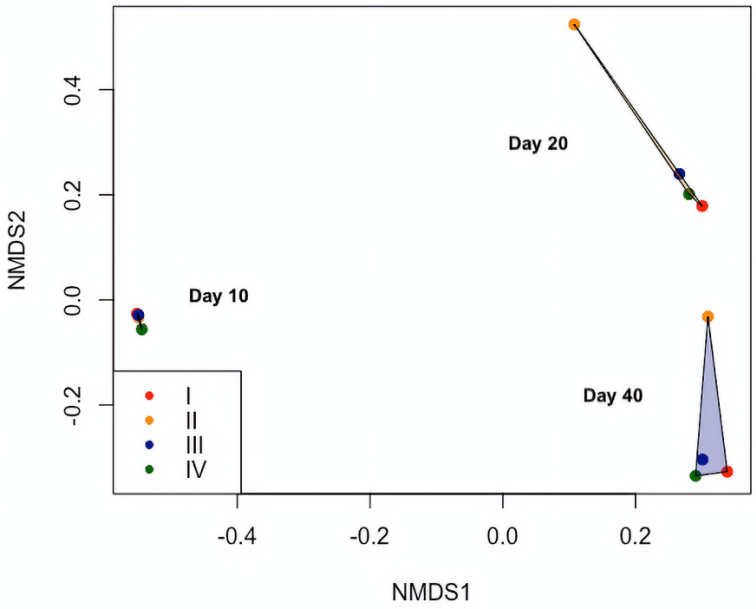

Figure 2.1 Comparison of microbial community differences based on ASV analysis over the time course (day 10, 20, 40) for each of the four adaptation experiments using nonmetric multidimensional scaling (NMDS) ordination (Bray-Curtis) dissimilarity, to quantify differences between samples based on abundance. The plot shows that at day 10, all samples are grouped together. By decreasing pH from day 10 to 20 and 40, microbial compositions are changing, with Experiment II showing the highest dissimilarity to other experiments.

Shannon and Simpson indices and the Chao1 index were used to assess shifts in the diversity and richness of microbial communities over time. Chao1 is a nonparametric method for estimating the number of species in a community. The Shannon index is an information statistic index, which means it assumes all species are represented in a sample and that they are randomly sampled. The Simpson index is a dominance index because it gives more weight to common or dominant species. In this case, a few rare species with only a few representatives will not affect the diversity. Shannon values in the original biofilters and water sample were 4.02 and 3.63, respectively (Supp. Table A-1). During the adaptation experiment, the average Shannon values were equivalent at days 10 (2.80), 20 (2.83), and 40 (2.77). Similarly, Simpson

values were calculated to be 0.86, 0.84, and 0.85 at days 10, 20, and 40, respectively. In contrast, Chao1 index indicated that concurrent with decreasing the pH of the media, the richness of the microbial communities decreased as well, and changes from day 20 (99.4) to day 40 (83.5) were statistically significant (Tukey's HSD test, $P < 0.05$) (Supp. Table A-1).

An average of 84.11% ± 9.07% of the total filtered amplicon reads could be assigned to taxonomic levels in the Silva database. For a more in-depth analysis and comparison between samples, only groups representing more than 1% of the total reads per sample were analyzed. The original water from the commercial scale aquaponics biofilter was dominated by members of the families *Fusobacteriaceae* (36%), *Microbacteriaceae* (15%), *Rhodocyclaceae* (12%), and *Verrucomicrobiaceae* (8%) (Fig. 2.2), whereas the biofilm carriers from the biofilter were dominated by members of the families *Chitinophagaceae* (33%), *Nitrososphaera* (15%), *Nitrospiraceae* (9%), *Chryseolinea* (7%), *Saprospiraceae* (4%), *Sphaerobacteraceae* (4%), and various *Proteobacteria* (16%) (Fig. 2.2). After 10 days of adapting the biofilm carriers to mineral medium at pH 7.6, only three major phylum-level groups were identified: *Proteobacteria* (40%), *Nitrospirae* (36%), and *Thaumarchaeota* (6.8%) (Supp. Table A-2, Supp. Figure A-2). The average relative abundance of *Proteobacteria* across the four adaptation experiments decreased by 18% upon shifting the pH from 7.6 to 5.6. Also, reads affiliating with *Thaumarchaeota* significantly decreased from 6.8 at pH 7.6 at day 10 to 1.3% at pH 5.6 on day 20 (Wilcoxon rank sum test, $P<0.05$). In contrast, the average abundance of *Nitrospirae* slightly increased from 36 on day 10 to 39% on day 40.

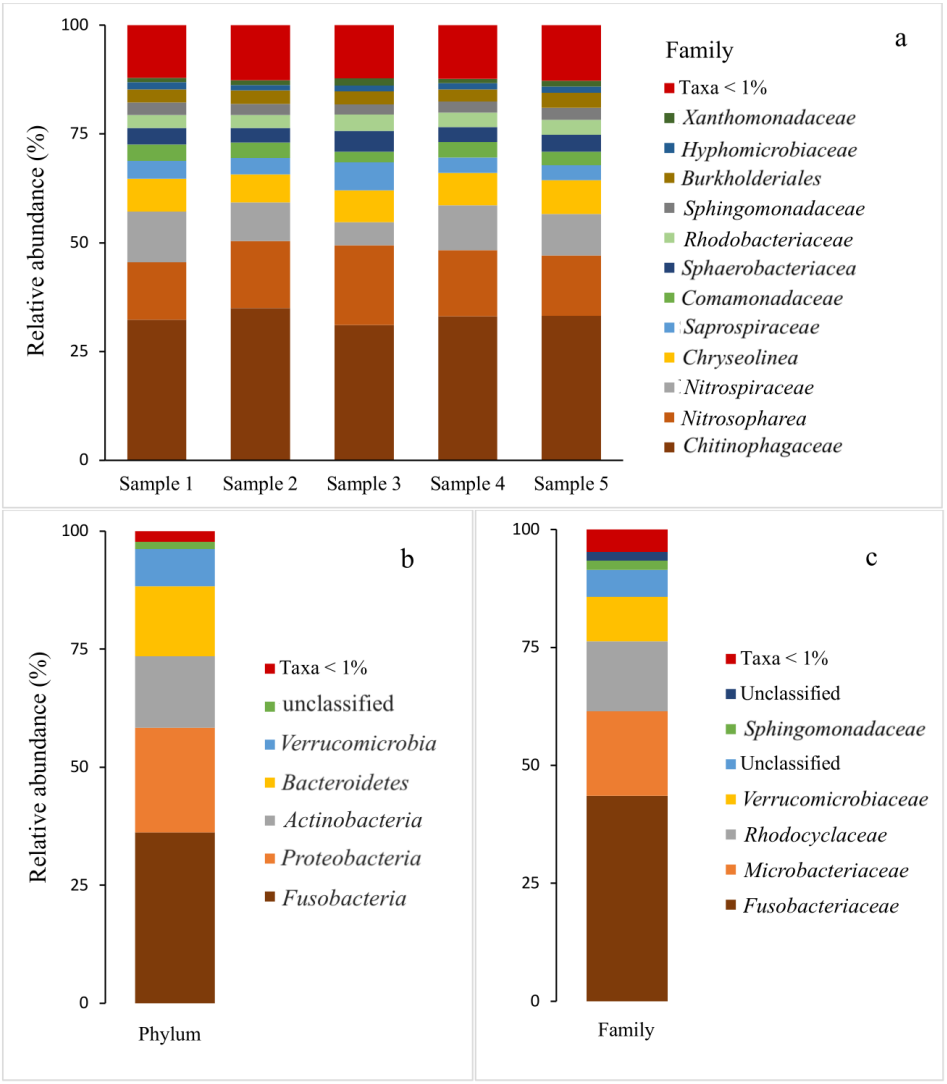

Figure 2.2 Microbial community composition of the original biofilm carriers (n = 5, individual samples) at the family level (a) and water samples at phylum (b) and family (c) levels.

Similar patterns were observed at the genus level (Fig. 2.3, Supp. Table A-2). The average relative abundance of the genus *Nitrosomonas* decreased from 26.2 at pH 7.6 on day 10 to 3.8% at pH 5.6 on day 20 and then increased slightly to 6.3% on day 40. The abundance of *Nitrosocosmicus*, the only *Thaumarchaeota* genus detected in the samples, significantly decreased from 8.5 at pH 7.6 on day 10 to 1.2% on day 20 (Wilcoxon rank sum test, $P<0.05$). Unlike AOB and AOA, the abundance of *Nitrospira* showed an average increase of 15% (Supp. Table A-2);

after 40 days, the highest abundance.

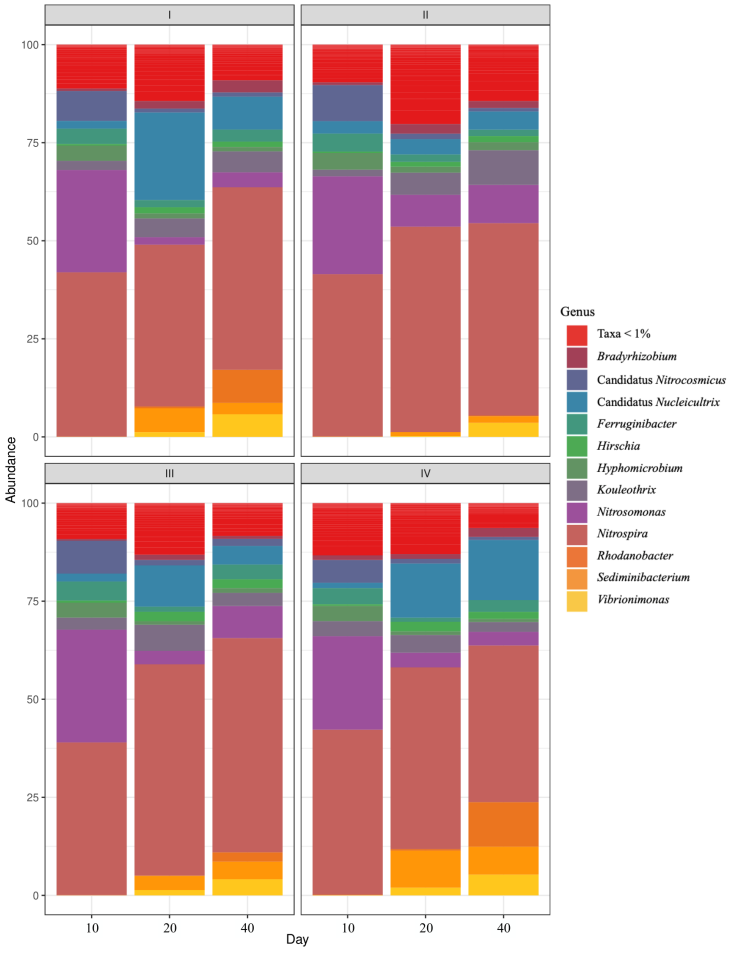

Figure 2.3 Changes in microbial community composition on days 10, 20, and 40 based on ASV analysis in each of the four adaptation experiments (I, II, III, and IV). The plot shows that ammonia oxidizing bacteria (*Nitrosomonas*) and archaea (candidatus *Nitrosocosmicus*) are disappearing from the sample over time, while the population of *Nitrospira* species are growing.

2.4.4 Changes in abundances of functional genes, amoA and nxrB, over the duration of the adaptation experiments

qPCR was used to determine changes in populations of nitrifying microorganisms over the duration of the adaptation experiments. Abundances of the *nxrB* gene, corresponding to nitrite oxidoreductase of canonical nitrite oxidizers and comammox bacteria, and the *amoA* genes,

corresponding to the three homologs of ammonia monooxygenase of AOB, AOA, and comammox bacteria, were measured at days 10, 20, and 40. AOB *amoA* genes were abundant in all samples on day 10 at pH 7.6 (Fig. 2.4).

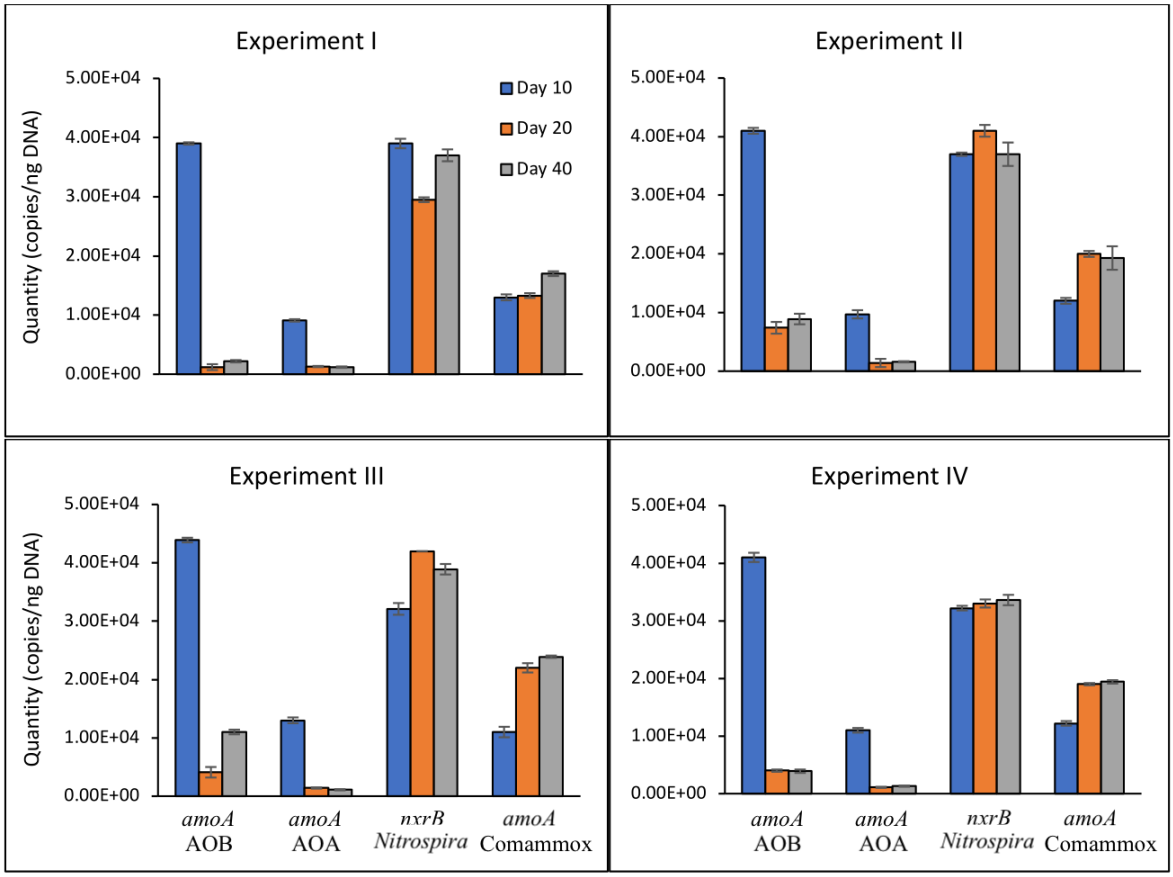

Figure 2.4 Abundances of AOA, AOB, and comammox *amoA* (ammonia monooxygenase) genes and *Nitrospira* nxrB (nitrite oxidoreductase) genes in each of the four adaptation experiments. The values are expressed as gene copy number per nanogram of DNA as quantified by qPCR. The plot shows that AOB-*amoA* and AOA-*amoA* are disappearing from the sample over time, while the comammox-*amoA* is growing.

However, by day 40, the average AOB *amoA* gene abundance across the four experiments decreased from 4.1×10^4 copies per ng DNA^{-1} to 6.5×10^3 copies per ng DNA^{-1}. In Experiments I,

II, and III, the quantity of AOB *amoA* genes gradually increased towards the end of the adaptation experiments but remained unchanged in Experiment IV. Similarly, the average abundance of AOA *amoA* genes decreased across the four experiments from 1.1×10^4 copies per ng DNA^{-1} on day 10 to 1.3×10^3 copies per ng DNA^{-1} on day 40 (Fig. 2.4). The quantity of AOA obtained from qPCR measurements compared to AOB and comammox also correlates with the relative abundances of AOA (*Thaumarchaea*) obtained from the 16S rDNA analysis.

In contrast to AOA and AOB *amoA* genes, the average quantity of *nxrB* genes was relatively stable with an average of 3.5×10^4 copies per ng DNA^{-1} on day 10 and 3.7×10^4 copies per ng DNA^{-1} on day 40. However, the average abundance of comammox *amoA* genes increased from 1.2×10^4 copies per ng DNA^{-1} at day 10 to 2.0×10^4 copies per ng DNA^{-1} at day 40, which was consistent with the increase in relative abundance of ASVs related to *Nitrospira* across the four adaptation experiments from day 10 to 40 (Fig. 2.4). As comammox bacteria encode both *nxrB* and *amoA*, it can be deduced that the ratio of comammox to canonical *Nitrospira* bacteria increased from day 10 to 40 across the four adaptation experiments. Results from a PCR experiment with primer sets that differentiate comammox *amoA* clades A and B showed that only clade B was present in the samples. Interestingly, although Experiment II maintained the highest level of nitrification activity following adaptation, there was no difference in nitrifier-related gene abundance at day 40 among any of the experiments (Fig. 2.4).

2.5 Discussion

Managing pH is a common concern among aquaponics growers as fish, plants, and microbial activity must be equally supported. Due to the higher solubility and availability of essential plant nutrients at lower pH values, operating aquaponics systems at a slightly acidic

pH can result in optimal nitrogen use efficiency and higher plant yield (Zou et al. 2016). However, decreasing the pH can also increase the proportion of ammonium to ammonia, which slows down ammonia detoxification and nitrate formation by nitrifiers. As most freshwater aquaponic fish (e.g. goldfish and tilapia) can tolerate pH > 5.5 (Crane 2006), the main aim of this research was to identify strategies for adapting nitrifying microorganisms to an optimal pH for plant growth, without compromising fish health, for use as an inoculum in recirculating aquaponics systems. There are several considerations for maintaining a stable microbial inoculum for aquaponics systems, including the sources of microorganisms, water source and quality, protection from pathogens, and nutrient (e.g., nitrogen) use efficiency, among others (Espinal and Matulić 2019). It has been hypothesized that intentionally colonizing recirculating aquaponics systems with microorganisms that are pre- adapted to a desired environment can improve system performance and plant growth while maintaining fish health (Espinal and Matulić 2019). In our experiments, we found that a slow stepwise reduction in medium pH to 5.6, along with slowly lowering and then raising ammonium concentration (from 1 to 0.1 to 1 mM), resulted in achieving a highly active inoculum operating at an acidic pH (i.e., 81% retention of activity from pH 7.6 to 5.6). Using pre-adapted and enriched biofilm carriers as inoculum, rather than mature biofilm carriers, also helps slow-growing nitrifiers to dominate the system and compete well with heterotrophic bacteria that originate mainly from fish feces.

Interestingly, all four of our pH adaptation experiments resulted in enrichment of comammox bacteria over AOB and AOA, but with varying levels of nitrification efficiency. The adapted microbial communities had lower species rich- ness (by ASV analysis) and a much higher proportion of nitrifying microorganisms when compared with microbial communities of the original water and biofilters from the commercial aquaponics facility and in comparison to

other examined recirculating aquaponics systems (Eck et al. 2019; Schmautz et al. 2017). The proportion of nitrifiers (AOB, AOA, *Nitrospira*, comammox) adapted in a mineral medium increased from an average of 25% on the original biofilm carriers to an average of 61% at the end of the adaptation experiments.

Our adaptation experiments validated that pH remains a major determinant of nitrifier composition as a former study has shown that weakly acidic media select for comammox while inhibiting AOB (Lu et al. 2020). Furthermore, our results showed that as the medium approached pH 5.6, the AOB and AOA microbial communities significantly decreased in abundance, whereas the abundance of comammox bacteria increased, as well as the proportion of comammox bacteria to canonical nitrite oxidizers. This shift indicates that comammox bacteria became largely responsible for the relatively high nitrification activities maintained by the pH 5.6 adapted microbial communities, especially in Experiment II. Comammox *Nitrospira* are oligotrophs that have increased competitive success under low substrate concentrations (Kits et al. 2017). In addition, unlike the low affinity Rh-type ammonium transporters encoded by AOB, comammox bacteria clade B use homologs of Amt-type transporters with a higher rate of ammonium uptake (Koch et al. 2019; Palomo et al. 2018), which may make them more competitive in acidic environments where ammonium is far more available than ammonia. Past studies indicate that acidic pH can have a negative impact on the abundance and activity of AOB in soils (Hu et al. 2013a; Nicol et al. 2008) and soilless cultivation media (Cytryn et al. 2012). On the other hand, one report noted that in acidic forest soils (pH<6), comammox *Nitrospira* are the most abundant nitrifiers and might play a more important role in nitrification over AOA and AOB (Hu and He 2017). Also, in an enrichment study using an ammonia-fed bioreactor, decreasing pH to 5.5 significantly increased the relative abundance of comammox *Nitrospira*

(Takahashi et al. 2020), which is consistent with our findings of enriched comammox bacteria over AOB and AOA.

