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Foodborne Pathogens and Water Quality in Commercial-Scale Aquaponic Systems with Rapid Micro Screen Solids Removal

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FOODBORNE PATHOGENS AND WATER QUALITY IN COMMERCIAL-SCALE AQUAPONIC SYSTEMS WITH RAPID MICRO SCREEN SOLIDS REMOVAL

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Approval signatures are on file with the University of New Hampshire Graduate School.

Dedication

To Harry, without whom

this undertaking would have been

neither possible nor worthwhile.

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Abstract

To meet global nutritional needs, humans need to produce an ever-increasing amount of food with dwindling natural resources. Demand for nutritious foods, particularly lean meat, fruits, and vegetables, is rapidly accelerating. To increase the intensity of production and utilize non-arable land, many growers are turning to controlled environment agriculture systems like hydroponic crop production and aquaculture (fish farming), but these are inherently inefficient in terms of water and nutrient utilization. Aquaponics is a relatively novel agricultural method that combines hydroponic and aquacultural production models to mitigate many of the costs—both realized and implicit, environmental and economic—associated with either production model on its own. Fundamental research is needed to determine optimal system designs, water quality conditions, and potential food safety considerations specific to aquaponics. Here we demonstrate the operation of replicated, greenhouse-scale aquaponic systems with a novel design growing tilapia (*Oreochromis* spp.) and lettuce (*Lactuca sativa* L.) in continuous production. Nitrification efficiency in these systems was greater than in previous studies, and water quality conditions suggested a relationship between the system design, dissolved carbon/nitrogen ratio, nitrogen use efficiency, and environmental impact through emission of N oxides. Food safety risk was evaluated in these aquaponic systems through culture-based screening of production water for *E. coli*, *Salmonella* spp., and *Listeria* spp. None of these indicator organisms were detected, in accordance with most of the aquaponic food safety research to date. However, taxa containing less-common zoonotic and opportunistic human pathogens were observed, including *Aeromonas hydrophila*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Citrobacter freundii*, and *Providencia* spp. Pathogenicity of the observed strains was not determined, but the persistent presence of these taxa suggests that aquaponics presents a unique environment in

terms of potential food safety hazards. With increasing regulation of food production environments in the United States, European Union, and United Kingdom, understanding the hazards in aquaponics will be necessary to ensure fair and effective water quality standard enforcement. Our results provide baseline physicochemical and microbial water quality data for a novel aquaponic system design with the potential to improve efficiency and food safety risk. This information will help to inform future research and commercial system designs and the development and enforcement of regulatory microbial water quality standards.

Chapter 1: Introduction

Aquaponics is a food production model that combines two types of controlled environment agriculture (CEA)—aquaculture and hydroponics. This integrated approach has the potential to mitigate several of the negative externalities and direct costs of both aquaculture and high-value crop production. Research interest in aquaculture, hydroponics, and aquaponics has grown rapidly in the previous decade (Figure 1), while at the same time population growth has fueled an ever-increasing need for nutritious foods including fish, fruits, and vegetables. To assist with the burgeoning development of the aquaponic industry, fundamental research is needed in several aspects of system design, management, and food safety.

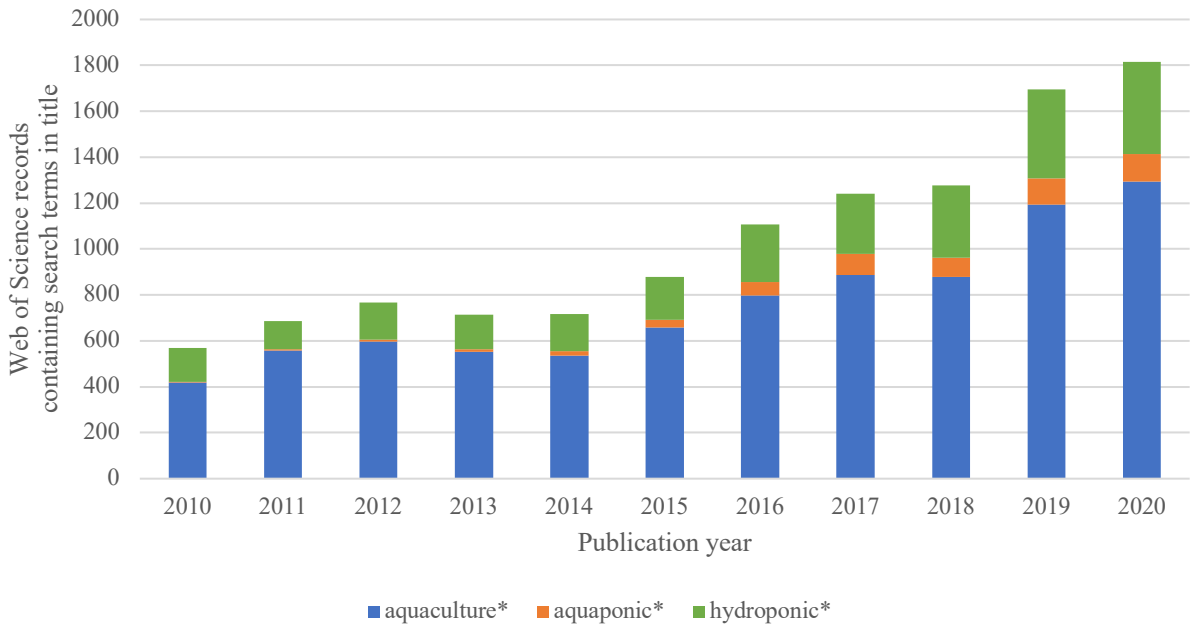

Figure 1. Research interest in aquaculture, hydroponics, and aquaponics, measured by the number of published article titles indexed by Web of Science that contained those root terms. Search conducted in April 2021.

State of global human nutrition

We are a world in nutritional crisis. More than 3 billion people cannot afford a healthy diet, and 229 million children under 5 experience malnutrition (FAO, IFAD, UNICEF, WFP and WHO, 2020). Nearly fifteen percent of infants are born underweight, and stunting—being too short for one's age—affects 144 million children under 5. Stunting is associated with significant impacts to health and economic welfare over the lifetimes of those afflicted (FAO, IFAD, UNICEF, WFP and WHO, 2020). Meanwhile, child overweight and adult obesity are on the rise in all regions of the world except Africa, where indicators of child overweight prevalence are on track to meet 2025 targets for the United Nations (UN) Sustainable Development Goals (SDGs) (FAO, IFAD, UNICEF, WFP and WHO, 2020). Following the Green Revolution of the mid-20th century, the focus of food security interventions was on supplying calories to meet dietary energy needs (FAO, 2018). The Green Revolution fueled growth in agricultural yields through innovations in synthetic fertilizers and pesticides, along with staple crops bred to grow optimally in monoculture with these new chemical agents (Glaeser, 2011). But the indicators of global nutrition status—especially stunting and obesity—have illuminated deficiencies in our ability to distribute calories equitably and generate enough healthy food for the population.

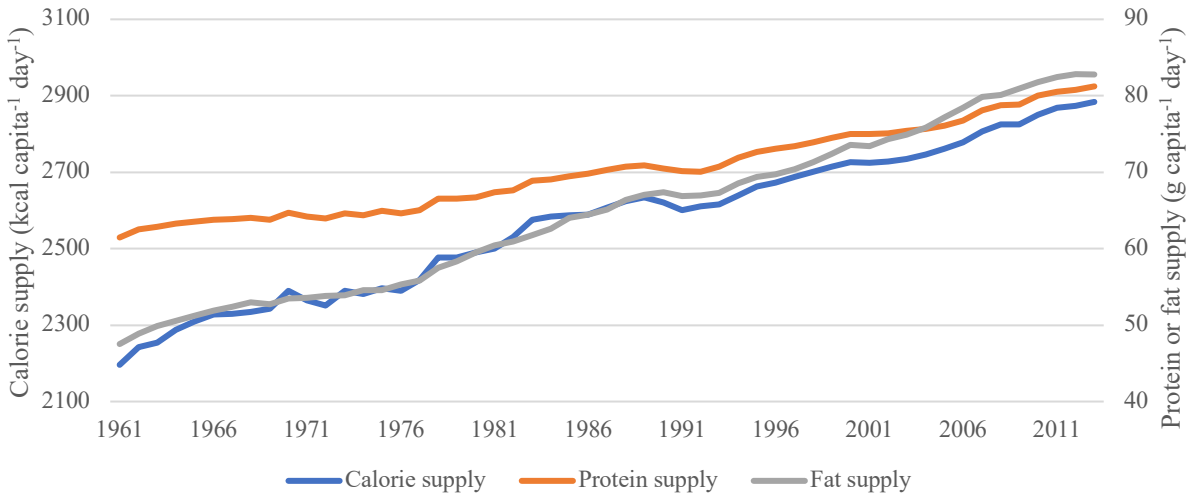

Figure 2. Calorie, fat, and protein supply per capita from 1961 to 2013. (FAO, 2018)

The composition of a healthy diet for any one person will vary depending on their location, culture, and access to ingredients. The UN Food and Agriculture Organization (FAO) recognizes 4 model healthy diets that also satisfy sustainability goals: flexitarian, pescatarian, vegetarian, and vegan. One common theme of these model diets when compared to actual food consumption is that they call for increasing intake of a diversity of vegetables, fruits, and lean protein, while reducing intake of processed foods, sugars, and saturated fats from foods like red meat and dairy (FAO, IFAD, UNICEF, WFP and WHO, 2020). Making these changes, if we choose to, will require a reversal of food macronutrient trends of the last 50 years with structural shifts in the makeup and outputs of global food production systems (Figure 2). The efforts that have led to rapidly increasing calorie and fat availability—in the forms of grains, roots, tubers, and oil crops—should be supplanted by efforts to more efficiently produce nutrient-dense crops (fruits and vegetables) and lean protein. Nutritional quality of foods will be the true indicator of agricultural productivity, inasmuch as agriculture serves the primary purpose of nourishing people.

Addressing global nutritional deficits on the production side of agriculture

FAO and the World Health Organization (WHO) recommend a minimum daily intake of 400 g of fruits and vegetables (FAO, IFAD, UNICEF, WFP and WHO, 2020). Estimates of the availability of fruits and vegetables in different regions and country-level income groups reveal that this amount is only available in Asia and in upper-middle-income countries (Figure 3). While the world average availability of 390 g in 2017 approached the nutrition-based target, this does not ensure that adequate amounts of nutrient-dense foods are equitably distributed or accessible. In low-income countries and in Africa, availability of these foods is less than half of the recommended intake (FAO, IFAD, UNICEF, WFP and WHO, 2020).

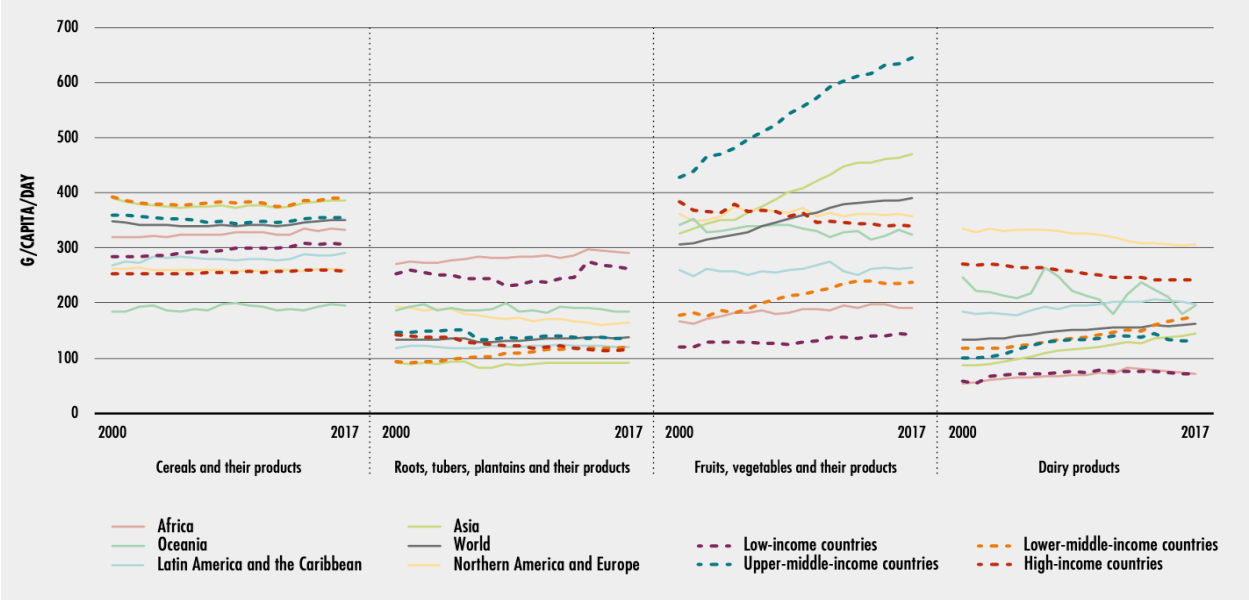

Figure 3. Edible supply of plant-based food groups and dairy from 2000 to 2017, by region and national income level. Adapted from FAO, IFAD, UNICEF, WFP, and WHO (2020).

Of the common vertebrate food animals, fish are the most efficient at converting feed into biomass (Fry et al., 2018). To increase the global availability of animal protein most efficiently, fish production should therefore be prioritized over other animal agriculture. The global production of fish has steadily increased since 1980 to a 2018 total of 178.5 million metric tons (FAO, 2020). All of this increase has been driven by less-developed countries (LDC), while fish production by developed countries has shrunk since 1980 (Figure 4). Production in LDC over the same period has increased through both wild capture and aquaculture, or fish-farming, but aquaculture in particular has risen at an average annual rate of 65%. Inland production of finfish represents the largest segment (57.2%) of this fast-growing sector (FAO, 2020). The value of the global trade in fish has grown more rapidly than actual production since 2000 to a 2018 total of $325 billion USD, reflecting increasing demand despite obstacles to increasing production (Figure 4).

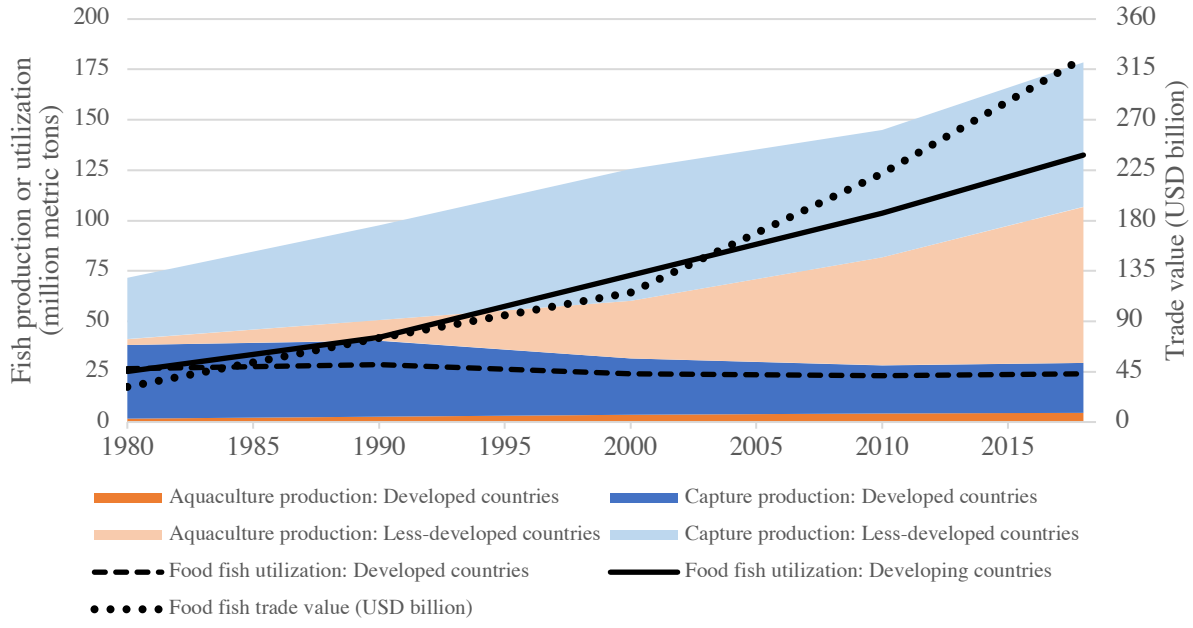

Figure 4. Global food fish production, utilization, and trade value from 1980 to 2018, by development category. Data from FAO (2020).

The world does not produce enough fish to meet dietary guidelines, even in relatively wealthy countries. Currently in the US, about 4.5 ounces (128 g) of fish is available and consumed per capita per week, while the USDA Dietary Guidelines for Americans recommends a weekly fish intake of 8 ounces (227 g) (US Department of Agriculture and US Department of Health and Human Services, 2020). Increasing fish supply in wealthy countries could increase food insecurity in lower-income countries and coastal communities by placing more of a burden on both marine ecosystems and international markets. The elasticity of demand for meats, fruits, and vegetables is greater than that for breads and cereals, indicating that people are more likely to forgo meats, fruits, and vegetables if their price is too high (FAO, IFAD, UNICEF, WFP and WHO, 2020). This means that increased demand for healthier foods in developed countries, as people attempt to address health issues through food choices, may engender food insecurity in LDC. As the growth in production from wild marine fisheries continues to slow, the primary way to generate an increasing supply of fish will be through aquaculture. Current trends suggest that inland rearing of finfish will continue to provide the largest segment of this growth needed to satisfy rapidly increasing demand (FAO, 2020). To promote equity of food access, this increase in terrestrial aquaculture should be globally distributed.

Obstacles to future growth in production of healthy foods

Over the coming decades, climate change will present increasing challenges for all agricultural systems, including those that produce the supply of nutrient-dense fruits and vegetables and lean animal proteins like fish. Of these challenges, fresh water supply is perhaps the most pressing, as reflected by the theme of the FAO 2020 State of Food and Agriculture report: “overcoming water challenges in agriculture.” Current production systems for terrestrial

meat animals utilize thousands of liters of water throughout the production process per kilogram of animal raised (Mekonnen & Hoekstra, 2012), while some conventional aquaculture systems use even more (Figure 5) (Verdegem et al., 2006). On the other end of the water footprint spectrum, intensive recirculating aquaculture systems (RAS) utilize about 10 times less water than chicken and cricket production, the least water-intensive major terrestrial food animals (Goddek et al., 2019). Of existing flesh food production models, RAS is by far the most water efficient.

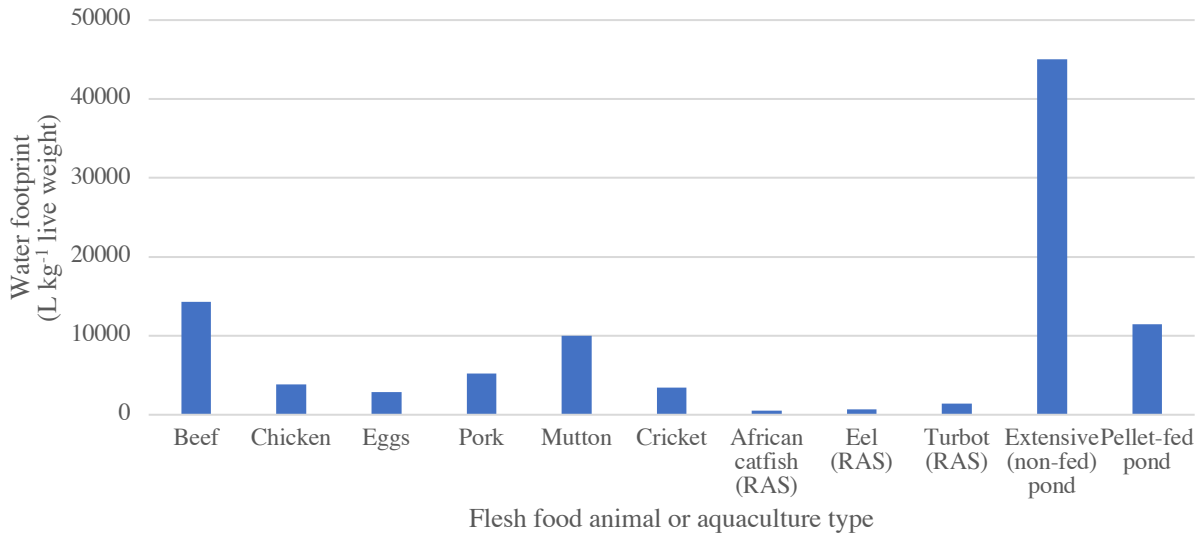

Figure 5. Water footprints for flesh food animals in conventional terrestrial production systems and aquaculture. Values include fresh water utilized throughout the production chain, from feed production through harvest. Data from Goddek et al. (2019), Mekonnen & Hoekstra (2012), and Verdegem et al. (2006).

Another major challenge for agriculture is the inefficient utilization of non-renewable nutrient resources, including nitrogen. Nitrogen use efficiency (NUE) in primary production systems describes the proportion of input N that is assimilated by a system and then harvested in agricultural products. This measure of productivity is one of the indicators of progress toward the UN SDGs due to the impacts of N extraction and waste on the environment and climate

(Leadership Council of the Sustainable Development Solutions Network (SDSN), 2015). In 2010, global average NUE for all crops was 42%. Fruit and vegetable production had the lowest NUE (14%) among major crop categories (Figure 6), indicating that 86 % of N fertilizers applied to these crops was wasted and released into the surrounding environment (Zhang et al., 2015).

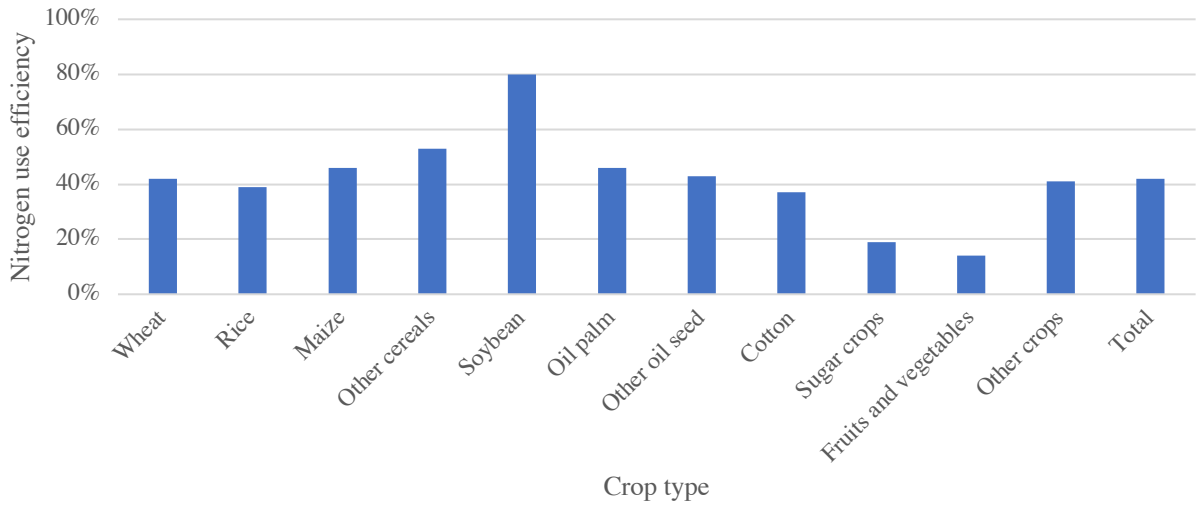

Figure 6. Global average nitrogen use efficiency (NUE) in primary crop production systems, by crop type. NUE is input N divided by harvest N.

Nitrogen utilization in aquaculture is also inefficient. Fish assimilate 10 to 49 % of the N contained in eaten feed, while the rest is excreted in feces (solids) or through the gills (Figure 7) (Piedrahita, 2003). Solids are removed from the water flow in RAS as soon as possible after excretion due to their negative impacts on several aspects of system operation. Dissolved waste nutrients—including ammonia, urea, uric acid, and amino acids—accumulate in the water, depending on water exchange rate. Because ammonia is toxic to fish, RAS utilize biological aerated filters (biofilters) to convert ammonia to nitrate, which fish can tolerate at much higher concentrations (Timmons et al., 2018). Typically, low rates of water exchange in RAS mean that

the solids and wastewater that emerge contain high concentrations of accumulated nutrients. The US Environmental Protection Agency (EPA), under the authority of the Clean Water Act, sets limits for aquaculture wastewater discharge parameters including suspended solids, biological oxygen demand, and dissolved nutrient concentrations (EPA, 2004). Local or state agencies may also enforce water quality standards for discharges to surface waters or charge for treatment via municipal sewage systems. The costs for treatment of wastewater to meet regulatory requirements must generally be borne by aquaculture producers (Timmons et al., 2018). Recirculating aquaponic systems (RApS) integrate aquaculture with hydroponic crop production to monetize the waste streams from aquaculture as the primary nutrient inputs for hydroponic crop production. This integrated approach improves both water use efficiency (WUE) and NUE for fish and plant production simultaneously.

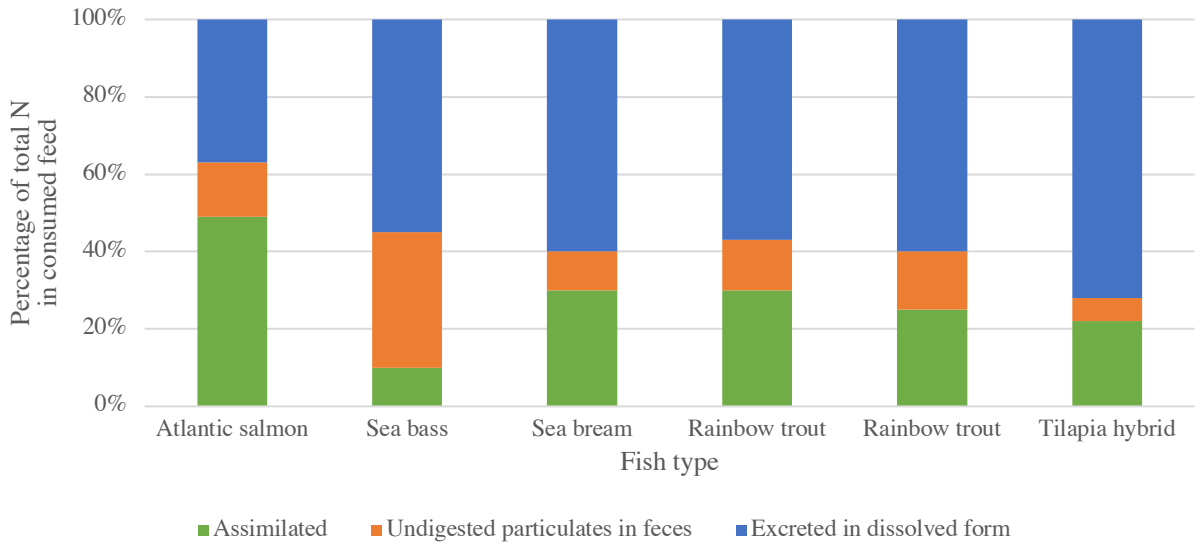

Figure 7. Nitrogen excretion and retention rates in aquaculture by fish type. Data from Piedrahita (2003).

