Thesis for the degree of Doctor of Philosophy

Nutrient Transfer in Aquaponic Systems

Optimizing microbial processes for greater circularity and economic viability

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Abstract

The trend towards sustainable process design in modern industries combines the goal of improving process efficiency with a conscientious shift towards resource conservation. Aquaponics, a system that involves co-cultivating fish and plants, is a waste-conscious food production system. The primary system inputs - water and fish feed - are supplied to the aquaculture component and then transferred downstream to an area of plant cultivation. The upstream aquaculture component is organized in the form of a recirculating aquaculture system (RAS), while the downstream portion is most often a hydroponic greenhouse and as such takes the form of two recirculating loops that have a variable degree of connectivity. Aquaponics, just as its parent fields of aquaculture and hydroponics, falls under the umbrella of closed environment agriculture (CEA) systems. Unlike aquaculture and hydroponic cultivation systems, aquaponics relies heavily on endogenous microbial communities to remineralize nutrients and eliminate fish-toxic waste products. To date, efforts to improve nutrient use efficiency in aquaponic systems have primarily focused on nitrogen metabolism within the biofilter, with research around the utilization of other nutrient streams (solid waste) relegated to waste disposal.

This dissertation addresses this shortcoming by investigating the processes underlying microbial colonization and nutrient remineralization in aquaponics, along with an analysis of the potential to improve system efficiency and sustainability through solids revalorization. These efforts demonstrate the capacity of bioprocess innovation to bridge the commercialization gap that has thus far limited widespread adoption of this type of high intensity, yet sustainable, food production systems. While there are already hundreds of aquaponics operations developing globally, achieving industrial scale production at similar scales to land-based aquaculture and hydroponic facilities has yet to be accomplished. Therefore, this dissertation aims to better understand nutrient flows and remineralization and how they can be utilized to improve food production and resource-use efficiency. Chapter 2 discusses how plants can guide microbial colonization in aquaponic systems, Chapter 3 reviews the advent of ecosystem-specific microbiota and microbiome databases, Chapter 4 introduces a novel nutrient remineralization system that converts fish solids into a fertilizer for CEA, and finally, Chapter 5 expands this technology to include the generation of methane from fish solids. In conclusion to these four chapters, a discussion section contextualizes the experiments within the larger umbrella of microbial and nutrient flow and how this relates to sustainable process design.

Sammanfattning på svenska

Övergången till hållbar processdesign inom moderna industrier kombinerar målet att förbättra processens effektivitet med en medveten prioritering av resursbevarande. Ett avfallsmedvetet livsmedelsproduktionssystem är akvaponik, där fisk och grödor odlas tillsammans. Systemets insatsvaror, såsom vattenförsörjning och fiskfoder, kommer in via vattenbrukskomponenten och överförs sedan nedströms till ett område för planodling, oftast jordbruk i sluten miljö (CEA). I enlighet med systemets cirkulära karaktär är vattenbruksdelen uppströms organiserad i form av ett recirkulerande vattenbrukssystem (RAS). Därför kan akvaponik betraktas som ett RAS-CEA-system med två recirkulerande kretsar med varierande grad av anslutning. Till skillnad från vattenbruk och hydroponiska odlingssystem är akvaponik beroende av endogena mikrobiella samhällen för att remineralisera näringsämnen under produktionen. Forskning på området har huvudsakligen fokuserat på kväveomsättningen i biofiltret.

Denna avhandling beskriver processer som ligger till grund för mikrobiell kolonisation och remineralisering av näringsämnen i akvaponiska system. Den innehåller också en analys av potentialen att förbättra systemets effektivitet och hållbarhet. Tillsammans visar dessa insatser på bioprocessinnovationens förmåga att överbrygga det kommersialiseringsgap som hittills har begränsat den utbredda användningen av denna typ av högintensiva, men ändå hållbara, livsmedelsproduktionssystem. Även om det finns hundratals akvaponiska verksamheter som utvecklas globalt har man ännu inte uppnått industriell produktion i liknande skala som landbaserat vattenbruk och hydroponiska anläggningar. För att uppnå detta mål fokuserar denna avhandling på att utveckla en bättre förståelse för näringsflöden och remineralisering samt hur de kan användas för att förbättra livsmedelsproduktionen och effektiviteten i resursanvändningen.

I kapitel 2 kommer jag att diskutera hur växter kan styra mikrobiell kolonisation i akvaponiska system. I kapitel 3 kommer jag att gå igenom tillkomsten av ekosystemspecifika mikrobiota- och mikrobiomdatabaser. I kapitel 4 kommer ett nytt system för remineralisering av näringsämnen att presenteras som omvandlar fiskfester till gödsel för CEA. Slutligen utvidgas denna teknik i kapitel 5 till att omfatta generering av metan från fiskföremålen. Som avslutning på dessa fyra kapitel finns ett diskussionsavsnitt som kontextualiserar experimenten inom det större paraplyet av mikrobiellt flöde och näringsflöde och hur detta relaterar till hållbar processutformning.

Slow is smooth; smooth is fast.

Anonymous sailing quote

Contents

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List of Papers

The thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Lobanov VP**, Keesman KJ, Joyce A. Plants Dictate Root Microbial Composition in Hydroponics and Aquaponics. Frontiers in Microbiology. 2022;13. https://doi.org/10.3389/fmicb.2022.848057.

VL conducted the laboratory experiment, analyzed the data, and led the writing of the manuscript. This paper is licensed under Creative Commons Attribution 3.0 License.

II. **Lobanov, VP**, Gobet A, Joyce A. "Ecosystem-specific microbiota and microbiome databases in the era of big data." Environmental Microbiome. 2022; 17.1:1-17. https://doi.org/10.1186/s40793-022-00433-1.

VL developed the concept and led the writing of the manuscript. This paper is licensed under Creative Commons Attribution 3.0 License.

III. **Lobanov VP**, Combot D, Pelissier P, Labbé L, Joyce A. "Improving Plant Health Through Nutrient Remineralization in Aquaponic Systems." Frontiers in Plant Science 12 (2021): 1064. https://doi.org/10.3389/fpls.2021.683690.

VL conducted the laboratory experiment, analyzed the data, and led the writing of the manuscript. This paper is licensed under Creative Commons Attribution 3.0 License.

IV. **Lobanov VP**, de Vrieze J, Joyce A. "Simultaneous Biomethane Production and Solids Waste Treatment in Aquaculture." Aquacultural Engineering. 2023; 102328. https://doi.org/10.1016/j.aquaeng.2023.102328.

VL conducted the laboratory experiment, analyzed the data, and led the writing of the manuscript. This paper is licensed under Creative Commons Attribution 3.0 License.

List of Abbreviations

Abbreviation	Description
BF	Biofilter
BOD	Biological oxygen demand
CEA	Controlled environment agriculture
CAMI	Critical Assessment of Metagenome Interpretation
CARD-FISH	Catalyzed Reporter Deposition FISH
CEA	Controlled environment agriculture
CHP	Combined heat and power
COD; tCOD, sCOD	Chemical oxygen demand; total, soluble
cDNA	Complementary DNA
DNRA	Dissimilatory nitrate reduction to ammonium
DO	Dissolved oxygen
DWMP	Drinking Water Microbiome Project
EBPR	Enhanced biological phosphorus removal
EC	Electric conductivity
Eh	Redox potential
EMP	Earth Microbiome Project
ES-DB	Ecosystem specific database
FID	Flame ionization detector
FISH	Fluorescence In Situ Hybridization
GC	Gas chromatography
HNS	Hydroponic nutrient solution
HTS	high-throughput sequencing
IMTA	Integrated multitrophic aquaculture
MCC	Microbiome Centers Consortium
MiDAS	Microbial Database for Activated Sludge
N-DAMO	Nitrate/nitrite dependent anaerobic methane oxidation
OTU	Operational taxonomic unit
PAO	Phosphate accumulating organisms
PCR	Polymerase chain reaction
PHA	polyhydroxyalkanoates
PPB	purple phototrophic bacteria
RAS	Recirculating aquaculture system
RM	Remineralization effluent
rRNA, mRNA	Ribosomal RNA, mitochondrial RNA
SBR	Sequential batch reactor
SCFA	Short chain fatty acid
SRT	Sludge retention time
SRT	Solids retention time
SVI	sludge volume index
TN	Total nitrogen
TS	Total solids

TSS	Total suspended solids
USEPA	US Environmental Protection Agency
UVI	University of the Virgin Island system
VFA	Volatile fatty acids
VFA	Volatile fatty acid
VOCs	Volatile organic compounds
VS	Volatile solids
WWTP	Wastewater treatment plant

Chemical Formulae

Percentage CH_4 as part of the headspace gas composition:

$$\%CH_4 = 100 * \frac{CH_4}{CO_2 + CH_4}$$

Volume of CH_4 produced per liter reactor at standard temperature and pressure:

$$Volume_{CH_4} = \%CH_4 * Volume_{biogas,daily} \frac{273\ K}{301\ K}$$

CH_4 yield calculated from the volume of CH_4 produced per liter reactor divided by the volume of feed sludge added (L) multiplied by its VS or COD (g/L sludge) content:

$$CH_{4_{yield,VS}} = \frac{Volume_{CH_4}}{\frac{g\ VS}{L\ sludge} * Volume_{feed}} \qquad CH_{4_{yield,COD}} = \frac{Volume_{CH_4}}{\frac{g\ COD}{L\ sludge} * Volume_{feed}}$$

Annual energy and electricity production was estimated assuming a CHP electricity conversion efficiency of 40% and a methane to electricity conversion of 1 m^3 CH_4 = 10 kWh and calculating the MJ energy produced as 3.6 MJ = 1 kWh:

$$Electricity\ yield\ \frac{KWh}{L\ sludge} = CH_{4_{yield,VS}} * \frac{10\ kWh}{1\ m^3 CH_4} * 40\%\ efficiency * \frac{g\ VS}{L\ sludge}$$

1. Introduction and Objectives

The logic of viewing production as a linear pathway contrasts with the circularity of natural systems. Since the industrial revolution, this shortcoming has resulted in the proliferation of unsustainable outputs in the name of profitable exploitation. While historically referred to as "waste," these outputs are increasingly being re-examined through the lens of a contemporary paradigm seeking to replicate the cyclical regeneration observed in the natural world. Aquaponics, the co-cultivation of plants and aquatic animals, is representative of this paradigm shift taking place in the context of food production. In aquaponic systems, nutrients are supplied primarily in the form of fish feed, which is consumed and transformed by the aquatic organism. A portion of the excreta fertilizes downstream crops. In practice, however, the loop is not as neatly closed. While it is universally accepted that the nitrogen-metabolizing microbial community plays a crucial role in the elimination of aqueous ammonia, it is not clear how that community interacts with other nutrients. This shortcoming limits the degree to which the community may be steered to optimize production. Secondly, the vast majority of installations utilize only the nitrogen-rich, soluble excreta for plant fertilization. The solids, collected and removed from the facility, are, thus, still being considered waste products. Accessing the nutrients within the solids presents an opportunity for a greater degree of sustainability and circularity. This thesis describes work unraveling these two challenges from the perspective of nutrient transfer.

1.1. The need for the circularization of nutrient flows

A seminal paper by Steffen et al. (2015) presented an outline for planetary boundaries and the potential risks faced by humanity (1). Most segments are insufficiently understood to create a realistic risk assessment, however, in terms of the biochemical flows of nitrogen and phosphorus, a fairly coherent picture has emerged. The introduction of reactive nitrogen and phosphorous species into aquatic ecosystems leads to significant perturbations in community structure as well as a heightened risk for the destabilization of global ecosystems. Proper management of agricultural systems can limit the flow of nutrients from land into water, alleviating pressure on multiple planetary boundaries: biochemical flows and biosphere integrity, with implications as well for climate change (1, 2).

Nitrogen and phosphorus are typically the limiting nutrients for algal growth in water ecosystems (3). Their excess in a water body – alongside other minerals - is a process referred to as eutrophication with disastrous environmental and health consequences (4-6). While anthropogenic sources of these nutrients are numerous, this thesis will narrow the discussion to aquatic food production systems. The motivation for this focus arises from both the necessity of ensuring a stable and efficient food supply for the future and mitigating the environmental impacts of increasing production through intensive aquaculture or agriculture. Aquaculture, an umbrella term encompassing any aquatic animal rearing system, is rapidly emerging as a replacement for the hunting of aquatic animals (fishing), an industry that has leveled off, due to decreasing wild supply and overexploitation (figure 1).

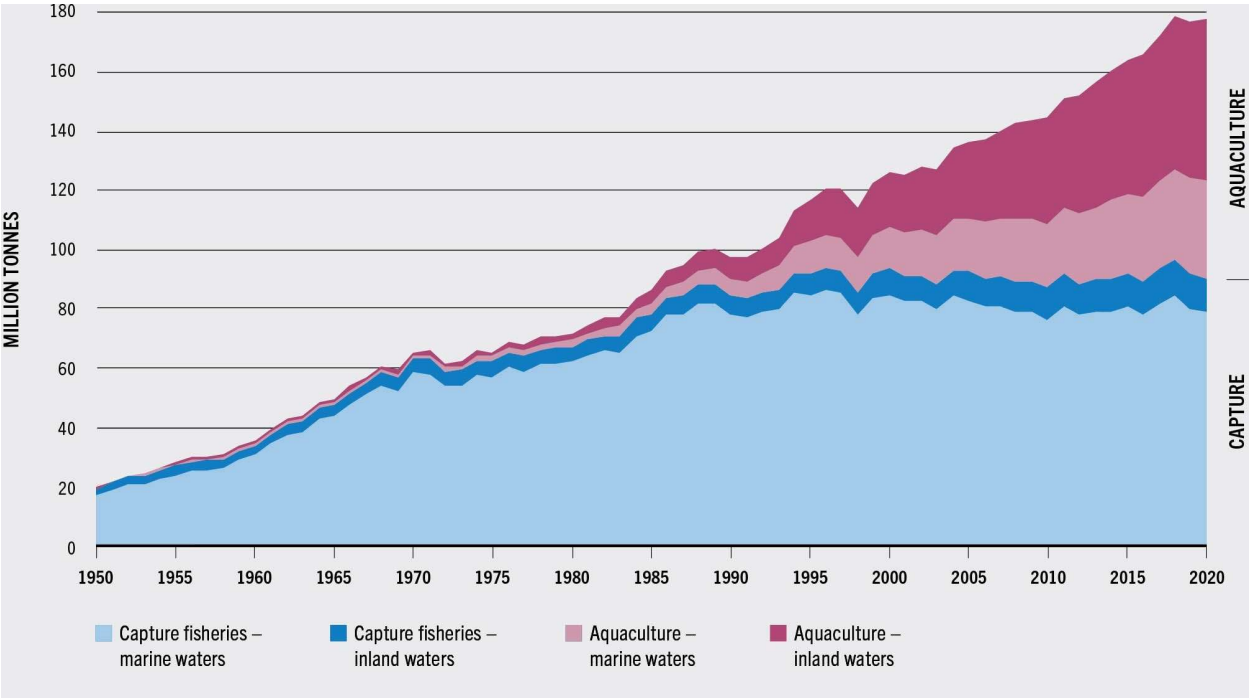


Figure 1. Recent trends in global industrial fisheries and aquaculture output (7). Reprinted "The State of World Fisheries and Aquaculture 2022," by the FAO Rome, 2022.