Because of their oligotrophic nature, enrichment of biofilm carriers with highly active comammox bacteria is possibly advantageous for reducing the load of nitrite and other toxic intermediates of nitrification (e.g., hydroxylamine) into the water supply (Sakoula et al. 2021). Comammox bacteria also encode urease genes and a higher diversity of urea transporters compared to other nitrifiers (Palomo et al. 2018). Urea hydrolysis releases bicarbonate that could further buffer recirculating aquaponics systems against acidification during active nitrification. N_2O production by our microbial communities adapted to pH 5.6 was consistently below our detection limit (100 nmol/mg biomass), which is consistent with a low production of N_2O observed by comammox bacteria relative to AOB (Kits et al. 2019; Prosser et al. 2020). N_2O emission in aquaponics is typically equal to or slightly higher than that of conventional aqua- culture or fertilized soil aquaculture (Hu et al. 2015, 2013b; Zou et al. 2016), probably due to operation at suboptimal pH for nitrification, or a disruption of complete denitrification activity (Zou et al. 2016). Greenhouse gas emission from agriculture soils is not easily controllable, but in closed systems such as recirculating aquaponics, controlling gas emissions is a feasible benefit. Low dissolved oxygen concentration (< 2 mg O_2/L) in the biofilter tanks is another factor that can increase N_2O production in AOB-dominated systems, mainly by promoting nitrifier denitrification (Ribera-Guardia and Pijuan 2017). In contrast, N_2O emission by comammox-enriched communities is not affected by low dissolved oxy- gen concentration (Li et al. 2021). Evaluation of N_2O pro- duction in a recirculating aquaponics system inoculated with comammox-enriched biocarriers is still necessary, however, as the increased availability of organic carbon from fish and plants may enrich for denitrifying microbial communities that could also be a

source of N_2O.

2.6 Conclusion

A major obstacle for increasing crop yields in aquaponics systems has been matching an optimal operating pH for nitrification activity with that for plant growth and fish health. We adapted a microbial inoculum that maintains 81% of its nitrification activity at pH 5.6 by gradually reducing the pH and ammonium concentration of an adaptation medium, and then gradually increasing the ammonium concentration over a 40-day period. In adapting the biofilm carriers from pH 7.6 to 5.6, the nitrifying microorganisms became preferentially enriched with comammox bacteria over AOB, AOA, and canonical *Nitrospira*, but with varying levels of activity. Using biofilters enriched in highly active comammox bacteria as an inoculum for recirculating aquaponics systems can have eco-friendly properties including less accumulation of toxic intermediates of nitrification, like hydroxylamine and nitrite, urea hydrolysis, and less N_2O production. The relatively slow growth rate of comammox bacteria as compared to most heterotrophic bacteria suggests that establishment of a recirculating aquaponics system at an acidic pH could be best achieved by providing a previously adapted microbial inoculum in a defined mineral medium, rather than attempting to alter the operating pH of a complete system in situ. Our high activity comammox-enriched biofilm carriers pre- adapted to a slightly acidic pH will be used as an inoculum for a recirculating aquaponics system to determine if it sup- ports higher plant yield, maintains high nitrification activity, reduces GHG production, and provides high nitrogen use efficiency relative to a system operating at neutral pH.

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Chapter 3: Effects of pH and microbial inoculum on the dynamics of microbial communities in bench-scale aquaponics systems

3.1 Abstract

Understanding the ecology of microorganisms involved in water treatment and recirculation is essential for optimizing and sustaining aquaponics systems. The effects of pH and microbial inoculum on nitrification and dynamics of microbial communities from microbial biofilters, plant roots, fish intestine and fish gills, from lab-scale aquaponics systems were examined. Although initial ammonium amounts in systems with comammox-enriched inocula were 47 (A2) and 69% (B2) that of systems with unenriched inocula (A1 and B1), respectively, the systems stabilized to between 72-87% ammonium removal per day by the end of operation, with higher removal efficiency systems with unenriched inocula. *Nitrosomonas* and *Nitrosospira* were dominant ammonia-oxidizers in systems operating at pH 7.6-7.8, whereas *Nitrospira* (comammox) and plant growth-promoting microbes were more abundant in systems operating at pH 5.8 - 6. In addition, higher quantities of plant growth-promoting bacteria were identified in the root samples in slightly acidic systems. However, pH did not have a significant impact on the microbiota of fish intestine and gills. This study demonstrated functional stability and redundancy of aquaponics microbiota as a function of pH, although enrichment of comammox nitrifiers operating at a reduced pH led to consistently higher plant biomass but with a decrease in nitrogen-removal efficiency. These results suggest that starting inoculum and operating are important considerations for aquaponics system optimization.

3.2 Introduction

Manufacture and usage of artificial fertilizer, as driven by a growing world population that has shifted towards a more protein-rich diet, is severely impacting the environment. The rapid increase of global nitrogen input in agriculture, estimated at 114.6 Mt by 2024 (IFA, 2019), has altered the balance of nitrogen at a global scale. Due to low nitrogen use efficiency, approximately 60% of reactive nitrogen, predominantly ammonium and nitrate, escape from soils via leaching and runoff resulting in eutrophication of freshwater and estuarine ecosystems, contamination of groundwater, and greenhouse gas emissions, mainly nitrous oxide (N_2O) (Zhang et al., 2015; Stein., 2022). In addition, population growth has recently tripled the demand for aquaculture production, from 34 Mt in 1997 to 112 Mt in 2017 (Naylor et al., 2021). Aquaculture effluent contains large amounts of suspended solids, dissolved organic matter, and nitrogenous and phosphorus compounds, which require appropriate treatment prior to discharge into receiving water bodies (Piedrahita, 2003). By combining recirculating aquaculture and hydroponics into aquaponics, nutrient-rich effluent can be used as a source of fertilizer for soilless plant cultivation, thus reducing water usage, fertilizer input, and waste discharge as compared to traditional agriculture.

Continuous water treatment to support healthy fish and crop production in aquaponics systems relies on the metabolic activity of microorganisms and their metabolic products. Nitrifiers are key microorganisms for improving water quality due to balancing reactive nitrogen pools. Nitrifiers are autotrophic microbes that oxidize ammonia released through fish gills to nitrate, which is less toxic for fish (Camargo and Alonso, 2006) and the preferred N-source by most plants (Hessini et al., 2019). Heterotrophic bacteria also supply micronutrients and growth-

promoting molecules for plants through decomposing organic matter and solubilizing phytates, among other activities (Kasozi et al., 2021).

Although heterotrophic bacteria are essential for effluent treatment in aquaponics systems, they can have negative impacts on ammonia oxidation by out-competing the slow-growing nitrifiers. Cycling the system with low fish density or cycling with ammonium chloride for 4 – 6 weeks before adding fish are common methods for establishing nitrifying populations in biofilters (Sallevane, 2016). Adjusting the water pH is also a major challenge for sustaining nitrification activity in aquaponics systems. While most plants prefer slightly acidic pH (Tyson et al., 2011), the activity of nitrifiers decreases at pH values below 7.5 and can result in enhanced nitrous oxide emissions (Zou et al., 2016). Enriching the biofilter with complete ammonia oxidizing *Nitrospira* (comammox) bacteria has been suggested to improve nitrification efficiency of aquaponics systems operating at acidic pH (Derikvand et al., 2021).

Developing more profitable and environmentally sustainable food production systems using aquaponics requires a comprehensive understanding of the structure, dynamics, and activities of microbial communities involved in water treatment. Evaluating microbial diversity and ecology of aquaponics systems can provide a basis for managing biofilter arrangements in start-up systems and operating them towards optimum nitrogen use efficiency. Although advances have been made towards understanding the microbial ecology of aquaponics systems (Schmautz et al., 2017; Bartelme et al., 2019; Heise et al., 2021), little is known about the effects of pH and the initial inoculum composition on the dynamics of microbial communities over time and the complex interplay among the three biological compartments: fish, biofilters, and plants.

One aim of this study was to evaluate the effect of operating pH and the initial microbial inoculum on ammonium removal efficiency and the spatiotemporal distribution of microbial

communities within and between the fish, biofilter, and plant compartments of recirculating aquaponics systems. A second aim was to identify an operating pH that can improve nitrogen use efficiency and plant growth in recirculating aquaponics systems.

3.3 Methods

3.3.1 Ethics statement, animal subjects

This research was conducted in accordance with institutional standards for animal care and use by The University Animal Policy and Welfare Committee (UAPWC), University of Alberta, Canada.

3.3.2 Aquaponics microcosms

Four floating raft aquaponics mini-systems were built and maintained in the Department of Biological Sciences, University of Alberta. Each mini-system consisted of a recirculating aquaculture unit (fish tank and biofilter tank: total of 320 L volume and 250 L water volume) and a hydroponic bed above the system (Fig. 3.1). Water from the fish tanks flowed to the biofilter tanks, and then to the hydroponic beds using submerged circulation pumps with a flow rate of 10 L/min, before returning to the fish tanks. Water loss due to evaporation and evapotranspiration was compensated by replenishing the systems daily with dechlorinated water. Suspended solids were continuously removed from fish tanks using mechanical filters (AquaClear 70 Power Filter). Air was supplied to the fish tanks and grow beds to a dissolved oxygen (DO) concentration of ~ 7 mg/L. Each hydroponic tank contained a single raft supporting 14 plants equipped with overhead LED lighting (Fluence RAZRx LED Grow Light System) operating 18 h/d. Tanks were covered with corrugated plastic sheets to prevent algal growth.

Figure 3.1 Four floating raft aquaponics mini-systems were built. Each mini-system consisted of a recirculating aquaculture unit and a hydroponic bed above the system. Each hydroponic tank contained a single raft supporting 14 plants equipped with overhead LED lighting operating 18 h/d.

Four aquaponics systems were operated side by side in a temperature-controlled room (23 °C) for 3.5 months. Two systems were maintained at pH 7.6-7.8 (A1 and A2) and two were maintained at pH 5.8-6.0 (B1 and B2). Two systems were inoculated with microbial biofilm carriers (Kaldnes, Veolia Water Technol., Sweden) derived from a commercial scale aquaponics system (NutraPonics Inc. Alberta, Canada; A1 and B1) and two were inoculated with microbial biofilm carriers previously adapted to pH 5.8 (Derikvand, et al. 2021; A2 and B2). Each system was stocked with five goldfish with initial weights of 20-25 g/fish approximating a density of 1 kg/m^3 (Lee, et all. 2019). Fish were fed 1 g floating fish pellets (Mazuri Bits) per day per tank for the first week, increasing to 4 g twice per day at 9 am and 5 pm for the remainder of operation. Total fish biomass in each system was calculated by subtracting the initial from the final fish

weight at the end of operation. Lettuce seeds were germinated in peat moss two weeks prior to transplant into the grow beds. Three rounds of crops were grown and harvested for biomass measurements: the first and second rounds after 4 weeks of growth and the third round after 6 weeks of growth.

3.3.3 Measurement of ammonium removal capacity

Water samples (n=3) were collected from the fish tanks 30 min, 2 h and 5 h after fish feeding every other day over the course of operation. Ammonium, nitrite, and total nitrite + nitrate was measured using colorimetric assays in 48-well microplates (Multiskan Spectrum, Thermo Fisher Scientific) (Derikvand et al., 2021). Differences in ammonium concentrations between the four systems, the point of highest ammonium load, were compared during the second week of operation using the Kruskal-Wallis test and Dunn's multiple comparison test. Changes in the ammonium removal rate per day from week to week was calculated by subtracting the average ammonium concentration measured over each week from the average ammonium concentration measured at week 2 for each system divided by 7. These measurements assume that the fish minimally produced the same average amount of total ammonium measured for each system during week 2 throughout the remainder of the operation time. A Tukey test was used to detect significant changes each week in the ammonium removal for each system.

3.3.4 Sample collection and DNA extraction

Biofilm carriers were collected from biofilter tanks after 10 days, one month, and three months of operation. Five biofilm carriers from each of the four systems were separately added

to sterile 50 ml falcon tubes containing 10 ml sterile Milli-Q water. Tubes were vortexed vigorously for 5 min to detach biofilm from the carrier surfaces. Biofilm samples were centrifuged at 10,000 × g for 10 min and the resulting biomass pellets were used for genomic DNA extraction (Derikvand et al., 2021). For characterizing plant root microbiota over the course of a lettuce growth cycle, samples of the root entire system were collected from lettuce seedlings grown for two weeks in peat moss and also from lettuce roots grown for four and six weeks in the hydroponics beds. Samples were frozen in liquid nitrogen and ground into small pieces prior to DNA extraction (Simmons et al., 2018).

At the end of the aquaponics cycle, three fish from each system were selected and euthanized. Whole intestine and gill samples were taken using sterile scalpel blades, air-dried in a laminar flow hood, and ground prior to DNA extraction (Clinton et al., 2021). Samples were also collected from water and sponges in the mechanical filters at the end of operation. 4L water from each fish tank was filtered (0.2 micron), and filters were treated the same as biofilm carriers for DNA extraction (Derikvand et al., 2021).

Genomic DNA from all samples were extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals Inc.) along with blank controls. Quality and quantity of the extracted DNA were measured using Nanodrop (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific) and a Qubit dsDNA HS Assay Kit, according to manufacturer's protocol (Invitrogen Qubit Fluorometer, Thermo Fisher Scientific). The extracted DNA was tested for the presence of enzymatic inhibitors via PCR amplification of 16S rRNA genes (515F and 806R primers) prior to sequencing.

3.3.5 Sequencing and statistical analysis

The V3-V4 region of 16S rRNA was shown to be optimal for profiling microbial communities (Mizrahi-Man et al., 2013), with maximum phylogenetic coverage (Klindworth et al., 2013). We targeted the V3-V4 region of 16S rRNA genes of our samples and a control mock community for library preparation and sequencing by a commercial sequencing provider (Genome Quebec, Canada) using the Illumina MiSeq platform (MiSeq PE 250bp). The unfiltered sequence data in this study was deposited in the NCBI Short Read Archive under accession number PRJNA941956.

The paired-end fastq files were processed with DADA2 pipeline version 1.16 as an R script (in R4.2.1). Filtering was performed with the FilterAndTrim function to keep the first 240 bases of the forward reads and 220 bases of the reverse reads. Error rates model learning [*(learnErrors)*] and ASV inference [*dada()*] were performed in R with the DADA2 default parameters. After clustering, chimeric sequences were identified and removed. The Silva database (silva_nr_v138) was used to assign taxonomy to the resulting amplicon sequence variants (ASVs). Sequences assigned to chloroplast and mitochondria were removed. Accuracy of the inferred sequence variants was evaluated by comparing to the accuracy of the control mock community.

Metrics of dissimilarity for samples were calculated using the Bray-Curtis distance metric. Nonmetric multidimensional scaling (NMDS) was used to visualize patterns of similarity among samples in two dimensions. Statistical support for differences between samples was calculated with the aid of permutational analyses of variance (PERMANOVA) using *pairwise.adonis()* function in the Vegan R package with Bonferroni corrections. Chao1 and Shannon indices were used to assess alpha diversity of the samples. Significant shifts in the richness and diversity

across sample groups were detected using ANOVA test and multiple comparisons by means of Tuckey (*HSD.test()* function, $P < 0.05$). Lastly, we used *ps_venn()* function to diagram the shared ASVs across the aquaponics compartments in the four systems.

3.3.6 Metagenomic sequencing and analysis

To distinguish between *amoA* genes of AOB and comammox *Nitrospira*, DNA samples extracted from the biofilm carriers of systems A2 and B2 were sequenced using circular consensus sequencing (CCS) method. SMRTbell prep kit 3.0 was used according to the manufacturer's recommendations, and libraries were sequenced on a PacBio® Sequel IIe instrument (Pacific Biosciences) using one SMRT cell 8M. Translation alignment of HiFi reads against protein database was performed using *DIAMOND* snakemake workflow (Portik et al., 2022). NCBI non-redundant protein database (NCBI-nr) was downloaded and indexed with *DIAMOND* prior to running the pipeline. DIAMOND workflow was run with default settings. The resulting alignments were summarized using MEGAN-CE. Reads identified as particulate methane monooxygenase (pMMO) were extracted and analyzed using *blastn*.

3.4. Results

3.4.1 Ammonium removal as a function of pH and initial inoculum

Ammonium removal to nitrite and nitrate was monitored over the course of operation in the four aquaponic systems: two operating at pH 7.6-7.8 (A1 and A2) and two operating at pH 5.8 – 6 (B1 and B2), each inoculated with either unenriched (A1 and B1) or comammox-enriched (A2 and B2) microbial biofilters (Table 3.1). The ammonium concentration in all four systems increased slightly upon increasing the fish feeding rate on day 7, achieving the

maximum average concentration over week two followed by varying rates of ammonium removal for each system (Table 3.1, Supp. Figure B-1). The B1 and B2 systems reached higher maximum ammonium loads (261.1 and 180.6 μM ammonium) than the A1 and A2 systems (193.7 and 91.6 μM), respectively. Furthermore, systems inoculated with comammox-enriched inocula had 47% (A2) and 69% (B2) the maximum ammonium load relative to systems with unenriched inocula (B1 and B2, respectively). Systems B1 and B2 (pH 5.8 - 6) showed slower ammonium removal rates and required more time to achieve stability compared to systems A1 and A2, respectively, although a Dunn's multiple comparison test indicated that only system A1 had a significantly higher ammonium removal rate than the other three systems by the end of operation ($P < 0.05$). A slight rise in ammonium concentration after lettuce harvesting and transplanting of new seedlings at day 28 and day 56 was observed in all four systems (Supp. Figure B-1).

Table 3.1 Average ammonium removal rates and percentage of ammonium removal for each week of operation in the four aquaponics systems. The maximum average ammonium concentration was measured over week 2 and is assumed to represent the minimum concentration of ammonium released by the fish each day for each system. The average rates of ammonium removal per day were determined by subtracting the average concentrations of ammonium over each week of operation from the maximum average concentration (measured over week 2) divided by 7 days. The % removal was calculated as the average rate of removal for each week divided by the average maximum ammonium concentration. Ammonium removal rates by system A1 (*) was significantly higher than the other three systems by the end of operation (P > 0.05).

Week of operation	Average ammonium removal rates (μM per day)							
	System A1		System A2		System B1		System B2	
	Avg. Rate	% Removal	Avg. Rate	% Removal	Avg. Rate	% Removal	Avg. Rate	% Removal
2 Max. Conc.	*193.7*		*91.6*		*261.1*		*180.6*	
3	-9.0	-5	7.1	8	31.6	12	31.7	18
4	56.7	29	48.9	53	49.1	19	55.7	31
5	86	44	51.6	56	93.6	36	70.9	39
6	97.6	50	55	60	116.4	45	86.2	48

7	125.3	65	55.8	61	130.2	50	95.4	53
8	140	72	59.3	65	161.3	62	95.3	53
9	144	74	58.6	64	173.1	66	106.9	59
10	135.9	70	55	60	169.1	65	74.6	41
11	136.2	70	58.1	63	188.2	72	119.1	66
12	168.7	87	60.3	66	179.3	69	124.9	69
13	170.7	88	68.3	75	204.1	78	133.6	74
14	168	87*	67	73	198.5	76	129.6	72

Nitrite accumulation was not observed for systems B1 or B2 while in systems A1 and A2, initial nitrite levels were substantial (ca. 5 μM), but rapidly declined to ca. 0.5 μM within 7 days of operation (Supp. Figure B-2). Nitrate concentrations increased from days 1 to 7 due to the increased fish feeding rate (Supp. Figure B-3). However, nitrate was lower in the B1 and B2 systems compared to the A1 and A2 systems until the microbial communities were established after 9 weeks, which coincided with the third round of lettuce cultivation. No significant difference in nitrate levels was observed among the four systems (ANOVA, $P < 0.05$). Similar to ammonium levels, nitrate levels also spiked between lettuce harvest and transplant of new seedlings, as uptake by the plants was disrupted during this interval. Levels of nitrate remained unchanged up to 5 h following fish feeding (Supp. Figure B-3).

3.4.2 Biofilter microbiome communities converge over time and sustain both AOB and comammox nitrifiers

To study the dynamics of biofilter microbial communities over time, ASV diversity was compared after 10, 30 and 90 days of aquaponics operation. Non-metric multidimensional scaling (NMDS) revealed that the microbial composition among the biofilm carriers was significantly different depending on the sampling date ($P < 0.05$) (Fig. 3.2). NMDS analysis based on Bray-Curtis distance showed significant differences between biofilters from systems with unenriched (A1 and B1) versus comammox-enriched inoculum (A2 and B2) at 10 days of

operation. These differences decreased over time, with higher similarity between all four biofilter communities after 90 days of operation. Biofilters from systems operating at different pH (A1/B1 versus A2/B2) also showed greater similarity to one another after 3 months of operation (Fig. 3.2). Both richness (Chao1) and diversity (Shannon) of the biofilter samples increased significantly over time for all biofilter communities (ANOVA, $P < 0.05$); however, the highest richness and diversity was measured for communities from system A2 (Fig. 3.3).