Nitrogen utilization and emission in aquaponics

While different combinations of fish and plant species in RApS will have different water quality requirements, a common theme is compromise between the optimal conditions for fish, plants, and nitrifying bacteria. Additionally, nitrogen production by fish should be balanced with uptake by plants to maintain a steady N concentration over time and prevent N toxicity to the fish (Timmons et al., 2018). NUE in experimental RApS has so far ranged between 34 % (Hu et al., 2015) and 78 % (Wongkiew et al., 2018). This range spans the gap between current global NUE and the improvements that will need to be made to feed the world in coming decades: NUE must increase from an average of 40 % in 2015 to 70 % in 2050 (Zhang et al., 2015). There are many proposed strategies to meet NUE targets over the coming decades while also increasing yield (see Tei et al., 2020; Zhang et al., 2015). While RApS increase the NUE of fish and crop production through integration, NUE may further be improved by careful management of microbial activity within the recirculating production water.

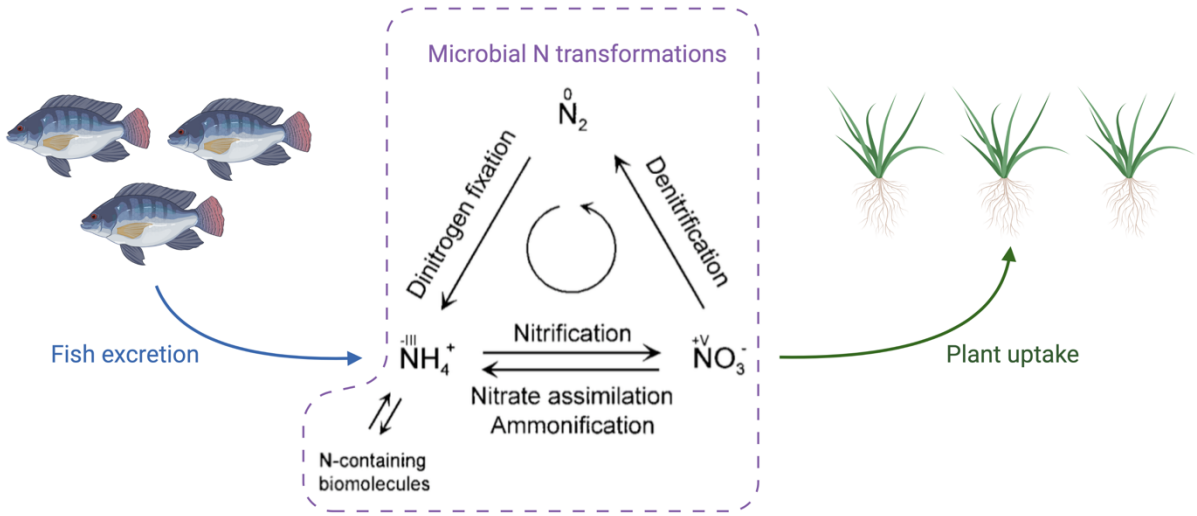

Figure 8. Dissolved N flow in aquaponics, in relation to microbially-facilitated N transformations. Denitrification competes with plant uptake for dissolved nitrate. N cycle adapted from Zumft (1997). Created with BioRender.com.

Since nitrification is actively facilitated in RApS, nitrate (NO_3^-) concentration in the production water tends to be much greater than that of ammoniacal nitrogen (NH_3 and NH_4^+) (Timmons et al., 2018). Plants in RApS thus compete with microorganisms for uptake and transformation of NO_3^- (Figure 8). Certain ubiquitous microbes can convert NO_3^- to dinitrogen gas (N_2)—mostly under anaerobic conditions—through a series of reduction reactions called denitrification (Zumft, 1997). One of the intermediaries of this process, nitrous oxide (N_2O), is a potent greenhouse gas (GHG) with radiative forcing 265 times greater than carbon dioxide (IPCC, 2013) and may be the most important anthropogenic ozone-depleting substance of this century (Ravishankara et al., 2009a). Nitrous oxide emissions from experimental RApS have ranged from 0.4 % to 3.6 %, indicating measurable rates of "incomplete" denitrification (Fang et al., 2017; Hu et al., 2015; Wongkiew et al., 2018; Zou, Hu, Zhang, Guimbaud, et al., 2016; Zou, Hu, Zhang, Xie, et al., 2016). Emission of gaseous N_2 and N_2O represents an economic loss for producers in addition to the environmental consequences. For these reasons, aquaponic practitioners and researchers should seek to minimize denitrification through system design choices and operational protocols that appropriately mediate conditions favorable to denitrification.

Food safety concerns in aquaponics

Soil-based crop production has utilized animal manure as a major nutrient source since at least the Bronze Age in Europe (Clark, 1952) and since the early Vedic period (second millennium BCE) in India (Ramprasad, 2012). In modern agriculture, which has developed around a small number of domesticated animals, the potential human health hazards associated with these animals and their wastes is well-studied (Allende & Monaghan, 2015; Hoagland et al.,

2018; Steele & Odumeru, 2004). Enteric pathogens that can live in the guts of warm-blooded animals are responsible for the majority of foodborne diseases (FBD) with known causes, entering the food production chain at primary production or at any point through consumption (Scallan, Hoekstra, et al., 2011). Leafy green vegetables and fruit crops are implicated in 22 % and 12 % of attributable FBD in the US, respectively (Painter et al., 2013). However, attribution of outbreaks to specific food commodities and causal agents is challenging, and many cases of FBD are not recognized as such. In nearly 80 % of US FBD the causative agent is not identified (Scallan, Griffin, et al., 2011), leaving much room for exploration of novel causal agents and routes of transmission.

Recirculating aquaculture and hydroponic crop production, on the other hand, are relatively new developments in the history of agriculture. While the waste stream from RAS is too dilute for land application, it is rich in nutrients and can be utilized in hydroponic crop production through aquaponics. In addition to plant-essential nutrients, however, RAS waste also contains elevated concentrations of organic C (OC) compared with typical hydroponic fertilizer solutions. This OC can support the growth of heterotrophic bacteria including foodborne disease organisms and bacterial indicators of fecal contamination. Thus, reducing the accumulation of OC in aquaponics through mechanical and biological treatment of the RAS waste may help to mitigate produce safety concerns in the hydroponic component. The contribution of organic carbon in fish feces to the produce safety risk associated with hydroponic production water is unknown. Fish don’t harbor the same enteric pathogens as humans or land animals, having gut microorganisms including some fecal indicator bacteria that are closely linked to that of their environment (Geldreich & Clarke, 1966). Thus, organisms routinely used as indicators of fecal

contamination—and by proxy, food safety risk—may not necessarily be the most effective indicators of risk in aquaponics.

Aquaponic producers in the US and places that export produce to the US currently face uncertainty due to the advent of production water regulation under the Food Safety Modernization Act (FSMA), passed in 2011 and enforced by the Food and Drug Administration (FDA). The Produce Safety Regulation (PSR) under FSMA contains two provisions that will impact the growth and development of the aquaponics industry: Subpart E and Subpart F (*Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption*, 2015). Both of these provisions are currently under review pending more scientific evidence (FDA, 2020). The current version of Subpart E, known as the "ag water rule," requires growers to monitor *E. coli* concentrations in water that is used for production activities if the water is intended or likely to come into contact with produce that is covered by the rule. If values exceed certain thresholds—borrowed from EPA regulations for recreational waters—then corrective actions must be taken to reduce *E. coli* concentrations (*Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption*, 2015). Subpart F of the PSR regulates the use of biological soil amendments of animal origin (BSAAOs), which includes manure and non-fecal animal products applied to soils and solid growing matrices utilized in greenhouse crop production. This provision, in its current state, requires that BSAAOs be treated using a scientifically validated process to "reduce microorganisms of public health significance." This requirement is operationalized with maximum thresholds for detectable *E. coli*, *Salmonella* spp., and *Listeria monocytogenes* in the treated BSAAO (*Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption*, 2015). As it stands, Subpart F suggests that aquaponic systems that utilize solid media for plant growth must meet this standard

as a scientifically validated water treatment process. Information about the likely revisions to these two rules is an urgent need among producers planning for the future (Wall et al., 2019). In the meantime, educational resources about best practices can help growers to prepare.

As N management in aquaponics is important for economic and environmental reasons, feces management has multiple impacts on aquaponic systems. Available organic carbon (OC) in production water encourages the growth of heterotrophic bacteria that utilize it as an energy source. These heterotrophs typically grow at faster rates than autotrophic bacteria, a group that includes the primary nitrifying bacteria relied upon in RAS and RApS to convert fish-toxic ammoniacal N to nitrate (Michaud et al., 2006; Nogueira et al., 2002; Satoh et al., 2000; Zhu & Chen, 2001a). So in RApS, accumulation of organic C favors heterotrophic growth that reduces nitrification potential. A higher C:N ratio is also correlated with a reduction in bacterial species richness and diversity (Michaud et al., 2014). That study found positive selection for *Gammaproteobacteria* as C:N increased, especially in systems with static biofilters. This class of the phylum Proteobacteria contains many potential pathogens of humans, plants, and animals. Moving bed biofilters, also tested in the above study, are self-cleaning, with reduced C accumulation compared to static biofilters. These reduced C conditions led to smaller reductions in richness and diversity as C was artificially enriched. Bacterial species richness and diversity in RApS were recently found to positively correlate with suppression of the plant pathogen *P. aphanidermatum*, suggesting a greater resilience of diverse bacterial communities to change in response to the introduction of a pathogen (Stouvenakers et al., 2020).

Experimental RApS designs

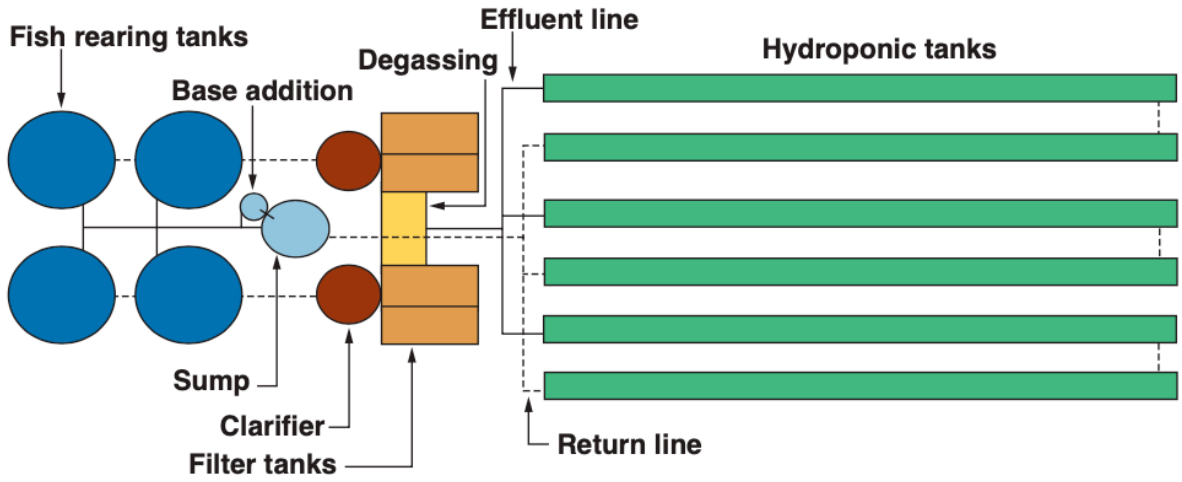

Figure 9. UVI aquaponic system schematic. Water flows in a single loop: Pipes represented by solid lines flow from the sump to the fish and hydroponic units, and pipes represented by dotted lines return water from the hydroponic and fish unit drains. Solid waste is retained in the clarifier and filter tanks and periodically discharged. Adapted from Rakocy et al. (2006).

Most experimental RApS to date have adapted the UVI design, first developed in the 1980s (Rakocy et al., 2006), without addressing some of its potential weaknesses. In temperate zones, year-round aquaponic production requires a greater degree of environmental monitoring and control as compared to lower-input tropical systems. Increased energy inputs to maintain acceptable conditions must be offset by increased productive intensity. In the UVI design (Figure 9), *in situ* mineralization of waste solids in "degassing tanks" encourages denitrification, reducing NUE, while also providing an organic carbon source for the growth of pathogenic bacteria that may be introduced to the system. Here we present a novel RApS system design (Figure 10) adapted from the optimal design principles espoused by Timmons et al. (2018). Two primary differences of the University of New Hampshire (UNH) Kingman Farm Recirculating Aquaponic Greenhouses (KFRAG) compared to other designs in the literature are 1) complete

solids removal and 2) the independence of unit processes. We define complete solids removal as the diversion of waste solids from all units to a separate storage or treatment system, which was implemented here utilizing a microscreen (54µm mesh) drum filter. As demonstrated at pilot scale by Tetreault et al. (2020), an additional loop incorporating controlled an-/aerobic microbial treatment could in future be added to solubilize nutrients from waste solids, improving the nutrient profile relative to plant requirements and overall nutrient use efficiency. A multi-loop design with independent unit processes at KFRAG meant that the fish, plant, and biofilter units could be isolated at any time in case of emergency, which is essential in temperate zones where failure of environmental controls could lead to major system disruptions and losses.

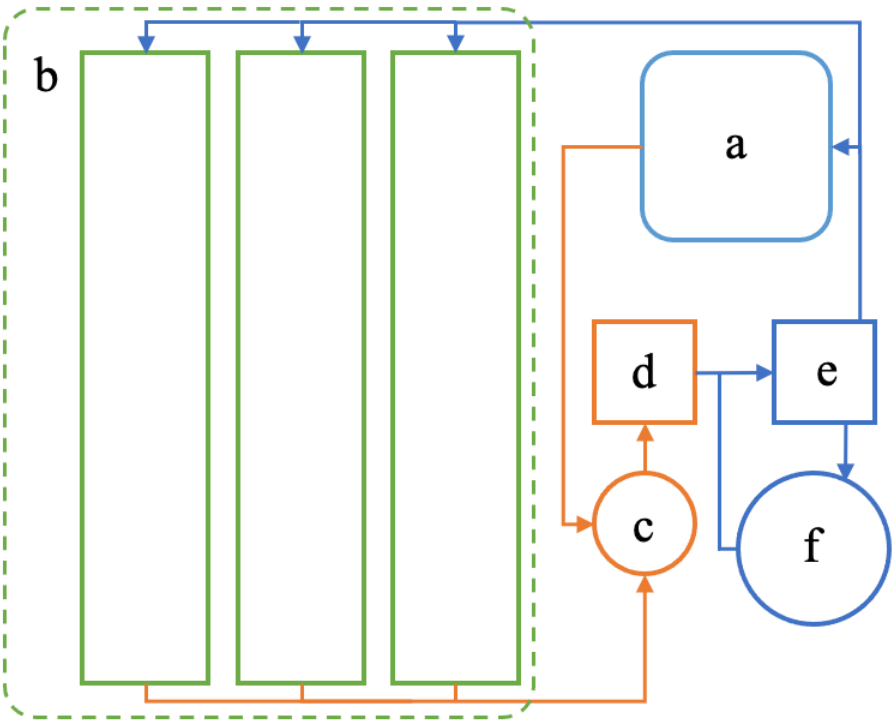

Figure 10. UNH KFRAG system schematic. Wastewater (in orange) from the fish (a) and plant (B) units flows to a central standpipe well (c) and then through a mechanical drum screen filter (d). Filtered water (in blue) flows to the sump tank (e), where it is pumped to the moving bed biofilter side loop (f) and back to the fish and plant units.

Objectives

The body of this thesis presents two case studies of recirculating water conditions in the replicated KFRAG systems (n = 3). First, we establish that the systems maintained appropriate physicochemical water quality for tilapia, lettuce, and nitrifying bacteria. This confirms that the facility duplicated typical intensive commercial production conditions and allows for discussion of the implications of physicochemical water quality on aquaponic ecosystem function and efficient N utilization. Next, we demonstrate a microbial water quality profile that is in accordance with FSMA requirements for both agricultural production water and treated BSAAO, with the first aquaponics study to screen for *E. coli*, *Salmonella* spp., and *Listeria* spp. We conclude with discussion of the potential interactions between physicochemical water quality and microbial activity, and implications of these interactions for productivity and food safety in aquaponics.

Chapter 2:

Water Quality in Experimental Commercial-scale Recirculating Aquaponic Systems

Introduction

Aquaponics is an integrated food production system that combines hydroponics and recirculating aquaculture systems (RAS) to efficiently produce both fish and plants. Recirculating aquaponic systems (RApS, also known as "coupled" aquaponic systems) share culture water that flows repeatedly through the fish and plant units. As the primary growing medium, this production water impacts all aspects of system operation and performance. The living components of RApS—fish, plants, and microbes—depend on physical and chemical water quality properties being within tolerable ranges for each organism. This multitrophic aquaponic ecosystem in turn has dynamic impacts on water quality parameters. These impacts may change over time, dependent upon environmental conditions and the developmental physiology of the plants, fish, and microbial community. There is a lack of information about the day-to-day variability and stability of important water quality parameters. Other researchers have reported measuring pH and dissolved oxygen (DO) as infrequently as weekly (C. Li et al., 2019; Liang & Chien, 2015; Nhan et al., 2019). Monitoring that is too frequent can lessen the ability to control parameters, leading to suboptimal water quality that may increase the risk of fish disease and mortality. Suboptimal fish health due to poor water quality can also reduce the feed conversion ratio (FCR) and the nutrient use efficiency (NUE) (Timmons et al., 2018). Many

studies have detected parameters deviating from optimal ranges for the organisms studied, indicating inadequate water quality controls (Espinosa Moya et al., 2016; C. Li et al., 2019; Ngo Thuy Diem et al., 2017; Nhan et al., 2019; C.-Y. Wang et al., 2016).

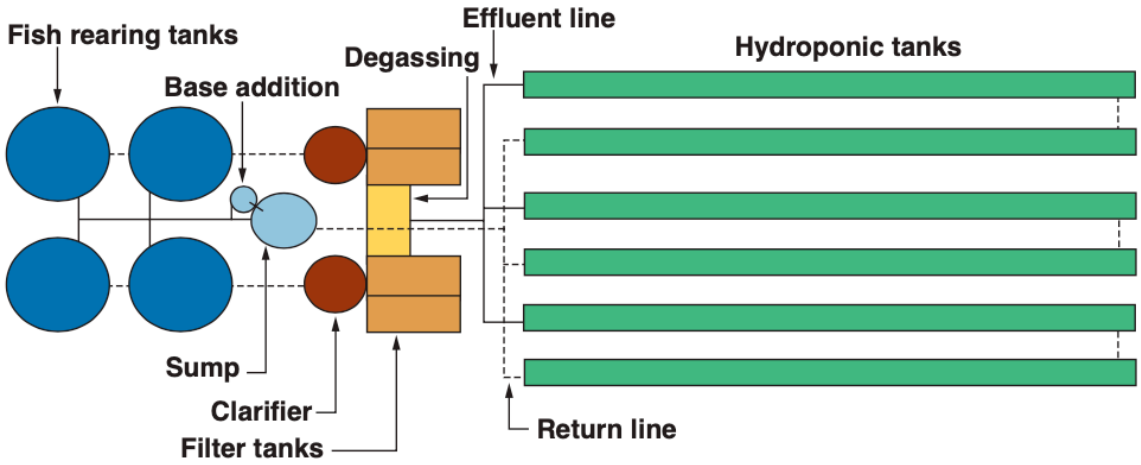

Figure 11. Single-loop aquaponic system design from the University of the Virgin Islands (UVI). Water flows from left to right through plumbing represented by solid lines and returns from the fish and hydroponic units through plumbing represented by dashed lines. (Adapted from Rakocy et al., 2006.)

There have been several studies using a variety of lab-scale RApS to replicate and integrate components of full-scale commercial RAS and hydroponics (Endut et al., 2009; Espinosa Moya et al., 2016; Liang & Chien, 2015; Maucieri et al., 2019; Moriarty et al., 2018; Ngo Thuy Diem et al., 2017; Pérez-Urrestarazu et al., 2019; C.-Y. Wang et al., 2016; Y.-J. Wang et al., 2020). Many of these experimental RApS designs have been derived from the array of 15,000 L experimental aquaponic systems at the University of the Virgin Islands (UVI) that have been operating since the 1980s (Danaher et al., 2011, 2013). This design is characterized by a single circular flow of production water with retention and periodic discharge of concentrated waste solids (Figure 11). These solids are retained in the system by a device such as a swirl separator, cylindro-conical clarifier, sand or gravel filter and by plastic netting in secondary filter tanks. The high concentration of carbon in the waste generates biological oxygen demand from

heterotrophic bacteria and can create anoxic zones where denitrification, and loss of nitrogen as nitrogen gas, can occur (Timmons et al., 2018).

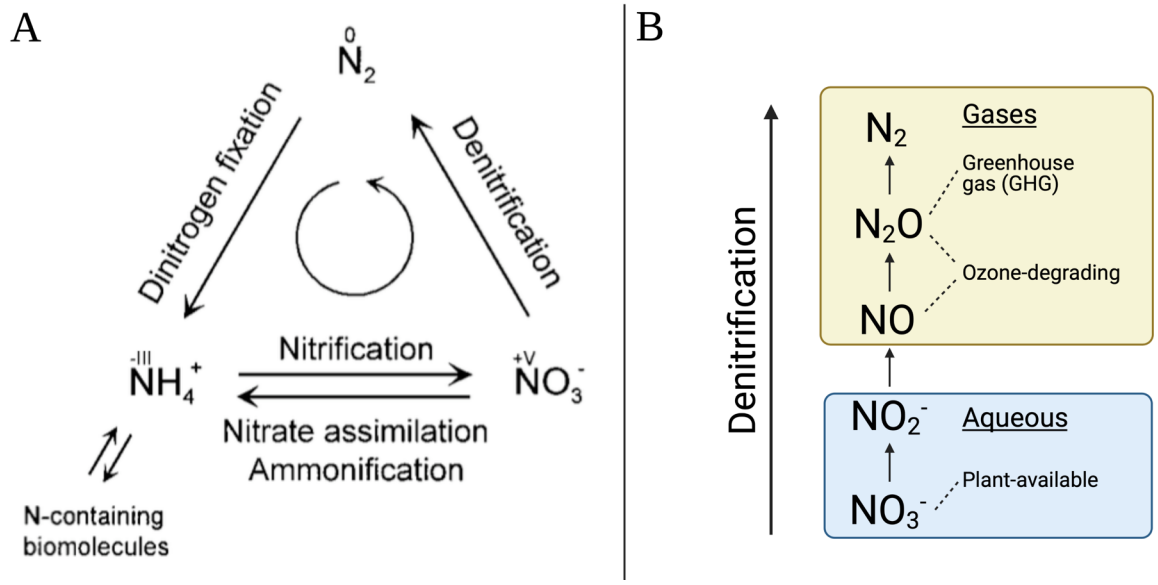

Figure 12. Biogeochemical nitrogen cycle (A) and denitrification (B). (A) was adapted from Zumft (1997). Created with BioRender.com.

Denitrification is a four-step chain of microbially-facilitated reduction reactions that together constitute one of the major branches of the biogeochemical nitrogen cycle (Figure 12A) (Zumft, 1997). The nitrogen oxyanions nitrate (NO_3^-) and nitrite (NO_2^-) and the gases nitric oxide (NO) and nitrous oxide (N_2O) are utilized successively as terminal electron acceptors instead of dioxygen (O_2), finally producing dinitrogen gas (N_2) that escapes to the atmosphere (Figure 12B). The intermediate gases nitric and nitrous oxide also escape to the atmosphere during this process, a phenomenon that occurs in a concentrated fashion from wastewater treatment plants, wetlands, continental shelves, and agricultural fields (Seitzinger et al., 2006). While denitrification can be operationally beneficial in RAS for maintaining desired nitrate concentrations, in RApS it represents a loss of the essential plant nutrient nitrogen, reducing the NUE of the system. The more nitrogen lost to microbial biomass and the atmosphere, the less

can be taken up by plants in the hydroponic unit. One justification for allowing denitrification to occur is that solids retention also allows for greater mineralization of other plant nutrients that are bound in the fish feces, creating a more balanced nutrient solution. For commercial aquaponic producers, however, the reduction of NUE represents an economic loss of the nitrogen added to the system through fish feed and may reduce the amount of plant growing area that can be supported by the fish system, unless costly nitrogen fertilizer is introduced. Furthermore, nitric and nitrous oxides released to the atmosphere by denitrification contribute to the degradation of the stratospheric ozone layer that protects Earth's surface from solar ultraviolet radiation (Crutzen & Ehhalt, 1977). Nitrous oxide is also a potent greenhouse gas, with 100-year global warming potential of 265 times that of carbon dioxide (IPCC, 2013). With N_2O concentrations predicted to continue increasing steadily in coming decades (IPCC, 2013; Ravishankara et al., 2009b; W. Wang et al., 2014) (Figure 13), any opportunity to reduce emissions should be taken.

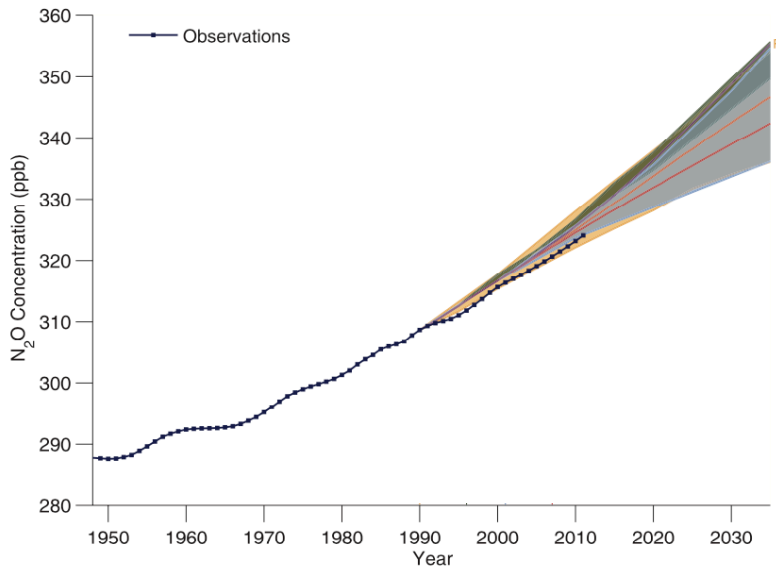

Figure 13. Globally and annually averaged atmospheric nitrous oxide concentrations observed from 1950 to 2011 and predicted through 2035. Observed values are indicated with dark blue squares. The dark gray shading shows the largest projected range of global N_2O concentrations under the scenarios considered by the Intergovernmental Panel on Climate Change (IPCC). (Adapted from IPCC, 2013.)