Currently, only one aquaculture production model allows for efficient waste collection - recirculating aquaculture systems (RAS) (8). This makes RAS ideal for nutrient reuse in subsequent farming practices.

1.2. Waste streams characterization in recirculating aquaculture systems

The aquaculture industry is praised for its lower environmental impact compared to cattle or fisheries derived protein (9). Due to the relatively small scale of aquaculture compared to other food industries, the distance of facilities from the public, and the low level of regulation in the nascent industry, aquaculture waste treatment is often shadowed by discussions around other animal food production models (*e.g.*, beef, pork, poultry production). Nonetheless, significant work has been done on assessing and modelling the impact of wastes on benthic environments (10, 11). Common solutions for agriculture-induced pollution tend to gravitate towards reducing production (*i.e.*, constricting permits, capacity). A more optimal solution to meet increasing food demands alongside minimal environmental perturbation requires active management of the nutrients classified as "waste". The development of closed containment systems for land and coastal cultivation facilities has been indicative of the paradigm shift towards increased water and nutrient-use sustainability (12, 13). Under pressure both from regulatory agencies and the public, solids waste management is an increasingly important issue for the further development of global aquaculture (14-16). With the exception of extractive aquaculture, such as seaweed or bivalve rearing, aquaculture relies on the addition of feed into the water column which is then consumed by the target aquatic organism (fish). Regardless the source of ingredients for fish feed, the nutrients not integrated into animal biomass are excreted and returned to the water column. Some portion of the biomass may shed (*e.g.*, scale loss); otherwise, polluting feed nutrition comes from uneaten feed and feed dust (figure 2).

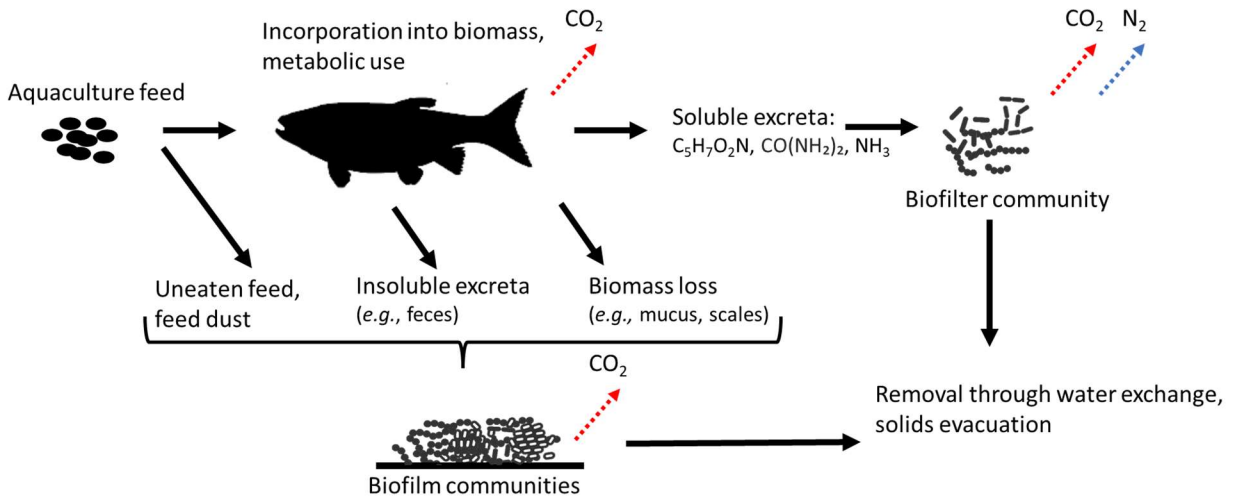


Figure 2. Schematic overview of nutrient flow in recirculating aquaculture systems.

It is easy to visualize why better waste stream management is necessary when considering the scale of modern aquaculture. In 2018, world salmon aquaculture production alone exceeded 2.2 million tons, corresponding to a nutrient loss of 888,800 tons of carbon, 1,113,200 tons of nitrogen, and 20,680 tons of phosphorus into coastal waters (12, 17). These values are a combination of discharge related to metabolism (the exhalation of ammonia from salmon gills and fecal discharge) and the fraction of uneaten feed decomposing in the water column (18).

From another perspective, these waste streams can be divided into two broad categories: dissolved and precipitated fractions. Treatment of the dissolved fraction focuses on the simultaneous removal and neutralization of nitrogenous species, resulting in the net oxidation of residual ammonia to nitrate and co-production of nitrogen gas (19-23). Other nutrients are also carried downstream to varying degrees, depending on their solubility at the neutral pH typical of the upstream water source and their complexation in the fish solids or feed (24-28). In freshwater aquaculture, fecal-derived wastes can be repurposed for agricultural applications (29), although the scope is limited due to a) the costs of transporting large volumes of liquid, b) the high sodium content, c) the seasonality for fertilization (only available in spring/early summer), and d) the high nitrogen load of the fish sludge, which may exceed local regulations if applied in excess. For these reasons, reutilizing the fish sludge in the context of more localized and highly productive systems, such as controlled environment agriculture, is the most promising output for aquaculture-derived nutrient streams. For marine wastes, some form of desalination would also be necessary.

1.3. Controlled environment agriculture

The proliferation of controlled environment agriculture (CEA) began in the late 19th century with the proliferation of modern greenhouse designs in Venlo, the Netherlands. Over a century later, CEA is the most rapidly expanding form of agriculture globally. The need for efficient, compact land-use incentivizes the increased production intensity that is only possible in controlled environments. Enclosing plants in a greenhouse environment is advantageous for several reasons: greater temperature regularity, the possibility for artificial lighting, protection from grazing and inclement weather, as well as perhaps most important: increased biosecurity from pests.

Controlled environment agriculture may include both soil-based and soil-less culture. Between the two, water-based (hydroponic) culture has received interest as a strategy that can inhibit the spread of soil-borne diseases by removing the soil component from the cultivation process. Hydroponics additional enables fine-tuned nutrient profiles for plants, ultimately requiring less resources (30). Accelerating a trend already underway, hydroponic greenhouses are generally monoculture environments where plants are grown independently of the microbial infrastructure they depend on in soil-based systems.

The removal of microbial support hampers the ability of plants to uptake nutrients and adapt to stressors. Hydroponic systems have optimized nutrient dosing based on plant life stage, species/cultivar grown, as well as ambient conditions, with the major caveat that cultivation takes place under conditions relatively void of microbial life. The priority placed on quick growth and appearance for better salability incentivizes the overapplication of some nutrients, such as nitrogen, which force plant cells to uptake more water to compensate toxicity at high concentrations – leading to leaves with a darker green color that consumers associate with vigor. Meanwhile, nitrogen excess locks out other nutrients, such as calcium and potassium (31, 32). Under natural conditions, nitrogen is virtually always deficient giving rise to microbial communities specialized in adressing the floral demand for nitrogen as an essential nutrient for growth. Excessive nitrogen compounds the diversity loss typical to hydroponic cultivation by removing the incentive for plants to trade with microbial symbionts. Typically, plants release significant fractions of their sequestered CO_2 through their roots in the form of secondary metabolites (mainly sugars and short chain fatty acids (SCFAs)). Besides nitrogen, the plant receives other mineral nutrients from the root-inhabiting (rhizospheric) microbial community, alongside a slew of secondary metabolites. CEA titrates nitrogen into plant cultivation systems with a far higher degree of precision than field fertilization, which must additionally account for loss through runoff and imprecise application.

Aquaculture, alongside other forms of animal husbandry, is encompassed under the umbrella of CEA (33). Aquaponics has the potential to combine the precision of hydroponics alongside the high protein production efficiency of aquaculture into a circular and value-adding CEA model by sourcing the nitrogen from the fish tanks - decoupling itself from industrial nitrogen fertilizer production. This paradigm shift in fertilization is a powerful driver of the aquaponics industry, representing a critical first step in establishing a circular food production network.

1.4. Aquaculture as an ideal CEA subset

Aquaponics derives its name from the portmanteau between the Latin “aquaculture” and Greek “hydroponics”. While the co-cultivation concept has existed in various forms for millennia, particularly in Asia where pond-based systems are common, the modern concept stems from the University of the Virgin Island (UVI) system established in 1981. The UVI system typically has several circular fish tanks filled to a maximal density based on the species cultivated, the feed used, and the ambient temperature. Water is continuously pumped in and out of the tanks with the drained water being passed into a treatment zone. Fish solids are removed from the water column, typically through mechanical filtration or settling in sump clarifiers, which are cleared out regularly – the fish solids are considered to be a waste product from the system. The supernatant passes through a biofilter with the goal of oxidizing dissolved ammonia. Despite an initial belief in the industry that the nitrogen profile was converted into nitrate through two stages of oxidation, research over the past thirty years has greatly expanded our knowledge of the nitrogen cycle (see section 1.8.2). The effluent then passes into a sump, from which is

it distributed throughout the hydroponics cultivation system. The UVI system includes regular base addition for pH control, however, this is not universally present across aquaponic systems. The plants thus receive a nitrogen-rich soluble fertilizer as well as a component of the available phosphorus and other dissolved nutrients that is not colloidally bound in the fish solids.

Two water circulation strategies are used with unique advantages depending on the conditions: decoupled vs. coupled systems. In decoupled systems, water entering the hydroponic unit from the fish tanks is re-used internally for a period of time for plant production but is ultimately discharged without passing back to the fish tanks. This creates more room for intervention in plant cultivation, such as fertilization and pesticide application, which may be otherwise toxic to aquatic animals. Because of the unilateral flow, microorganisms travel from the aquaculture unit to the plants, but not back, thus, they cannot influence fish health.

In contrast to the decoupled approach, coupled systems function within a fundamentally different paradigm. Water leaving the biofilter enters the hydroponic system where nutrients are taken up by the plants before returning to the aquaculture unit for re-use. Whether all water is recirculated or just a fraction depends on the site-specific conditions. A high salinity environment, for example, may require the hydroponic water to be diluted with water from a less saline source so as not to stress the plants, or in conditions with low evapotranspiration, excess water may be discharged outwards to other sources, such as fruit trees where it can be used for irrigation. While a minor amount of plant-specific fertilization is possible without adversely impacting the aquaculture unit, coupled systems tend to operate with minimal intervention, thus, treatments such as foliar sprays are the preferred method of nutrient supplementation for plants when required. Mounting evidence suggests that a common microbiome forms for coupled systems (25, 34-36). Under emergency situations (*e.g.*, disease outbreak in the aquaculture unit), the system is often decoupled to isolate the affected segment.

To date, there is a paucity of data on the commercial prevalence of coupled vs. decoupled vs. alternating systems, although the number of aquaponic facilities in Europe and the USA likely numbers in the thousands (François Latrille, Hydroccitanie, FR; Gundula Proksch, University of Washington, USA. Unpublished data).

1.5. Waste revalorization in an aquaculture context

Within an aquaponics system, dissolved nutrients are carried between fish and plant components for use as a liquid fertilizer. However, there is an additional form of waste that has proven more difficult to re-use, including particulate and insoluble portion of the feces and uneaten feed, known as fish solids. This fraction nonetheless contains a significant portion of the total carbon and nitrogen in the system (figure 3). There are intrinsic challenges when handling fish solids, the first of which is the low specific gravity (*e.g.*, 1.05 g/cm^3 for rainbow trout (*Oncorhynchus mykiss*) (37)), however, this value varies greatly across species (38). Solids can be subdivided into light particles, consisting of fish mucus and gelatinized starches (1.05 g/cm^3) and dense particles (1.15 g/cm^3), consisting of undigested cellulose present in the feed (39). In terms of particle size, roughly half of particles exceed a diameter of 100 µm, while the other half fall between 40-100 µm (40). For instance, in rainbow trout, the majority of particles are between 300-650 µm, with densities ranging from 1.01 to 1.05 g/cm^3 (41). Total suspended solids in the water range up to 20 mg/L in a healthy RAS, correlated linearly with stocking density. This results in an average total suspended solids concentration of around 7.5 mg/L for 100 kg/m^3 of trout (41). In terms of the elemental composition, a recent review by Schumann and Brinker (2020) summarizes typical total

carbon, nitrogen, and phosphorus for solids from salmonid culture as averaging 336, 35.2, and 27.5 mg/g dry weight, respectively. Fish solids average 452, 38.3, and 23.9 mg/g dry weight (41). Beyond this, the trace element load varies considerably, depending on the choice of feed and fish (42). Recent reviews of solids derived from aquaculture discuss in detail factors influencing the quantity and composition of these solids with respect to the choice of feed (38, 39). Given these properties, a biologically mediated process could be better suited for waste revalorization compared to a physical solids collection system. Biological treatment is generally more cost-effective than chemical or physical treatment, is by definition an environmentally friendly process not requiring the use of harsh chemicals or producing toxic byproducts. Under optimized conditions, microbial communities may adapt to a wide range of contaminants as well as be customized to meet the specific needs of the facility. Biological treatment is a scalable process that can be easily adjusted to handle changes in the volume and quality of wastewater. And finally, it is typically less energy-intensive than other treatment methods.

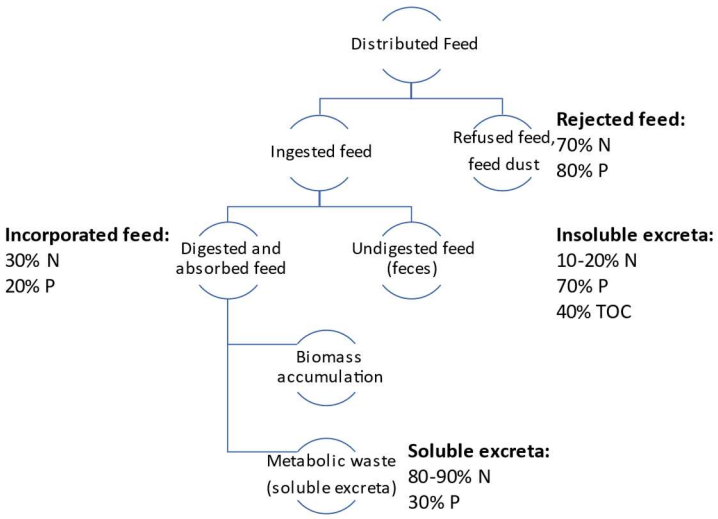


Figure 3. Downstream pathways for distributed feed with approximate values of N, P, and total organic carbon (TOC) attributed to each outcome. Adapted from d'Orbcastel et al. (2008) (13).