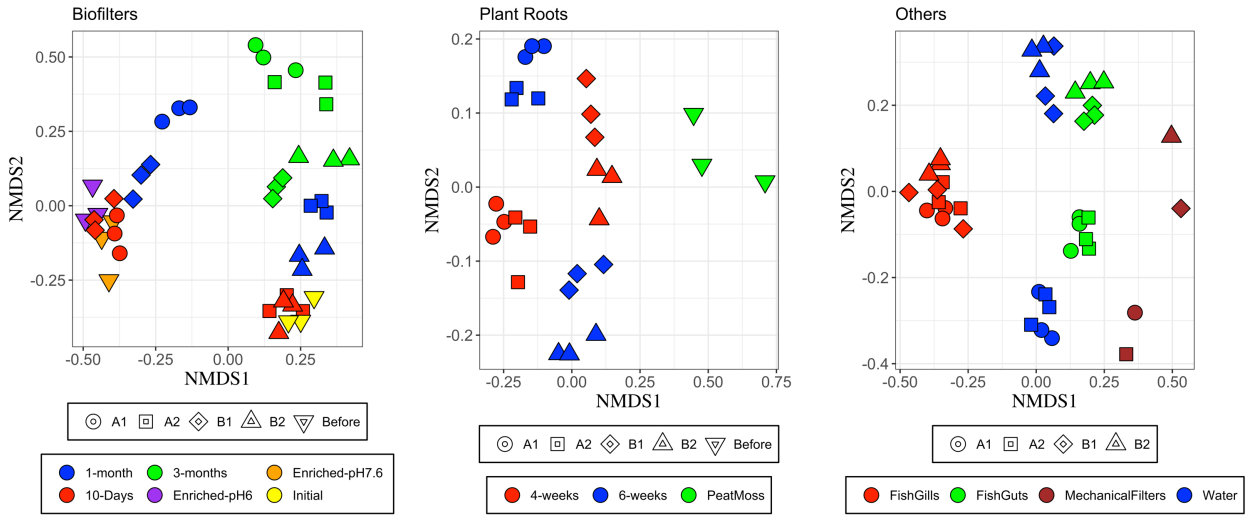

Figure 3.2 Nonmetric multidimensional scaling ordination (Bray-Curtis dissimilarity) representing the grouping of samples per compartment. Shapes represent aquaponics systems. Shifts in the beta-diversity of biofilters and plant roots were investigated over time, and colors show different time intervals. Fish intestines/gills, water samples, and mechanical filters were investigated at the end of operation. showed significant differences between biofilters from systems with unenriched (A1 and B1) versus comammox-enriched inoculum (A2 and B2) at 10 days of operation. These differences decreased over time, with higher similarity between all four biofilter communities after 90 days of operation.

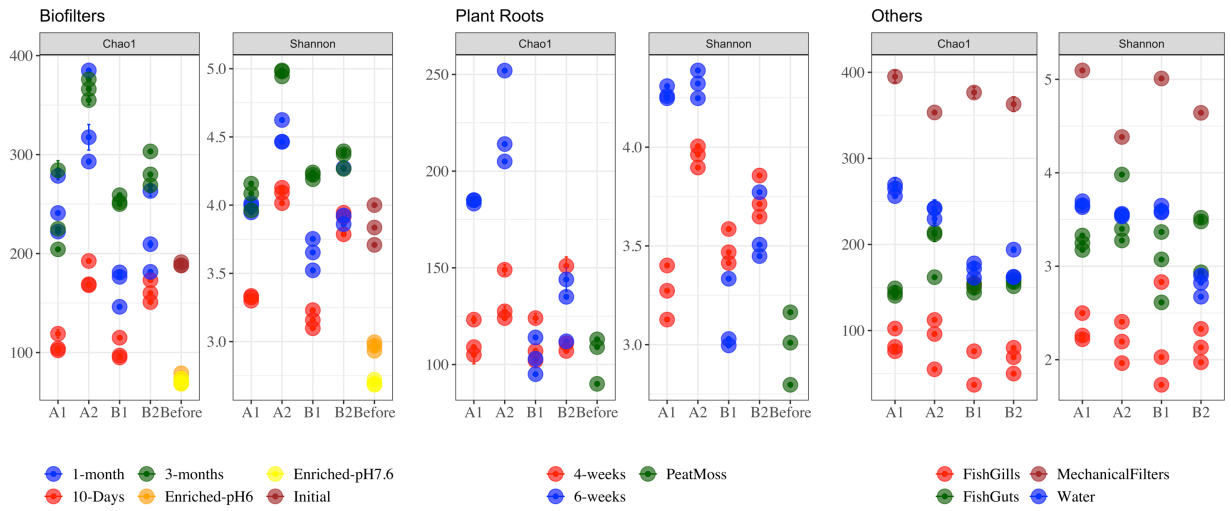

Figure 3.3 Richness (Chao1) and diversity (Shannon) distribution for recovered ASVs of different aquaponics compartments. Shifts in the alpha-diversity of biofilters and plant roots were investigated over time, and colors show different time intervals. Fish intestines/gills, water samples, and mechanical filters were investigated at the end of operation. Both richness and diversity of the biofilter samples increased significantly over time for all biofilter communities (ANOVA, $P < 0.05$); however, the highest richness and diversity was measured for communities from system A2.

After 3 months of operation, the proportion of Actinobacteria and Choloroflexi was higher in biofilter communities from systems B1 and B2 than in systems A1 and A2. (Supp. Figure B-4). The abundance Planctomycetota increased over time in systems A2, B1, and B2, which might be attributed to growth of *Pirellulaceae* spp. (Table 3.2), whereas Cyanobacteria increased in abundance only in system A1. The proportion of Proteobacteria populations from systems A2 and B2 were generally more stable than those from systems A1 and B1, which declined from day 10 to 3 months. ASVs related to the Hydrogenedentes phylum were abundant in system B1 at day 10 and 1 month, but largely disappeared by 3 months.

Table 3.2 Relative abundances of the dominate microbial families in different compartments of four aquaponics systems.

Family	% ASV Groups: Biofilters											
	A1			A2			B1			B2		
	10 days	1 month	3 months	10 days	1 month	3 months	10 days	1 month	3 months	10 days	1 month	3 months
Nitrosomonadaceae	4.2	2.2	2.3	16.8	2.3	2.8	3.1	2.5	0.4	0.1	0.4	0.5
Nitrospiraceae	0.5	5.4	1.6	1.9	5.9	3	0.3	2	1.3	1.1	2.1	4.7
Nitrososphaeraceae	0.03	0.03	0.01	0.1	0.06	0.04	0.01	0.08	0.05	0.2	0.07	0.06
Mycobacteriaceae	0	0.4	4.4	0.4	0.9	2.6	0.6	3.3	8.8	0.7	1.4	5.7
Caldilineaceae	0.02	5.2	2	0.3	0.9	2.3	0	0.2	1.2	0.3	0.8	3.2
Flavobacteriaceae	0	0.01	1.5	0	0.04	1.4	0	0.02	0.2	0	0	0.1
Comamonadaceae	1	1.9	4.8	1.4	1.6	2.1	2.1	1.9	3.7	1.4	0.9	2.6
Aeromonadaceae	0	3.2	7.8	0	0.3	3.9	0	0	0	0	0	0.4
Pirellulaceae	1.7	3.9	6.6	3	5.3	7.8	1.9	3.1	9.6	2.8	4.5	8.7
Pseudomonadaceae	0	0.9	6.7	0.1	0.8	2	0	0	0.09	0.01	0.04	0.05
Nocardiaceae	0.2	1.1	1.6	0	0.4	1.5	0.03	0.3	0.2	0	0.2	0.3

	% ASV Groups: Plant roots								
	Peat moss	A1		A2		B1		B2	
		4 weeks	6 weeks	4 weeks	6 weeks	4 weeks	6 weeks	4 weeks	6 weeks
Mycobacteriaceae	3.4	0.5	1.8	0.4	2	8.3	2.9	10.7	3.1
Micromonosporaceae	3.6	0.1	4.9	0.1	3.8	0.2	35	0.4	29
Pirellulaceae	0	2.7	19.3	0.9	21.9	2.1	6.6	1.5	6.2
Gemmataceae	0.1	7.9	11.4	13	11.2	15.6	9.9	12.9	8.9
Aeromonadaceae	0	0.8	1.1	0.1	0.9	0.1	0.4	0.2	0.2
Nitrospiraceae	0	0.4	2.1	0.9	1.9	0.1	0.2	0.1	0.1
Roseiflexaceae	0	0.1	5.2	1.8	4.1	0.7	6.4	1.5	9.2
Microbacteriaceae	10.6	0.1	0.4	0.1	0.3	4.8	2.1	3.4	1.4
Rhodobacteriaceae	5.1	4.4	5.3	6.1	4.5	4.3	4.4	4.2	3.9
Ilumatobacteriaceae	0	1.3	4.6	1.1	4.1	0.6	0.3	0.2	0.4
Rubritaleaceae	0	4.3	3.9	3.1	3	0	0.09	0	0
Comamonadaceae	0.5	6.1	5.1	7.2	5.4	3.1	3.7	5.2	4.8
Verrucomicrobiaceae	2.2	1.6	2.3	1.9	1.7	0	0	0	0.06
Beijerinckiaceae	0	0	1.2	0.1	1.1	0.1	1.9	0.4	1.2
Frankiaceae	8.1	0	0	0	0	0	0	0	0

	% ASV Groups: Fish intestines				% ASV Groups: Fish gills			
	A1	A2	B1	B2	A1	A2	B1	B2
Pseudomonadaceae	0.1	0.4	0.09	0.1	43	58	50	54
Weeksellaceae	0	0	0	0	29	26	31	25
Caulobacteracea	0	0	0	0	10.8	7.5	7.1	9.4
Comamonadaceae	0.1	0.1	0.1	0	6.3	2.9	3.7	5.5
Moraxellaceae	0	0.1	0	0.1	2.2	1.2	3.1	1.7
Nitrospiraceae	0	0	0	0	1.1	0.6	0.6	0.7
Nostocaceae	40	32	38	19	0.2	0.8	0.4	0.2
Mycobacteriaceae	23	11	20	39	0.2	0.4	0.4	0.3
Rhodobacteraceae	4.2	6.1	5.5	5.9	0.3	0.2	0.2	0.3
Nocardiaceae	1.9	7.9	1.8	2	0.2	0.2	0.4	0.1
Gemmataceae	5.6	5.9	5.6	4.4	0	0	0	0
Pirellulaceae	3.4	6.1	3.7	3.9	0.07	0.05	0.2	0
Isosphaeraceae	3	2.7	2.1	5.6	0	0	0	0

Among the nitrifier populations, AOB in the Nitrosomonadaceae, including the genera *Nitrosomonas* and *Nitrosospira*, made up 4.2% (A1), 16.8% (A2), 3.1% (B1) and 0.1% (B2) of the ASVs at day 10, and reached 2.3% (A1), 2.8% (A2), 0.4% (B1) and 0.5% (B2) of ASVs after 3 months of operation (Table 3.2). ASVs related to *Nitrospira* represented 0.5% (A1), 1.9% (A2), 0.3% (B1) and 1.1% (B2) at day 10 and increased to 1.6% (A1), 3% (A2), 1.3% (B1) and 4.7% (B2) by 3 months (Table 3.2).

Metagenomic analysis of biocarriers samples confirmed that 74% of the ammonia monooxygenase subunit A (*amoA*) genes in system A2 belonged to AOB species and 26% belonged to *Nitrospira* comammox clade B, while in system B2 comammox-*amoA* was dominant (97% of *amoA* gene sequences) and only 3% of the *amoA* gene sequences were assigned to AOB (Fig. 3.4). No *amoA* gene sequences related to canonical nitrite-oxidizing *Nitrospira* were identified, suggesting that all of the *Nitrospira* ASVs were most likely related to comammox *Nitrospira*. Similarly, consistently low quantities (<1%) of ASVs and zero *amoA* genes related to ammonia oxidizing archaea (AOA) were detected across all four systems, indicating negligible contributions to nitrification activity from this microbial group.

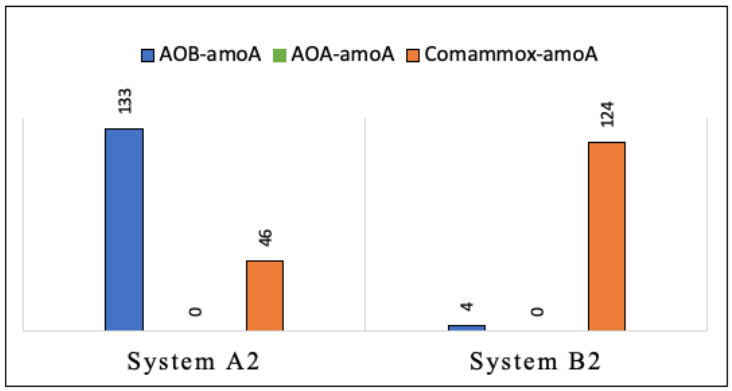

Figure 3.4 Number of HiFi reads assigned to AOA, AOB, and comammox amoA genes quantified in metagenomes of biocarrier samples from systems A2 and B2. PacBio® metagenomic analysis was conducted on DNA samples collected at the end of the operation period. The plot indicates that both

AOB and comammox *Nitrospira* are present in the system with pH 7.6-7.8, while comammox *Nitrospira* are the dominant nitrifiers at slightly acidic pH.

3.4.3 Plant biomass increases at lower pH and root microbiome is altered by pH

Three rounds of lettuce were cultivated in each of the four aquaponics systems to compare the effect of microbial inoculum and operating pH on plant biomass and root microbiota. Lettuce was harvested after 4 weeks growth in the first and second rounds, and after 6 weeks growth in the third round. No relationship was found between microbial inoculum and effects on final plant biomass, but consistently higher biomass was achieved in systems B1 and B2 (pH 5.8 - 6) versus systems A1 and A2 (pH 7.6-7.8) for each round of lettuce growth, although the combined difference over the three rounds was not significant (ANOVA, $P < 0.05$), likely due to the two weeks of additional growing time for lettuce round three (Supp. Figure B-5).

NMDS plots showed that rhizosphere microorganisms sampled after 4 weeks or 6 weeks of lettuce growth clustered into distinct groups relative to seedlings in peatmoss (Adonis, $P < 0.05$), indicating that time was an important factor for microbiome development (Fig. 3.2). Comparisons between systems A1 and A2 with B1 and B2 revealed a significant impact of pH on root microbiota composition ($P < 0.05$). Based on the Chao1 index, richness of the root microbiota in systems A1 and A2 significantly increased over time, but not in systems B1 and B2 (Fig. 3.3). Conversely, the Shannon index measurements indicated a significant increase in the diversity of root microorganisms over time at both pH levels. Development of root microbiota was not significantly influenced by the source of the initial inoculum, suggesting that the rhizosphere microbiome develops independently.

In terms of community composition, the two major ASV phyla observed in the lettuce rhizosphere from seedlings growing in peat moss were Actinobacteriota (31%) composed of the

families Microbacteriaceae (10.6%) and Frankiaceae (8.1%), and Proteobacteria (64%) (Supp. Figure B-6, Table 3.2). Lower pH favored the growth of Actinobacteriota, predominantly Micromonosporaceae, comprising 43% of ASVs from the 6 weeks lettuce rhizosphere in systems B1 and B2 (Supp. Figure B-6, Table 3.2). Interestingly, nitrogen-fixing Frankiaceae were absent from the rhizosphere of all systems, likely due to the ready availability of nitrate (Table 3.2). In contrast, Planctomycetota, mainly Gemmataceae, were higher in systems A1 and A2 than systems B1 and B2, increasing to 29% of ASVs in the 6 week lettuce rhizosphere. Proteobacteria were abundant at 29% and 25% in the 4 week and 6 week lettuce rhizosphere, respectively.

3.4.4 Fish biomass and intestine/gill microbiomes were unaffected by pH

The four aquaponics systems were stocked with five goldfish per tank with initial weights between 20-25 g/fish. No fish mortality was observed for the duration of the experiment. Fish biomass in systems A1 and A2 reached 31.9 and 31.4 g, respectively, slightly higher than in the B1 (23.7 g)and B2 (27.4 g) systems, although not statistically significant (Kruskal-Wallis test, $P < 0.05$) (Supp. Figure B-7).

NMDS plots showed that microorganisms collected from water, fish feces (collected in mechanical filters) and fish digestive systems in the A1 and A2 versus the B1 and B2 tanks clustered into distinct groups (Fig. 3.2). Despite these differences, the microbial inoculum source did not influence either water or fish-associated microbial populations. The diversity of microorganisms from water, fish gills and fish intestines were very similar in all systems based on the Shannon index, whereas the Chao1 index indicated that the A1 and A2 microbiomes had higher species richness than the B1 and B2 microbiomes (Tuckey, $P < 0.05$; Fig. 3.3).

The major microbial phyla of ASVs in fish intestines were Cyanobacteria (37%), Actinobacteria (34%), Proteobacteria (17%), and Planctomycetota (13%) (Supp. Figure B-8). These same phyla with ASV relative abundances of 1.5%, 10%, 16%, and 20%, respectively, were detected in the mechanical filters collecting fish feces (Supp. Figure B-9). Mechanical filters were dominated by ASVs related to Firmicutes (31%) but also contained ASVs related to *Nitrospira* (2.7%). Compared to fish intestines, a higher diversity of phylum-level ASVs was observed in the mechanical filters, which shared similar phyla with the bulk water samples (Supp. Figure B-10). High abundances of ASVs related to Bacteroidota and Proteobacteria were found in both water and fish gill samples (Supp. Figure B-10 and B-11), but with higher relative abundances in water samples at pH 5.8 – 6 than at pH 7.6 - 7.8 water samples. Also, Actinobacteria dominated ASVs in the pH 5.8 - 6 (54%) compared to pH 7.6 - 7.8 water samples (10.5 %). A low percentage of ASVs related to *Nitrospira* were detected in all fish gills (Table 3.2). Unlike the biofilter and rhizosphere microbial communities, the microbiome of fish intestines/gills were not influenced by pH.

3.4.5 The majority of ASVs were shared among different compartments in aquaponics systems

Plotting the distribution of ASVs across all samples provided evidence for unique and shared microbial groups between and among the aquaponics compartments (Fig. 3.5). The core microbiome detected in all three biological compartments, including fish intestines/gills, lettuce rhizosphere, and microbial biofilters, were mainly attributed to the genera *Mycobacterium*, *Nocardia*, *Pirellula*, and *Bacillus*. 88% of the microbiota were unique for each compartment, with 52.5%, 19.8%, 10.2%, and 5.5% belonging to biocarriers, plant roots, fish intestines, and fish gills, respectively. Biocarriers shared 684, 383, and 126 ASVs with plant roots, fish

intestines and fish gills, respectively. The smallest number of shared ASVs was between fish intestines and fish gills. In addition to the core microorganisms, fish intestines and gills samples also shared ASVs in the *Pseudomonas*, *Aeromonas*, *Acinetobacter*, and *Serratia* genera.

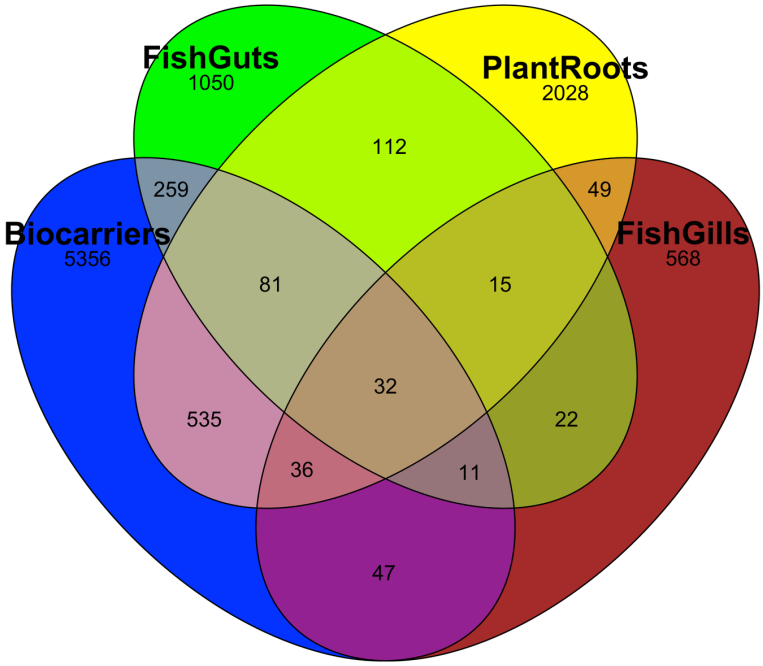

Figure 3.5 Venn diagram representing the proportion of common and unique ASVs in different biological compartments of aquaponics. The highest number of ASVs were shared by biocarriers and plant roots. Fish intestines and fish gills were discovered to share the slightest similarities.

3.5 Discussion

The aim of this study was to investigate how the source of microbial inocula and operational pH influenced both nitrogen removal rates and microbial community composition in an aquaponics system. Nitrogen (ammonium, nitrite and nitrate) measurements in the fish tanks revealed that systems A2 and B2 inoculated with comammox-enriched biocarriers achieved a smaller maximum concentration of ammonium, but were also slower to remove it relative to

systems A1 and B1 that were inoculated with biocarriers harboring AOB nitrifiers. The benefits of initiating aquaponics systems with microbial communities enriched with nitrifiers has been reported in other studies (Ajijah et al., 2021; Day et al., 2021). Thus, selection of a nitrifier-enriched inoculum, whether by comammox or AOB, can be a useful strategy to enable desired start-up conditions and stabilization time for aquaponics systems rather than operating at low fish density or amending with ammonium chloride for several weeks (Sallevane, 2016). In terms of operating pH, lower quantities of nitrite and nitrate were measured in systems B1 and B2 (pH 5.8 - 6), which was likely due to the higher rate of plant growth. These results were similar to those of Wongkiew et al. (2018) where lower nitrate and higher ammonium concentrations were measured in a system operating at pH 6 versus pH 7. The quantity of nitrite and nitrate represents an equilibrium between nitrification activity and plant absorption, which also explains the temporary increases in nitrate concentration observed between plant harvest and re-planting (Supp. Figure B-3).