Nitrification—another branch of the biogeochemical N cycle (Figure 12)—is a microbially-mediated two-step process whereby ammoniacal nitrogen (NH_4^+) is converted to nitrate (NO_3^-) in the presence of oxygen. First, NH_4^+ is oxidized to NO_2^- by ammonia-oxidizing bacteria and archaea. In aquaponic systems, bacteria such as *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, and *Nitrosolobus* spp. dominate this process (Wongkiew et al., 2017). Second, NO_2^- is oxidized to NO_3^- by nitrite-oxidizing bacteria including *Nitrobacter*, *Nitrococcus*, *Nitrospira*, and *Nitrospina* spp. Because NH_4^+ is much more toxic to fish than NO_3^-, ammonia- and nitrite-oxidizing bacteria are intentionally cultivated in RAS and RApS in biological aerated filters (biofilters) (Timmons et al., 2018). These autotrophic bacteria grow at slower rates than heterotrophic bacteria, which can overtake the surfaces of biofilms in the biofilter if there is available organic carbon (Michaud et al., 2006; Nogueira et al., 2002; Satoh et al., 2000; Zhu & Chen, 2001b). Studies in RAS have shown that the addition of organic C negatively impacts nitrification rates (Chen et al., 2006; Guerdat et al., 2011; Ling & Chen, 2005; Michaud et al., 2006) and can potentially stimulate denitrification and thus N_2O emissions (Hu et al., 2015). From this it can be inferred that limiting the accumulation of organic carbon in aquaponic production water would improve both the productivity and sustainability of the system by increasing NUE and reducing GHG emissions.

In contrast to the UVI systems, the University of New Hampshire Kingman Farm Recirculating Aquaponic Greenhouses (UNH KFRAG) were designed based on the most current water treatment principles employed in large-scale RAS in the continental US. Three 11,000 L replicate aquaponic systems featured independent circulation loops for plant, fish, and biofilter unit processes and rapid filtration of waste solids. Solids were diverted to an external storage

tank and not recirculated to the system. Future research could implement further solids processing by aerobic/anaerobic digestion as demonstrated at pilot scale by Delaide et al. (2019) and Tetreault (2020). UNH KFRAG was in continuous lettuce and tilapia production from June 2018 through February 2020. The objective of this study was to evaluate the impact of rapid solids removal on physicochemical water quality in the UNH KFRAG systems. Dissolved oxygen, water temperature, pH, dissolved nitrogen species, alkalinity, and dissolved organic carbon were prioritized due to their outsized roles in key ecological and physiological processes in recirculating aquaponics. In this case study, we report variability, deviations from acceptable ranges, and how well water quality parameters were maintained within acceptable ranges in the triplicate systems.

Methods

Experimental recirculating aquaponic systems

Figure 14. Aerial view of the University of New Hampshire Kingman Farm Recirculating Aquaponic Greenhouses (UNH KFRAG), with adjacent outbuildings and active research farmland. Photo credit: UNH Media

This study took place at the University of New Hampshire (UNH) Kingman Farm Recirculating Aquaponic Greenhouses (KFRAG). KFRAG consists of three identical 9.0 x 14.6 m polycarbonate greenhouses (Figure 14), with each greenhouse containing a single identical coupled aquaponic system (Figure 15). In a coupled aquaponic system, the fish unit and plant unit share culture water that is recirculated between them. Culture water was circulated through each 11,000 L system using a single 3 HP Pentair Sparus™ pump (Pentair, Minneapolis, Minnesota, USA) set to a constant flow rate of 376 L min^{-1}. Flow to each unit process was controlled with ball valves at each inflow manifold. One inline water heater per greenhouse was used to maintain water temperature at 25.5 °C (± 0.5 °C).

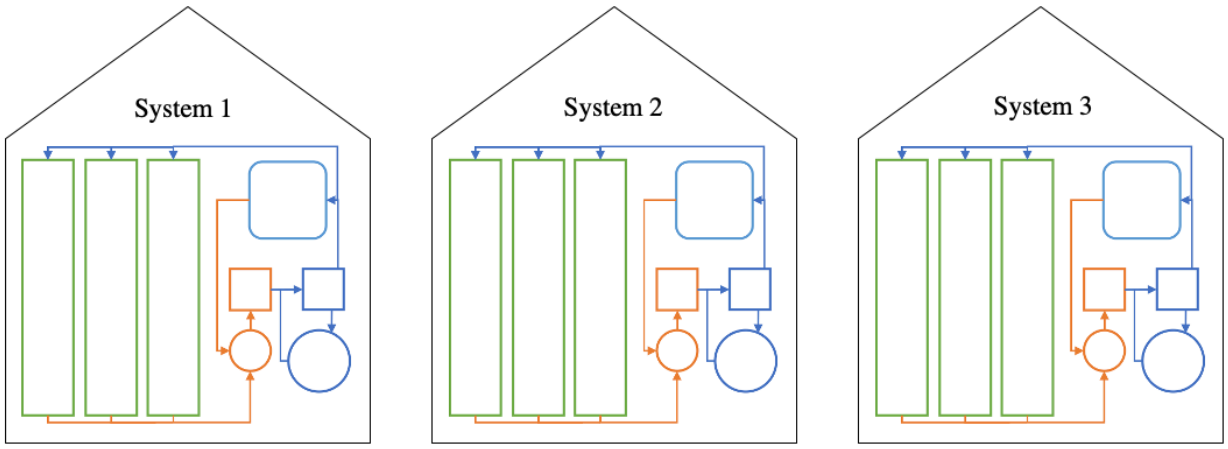

*Figure 15. UNH KFRAG schematic. Each greenhouse contained a single, large-scale experimental aquaponic system in continuous production of tilapia (*Oreochromis *spp.) and lettuce (*Lactuca sativa *L. cv. 'Rex').*

Fish unit description

Tilapia (*Oreochromis* spp.) fingerlings of 0.5 g per fish average weight were sourced from Aquasafra, Inc. (Bradenton, Florida, USA). They were grown out in experimental RAS quarantine systems at the Anadromous Fish and Invertebrate Research (AFAIR) facility at UNH. Fingerlings were fed Finfish Gold floating crumble, then pelleted feed (Zeigler Brothers, Inc., Pennsylvania, USA) using vibrating feeders on hourly timers. Feed rates were calculated for optimum growth based on fish weight according to DeLong et al. (2009). The quarantine system was thoroughly sanitized before introducing the tilapia fingerlings, and AFAIR systems and staff utilized strict biosecurity protocols to prevent the incursion of fish pathogens. Similar protocols will be detailed below. Tilapia were transferred to the KFRAG systems after 90 days in quarantine, at 80 g average per fish weight.

At KFRAG the fish were reared in a 3 m^3 rounded square tank (Figure 16a), filled to a volume of 2,500 L and stocked at a starting density of 23.7 – 25.5 kg m^{-3}. The fish tank in each system was supplied with culture water at a rate of 222 L min^{-1}, with a hydraulic residence time

(HRT) of 15 m. Over the 7-week study period, tilapia biomass nearly doubled, to a density of 43.5 – 44.2 kg m^{-3}. The same cohort of fish was present in each system for the duration of this study, meaning that no fish were added to the systems and all fish present throughout the study were of the same age and size.

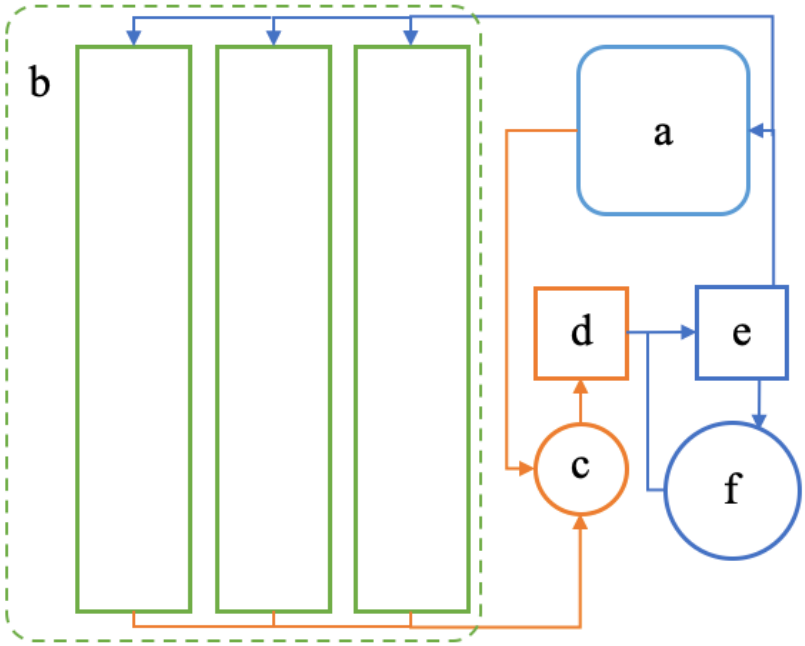

Figure 16. UNH KFRAG coupled aquaponic process flow. From the pump sump (e), water flowed to the fish tank (a) and deep-water culture plant beds (b). Effluent from both units drained to the standpipe well (c) before flowing through the rotating mechanical drum filter (d). Filtered water was returned to the pump sump. The biofilter (f) operated on a side loop, drawing 29% of the flow. Orange lines indicate fish and plant unit effluent prior to treatment through the mechanical drum filter.

Plant unit description

The vegetative component of each system consisted of three deep water culture (DWC) beds, each with a filled volume of 2.5 m^3 and surface (growing) area of 11.9 m^2. Each DWC bed was supplied with culture water at a rate of 14 L min^{-1}, with an HRT of 4 h.

Lettuce seedlings were grown in Horticubes® XL, an inert synthetic foam substrate with ideal water holding characteristics (holding 50% water and 50% air at saturation) and no cation exchange capacity (Oasis Grower Solutions, Kent, Ohio, USA). Sheets of this medium in web-

bottomed trays were seeded with Bibb lettuce (*Lactuca sativa* cv. 'Rex' pelleted seed, Johnny's Selected Seeds, Winslow, Maine, USA), then leached of residual salts with clear water per manufacturer's instructions. Seedling trays were fertigated by overhead watering with 5-11-26 HydroSpecial and calcium nitrate (JR Peters, Allentown, Pennsylvania, USA) at a rate of 150 mg L^{-1} nitrogen for 14 days.

At 14 days after seeding, seedlings were transplanted into high-density polystyrene floating rafts (Beaver Plastics, Ontario, Canada) in the aquaponic DWC beds. Each cohort—216 plants of the same age split across the 3 DWC beds—remained in each aquaponic greenhouse for 4 weeks until harvest at 42 days (Figure 16b). Four cohorts of lettuce were present in the system at any given time, and seeds were sown weekly to replace plants as they were harvested. This continuous production system resembled an automated hydroponic greens operation observed in New Hampshire, where plants at all stages of growth were present simultaneously in the same environment. In order to limit contact between the crop and culture water at harvest, lettuce heads were cut and removed from the system before the rafts were removed. Rafts containing the younger cohorts were then floated gently down the beds to minimize splashing. The harvested rafts were scrubbed of gross organic material and sanitized by coarse spray application of 500 ppm peroxyacetic acid (Sanidate® 5.0, BioSafe Systems, East Hartford, Connecticut, USA), then allowed to air dry before reuse. After each harvest, sanitized boards were placed into the inflow end of each bed and a new cohort of 216 lettuce plants was transplanted into each system. Note that complete sanitization of lettuce boards was not expected or necessary; boards were re-inoculated with endemic organisms upon reentry into the DWC beds. The primary intention of the sanitization procedure was to minimize the accumulation of microbial biofilm on the board surfaces.

Solids removal

Effluent from the fish and plant units flowed to a standpipe well and then through the mechanical drum screen filter (RFM2014, PR Aqua, USA), where solid wastes including fish feces and detritus from the hydroponic unit were actively removed (Figure 16c-d). Culture water flowed from the drum filter to the pump sump (Figure 16e). To minimize the accumulation of biofilm in the tanks, surfaces were scrubbed weekly. In the plant unit, this weekly cleaning included sweeping biofilm and root detritus down the bed and towards the drain at harvest time, while the rafts were out of the beds. This material was subsequently removed from the system by the drum filter. Drum screens were cleaned monthly with muriatic acid to maintain efficient solids removal.

Midway through the study period, there was a mechanical failure in system 1 that caused water loss of about 5600 L over the course of 3 days. A metallic component broke down over a weekend and sent shards of metal into the drum filter spray nozzle switch, keeping the nozzles open and draining culture water from the system. This lost water was replaced automatically with well water, which had an impact on the water quality parameters described below.

Biofilter unit description

A 1.1 m^3 moving bed bioreactor, containing 0.75 m^3 of Kaldnes K1 media (Veolia North America, Boston, Massachusetts, USA), was operated as a recirculating side loop from the pump sump (Figure 16f). The biofilters in each system received culture water at 112 L min^{-1}. At startup, biofilters were seeded with media from the established experimental RAS systems at AFAIR. The biofilters in systems 1, 2, and 3 took 54, 49, and 52 days, respectively, to achieve stable, near-complete nitrification, at which point plants were introduced to the DWC beds.

System inputs and biosecurity

The sole inputs to the UNH KFRAG systems were fish feed (Finfish Silver, floating, Zeigler Brothers, Inc., Pennsylvania, USA), chelated DTPA iron (Sprint 330, BASF SE, Ludwigshafen, Germany), potassium carbonate, and well water. Fish were fed 1,300 g day^{-1} with automatic vibrating feeders that dispensed feed hourly during daylight hours. Iron was supplemented as needed to maintain a system-wide concentration of 1.8 – 2.8 mg L^{-1}, and potassium carbonate was added daily to maintain alkalinity of 20 – 40 mg L^{-1} (measured as mg L^{-1} $CaCO_3$). To ensure adequate mixing throughout the system, iron and potassium carbonate were added to the pump sump.

To reduce the introduction of exogenous microbes to the KFRAG systems, all workers were trained in biosecurity and food safety protocols. These measures included frequent handwashing, a Virkon-S footbath to disinfect shoes upon entering and exiting the facility, and sanitary harvest practices. See Chapters 1 and 4 for in-depth discussion of good agricultural practices (GAPs) for aquaponic growers.

Aquaponic water quality and system design

In RAS or hydroponic systems, water quality is typically maintained within optimal ranges for the health of a single crop species or variety. Since this is not possible for most fish-plant combinations in aquaponics, the aquatic environment must be maintained at a compromise point that is as close as possible to the optimal environment for fish, plants, and the nitrifying microbes in the biofilter. Acceptable ranges for water quality parameters were determined utilizing optima from the literature for tilapia in RAS and hydroponic lettuce (Table 1).

Table 1. Acceptable ranges for aquaponic water quality parameters at UNH KFRAG.

Parameter	Acceptable range
Dissolved oxygen (DO) (mg L^{-1})	> 5.0
Temperature (°C)	24 – 28
pH	6.7 – 7.2
Alkalinity (as mg L^{-1} $CaCO_3$)	20 – 40
Electrical conductivity (EC) (mS cm-1)	1.0 – 2.0
Iron (mg L^{-1})	1.8 – 2.8
Nitrate nitrogen (mg L^{-1})	100 – 200
Nitrite nitrogen (mg L^{-1})	< 0.1
Total ammoniacal nitrogen (mg L^{-1})	< 1.0
DOC:TDN	< 0.5

The most important water quality standard was that for dissolved oxygen (DO) concentration. Under normal atmospheric conditions, water at 25.5 °C becomes saturated with oxygen at 8.2 mg L^{-1}. Plant roots work most efficiently in hydroponic solution when it is saturated with oxygen (Chun & Takakura, 1994). DO deficiency in hydroponic systems is associated with reduced root respiration, which in turn reduces plant growth rates and increases susceptibility to root pathogens (DeWit, 1978). Meanwhile, tilapia require oxygen concentrations above 5.0 mg L^{-1} for optimal growth and health (Timmons et al., 2018). Thus, aeration was included in every unit process. This included stone aerators in the fish tank and at several positions (every 60 cm) along each plant bed. The flow of turbulent water with exposure to the air provided adequate aeration in both filters and the pump sump.

The operating temperature of 25.5 °C and acceptable range of 24 – 28 °C were determined as a compromise between disparate optima. Hydroponic lettuce fares best with water temperatures of 20 – 21 °C (F. Li & Chen, 2017), but tilapia in RAS have demonstrated optimal

growth rates near 30 °C (Brahmane et al., 2017). Similarly, optimal pH for hydroponic nutrient solution is 5.5 – 6.5, while optimum nitrification efficiency is achieved at a pH close to 9.0 (Timmons et al., 2018). To promote both plant nutrient availability and biofilter function, the target pH in the KFRAG systems was between 6.7 and 7.2. Preliminary data showed that this was best achieved by maintaining alkalinity at 20 – 40 mg L^{-1} $CaCO_3$, which was a lower target than reported in other studies (Danaher et al., 2011, 2013; Elumalai et al., 2017).

Dissolved nitrogen species including ammonia, nitrite, and nitrate have distinct effects on the health of both fish and plants in aquaponics. The accumulation of nitrogenous wastes is problematic for fish health in RAS and mitigated by the use of nitrifying biofilters. Total ammoniacal N (TAN) and nitrite-N concentrations were utilized at KFRAG as indicators of biofilter efficacy, with values above 1.0 and 0.1 mg L^{-1}, respectively, indicating reduced nitrification efficiency (Timmons et al., 2018). The product of nitrification, nitrate, is non-toxic to fish and is the preferred form of nitrogen for uptake in most agricultural crops (Claussen & Lenz, 1999; Guo et al., 2002; Raab & Terry, 1994; Savvas et al., 2006). In an efficient RAS or RApS with near-complete nitrification, nitrate comprises the vast bulk of total dissolved N (TDN). Nitrate concentrations vary widely in hydroponic nutrient solutions, with 150 mg L^{-1} recommended for herbs and leafy green vegetables (Mattson & Peters, n.d.). The KFRAG systems were run with a target of 120 mg mL^{-1} NO_3 and an acceptable range of 100 to 200 mg mL^{-1} NO_3.

Ideal electrical conductivity (EC) in aquaponics, like nitrate concentration, is a matter of some debate. Given the constant addition of nutrients through fish excretion, it is possible to operate recirculating aquaponics systems with an EC as low as 0.3 to 0.6 mS cm^{-1} (Danaher et al., 2013; Rakocy et al., 2004; Wongkiew et al., 2018). However, greater growth rates have been

observed at EC above 1.2, and hydroponic nutrient solutions typically range from 1.0 to 4.0 mS cm^{-1} (Resh, 2013). For KFRAG, we determined the acceptable EC range to be 1.0 – 2.0 mS cm^{-1}. Preliminary data showed no clear effect of EC on plant growth. Iron concentration did influence lettuce plant health and growth rates, and through experience an acceptable range of 1.6 – 2.8 mg mL^{-1} was determined (data not shown).

Dissolved organic carbon (DOC) concentrations have not been reported in experimental aquaponic systems to date, so no target range was set for DOC. The KFRAG systems and operational protocols were designed to limit C accumulation due to the negative impact of organic C on nitrification kinetics and biofilter efficacy (Chen et al., 2006). It has been demonstrated in both lab scale (Michaud et al., 2006; Zhu & Chen, 2001b) and commercial scale RAS (Guerdat et al., 2011) that as C:N increases from 0 to 0.5, there is a sharp decline in nitrification rate. In the KFRAG systems with active, complete solids removal, a C-limited aquatic environment was expected, with C:N < 0.5.

Water sample collection and analysis

Regular water quality monitoring was conducted as part of operations at UNH KFRAG in order to maintain a stable aquatic environment (described above) with acceptable conditions for fish, plants, and microbes. Water temperature was measured continuously using a Maxim (Sunnyvale, California, USA) DS18B20 temperature probe connected to the Seed V2 environmental control system (Wadsworth Controls, Arvada, Colorado, USA), which recorded instantaneous temperatures every 15 minutes. Dissolved oxygen (DO) and electrical conductivity (EC) were measured daily in the fish effluent standpipe with a YSI Pro2030 instrument (YSI Inc., Yellow Springs, Ohio). Pump sump grab samples from each system were also analyzed

daily for pH and alkalinity (Method 2320B, American Public Health Association, 2012) using an Accumet™ AB150 benchtop pH meter (Fisher Scientific, Pittsburg, Pennsylvania). From the same grab sample, nitrite and ammonia-nitrogen concentrations were measured four times per week by EPA methods 8507 and 8038, respectively, using a DR3900 spectrophotometer (HACH Company, Loveland, Colorado). The same grab sample and instrument were used twice weekly to measure iron concentrations using EPA method 8008. Replicate samples were not analyzed, however, the high frequency of sampling relative to other work in the literature (C. Li et al., 2019; Maucieri et al., 2019; Ngo Thuy Diem et al., 2017; Nhan et al., 2019) provided adequate data to characterize trends for water quality measurements over time.

Culture water from multiple sites within each aquaponic system was filtered weekly during the study period for microbial analysis (see Chapter 3). Dissolved carbon and nitrogen concentrations were measured in a subset of these samples from the first and last weeks of the study period. For each sample, a 50 mL aliquot of the filtrate was stored at -4 °C. Filtrate samples were submitted to USDA Forest Service (Durham, NH) for dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) analysis. DOC was measured by high-temperature catalytic oxidation (HTCO) (Method 5310 B, American Public Health Association, 2012), and TDN was measured by HTCO with chemiluminescent N detection (ASTM International, 2016). Nitrification efficiency for each system was calculated as mean nitrate-nitrogen divided by mean TDN.

Statistical analysis

Statistical analysis of measured water quality parameters was performed using JMP Pro v 15.0.0. There were 24 missing data points for water temperature, out of 14,814 total

measurements, and these were excluded from analysis. Data for each system were assessed for normality using the Anderson-Darling test and for homoscedasticity with Levene's test (Anderson & Darling, 1954; Levene, 1960). Transformation of variables that were not normally distributed did not improve the distribution, so the Kruskal-Wallis non-parametric test was used to determine differences between system means. Analysis of variance was conducted for differences between system means for normally distributed variables. Where significant differences were found, the Tukey-Kramer test for honestly significant differences was used to perform pairwise comparisons (Kramer, 1956; Tukey, 1949). JMP Pro and Excel v 16.41 were used to create tables and visualize the results.

Results

Physical and chemical water quality analysis

Environmental parameters for KFRAG aquaponic culture water that are critical for maintaining the health of the fish and biofilter were monitored. The mean, standard deviation and minimum and maximum values for each parameter were determined for the 7-week study period from September 9 to October 30, 2019 (Table 1).

Table 2. Summary statistics for UNH KFRAG culture water in each of the 3 replicated aquaponic systems. Data represent measurements taken during the 7-week study period in Sept.-Oct. 2019.

Parameter	Acceptable range	Mean ± standard deviation (minimum, maximum)		
		System 1	System 2	System 3
DO concentration (mg L^{-1})	> 5.0	6.4 ± 0.55 (5.5, 7.6)	6.5 ± 0.56 (5.5, 7.7)	6.3 ± 0.49 (5.4, 7.2)
Temperature (°C)	24 – 28	25.9 ± 0.85 (24.9, 29.7)	24.6 ± 0.90 (23.1, 28.6)	25.4 ± 0.88 (24.0, 29.2)
pH	6.7 – 7.2	6.8 ± 0.25 (6.3, 7.4)	7.0 ± 0.20 (6.4, 7.4)	7.0 ± 0.18 (6.5, 7.3)
Alkalinity (as g $CaCO_3$ m^3)	20 – 40	29 ± 5.3 (17, 44)	30 ± 4.4 (20, 41)	29 ± 3.3 (19, 38)
EC (mS cm^{-1})	1.0 – 2.0	1.64 ± 0.11 (1.46, 1.83)	1.36 ± 0.21 (0.99, 1.66)	1.76 ± 0.16 (1.46, 1.98)
Iron (mg L^{-1})	1.8 – 2.8	2.13 ± 0.27 (1.71, 2.72)	2.04 ± 0.26 (1.61, 2.63)	2.11 ± 0.19 (1.73, 2.53)
TDN concentration (mg L^{-1})		135.5 ± 13.9 (112.2, 165.6)	119.5 ± 23.6 (95.1, 154.5)	128.2 ± 32.1 (84.9, 184.5)
NO_3-N concentration (mg L^{-1}) *	100 – 200	135.0	118.9	127.6
NO_2-N concentration (mg L^{-1})	< 0.1	0.05 ± 0.02 (0.02, 0.11)	0.07 ± 0.03 (0.04, 0.15)	0.07 ± 0.02 (0.04, 0.10)
TAN concentration (mg L^{-1})	< 1.0	0.5 ± 0.07 (0.4, 0.7)	0.5 ± 0.09 (0.4, 0.7)	0.6 ± 0.08 (0.48, 0.82)
DOC concentration (mg L^{-1})		20.9 ± 2.83 (16.0, 25.8)	20.4 ± 1.86 (18.9, 24.1)	19.4 ± 4.22 (14.6, 26.0)
DOC:TDN	< 0.5	0.15 ± 0.011 (0.14, 0.18)	0.17 ± 0.023 (0.15, 0.18)	0.15 ± 0.019 (0.13, 0.18)

* Mean NO_3-N was calculated as mean TDN – mean NO_3-N – mean TAN.

Dissolved oxygen

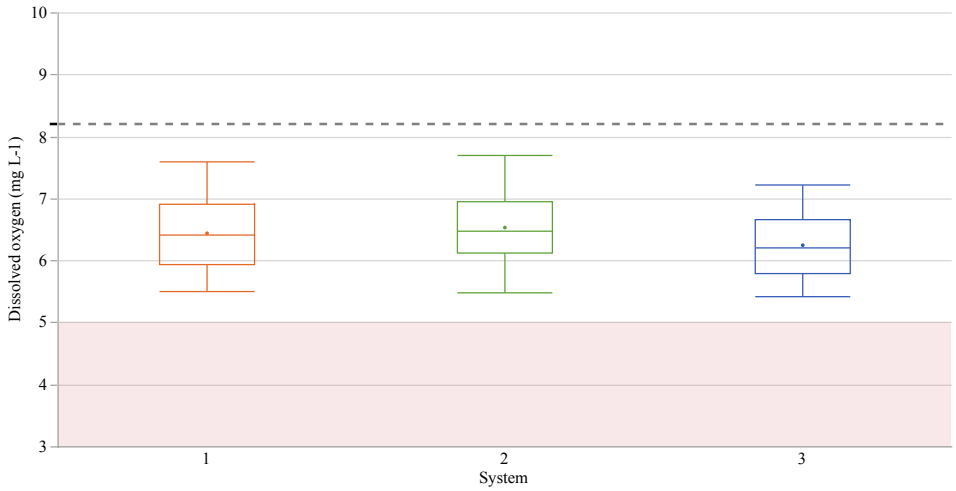

Figure 17. Dissolved oxygen concentrations in the KFRAG systems during the study period. Values in the red shaded area are below the acceptable minimum value of 5 mg L^{-1}. The gray dashed line indicates DO at 8.2 mg L^{-1}, where water at 25.5 °C becomes saturated. Horizontal lines in the centers of boxplots indicate median values and points within the boxes indicate means. The top and bottom of each box represent the third and first quartile, respectively, and whiskers indicate the highest and lowest values.

Dissolved oxygen values in all systems were observed to be within acceptable limits for the duration of the study (Figure 17). ANOVA results (n = 172) indicated significant differences between systems. Pairwise comparison showed only a difference between systems 2 and 3 ($p = 0.0130$), while pairs 1-2 and 1-3 were not significantly different ($p = 0.6200$ and $p = 0.1353$, respectively).