Once diverted from the water recirculation system, aquaculture facilities have two terminal options for fish solids: neutralization (*e.g.*, biological degradation and disposal of residual sludge, where they are often redirected to municipal waste treatment streams), or the possibility for revalorization of nutrients in the effluent as part of other bioprocesses (*e.g.*, use as fertilizers, or in biogas/biofuel production). The inherent financial costs associated with the collection and removal of fish solids is increasingly creating incentives for growers to resell sludge as organic fertilizer or explore uses (43-45). Of the nutrient revalorization strategies currently available, microalgal-based biomass production has received much attention, although high downstream processing costs continue to limit the widespread adoption and applicability of such techniques (46-48).

1.6. Solids removal in closed aquaculture systems

Solids management in closed aquaculture systems is essential, due to pernicious direct and indirect effects of suspended solids on finfish health (38, 41, 49, 50). As thoroughly reviewed elsewhere, there are many types of solids collection systems available to modern freshwater aquaculture facilities today, each with unique advantages and disadvantages (41, 51). Solids are taken out of the circulation loop

through diverse and often facility-specific, collection designs (drum, swirl separator, or radial flow settlers) (38, 52). Once diverted from the water recirculation system, aquaculture facilities have two terminal options for fish solids: neutralization (*e.g.*, biological degradation and disposal of residual sludge, where they are often redirected to municipal waste treatment streams), or the possibility for revalorization of nutrients in the effluent as part of other bioprocesses (*e.g.*, use as fertilizers, or in biogas/biofuel production).

The financial costs associated with the collection and removal of fish solids incentivizes growers to resell sludge as organic fertilizer or explore other uses (43-45). Of the nutrient revalorization strategies currently available, microalgal-based biomass production has received much attention, although high downstream processing costs continue to limit the widespread adoption and applicability of such techniques (46-48). Anaerobic biorefineries have been shown to integrate well with aquaculture systems, although their current stage of development suggests that considerable time and innovation is still needed before they become economically viable at a commercial scale (53-56). Besides microbial strategies for waste revalorization, there are two branches of aquaculture that exploit the co-cultivation of symbiotic organisms to offset waste treatment costs by diversifying production. Integrated multitrophic aquaculture (IMTA) seeks to maximize nutrient use efficiency whereby waste from higher trophic species is used as nutrients for species at lower trophic levels (57). Currently, however, IMTA has not been widely adopted in the industry owing to the complexity of cultivation parameters (different stocking densities, time to harvest, and a range of geographic constraints) that lead to both economic and logistical disincentives. Nutrients are also lost from IMTA systems, thus making them much less efficient – albeit much cheaper to install and operate - than land-based contained aquaculture system such as a RAS.

1.7. Anaerobic digestion

Anaerobic digestion is a process that involves the breakdown of organic matter in the absence of oxygen. The digestion of organic wastes from aquaculture or agriculture is considerably more straightforward than municipal wastewater sludge owing to the more narrowly defined feedstock and lack of potentially toxic contaminants (*e.g.*, heavy metals, pharmaceutical biproducts, industrial wastes). Organic feedstocks differ mainly in their carbon: nitrogen ratio, chemical oxygen demand (COD), and the biodegradability of the sludge as determined by the biological oxygen demand (BOD). Regardless of the feedstock, anaerobic digestion involves interconnected groups of microorganisms that coordinate to perform a range of tasks: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (58). During hydrolysis, complex organic compounds are broken down into simpler sugars and organic acids. In acidogenesis, these sugars and organic acids are further metabolized into more simple organic acids, alcohols, aldehydes, and gases like carbon dioxide and hydrogen. In acetogenesis, the organic acids are converted into volatile fatty acids, especially short chain fatty acids. The methanogens themselves are typically limited to a narrow range of carbon and energy sources: CO, CO_2/H_2, formate, acetate, methanol, and methylamines, and CO (59).

The ideal anaerobic digester must allow for a high organic loading rate that minimizes the hydraulic retention time and maximizes the volume of methane production. Environmental parameters relevant to those goals are temperature, pH, and volatile fatty acid concentrations. While anaerobic digesters may operate under colder conditions (<20 °C) (60), biogas production decreases as the temperature drops below 35 °C (61). Thus, alongside the initial cost of insulation for the digester, there is the

potential need for heating of the reactor. Nonetheless, mesophilic digesters, in general, can operate at 37°C if well isolated, keeping itself warm from metabolic heat. Thermophilic digesters can keep 50-54°C if installed with a good heat coupling system, isolation, and mild weather. Heating beyond mesophilic temperatures (37 °C) into the thermophilic range (55 °C) does not always increase methane production, although it can reduce the hydraulics retention time necessary to decompose a given substrate (62, 63).

Ideally, the pH range should be maintained between 6.8–7.2 (64). Methanogens are inhibited below pH 6.6, which is above the ideal pH range for hydrolysis and acidogenesis (pH 5.5 and 6.5, respectively). The compartmental separation of these segments is possible, however, must be validated to determine if it is economically sensible for a given application. The pH will affect the ionization state of two potential inhibitors to methanogenesis, hydrogen sulfide and ammonia, which are both toxic in their non-ionized forms, with the former becoming problematic at acidic pH and the latter at alkaline pH (65). As such, both should be characterized for the feedstock to know the specific risks for deviating outside of the optimal pH zone.

Volatile fatty acids (VFAs) alongside H_2 are a critical substrate for methanogens. At high concentrations, however, they may inhibit methanogenesis. Acetic acid is the most abundant; the accumulation of longer chain SCFAs (especially C3 and C4 acids) typically signifies an inhibition in methanogenesis (figure 4). The fermentation of readily bioavailable carbon such as sugars, is inhibited when total VFA concentrations exceed 4 g/L (66). Whereas feedstocks rich in readily bioavailable carbon may be limited by methanogenesis (SCFA production outpaces methane production), poorly degradable feedstocks are typically limited at the hydrolysis stage (65). Furthermore, high SCFA concentrations will decrease the buffering capacity of the digester before the pH decreases.

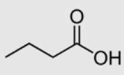
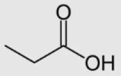
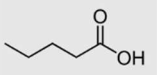
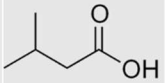
Name	Formic acid	Acetic acid	Propionic acid	Butyric acid	Isobutyric acid	Valeric acid	Isovaleric acid
Lipid number	C1:0	C2:0	C3:0	C4:0	C4:0	C5:0	C5:0
Lewis structure							

Figure 4. List of short chain fatty acids typically present in anaerobic digestion.

1.8. Tracing the paths of nutrients

In the background of any microbial process is a complex web of nutrient trading, regulated by physicochemical parameters (redox, pH, temperature), substrate availability (loading rate), competition, and growth kinetics. In aquaponics, all nutrients take the general path described in figure 5.

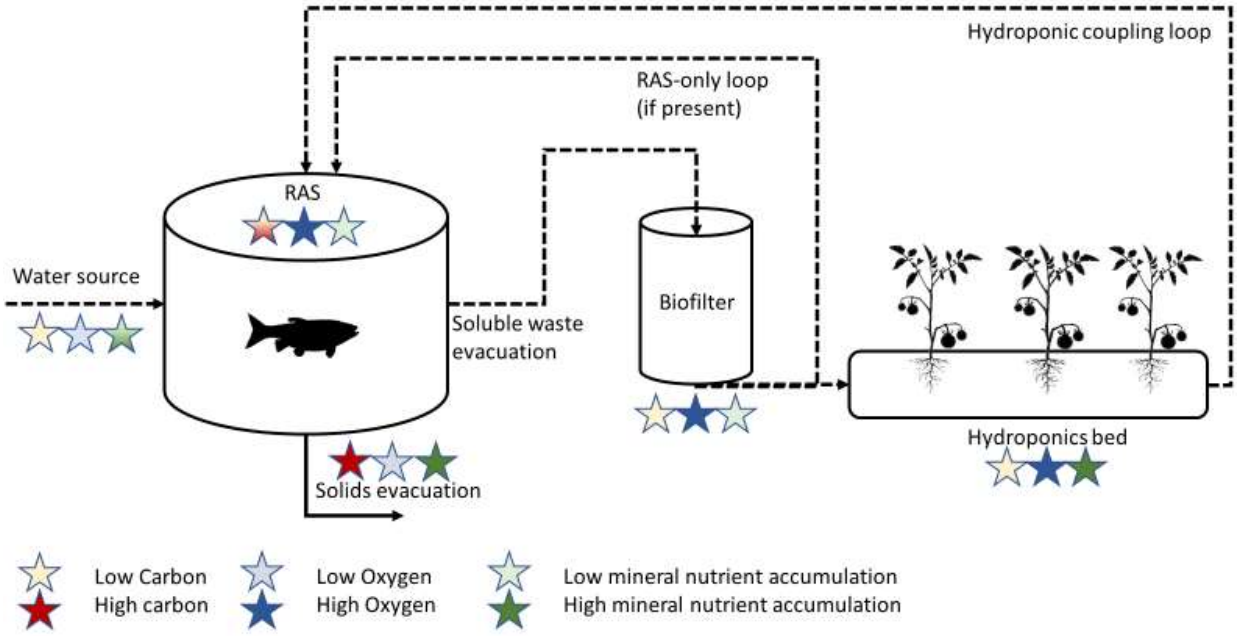


Figure 5. Schematic of water and nutrient flow in a typical aquaponics system.

Understanding how individual nutrients change state or form can help pinpoint regions for further optimization. In the first phase, the feed is metabolized by the fish and released back into the water column, at which point there is an increase in the heterogeneity across how nutrients are bound (figure 6). The following section will briefly cover the path taken by carbon, nitrogen, and phosphorus – the three most dominant elements in biological systems – before discussing mineral nutrients as a collective group.

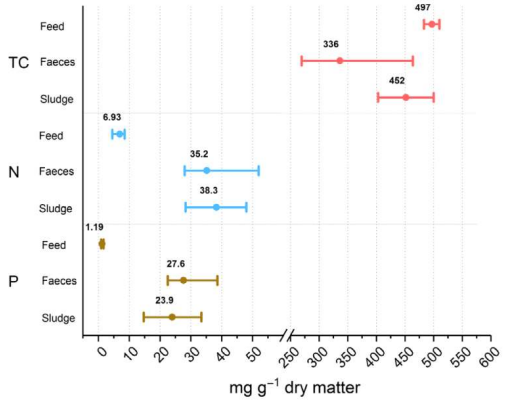


Figure 6. Relative concentration of total carbon (TC), nitrogen (N), and phosphorus (P) in commercial salmonid farms. Graphic taken from Schumann and Brinker (2020) (41).

1.8.1. Carbon

In aquafeed, carbon is present in the form of oils (main source of calories for fish metabolism) and plant-based meal. Because of the high energy and nutrient density, uneaten feed in the form of uneaten pellets and feed dust contributes disproportionately higher to deteriorating water quality than feces. In a well managed farm, however, the main source of carbon into the water column is through fish feces, the composition of which is dependent on the species being farmed, and the type (target life stage) and quality of feed. Regardless the species, feces comprise a soluble fraction made up of water and bodily secretions and a solid fraction of undigested or otherwise unabsorbed material, microorganisms and sloughed gastrointestinal cells (67).

The next phase of the carbon cycle in aquaponics is microbial. Microorganisms are present in the water column (*i.e.,* heterotrophic bacteria directly consume soluble carbon), although most bacterial diversity is present in biofilms (68, 69). The most important biofilm from the perspective of aquaculture is the biofilter. While nitrogen metabolizing microorganisms comprise the majority of biofilter environment, they cohabitate with heterotrophic bacteria. As such, the main carbon sinks are microbial biomass and CO_2. In a study monitoring carbon in a saltwater RAS, 34% of the carbon was calculated to be released aerobically as CO_2 of which 10% was from the biofilter and 24% from the fish themselves (70). The majority of carbon becomes sludge (39% according to the same study), a term which can broadly include wastes from the fish, sloughed microbial biomass, and uneaten feed, which is removed from the system using a waste containment technology. Solids are taken out of the circulating system through diverse, and often facility-specific, collection designs (drum, swirl separator, or radial flow settler) (38, 52). Mesh lined drum filters (20 – 100 μm) are a the most common waste collection system for RAS (51, 71).

Anaerobic biorefineries have been shown to integrate well with aquaculture systems, although their current stage of development suggests that considerable time and innovation is still needed before they become economically viable at a commercial scale (53-56). Thus, carbon leaves the system either as part of fish biomass, CO_2, or sludge with minimal transferred downstream into a hydroponics bed when aquaculture systems are coupled to CEA.

1.8.2. Nitrogen

Aquafeed is rich in nitrogen, due to a high protein content (25–65% depending on the species, corresponds to 4.1–10.7% organic nitrogen) (70, 72). While virtually all of the oil fraction in aquafeed is metabolized by the fish, only about 20–30% of the nitrogen is retained in the biomass (73, 74). Part of this nitrogen is secreted through the gills as ammonia, while the rest leaves through the feces – together amounting to approximately ¾ of the total nitrogen consumed (75, 76).

Aerobic nitrification via a biofilter is the industrial standard to mitigate ammonia toxicity, whereby constant aeration over biofilm-laden carriers support a diverse nitrogen metabolizing community (51). In a study tracking the fate of carbon and nitrogen in a closed system RAS, Yogev et al. determined that 45% of the total nitrogen added into the system left as N_2 and 1.3% as N_2O. With 28% incorporated into the fish biomass, 25.7% remained in their system as soluble NO_3 following aerobic nitrification (70). Simultaneously, approximately each mole of reduced nitrate creates a mole of alkalinity (77).

Oxygen is unable to penetrate deeply (> 100 μm) into biofilm, thus, leading to anoxic conditions and denitrification, a process by which nitrogen oxides are reduced to nitrogen gas (N_2) through a series of intermediate compounds (figure 6). Anammox, short for anaerobic ammonium oxidation, is a process in

which nitrogen is oxidized to nitrogen gas utilizing ammonia and nitrite as substrates. In RAS, anammox is responsible for removal of around a fifth of the introduced nitrogen (70). Other recently discovered nitrogen metabolic lifestyles, such as complete ammonia oxidation (comammox), dissimilatory nitrate reduction to ammonia (DNRA), and nitrate/nitrite dependent anaerobic methane oxidation (N-DAMO) are yet poorly studied in aquaculture systems. Comammox, alongside ammonia oxidizing archaea, are suspected in forming a stable consortium in biofilters, which would help explain the high rate of nitrogen gas removal despite very low hydraulic retention times (78). The N-DAMO bacteria certainly would exist in an anaerobic sludge treatment system (79), although the practical contribution to methanogenesis has not yet been explored. The DNRA bacteria are considered to be prevalent under anaerobic conditions with a ready supply of labile organic carbon relative to nitrate as well as sulfidic conditions (80), and, thus, may also be present in RAS – especially saltwater RAS.