Systems B1 and B2 (pH 5.8 - 6) consistently yielded higher lettuce biomass, likely due to the optimized pH for plant nutrient uptake (Wortman., 2015). The positive effect of lower pH on lettuce growth was less pronounced in the 6 week old plants (29% difference) compared to the 4 week old plants (58% and 53% differences), which is consistent with Tyson et al. (2008) where lower pH had a greater effect on early crop yield than on later yields. In contrast, slightly lower fish biomass was obtained in the B1 and B2 systems, perhaps due to the higher ammonium concentrations than in the A1 and A2 systems (El-Sherif and El-Feky, 2008) or the negative impact of acidic pH on fish growth rates (Yildiz et al., 2017). Despite the difference in fish growth, no mortality or negative behavior was observed.

This study validated that pH remains a strong determinant of microbial community composition in aquaponics bio-compartments (Zou et al., 2016; Wongkiew et al., 2018). Comparisons of beta diversity and microbial composition associated with biofilm carriers at two pH levels showed convergence from initial populations to those after 3 months of operation. Interestingly, nitrifiers in the genera *Nitrosomonas* and *Nitrosospira* made up 2 - 3% of the biofilter microbiota at pH 7.6-7.8, which is similar to other aquaponic systems (Wongkiew et al., 2018; Eck et al., 2019), whereas the biofilter microbiota at pH 5.8 – 6 were mainly *Nitrospira* (1.3-4.7%). Despite the lower nitrification rate of comammox *Nitrospira*, their presence could be advantageous for reducing nitrite accumulation in the water (Sakoula et al., 2021) and lowering the emission of nitrous oxide (Kits et al., 2019). Nitrification by comammox *Nitrospira* has been reported in highly efficient aquaponics systems, similar to what is reported here (Schmautz et al., 2017; Heise et al., 2021). Nevertheless, ASVs and metagenome signatures of comammox bacteria were present in the biofilter communities in all four systems after 3 months of operation. Ammonia-oxidizing archaea, however, were not a major population in any of the systems.

Higher abundances of ASVs related to Actinobacteria, characterized as plant growth-promoting microbes (PGPM), were observed in the microbial biofilters, water, and lettuce rhizosphere samples in the pH 5.8 - 6 systems. Members of this phylum including strains of *Mycobacterium* (Egamberdiyeva, 2007), *Nocardia* (Ghodhbane-Gtari et al., 2019), *Leifsonia* (Nordstedt et al., 2021), and *Sporichthya* (Zhang et al., 2019) were identified. These microbes can significantly promote nutrient uptake and plant growth. Water samples from the systems at pH 5.8 - 6 especially favored ASV presence of the endophytic Actinobacterial genera *Micromonospora* and *Actinoplanes* (Micromonosporaceae family), also known to enhance plant growth (Trujillo et al., 2015). Rhizobacteria appeared to thrive at the lower pH, which also

correlated with more robust plant growth. Upon transferring lettuce seedlings to the hydroponics grow beds, ASVs related to *Frankia* bacteria disappeared, and *Gemmata* and *Pirellula* ASVs appeared, which could be in correlation with increased flavonoid content of plants (Singha et al., 2021). Furthermore, ASVs related to *Bacillus*, *Paenibacillus*, and the Proteobacteria *Rhodobacter* and *Pseudomonas* were present in all rhizosphere samples, including those from peat moss. Some strains of these genera are among the most widely reported PGPM that stimulate plant growth mainly through solubilizing phosphate and producing siderophores (Adesemoye et al., 2008; Kang et al., 2022).

Compared to biofilters and rhizospheres, low phylogenetic diversities and similar ASV groups were observed in the fish intestines and gills at both pH levels. The major fish-associated bacterial phyla were consistent with previous studies (Liu et al., 2016; Wang et al., 2022) reporting the presence of Proteobacteria, Cyanobacteria, Planctomycetes, and Actinobacteria as core microbiota of carp species. Some aquaponics studies sampled fish feces to represent fish intestine microbiota (Schmautz et al., 2017; Day et al., 2021). Our results showed that fish intestine microbiota resembled the water microbiota. Similar to the fish intestines, low phylogenetic diversities among ASVs were identified in the fish gill samples. Despite direct contact with water, the microbiome of fish gills were not affected by pH. Similar phyla with the same relative abundances in all fish gills samples indicate that these microorganisms likely contribute to gill function. Low quantities of *Nitrospira* on fish gills may benefit from ammonium supplied by the host. Bacterial ammonia oxidation and the presence of nitrifying microbes (AOB) in fish gills has been reported previously (van Kessel et al., 2016).

3.6 Conclusion

Aquaponics are constructed ecosystems designed for the efficient farming of fish and crops. The three biotic compartments of these systems: fish, plants and microorganisms, are connected and dependent on one another through the recirculation of water and flow of nutrients. Along with researching aquaponics microorganisms to enhance nitrogen use efficiency, mesocosm studies provide a tool for examining the potential influence of environmental factors, like pH and microbial inocula, on functional groups of microorganisms like nitrifiers and PGPM in larger scale systems. This study showed that despite differences in the abundance of particular phyla, the core microbiota tended to converge, regardless of operating pH and source of inoculum. The results suggests that groups of microorganisms with defined functions are supported by aquaponics operations that are selected for and maintained within a range of operating conditions.

This study also showed that while both comammox and AOB nitrifiers can be sustained in aquaponics systems, the lower pH systems favored comammox over AOB. Thus, decreasing the pH in systems with low abundances of comammox *Nitrospira* could result in transient accumulation of ammonia until the population can grow and stabilize. Nevertheless, using mini-systems with particular types of fish and plants at bench scale makes it difficult to determine whether similar effects will be observed in commercial scale aquaponics systems with different combinations of fish and plants.

A lower pH also altered the composition of microbial communities in fish intestines, lettuce rhizospheres and the heterotrophic bacteria associated with the biofilters, and also resulted in increased plant growth. Further investigations using metagenomics could provide valuable information about other important microbes and their functions like those involved in

iron and phosphorous availability, other N-cycle processes, the presence of pathogens, and detailed functions of PGPM in aquaponics systems.

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Chapter 4: Effect of inoculum and pH on microbial activity and metagenomic diversity from biofilters and plant roots in bench-scale aquaponics systems

4.1 Abstract

Aquaponics is an efficient food production system that integrates recirculating aquaculture system (RAS) and hydroponics to obtain high crop yields. The development of aquaponic food production systems requires a thorough understanding of the make-up and roles of the microbial communities involved in the water treatment and plant growth processes. Using the PacBio® sequencing platform, we examined the metagenomes of lettuce roots and biofilters in two aquaponic mini-systems operating at pH 5.8 - 6 and pH 7.6-7.8. Taxonomy analysis of HiFi reads using DIAMOND-MEGAN tools indicated that pH is a determinant of the composition of *Nitrospira* communities. Moreover, *Nitrosospira* (ammonia-oxidizing bacteria, AOB) and *Nitrosocosmicus* (ammonia-oxidizing archaea, AOA) species had different pH sensitivity patterns, populating the systems at pH 7.6-7.8 and pH 5.8 - 6, respectively. Annotating the reads against the SEED protein database revealed higher numbers of genes involved in siderophore production in the system at pH 7.6-7.8. This higher pH condition may impede iron solubility, as higher plant growth was observed in the system operating at pH 5.8 - 6. In addition, sequence assembly and contig binning using MetaBat2 and SemiBin2 tools resulted in five high quality MAGs from biofilter samples.

4.2 Introduction

Human population growth worldwide and increasing demand for food has stimulated research into engineered agriculture systems to supplement or replace traditional food production methods (Junge et al., 2017). Aquaponics is an efficient food production system that integrates recirculating aquaculture system (RAS) and hydroponics to obtain high crop yields and meet global food demand. This innovative technique reduces synthetic fertilizer input and environmental impacts of traditional agriculture via using nutrient-rich effluent of fish farming as a source of fertilizer for organic plant cultivation (Lennard and Goddek., 2019).

Microorganisms constitute a bridge between the two food-producing compartments of aquaponics. The activity of microorganisms in effluent treatment supports healthy fish and crop production. Heterotrophic bacteria supply micronutrients for plant growth through decomposing organic matter and solubilizing essential elements (Kasozi et al., 2021). Also, nitrifiers improve water quality by detoxifying ammonia, released through fish gills, and convert it to nitrate which is less toxic for fish (Camargo and Alonso, 2006) and is the preferred N-source for most plants (Hessini et al., 2019). In addition, microorganisms in aquaponics systems are assumed to have other beneficial effects, such as production of plant growth-promoting microorganisms (PGPM) that enhance nutrient uptake and stimulate plant growth (Chapter 3).

Developing more profitable and environmentally sustainable food production systems using aquaponics systems requires a comprehensive understanding of the structure and activities of microbial communities involved in water treatment. The diversity of microorganisms from different aquaponics compartments and their responses to shifts in environmental conditions have been evaluated using 16S rRNA gene sequencing (Schmautz et al., 2017; Bartelme et al., 2019; Heise et al., 2021; Chapter 3). Nevertheless, little is known about aquaponics microbial

functions, like those involved in the nitrogen cycle or iron and phosphorous availability. Obtaining a high-resolution profile of microbial activities is necessary to accurately understand the impact of physicochemical factors on the functional capacity of microbiota in aquaponics systems.

Shotgun metagenome sequencing (metagenomics) can provide unbiased classification of microbial communities with high taxonomic resolution as well as functional profiling. Third-generation sequencing technologies like the circular consensus sequencing (CCS) method developed by Pacific Biosciences (PacBio®) provides high fidelity (HiFi) long sequencing reads with > 99% per-base accuracy which can surmount many difficulties in gene alignment and metagenome assembly via spanning multiple genes and repetitive regions (Portik et al., 2022). Also, many pipelines have specifically been developed for assembly and binning of HiFi long reads to investigate the structure and functionalities of microbial communities (Feng et al., 2022).

In Chapter 3 of this thesis, 16S rRNA gene sequencing from different compartments of four bench scale aquaponics systems revealed that pH impacted the microbial composition of biofilters and lettuce roots. Therefore, we aim here to more deeply investigate the potential functionality of microorganisms associated with the biofilters and lettuce roots from aquaponics systems operating at two pH levels using PacBio® sequencing and metagenomics analysis.

4.3 Materials and methods

4.3.1 Sample collection and DNA extraction

Biofilm carriers were collected from biofilter tanks A2 and B2 as described in Chapter 3 after three months of operation. Five biofilm carriers from each of the two systems were

separately added to sterile 50 ml Falcon tubes containing 10 ml sterile Milli-Q water. Tubes were vortexed vigorously for 5 min to detach biofilm from carrier surfaces. Biofilm samples were centrifuged at 10,000 × g for 10 min and the resulting biomass pellets were used for genomic DNA extraction (Derikvand et al., 2021). For characterizing plant root microbiota, samples of the entire root system were collected from lettuce roots grown for six weeks in the hydroponic beds as described in Chapter 3. Samples were frozen in liquid nitrogen and ground into small pieces prior to DNA extraction (Simmons et al., 2018).

Genomic DNA from all samples was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals Inc.) along with blank controls. Quality and quantity of the extracted DNA were measured using Nanodrop (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific) and a Qubit dsDNA HS Assay Kit, according to manufacturer's protocol (Invitrogen Qubit Fluorometer, Thermo Fisher Scientific). The extracted DNA was tested for enzymatic inhibitors via PCR amplification of 16S rRNA genes (515F and 806R primers) prior to sequencing.

4.3.2 Library preparation and sequencing

High quality DNA samples were sequenced using the circular consensus sequencing (CCS) method. 500 ng of genomic DNA was used to make unamplified libraries using the SMRT-bell prep kit 3.0, according to the manufacturer's recommendations. gDNA was sheared to a targeted fragment size of 12 kb using Megaruptor and Long Hydropores (Diagenode, Denville, NJ, USA). Sheared gDNA were concentrated using AMPure PB Beads according to the manufacturer recommendations (Pacific Biosciences, Menlo Park, CA, USA) and underwent two treatment procedures for DNA damage repair and end-repair. Barcoded overhang Hairpins adapters from the manufacturer were ligated to the fragment ends to create SMRT-bell templates used for

sequencing. SMRT-bell templates were purified using an exonuclease procedure to remove any free ends molecules or no adapter templates. Libraries were sequenced on a PacBio RS II Single Molecule, Real-Time (SMRT®) DNA Sequencing System (Pacific Biosciences, CA, USA) using one SMRT cell 8M. A total of raw ~1.2 Gb of long-read data were obtained, and high-fidelity reads were then generated with the "ccs" module within the SMRT link v10.0 package.

4.3.3 Taxonomic and functional profiling

Translation alignment of HiFi reads against protein databases was performed using *DIAMOND* snakemake workflow (Portik et al., 2022). NCBI non-redundant protein database (NCBI-nr) was downloaded and indexed with *DIAMOND* prior to running the pipeline (diamond makedb –in nr.gz –db diamond_nr_db –threads 24). Default settings were used for configuring the analysis (chunks: 4, block_size: 12, threads: 24, hit_limit: --top 5, readassignmentmode: readCount, minSupportPercent: 0.01). DIAMOND workflow was run on a local system using long-read settings (--range-culling). This snakemake analysis identifies and removes CIGAR strings with illegal frameshift characters. *sam2rma* (-alg longReads) and *rma2info* are the executable tools required for this workflow. The resulted unfiltered RMA files were used to summarize the resulting alignment using MEGAN-CE with minimum threshold-filtering for hits. Read counts and read-based profiling of the NCBI taxonomy was computed using the *Long Read LCA* algorithm. Also, *best hit* algorithm with SEED, eggNOG, and InterPro2GO was used for functional classification (Huson et al., 2016). Bray-Curtis distances among samples were visualized with Principal Coordinate Analysis (PCoA) at both taxonomic and functional levels.

4.3.4 Contig assembly, MAG binning and genome annotation

Assembly of contigs was performed using the de novo metagenome assembler hifiasm-meta (https://github.com/xfengnefx/hifiasm-meta, version 0.3.1-r63.2, default parameters) (Feng et al., 2022). Assembled contigs and HiFi reads were both used as inputs in a workflow with a "circular-aware" binning strategy for maximizing MAGs from hifi reads (https://github.com/PacificBiosciences/pb-metagenomics-tools/tree/master/HiFi-MAG-Pipeline). The snakemake analysis was run on a local system (snakemake --snakefile Snakefile-hifimags.smk --configfile configs/Sample-Config.yaml -j 48 --use-conda). In this pipeline, long contigs larger than 500 kb are spotted and put into separate fasta files. Then, CheckM2 is used to examine the percent completeness of the contigs. The initial set's shorter incomplete contigs are pooled with all long contigs that are >93% complete, and this contig set is then subjected to binning. MetaBat2 and SemiBin2 are the two algorithms (long read setting) used for binning. DAS_Tool is used to compare and combine the two bin sets. The dereplicated bin set consists of the merged bin set from above and all long complete contigs found. Quality of the dereplicated bins was then assessed and subsequently filtered using CheckM2 with default parameters (>70% completeness, <10% contamination, <20 contigs). Finally, GTDB-Tk was used to assign taxonomy to the filtered bins/MAGs.

4.4 Results

4.4.1 Sequencing statistics

Genomic DNA was isolated from the roots and biofilm carriers of the two aquaponic systems operating at pH 7.6-7.8 (A2) or 5.8 - 6 (B2). Using a SMRT cell 8M and a Sequel IIe device, the libraries were sequenced. The result of high molecular weight DNA sequencing was

1.2 Gb with 417,240 HiFi reads. With an average N50 value of 5711 bp, the mean read length was around 5000 bp. Table 4.1 shows specifics of the HiFi sequencing data.

Table 4.1 Statistics of PacBio® HiFi sequencing data. Systems A2 and B2 were operated at pH 7.6-7.8 and 5.8 - 6, respectively as described in Chapter 3.

Samples	Mean Length (bp)	Mean Quality (Q-score)	NC50 length (bp)	Total bases	Number of reads
Biocarriers (system A2)	5905.3	58.4	6732	1,123,137,941	190,192
Biocarriers (system B2)	6733.6	56.1	3940	657,665,865	97,669
Roots pH (system A2)	3290	66.9	7560	258,856,820	78,681
Roots (system B2)	3862	64.3	4614	195,840,276	50,698

4.4.2 Taxonomy classification of HiFi reads

The MEGAN-LR taxonomic sequence classifier annotated the metagenome samples using NCBI taxonomy. Using LCA of the hits, a homology search of the raw reads against the database was conducted. For nitrifiers, relative abundances were calculated at the species level (Table 4.2). From all HiFi reads collectively 32,771 16S rRNA gene sequences were identified, of which 4255 were categorised as belonging to nitrifiers (~ 13%). Sequences related to *Nitrospira* accounted for 54% of the total 16S rRNA gene sequences among the nitrifiers, of which 32.3%, 61.1%, 0.2%, and 6.3%, respectively, were found in system A2 Biocarriers, system B2 biocarriers, system A2 Roots, and system B2 Roots (Fig 4.1). Among the comammox *Nitrospira* representatives, *N. nitrosa* was most prevalent in metagenomes of system A2 biocarriers, while other species including *N. inopinata*, *N. kreftii*, *N. nitrificans*, and *Nitrospira* sp. UW-LDO-01 were more abundant in metagenomes of system B2 biocarriers.

Table 4.2 Number of reads assigned to nitrifiers at species level (* = comammox *Nitrospira* species).

Nitrifiers	Biocarriers pH 7.6	Biocarriers pH 6	Roots pH 7.6	Roots pH 6
Nitrospira				
*inopinata**	14	94	2	0
*kreftii**	0	9	0	2
*nitrificans**	1	16	1	6
*nitrosa**	154	7	8	34
moscoviensis	44	69	0	7
defluvii	21	3	0	1
japonica	20	0	0	1
lenta	53	5	1	2
sp.	79	332	10	40
sp. CG24B	0	38	0	0
sp. CG24D	18	1	0	0
sp. ND1	3	2	0	0
sp. KM1	256	2	0	0
sp. SCN 50-13	19	0	0	0
sp. SG-bin1	2	69	0	9

sp. ST-bin4	0	316	0	3
sp. ST-bin5	67	18	0	0
sp. UW-LDO-01*	31	422	21	40
Nitrosomonas				
oligotropha	1	0	1	1
unclassified	45	4	0	1
Nitrosospira				
briensis	29	0	0	0
lactus	131	0	0	0
multiformis	25	0	0	0
unclassified	1358	43	1	0
Nitrosovibrio				
tunuis	7	0	0	0
unclassified	17	2	1	0
Nitrosocosmicus				
arcticus	0	19	0	0
franklandus	0	21	0	0
hydrocol	1	55	0	0
oleophilus	1	49	0	0
unclassified	8	139	0	0

16S rRNA genes related to ammonia-oxidizing bacteria (AOB) were largely present in metagenomes from system A2 Biocarriers but absent or present in small numbers in other samples (Table 4.2). More than 95% of AOB found in the aquaponics systems belonged to the *Nitrosospira* species (Table 4.2, Supp. Figure C-1). Contrarily, 16S rRNA genes related to ammonia oxidizing archaea (AOA), which were all related to species of *Nitrosocosmicus* and were more abundant in metagenomes from system B2 biocarriers (Supp. Figure C-2). 16S rRNA gene seqences related to AOA were not detected in lettuce root metagenomes.

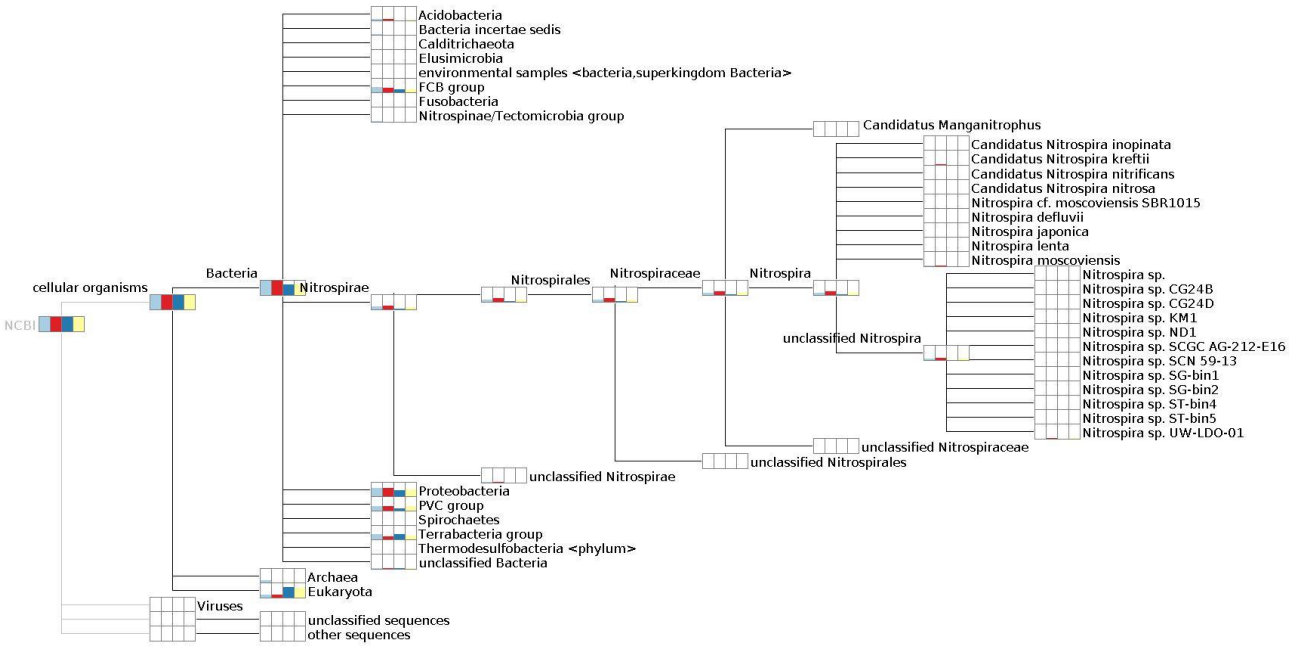

Figure 4.1 Phylogeny of the HiFi reads assigned to *Nitrospira* species. NOB *Nitrospira* and comammox *Nitrospira* dominate biofilm carriers at pH 7.6-7.8 and pH 5.8-6, repectively (cyan: system A2 biocarriers, red: system B2 biocarriers, blue: system A2 Roots, yellow: system B2 Roots).