Water temperature

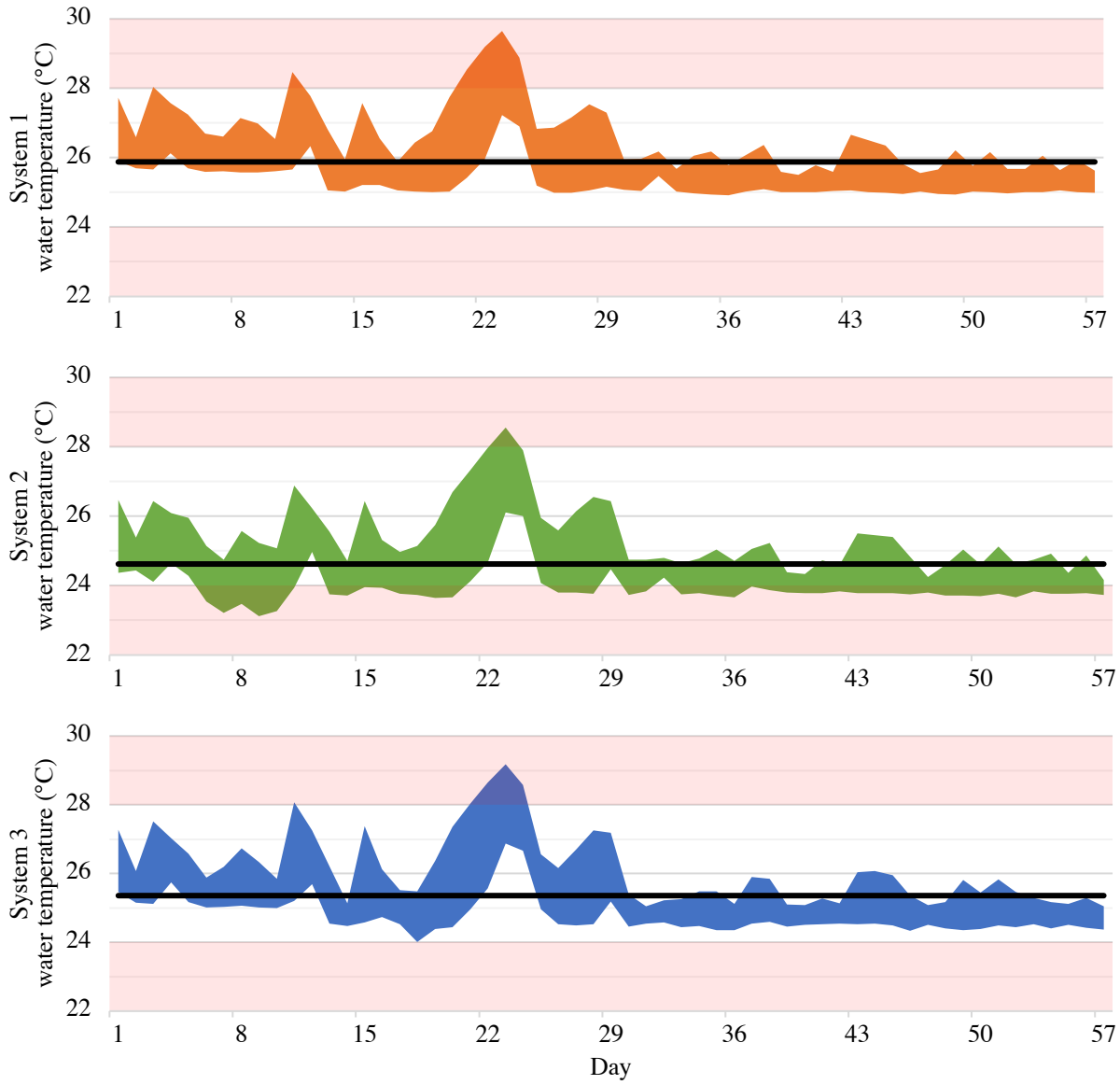

Figure 18. Daily water temperature ranges for KFRAG systems 1, 2, and 3. The top of the plotted area indicates daily temperature maximums, while the bottom represents daily minimums. Mean values for each system are shown with black lines. Red shading indicates areas outside the target temperature range of 24 – 28 °C.

For most of the study period, water temperatures were kept within the target range of 24 – 28 °C (Figure 18). System 2 was the only one to dip below the acceptable minimum, which happened on most nights (n = 41) when ambient air temperatures were lowest during the study. Water temperature in system 2 was below the 24 °C threshold for 26.0% of measurements (n =

1,283), with a minimum water temperature of 23.1 °C, which still falls between the optimal values for fish and plant health. The longest duration of temperatures below 24 °C in system 2 was 12 hours. Temperatures above our upper threshold of 28 °C were less common and occurred entirely in September when ambient air temperatures were high. Systems 1, 2, and 3 were above 28 °C for 4.4, 0.7, and 2.3% of measurements, respectively. The longest duration of temperatures exceeding the upper threshold was 18.25 hours.

Mean temperatures for systems 1, 2, and 3 (26.0, 24.7, and 25.4 °C, respectively) were each within 1 °C of the set point of 25.5 °C and were significantly different ($p = <0.0001$; $n = 14,790$). Since there was no active water-cooling mechanism in the system design, temperatures above the acceptable range were not adjusted.

Alkalinity and pH

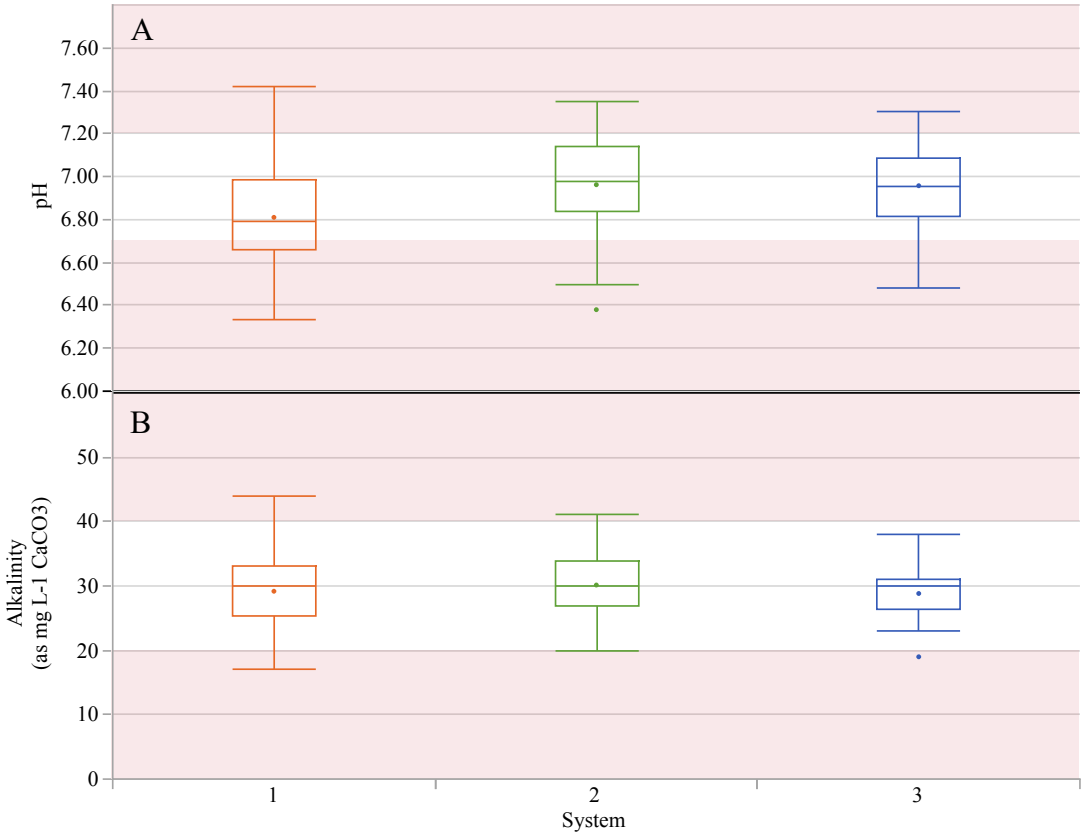

Figure 19. A) pH and B) alkalinity in KFRAG systems 1, 2, and 3. Red shaded areas are outside the target ranges of 6.7 – 7.2 and 20 – 40 mg L^{-1}, respectively. Horizontal lines in the centers of boxplots indicate median values and points within the boxes indicate means. The top and bottom of each box represent the third and first quartile, respectively, and whiskers indicate the highest and lowest values, excepting outliers indicated by dots.

pH values (Figure 19A) were significantly different between all systems ($p = 0.0002$; $n = 180$) but were similar in a pairwise comparison between systems 2 and 3 ($p = 0.9880$). pH values were within the acceptable range most of the time but exceeded the maximum threshold 3.3, 5.0, and 3.3% of the time and the minimum threshold 22, 6.6, and 3.3% of the time in systems 1, 2, and 3, respectively. Mean alkalinity (Figure 19B) did not differ between systems ($p = 0.1764$; $n = 180$), despite differences in the range of values. Alkalinity, which was controlled through K_2CO_3 addition, remained within the acceptable range for the majority of the study period, with

greater departures from that range in system 1. Similarly, the pH in system 1 had the greatest range. There was a previously described mechanical failure in system 1 midway through the study that caused significant water loss and an increase in alkalinity and increased variability in pH due to the hardness of the source water.

Electrical conductivity and iron

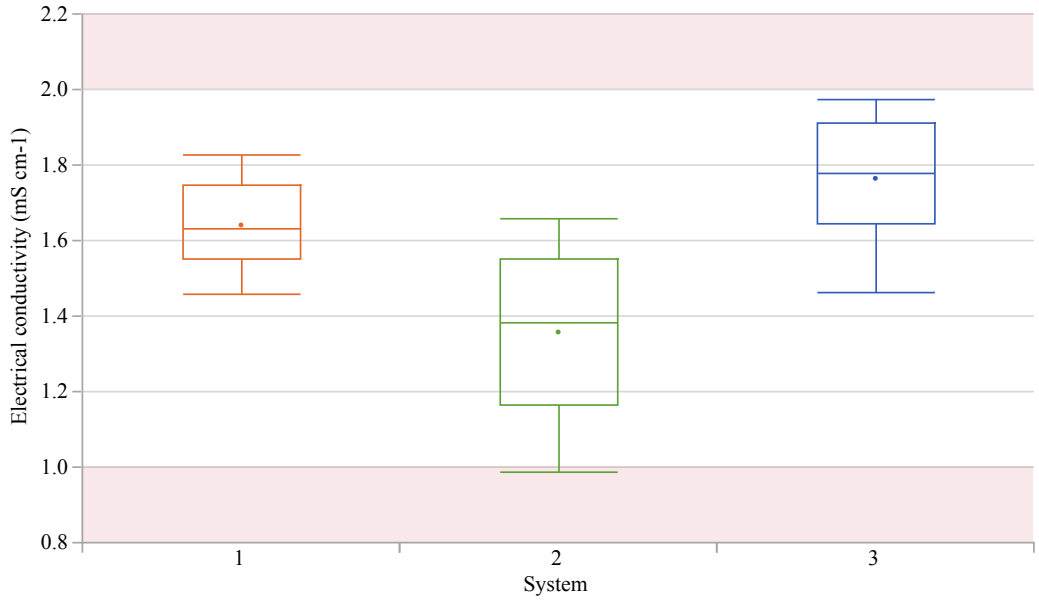

Figure 20. Electrical conductivity (EC) in KFRAG systems 1, 2, and 3. Horizontal lines in the centers of boxplots indicate median values and points within the boxes indicate means. The top and bottom of each box represent the third and first quartile, respectively, and whiskers indicate the highest and lowest values.

Means for EC were significantly different between all systems ($p < 0.0001$; $n = 172$) but were within acceptable range for the vast majority of the study period (Figure 20). Only 1.7% of measurements for EC in system 2 ($n = 1$) fell below the minimum threshold. No significant differences were observed for iron concentration ($p = 0.3034$; $n = 98$), which was managed by addition of chelated iron. Iron values were within the acceptable range for the duration of the study in all three systems (Figure 21).

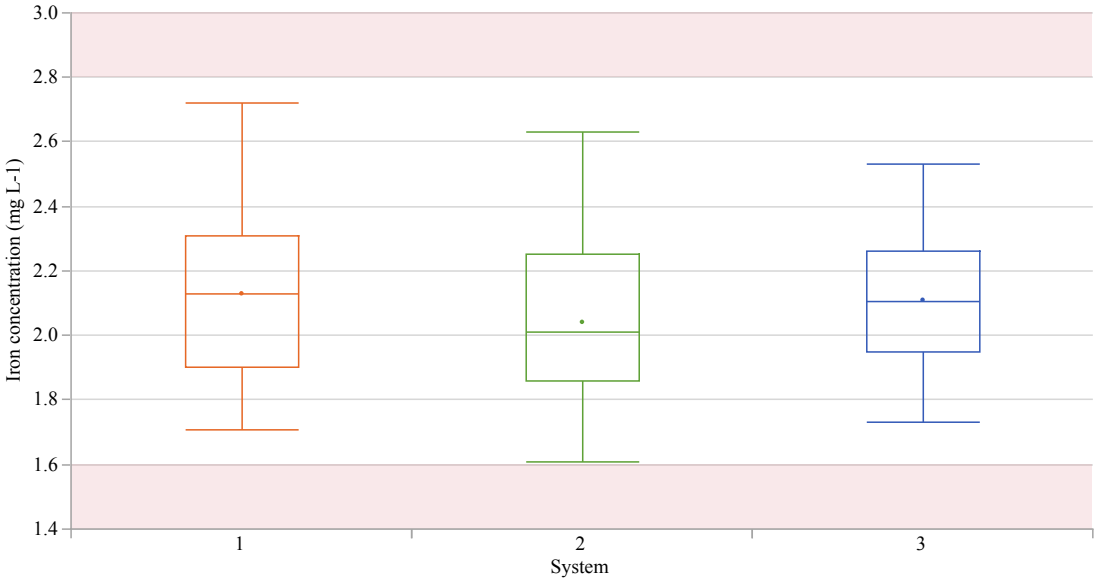

Figure 21. Iron concentrations in KFRAG systems 1, 2, and 3. Horizontal lines in the centers of boxplots indicate median values and points within the boxes indicate means. The top and bottom of each box represent the third and first quartile, respectively, and whiskers indicate the highest and lowest values.

Nitrogen species

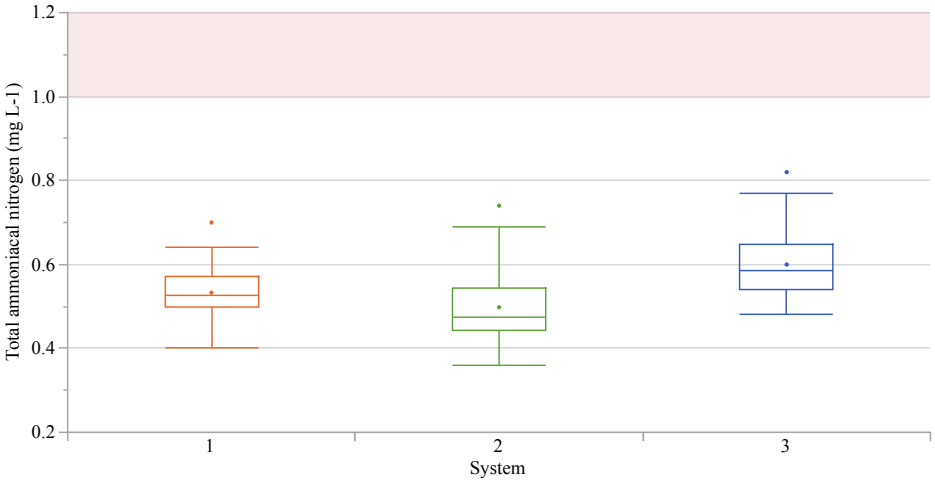

Figure 22. Total ammoniacal nitrogen concentration in KFRAG systems 1, 2, and 3. Red shaded area is above the target threshold of 1.0 mg L^{-1}. Horizontal lines in the centers of boxplots indicate median values and points within the boxes indicate means. The top and bottom of each box represent the third and first quartile, respectively, and whiskers indicate the highest and lowest values, excepting outliers indicated by dots.

Total ammoniacal nitrogen concentrations (n = 96) remained below the target maximum of 1.0 mg L-1 in all systems for the duration of the study (Figure 22). While the majority of nitrite measurements (n = 99) fell below the target maximum of 0.1 mg L-1, there were values that exceeded this threshold (Figure 23). In system 1, 3% of nitrite measurements (n = 1) were greater than 0.01 mg L-1, and in system 2 18% exceeded the threshold (n = 6). For both TAN and nitrite, system means were significantly different ($p < 0.0001$). However, pairwise comparison of systems 1 and 2 did not show a difference for TAN ($p = 0.1984$), and systems 2 and 3 had similar means for nitrite ($p = 0.8869$).

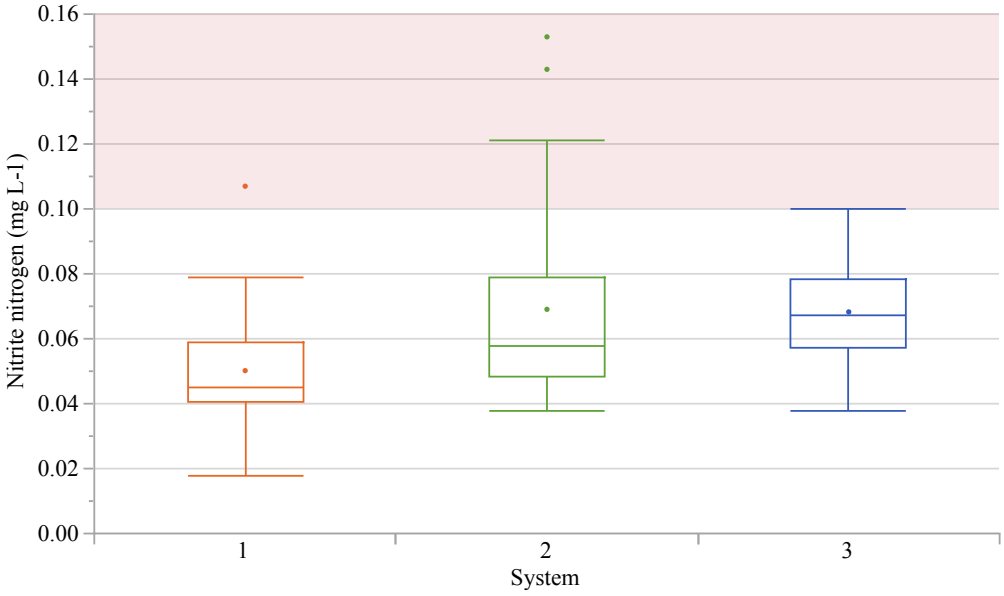

Figure 23. Nitrite nitrogen concentration in KFRAG systems 1, 2, and 3. Red shaded area is above the target threshold of 0.1 mg L^{-1}. Horizontal lines in the centers of boxplots indicate median values and points within the boxes indicate means. The top and bottom of each box represent the third and first quartile, respectively, and whiskers indicate the highest and lowest values, excepting outliers indicated by dots.

DOC, TDN, and nitrification efficiency

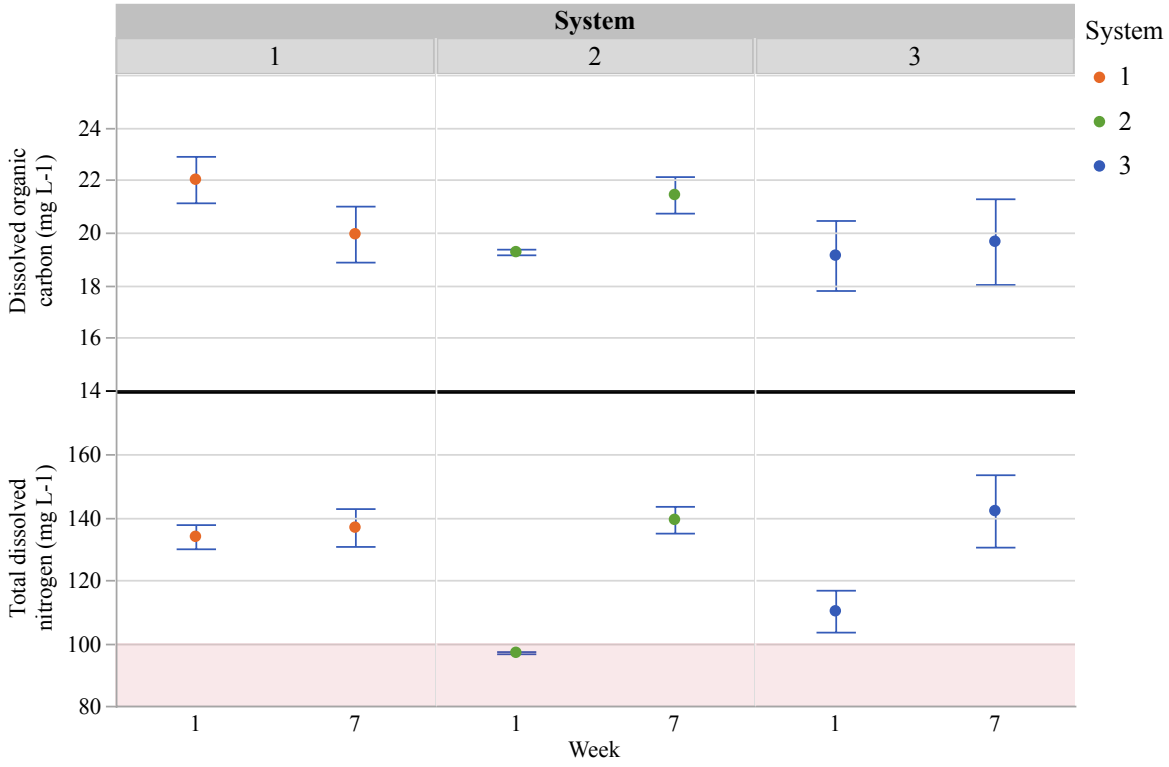

Figure 24. Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) in KFRAG systems 1, 2, and 3 at the beginning and end of the study period. Error bars indicate standard error.

Concentrations of dissolved organic carbon (DOC) in the KFRAG systems ranged from 14.6 to 26.0 mg L-1 (n = 48), and concentrations of total dissolved nitrogen (TDN) ranged from 84.9 to 184 mg L-1 (n = 48; Figure 24). System 2 started the study with TDN concentrations below our acceptable range for nitrate (a component of TDN) but reached the target range by the end. Mean concentrations that included both weeks of measurement were not significantly different between systems for DOC or TDN ($p = 0.2686$ and $p = 0.1457$, respectively).

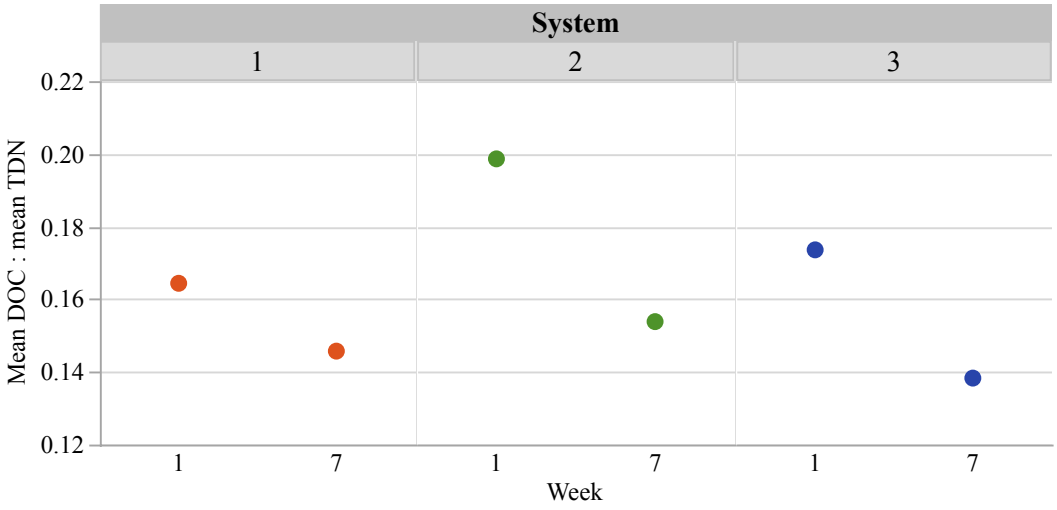

Figure 25. Ratio of mean dissolved organic carbon (DOC) to mean total dissolved nitrogen (TDN) in the KFRAG systems at weeks 1 and 7.

In all systems the ratio of DOC to TDN (DOC:TDN) decreased from week 1 to week 7, but the overall range of DOC:TDN values was small (0.13 – 0.20; Figure 25) and fell below the target threshold of 0.5. In systems 2 and 3, the decrease in DOC:TDN over time was driven by an increase in mean N concentrations. This accumulation of TDN indicates an imbalance between N inputs and plant uptake over the study period. Though System 1 started with the highest TDN concentration, it did not see overall TDN accumulation from week 1 to 7 due to the previously described mechanical failure; accumulation of TDN until the failure point was not captured by our measurements. Thus in system 1, the smaller decrease in DOC:TDN was driven by a decrease in DOC through accidental water loss. Nitrification efficiency in systems 1, 2, and 3 was 99.6%, 99.5%, and 99.5%, respectively, indicating near-complete conversion of TAN to NO_3.

Discussion

Aquaponic system performance

Water quality in the UNH KFRAG systems remained within acceptable ranges for DO, water temperature, N species, pH, alkalinity, and DOC:TDN for the majority of the 7-week study period. Other researchers have observed low DO in systems with high stocking densities, poor aeration, and accumulation of organic material in the hydroponic unit (Espinosa Moya et al., 2016; Ngo Thuy Diem et al., 2017; Nhan et al., 2019). These problems were addressed in the KFRAG systems through both system design and operational protocols, particularly aeration throughout the systems and rapid solids removal from both fish and plant units.

Ambient air temperatures play a critical role in influencing plant physiology and evapotranspirative water dynamics in aquaponics. Elevated ambient air temperatures in the greenhouses caused daily maximum water temperatures to exceed the target range for a few days in September. These water temperatures were observed to be within the optimal range for tilapia growth but were a concern because of the potential for short-term negative impacts on the lettuce. However, no temperature effects on lettuce health were observed. Prolonged periods of elevated water temperatures could be mitigated by installing chillers in the pump sumps. Temperatures below the target range were only observed in one system, and this consistent observation was likely the result of incidental and temporary operational aberrations. These results are encouraging because it provides useful information about temperature tolerance for lettuce in aquaponics.

High or low pH conditions can have negative effects on plant nutrient availability and nitrification rates. Compared to other parameters, pH deviated more often from our target acceptable range of 6.7 to 7.2, but this was expected for a parameter that is affected by many

biological and physicochemical variables within the system. In this study, pH ranged from 6.3 to 7.4. pH was not measured in the root zone, however, which is naturally acidified as a byproduct of anion uptake. Low pH could negatively affect biofilter function, however TAN values indicated that nitrification was occurring at appropriate rates for maintaining optimal system health. Resilience of biofilter function was likely supported by a well-established and resilient biofilm of nitrifying bacteria in the biofilter itself and on other surfaces within the systems. Since TAN—the nitrogen species most toxic to fish—remained within the target range throughout the study, occasional high nitrite measurements were not of concern. Lettuce yield was not significantly different between systems ($p = 0.060$, data not shown) and fish mortality was low, reflecting physicochemically appropriate water quality and adequate water quality management.