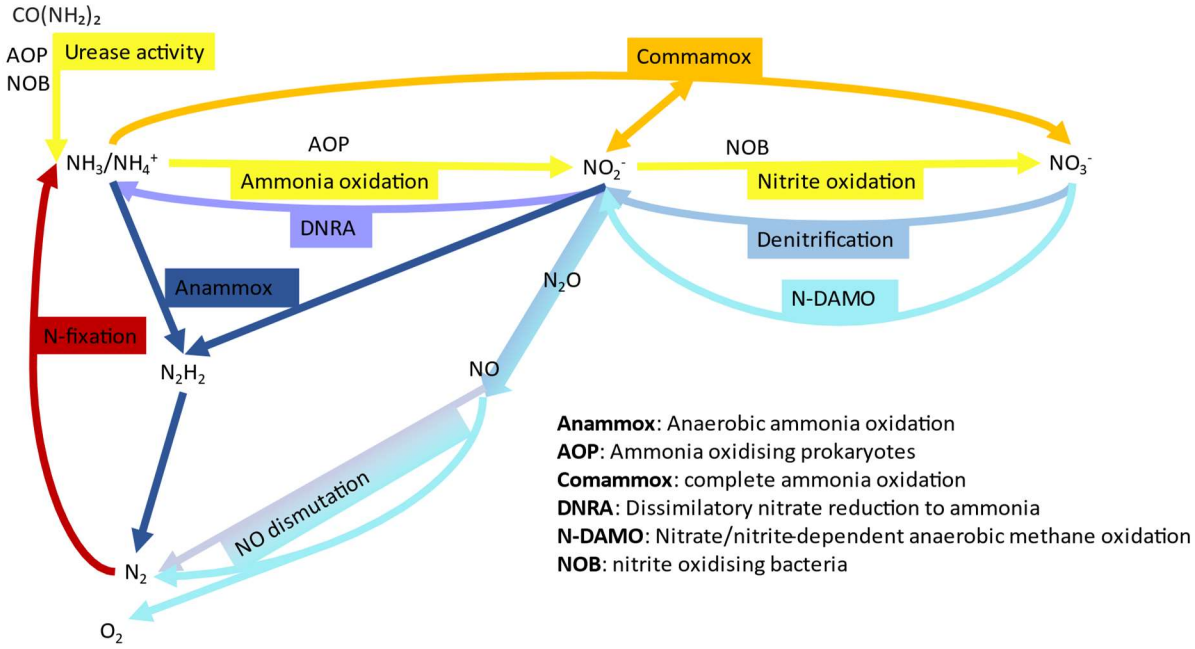


Figure 7. Interplay of nitrogen metabolic pathways discovered to date.

With respect to aquaponics, the distribution of nitrogen between gas, liquid, and solid phases directly impacts its transferability from aquafeed to downstream plants. Following the estimations by Yogev et al. (2017), around a quarter of the original nitrogen can theoretically make its way into the hydroponics system using conventional aerobic nitrification. In their calculations, an additional 23% was removed as "stabilized solids". The next generation of circular waste treatment, and the focus of this dissertation, is in unlocking the nutrient contained within that fraction for the purposes of on-site plant cultivation.

1.8.3. Phosphorus

After carbon and nitrogen, the remaining nutrients are far less consequential in terms of the total amounts presents. Phosphorus, however, is unusual in the disproportional effect it engenders on photosynthetic organisms at low concentration. The US Environmental Protection Agency (USEPA) regulations aim between 8- 37 µg TP/L in lakes and reservoirs and 10 – 128 µg TP/L for rivers and streams depending on ecoregion in the USA, although these are not legally binding criteria (81). From an

environmental perspective, biofilms become phosphorus limited when reactive P concentrations drop below 100 µg/L (82). There is furthermore an economic argument behind responsible phosphorus management. Phosphate is a key agricultural fertilizer import, with global P resources being rapidly depleted (83). The USA currently has less than 25 years of domestic phosphate rock available. Meanwhile, much of the world, including all EU countries, are entirely dependent on phosphate imports. Morocco, including occupied possessions in West Sahara, provides a third of the world's phosphate demands. China has recently imposed a 135% export tariff to preserve its reserves for domestic use, which has led to an increase in phosphate prices worldwide, especially in Scandinavia, which currently boycotts Moroccan phosphate for political reasons (84, 85). Phosphorus sequestration is, thus, not only a nutrient recovery strategy to reduce pollution of waterbodies but is imperative to ensure food production in the future.

As with carbon and nitrogen, phosphorus enters the system via the feed. Dissimilarly, phosphorus has no gas phase, and, thus, remains bound in the solids matrix or microbial biomass, or is liberated into the water column as orthophosphate (PO_4^{3-}). Inorganic phosphorus precipitates readily with metals, such as iron, important when considering the use of iron-based coagulants. To limit environmental discharge, many P removal strategies targeting soluble phosphorus removal have been developed over the past decades (86-91). This diversity is largely due to hidden costs behind most treatment strategies. Reactive filters may be suitable for P capture (92, 93), however, replacement filter costs often make this option undesirable. Alum, especially in combination with additional polymers, can be effective at precipitating soluble P (up to 99%) (94-96). Drawbacks to this strategy include potential toxicity from unprecipitated alum, additional safe disposal/reuse steps, and cost factors, as these types of industrial compounds required for removal may only be purchases in large quantities, thus, making them mostly useful in large-scale waste-treatment plants. Other industrial byproducts have been shown effective at removing P (89, 97, 98), although similarly, these must be further validated to address consumer safety concerns. The use of industrially-sources chemicals may introduce exogenous metals or create metal-chelating environments, potentially elevating concentrations above daily intake recommendations. Outdoor constructed wetlands and the related woodchip bioreactors have also been explored as a low-cost filter technology for wastes that don't pose significant pathogen risks, and don't contain other industrial chemical or heavy-metal contaminants that are risks to the environment. Wetlands typically reduce P in the effluent by ca. 22%, and, thus, are often utilized as a minimal waste treatment strategy for RAS facilities (99). One type of constructed wetland, known as woodchip biofilters, can lead to 15 – 54% P removal (100). While reducing nutrient transfer into the downstream environment, these strategies do not allow for easy recovery.

The past couple decades have seen a trend towards biological strategies for general water treatment in closed containment systems, especially in the context of recirculating aquaculture systems (101). As most (> 94%) particulate matter in water recirculation systems are smaller than 20 µm in diameter (102), biological strategies are more suitable than mechanical separation or filtration at cleaning the water column. This trend has little impact in the context of phosphorus removal, because although particle size is not related to P content, particles < 100 µm in size only account for 20% of all P (103). This indicates that most P is not present in the water column, but instead is bound, and, thus, not bioavailable.

The relevancy of soluble phosphorus recovery through downstream biological systems is controversial. Cerozi et al. (2017) showed that 71.7% of the excreted soluble P_i can be taken up by lettuce in an

aquaponic operation (25), with similar values reported elsewhere (104, 105). Jaeger et al. (2019), however, demonstrated that P-uptake by lettuce represented only 0.42% of the total feed-P originally provided (106). In contrast, Schneider et al. (2005) demonstrated that up to 90% of feed-derived P could be removed through a combined microalgae/aquatic herbivore cultivation on RAS effluent (107). In the same vein, integrated multi-trophic aquaculture has been proposed as a solution to both N and P waste in open and closed contained systems alike (108-110). The complexity of maintaining additional culture species, however, inhibits widespread adoption of this strategy by most RAS facilities.

The proportion of P ending up in settling tanks varies widely (21 – 86% in solid waste (29, 51)) in a manner dependent on species and growing conditions. Strictly speaking, around 18% of the original feed-P ends up as dissolved inorganic P (orthophosphate; P_i), while 44% remains bound in insoluble fecal particles (17), ultimately accumulating in settling tanks for removal. Recently, Yogev et al. (2020) reported 69.8% of feed-P left the fish as sludge, while 3.8% left as soluble P_i in an aquaponics operation growing African catfish and lettuce (111). Phosphorus solubility is generally not affected by chemical, mechanical, thermal, and biological pretreatment prior to anaerobic fermentation (112). Rather, P is slowly liberated from fecal particles over time, as they are broken down by heterotrophic bacteria. Fish farm facilities elevate downstream P loads by 0.02 – 0.13 mg TP/L; 44% of TP is present as orthophosphate (113). This is not due to feces alone, but also uneaten feed pellets, contributing 40-88% of the downstream benthic community nutrition from open containment systems diets (114).

The above sections paint a complicated picture for phosphorus. On the one hand, there are environmental and economic incentives to extract it from wastes including fish solids. On the other hand, it is less mobile than carbon or nitrogen, remaining more tightly bound within the sludge matrix.

1.8.4. Trace minerals

Following the big three, the remaining nutrients can be neatly summed up at mineral nutrients. Their concentrations are negligible beside C, N, and P, yet a few still exert an important role both in sludge treatment and eventually, plant cultivation. From a microbial perspective, these elements play an important role as catalysts. Iron (II) is oxidized to iron (III), which is then returned to iron (II) by iron-reducing bacteria as part of a promiscuous dance of oxidation states. Iron in particular plays a special role for solids treatment as a coagulant and in sustaining healthy anaerobic digestion. Furthermore, iron deficiencies in aquaponic systems are common, given that iron levels in fish feeds are relatively low to what is believed to be desirable levels of iron for plants in hydroponic systems (115). Experiments presented in chapter 3 and 4 demonstrate a potential way to use iron to fulfill multiple rolls within an aquaponics nutrient remineralization system: first as a coagulant, then as an essential nutrient for methanogens, and finally to supplement plant nutritional demands.

1.9. Broadening the scope

The above sections discuss the topic of nutrient remineralization from the perspective of nutrient-use optimization. While this thesis limits the scope to the topic of aquaculture, the fundamentals of the process may be applied to any nutrient-rich waste process. Agricultural waste from livestock manure, crop residues, or food processing waste are all suitable substrates for anaerobic digestion. Much of the underlying principles in the waste treatment system developed in chapters 4 and 5 originate from work done on municipal waste streams. Lastly, depending on the sector, the trends described in this dissertation may apply to industrial waste streams (with food and beverage processes being most optimal). Depending on the carbon: nitrogen ratio, many options exist for downstream treatment of the

anaerobic digestate either for the purpose of reducing the total nutrient load in the effluent or solubilizing nutrients for hydroponics cultivation. Ultimately, while the context here is constrained to aquaculture and aquaponics, the information and conclusions are more broadly applicable and can serve to facilitate research into the revalorization of other waste streams.

1.10. Aims of the thesis

The aim of this thesis was to experimentally and analytically evaluate the potential to modulate microbial communities for the purpose of waste treatment and nutrient remineralization in aquaculture and aquaponics food production system. In addition to studying the underlying biological processes underpinning waste mitigation and nutrient remineralization, this thesis prioritizes economically viable and circular solutions. Achieving the aims of the thesis involved:

- Tracking the colonization capacity of aquaculture-derived microorganisms as they enter downstream (hydroponic) components;
- Establishing a framework for the systematic study of microorganisms across compartmentalized systems;
- Assessing the capacity for nutrient remineralization using the native community present in aquaculture solids;
- Assessing the capacity for biogas production alongside nutrient remineralization.

The outcomes of this thesis emphasize a focus on economically viable solutions. While it is technically possible to remove and treat waste from aquaculture production in a variety of ways, only economically sustainable systems will outlast the research phase. The first sections of this thesis present a series of experiments carried out to achieve the aforementioned goals followed by a discussion of the practical and economic scalability of solids treatment systems for aquaculture and aquaponics.

2. Plants Dictate Root Microbial Composition in Hydroponics and Aquaponics

In this chapter we examined the capacity of upstream microbial communities to colonize plant roots in downstream hydroponic cultivation systems. The novelty of this study was that it corroborated similar studies in soil-based systems on the capacity of plants to screen microorganisms before they can colonize the roots (collectively referred to as the rhizosphere). These observations contrasted with "common sense" in the fields of aquaculture, hydroponics, and by extension – aquaponics – where it was commonly assumed that microorganisms in the water column colonize all surfaces much in the same way they colonize carriers in the biofilter, or the surfaces of tanks and pipes. In hydroponic cultivation in particular, the fear of disease has led to routine sterilization of the water source with the notion that sustaining a local facility "microbiota" is unnecessary and dangerous. Below the publication is presented as published, followed by a discussion on the influence this research had in steering the direction of later research in this thesis and the broader implications of its findings.

2.1. Abstract

The role of the microbial community in mediating fish and plant co-culture is often considered the black box of aquaponics. Despite widespread recognition of the dependency of plants on their rhizosphere, the extent to which upstream aquaculture influences downstream hydroponic root communities has been poorly described in the literature. In this study, we performed a taxonomic survey (16S rRNA gene metabarcoding) of microbial communities originating in the facility water source, hydroponic nutrient solution (HNS) sump, nutrient supplemented biofilter effluent (BF) sump, and recirculating aquaculture system tanks stocked with Nile tilapia (*Oreochromis niloticus*). Lettuce (*Lactuca sativa*) was then grown using the HNS and BF effluent under sterilized or mature (prior aquaponics/hydroponics lettuce culture water) conditions. Likewise, the influence of probiotic addition or inoculation with soil-grown lettuce rhizosphere was assessed. Compositional similarities across treatments suggest that under soil-less conditions, plants are able to exert a stronger discriminatory influence on their rhizosphere composition than is done by colonization from upstream sources. Furthermore, cluster dendrograms grouped the sterilized and unsterilized treatments more consistently together than hydroponics and aquaponics treatments. These findings contradict conventional beliefs that microbial communities in the water column colonize roots based on their presence alone, ignoring the role that plants play in rhizosphere community selection.

2.2. Introduction

The region in and around plant roots, the rhizosphere, is an interspecies nutrient and electron trade zone with stakeholders representing all kingdoms (116-121). Recent studies have shown that soil-based plants exert significant pressure in terms of nutrient composition on their rooting communities (122-124). The extent to which these findings may be transposed onto plants grown in soil-less cultivation conditions is less clear for two reasons. Firstly, it is unclear whether the release of soluble plant exudates into an aqueous milieu diminishes their effect on the microbial community. Secondly, the greater ease by which the microbial community may be transferred within the aqueous environment could contribute to a greater capacity for root colonization.