4.4.3 Functional classification of HiFi reads

Translation alignment of HiFi reads against different protein databases was performed using *DIAMOND* and summarized using MEGAN-CE. Principal coordinate analysis (PCoA) plot using the Bray-Curtis dissimilarity calculation showed that based on taxonomy, biocarriers and root metagenomes are separated from each other along the PCoA axes (Fig 4.2-a). However, based on the functional structure of samples from SEED genome annotations, root metagenomes were better clustered and showed more functional similarities as compared to the biocarrier metagenomes (Fig 4.2-b).

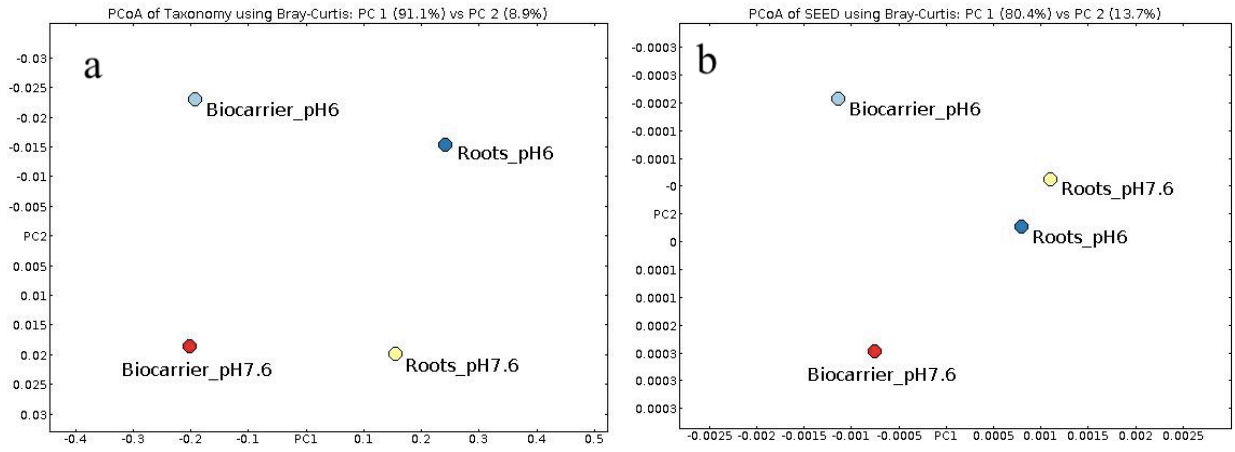

Figure 4.2 Dissimilarities of microbial community diversity (a) and functional structure (b) based on the Bray-Curtis distance metric. Different microbiota with similar functionality were identified in the roots samples collected from alkaline and acidic systems (cyan: system A2 biocarriers, red: system B2 biocarriers, blue: system A2 Roots, yellow: system B2 Roots).

To investigate the metabolic divergence of microorganisms withing the metagenomes, a SEED subsystem-based gene-centric comparative analysis was conducted (Supp. Fig C-3). The highest percentage of functional genes among all metagenomes were those involved in cell metabolism (38.4%), protein processing (14.5%), energy (12.9%), and DNA processing (7.2%).

Read count analysis of biocarrier metagenomes showed that 74% of the ammonia monooxygenase subunit A (*amoA*) genes in system A2 belonged to AOB species, while in system B2 the comammox-*amoA* genes were dominant with only 3% of *amoA* genes assigned to AOB (Chapter 3, Fig 3.3). The samples also contained several genes that encoded denitrification-processing enzymes, such as nitrate reductase, nitrite reductase, and nitrous oxide reductase. The metagenomes from system A2 biocarriers had more denitrification-related genes than metagnomes from system B2. No genes encoding nitric oxide reductase (*nor*) were detected using the search algorithms, and will need to be revisited using other search strings as most AOB encode several types of nitric oxide reductase (Kozlowski et al., 2016). Gene related to denitrification were also not identified in either root metagenome. In all samples, genes encoding

proteins involved in the metabolism of iron and phosphorus were found. In comparing the two pH levels, similarities were found in the quantities of phosphonate (phosphite) dehydrogenase, the ABC-type iron transport system, and the ferrous iron transporter EFeUOB. In contrast, genes encoding siderophore biosynthesis were ~9-fold higher in metagenomes from system A2 than system B2 for both biocarriers and roots (Table 4.3).

Table 4.3 Quantities of genes assigned to proteins involved in nitrogen, iron and phosphorous metabolism. Read count obtained from SEED annotation of samples.

Enzymes	Biocarriers A2	Biocarriers B2	Roots A2	Roots B2
Nitrate reductase	39	31	0	0
Nitrite reductase	30	29	0	0
Nitrous oxide reductase	27	17	0	0
Phosphonate (phosphite) dehydrogenase	16	12	2	0
ABC-type iron transport system	1	3		
Ferrous iron transporter EFeUOB, low pH induced	25	24	3	11
Siderophores	168	15	34	7

4.4.4 Sequence assembly and MAG retrieval

The program HiFiasm-meta was used to assemble the PacBio® HiFi reads. The assembly process generated 43687, 17682, 3034, and 1234 contigs from system A2 biocarriers, system B2 biocarriers, systeme A2 roots, and system B2 roots, respectively, with maximum contig sizes of ~3.6 Mb. MAGs binning was performed in a "circular-aware" pipeline using MetaBat2 and SemiBin2 tools.

The binning process resulted in 53 MAGs from the biocarrier metagenomes as no MAGs from the root metagenomes passed the CheckM2 criteria as high quality (>70% completeness, <10% contamination, <20 contigs). From the system A2 biocarrier metagenome, four MAGs passed the CheckM2 parameters (Fig. 4.3) with a completeness score of 100% (4 contigs), 99%

(3 contigs), 90% (15 contigs), and 86% (9 contigs). Based on the GTDB-Tk, the MAGs were assigned to order Flavobacteriales PHOS-HE28 (unclassified family), genus *Nitrospira* (unknown species), genus *Terrimesophilobacter* (unknown species), and genus *Ginsengibacter* (unknown species), respectively.

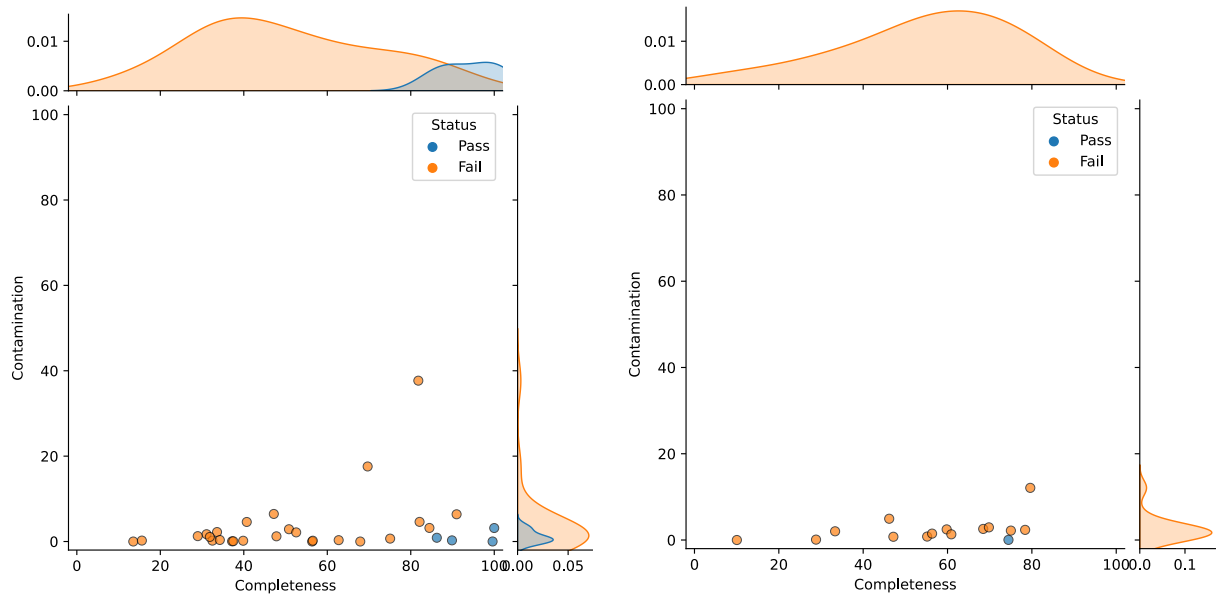

Figure 4.3 MAG characteristics representing completeness versus contamination scores for high quality MAGs. Each circle represents a MAG and colors indicate whether they pass CheckM2 criteria (>70% completeness, <10% contamination, <20 contigs). Totally, 43 MAGs retrieved from these two samples and 5 MAGs identified as high-quality MAGs; left: system A2 Biocarriers, and right: system B2 Biocarriers.

One MAG from the metagenome of system B2 biocarriers passed the CheckM2 parameters with a completeness score of 74% (2 contigs) (Fig. 4.3). This MAG was assigned to the order Chitinophagales (unclassified family). GC contents and contamination scores of the MAGs are shown in Supp. Figure C-4 and Supp. Figure C-5.

4.5 Discussion

This study investigated the influence of pH on the functional and high-resolution taxonomy of aquaponics microbial communities operating at pH 7.6-7.8 (A2) and pH 5.8 - 6 (B2) from biofilters and lettuce roots using metagenomics generated by PacBio® Sequel IIe sequencing. NCBI taxonomy of metagenomes using DIAMOND-MEGAN revealed that while AOA were absent in systems operating at pH 7.6-7.8, both AOB and comammox *Nitrospira* were present. On the other hand, AOB were less prevalent in the system operating at pH 5.8 - 6, whereas the abundance of comammox bacteria increased, confirming results from the amplicon analysis in Chapter 3. This change suggests that comammox bacteria drove effective nitrification at pH 5.8 - 6, which could benefit nitrogen balance in engineered systems and prevent N_2O emissions (Derikvand et al., 2021). The quantity of amplicons in metageomes related to AOA *Nitrosocosmicus* species also increased in the B2 system. This is in line with earlier studies reporting the ability of AOA to occupy low pH habitats (Zhang et al., 2012).

The dominant AOB in the system was related to the genus *Nitrosospira* that has been found previously as a major contributor to nitrification in aquaculture and aquaponics systems (Sanchez et al., 2019; Schmautz et al., 2022). Unlike soil environments, organic molecules in aquaponics systems are continuously eliminated by mechanical filters and heterotrophic bacterial hydrocarbon breakdown. This is to maintain water quality for fish farming. Low C/N ratios can stimulate *Nitrosospira* growth over *Nitrosomonas* in these systems (Xia et al., 2010).

In the A2 system operating at pH 7.6-7.8, there was a higher abundance of comammox *Nitrospira nitrosa*. In the B2 system operating at pH 5.8 - 6, other comammox species including *N. inopinata*, *N. kreftii*, *N. nitrificans*, and *Nitrospira* sp. UW-LDO-01 and AOB nitrifiers were more abundant in the metagenomes. Comammox *Nitrospira* have greater competitive success

under low substrate concentrations (Kits et al. 2017), such as acidic environments where ammonium is favored over ammonia. Additionally, transporters in some comammox strains for rapid ammonium uptake may make them more competitive than AOB in acidic habitats (Koch et al. 2019). However, more research is needed to better understand the physiological differences between *N. nitrosa* and other comammox species.

Similar numbers of reads assigned to denitrification and phosphorous metabolism were detected in both aquaponics systems, indicating that these functions were not affected by pH. As a non-renewable resource, phosphorus (P) presents one of the biggest obstacles to sustainable agriculture. Phosphonate dehydrogenase (PtxD) produced by microorganisms can catalyze the oxidization of reduced phosphonates through breaking the C-P bond and releasing phosphate (White and Metcalf, 2007). Production of this enzyme by microorganisms can enhance phosphorus metabolism by plants (Lie et al., 2021). Denitrification is also an important microbial activity in engineered systems, such as wastewater treatment plants, that balance fixed nitrogen in anoxic habitats (Sriwiriyarat et al., 2008). Due to the high DO concentration in aquaponic systems to support fish survival, denitrification may not be a major metabolic process in aquaponics, leading to low numbers of genes related to denitrification. However, the low abundances of these genes may also be attributed to an insufficient search engine to identify homologues, particularly for nitric oxide reductase (*nor*) genes.

A neutral to alkaline pH can lead to Fe concentrations in the system far below those required for the optimal growth of plants and microbes (Wortman., 2015). Microorganisms have evolved secretion mechanisms to mine Fe via solubilization, chelation and reduction processes (Sisó-Terraza et al., 2016). The quantity of genes encoding siderophores was nine times higher in the metagenomes of biocarriers and plant roots in the A2 than in the B2 aquaponic system. This

result indicates that iron deficiency is a challenge in aquaponic systems operating at higher pH values as high siderophore production indicates a need to make Fe more available for microbial growth.

4.6 Conclusion

Aquaponic food production systems must be developed with a thorough understanding of the composition and functions of microbial communities engaged in the water treatment process and to support plant growth. This study showed that despite the observation that comammox *Nitrospira* is present in both alkaline and acidic environments, pH is an effective parameter for the composition and diversity of this group, and distinct comammox species seem to prevail at different pH values. Genes related to the AOA *Nitrosocosmicus* species was also observed more frequently in B2 than the A2 system even though archaeal nitrifiers have not previously been identified as important contributions to aquaponics systems. AOA may be detrimental in aquaponics systems, particularly at lower pH, as they may increase nitrite buildup and create toxicity for plants and fish.

This study also demonstrated that siderophore production increased at higher pH levels to overcome iron insolubility. Iron deficiency, which has been found in numerous industrial systems, may be improved by the addition of probiotic bacteria with high siderophore synthesis.

Deeper analysis of the metagenome sequences along with statistical comparisons are needed to discover more information about the influence of pH on microbial metabolism in aquaponics, including PGPMs that were identified as major populations in Chapter 3. Future work on this and other aspects of bench scale aquaponics systems are discussed in the next chapter of this thesis.

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Chapter 5: Conclusions, future directions, and recommendations

5.1. Overall conclusions

Aquaponics systems are constructed ecosystems designed for the efficient farming of fish and crops. The three biotic compartments of these systems —fish, plants, and microorganisms— are interconnected and reliant on one another through the recirculation of water and flow of nutrients. Along with researching aquaponics microorganisms to enhance nitrogen use efficiency, bench scale mesocosm studies provide a tool for examining the potential influence of environmental factors, like pH and microbial inocula, on functional groups of microorganisms like nitrifiers and PGPM in larger scale systems. A major obstacle for increasing crop yields in aquaponics systems has been matching an optimal operating pH for nitrification activity with that for plant growth.

In Chapter 2, we found that greater than 80% of the nitrification activity of microbial biofilms can be retained by gradually reducing the pH of an adaptation medium that the carriers are exposed to over a 40 d period. In adapting the biofilm carriers from pH 7.6 to 5.6, the nitrifying microorganisms became preferentially enriched with comammox bacteria over AOB, AOA, and canonical *Nitrospira*. Using biofilters enriched in complete nitrifiers as inoculum for aquaponics have eco-friendly properties including less accumulation of toxic intermediates of nitrification, like hydroxylamine and nitrite, urea hydrolysis, and less N_2O production. Slow growth of comammox *Nitrospira* makes it challenging to start running systems at acidic pH without pre-adaption of biofilters. Creating an acidic pH-adapted microbial inoculum to seeds the aquaponics systems can speed up the cycle to complete. Comammox-enriched biofilm carriers pre-adapted to a slightly acidic pH can be used as inoculum for commercial scale

aquaponics facilities to obtain higher plant yield, high nitrification activity, reduced GHG production, and maintain high nitrogen use efficiency.

Chapter 3 of this study showed that despite differences in the abundance of particular phyla, the core microbiota across all aquaponics compartments tended to converge, regardless of operating pH and source of inoculum. The results suggest that groups of microorganisms with defined functions are supported by aquaponics operations that are selected for and maintained within a range of operating conditions.

This study also showed that while both comammox and AOB nitrifiers can be sustained in aquaponics systems, the lower pH systems favored comammox over AOB. Thus, decreasing the pH in systems with low abundances of comammox *Nitrospira* could result in transient accumulation of ammonia until the population can grow and stabilize, but also result in no nitrite accumulation and, as shown in Chapter 2, no N_2O production. Nevertheless, using bench scale systems with particular types of fish and plants makes it difficult to extrapolate results to large-scale systems. Thus, similar investigations using commercial scale aquaponics systems with different combinations of fish and plants is an important future work.

A lower pH also altered the composition of microbial communities in fish intestines, lettuce rhizospheres and the heterotrophic bacteria (especially PGPM) associated with the biofilters. However, the consequences of these microbial shifts on nutrient flow were inconsequential, and better plant growth was observed at the lower operating pH. The investigation using metagenomics in Chapter 4 was initiated to provide valuable information about other important microbes and their functions like those involved in iron and phosphorous availability, other N-cycle processes, the presence of pathogens, and detailed functions of PGPM in aquaponics systems.

The results from Chapter 4 showed that While comammox *Nitrospira* can thrive at pH 5.8 - 6 and 7.6-7.8, pH remains is a key determining factor for species distribution of this group. Certain comammox species seem to prefer a specific pH level. This study also showed that genes related to siderophore synthesis increased at pH 7.6-7.8, likely to overcome iron insolubility. Probiotic bacteria with high siderophore production have been added to treat iron shortage in industrial systems (Radzki et al., 2013). Deeper analysis of the metagenome sequences along with statistical comparisons are needed to discover additional information about the influence of pH on microbial communities and their functions in biofilters and lettuce roots from aquaponics systems. Future work will focus on identification and abundances of genes and microbial species that support plant growth (PGPM) in the metagenomes. In addition, the high-quality MAGs obtained from the biocarrier metagenomes will be analyzed in detail to describe potential functions of the associated microorganisms, particularly the MAG related to comammox *Nitrospira*.

5.2. Recommendations

This research demonstrated that pH is a strong determinant of nitrification activity and microbial community composition in aquaponics systems. Aside from nitrification, other microbial activities such as iron metabolism were also affected by pH. The following recommendations can be further explored:

- Study the effects of different combinations of fish and plants on microbial dynamics in aquaponics operating at different pH values;
- Test the comammox-enriched biofilters on nitrification rate and nitrogen balance in commercial-scale systems;

- Low NH_3/NH_4^+ ratio is most likely the cause of *Nitrospira* to overcome AOB at acidic pH. Certain strains of comammox bacteria use homologs of ammonium transporters of the Amt-type, which have a faster rate of ammonium uptake than the low affinity Rh-type ammonium transporters of AOB. As a result, they may be better able to compete in situations where ammonium is abundant compared to ammonia, such as lower pH. Considering the selectivity of ammonia monooxygenase for ammonia, more research is required to determine how comammox bacteria convert ammonium to ammonia for oxidation.

- Chapter 3 of this thesis showed that *Nitrospira* can grow on fish gills and plant roots. It is unclear whether the relation between fish and plants with *Nitrospira* is a transient relationship or a mutualism/commensalism symbiosis. Further genome comparison studies of gill- and root-residing strains of free-living *Nitrospira* are required for identifying differences in comparison to symbiotic *Nitrospira*.
- Chapter 4 of this thesis showed that genes related to siderophore synthesis increased likely to overcome iron insolubility at the higher operating pH. Future research can examine the effects of adding probiotic bacteria with strong siderophore production to treat iron deficiency similar to previous studies (Radzki et al., 2013).