In a biofilter, heterotrophic and autotrophic bacteria coexist in biofilms on the surfaces of the biofilter media (Ohashi et al., 1995). These organisms compete for space and oxygen, and when particulate or dissolved organic C is available in the water, faster-growing heterotrophs form an outer layer to the biofilm that reduces the diffusion rate of nitrogenous substrates and oxygen to the underlying nitrifying autotrophs (Chen et al., 2006; Michaud et al., 2006; Nogueira et al., 2002; Satoh et al., 2000). The weight ratio of DOC to TAN (DOC:TAN) in bench-scale biofilter influent has been manipulated in previous studies to investigate the impact of this heterotrophic competition phenomenon on biofilter performance, i.e. TAN removal rate or nitrification efficiency. However, these were laboratory experiments, for the most part utilizing synthetic biofilter inputs where all of the organic C was either dissolved sucrose or acetate, and thus did not reflect the complexity of C sources in actual RAS production conditions. For this reason, DOC = total organic C (TOC) in these lab studies. Optimal nitrification efficiency was consistently observed when influent was not amended with organic C (e.g., sucrose or acetate),

with rapid reductions in efficiency as the ratio of amended DOC:TAN increased from 0 to 2 (Ling & Chen, 2005; Nogueira et al., 2002; Satoh et al., 2000; Zhu & Chen, 2001b). The only published studies to manipulate organic C under actual production conditions were by Guerdat et al., utilizing three 60 m^3 tilapia RAS systems stocked with about 5,000 fish each. These systems were first operated for approximately one year under normal operating conditions (NOC) to establish baselines for water quality parameters (Guerdat et al., 2010). Lower TAN removal rates under NOC established that lab-scale studies were not representative of conditions in commercial RAS. After the NOC period, the systems were dosed with sucrose to increase DOC (Guerdat et al., 2011). While DOC was manipulated in the second study in order to compare results to previous work, total organic C (TOC) was measured and reported in water quality summary statistics. This revealed that mean TOC:TAN in the actual RAS was 36.9 under NOC and 79.4 with sucrose amendment, much higher than the ratios studied at lab scale. We observed mean DOC:TAN at KFRAG (32.4 – 41.0) in accordance with those NOC conditions. The similarity between Guerdat's TOC:TAN and our DOC:TAN was likely because the Guerdat systems utilized 40µm micromesh drum screen filters, similar to the 56µm mechanical filters at KFRAG. The KFRAG nitrification efficiencies of > 99 % (Figure 26C) confirmed that that biofilters were operating at maximum efficiency under this solids management regime.

In addition to biofilter effects, achieving a low C:N ratio reduces biological oxygen demand from heterotrophs in the bulk water column, helping to ensure sufficient DO in the root zone for optimal plant vigor. A low C:N ratio is also desirable for other reasons. Higher C:N ratios—between 0.5 and 4—have been correlated with reduced bacterial species richness and diversity in lab-scale RAS biofilters (Michaud et al., 2014) and in the coconut coir medium of strawberries fertigated with a synthetic nutrient solution (Hardesty-Dyck, 2020). This is

important because Stouvanakers et al. (2020) found that suppression of the lettuce root pathogen *Pythium aphanidermatum* on plants grown in aquaponic water was positively correlated with fungal and bacterial diversity in the rhizoplane. Together, these results suggest that a low C:N in the recirculating water is advantageous to productivity due to the impact on microbial diversity and, consequently, pathogen pressure and nitrification efficiency.

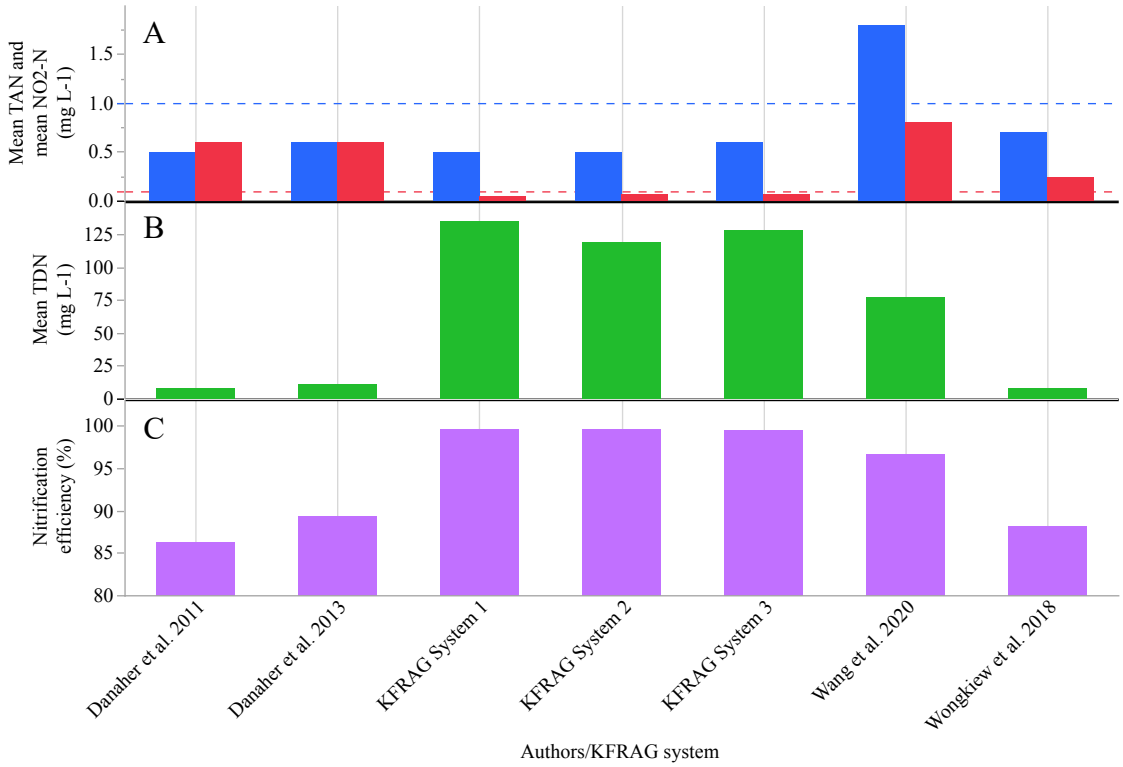

Figure 26. Mean total ammoniacal nitrogen (TAN) and mean nitrite-nitrogen (NO_2-N) (A), mean total dissolved nitrogen (TDN) (B), and nitrification efficiency (C) reported in KFRAG and other experimental aquaponic systems. Dashed lines in (A) represent upper thresholds used in this study for TAN (blue) and NO_2-N (red). Nitrification efficiency was calculated as nitrate-nitrogen (not shown) divided by TDN. Data from Danaher et al. 2011 and 2013, Wang et al. 2020, and Wongkiew et al. 2018.

The constraints of the KFRAG facility, did not allow for manipulation of DOC in order to compare nitrification efficiency at different C:N ratios. However, several other experimental systems utilizing the UVI design can be inferred to have had higher circulating DOC based on

the fact that waste solids were intentionally retained within the systems (Danaher et al., 2011, 2013; Y.-J. Wang et al., 2020; Wongkiew et al., 2018). No data on DOC were reported, but the overall C/N balance is reflected in the nitrification efficiency of each system (Figure 26C). Though these other systems had much lower TDN concentrations than the KFRAG systems (B), mean TAN and NO_2-N concentrations (A) were generally at or above those achieved at KFRAG. Thus, the rate at which TAN was converted to NO_3 was lower in these systems than at KFRAG.

This study yielded useful information about the acceptability of some degree of deviations in water temperature and pH from desired ranges. Brief periods of higher water temperatures (up to 1.7 °C above our optimal range) did not negatively impact lettuce growth, while occasional low pH (up to 0.4 units below our optimal range) had no noticeable effect on nitrification efficiency. Active water chilling in temperate aquaponic systems, which are already energy-intensive, may be undesirable, so producers benefit from a better understanding of temperature tolerances for crops in aquaponics. The resilience of the nitrifying bacterial community to pH lower than the optimal range could be related to the C-limited environment and possibly to overall bacterial diversity. Future research should investigate the link between C:N and productivity in aquaponics in lab-scale systems where other water quality parameters can be manipulated to determine optimal ranges. Information about the magnitude of DOC effects on plant, fish, and microbial performance, and the possible interactions of these effects with other water quality parameters, would help producers make important operational decisions around water quality and carbon management.

Chapter 3:

Culture-based Pathogen Detection in Aquaponic Production Water

Introduction

Outbreaks of foodborne disease (FBD) associated with enteric pathogens and the consumption of fresh produce have been increasing in recent decades (Deering et al., 2012; Hoagland et al., 2018; Painter et al., 2013; Solomon et al., 2002; Strawn et al., 2013). Produce can become contaminated at any point in the supply chain from seed to table, but a major source of human pathogens that are associated with FBD is contaminated production water, either as fecal runoff from animal agriculture operations (Levantesi et al., 2012; Steele & Odumeru, 2004), or contaminated surface waters utilized for irrigation or other production activities. In field crops, precipitation can splash contaminated soil particles up onto the edible portions of crops (Lee et al., 2019; Monaghan & Hutchison, 2012). In hydroponic systems, contaminated irrigation water may come into contact with the edible portion of a crop during production or at harvest.

Aquaponics combines two well-studied models—recirculating aquaculture systems (RAS) and hydroponics—into a multitrophic food production system for fish, fruits, and vegetables (Timmons et al., 2018). The integration of aquaculture with hydroponics allows for the utilization of nitrogenous fish wastes as fertilizer, monetizing the waste stream. Of fish, fruits, and vegetables, cultivated vegetables carry by far the greatest risk of foodborne illness

(Painter et al., 2013). Leafy greens that are typically consumed raw are particularly well-suited to aquaponic system designs but are also the food agent responsible for nearly two-thirds of foodborne illnesses that are attributed to vegetables. In contrast to fresh produce, fewer than 3% of foodborne illnesses are attributed to fish (Painter et al., 2013). For this reason, fresh produce is of greater food safety concern than fish in aquaponics (Table 3).

Table 3. Estimated annual attributions of domestically acquired foodborne illnesses to fish and produce. Estimates were derived from data from 2000 – 2008. Adapted from Painter et al., 2013..

Commodity or commodity group	*# of illnesses (% of total illnesses)*
Fish	258,314 (2.7)
Produce†	4,423,310 (45.9)
Fruits & nuts	1,123,808 (11.7)
Vegetables†	3,299,501 (34.2)
Leafy	2,152,652 (22.3)
Vine-stalk	759,889 (7.9)
Root	349,715 (3.6)
Sprout	32,703 (0.3)
Fungi	4,542 (0.0)

† Indicates a commodity group.

Produce safety is federally regulated in the United States under the Food Safety Modernization Act (FSMA) Produce Safety Regulation (PSR), administered and enforced by the US Food and Drug Administration (USFDA). The FSMA PSR regulates water that is used in agricultural production if that water is intended or likely to come into contact with the edible portion of a crop. While the rule has not been finalized and USFDA has extended compliance dates to 2022 (FDA, 2019), there are proposed requirements to consider, including two primary regulatory hurdles for aquaponic producers. First, microbial water quality in agricultural production water is regulated based on concentrations of *Escherichia coli*, which may include

pathogenic and/or non-pathogenic strains and is utilized as an indicator of fecal contamination. The regulatory standard (PSR Subpart E) requires growers to establish a microbial water quality profile (MWQP) for total *E. coli* concentrations over time, with the frequency of testing dependent upon water source and its potential for contamination—surface, or municipal. Water that is drawn from its source and then held, as in a holding pond, becomes "surface" water under the rule, triggering the requirement for the greatest testing frequency (Food Safety Modernization Act, 2011; *Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption*, 2015). The classification of recirculating water in an aquaponic system is unclear, pending the finalized PSR and subsequent guidance from USFDA.

The second regulatory hurdle for producers (PSR Subpart F) is that the PSR places restrictions on the use of soil amendments, in particular biological soil amendments of animal origin (BSAAO). This category includes amendments such as manure, fishmeal, and compost containing animal waste or parts. The FDA has clarified that for hydroponic and aquaponic systems where the plant growing medium is a liquid matrix, as in deep water culture (DWC) or nutrient film technique (NFT) systems, that water will be subject to the microbial standard for production water as above. However, it is unclear how the BSAAO rule applies to systems where the plants are grown in a solid or semisolid medium such as potting mix and fertigated with aquaponic production water. Aquaponic producers utilize both types of systems (Figure 27). In the latter case, aquaponic production water could be classified as an "agricultural tea" made with un-composted manure. Under the law, this agricultural tea or its components must undergo scientifically validated treatment processes to reduce the concentration of *Listeria monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7. These three organisms are used as indicators of contamination for regulatory purposes as they are also significant food safety

concerns in the US (Table 4). Theoretically, an aquaponic system design that met these standards for waste treatment could qualify as a scientifically validated treatment process, allowing unrestricted use of the culture water in the production of fresh fruits and vegetables.

Figure 27. Two different aquaponic scenarios: Lettuce in deep water culture beds (far left) and "drip-to-drain" strawberries in potting media fertigated with aquaponic production water (near right).

Table 4. Number of domestically acquired foodborne cases, hospitalizations, and deaths attributed to major foodborne pathogens (those with ≥ 2,000 hospitalizations or ≥ 50 deaths annually). Estimated were derived from data from 2000 – 2008 using statistical models and based on the US population in 2006. Adapted from Scallan et al. (2011).

Pathogen	Pathogen type	Domestically-acquired foodborne illnesses	Hospitalizations (% of cases)[1]	Deaths (% of cases)
Salmonella spp.	Bacterial	1,029,382	19,533 (1.9)	378 (0.036)
Toxoplasma gondii	Parasitic	86,686	4,428 (5.1)	327 (0.37)
Listeria monocytogenes	Bacterial	1,591	1,455 (91)	255 (16)
Norovirus	Viral	5,461,731	14,663 (0.27)	149 (0.0027)
Campylobacter spp.	Bacterial	845,024	8,463 (1.0)	76 (0.0090)
Escherichia coli	Bacterial	205,781	2,429 (1.2)	20 (0.0097)

[1] Hospitalization and death percentages were calculated by dividing the number of hospitalizations by the mean estimated number of cases and differ from reported hospitalization and death rates for laboratory-confirmed cases.

Well-designed aquaponic systems that use potable source water present a low direct risk for produce safety if biosecurity and farm hygiene are maintained. Biosecurity in aquaponics includes the use of sanitary foot baths or boot covers and quarantining fish before their introduction into the system. Farm hygiene includes worker training and hygiene, regular cleaning and sanitization of tools and equipment, and harvest practices that minimize food safety risk. The detection of fecal contamination in aquaponic operations would most likely indicate the use of contaminated source water, incursion of wildlife, or poor enforcement of biosecurity and farm hygiene measures. However, due to the novelty of aquaponics and lack of standardization, the level of risk associated with recirculation of water containing fish feces—a source of a unique microbial community and labile carbon—is unknown.

Early research of commercial aquaponic systems in Hawaii found low levels of *E. coli* in the culture water of most systems but did not detect *E. coli* or *Salmonella* spp. in samples of produce or fish tissue collected and analyzed over a one-year period (Fox et al., 2012). Samples with significant *E. coli* concentrations were attributed to farms that utilized poor-quality source water. Subsequent studies focused heavily on the potential for UV sterilization to reduce contaminant loads in aquaponic production water, with mixed results depending on the environment and specific system design (Elumalai et al., 2017; Moriarty et al., 2018; Pantanella et al., 2015).

Moriarty (2018) and Wang (2020) both found *E. coli* in their experimental aquaponic and hydroponic systems but did not observe internalization—uptake of microbes through plant roots and translocation to the leaves—of *E. coli* by lettuce plants. In a follow-up hydroponic study, Moriarty (2019) reported internalization of *E. coli* O157:H7 at 2.4 – 4.0 log CFU g^{-1} when irrigation water was spiked with 5.3 – 6.0 log CFU mL^{-1}. These recent results suggest that

internalization of *E. coli* by lettuce in most aquaponics systems is possible, although unlikely in systems that typically have low *E. coli* concentrations. Wang (2020) demonstrated the importance of biosecurity and farm hygiene through their observations of Shiga toxin-producing *E. coli* (STEC) in both hydroponic and aquaponic systems and in all sampled locations except within lettuce tissue. STEC was also found in fish-only systems containing the same cohort of fish. The authors' investigation concluded that the tilapia in these systems were likely contaminated through handling during the 10-mile transport process from their source to the lab-scale systems (Y.-J. Wang et al., 2021). The subsequent findings suggest that fish feces were then a vehicle for spread of STEC throughout the experimental systems, though source tracking was not within the scope of the study. Since no STEC was observed in the edible portions of the hydroponic and aquaponic plants, this serves as further evidence for the importance of separating the production water from edible portions of crops as recommended by the PSR.

As aquaponic food production expands, it is important to understand the food safety hazards and potential critical control points in large-scale systems. The University of New Hampshire (UNH) Kingman Farm Aquaponic Greenhouses (KFRAG) were established to evaluate a new system design for optimal plant and fish growth that addresses some of the weaknesses of previous experimental systems. The objective of this study was to determine whether the UNH KFRAG systems were susceptible to the presence or conducive to the proliferation of common foodborne pathogen species and fecal indicator bacteria. Culture-based assays were utilized to detect *E. coli*, pathogenic *Listeria* spp., and *Salmonella* spp. in production water from throughout the KFRAG systems to investigate potential unit process effects on pathogen presence.

Methods

Experimental recirculating aquaponic systems

Figure 28. Aerial view of the University of New Hampshire Kingman Farm Recirculating Aquaponic Greenhouses (UNH KFRAG), with adjacent outbuildings and active research farmland. Photo credit: UNH Media

This study was conducted at the UNH KFRAG in September and October 2019 (Figure 14). At the beginning of the study period, each of the greenhouse-scale aquaponic systems had been in lettuce (*Lactuca sativa* cv. 'Rex') and tilapia (*Oreochromis* spp.) co-production for at least one year. KFRAG system design and operation, including biosecurity protocols and food-safe harvest practices, are described in detail in Chapter 2.

The KFRAG food safety program consisted of two primary arms: system design and operational protocols. System design priorities included efficient solids removal and aeration in every unit process. A mechanical drum screen was utilized to filter effluent from the fish and

plant units (Figure 16), removing particulate organic matter that could serve as a carbon source to potentially alter the microbial community and support unwanted microorganisms, including pathogens. Aeration in every unit process served a dual purpose: 1) to oxygenate the water and 2) to add turbulence, increasing mixing in the water column. Both of these functions helped to reduce the occurrence of anaerobic conditions anywhere in the systems. Filtered culture water was circulated to the biofilter on a side loop, ensuring the conversion of potentially toxic nitrogenous wastes to non-toxic nitrate for maintaining fish health. Operational protocols contributing to food safety included biosecurity measures and daily water quality monitoring and adjustment to maintain optimal ecosystem health. These measures were intended to reduce pathogen pressure on the fish and plants, but also reduced the probability of the culture water becoming a reservoir for incidentally introduced human pathogens by instead creating a stable microbial ecosystem in which all critical conditions and processes were managed.

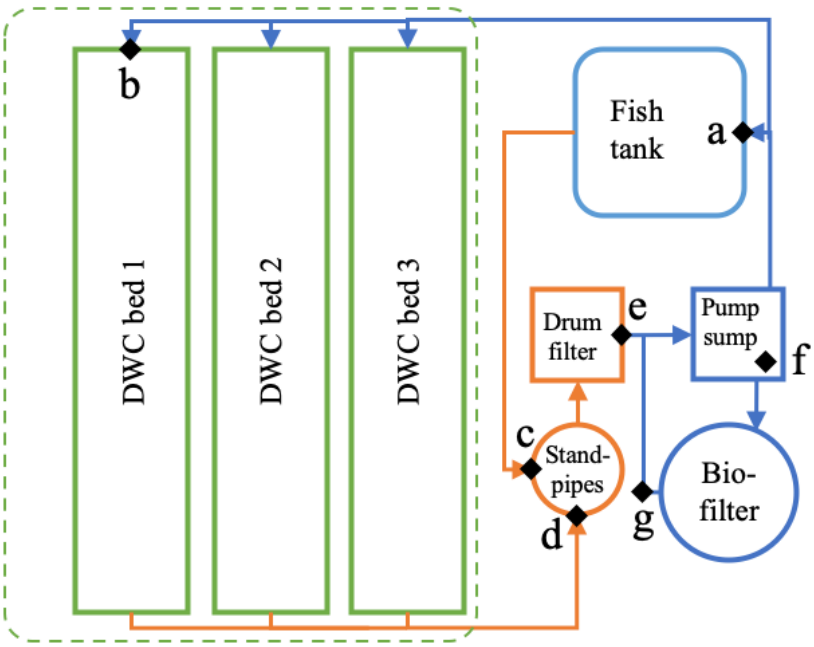

Figure 29. UNH KFRAG coupled aquaponic process flow. Orange lines indicate fish and plant unit effluent prior to treatment through the mechanical drum filter. Water sampling sites are indicated with black diamonds: a) fish tank inlet, b) plant bed inlet, c) fish tank outlet, d) plant bed outlet, e) drum filter outlet, f) pump sump, and g) biofilter outlet.

Sample collection

Production water samples were collected from each of the 3 UNH KFRAG systems weekly during September and October 2019. Sites for grab sample collection were chosen to isolate possible variation in pathogen presence across unit processes (Figure 16). For the fish and plant units, samples were collected at both the inlet manifolds and the unit outlets in the standpipe well. The farthest plant bed inlet from the sump (the inlet for DWC bed 1) was chosen to capture effects of the culture water distribution system. Outlet samples from the fish and plant units were considered as inlet samples for the mechanical drum filter, while samples from the sump were considered inlet samples for the biofilter. Biofilter outlet samples were collected from an open section of pipe in the return line to the sump. The eight sites within each greenhouse were sampled immediately prior to the routine system disturbance caused by harvest each week, to reflect steady-state conditions. Sample water was collected in autoclaved 1 L HDPE bottles (Nalgene, Rochester, New York) using aseptic technique, then stored in an insulated container on ice for ≤6 hours prior to analysis per EPA Method 1603 sample storage protocols. See Appendix A for the full sample collection protocol.

The sample collection schedule with which bacterial assays were conducted is detailed in Table 5. To verify the representative nature of sample collection at each site after Week 3, a duplicate sample was collected and processed identically to the others.

Table 5. Water sampling schedule for pathogen and indicator organism detection in UNH KFRAG production water. mTEC' = modified membrane-thermotolerant E. coli *agar, Brilliance = Oxoid chromogenic Brilliance™* Listeria *agar, and XLD = xylose lysine deoxycholate agar.*

Week	System	N	Pathogen detection media	Organism(s) of interest	Duplicate sample(s)
1	1	8	mTEC'	*E. coli*	none
	2	8	mTEC'	*E. coli*	none
	3	8	mTEC'	*E. coli*	none
2	1	8	mTEC', Brilliance	*E. coli*, *Listeria* spp.	none
	2	8	mTEC', Brilliance	*E. coli, Listeria* spp.	none
	3	7	mTEC', Brilliance	*E. coli, Listeria* spp.	none
3	1	9	XLD	*Salmonella* spp.	Sump
	2	9	XLD	*Salmonella* spp.	Sump
	3	9	XLD	*Salmonella* spp.	Sump
4	1	9	mTEC', Brilliance	*E. coli, Listeria* spp.	Fish outlet
	2	9	mTEC', Brilliance	*E. coli, Listeria* spp.	Fish outlet
	3	8	mTEC', Brilliance	*E. coli, Listeria* spp.	Fish outlet
5	1	9	XLD	*Salmonella* spp.	Plant inlet
	2	9	XLD	*Salmonella* spp.	Plant inlet
	3	8	XLD	*Salmonella* spp.	Plant inlet
6	1	9	mTEC', Brilliance	*E. coli, Listeria* spp.	Plant outlet
	2	4	mTEC', Brilliance	*E. coli, Listeria* spp.	none
	3	8	mTEC', Brilliance	*E. coli, Listeria* spp.	Plant outlet
7	1	9	XLD	*Salmonella* spp.	Sump, fish outlet
	2	9	XLD	*Salmonella* spp.	Sump
	3	9	XLD	*Salmonella* spp.	Sump, fish outlet
	Total n =	174			

Physical and chemical water quality analysis

Water quality was monitored daily at UNH KFRAG to inform the maintenance of a stable aquatic environment with optimal conditions for fish, plants, and nitrifying microbes (see Chapter 2). Temperature, dissolved oxygen (DO), and electrical conductivity (EC) were measured daily in the fish effluent standpipe with a YSI Pro2030 instrument (YSI Inc., Yellow

Springs, Ohio). Pump sump samples from each system were also analyzed daily for pH and alkalinity using an Accumet™ AB150 benchtop pH meter (Fisher Scientific, Pittsburg, Pennsylvania; Method 2320 B, American Public Health Association, 2012). Nitrite, ammonia-nitrogen, and iron concentrations were measured four times per week by EPA methods 8507, 8038, and 8008, respectively, using a DR3900 spectrophotometer (HACH Company, Loveland, Colorado).

Culture water was filtered for microbial analysis, as described below. A 50 mL aliquot of the filtrate was stored at -20 °C. Filtered sample water from the first and last weeks of the study was submitted to USDA Forest Service (Durham, NH) for analysis of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN). DOC was measured by high-temperature catalytic oxidation (HTCO) (Method 5310 B, American Public Health Association, 2012), and TDN was measured by HTCO with chemiluminescent N detection (ASTM International, 2016).

Bacterial culture and enumeration

Samples were transported on ice to the UNH Jackson Estuarine Laboratory (Durham, NH) for bacterial culture assays. Prior to filtration, samples were inverted manually 30 times to evenly distribute bacteria. For each assay, a 100 mL aliquot of culture water was filtered under vacuum (<15 mm Hg) using a 47 mm sterile membrane filter of 0.45 µm pore diameter (Sigma-Aldrich, St. Louis, Missouri). These filters were plated directly using flame-sterilized forceps to agar media appropriate for detection of each organism of interest or used for enrichment processes prior to plating. See Appendices B – E for the full protocols for sample filtration and each microbial analysis.

Escherichia coli analysis (Appendix C)

Assays for *E. coli* were conducted using modified membrane-thermotolerant *E. coli* (mTEC) agar (adapted from Dufour et al., 1981). Filters were placed directly on modified mTEC (Difco Laboratories, Detroit, Michigan), then incubated at 44.5 °C for 18 ± 2 h. Red/magenta colonies were enumerated as presumptive positive isolates of *E. coli*.

Listeria spp. analysis (Appendix D)

Listeria assays were conducted using chromogenic Brilliance™ *Listeria* agar (Oxoid, Hampshire, United Kingdom). This medium was selected for its ease of use, high specificity (96.5%), high sensitivity (92.0%), and high confirmation rate (98.7%) (Park et al., 2014). Filters were placed directly on Brilliance™ *Listeria* agar, then incubated at 34 °C for 22 ± 3 h. The protocol described by Lopez-Galvez et al. (2014) was modified to shorten the incubation time based on preliminary results, where overgrowth was observed after 48 h. Blue colonies were enumerated as presumptive positives for *Listeria* spp., while blue colonies with opaque white haloes of cleared media were enumerated as potential pathogens, specifically, presumptive positives for *Listeria monocytogenes* or pathogenic *L. ivanovii*.