The rhizosphere community (rhizobiome) manages nutrient uptake needs (116, 125-127), abiotic stress resistance (124, 128, 129), and host defense (130-132). It is composed of a core component fulfilling

essential functions required by the plant at each stage of its growth, and a satellite component consisting of strains present at low abundances (133). The core community consists of taxa that are necessarily drawn to the root environment in contrast to bulk soil (134). As only 7% of bulk soil microorganisms are found in the rhizosphere (135), the carbon-rich environment of the rhizosphere has been described as a precursory selection pressure. The relatively stable flow of 10-250 mg/g organic acids from the plant into the rhizosphere enriches microbial taxa two orders of magnitude greater than surrounding soil (136), with root exudates including amino acids, organic anions, sugars (137-140). The complex dynamics of rhizobiome development has given rise to many metagenomic studies on the rhizosphere (122, 124, 126, 141, 142). Research on soil-based studies indicates that investments into the root community is a high priority for terrestrial plants, but it is not evident how well this relationship is preserved in a nutrient solution environment such as soil-less hydroponic or integrated agriculture systems (*e.g.*, aquaponics). Furthermore, the capacity of probiotics to mediate host plant/ rhizosphere interactions was explored through the application of the commercially relevant bacterium *Bacillus amyloliquefaciens*, which has been developed as a probiotic in hydroponics but not aquaculture (143-145).

In this study, a decoupled aquaponics design was used to study downstream colonization of the rhizosphere by upstream microbial communities (146). From a nutrient perspective, there are two inputs: fish feed for the aquaculture unit and any fertilizer addition in the hydroponics unit. Sources for microbial inoculation may arise from the local aqueous or airborne environment, as well as through the import of foreign material into the system (*i.e.*, via feed). Recent publications focusing on the diversity of microorganisms in aquaponic systems have given rise to many hypotheses as to how the microbial community may lead to increased performance based on the increased abundance of chelating agents, cofactors, enzymes, or hormones facilitating nutrient bioavailability, either directly or indirectly (147-151). While the microbial community is widely recognized as important to the success of aquaponic systems (34, 152-157), it has likewise been suspected as a vector for pathogen proliferation (151, 158).

With the objective of determining the source of the microbial community colonizing the rhizosphere, lettuce (*Lactuca sativa*) was grown under a variety of hydroponic conditions including nutrient supplementation with a commercial hydroponic solution alone, nutrient supplemented aquaculture-derived water stocked with Nile tilapia (*Oreochromis niloticus*) and after inoculation with a probiotic or soil culture. Through multiple discriminating analyses (cluster dendrogram, principal component analysis), this study highlights the important role of plants in determining their own rhizosphere composition in soil-less cultivation systems.

2.3. Methods

A decoupled (unidirectional flow) aquaponics system was stocked with Nile tilapia (*Oreochromis niloticus*) and Batavian lettuce (*Lactuca sativa*) Exaudio RZ 79-43 (Rijk Zwaan, Netherlands) grown at the Wageningen UR Greenhouse Horticulture Unit (Bleiswijk, Netherlands). The lettuce was grown in hydroponic boxes (3 plants/ea.) with three replicates per treatment. Boxes, insomuch as they were self-contained provided better control over microbial exposure to the plants than normal media-based, raft or nutrient film systems, but did not completely prevent bacterial transfer as growth conditions were not sterile, nor were seeds sterilized prior to planting. Each box contained a Styrofoam sheet floating on nutrient solution, mimicking a deep-water culture environment. Four microcentrifuge tubes with sheared tips were filled with 2% w/v agar-agar (Sigma, Netherlands) and inserted into the sheet with

seeds immersed in the agar. Roots growing into the aqueous milieu were considered to be representative of the plants' rhizosphere, as this most closely resembles root structure in hydroponic cultivation conditions.

For all treatments, seeds were incubated in darkness overnight (8 h) at 25° C. Filter sterilized (0.22 µm) hydroponic nutrient solution (HNS) was added to each box at the beginning of cultivation and exchanged for the treatment-specific nutrient solution after two days. Nutrient solutions were prepared weekly, at which time half of the volume was exchanged. Supplementation of the sump solution was done as necessary to maintain the following approximate macronutrient composition (mmol/L): 15.0 NO_3, 1.5 NH_4, 5.0 K, 1.5 Na, 3.0 Ca, 1.5 Mg, 0.1 Si, 0.1 Cl, 1.5 SO_4, 0.5 HCO_3, 0.5-1.0 P. The following trace elements set points were also maintained (µmol/l): 20.0 Fe, 7.0 Mn, 5.0 Zn, 20.0 B, 0.5 Cu, 0.1 Mo, while pH (set to 6-7) and electric conductivity (set to 2-2.5 mS/cm) were adjusted as needed to maintain desired ranges. Studies directly comparing yields between aquaponics and hydroponics have proven difficult to reproduce (141, 159). As most aquaponic and hydroponic systems strive to maximize crop productivity through the same conventional means (greenhouse design, cultivar selection, etc.), nutrient concentrations were kept constant in this study to avoid confounding the relationship between nutrient loading and plant health.

Treatments were watered from either the aquaponics system (BF) or a commercial hydroponic nutrient solution (HNS) (figure 8). Aquaponics crops received effluent from the biofilter, with nutrient supplementation carried out in a decantation tank prior to the hydroponics unit. Here, we refer to HNS from two full crop cycle as mature HNS (HNS.m). To make sterilized HNS (HNS.s) or BF (BF.s), freshly made nutrient stock solutions were filter sterilized (0.22 µm). The probiotic effect of *B. amyloliquefaciens* was added to sterilized HNS and to unsterilized BF (corresponding to treatments Probio.s and Probio.m, respectively). A DSMZ (Germany) culture stock of *B. amyloliquefaciens* (ex Fukumoto 1943) grown in pure culture to $5x10^{11}$CFU/g stock was applied to achieve a final concentration of 2 mg/L. Soil inoculum (ca. 50 mg) was sourced from Batavian lettuce grown in potting soil for 4 weeks. The soil sample was sequenced as a control (referred to as "Soil"), inoculated treatments are referred to as Soil.inoc. The water column from Batavian lettuce grown aquaponic (BF.aqueous) and hydroponic (HNS.aqueous) basins were furthermore sampled as a control for the pelagic microbial community, as was the facility water source (WS) and the aquaculture tanks (RAS).

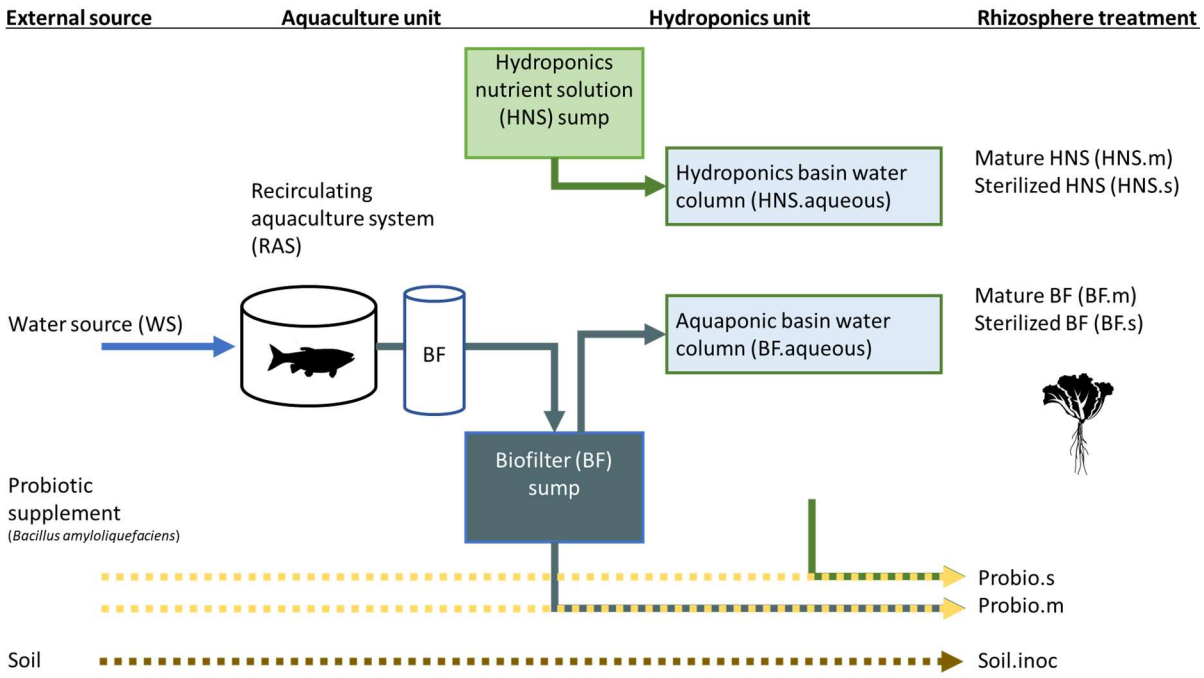


Figure 8. Summary of treatments in the current study.

Water samples during all three trials were analyzed weekly for nutrient concentrations, pH, and EC (Groen Agro Control, Netherlands). Dissolved oxygen (DO) was kept saturated for both experiments. Temperature was controlled at 16°C. Broad spectrum lighting was maintained at 200 µmol/s/m^2 for 16 h/day for all trials, although supplemental lighting was not used for trial 2 (due to summer conditions providing adequate irradiation). Crops were harvested after 6 weeks.

For microbial community profiling, DNA was isolated from the roots of each technical replicate using the DNeasy PowerSoil Kit (Qiagen, Germany). All plant roots in an individual box (technical replicate) were combined for DNA extraction. Roots were lightly shaken but not directly dried so as not to influence the rhizosphere community prior placement inside the microcentrifuge tube used in the protocol. A noticeable film of water enveloped the roots after shaking; 0.25 g of wet roots were used for the DNA extraction. The PowerSoil kit was chosen as it is well adapted to extract DNA from complex matrices such as the extracellular polymeric substances consistent with biofilm structure. For soil samples (soil inoculum referred to above as “Soil”), 0.25 g of soil from around the root was used. Purified DNA was PCR amplified using universal 16s rDNA bacterial primers (table 1) targeting the V3-V4 region of the 16s rRNA gene. Primers were provided by BaseClear B.V. (Netherlands) and sequenced using their MiSeq system. Sequenced operational taxonomic units (OTUs) were processed as per BaseClear protocols whereby sequenced amplicons are merged into overlapping pseudo-reads and subsequently aligned against the NCBI 16s rRNA database for putative taxonomic identification.

Table 1. Primer sequences used for the taxonomic community analysis in this study.

Domain target	Direction	Sequence	Length (bp)	Melting temperature	GC%
Bacteria	Fwd primer	AGAGTTTGATCCTGGCTCAG	20	56.92	50.00
	Rv primer	ATTACCGCGGCTGCTGG	17	60.18	64.71

In R, the OTU data set was subdivided into six data frames related to the taxonomic rank using the Tidyverse packages tidyr and dplyr. Subsequent analyses were restricted to the genus, family, and order ranks as a compromise between the large amount of OTUs generated in the data set (obscuring clear visualization of the data) and to avoid a lack of resolution as occurring at higher ranks. Firstly, vegan was used for the diversity analysis, ade4 and labdsv were used for multivariate data analyses, pvclust for hierarchical cluster analysis, vegclust and vegsoup for data clustering, picante for community analysis, and finally corrplot for the correlation plot. Packages for visualization of the data included gclus to generate the clustering graphics, dendextend for dendrograms, and ggplot2 for the correlation plot.

Due to the effect of outliers, several normalization strategies were explored: presence/absence, maximum abundance per treatment, relative abundance per species, relative frequency per site, normalization to the Euclidian norm (Chord transformation), normalization to the relative frequency per site (Hellinger transformation), double profile normalization (Chi-squared transformation), and normalization first by species maxima then by site totals (Wisconsin standardization) (160). Normalization by Hellinger transformation were chosen for this study based on the tightness of the variance range in the processed data sets.

Three types of neighbor clustering were used to organize the data: nearest, furthest, and Ward. Nearest neighbor clustering agglomerates groups based on the shortest pairwise dissimilarities between members, while the furthest neighbor method defines the group membership based on the maximum distance between any two clusters. Ward's minimum variance clustering minimizes the total within-cluster variance and appeared the most logical to follow based on the robustness of the groups. The optimal number of clusters were calculated using Ward correlation, Pearson correlation, IndVal method, simple structure index (ssi) criteria, and Calinski criteria (161, 162). While the range of optima was fairly consistent across taxonomic ranks, the optimization algorithms never converged on a single figure. The clustering result was then independently confirmed by a principal component analysis and correlation plot of the treatments. Finally, co-occurrence network analyses of both the treatments and microbial taxa allowed us to visualize which treatments most closely resemble each other at different taxonomic ranks.

2.4. Results

In terms of plant health, treatments were not nutrient limited nor displayed signs of disease. Lacking obvious indications of stress, it was assumed that plants interacted with the surrounding microbial environment under homogenous circumstances across treatments with differences in community composition originating from the source water and not physicochemical or stress factors. To elucidate the relationship between the host plants and the composition of the rhizosphere microbial community, this study investigated patterns in taxonomic prevalence across treatments through hierarchal classification and clustering analyses.

Plotting the distribution of OTUs across the treatments provided evidence for the existence of a core microbiome present in many (9-10 out of 28 treatments), although most OTUs are unique to 1-2 treatments as the genus rank (figure 9); plots of the family and order rank were similar but not identical (figures 10-11). Approximate unbiased (red, significant ≥ 0.95) and bootstrap probability (green, value indicates the amount of bootstrapping until robust) p-values for the edge dendrograms (edge number in grey) are indicated. Blue squares indicate significance at $p \geq 0.90$ with red bars indicating high robustness at $p \geq 0.95$). At the genus rank it is visible that the source of colonization does not strongly predict clustering. Aquaculture derived water appears to influence community dynamics whether sterilization is imposed (BF.s) or not (BF.m) (group 2), however mature BF or HNS box communities (group 3) were not closely related to the aqueous community used to inoculate the boxes. The control treatments (BF, WS, HNS, RAS, Soil) cluster similarly (group 4), with the aqueous communities (aquaculture linked or independent) clustering closely together. Probiotic supplementation mostly clustered in group 5, however some branches were mixed with other treatments. At the family rank (figure 10) no clear pattern was visible, although it is visible that the probiotic treatments populated one branch at the first fork, while the controls and most mature and sterilized HNS treatments populated the second fork.