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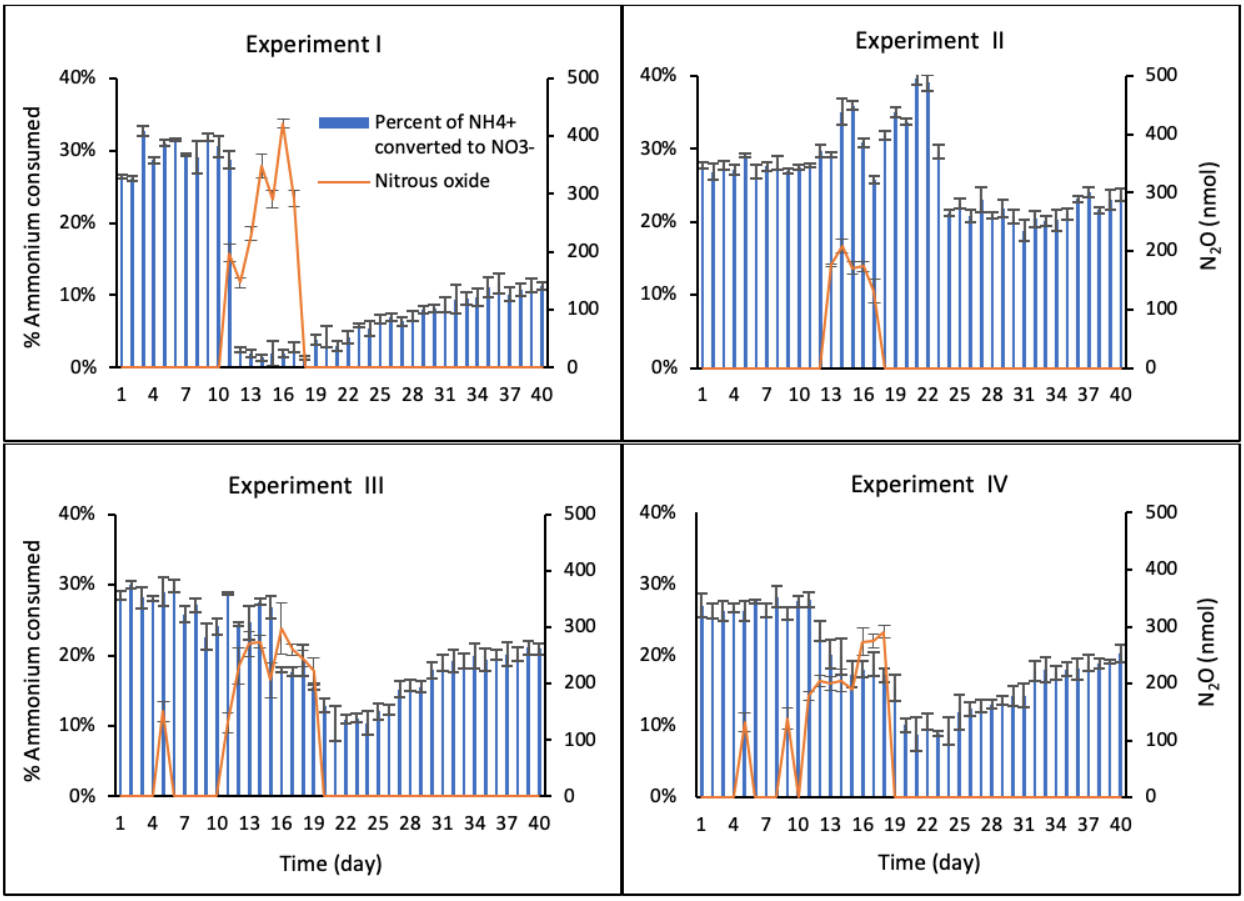
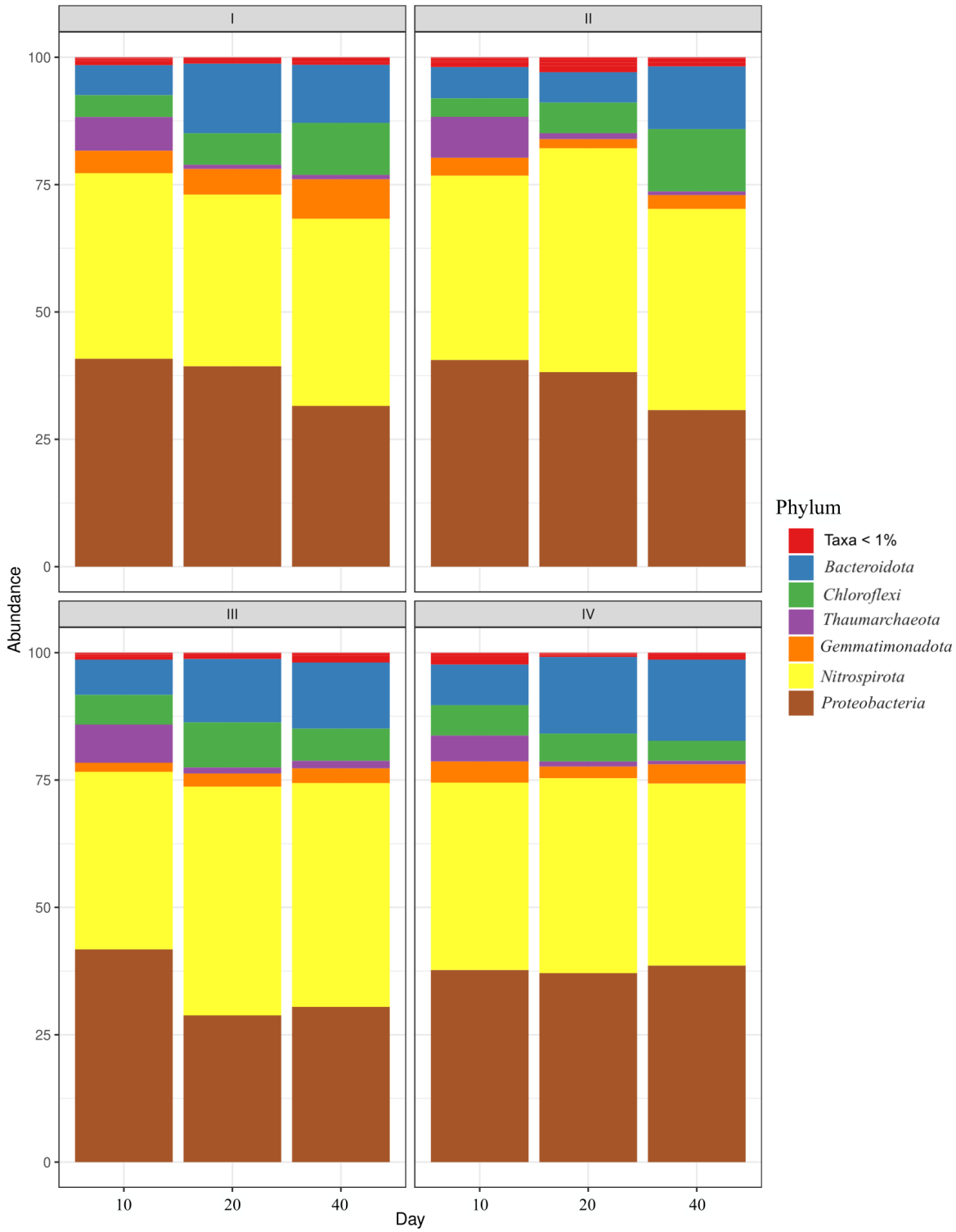
Appendices

Appendix A) Supplementary Material for Chapter 2



Supp. Figure A-1. Nitrification activity of microbial communities during adaptation from pH 7.6 to 5.6 in each of the four adaptation experiments (I-IV). In the first 10 days, the pH was kept at 7.6 for all Experiments. The percentage of ammonium (out of 1 mM for Experiments I, III, and IV; out of 1 mM for days 1-10 and 21-40 and 0.1 mM days 11-20 for Experiment II) oxidized to nitrate (blue bars) and the amount of N 2 O (orange line) measured in the gas headspace was measured at the end of each 24 h incubation.



Supp. Figure A-2. Changes in microbial community composition on days 10, 20 and 40 based on ASV analysis in each of the four adaptation experiments. ASVs were determined at the phylum level.

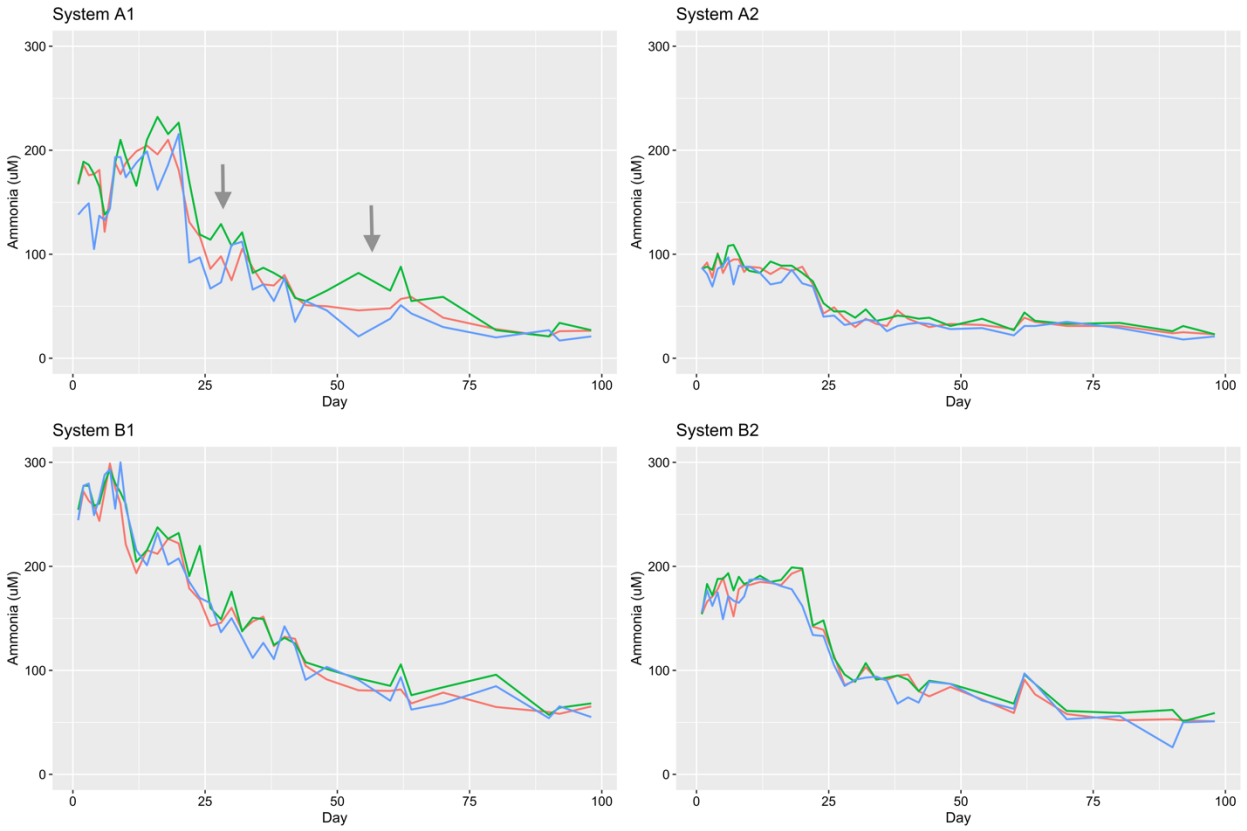
Supp. Table A-1. Richness (Chao1) and diversity (Shannon and Simpson) of biofilm and water samples before and during adaptation experiments.

Sampling day	Adaptation type	Chao1		Shannon		Simpson	
Original biofilters	-	112.6		4.02		0.81	
Water sample	-	108.1		3.63		0.82	
10	I	109.93	112.25	2.8	2.80	0.86	0.87
	II		112.00		2.80		0.87
	III		106.25		2.73		0.86
	IV		109.25		2.87		0.87
20	I	94.4	99.00	2.83	2.81	0.84	0.85
	II		103.00		3.02		0.87
	III		97.60		2.78		0.82
	IV		98.00		2.74		0.83
40	I	83.5	75.00	2.77	2.76	0.85	0.85
	II		99.00		2.92		0.87
	III		87.00		2.81		0.84
	IV		73.00		2.61		0.84

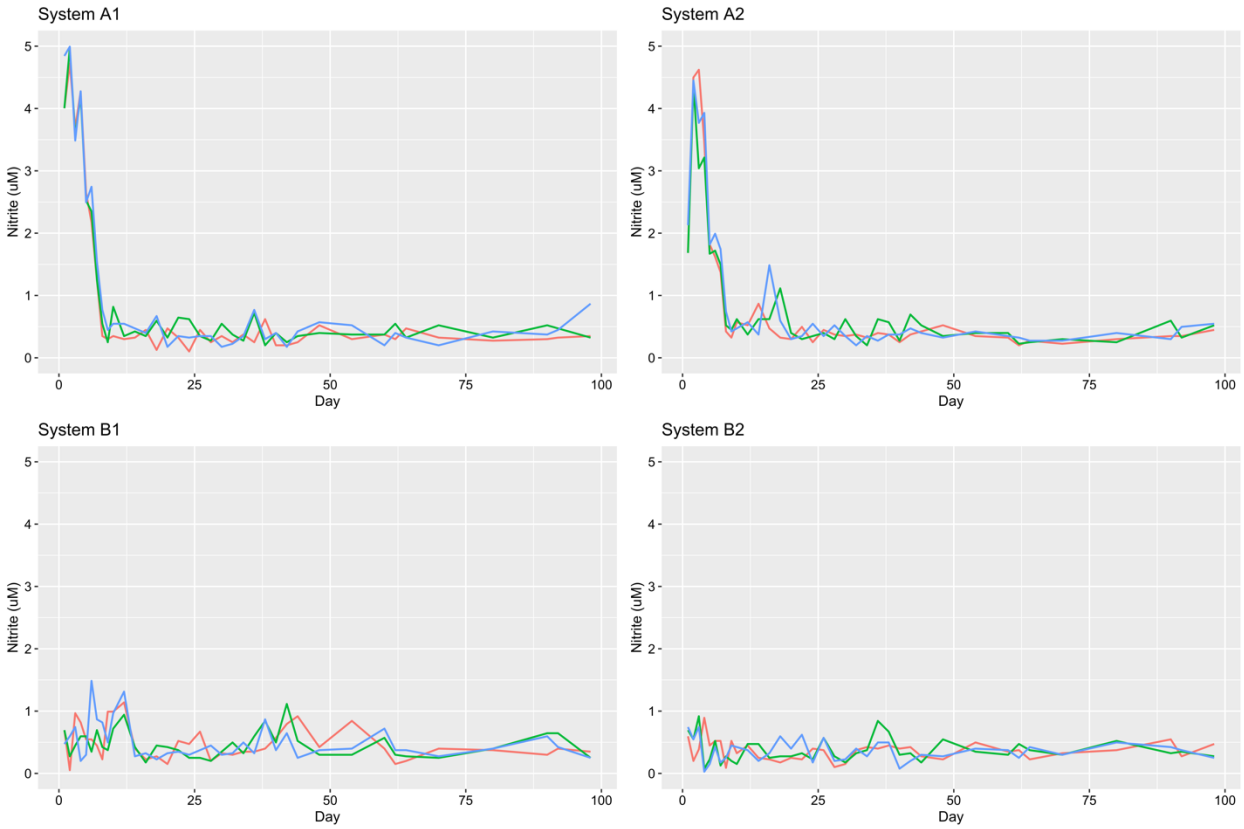
Supp. Table A-2. Relative abundance of nitrifying microorganisms as determined by 16S rRNA gene amplicon analysis. Shifts in the quantity of Nitrosomonas and Candidatus-Nitrosocosmicus (Genus level) and Thaumarchaeota (Phylum level) amplicons from day 10 to day 20 were statistically significant (Wilcoxon rank sum test, P<0.05).

		Relative abundance (%)					
		Phyla			Genus		
Sampling day	**Exp. Variation**	**Proteobacteria**	**Nitrospirota**	**Crenarchaeota***	***Nitrosomonas****	***Nitrospira***	**Candidatus-*Nitrosocosmicus****
10	I	40.81	36.42	6.64	25.90	40.52	7.52
	II	40.58	36.18	8.05	26.17	40.11	10.84
	III	41.76	34.84	7.52	28.73	38.47	10.09
	IV	37.71	36.78	5.09	23.94	40.05	5.51
20	I	39.36	33.69	1.09	1.64	39.88	1.29
	II	38.19	43.94	1.35	7.35	52.17	1.31
	III	28.79	44.92	1.36	3.26	53.35	1.11
	IV	37.09	38.27	1.27	3.11	47.33	1.17
40	I	31.55	36.75	1.23	3.89	42.07	1.22
	II	30.73	39.51	1.09	9.18	48.19	1.46
	III	30.47	43.94	1.45	8.41	53.29	2.17
	IV	38.58	35.74	1.07	3.61	43.61	1.02

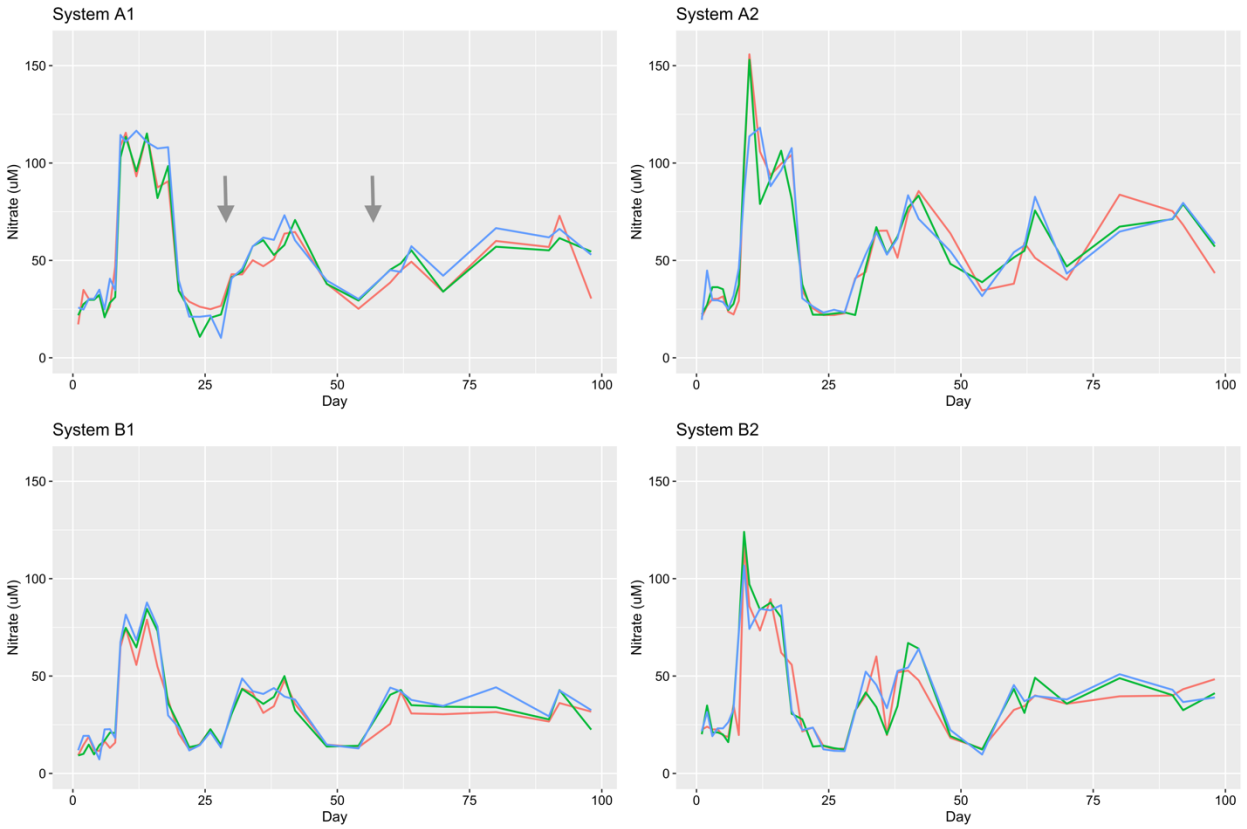
Appendix B) Supplementary Material for Chapter 3



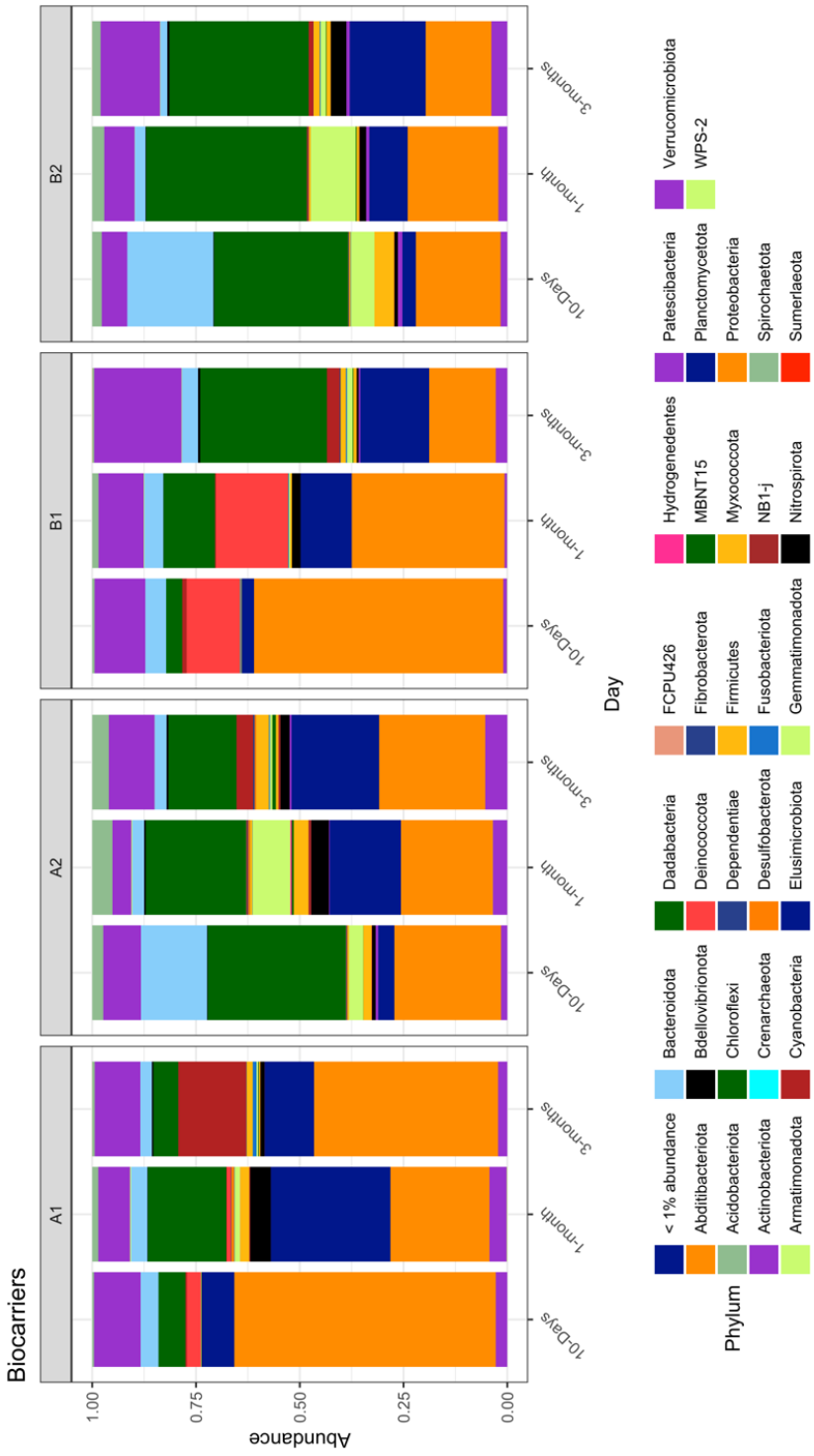
Supp. Figure B-1. Variation of ammonia concentrations in four different aquaponics systems over three plant harvesting rounds. Arrays indicate harvesting time points. Green: 30 min, red: 2 h, and blue: 5 h after feeding.