Salmonella spp. analysis (Appendix E)

Salmonella assays were conducted using two enrichment steps followed by plating on xylose lysine deoxycholate (XLD) agar according to the FDA Bacteriological Analysis Manual (BAM; W. H. Andrews et al., 2018), as adapted by Li et al. (2014). Filters were folded radially 3 times using two pairs of flame-sterilized forceps and placed in tubes containing tryptic soy broth (TSB; Difco). Tubes were capped, vortexed at high speed for 30 s, and then incubated at 34 °C with constant shaking at 200 rpm for 24 ± 2 h. Then, 0.5 mL of pre-enriched TSB was added to a test tube containing 4.5 mL tetrathionate (TT) broth (Difco). TT tubes were incubated at 34 °C

with constant shaking at 200 rpm for 24 ± 2 h. A loopful of enriched TT broth was then streaked to XLD agar. XLD plates were incubated at 34 °C for 24 ± 2 h.

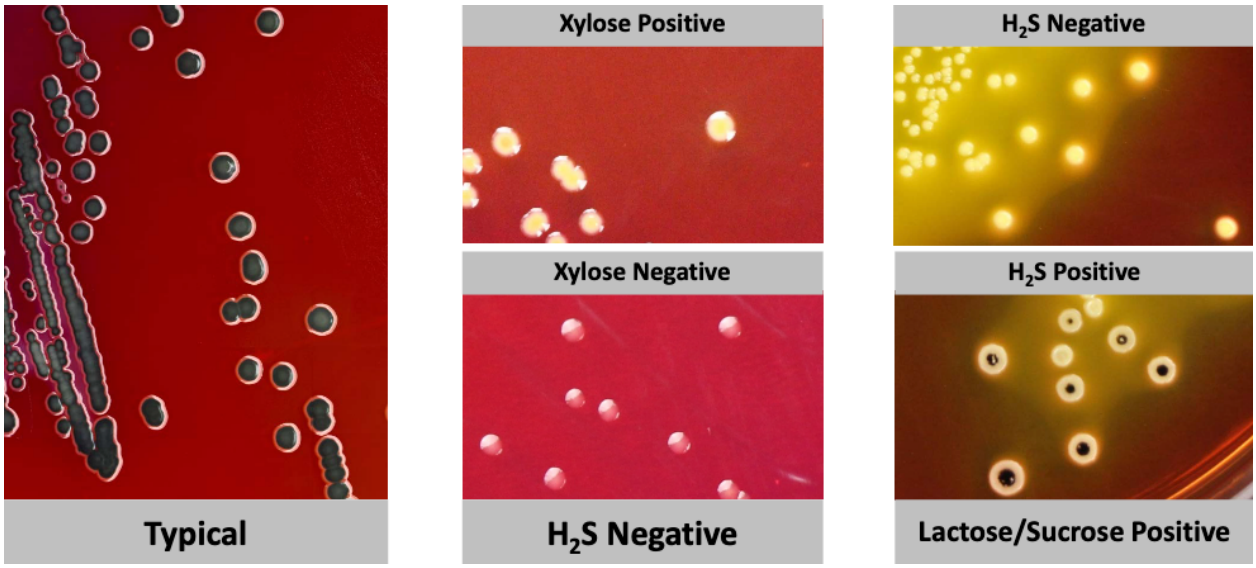

Figure 30. Five different colony morphologies can indicate Salmonella *spp. on xylose lysine deoxycholate agar. Adapted from Forstner (2016).*

Salmonella spp. can appear with five different morphologies on XLD agar (Figure 30). Presumptive positive colonies for Salmonella were identified according to the Bacteriological Analysis Manual (Andrews et al., 2018) and with the assistance of the USFDA Salmonella Flipbook (Forstner, 2016). All assays (n = 81) detected presumptive positive colonies. Since this detection method included two enrichment steps, concentrations of presumptive positives in the original sample were not determined. During weeks 5 and 7, eleven colonies representative of the five different observed morphologies were selected for isolation on tryptic soy agar (Difco). Four of these isolates originated from the sump tanks; two each from the plant bed inlets, fish tank inlets, and fish tank outlets; and one from source (well) water. The identities of these

isolates were confirmed by RapiD 20 E biochemical assays (BioMérieux, Marcy-l'Étoile, France), each consisting of 20 individual biochemical tests on a single-use strip.

The RapiD 20 E is used to quickly identify a bacterial isolate by comparing its biochemical profile to a database (APIweb) of 65 taxa containing enteric pathogens isolated from food and beverage, pharmaceutical, and clinical environments. For each taxon in the database, a profile was generated consisting of the frequency of positive and negative results for each biochemical test on the strip. The reference database for RapiD 20 E contains 7 *Salmonella* strains belonging to different species, subspecies, and serovars (*S. enterica* ssp. *arizonae, S. enterica* ssp. *enterica, S. serovar Gallinarum, S. ser. Paratyphi A, S. ser. Pullorum, S. ser. Typhi,* and *Salmonella* spp.).

RapiD 20 E strip results—in the form of negative or positive reactions for each of the 20 biochemical tests—were used to calculate 2 test statistics for each sample through the APIweb online platform. The first statistic, %ID, represents the relative spatial proximity of the sample profile to different taxa in the database in *n*-dimensional space, where *n* is the number of taxa in the database (Figure 31-1). Then, a T index is calculated which similarly reflects the proximity of the sample profile to the most typical profile for each taxon identified by %ID (Figure 31-2). APIweb finally assigns a comment to each set of results describing the quality of the identification based on %ID and T index (e.g., “Excellent identification” if %ID ≥ 99.9 and T ≥ 0.75). Identification is said to be “not reliable” if the sum of %ID values is less than 80.0.

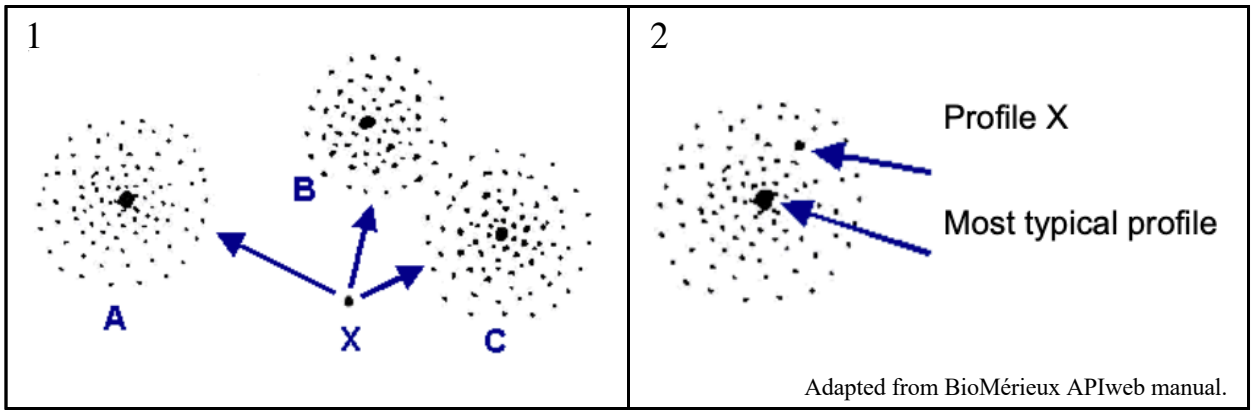

Figure 31. Abstract representation of APIweb methods for calculating %ID and T-index. 1) %ID indicates the relative proximity of the sample profile (X) to clusters of profiles for each taxon in the database (A, B, and C). 2) T-index indicates the proximity of the sample profile to the most typical profile for each taxon identified as a potential match in step 1.

Three additional *Salmonella* strains (*S. serovar Enteriditis, S. ser. Paratyphi B, and S. ser. Typhimurium*) that were not included in the RapiD 20 E database were included in the database for Bio-Mériuex's API 20 E platform, which was not used due to time constrains. In addition to saving time, Rapid 20 E outperformed API 20 E in manufacturer testing, correctly identifying 95.52% of 2,365 known test strains compared to 86.2% out of 5,544 known strains for API 20 E (BioMérieux, 2016). We also assumed that these other *Salmonella* strains, if present, would later be identified through DNA sequencing.

Bacterial isolate DNA extraction and sequencing

Isolate samples for DNA extractions were maintained on TSA by restreaking every 7 to 10 days. After each restreak, TSA plates were incubated at 34 °C for 22 ± 2 h, then wrapped in parafilm and stored at 4 °C. DNA was extracted from individual isolate colonies using Qiagen PowerSoil Pro kits on a Qiacube® Connect automated nucleic acid extraction platform (Qiagen, Venlo, Netherlands). Growth from an individual colony on each TSA plate was transferred with a sterile pipette tip into a bead tube and processed on the QiaCube according to Qiagen's

protocol. Negative controls containing no samples were included in each Qiacube run. A Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts) was utilized to quantify concentrations of extracted DNA, which ranged from 1.17 to 26.6 ng μL^{-1}. Samples were stored at -20 °C until sequencing.

DNA samples were submitted for whole genome sequencing at the Hubbard Center for Genome Studies (University of New Hampshire, Durham, NH) on an Illumina HiSeq2500 with Rapid Run© chemistries (Illumina, San Diego, CA). Each sample was diluted with nuclease-free water to a concentration of 1 ng μL^{-1} for preparation of Illumina TruSeq PCR-free DNA libraries. Reads were converted to fastq format and demultiplexed using bcl2fastq (v1.8.4, Illumina).

Bacterial isolate sequence analysis

Analysis of DNA sequences from XLD isolates was executed using an original python script to automate the steps outlined below (see Appendix F). Reads were checked for quality with FastQC (v0.11.9, Andrews, 2010) and an aggregated quality report generated using MultiQC (v1.8, Ewels et al., 2016). Trimmomatic (v0.38, Bolger et al., 2014) was used to remove adapter sequences, the first and last 3 nucleotides in each read, sequences with average quality scores below 15 in a sliding window of 4 nucleotides, and reads less than 36 nucleotides long. Trimmed reads were aligned and assembled with SPAdes (v3.13.0, Bankevich et al., 2012) and the assemblies were evaluated with QUAST (v5.0.2, Gurevich et al., 2013). PROKKA (v1.13.3, Seemann, 2014) was used to annotate the assembled contigs. To determine taxonomic identities for isolates, contigs containing segments of 16S genes, as mapped by PROKKA, were extracted from the assemblies. Samples contained between 1 and 4 complete or partial 16S

sequences. NCBI BLASTn was utilized with default settings to align these contigs to known sequences in the NCBI nucleotide collection (Altschul et al., 1990).

Data management and analysis

All bacterial culture assays described above utilized a 100 mL sample, so the detection limit for *E. coli*, *Listeria* spp., and *Salmonella* spp. was 1 CFU per 100 mL. The frequency of detection for *E. coli* and pathogenic *Listeria* spp. was zero, so no statistical analyses were performed to compare occurrence between systems.

Results

Physical and chemical water quality conditions

Water quality conditions related to optimal plant and fish growth in the UNH KFRAG systems remained within acceptable ranges for DO, water temperature, N species, pH, alkalinity, DOC:TDN, EC, and iron concentration for the majority of the 7-week study period (Table 6). Deviations from acceptable ranges were brief and did not co-occur, indicating that daily water quality monitoring and adjustment protocols were effective in maintaining a productive aquaponic environment. Since acceptable ranges were chosen at compromise points within optimal growth ranges for plants, fish, and nitrifying microbes, it was possible that deviations for a single parameter could affect more than one biological component of the system. Brief periods of elevated water temperature, caused by high ambient air temperatures, were not observed to negatively affect lettuce growth. Although there were occasional elevated values for nitrite, acceptable TAN concentrations at the same sampling event provided evidence of effective biofilter nitrification function. pH varied the most in system 1, where a mechanical failure caused large water losses halfway through the study. It took several weeks after this event for pH and alkalinity to stabilize through daily refinement of potassium carbonate addition amounts.

Table 6. Summary statistics for UNH KFRAG culture water in each of the 3 replicated aquaponic systems. Data represent measurements taken during the 7-week study period in Sept.-Oct. 2019.

Parameter	Mean ± standard deviation			
	GH1	GH2	GH3	Acceptable range
Temperature (°C)	25.9 ± 0.85	24.6 ± 0.90	25.4 ± 0.88	24 – 28
DO concentration (mg L^{-1})	6.4 ± 0.55	6.5 ± 0.56	6.3 ± 0.49	> 5.0
EC (mS cm^{-1})	1.64 ± 0.11	1.36 ± 0.21	1.76 ± 0.16	1.0 – 2.0
pH	6.8 ± 0.25	7.0 ± 0.20	7.0 ± 0.18	6.7 – 7.2
Alkalinity (as g $CaCO_3$ m^3)	29 ± 5.3	30 ± 4.4	29 ± 3.3	20 – 40
TDN concentration (mg L^{-1})	135.5 ± 13.9	119.5 ± 23.6	128.2 ± 32.1	ND
NO_3-N concentration (mg L^{-1}) *	135.0	118.9	127.6	100 – 200
NO_2-N concentration (mg L^{-1})	0.05 ± 0.02	0.07 ± 0.03	0.07 ± 0.02	< 0.1
TAN concentration (mg L^{-1})	0.5 ± 0.07	0.5 ± 0.09	0.6 ± 0.08	< 1.0
DOC concentration (mg L^{-1})	20.9 ± 2.83	20.4 ± 1.86	19.4 ± 4.22	ND
DOC:TDN	0.15 ± 0.01	0.17 ± 0.023	0.15 ± 0.019	< 0.5

* Mean NO_3-N was calculated as mean TDN – mean NO_3-N – mean TAN.
ND = not determined.

Bacterial assessment

E. coli was not detected in any samples (n = 94) using culture-based methods on modified mTEC agar, where no isolates contained β-D-glucuronidase (Figure 32). Putative *Listeria* spp. were observed (blue colonies on Brilliance agar), yet no colonies formed the opaque halo of cleared media indicative of pathogenicity (n = 70).

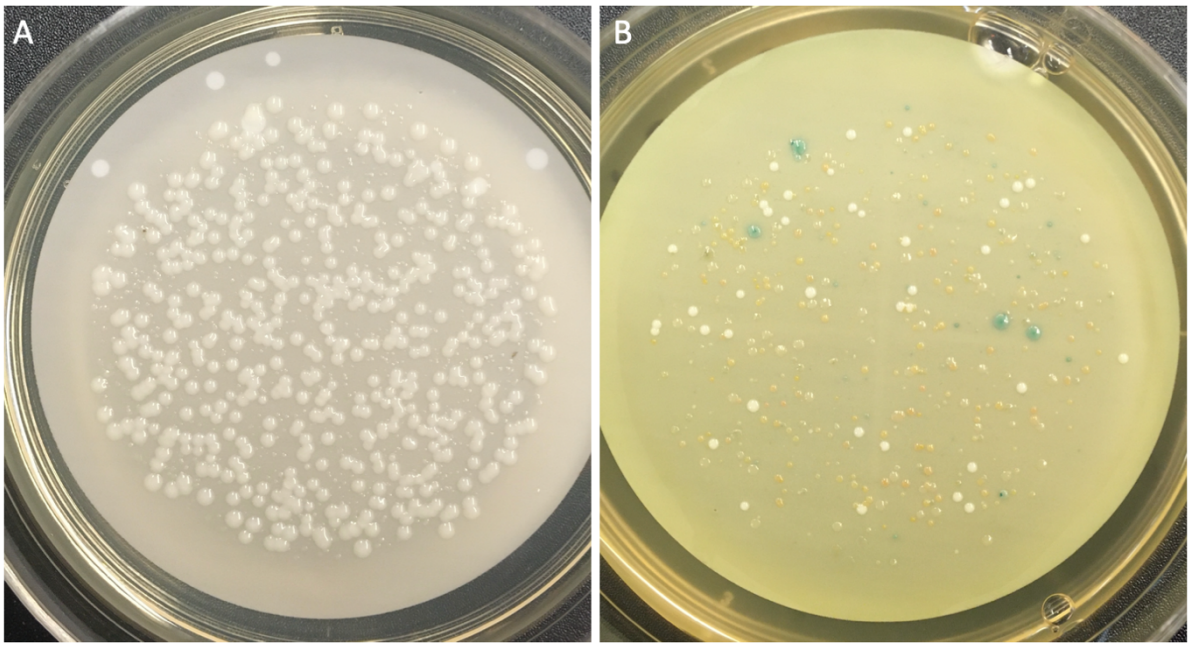

Figure 32. Bacterial culture results for water samples from UNH KFRAG. A negative E.coli *result in which no magenta colonies were detected on the modified mTEC media (A) and a positive result for* Listeria *spp. as indicated by the blue colonies using Brilliance agar (B). No* E. coli *and no pathogenic* Listeria *spp. were observed.*

All *Salmonella* screening using XLD agar (n = 80) resulted in colonies with morphologies indicating presumptive positives for *Salmonella* spp. Two analyses—from different weeks, greenhouses, and sampling sites—resulted in the growth of isolated pink colonies with black centers that are most typical of *Salmonella* on XLD (Figure 33a). This appearance is characteristic of colonies that produce lysine decarboxylase, don't ferment lactose or sucrose, and produce hydrogen sulfide. The remaining presumptive positive colonies displayed morphologies that are atypical for *Salmonella* on XLD (W. H. Andrews et al., 2018). Figure 33 and Table 7 are linked to provide visual examples and descriptions of the five colony morphologies that were observed.

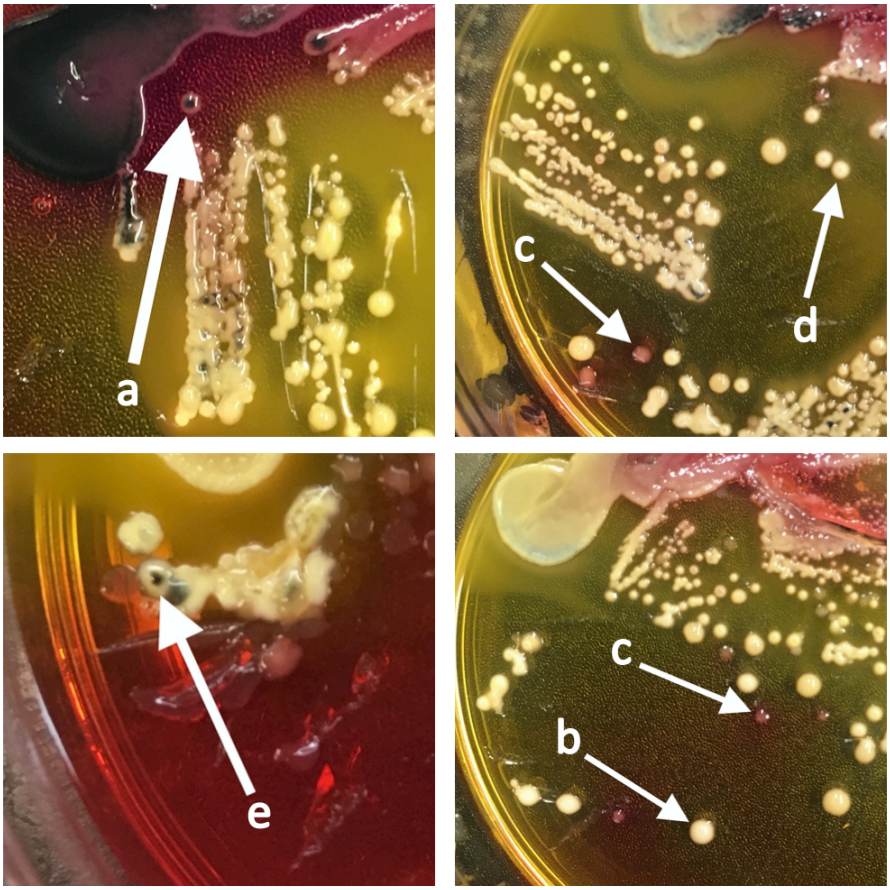

Figure 33. Examples of observed morphologies of colonies cultured on XLD plates from UNH KFRAG aquaponic culture water. All 5 of these morphologies are considered presumptive positives for Salmonella *spp. (W.H. Andrews et al., 2018). See Table 7 for descriptions.*

Table 7. Descriptions and biochemical characteristics of presumptive positive colonies for Salmonella *spp. observed on XLD from UNH KFRAG aquaponic culture water. See Figure 33 for examples of each colony type.*

Figure 33 label	*Colony code*	*Colony code description*	*Lysine decarboxylase production*	*Lactose/ sucrose fermentation*	*H_2S production*
a	pb	Pink with black center on red agar	+	-	+
b	yr	Opaque yellow on red agar	+	+	-
c	pr	Opaque pink/red on red agar	+	-	-
d	yy	Opaque yellow on yellow agar	-	+	-
e	yb	Opaque yellow with black center on yellow agar	-	+	+

Isolates representing each observed morphology were further analyzed using the RapiD 20 E biochemical assays (n = 10) and BioMérieux's APIWEB™ service to verify identification of the isolates (Table 8). *Salmonella* was not identified as a potential match for any isolate. Five of the 11 isolates were identified as *Klebsiella pneumoniae* spp. *pneumoniae* and one as *Serratia marcescens*, with good identification quality. The *Klebsiella pneumoniae* spp. *pneumoniae* were very close to the typical profile for this subspecies (T = 0.96). Four isolates were identified with very good quality (T ≥ 0.67), including two colonies that were identified as *Acinetobacter/Pseudomonas* spp., one *Aeromonas hydrophila*, and one *Providencia alcalifaciens*. The remaining isolate was identified as a member of the *Citrobacter freundii* group, and despite low %ID (64.3) the profile was in relatively close proximity to the typical profile for the taxon (T = 0.91). Two other genera—*Pantoea* and *Enterobacter*—were also identified as significant taxa for this sample, but none met the %ID threshold of 80.0 for an acceptable identification.

Isolate identities determined by 16S BLASTn queries were generally in agreement with biochemical assays (Table 8). The two methods agreed to the species level in 7 of the 10 comparisons, for *Klebsiella pneumoniae*, *Aeromonas hydrophila*, and *Citrobacter freundii*. The comparisons agreed at the genus level for the 3 pink colonies, including 2 as *Pseudomonas* spp. and another as two different *Providencia* species.

There were some trends for identification of strains of different colors. Two of the pink colonies (lysine decarboxylase positive) were identified as *Pseudomonas aeruginosa*, and another pink colony was identified as belonging to the genus *Providencia*. Four of the *Klebsiella* isolates were yellow colonies on yellow agar (lactose/sucrose positive), while another was pink with a black center (lysine decarboxylase and hydrogen sulfide positive). Though none of the

organisms identified were *Salmonella* spp., all of them have the potential to be human and/or plant pathogens. This result has implications for future pathogen screening targets along with the suitability of XLD as a surveillance mechanism in aquaponics.

Table 8. Identities of presumptive positive "Salmonella" isolates from BAM XLD assay, as determined by RapiD 20 E biochemical assays and NCBI BLAST queries of fragments of 16S rRNA genes extracted from the isolates.

Sample			**Biochemical ID confirmation**				**Molecular ID confirmation [4]**			
Sampling site	System	Colony code[1]	RapiD 20 E identity #1 [2]	% ID	T-index	ID quality [3]	BLAST genus	Genus % of hits	BLAST species	Species % of hits
Sump	1	yr	*Klebsiella pneumoniae* ssp. *pneumoniae*	90.5	0.96	Good	*Klebsiella*	100.0	*pneumoniae*	96.0
Sump	2	yr	*Klebsiella pneumoniae* ssp. *pneumoniae*	90.5	0.96	Good	*Klebsiella*	100.0	*pneumoniae*	95.0
Sump	2	pr	*Acinetobacter/Pseudomonas* spp.	99.4	0.90	Very good	*Pseudomonas*	100.0	*aeruginosa*	99.0
Sump	3	pb	*Aeromonas hydrophila*	99.9	0.68	Very good	*Aeromonas*	100.0	*hydrophila*	85.3
Plant bed inlet	3	pr	*Providencia alcalifaciens*	99.5	0.67	Very good	*Providencia*	98.9	*rettgeri*	26.3
Plant bed inlet	3	yy	*Citrobacter freundii* group	64.3	0.91	Low discrimination	*Citrobacter*	95.7	*freundii*	50.0
Fish tank inlet	1	pr	*Acinetobacter/Pseudomonas* spp.	99.4	0.90	Very good	*Pseudomonas*	100	*aeruginosa*	99.0
Fish tank inlet	1	yy	*Klebsiella pneumoniae* ssp. *pneumoniae*	90.5	0.96	Good	*Klebsiella*	100	*pneumoniae*	95.0
Fish tank outlet	1	pb	*Klebsiella pneumoniae* ssp. *pneumoniae*	90.5	0.96	Good	*Klebsiella*	100	*pneumoniae*	89.0
Fish tank outlet	3	yy	*Klebsiella pneumoniae* ssp. *pneumoniae*	90.5	0.96	Good	*	*	*	*
Source water	2	yr	*Serratia marcescens*	92.5	0.58	Good	*Serratia*	100.0	*marcescens*	86.8

[1] For colony code definitions, see Table 2.

[2] Where multiple significant taxa were identified, only the primary identification was included here. None of the secondary significant taxa were identified to an acceptable degree (see note 3).

[3] %ID ≥ 99.9 and T ≥ 0.75 indicates "excellent identification," %ID ≥ 99.0 and T ≥ 0.50 indicates "very good identification," %ID ≥ 90.0 and T ≥ 25% indicates "good identification," and %ID ≥ 80.0 and T ≥ 0 indicates "acceptable identification." "Low discrimination" means that more than one genus was identified, and the primary identification was not acceptable.

[4] BLAST genus and BLAST species are the genus and species with the greatest number of hits in the NCBI nucleotide database, respectively. Genus % of hits and species % of hits are the proportions of total hits belonging to these identified taxa.

* Indicates unavailability of molecular results due to user error.

Discussion

Aquaponic system performance

Environmental and water quality parameters for the aquaponic culture water were monitored to help track culture conditions that may affect the health of the fish, plants, and biofilter. See Chapter 2 for a comprehensive overview of water quality data. Overall, water quality was within target ranges for measured parameters with few, short term minor deviations that did not co-occur among variables. These deviations appeared to be insignificant because lettuce plants were healthy throughout the study, productivity was similar across systems, there were only three tilapia mortalities, and tilapia did not show any other signs of disease or disorder. This confirms that the food safety assessment was conducted under conditions that would be considered acceptable at a production facility. Establishing acceptable water quality conditions is important to assessing food safety because these conditions influence the makeup of the microbial community both directly to the physiology of the taxa making up the microbial community and indirectly through plant, fish, and biofilter health, resilience, and associated feedback effects to microbial physiology. Plants, for example, can become nutrient deficient if the pH of the solution reduces nutrient availability. In addition to reducing plant growth, this stress can increase carbon exudation by roots, altering the rhizosphere microbiota (Strayer, 1994). Excessive ammoniacal N in the recirculating solution can be toxic to fish, an effect that is also dependent upon pH (Timmons et al., 2018). Fish that are physiologically stressed due to suboptimal water quality conditions—such as high ammonia-N or low DO—are more susceptible to disease (Yavuzcan Yildiz et al., 2017) and less productive (Maucieri et al., 2017; Nhan et al., 2019; C.-Y. Wang et al., 2016). These conditions along with poor fish health in turn

alter the microbial community in ways that could potentially enable the growth of zoonotic disease organisms (Derome et al., 2016).