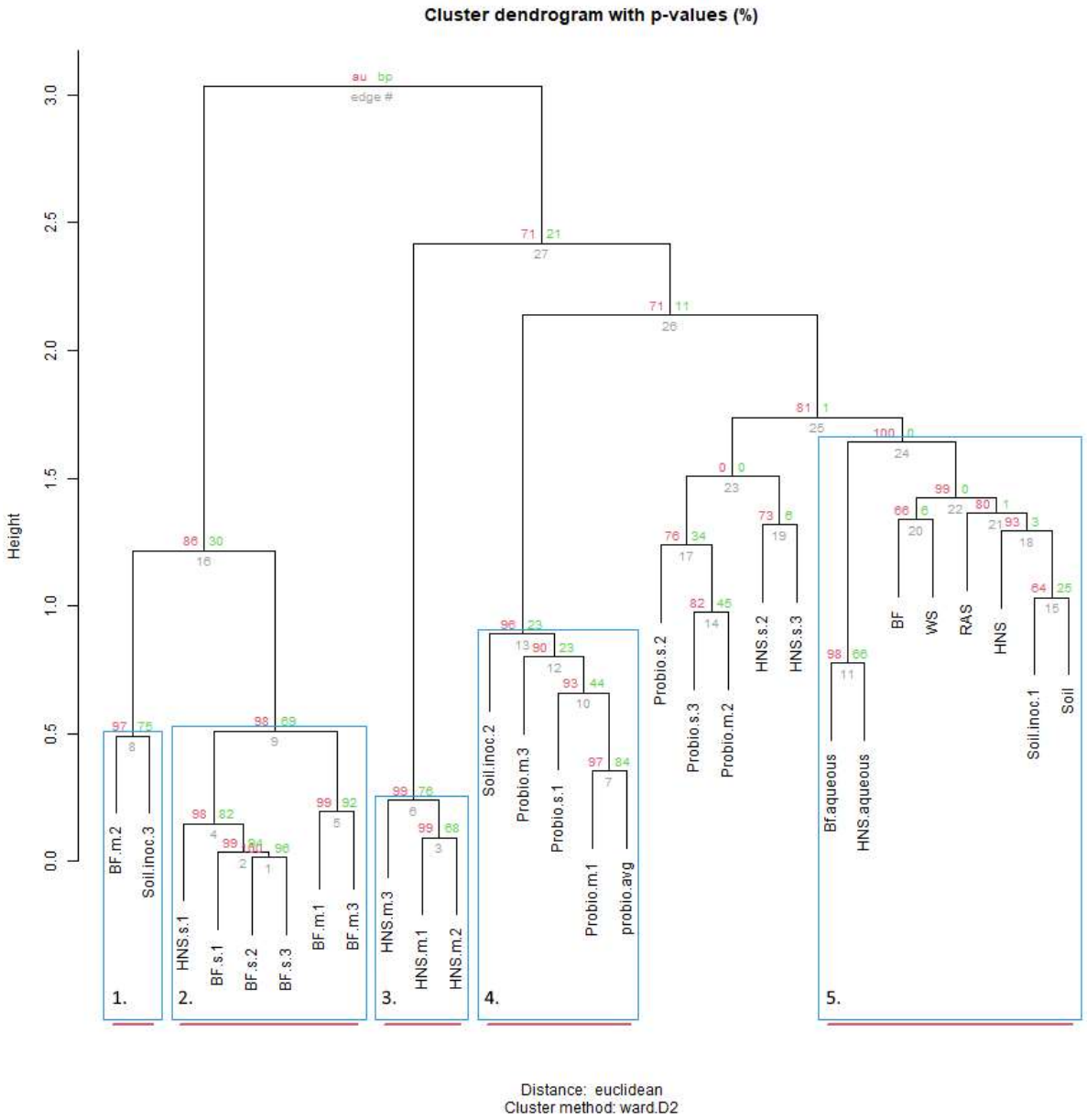


Figure 9. Cluster dendrogram of the distribution of microbial communities at the genus rank across treatments with the five most robust clades highlighted. Similar patterns were observed at higher ranks. Treatments include hydroponic nutrient solution sump (HNS) and biofilter effluent sump (BF) under mature (.m), sterilized (.s), and basin water column (.aqueous) conditions. Additionally, soil inoculum (Soil) and HNS inoculated culture (soil) and probiotic (probio) inoculated sterilized (.s) and unsterilized biofilter effluent (BF) samples, as well as the facility water source (WS) and recirculating aquaculture system water column (RAS) are also included.

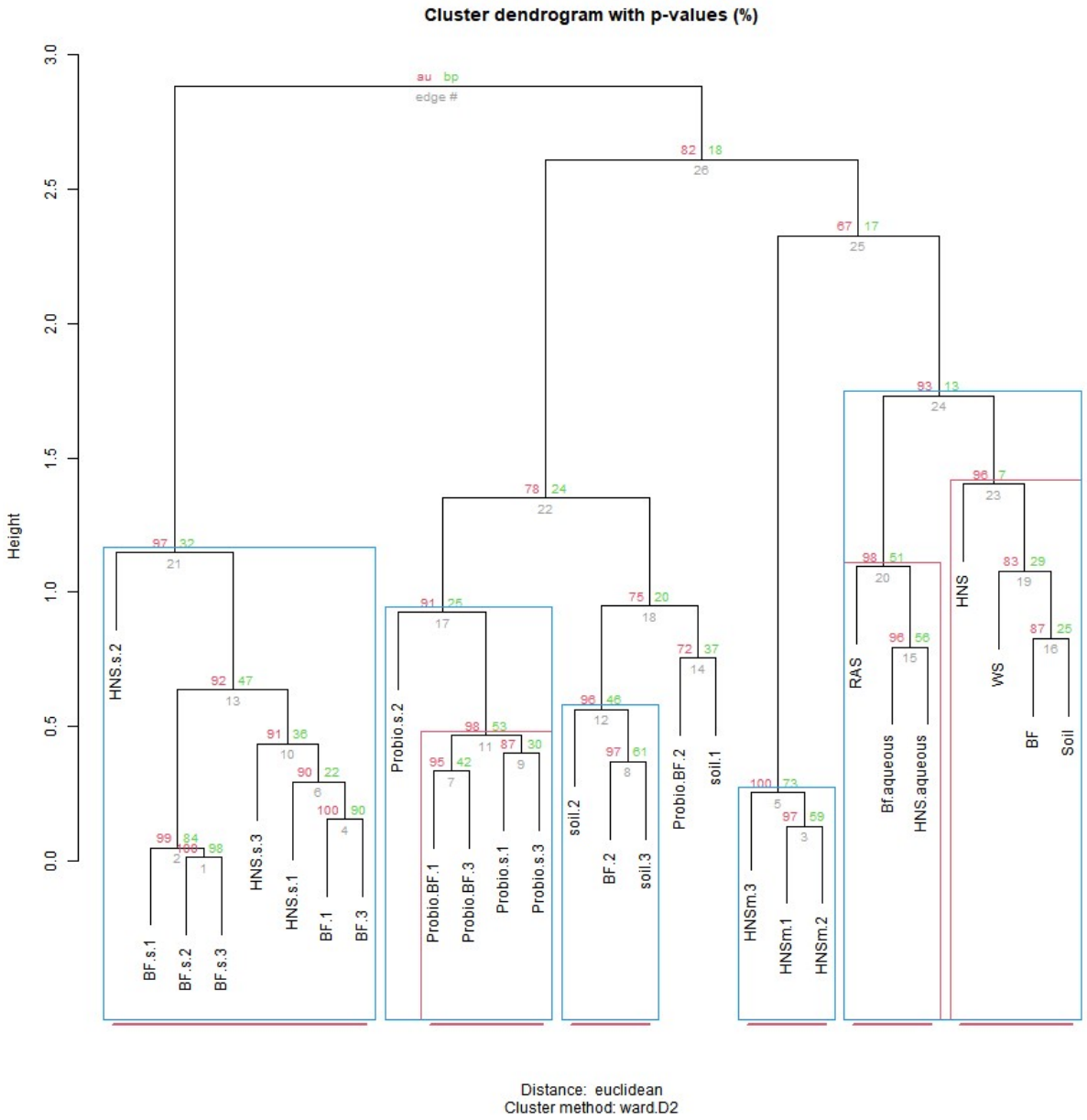


Figure 10. Cluster dendrogram of the distribution of microbial communities at the order rank across treatments with the five most robust clades highlighted. Similar patterns were observed at higher ranks. Treatments include hydroponic nutrient solution sump (HNS) and biofilter effluent sump (BF) under mature (.m), sterilized (.s), and basin water column (.aqueous) conditions. Additionally, soil inoculum (Soil) and HNS inoculated culture (soil) and probiotic (probio) inoculated sterilized (.s) and unsterilized biofilter effluent (BF) samples, as well as the facility water source (WS) and recirculating aquaculture system water column (RAS) are also included.

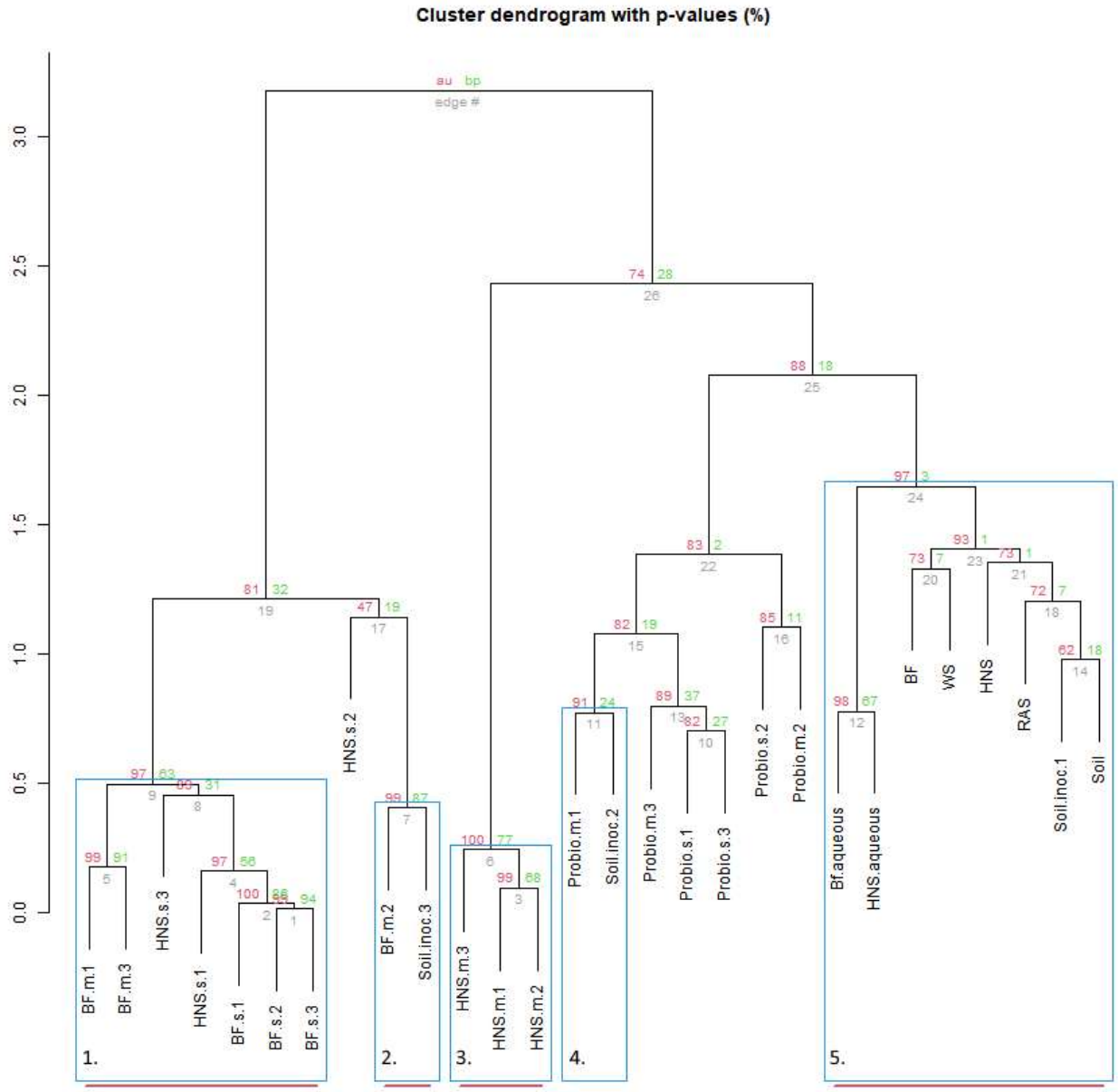


Figure 11. Cluster dendrogram of the distribution of microbial communities at the family rank across treatments with the five most robust clades highlighted. Similar patterns were observed at higher ranks. Treatments include hydroponic nutrient solution sump (HNS) and biofilter effluent sump (BF) under mature (.m), sterilized (.s), and basin water column (.aqueous) conditions. Additionally, soil inoculum (Soil) and HNS inoculated culture (soil) and probiotic (probio) inoculated sterilized (.s) and unsterilized biofilter effluent (BF) samples, as well as the facility water source (WS) and recirculating aquaculture system water column (RAS) are also included.

Partitioning based on ssi criteria resulted in multiple equally optimal partitions for a range of cluster objects. Ultimately, this indicates a high degree of interchangeability between most treatments, suggesting that the microbial communities present are more similar than different. Looking at a dissimilatory matrix of the treatments (figure 12), we see that the aquaculture impacted (BF series) and probiotic supplemented (Probio) treatments tend to be more similar within themselves that to each other, with the soil and standard hydroponics (HNS series) being less cohesive groups. The principle component analysis (figure 13) places the mature HNS treatments (HNS.m) at the center of the

distribution, with the two most discriminating factors at 23.8% (dimension 1) and 15.5% (dimension 2) causing a split between the controls (WS, RAS, BF, HNS, Soil, HNS.aqueous, and BF.aqueous) and experimental treatments (HNS.m, HNS.s, BF.m, and BF.s). The probiotic (probio) and soil inoculated (soil) treatments were less dependent on the two principal dimensions.