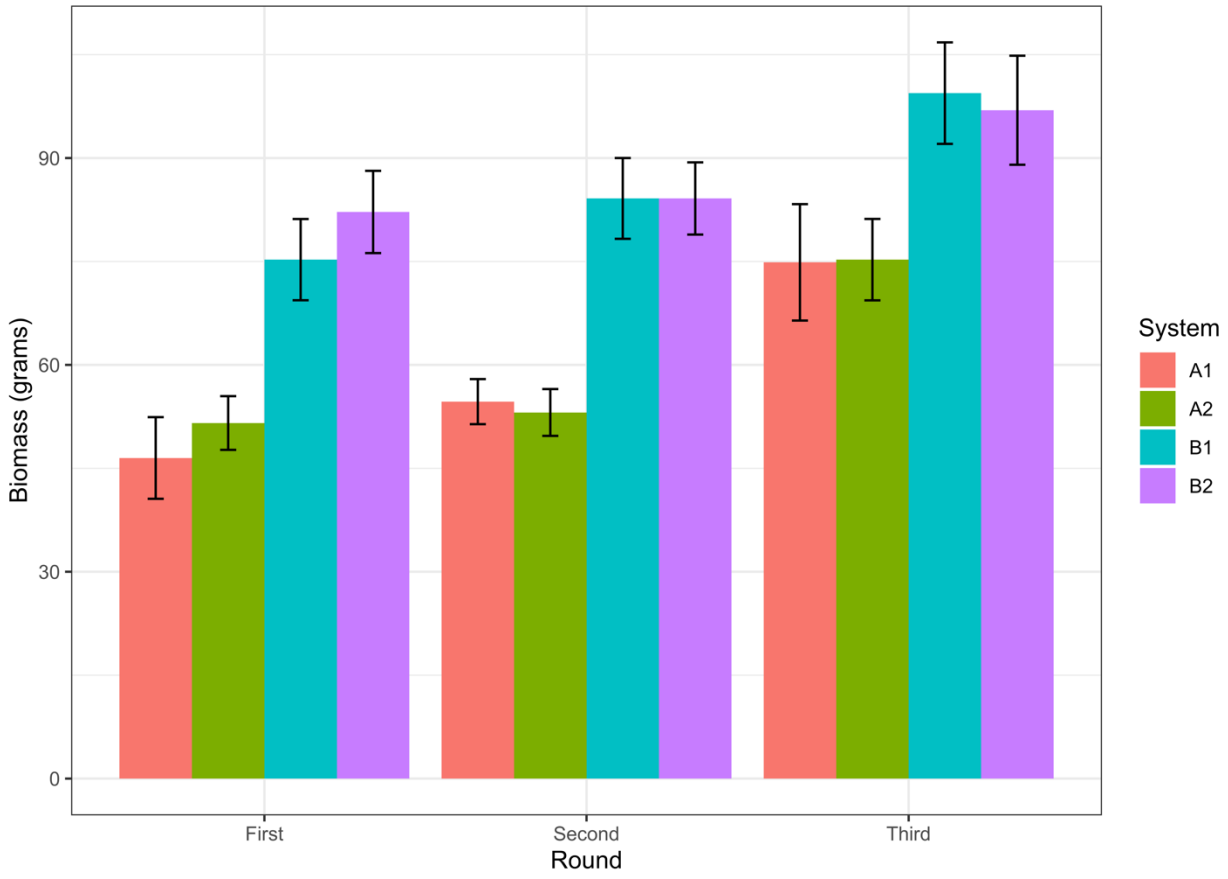
Supp. Figure B-2. Variation of nitrite concentrations in four different aquaponics systems over three plant harvesting rounds. Green: 30 min, red: 2 h, and blue: 5 h after feeding.



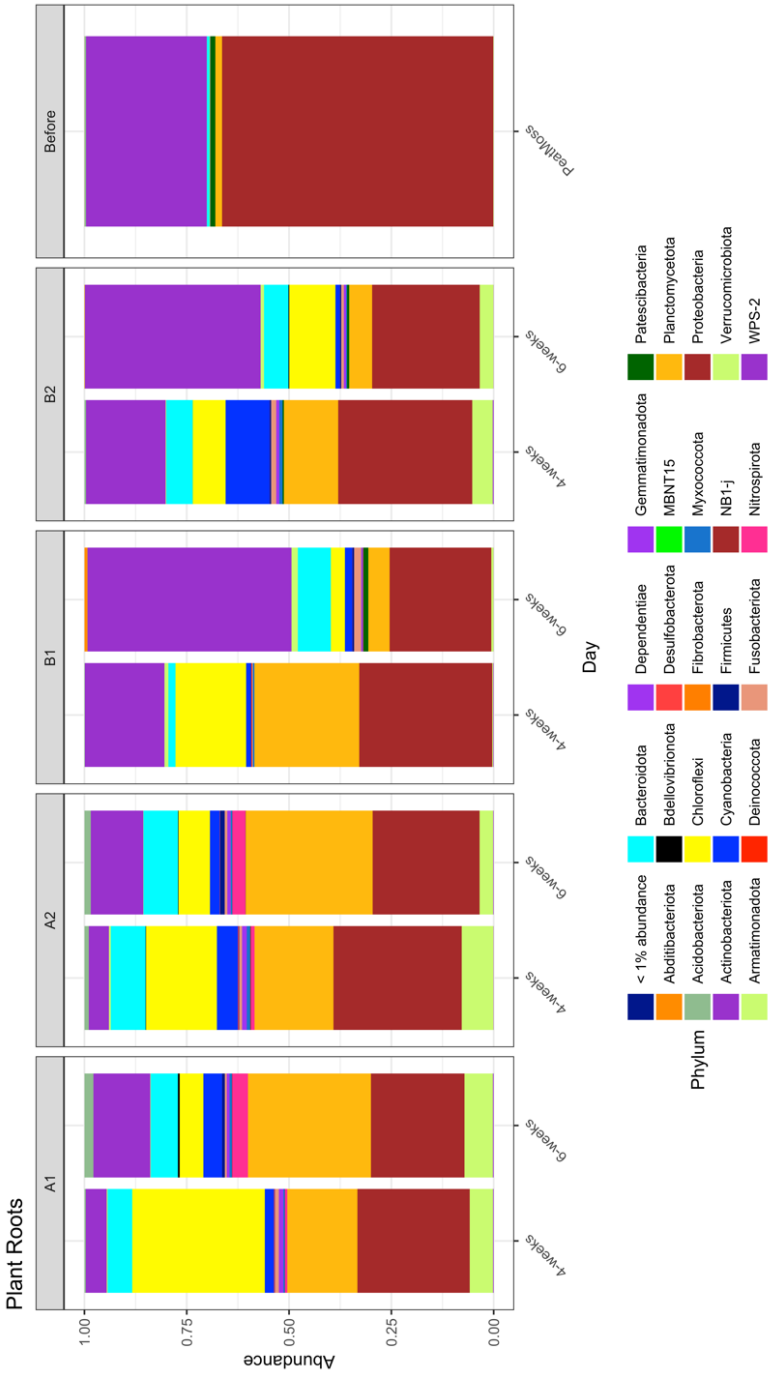
Supp. Figure B-3. Variation of nitrate concentrations in four different aquaponics systems over three plant harvesting rounds. Arrays indicate harvesting time points. Green: 30 min, red: 2 h, and blue: 5 h after feeding.



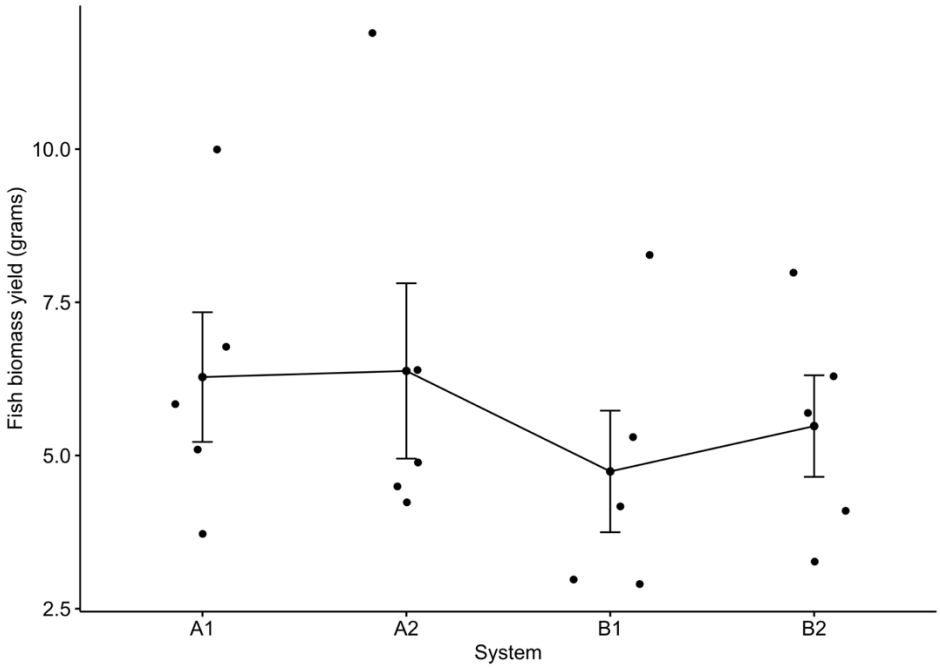
Supp. Figure B-4. Changes in microbial community compositions, 10 days, one month and three months after aquaponics operation based on ASV analysis. ASVs were determined at phylum level. Only phyla representing more than 1% of the total reads are presented.



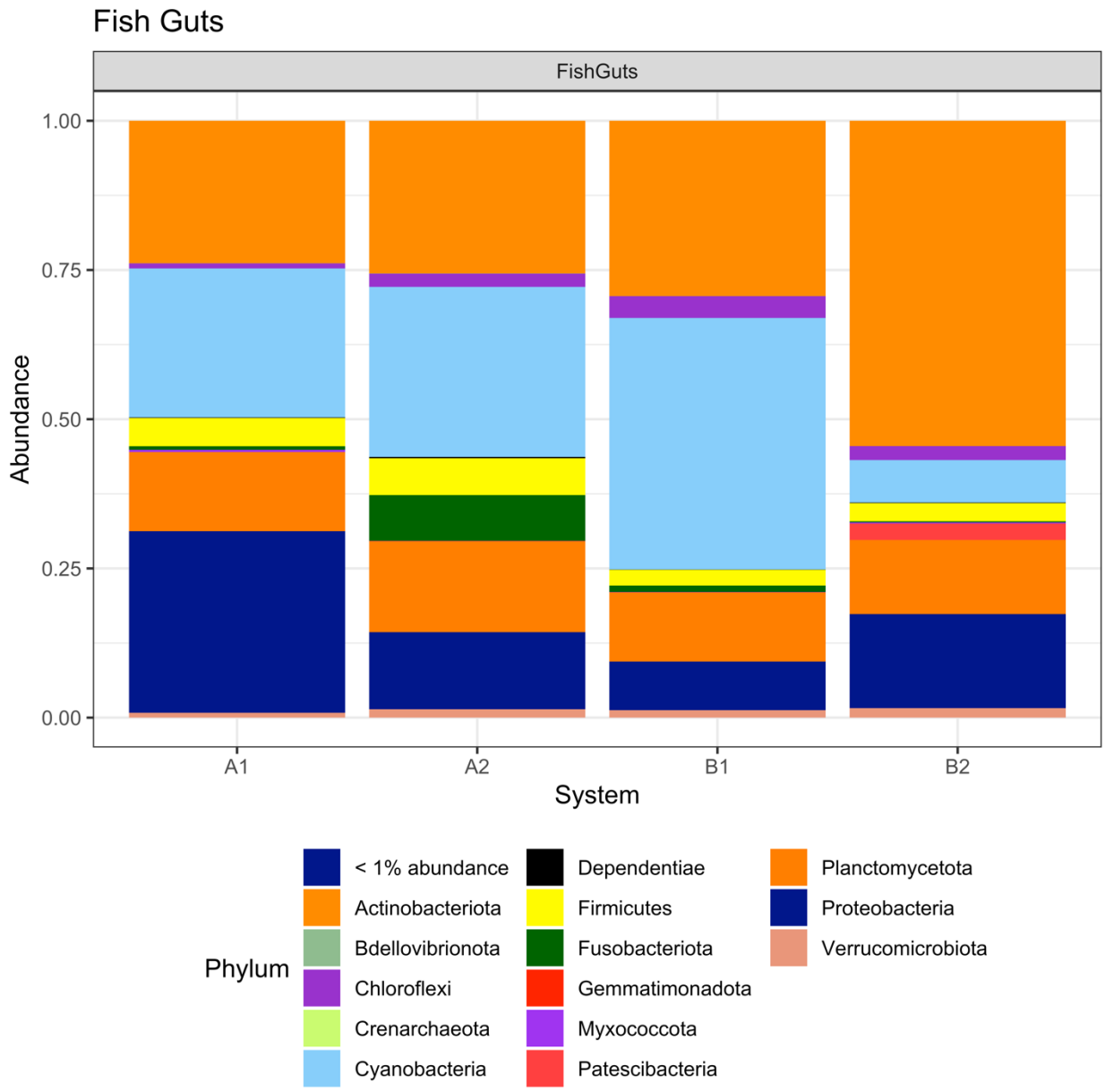
Supp. Figure B-5. Wet biomass of lettuce at three harvesting rounds from four aquaponics systems.



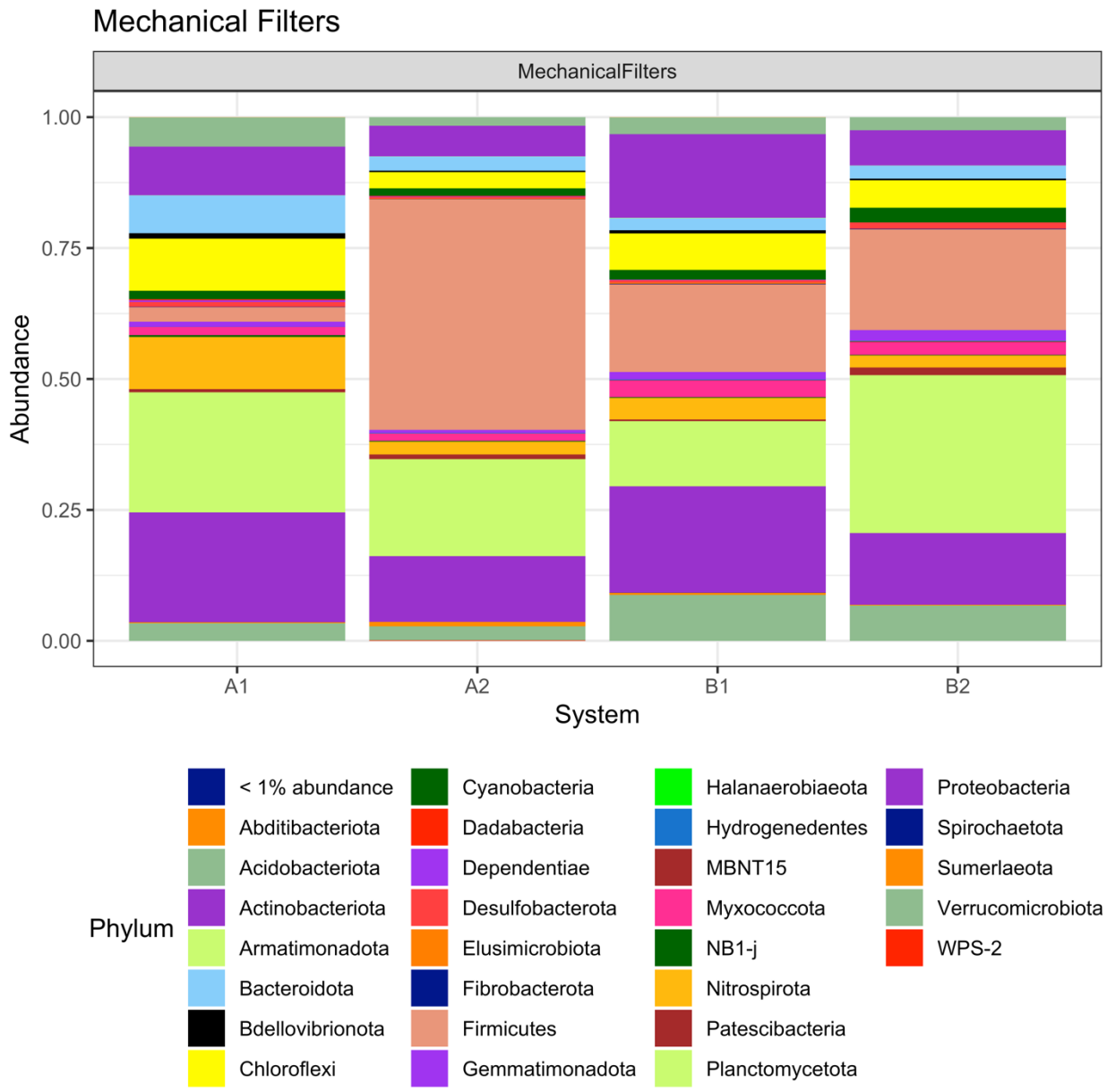
Supp. Figure B-6. Overview of the taxonomic profile of lettuce roots over time at phylum level. Only phyla representing more than 1% of the total reads are presented.



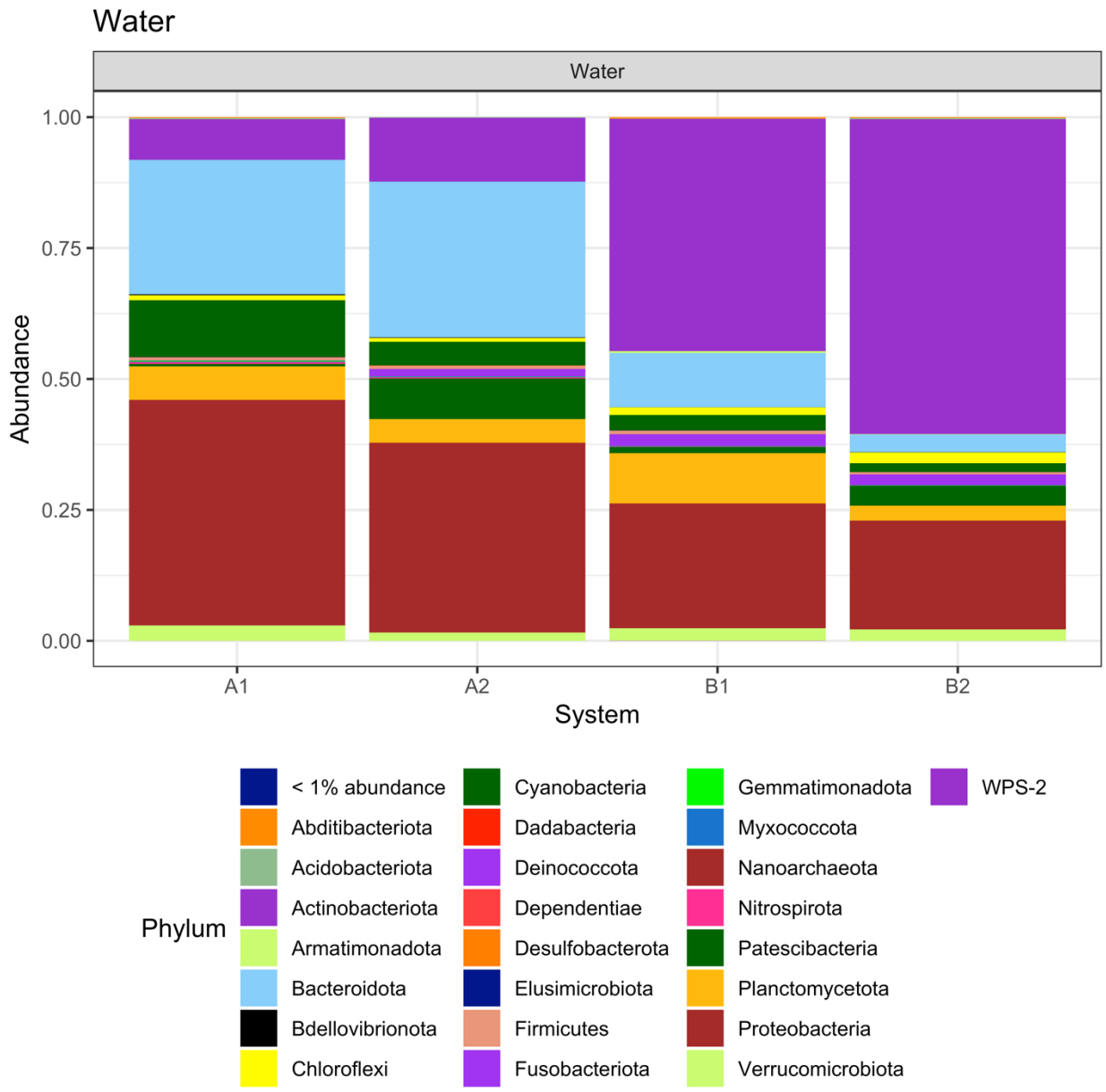
Supp. Figure B-7. Fish biomass yields of four aquaponics systems.