Pathogen screening results

The results of this bacterial assessment of aquaponic culture water validated the UNH KFRAG system design and operational protocols in relation to the microbial water quality requirements for BSAAO treatment processes under Subpart F of the PSR (Food Safety Modernization Act, 2011). The lack of detectable *E. coli* was in accordance with previous studies that detected no *E. coli* or low concentrations (Elumalai et al., 2017; Fox et al., 2012; Moriarty et al., 2018; Pantanella et al., 2015) and suggests two possibilities: that no *E. coli* was introduced to the facility, or *E. coli* were introduced but did not establish detectable populations within the aquatic microbiome. This finding differs substantially from that of Wang et al. (2020), where Shiga toxin-producing *E. coli* (STEC) were found in production water of both hydroponic and aquaponic systems, fish feces, and on the surfaces of plant roots. In that study, TAN and nitrite-N concentrations (1.8 – 3.0 mg L-1 and 0.3 – 1.2 mg L-1, respectively) were above our acceptable ranges and TAN exceeded recommended values for tilapia in aquaponics (Somerville et al., 2014). Wang et al. suggested many possible routes of contamination that were not controlled during that study, including the potential introduction of contaminated fish, inadequate on-farm hygiene practices, or inadequate sanitization of the physical system components prior to startup. The same routes of contamination were, however, addressed in the present study through system design and operational protocols that were used both to provide a healthy ecosystem for fish and plant production and to prevent introduction of fecal contamination sources. Active removal of waste solids reduced the available carbon in

production water which could help to minimize the potential for *E. coli* proliferation, were it to be introduced. The lack of *E. coli* detection under these controlled conditions similar to commercial RAS systems suggests that recirculating aquaponic systems where external contamination is excluded may not pose inherent human health risks, at least concerning *E. coli*, the primary indicator of fecal contamination.

It appears that pathogenic *Listeria* spp. and *Salmonella* spp. were also not introduced, or if they were they did not survive within the systems. Pathogenic *Listeria* spp., including *L. monocytogenes* and *L. ivanovii*, are of particular concern in temperate regions and in the production of crops that will be refrigerated, since they can survive and proliferate at much lower temperatures than most bacterial agents of FBD (Ryser & Marth, 2007). *Listeria* infections in humans, when they do occur, frequently lead to hospitalization and death (Scallan, Hoekstra, et al., 2011) (see Table 4). For these reasons environmental *Listeria* spp. screening is common in commercial produce storage and distribution systems. Although no assessment of the presence of *Listeria* spp. under produce storage and distribution system was undertaken, these results further validate the UNH KFRAG system as a safe system for cultivating lettuce and tilapia.

Salmonella spp. are the most common bacterial agent causing FBD in the US and are frequently implicated in outbreaks associated with produce that is eaten raw (Painter et al., 2013; Scallan, Hoekstra, et al., 2011). Even though bacterial colonies with presumptive positive *"*Salmonella" colony phenotypes were observed on XLD plates for all samples from the UNH KFRAG systems, no *Salmonella* spp. were identified. This observation is in accordance with the Hawai'i survey of commercial systems by Fox et al. (2012) that found no detectable *Salmonella* spp. in tissue samples from plants and fish. However, the consistent observation in the present study of presumptive positive colonies on XLD agar suggests that this culture method for

surveillance of *Salmonella* may not be optimal for monitoring the UNH KFRAG and other similar systems. Alternative *Salmonella* culture media in the BAM include Hektoen enteric (HE) agar and bismuth sulfite agar (W. H. Andrews et al., 2018). HE agar may be a more appropriate choice in aquaponics since it discriminates between *Salmonella* and *Shigella* and other organisms through the preferential use of peptone as a carbon source by *Salmonella* and *Shigella* (King & Metzger, 1968).

The routine follow-up analyses conducted on the presumptive positive *Salmonella* isolates were undertaken to confirm initial method results as well as to gain more information about what bacteria were in the system, given the lack of any identified target species. All except one of the presumptive positive "Salmonella" isolates were identified as *Enterobacteriaceae*. Some of the bacterial isolates from these colonies were unexpectedly identified as unmonitored taxa known to include strains that may be human and plant pathogens. This suggests that the experimental aquaponics system may harbor within the microbial ecosystem persistent bacterial species that may pose human or crop disease risks. One isolate was identified as *Aeromonas hydrophila*, which has long been known as a pathogen of animals in many phyla, including fish and humans (Hazen et al., 1978). *A. hydrophila* was identified as the causal agent for zoonoses resulting from the use of medicinal leeches on plastic surgery wounds (Snower et al., 1989), and a highly virulent strain was responsible for outbreaks in the catfish aquaculture industry in the southern US (Griffin et al., 2013; Pridgeon & Klesius, 2011). More common strains are not typically of concern as primary disease agents and are found as normal flora of freshwater fish. Since no tilapia disease was evident, the *A. hydrophila* strains identified here were likely not pathogenic to healthy tilapia or were not present in sufficient numbers to cause disease. Further analysis of genomic data could allow for comparison of this isolate to

known strains for determination of potential pathogenicity. If this species does become a concern, specific assays for its detection could then be included in water quality monitoring of aquaponics systems.

The isolates identified as *Enterobacteriaceae* most frequently were *Klebsiella pneumoniae*, which is commonly found in soils, water, and the human microbiome. Although *K. pneumoniae* is a serious human pathogen (Bengoechea & Sa Pessoa, 2019), about 30% of strains can fix nitrogen, and these have been shown to increase maize yields in field trials (Riggs et al., 2001) and may even play a role in human nutrition (Igai et al., 2016). *Pseudomonas aeruginosa* was also identified. Both *K. pneumoniae* and *P. aeruginosa* are opportunistic pathogens of humans and can cause nosocomial infections, especially in immunocompromised individuals (Bengoechea & Sa Pessoa, 2019; Ryan et al., 2004). *P. aeruginosa* is also a pathogen of plants—causing soft rot in lettuce—and forms a biofilm on roots that helps it to resist plant defenses (Walker et al., 2004). Another identified species, *Serratia marcescens*, is ubiquitous in the environment, but can also cause opportunistic infections in humans (Madigan et al., 2018). Most pathogenic strains of *K. pneumoniae*, *P. aeruginosa*, and *S. marcescens* are resistant to multiple antibiotics, and they are important pathogens of concern in healthcare settings (Gupta et al., 2011; Iguchi et al., 2014; Poole, 2004). *Citrobacter freundii* and *Providencia* spp. are also ubiquitous, found in water, soil, and the human gut. Most strains of *C. freundii* are beneficial, but some can cause opportunistic and nosocomial infections (Morowitz et al., 2011). One presumptive positive isolate was identified as *Providencia alcalifaciens* or *Providencia rettgeri*. *P. alcalifaciens* was recently identified for the first time as a foodborne pathogen in Kenya, in an outbreak that was attributed to poor hygiene before and during food preparation (Shah et al., 2015). *P. rettgeri* is rarely involved in human disease, but can cause nosocomial infections

(Sagar, 2017). Thus, the UNH KFRAG systems contained an array of bacterial taxa that tend to be found in many environments. These taxa include pathogenic strains, though no evidence of human pathogens was detected.

Overall, the results of the culture-based approach used to screen for pathogens and indicator organisms suggested that the experimental system was acceptable in terms of routine water quality concerns, while the detection of potentially-pathogenic taxa using methods that were not specific for these other organisms suggests a more in-depth understanding of the aquaponic microbiome could be highly informative. Many of the identified taxa are widespread in the environment so it is not surprising that they were present in the study system. The capacity for the microbiome of these systems to maintain or allow for the proliferation of these potential pathogens may require further study, especially if they in fact do cause problems with fish and plant production. The healthy nature of the plants and fish produced in this system, however, suggests that the potentially pathogenic taxa identified either did not include strains capable of causing diseases in the system, or that the overall system microbiome with the healthy plants and fish did not allow for proliferation of pathogenic strains. The apparent absence of any targeted pathogens is a significant finding that supports the notion that productive, healthy and well-run aquaponics systems can both maintain water quality and environmental conditions that meet requirements and produce food that is safe for human consumption.

Implications related to US produce regulation under FSMA

For US aquaponic producers, their most pressing questions relate to the definitions attributed to different types of agricultural production water under the PSR production water (Subpart E) and BSAAO (Subpart F) requirements. In many existing aquaponic systems,

production water inevitably contacts edible portions of the crop, which triggers the need for *E. coli* screening under the law (G. L. Wall, personal communication, March 31, 2021). Comparison with typical lettuce production in the western US elucidates the appropriate regulatory burden for aquaponic growers. California and Arizona together produce 99 % of US lettuce, which is irrigated with surface reservoirs sourced from the Colorado River. These surface waters provide habitat for fish and other aquatic organisms but may also contain pathogen contamination in runoff from animal agriculture, wildlife waste, and industrial and municipal wastewater discharge (Hansen et al., 2020; Jongman & Korsten, 2018; Kintz et al., 2019; Malakar et al., 2019; Rodrigues et al., 2019; Uyttendaele et al., 2015). In many instances water from surface sources is applied to fields with no filtration or treatment. Overhead irrigation systems are used in 46.7% of lettuce fields (USDA National Agricultural Statistical Service, 2019), which has been shown to increase the risk of *E. coli* contamination on the edible portions of the produce (Solomon et al., 2002). Further, rain events splash microbes from the soil on to the produce (Lee et al., 2019; Monaghan & Hutchison, 2012). Under the PSR agricultural water rule, farms covered by FSMA and using such surface waters to irrigate crops that are typically eaten raw are required to perform 20 initial tests for *E. coli* over a 2 to 4-year time period, after which 5 tests per year must be rolled into the microbial water quality profile (MWQP). Irrigation of crops with contaminated surface waters has been implicated in *E. coli* and *Salmonella* outbreaks globally (Levantesi et al., 2012). Results from this study demonstrate that aquaponic culture water from a groundwater source and in a controlled environment can be safe, despite the close proximity of fish waste to the growing produce. For this reason, culture water in commercial aquaponic systems that are covered by the FSMA PSR should probably be regulated no more intensively than surface waters, whether the produce is grown in a liquid or solid matrix,

although this will require more studies to confirm. Aquaponic producers should meanwhile keep records of physiochemical water conditions as well as *E. coli* as further evidence of good system design, operational control, and prevention of contamination. Fortunately, it appears that maintaining optimal conditions for vegetable and fish production also maintains safe water, and thus crop quality.

The results of this study indicate that it is possible for RApS to meet the BSAAO treatment requirements under the PSR, which set standards for *E. coli*, *Salmonella* spp., and pathogenic *Listeria* concentrations (*Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption*, 2015). However, to meet the legal standard for "scientific validation" of such treatment processes, each different system design, set of water quality conditions, and combination of cultivated species may have to be validated separately. Further guidance will be required from FDA regarding Subpart E and Subpart F compliance for aquaponic producers (Wall et al., 2019).

For regulatory agencies, it is important to keep in mind that the standard that is utilized for field crop irrigation water may not be the most appropriate to controlled environment agriculture (CEA) operations, and aquaponics in particular. These systems are not subject to the same environmental contamination sources, and physicochemical quality under controlled conditions may vary significantly compared to those in less controlled field systems, impacting microbial survival and growth. Research casting a wider net for potential pathogens in aquaponics would help to inform meaningful pathogen screening protocols for these unique systems.

Lesser-known pathogens in aquaponics

Food safety assessments of aquaponic systems have so far only screened for *E. coli*, pathogenic *Listeria* spp., and *Salmonella* spp. The presence of other potentially pathogenic organisms in the KFRAG systems suggests that RApS, and probably other culture systems, can have more complex microbial communities that should be further investigated. Studies in Europe by Schmautz et al. (2017) and Eck et al. (2019) have provided early glimpses into the composition of bacterial communities in aquaponics, focused on potential effects on plant productivity rather than potential pathogen presence. Their results did, however, provide some relevant information. As in the present study, Schmautz et al. identified potentially pathogenic members of the genus *Aeromonas* in fish feces, accounting for 0.25% of total DNA reads. Since fish in the system were healthy, the authors proposed that the *Aeromonas* observed were not present in sufficient numbers to cause disease or were non-pathogenic strains, as in the current study. Eck et al. (2019) conducted a broader survey two years later, sampling sump water and biofilter biofilm from 8 systems, including RAS and both coupled and decoupled aquaponic systems. Like Schmautz et al., they observed Proteobacteria and Bacteroides to be the dominant phyla in water and biofilm samples and identified nitrifying genera including *Nitrospira*. Of particular relevance to food safety, DNA reads assigned to the *Campylobacteriaceae* genus *Arcobacter* were found in two RAS and one RApS. *Arcobacter*, first described in 1977 by Ellis et al. (1977), belongs to the rRNA superfamily VI or *Epsilonproteobacteria*, along with the genera *Campylobacter* and *Helicobacter* (Vandamme et al., 1991). Unlike *Campylobacter* and *Helicobacter* spp., *Arcobacter* spp. can grow aerobically at 25°C (On, 1996), so it may be of concern in warm-water recirculating systems. Since the creation of the genus, *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus* are three *Arcobacter* species that have been associated with

enteric illness and bacteremia in humans and animals (Bücker et al., 2009; Lehner et al., 2005; Vandamme et al., 1992). Pathogenic *Arcobacter* have been isolated from a wide range of environmental water samples (Dhamabutra et al., 1992; Musmanno et al., 1997; Rice et al., 1999), from drinking water treatment plants (Jacob et al., 1998) and a sewage treatment plant (Stampi et al., 1993), and from water and mussels in a brackish lake (Fera et al., 2004). *Arcobacter* isolates from retail meats were shown to induce cytotoxic effects in Vero cells *in vitro* (Villarruel-López et al., 2003). In addition to contaminated meat and seafood, exposure to water contaminated with *Arcobacter* spp. may be an important mode of transmission to humans (Kiehlbauch et al., 1991), so these pathogens should be considered in food safety assessments of agricultural production water. The obvious reason these taxa are not monitored is because they have not yet been found to cause significant food safety problems. With the increase in less studied RAS and RApS systems, novel foodborne pathogens of concern may emerge over time.

Potential microbial community influences on pathogen persistence and aquaponic productivity

There are a variety of system design options that could help to reduce health risks in aquaponic systems. For example, much of the previous research in aquaponic food safety has focused on reducing concentrations of coliforms and *E. coli* through UV sterilization of production water (Elumalai et al., 2017; Moriarty et al., 2018; Pantanella et al., 2015). There are multiple reasons this option was not used for the UNH KFRAG system that these studies did not directly address. Even with rapid particulate removal, dissolved organic matter in the form of humic substances, proteins, phenols, lipids, and carbohydrates reduces the transmission of UV radiation, meaning that UV systems must be designed and maintained to compensate for

changing transmissivity over time (Timmons et al., 2018). Inadequate UV output from the disinfection system may select for organisms resistant to disinfection, including several known fish viruses that have high UV tolerance (Liltved et al., 1995; Momoyama & Sano, 1989; Sako & Sorimachi, 1985; Wedemeyer, 1996; Yoshimizu et al., 1986). Bacteria, including pathogenic strains, that are damaged by UV radiation may repair themselves through photoreactivation and dark repair (Friedberg et al., 1995; Liltved & Landfald, 1996, 2000; Miller et al., 1999). UV disinfection also has the potential to reduce concentrations of beneficial organisms that perform important ecosystem functions in both RAS and RApS. Instead of attempting to directly reduce microbial populations, we utilized system design and operational protocols to reduce available resources and niche space for pathogens as much as possible.

Maintaining populations of the naturally-occurring microbes in RApS may improve productivity, in part by preventing pathogen proliferation. Gravel et al. (2015) was the first study to demonstrate suppressive effects of RAS effluent on plant pathogens. They inoculated greenhouse tomatoes with root pathogens *Pythium ultimum* and *Fusarium oxysporum* and observed reduced mycelial growth and root colonization on plants treated with a combination of liquid and solid fish waste. Shortly thereafter, Sirakov et al. (2016) identified 42 bacterial isolates from a model aquaponic system that showed inhibitory effects on fungal pathogens of both plants and fish, *P. ultimum* and *Saprolegnia parasitica*, respectively. Though the pathogen-inhibiting isolates were not taxonomically classified, the authors inferred from the enrichment and culture methods that many were *Pseudomonas* spp. and lactic acid bacteria. Recently, Stouvenakers et al. (2020) demonstrated suppression of *P. aphanidermatum* infection on lettuce roots grown in aquaponic water compared to hydroponic or complemented (augmented) aquaponic solutions. They did not detect taxa known to have direct antagonistic effects on *P.*

aphanidermatum but did identify many taxa in the rhizoplane that they posit played complementary roles in pathogen suppression. The most abundant taxa correlated with suppression were the genus *Methyloversatilis* and the family *Burkholderiaceae*, which contains the plant growth-promoting and nitrogen-fixing genus *Hydrogenophaga*. These results together suggest that pathogen suppression attributed to the aquaponic microbiome may not be specific to the pathogen introduced, but a function of the resilience of the community against the incursion of allochthonous pathogenic organisms. This community resilience effect, if substantiated in future studies, would apply equally to plant, fish, and human pathogens, and may prove valuable to both aquaponic productivity and food safety.

Studies have shown equivalent plant growth in (fertilized) hydroponic and (potentially nutrient-limited) aquaponic systems with disparate concentrations of soluble plant nutrients (Graber & Junge, 2009; Pantanella et al., 2012). Nitrogen, phosphorus, and potassium levels in aquaponic systems typically range between 4 and 30 times less than optimal concentrations in fertilized hydroponic systems (Bittsánszky et al., 2016; Delaide et al., 2016). This suggests that beyond pathogen suppression, the aquaponic microbiome may contain plant growth-promoting microbes that may promote plant production through mechanisms including the direct exudation of plant hormones, antagonism of plant pathogens, and transformation of [non-labile] plant nutrients in the root zone to plant-available forms (Bartelme et al., 2018; Bashan & de-Bashan, 2010; Bona et al., 2017; Lingua et al., 2013). More in-depth analysis of microbial community composition and function in different types of aquaponic systems may reveal, in addition to effects on bacterial pathogens, how system design and operational decisions can be utilized to improve system performance by manipulating the microbiome.

There is a growing body of studies that have provided some evidence of the benefits that what may be called a “healthy” microbiome can have on all three of the important biological components in aquaponics—fish, plants, and microbes. Such a healthy and diverse aquaponic microbiome may also have suppressive effects on human pathogens when they are incidentally introduced due to the resistance of the microbiome to change. The future regulatory environment for aquaponic production water under the Food Modernization Act Produce Safety Rule is uncertain, and the possibility of a pathogen-suppressive microbiome should be considered in the implementation and enforcement of the regulation (*Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption*, 2015). However, commercial aquaponic producers must also demonstrate water quality management that promotes the diverse microbial communities associated with suppression. Future research on the effects of physicochemical water quality on microbial activity could inform aquaponic producers about the tolerances of beneficial microbial consortia to variation in conditions, potentially improving productivity and food safety simultaneously.

Chapter 4:

Conclusion

The second of the UN SDGs is to "end hunger, achieve food security and improved nutrition and promote sustainable agriculture" by 2030 (United Nations, 2015). This lofty goal will require innovation in all aspects of agriculture, including decentralization and integration of food production systems, technology transfer to LDC, and investment to enable development in the global agricultural sector. Aquaponics offers an integrated solution to sustainability for aquaculture and hydroponic crop production, which could be important in providing people with nutrient-dense locally produced foods.

Growth of the aquaponics industry is constrained by a lack of publicly available fundamental research regarding optimal system designs, species combinations, water quality and environmental conditions, and food safety. Free availability of this fundamental knowledge, which has mostly to date been developed by a few successful private companies, would allow for innovation and standardization that could lead to widespread distribution and scaling of aquaponic food production. As this industry grows, it has to potential to perpetuate some of the negative externalities of its predecessors, including environmental and public health impacts.

The potential nitrogen use efficiency of aquaponics depends on maximal plant uptake of waste N from fish feed which can be accomplished by converting all available ammonia-N to plant-available nitrate and preventing denitrification. Based on comparisons between the performance of the UNH KFRAG systems to other types of aquaponic systems in the literature, it appears system design, cleaning protocols, and consequent efficacy of solids removal from production water may be positively correlated with nitrification efficiency. Other experimental

systems in the literature employed solids capture devices that retained the captured solids within the production water flow, suggesting greater organic C accumulation than what occurred with the KFRAG design. Nitrogen species data from those other studies showed that their biofilter function was partially impaired (86-97 % efficient) compared to KFRAG (>99 % efficient). Observed pH values below 6.5 did not impede nitrification at KFRAG, perhaps due to the carbon-limited environment. This observation suggests that the conditions in aquaponics may allow or facilitate nitrification outside the normal optimal pH range. Water quality results from this study will contribute to the establishment of acceptable and optimal conditions for tilapia and lettuce in aquaponics. Future research should consider the interactions between C:N, productivity, and physicochemical water quality conditions for different combinations of crops and fish to develop more detailed guidance for diverse aquaponic enterprises. Distributed water quality research projects across geographic regions could be facilitated through automated data aggregation software (e.g. Aquaponics AI).

In water-based agricultural systems such as aquaponics, production water quality is a key food safety concern due to its ubiquity and proximity to produce. The UNH KFRAG systems met the most stringent microbial safety standards under FSMA, the first reported evaluation of an aquaponic system as a “scientifically-validated treatment process” for biological soil amendments of animal origin. Acceptance by the FDA of similar process validation studies, with the addition of solids digestion processes to complement the KFRAG system design, would enable aquaponic practitioners to produce a wider range of crops including those that contact the production water, like root and stem crops such as carrot and radish.

Evidence in the literature to date suggests that aquaponic systems do not pose any particular food safety threat in terms of *E. coli*, but the marked differences in results between this

study and that by Wang et al. (2020) demonstrate the potential for contamination and proliferation of pathogens in both hydroponics and aquaponics. This is influenced by system design, previous uses of facilities, hygiene, and fish handling practices. If contamination is prevented in the production setting, the shortening of supply chains through a distributed network of aquaponic facilities could help to dramatically reduce contamination risk throughout the farm-to-fork continuum. However, less-common foodborne pathogens and zoonoses may present novel hazards to consumers of aquaponic produce and employees of aquaponic facilities. Organisms like *Arcobacter* spp. that have been identified in RAS and RApS should be included in food safety studies as these production systems become more prevalent. On the other hand, naturally occurring microbes in RApS may improve productivity and potentially exclude pathogens, so strategies of overall microbial reduction such as UV irradiation of production water may be undesirable. Aquaculture is the most dangerous industry in the US (Fry et al., 2019), so new aquaponic practitioners must be educated regarding the potential biological, chemical, and physical hazards to their employees. Meanwhile, researchers should seek to characterize aquaponic microbiomes to understand the risks and rewards associated with different types of autochthonous microbes.

The observation of presumptive positive “Salmonella” colonies on XLD agar from all KFRAG samples illuminates a weakness in that method for detecting *Salmonella* presence and persistence in aquaponic contexts. Care should be taken to scrutinize methods accordingly when evaluating and utilizing information from relevant studies. Moving forward, molecular confirmation of presumptive positives will become more widely available as costs come down and infrastructure is developed for genomic analysis. Sequencing of isolates from produce, water, and environmental samples will not only confirm microbial identities, but will over time

build a library of genomic data that will transform food safety research and practice. Analyzing metagenomic data from aquaponics will in turn elucidate the magnitude of food safety risk while also generating invaluable data regarding the microbial ecosystem dynamics that drive agricultural productivity and sustainability. Co-management of RApS for food safety and productivity seem to be naturally aligned, based on existing information. Further research into the impacts of microbial activity in RApS on productivity, safety, and sustainability may reveal the mechanisms of these synergies between microbial consortia and agroecosystems.

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Appendix A: Aquaponic Culture Water Sample Collection Protocol

Materials

1 L Nalgene HDPE bottles (autoclaved)
PVC fittings for PI, FI, PO, FO (soaked in 1.5% hypochlorite solution for 10 minutes, then rinsed. See note in procedure, step 4.)
BO sample collector (250mL HDPE bottle with holes in neck, soaked in 1.5% hypochlorite solution for 10 minutes, then rinsed. See Figure 35.)
Permanent marker
Clean trash bags
Cooler and ice packs
1.5% hypochlorite solution
Nitrile powder-free single-use gloves
Label tape

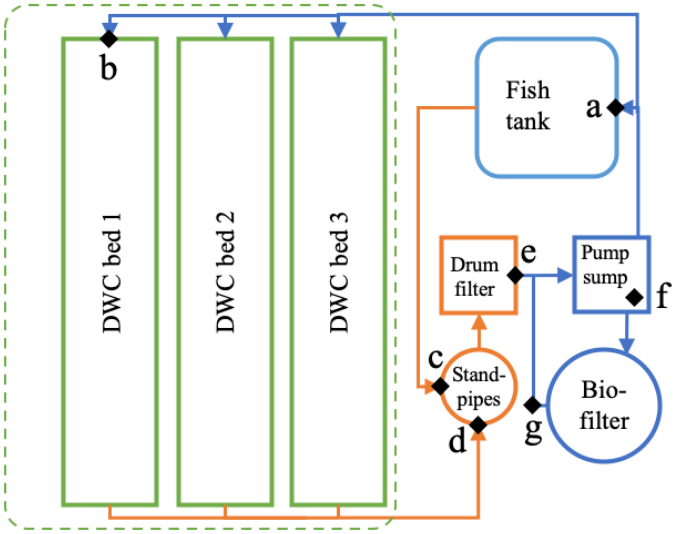


Figure 34. UNH KFRAG coupled aquaponic system schematic with water sampling sites. Orange lines indicate fish and plant unit effluent prior to treatment through the mechanical drum filter. Water sampling sites are indicated with black diamonds: a) fish tank inlet FI, b) plant bed inlet PI, c) fish tank outlet FO, d) plant bed outlet PO, e) mechanical drum filter outlet MO, f) pump sump BI, and g) biofilter outlet BO. Source water (SW) is not shown.

Table 9. Sampling site names and abbreviations.