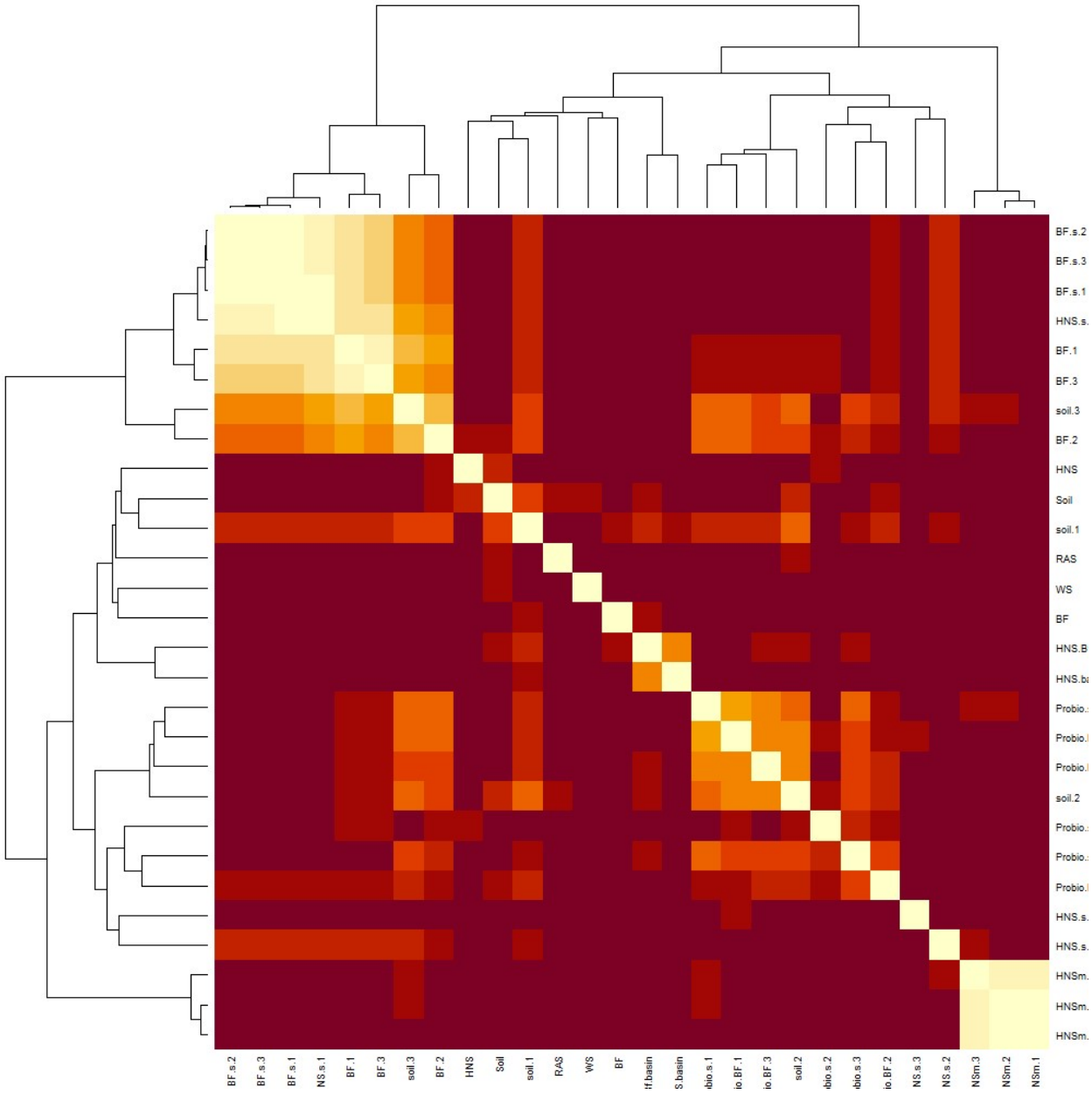


Figure 12. Dissimilatory matrix between microbial communities of the treatments in the study at the genus rank. Similar patterns were observed at higher ranks. Treatments include hydroponic nutrient solution sump (HNS) and biofilter effluent sump (BF) under mature (.m), sterilized (.s), and basin water column (.aqueous) conditions. Additionally, soil inoculum (Soil) and HNS inoculated culture (soil) and probiotic (probio) inoculated sterilized (.s) and unsterilized biofilter effluent (BF) samples, as well as the facility water source (WS) and recirculating aquaculture system water column (RAS) are also included.

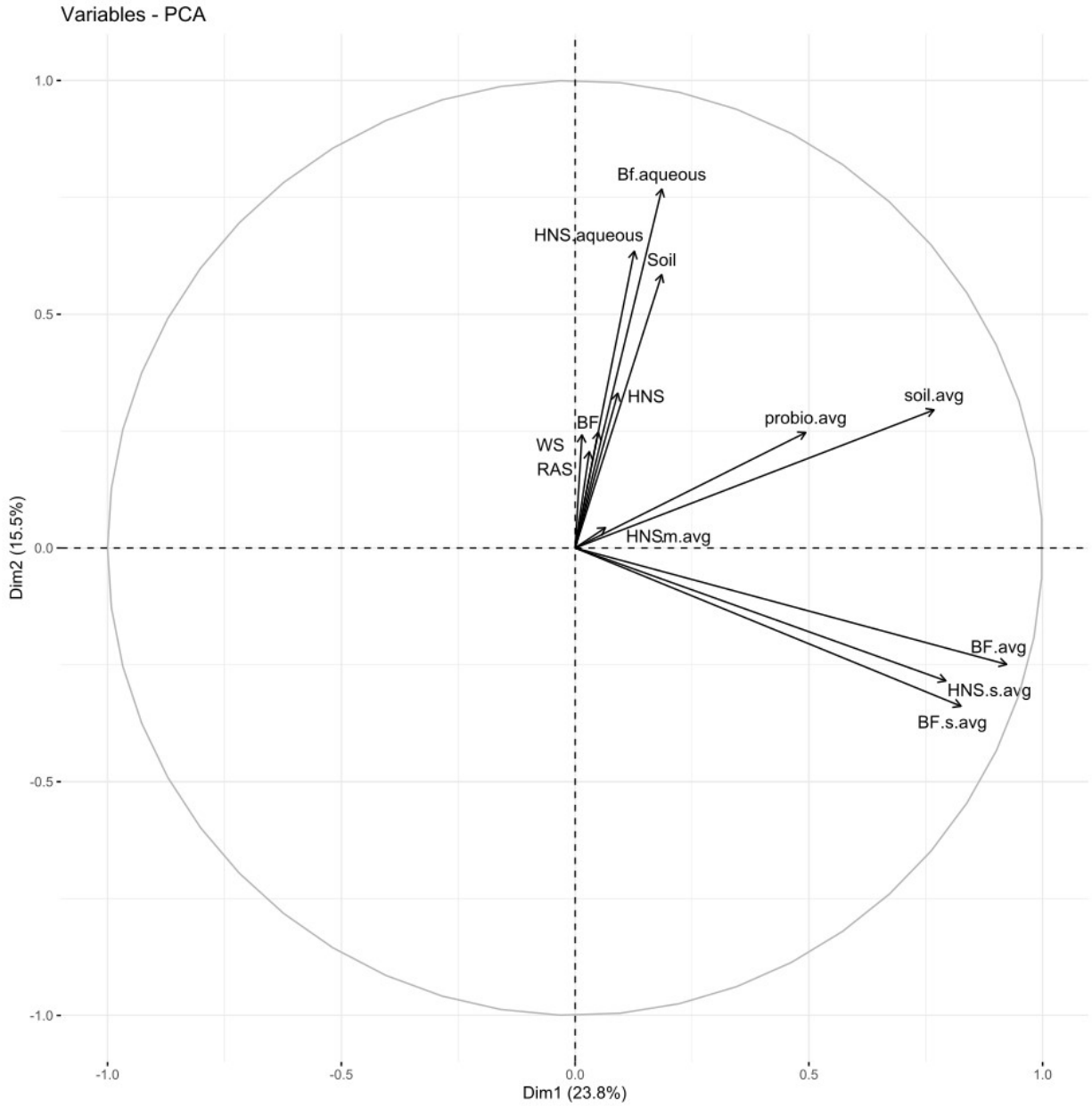


Figure 13. Principle component analysis for all treatments; abundance data across technical replicates were averaged for each set. Treatments include hydroponic nutrient solution sump (HNS) and biofilter effluent sump (BF) averaged for each technical replicate and basin water column (.aqueous) conditions. Additionally, soil inoculum (Soil) and HNS inoculated culture (soil) and probiotic (probio) samples averaged for all technical replicates, as well as the facility water source (WS) and recirculating aquaculture system water column (RAS) are also included.

Finally, co-occurrence networks were generated for microbial communities. At higher ranks the superstructure for community similarity across treatments is more clearly defined. At the order rank this appears as three clusters, two of which are more closely related (figure 14a). At lower ranks (figure 14b) these clusters begin to splinter as the quantity of unique labels corresponding to microbial taxa increases exponentially. Figure 15 shows the taxonomic clustering; however no functionality is discernable here as the taxa were identified through the NCBI reference database based on the metabarcoding reads.

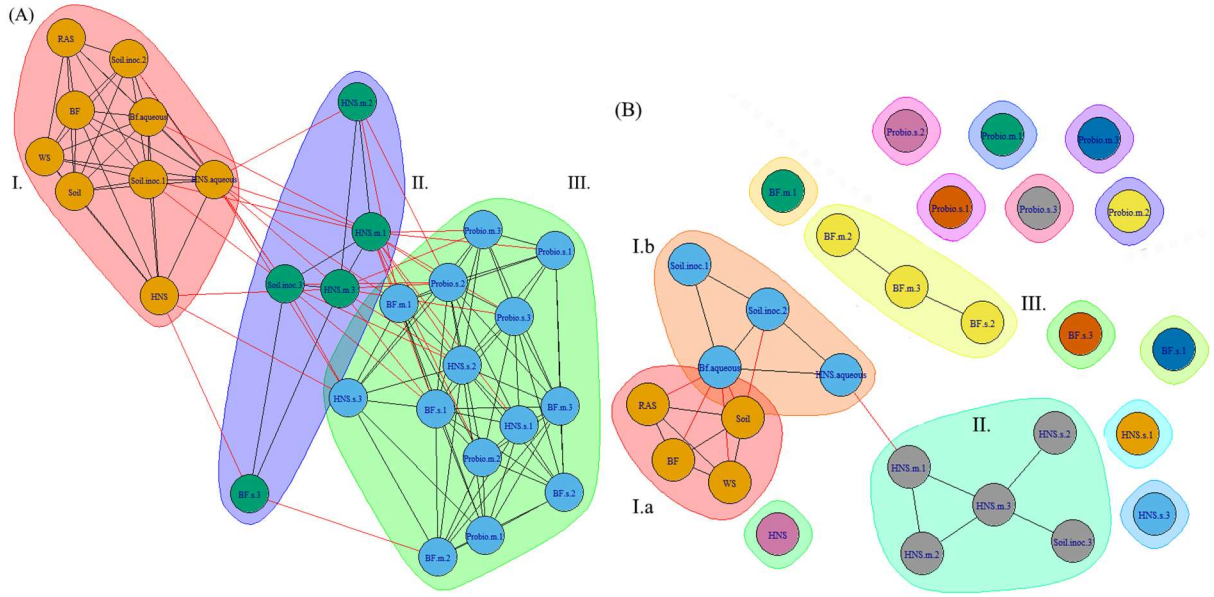


Figure 14. Co-occurrence network of microbial communities across treatments at the class (A) and family (B) ranks. Treatments include hydroponic nutrient solution sump (HNS) and biofilter effluent sump (BF) under mature (.m), sterilized (.s), and basin water column (.aqueous) conditions. Additionally, soil inoculum (Soil) and HNS inoculated culture (soil) and probiotic (probio) inoculated sterilized (.s) and unsterilized biofilter effluent (BF) samples, as well as the facility water source (WS) and recirculating aquaculture system water column (RAS) are also included.

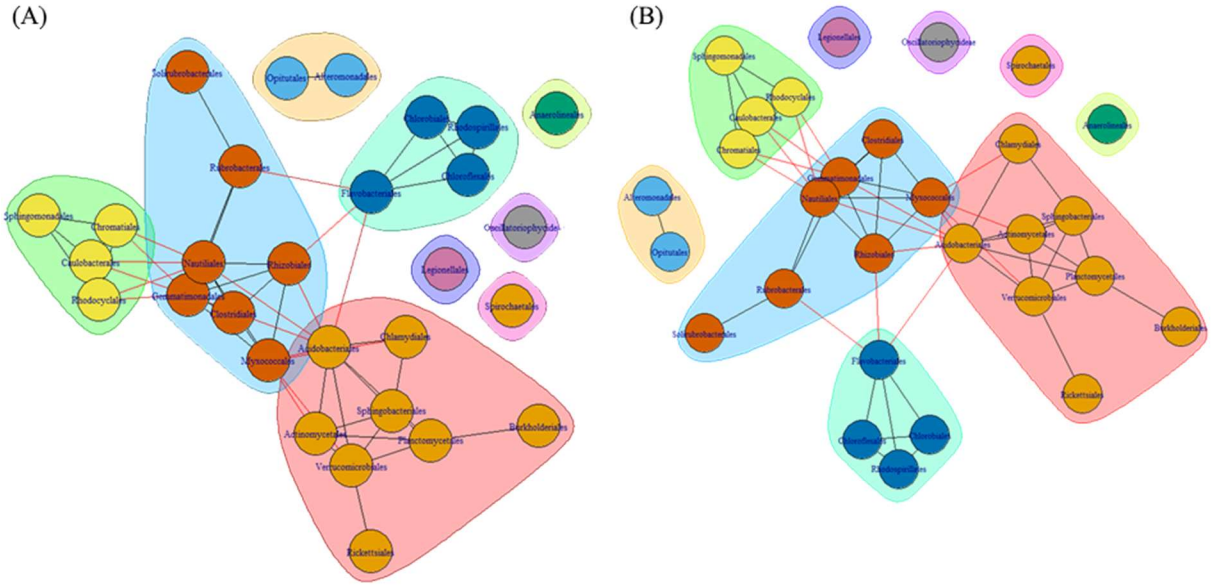


Figure 15. (A) Distribution of phyla across treatments and controls. (B) Co-occurrence network of microbial taxa at the order rank across treatments.

2.5. Discussion

2.5.1. Rhizosphere colonization patterns

This study is the first to investigate how the rhizosphere microbial community is shaped by upstream influences under soil-less cultivation conditions. Lettuce (*Lactuca sativa*) was grown hydroponically or in aquaponics co-culture with Nile tilapia (*Oreochromis niloticus*). As shown in figure 8, treatments included nutrient supplementation with a commercial hydroponic solution alone, nutrient-supplemented aquaculture-derived water, or the commercial nutrient solution inoculated with a probiotic or soil culture. Filter sterilization vs. inoculation with mature media (nutrient solution derived from a previously harvested lettuce culture) were tested for both hydroponic and aquaponic treatments as well as the probiotic addition.

As indicated by the cluster dendrogram (figure 9), no divisive split grouping all aquaponic (BF.m and BF.s) apart from all commercial hydroponic (HNS.m and HNS.s) treatments exists at the genus rank despite a highly robust clustering model with a cophenetic correlation of 0.93, a pattern consistent across different clustering methods and at higher taxonomic ranks. The number of optimal clusters, however, varied from 2-9 clusters between the five methods tested, mirroring the overall dendrogram shape when viewed based on cluster height. After an initial branching into 2-4 groups, the height difference between clusters drops sharply – reflecting a higher degree of replaceability. Either as part of a main or sub-branch, the controls (BF, WS, RAS, HNS, and Soil) tended to cluster closely. These controls mainly serve to identify environmentally prevalent microorganisms from the water supply (WS), aquaculture unit (RAS), nutrient-supplemented biofilter effluent sump (BF), hydroponic nutrient solution sump (HNS), and local soil-based lettuce rhizosphere (Soil). Their high degree of similarity at low taxonomic ranks suggests that most microorganisms are ubiquitously present, in agreement with the rare biosphere ecological model (163, 164).