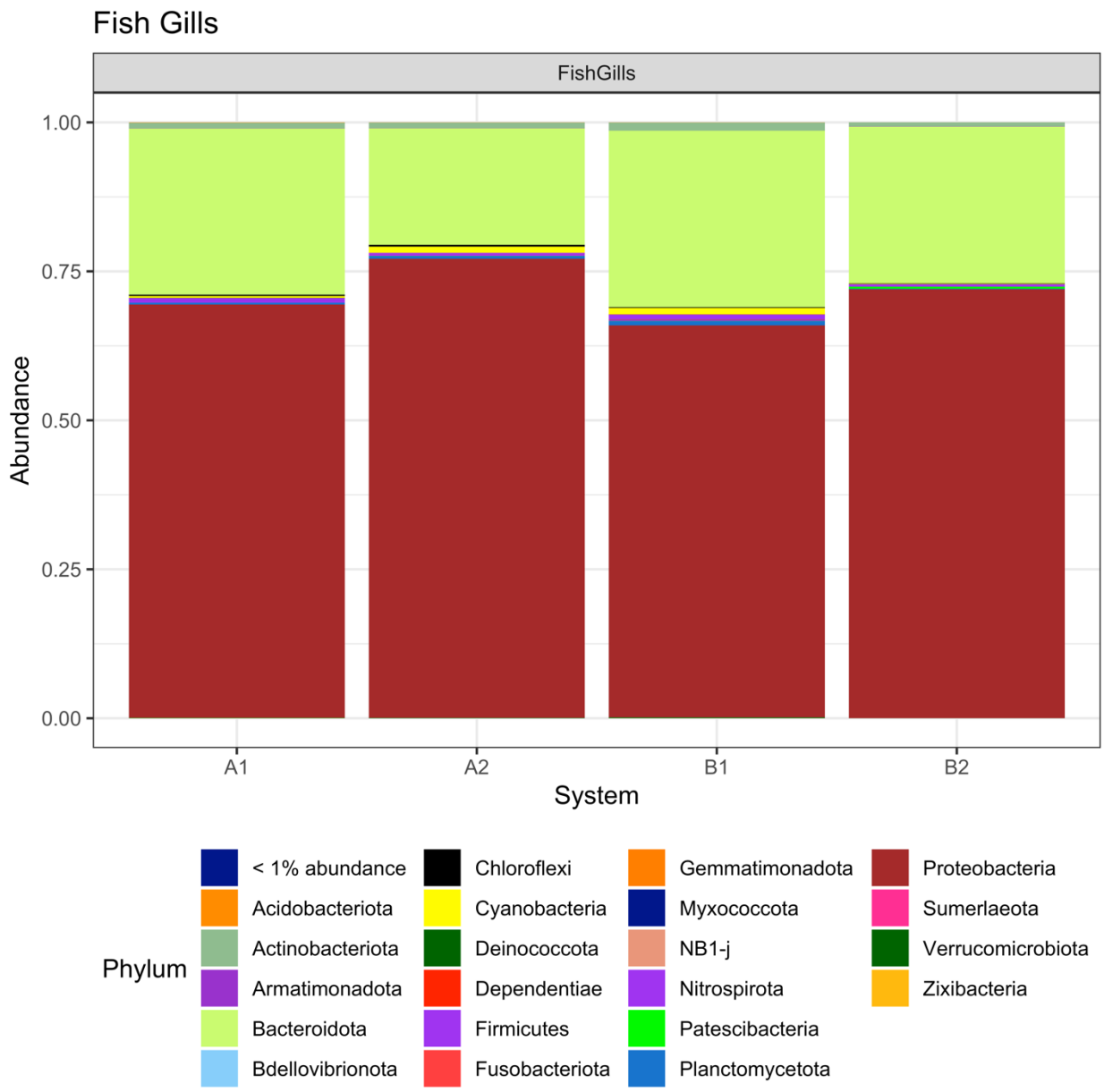
Supp. Figure B-8. Classification of reads from fish intestine samples to phylum indicated as percentage of populations.



Supp. Figure B-9. Bar charts of phylum level abundances of microbial communities of samples collected from mechanical filters.

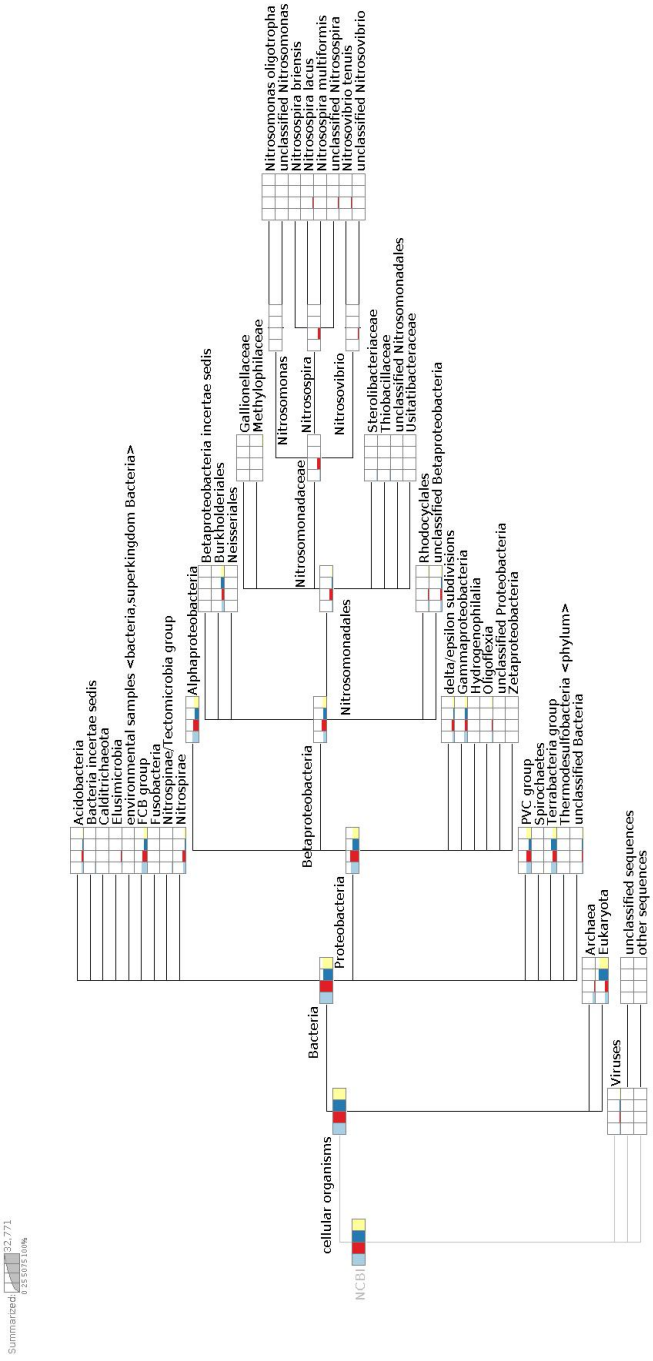


Supp. Figure B-10. Microbial community composition of water samples at phylum level.

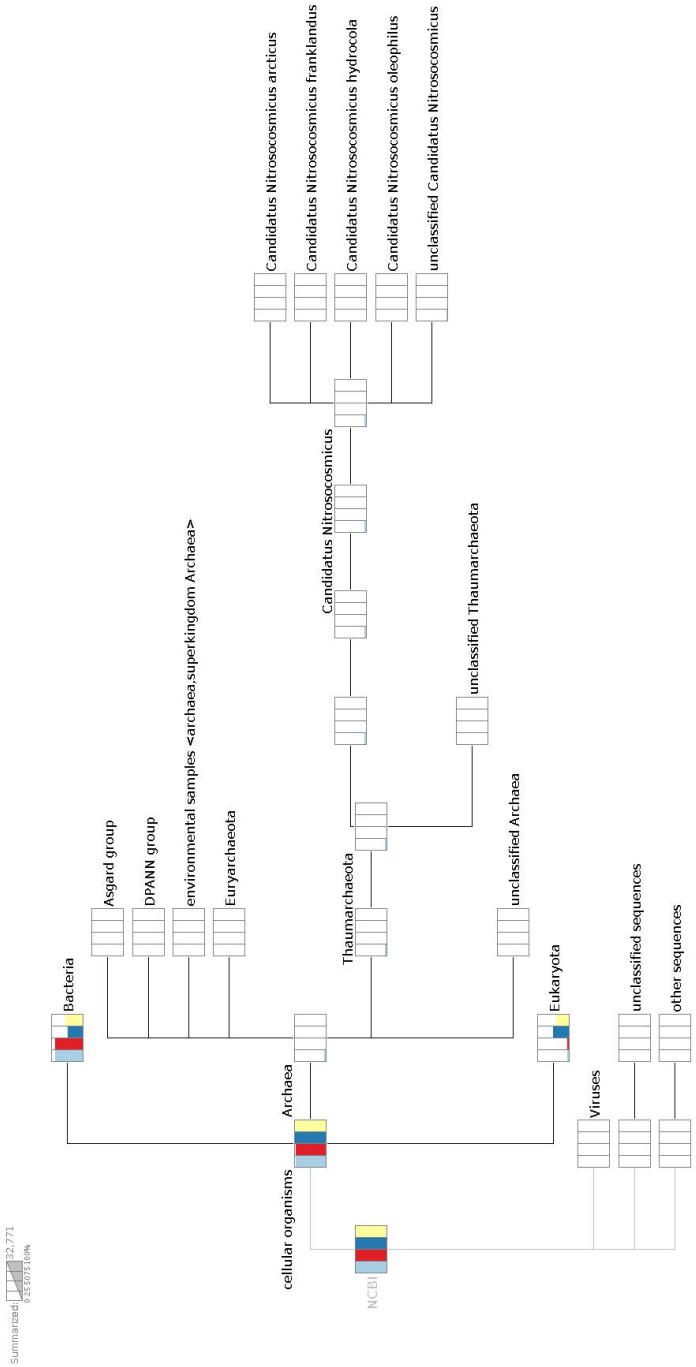


Supp. Figure B-11. Bar plots representing the relative abundances of phyla from fish gill samples.

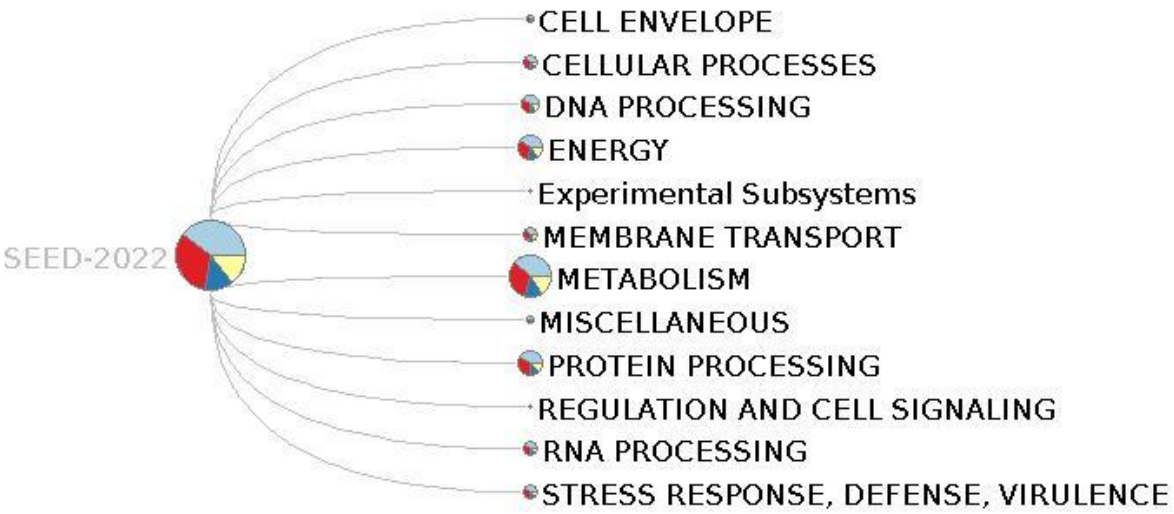
Appendix C) Supplementary Material for Chapter 4



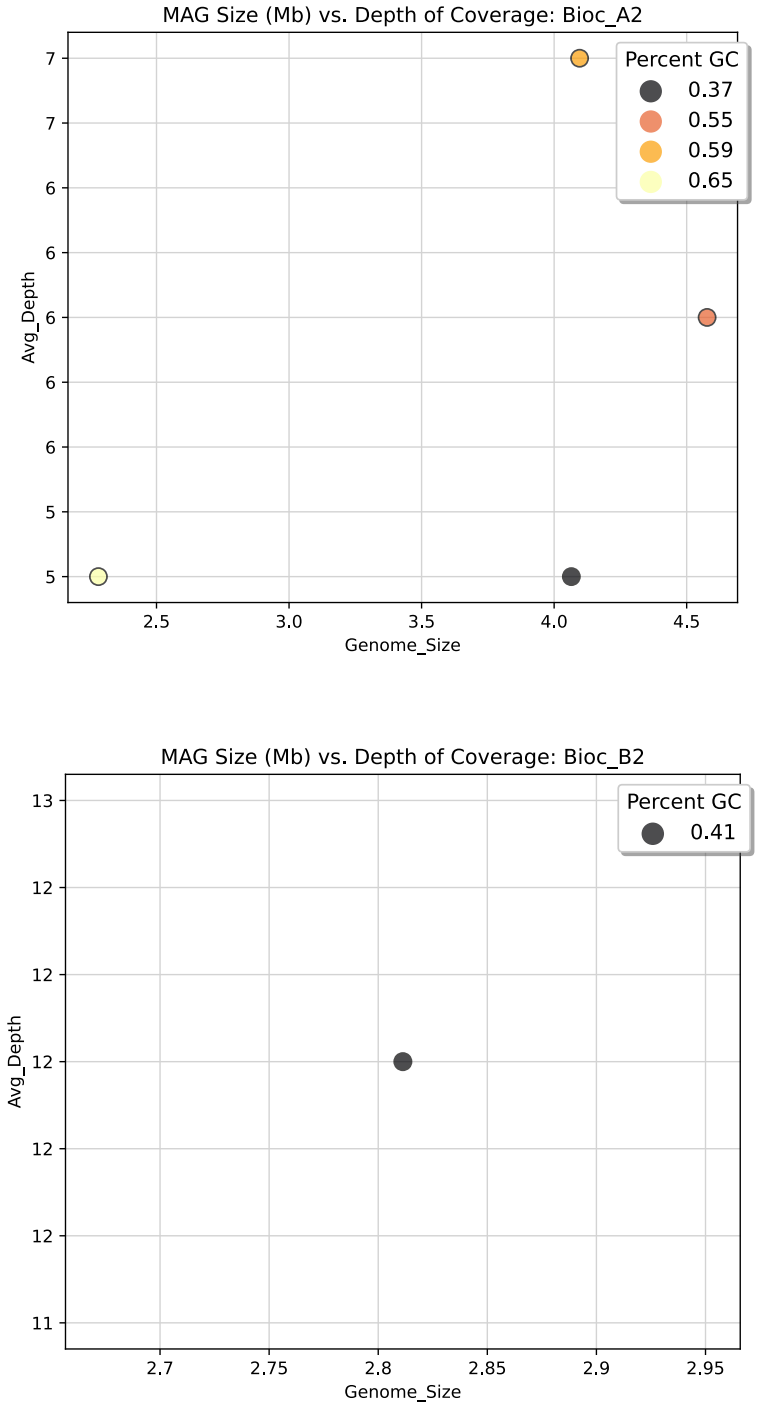
Supp. Figure C-1. Phylogeny of the HiFi reads assigned to ammonia oxidizing bacteria (cyan: Biocarriers pH 7.6, red: Biocarriers pH 6, blue: Roots pH 7.6, yellow: Roots pH 6).



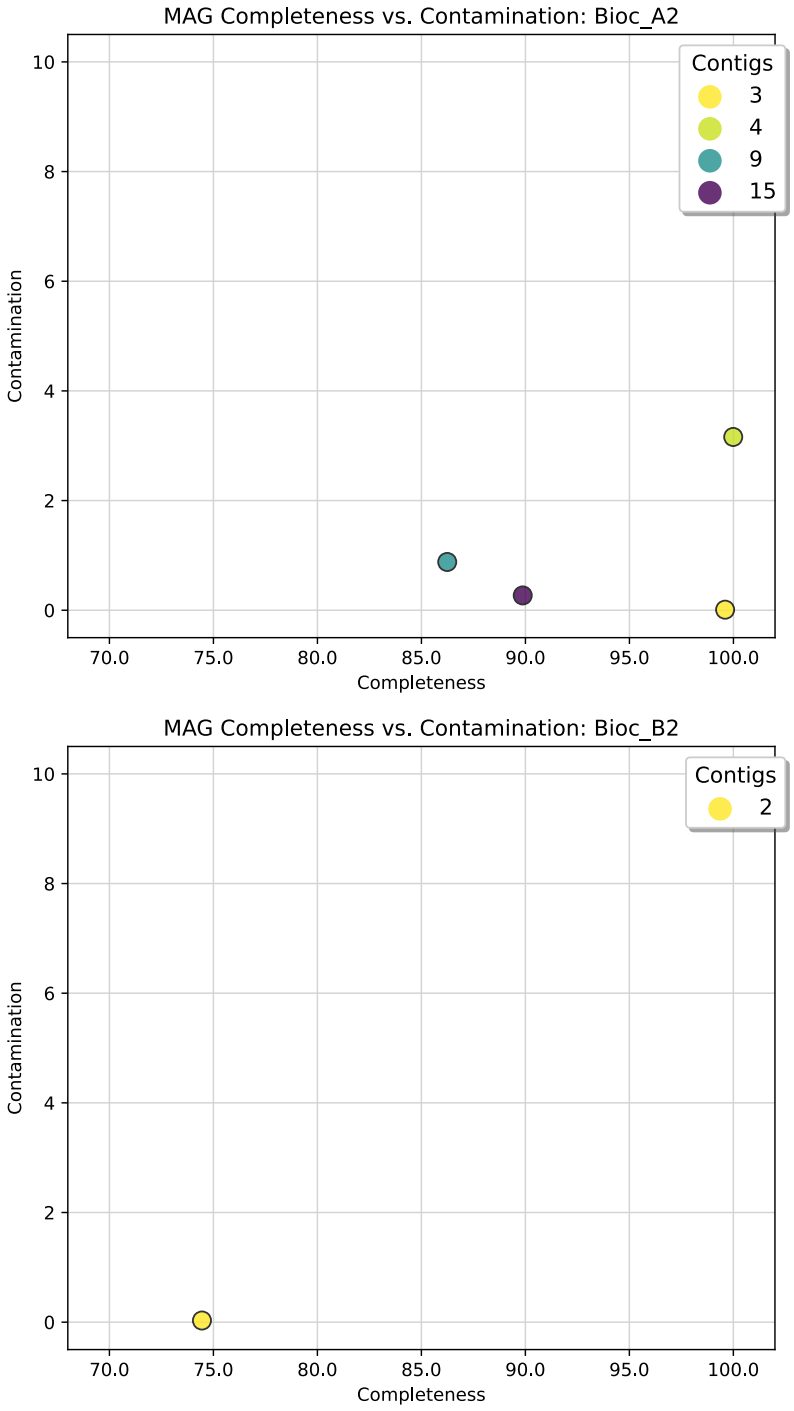
Supp. Figure C-2. Phylogeny of the HiFi reads assigned to ammonia oxidizing archaea (cyan: Biocarriers pH 7.6, red: Biocarriers pH 6, blue: Roots pH 7.6, yellow: Roots pH 6).



Supp. Figure C-3. Subsystem proportion of HiFi reads from SEED annotation of the metagenomes (cyan: Biocarriers pH 7.6, red: Biocarriers pH 6, blue: Roots pH 7.6, yellow: Roots pH 6).



Supp. Figure C-4. MAG characteristics representing genome size versus depth of coverage scores for high quality MAGs. Each circle represents a MAG and colors indicate GC contents; top: Biocarriers pH 7.6, and bottom: Biocarriers pH 6.



Supp. Figure C-5. MAG characteristics representing completeness versus contamination scores for high quality MAGs. Each circle represents a MAG and colors indicate number of contigs in each MAG; top: Biocarriers pH 7.6, and bottom: Biocarriers pH 6.

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