Site	Abbr.
Source (well) water	SW
Sump (biofilter influent)	BI
Biofilter effluent	BO
Drum filter effluent	MO
Fish tank inlet	FI
Plant bed inlet (bed 3)	PI
Plant bed effluent	PO
Fish tank effluent	FO

Procedure

0. Don nitrile gloves. Scrub and rinse away any debris in cooler, then wipe down entire cooler and ice packs with hypochlorite solution. Place cooler on elevated surface in the greenhouse.
1. Using clean gloved hands, label a 1L bottle for each sample site with the following:
    a. [Week#]-[GH#]-[Site] (e.g. 1-1-PI)
    b. Each week choose one random site for analytical replication. Label an additional bottle for that site, adding a lowercase (a) to the label name.
2. Place labeled bottles in cooler.
3. Gather clean PVC fittings into a clean trash bag. Label clean bag as “CLEAN,” and another bag for “DIRTY” materials.
    a. Note: Utilize appropriate PVC fittings in order to direct the flow of water from the sample collection site so that it can be captured in a sample bottle. These will differ slightly between greenhouses due to minor differences in the plumbing of each system.
4. Collect samples before morning standpipe plunging and harvest.
5. Use aseptic technique throughout sample collection, wearing clean gloves and replacing if soiled.
6. For PI and FI:
    a. Do not change inlet flow rates during sample collection.
    b. Remove manometer tube and attach clean ¾” PVC fitting, pointing it towards the tank. Allow water to flow for 5 seconds.
    c. Rinse sample bottle with sample water 3 times.
    d. Fill bottle with sample water and cap immediately.
    e. Remove PVC fitting and replace manometer tube. Place used fitting in DIRTY bag.
    f. Place sample in cooler with ice packs.
    g. Use new clean PVC fitting for the next site.
7. For PO and FO:
    a. Attach clean PVC fitting and allow water to flow for 5 seconds.
    b. Rinse sample bottle with sample water 3 times.
    c. Fill bottle with sample water and cap immediately.
    d. Remove PVC fitting and place in DIRTY bag.

   e. Place samples in cooler with ice packs.
   f. Use new clean PVC fitting for next site.
8. For MO:
   a. Water level in sump must be below mechanical filter outlet for sample collection.
   b. Rinse sample bottle with culture water 3 times.
   c. Fill bottle with sample water and cap immediately.
   d. Place sample in cooler with ice packs.
9. For BI (sump):
   a. Rinse sample bottle with sample water 3 times.
   b. Fill bottle with sample water and cap immediately.
   c. Place samples in cooler with ice packs.
10. For BO:
   a. Use clean sample collector for each site.
   b. Using attached wire, lower sample collector into biofilter outlet pipe until effluent is flowing into bottle (see Figure 35).
   c. Raise full sample collector and empty into sample bottle. Cover sample bottle with cap while repeating procedure with sample collector. Repeat until sample bottle is full.
   d. Place sample in cooler with ice packs.
11. For SW:
   a. Open well water valve partially and allow water to flow for 5 seconds.
   b. Rinse sample bottle with sample water 3 times.
   c. Fill bottle with sample water and cap immediately.
   d. Place samples in cooler with ice packs.
12. Transport cooler to lab within 6 hours for processing.

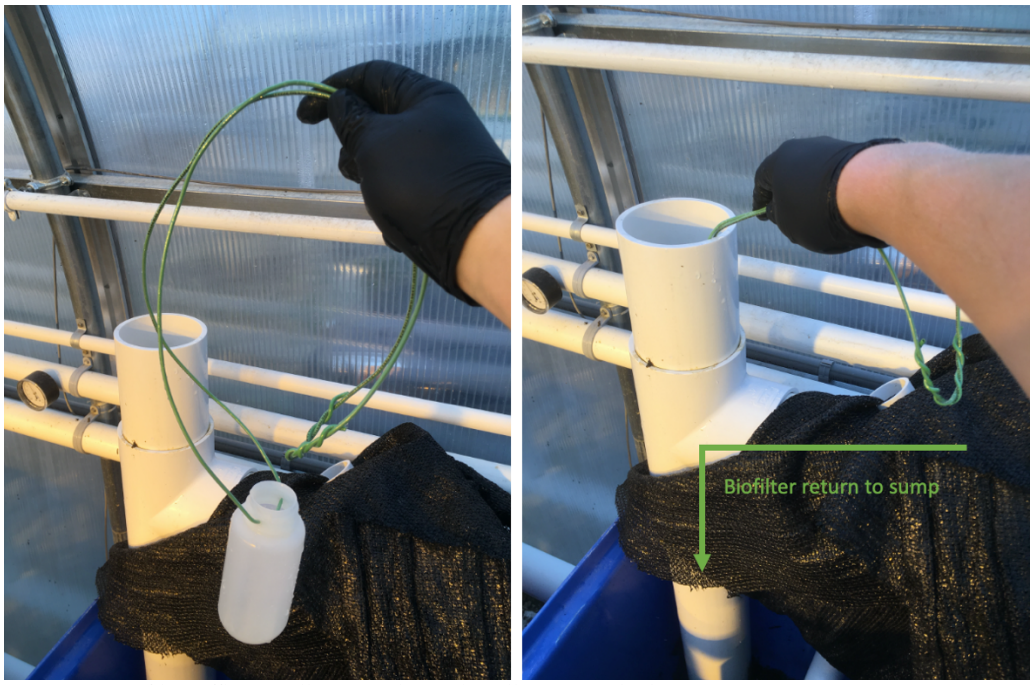


Figure 35. Biofilter effluent collector and sample collection method.

Appendix B: Aquaponic Culture Water Sample Filtering Protocol

Materials

Vacuum pump with flask for waste
Magnetic filter towers and bases (autoclaved)
Graduated cylinders (autoclaved) [500mL, 100mL]
Forceps
70% ethanol
Lighter
Media:
- XLD agar plates [15x60mm]
- mTEC' agar plates [15x60mm]
- Brilliance Listeria agar plates [15x60mm]
- Tryptic soy broth (TSB) [10mL broth in 50mL conical tubes]
- Tetrathionate (TT) broth [4.5mL broth in glass test tubes]

2mL sterile cryotubes
60mL HDPE bottles
Sterile filters [0.45µm pore size, 47mm diameter]
Permanent marker
-20°C freezer
-80°C freezer
Plastic incubator bin
Nitrile powder-free single-use gloves

Procedure

0. Don nitrile gloves. Wipe down lab bench with 1.5% hypochlorite, then with 70% ethanol. Spray gloves with ethanol and rub together, getting between fingers, until it evaporates.
1. Label 2mL cryotubes, mTEC' plates, Brilliance plates, TSB tubes, and/or 60mL HDPE bottles, depending on which analyses will be done. Arrange on lab bench in the order in which samples will be filtered (FIGURE).
2. Attach rinsed vacuum flask to pump with ¼" I.D. tubing. Place a sterile filter tower base on the vacuum flask (FIGURE). Flame sterilize forceps and use them to place a sterile filter on the filter tower base. Attach tower to base.
3. For mTEC' (*E. coli*) and Brilliance (*Listeria* spp.):
    a. Shake sample 30 times. Measure 100mL using 100mL graduated cylinder and pour into filter tower. Turn pump on and run until entire sample has passed through filter.
    b. Use flame-sterilized forceps to place filter, grid side up, on labeled mTEC' plate. Turn plate upside down and place in incubator bin.

   c. Repeat steps a and b, placing filter on labeled Brilliance plate instead of mTEC'. Place plate into a different incubator bin.
   d. For each sample, repeat steps 4 – 5c.
4. For XLD (*Salmonella* spp.):
   a. Shake sample 30 times. Measure 100mL using 100mL graduated cylinder and pour into filter tower. Turn pump on and run until entire sample has passed through filter.
   b. Remove filter tower. Use flame-sterilized forceps to fold filter radially 4 times. Place filter into labeled TSB tube. Cap tube and place in rack.
5. For DOC/TDN analysis:
   a. Before filtering sample, rinse waste flask 3 times with DI water.
   b. Filter sample as above for mTEC', Brilliance, or XLD.
   c. Measure 50mL of filtrate using 50mL graduated cylinder and pour into labeled 60mL HDPE bottle. Place in -4°C freezer until ready to send to USDA Forest Service for dissolved CN analysis.

Appendix C: *Escherichia coli* in Water Using Modified Membrane-Thermotolerant *E. coli* Agar

Adapted from EPA Method 1603, Section 11.

Materials

Water samples
Nitrile gloves
10% bleach
70% ethanol
Paper towels
Sterile filters (0.45µm pore size, 47mm diameter)
Modified membrane-thermotolerant *Escherichia coli* (mTEC') agar – one 15x60mm plate per sample

Sterile (autoclaved) magnetic filter towers – one per sample
Vacuum pump and flask
Forceps
Lighter

Procedure

Use aseptic technique and wear clean gloves throughout this procedure.
Store water samples in a cooler with lots of ice packs for no more than 6 hours until you begin.

0. Use paper towels to wipe down the lab bench with 10% bleach, then with 70% ethanol. Label one mTEC' plate for each sample to be processed.
1. Spray your gloves with 70% ethanol and rub your hands together to coat your gloves until the ethanol evaporates.
2. Place filter tower base in neck of vacuum flask.
3. Using flame-sterilized forceps, carefully place a sterile filter on the filter tower base, ensuring that the filter settles within the lip around the filter area.
4. Attach the filter tower.
5. Remove the first sample to be processed from the cooler and shake it 30 times to redistribute settled material.
6. Pour 100mL of sample water into the filter tower, using the gradations on the side of the tower to measure the volume.
7. Turn the vacuum pump on and let it run until all of the sample has passed through the filter, then turn the pump off.

8. Using flame-sterilized forceps, roll the filter on to the mTEC' agar with the filtered material facing up. Start with one edge of the filter, and slowly lay it on to the agar to avoid bubble formation. The filter can be reseated if bubbles occur. Use forceps to push the air from any large bubbles that form around the edge of the filter outside the area of filtration.
9. Repeat steps 1 through 8 for each sample to be processed.
10. Invert all of the plates and incubate at 44.5°C for 18 ± 2 h.

Presumptive positives on mTEC' agar

After incubation, colonies of *E. coli* will appear red or magenta. Isolates of presumptive positives can be streaked to TSA for storage until confirmation. For verification procedure, see EPA Method 1603, Section 12.

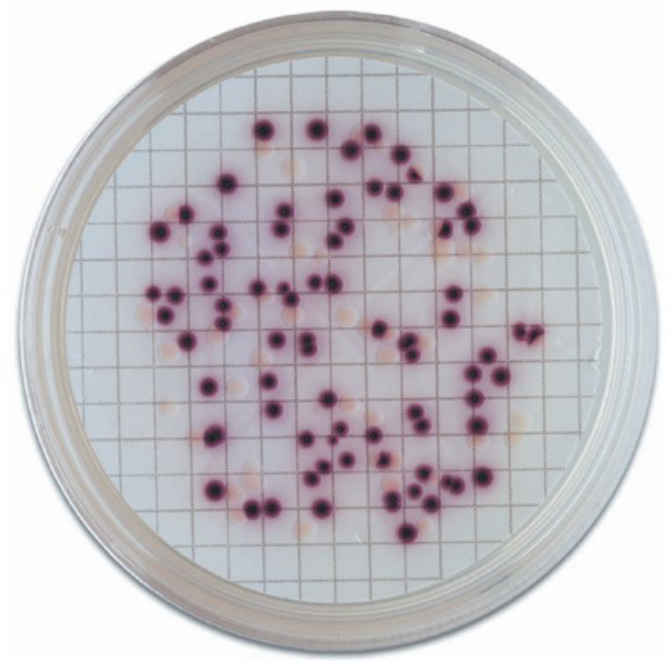


Figure 36. E. coli colonies on mTEC' are red to magenta. Photo: https://www.fishersci.com/shop/products/bd-difco-chromogenic-dehydrated-culture-media-modified-mtec-agar-2/p-4766393

References

U.S. Environmental Protection Agency, 2006, Method 1603—*Escherichia coli* in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar: Washington, D.C., EPA 821-R-06-011, 42 p.

Appendix D: *Listeria* spp. Using Oxoid Brilliance™ *Listeria* Agar

Adapted from Lopez-Galvez, Allende, Pedrero-Salcedo, Alarcon, & Gil, 2014.

Materials

Water samples
Nitrile gloves
10% bleach
70% ethanol
Paper towels
Sterile filters (0.45µm pore size, 47mm diameter)
Oxoid Brilliance *Listeria* agar – one 15x60mm plate per sample

Sterile (autoclaved) magnetic filter towers – one per sample
Vacuum pump and flask
Forceps
Lighter

Procedure

Use aseptic technique and wear clean gloves throughout this procedure.
Store water samples in a cooler with lots of ice packs for no more than 6 hours until you begin.

0. Use paper towels to wipe down the lab bench with 10% bleach, then with 70% ethanol. Label a Brilliance agar plate for each sample to be processed.
1. Spray your gloves with 70% ethanol and rub your hands together to coat your gloves until the ethanol evaporates.
2. Place filter tower base in neck of vacuum flask.
3. Using flame-sterilized forceps, carefully place a sterile filter on the filter tower base, ensuring that the filter settles within the lip around the filter area.
4. Attach the filter tower.
5. Remove the first sample to be processed from the cooler and shake it 30 times to redistribute settled material.
6. Pour 100mL of sample water into the filter tower, using the gradations on the side of the tower to measure the volume.
7. Turn the vacuum pump on and let it run until all of the sample has passed through the filter, then turn the pump off.
8. Using flame-sterilized forceps, roll the filter on to the Brilliance agar with the filtered material facing up. Start with one edge of the filter, and slowly lay it on to the agar to avoid bubble formation. The filter can be reseated if bubbles occur. Use forceps to push

the air from any large bubbles that form around the edge of the filter outside the area of filtration.

9. Repeat steps 1 through 8 for each sample to be processed.
10. Invert all of the plates and incubate at 37°C for 24 ± 2 h.

Presumptive positives on Brilliance agar

After incubation, colonies of *Listeria* spp. will appear blue-green. Pathogenic *L. monocytogenes* or *L. ivanovii* can be differentiated by the appearance of an opaque white halo around the colony. Isolates of presumptive positives can be streaked to TSA for storage until confirmation. For verification, use O.B.I.S. mono test (alternatively traditional ISO 11290-1:1997 confirmation tests may be used).

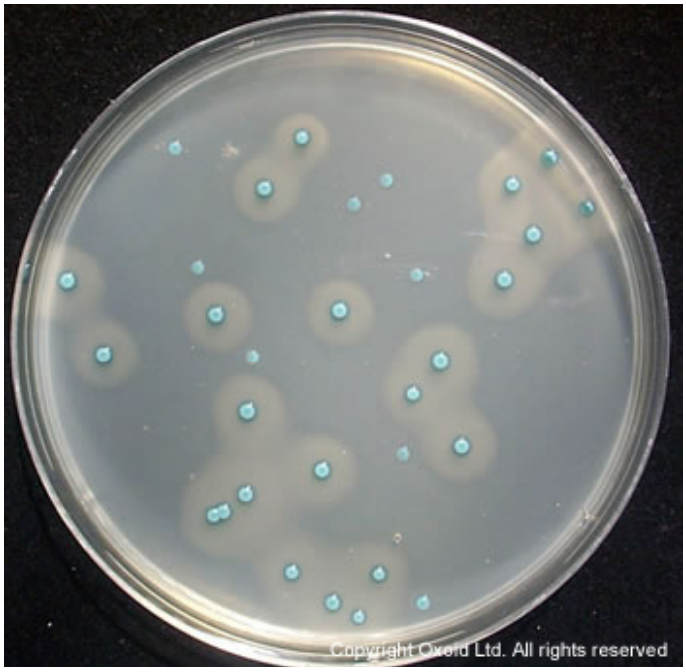


Figure 37. Listeria spp. on Brilliance agar appear blue-green, while pathogenic L. monocytogenes or L. ivanovii are differentiated by an opaque white halo.

References

Lopez-Galvez, F., Allende, A., Pedrero-Salcedo, F., Alarcon, J. J., & Gil, M. I. (2014). Safety assessment of greenhouse hydroponic tomatoes irrigated with reclaimed and surface water. *International Journal of Food Microbiology*, *191*, 97–102. https://doi.org/10.1016/j.ijfoodmicro.2014.09.004

Appendix E: Selective Differential *Salmonella* spp. Culture and Confirmation

Derived from Andrews et al., 2018 and Li et al., 2014

Materials

Water samples
Nitrile gloves
10% bleach
70% ethanol
Paper towels
Sterile filters (0.45 µm pore size, 47 mm diameter)
Sterile 500 µL pipette tips
500µL micropipettor

Sterile (autoclaved) magnetic filter towers – one per sample
Vacuum pump and flask
Forceps – 2 pair
Lighter
Tube racks for 50mL conical tubes and glass test tubes
Vortexer
Incubator at 34 °C
Shaker table
Inoculation loops
Ceramic loop sterilizer
Sterile toothpicks (autoclaved)

Tryptic soy broth (TSB) – 10mL per sample
    Note: It's easiest to autoclave the broth in autoclavable conical tubes rather than autoclaving and then measuring out the hot liquid. You'll need 10mL TSB in a 50mL conical tube for each sample. Be sure to only loosely cap the tubes to allow for gas exchange.
Tetrathionate (TT) broth – 4.5mL per sample
Xylose lysine deoxyxholate (XLD) agar – one 15x60mm plate per sample

Procedure

Use aseptic technique and wear clean gloves throughout this procedure.

Day 1: Pre-enrichment

Collect water samples this day and store them in a cooler with ice packs for no more than 6 hours until you begin.

0. Use paper towels to wipe down the lab bench with 10% bleach, then with 70% ethanol. Label a 50mL conical tube containing 10 mL TSB with sample name and date for each sample to be processed.
1. Spray your gloves with 70% ethanol and rub your hands together to coat your gloves until the ethanol evaporates.
2. Place filter tower base in neck of vacuum flask.
3. Using flame-sterilized forceps, carefully place a sterile filter on the filter tower base, ensuring that the filter settles within the lip around the filter area.
4. Attach the filter tower.
5. Remove the first sample to be processed from the cooler and shake it 30 times to redistribute settled material.
6. Pour 100mL of sample water into the filter tower, using the gradations on the side of the tower to measure the volume.
7. Turn the vacuum pump on and let it run until all of the sample has passed through the filter, then turn the pump off.
8. Using 2 pair of flame-sterilized forceps, fold the filter radially 3 times (into eighths; it should look like a pizza slice).
9. Place the filter into prepared 50mL conical tube containing 10mL TSB and screw the cap on tightly.
10. Vortex the tube on high for 30s.
11. Repeat steps 1 – 11 for each sample to be processed.
12. Place the rack of vortexed TSB tubes on a shaker at 200rpm inside a 34 °C incubator for 24 h ± 2 h.

Day 2: Enriching the pre-enriched sample

0. Use paper towels to wipe down the lab bench with 10% bleach, then with 70% ethanol.
1. Label a 10 mL tube containing 4.5 mL of TT broth with the sample name and date for each sample to be processed.
2. Remove yesterday's TSB tubes from 34 °C incubator. For each sample:
3. Spray your gloves with 70% ethanol and rub your hands together to coat your gloves until the ethanol evaporates.
4. Briefly vortex tube to evenly distribute contents.
5. Use sterile pipette to transfer 500 µL (0.5 mL) of pre-enriched TSB to labeled glass tube with TT broth.

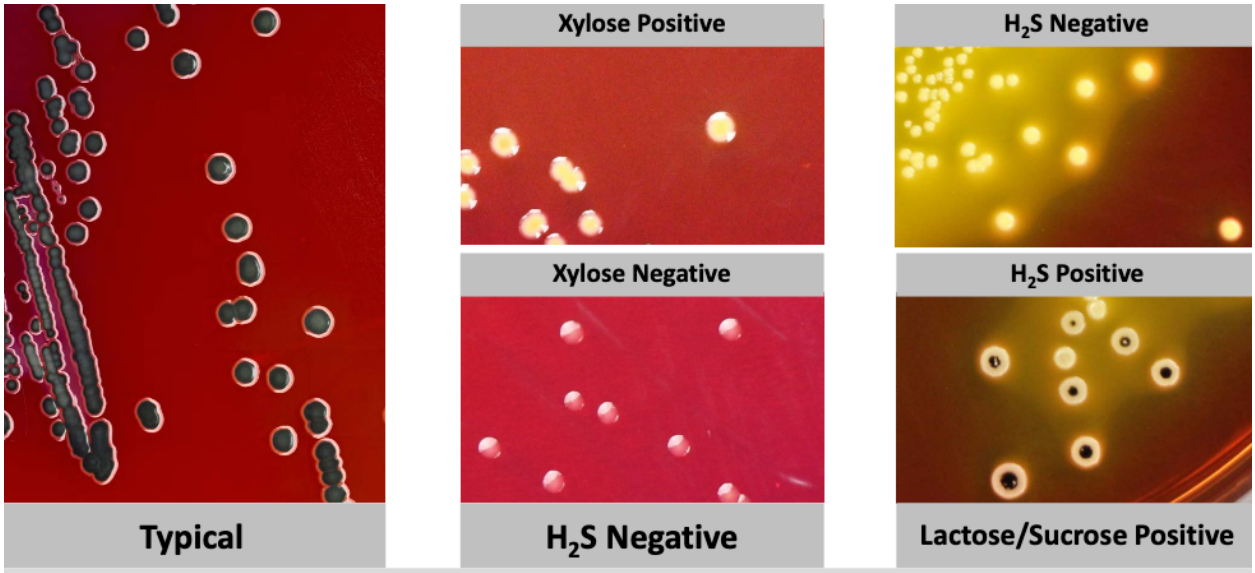
6. Repeat steps 3 – 5 for each sample to be processed.
7. Place rack of inoculated TT broth tubes on shaker table set at 200 rpm in incubator at 34 °C. Incubate for 24 h ± 2 h.

Day 3: Inoculating XLD plates

0. Use paper towels to wipe down the lab bench with 10% bleach, then with 70% ethanol.
1. Label an XLD plate with the sample name and date for each sample to be processed.
2. Remove yesterday's TT broth tubes from 34 °C incubator. For each sample:
3. Spray your gloves with 70% ethanol and rub your hands together to coat your gloves until the ethanol evaporates.
4. Briefly vortex tube to evenly distribute contents.
5. Use sterile inoculating loop to transfer a loopful of enriched TT broth to labeled XLD plate.
6. Use sterile toothpicks to streak sample over each quadrant of the plate using standard technique. Cover plate.
7. Repeat steps 3 – 6 for each sample to be processed.
8. Invert plates and place in incubator at 34 °C for 24 h ± 2 h.

Presumptive positives on XLD

Utilize the *Salmonella* Flipbook to interpret XLD plates after incubation (Forstner, 2016). Typical presumptive positive colonies appear as pink colonies with black centers, but atypical colonies can take several forms depending on the sugar and sulphur metabolism of the strain cultured (Figure 38). Presumptive positive colonies should be confirmed by biochemical or molecular means.



Note: *Salmonella* are typically xylose positive, which results in colonies with a dull yellow color after 22-26 hours of incubation, instead of the "pink colonies" described by the BAM. However, atypical xylose negative *Salmonella* will produce pink – red colonies on XLD after 22-26 hours of incubation. The yellow color caused by xylose fermentation is less intense than the yellow color expressed from atypical lactose and/or sucrose fermenting isolates.
Note: With prolonged incubation, or even letting the plates sit out on the bench, the yellow color caused by xylose fermentation, changes to a pink to red color.

Figure 38. Morphologies of presumptive positive colonies for Salmonella *spp. on XLD agar. Adapted from the Salmonella Flipbook (Forstner, M. J., 2016). Minnesota Department of Agriculture https://www.fda.gov/files/food/published/%3Ci%3ESalmonella%3C-i%3E-Flipbook.pdf).*

References

Andrews, W. H., Wang, H., Jacobson, A., & Hammack, T. (2018). Bacteriological Analytical Manual (BAM) Chapter 5: Salmonella. In *Bacteriological Analytical Manual (BAM)*. U.S. Food and Drug Administration. http://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam-chapter-5-salmonella

Forstner, M. J. (2016). *Salmonella Flipbook*. Minnesota Department of Agriculture. https://www.fda.gov/files/food/published/%3Ci%3ESalmonella%3C-i%3E-Flipbook.pdf

Li, B., Vellidis, G., Liu, H., Jay-Russell, M., Zhao, S., Hu, Z., Wright, A., & Elkins, C. A. (2014). Diversity and Antimicrobial Resistance of Salmonella enterica Isolates from Surface Water in Southeastern United States. *Applied and Environmental Microbiology*, *80*(20), 6355–6365. https://doi.org/10.1128/AEM.02063-14

Appendix F: Bacterial Isolate DNA Processing Script

quatasan.py

Script steps

1. Take the path to the *sample_forward_reads* and *sample_reverse_reads* file as command-line arguments.
2. Run Fastqc on the input files and generate a subdirectory *sample_qc_results*.
3. Run Trimmomatic on the input files and generate four output (*forward_paired*, *reverse_paired*, *forward_unpaired* and *reverse_unpaired*) files with <*base_name*> as <*sample_name*>
4. Run SPAdes on the four output files generated by Trimmomatic and make a subdirectory *sample_spades_results* with the results.
5. Take the *contigs.fasta* generated by SPAdes and run Quast using the *forward_paired*, *reverse_paired* files as inputs. Make a subdirectory *sample_quast_results* with the results.
6. Take the *contigs.fasta* generated by SPAdes, run Prokka on it, and make a subdirectory *sample_prokka_results* with the results.

Script metadata

Location: *home/unhTW/share/mcbs913_2020/aquaponics/conda/project_data/quatasan.py*
Input: Path to the forward and reverse read files for a sample.
Output: Subdirectories with results of Fastqc, Trimmomatic, SPAdes, Quast and Prokka for the sample, stored in the input directory.
Sample run: *$ python3 quatasan.py /path/forward_file /path/reverse_file*

Script

```
#!/usr/bin/env python3

import sys
import os
import subprocess

def main():

    forward = sys.argv[1]    # forward reads file
    reverse = sys.argv[2]    # reverse reads file

    base = forward.split("_")[0]
```

```
    #Fastqc
    print("\nRunning Fastqc...\n")
    outdir = base + "_qc_results"
    subprocess.call('mkdir {}'.format(outdir),shell=True)
    subprocess.call('fastqc {} {} -o {}'.format(forward,reverse,outdir),shell=True)

    #Trimmomatic
    print("\nRunning Trimmomatic...\n")
    baseout = base + ".fastq"
    outdir = base + "_trimmomatic_results"
    subprocess.call('trimmomatic PE -phred33 {} {} -baseout {} LEADING:3 TRAILING:3
SLIDINGWINDOW:4:15 MINLEN:36'.format(forward,reverse,baseout),shell=True)

    #SPAdes
    print("\nRunning SPAdes...\n")
    outdir = base + "_spades_results"
    fpaired = base + "_1P.fastq"
    rpaired = base + "_2P.fastq"
    funpaired = base + "_1U.fastq"
    runpaired = base + "_2U.fastq"
subprocess.call('spades.py -1 {} -2 {} -s {} -s {} -o
{}'.format(fpaired,rpaired,funpaired,runpaired,outdir),shell=True)

    #Quast
    print("Running Quast...\n")
    contigs = base + "_spades_results/contigs.fasta"
    outdir = base + "_quast_results"
    subprocess.call('quast.py -1 {} -2 {} -o {}
{}'.format(fpaired,rpaired,outdir,contigs),shell=True)

    #Prokka
    print("Running Prokka...")
    outdir = base + "_prokka_results"
    subprocess.call('prokka {} -o {}'.format(contigs,outdir),shell=True)

if __name__ == '__main__':
    main()
```

Appendix G: IACUC Approval

revised

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

08-Jan-2019

Guerdat, Todd C
ANFS, Spaulding G42
Durham, NH 03824

IACUC #: 181205
Project: Agriculture, Nutrition, and Food Systems
Approval Date: 02-Jan-2019

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category E in Section V of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are not used.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact me at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Dean Elder, D.V.M.
Vice Chair

cc: File