Another perspective of community similarity is portrayed in the dissimilatory matrix (figure 12), comparing treatments by virtue of their degree of similarity instead of being clustered based on a threshold consensus as well as the co-occurrence networks (figure 14a, b), where clustering is allowed to overlap if treatments are sufficiently similar. At the class rank (figure 14a), an agglomeration of the aquaponics treatments (3 BF.s, 2 BF.m) is visible in cluster III. The BF control and BF water column samples were distinct from this group (cluster I), sharing a greater degree of similarity with the other controls instead. While the mature HNS treatments (HNS.m, cluster II) clustered together, their cluster partially overlapped with cluster III containing the three HNS.s treatments. The probiotic treatments clustered together (cluster III), however the soil treatments were distributed between clusters I and II. Much of the cluster similarity disappeared at the genus rank, albeit the nodes of clusters I and II are still visible (figure 14b).

From the PCA (figure 13) and the co-occurrence network analysis (figure 14b), it is visible that the probiotic treatments poorly grouped together on average, meaning that their taxonomic composition was the least consistent within technical replicates. One may speculate that the colonizing influence of the probiotic shifted the community as a whole, rearranging the rhizobiome into a different configuration than in other treatments. Insofar as this may be attributed to the probiotic itself is outside the scope of this study.

Soil treatments did not consistently cluster together, ostensibly reflecting the shift in community composition from bulk soil to the rhizosphere environment as described elsewhere (135). Community

diversity was poorly retained when soil-based lettuce roots were used to inoculate sterile HNS. These treatments gravitated towards the same global consensus as the other hydroponic treatments rather than forming a robust branch independently, despite filter sterilization of the HNS and no direct contact between media. Although a portion of the HNS sump microbiome is shared with HNS.m and HNS.s treatments, several taxa undergo major shifts in abundance during this transition. As a model this suggests that under similar nutrient concentrations, rhizosphere involvement plays a greater role in driving microbial community composition than water source.

2.5.2. Factors influencing rhizobiome composition

An array of factors influencing rhizobiome composition have been identified, originating both from the plant (genotype, life stage) and the environment (water source, nutrient profile) (122, 165). As shown by Bartelme et al. (2019) (35), facility conditions strongly dictate the microbial populations present in RAS and aquaponic systems. Our results suggest that a similar facility-specific microbiome forms within the rhizosphere in hydroponic systems. Studies on the rhizobiome in other type of cultivation systems such as soil or air have indicated a similar pattern of consolidation. For instance, Schreiter et al. (2014) observed that the lettuce rhizobiome was consistent across varying soil types (166), while Edmonds et al. (2019) observed a rhizobiome unique from the circulating nutrient solution that formed after 12 days of plant growth in aeroponic conditions (167). This trend appears to be a hallmark of terrestrial plants (124, 168-170). In combination with the results from this study, it appears that selection pressures exerted by the plant to consolidate the rhizobiome around a particular profile are a fundamental aspect of plant physiology despite the influence of the exogenous microbial environment. That profile, although observed as a collection of taxa, mirrors the functional needs required by the plant at a particular life stage and under particular environmental conditions.

At a more global level, microbial communities will occupy all available niches as they become available. For instance, among its many discoveries, the Tara Oceans project revealed that physiochemical parameters such as pH and temperature play a more decisive role in the relative taxonomic abundance than does taxonomic presence (171, 172). Co-occurrence networks at the family and order rank indicate consistent grouping of certain microbial clades (figure 15). However, further research should combine our top-down approach with a bottom-up strategies to study community organization (*e.g.*, identification of keystone species (173)), as well as omics based techniques for community functional analysis, to elucidate how select microorganisms or clades may impact facility productivity through their disproportionate influence on community structure.

Understanding the potential impact of upstream microbial communities on downstream hydroponic units has direct implications for preventative disease management. Demonstrating that the rhizosphere community composition is associated with the plant more strongly than the presence of exogenous colonizing bacteria implies that focusing efforts on supporting plant health rather than on water sterilization will better protect crops. Sterilization of incoming water and media is widely used in hydroponics to discourage the proliferation of pathogens (174-178) albeit at the cost of reducing overall microbial diversity - both beneficial and harmful microorganisms – potentially opening niches for rapid colonization by r-strategists (126).

Some aquaponic studies advocate for continuous cycling of water between RAS and HP components (coupled aquaponics) (27, 179), while others have advocated for a discrete separation (decoupled aquaponics) with no return of water and hence microorganisms from the HP to the RAS (153, 180-184).

In this context, we sought to determine whether sterilization (reducing microbial proliferation across units) succeeds in significantly shaping the microbial community structure. Clustering did not indicate a mature/sterilization split at the genus, family, or order ranks (figures 9-11), nor was a strong split visible via the dissimilatory matrix (figure 12). Most treatments furthermore clustered together at the class rank (figure 14a), with the notable exception being the mature HNS treatments in cluster II. In a prior investigation into the effect of sterilization in the context of RAS coupling, Wielgosz et al. (2017) concluded that the beneficial effects on plant growth from RAS effluent were most likely conferred through microbial exudates, and thus unaffected by the sterilization process itself (157). While the identity of those exudates remains unknown, our results further support their hypothesis by showing that the community composition is not principally determined by the source water (HNS/BF) or source community (mature/sterilized).

2.5.3. microbial compositional diversity

In terms of microbial compositional diversity, the most profound shift occurred between controls and treatments in a stepwise manner (figure 15). The soil control indicated a high level of diversity with a couple phyla disappearing in soil-inoculated treatments (*Fibrobacteres, Nitrospinae*), however the majority of phyla were present at reduced concentrations. The facility water supply control (WS) was relatively enriched with some phyla compared to the recirculating aquaculture system (RAS) and biofilter (BF): *Bacillariophyta*, *Chlamydiae, Aquificae, Candidatus Saccharibacteria*. The RAS and BF conditions enriched the phyla *Fusobacteria, Nitrospirae,* and *Lentisphaerae*. Few members of these phyla could be detected in subsequent aqueous (BF.aqueous) or rhizosphere (BF.1-3) environments suggesting a lack of viability in the oligotrophic, ammonia-poor, hydroponic environment. Probiotic treatments (Probio.m.1-3, Probio.s.1-3) most significantly perturbed the total microbial composition. While no mechanism could be determined within the context of this study, probiotics have been shown to restrict microbial diversity in the gut microbiome (185). Regardless, further studies must corroborate our results to elucidate the relationship between probiotic administration and community diversity. In terms of co-occurrence, no clear patterns of taxonomic clustering could be discerned. At high taxonomic ranks, the amount of overlap consolidates most taxa together while at lower ranks the diversity creates an unmanageable number of sub-groups. At the order rank, some discernable clustering is visible (figure 11), however the significance of these co-occurrences could not be determined within the scope of this study.

2.5.4. microbial community dynamics

Our study focused on the microbial community dynamics at the main interface between the aqueous milieu and the plant in soil-less cultivation systems – the rhizosphere. The above trends indicate community consolidation in our system, suggesting that prioritizing plant health metrics will likewise reduce the potential for disease. We have recently demonstrated that trace nutrients are not taken up by plants proportionally to their external aqueous concentrations (186), which suggests that fundamental issues such as plant nutritional needs should be prioritized. Given the slow growth requirements of k-strategists (*e.g.,* anammox (187-189), archaea (19, 190)), system-wide maturation of the microbial population may take months or years (191). It would not be unreasonable to expect successive waves of colonization to mark this period, as is similarly observed within the rhizobiome during plant growth (192-195). Archaea and eukaryotic phyla (algae) were observed in the study at the phylum rank (figure 15), however their contribution to rhizosphere structure, organization, and nutrient flow in aqueous environments remains an open question. While not investigated here, community

succession in the rhizobiome during facility maturation may indicate the duration within which a facility microbiome stabilizes and thus is able to maximally resist pathogen colonization.

2.6. Conclusion

In this study, we have provided evidence that plant crop health cannot be predicted by exposure to upstream microbial communities in soil-less aquaponic cultivation systems. This study is the first to address the question of rhizosphere-colonizing microbial transfer in aquaponics by selectively exposing hydroponically grown plants to a range of treatments intended to shape the root microbiome. Prior literature has suggested that upstream aquaculture directly contributes to crop productivity through microbial colonization (34, 152), or in other cases, may represent an entry point for pathogens into the system (151, 158). While our data do not exclude these possibilities, they do suggest that the introduction of upstream bacteria is less impactful than previously assumed. More likely, plant health weaknesses are exploited by pathogenic microorganisms ubiquitous in the local environment, thus, not uniquely introduced through the water column. We expect the findings of this study to be transferable to cultivation conditions where healthy plants are not subject to excessive stress (*i.e.*, due to nutrient deficiency or other water quality perturbations), however, future research must investigate how these systems respond to acute abiotic or biotic stressors.

This work paves the way for two important future directions. First, our study suggests that aqueous nutrient concentration play a more predicative role in determining community composition than sterilization. While sterilization is a routine technique in aquaculture as well as hydroponics, it is nonetheless a tradeoff between pathogen suppression and total microbial diversity reduction. Future studies must likewise determine whether aquaponic facilities benefit from sterilization, or whether the co-cultivation of plants and fish in an environment promoting diversity leads to a more resilient facility-wide microbiome. Second, in line with previous work on the relationship between aqueous nutrient concentrations and plant health (196), more research is needed to determine whether a greater focus on maintaining plant health as opposed to only maximizing yield will lead to more disease-tolerant crops, and ultimately more productive crops.

2.7. Contextualization in the thesis

This study provides evidence for a greater resilience of plants to direct colonization of their roots than previously considered. From the perspective of waste treatment and nutrient remineralization, it suggests that by remineralization and, therefore, solubilizing nutrients, it is possible to meet plant nutritional needs without endangering them. Disinfection and sterilization in hydroponics is considered essential to suppress pathogens, yet, as established in chapter 2 and references within, many microorganisms are integral to nutrient uptake by plants. Lacking this community, plants are more susceptible to stressors (biotic or abiotic). There is evidently a cost-benefit trade-off between promoting a healthy rhizosphere (taking into consideration measures to develop a more favorable substrate around the roots, introduction of microorganisms, and attune nutrient loads experienced by the plant to simulate more natural conditions) against actively suppressing externalities to fruit/ biomass (excessive nutrient application, suppression of root growth and microbial biodiversity). The challenge lies in an inability to assess the relative role of auxiliary microorganisms towards productivity, an issue which itself belies a more fundamental challenge in the field of applied microbiology – the difficulty in linking taxonomic observations to other data types describing community functionality. Chapter 3 addresses

this issue by describing the growth and development of a novel database type specifically designed to tackle this methodological challenge.

3. Ecosystem-specific microbial databases in the era of big data

3.1. Abstract

The spread of sequencing methods over the past decades has accelerated the pace of microbiota and microbiome studies in both scope and depth. Recent developments in the field have been marked by an expansion away from purely categorical studies towards a greater investigation of community functionality. As in-depth genomic and environmental coverage is often distributed unequally across major taxa, it can be difficult to identify or substantiate relationships within microbial communities. A major challenge to-date is the integration of -omics data (*e.g.*, metabolomics, proteomics) with community expression studies (metatranscriptomics) and sequence data along habitat-specific standards. A special case of large genomic repositories, ecosystem-specific databases (ES-DBs) have emerged to consolidate and standardize sample processing and analysis protocols around individual ecosystems under study, allowing independent studies to produce comparable datasets. Here, we provide a comprehensive review of this emerging tool for microbial community analysis in relation with current trends in the field. Specifically: the factors leading to the formation of ES-DBs, their comparison to traditional microbial databases, the potential for ES-DB integration with multi-omics platforms, as well as inherent limitations in the applicability of ES-DBs.

3.2. Introduction

Interest in categorizing microbial communities across accessible habitats has exposed the vast complexity of microbial life (197-199). What had started initially with the laboratory isolation of microbial species from a habitat of interest has expanded, following the advent of genomics techniques, into the metabarcoding of samples: systematic cataloguing of microorganisms using identifying biomarkers (200-202). Technological developments over the past couple of decades have broadened community ecological analyses to encompass genomic data on a regular basis, either of representative genes (metabarcoding) or entire genome sequences within a sample (metagenomics). These deep dives into the microbial community allow a higher level of taxonomic precision as well as further opportunities to assess the functional capacity of the system (203-206). Coupled to this has been an expansion of gene expression studies from a focus on singular genes (transcriptomics) to all genes across microbial community constituents within a sample (metatranscriptomics). Recent decades have further seen the integration of diverse analytical tools into community ecological studies, collectively referred to as “omics” data. Ranging from metabolomics and proteomics to physiochemical parameters of the environmental sample (*i.e.*, pH, EC, E_h, temperature), omics data allows researchers to characterize microbial functionality within a community sample (207, 208). The ability to integrate measures of microbial functionality with taxonomic identification is essential to understanding inter-microbial relationships and their role as constituents in a particular environment. Nonetheless, databases have largely been organized around datatypes and not environments (*e.g.*, sequence information for taxonomy, spectral information for metabolites, morphological data for laboratory-isolated specimens). With respect to community ecology analysis, this practice results in less coordination across studies utilizing different investigative strategies on the same habitat, ultimately creating obstacles to the integration of multiple data types for community ecology analysis.

Several studies have highlighted concerns over the validity of sequencing data accruing from the ever-expanding body of microbial surveys and microbiome studies (209-212). One group of reviews has addressed this issue by proposing standards for studies to follow. Standardization in the collection and

processing of data for microbiome studies have been reviewed in different capacities from general guidelines (210, 213-215) to specific environmental situations (216-221). Another group of reviews have focused on the efforts in the integration of other data types (e.g., mass-spectroscopy spectra, environmental physiochemical data) into sequencing studies (222-227). These efforts notwithstanding, there has been limited focus in recent literature on the evolution of the fundamental profile of microbial database collections from a datatype orientation to an environment-specific one. A recent commentary in Nature Microbiology addressed the topic of data type integration from the perspective of "microbiome centers" – institutions or consortia designed to accelerate microbiome research by facilitating collaborations between personal and infrastructure resources (228). While the inception of the Microbiome Centers Consortium (MCC; http://microbiomecenters.org/) in 2019 marks a milestone for more coordinated standardization across microbiome studies, database resources are still developed largely independent of one another.

In this article, we present a review on the evolution of ecosystem-specific databases (ES-DBs) as an adaptation to address the unique challenges that arise when working with heterogenous datatypes inherent to community ecology analysis.

3.3. Unravelling microbial community diversity and function with omics-based data

Ranging from a generalized to focused lens, microbial ecosystems may be studied as an aggregate (community ecology), as a process for the modification of chemical constituents (microbial functionality), or from the perspective of a single community constituent (single strain approach) (figure 16). While unravelling the complex interspecies relationships in microbial communities lies at the heart of community ecology analysis (229-231), current strategies are limited in their capacity to compensate for the extreme taxonomic diversity, lineages (phylogenetic diversity), metabolites, and chemical speciation. Simplifying ecological samples to isolated strains or extracted compounds greatly reduces the background noise during analyses at the cost of unique biases (removal of unique changes brought about by biotic and abiotic interactions, changes to the microenvironment originally present in the sample being studied). The following section summarizes the status quo of community ecology studies with respect to information present in microbial collections and databases.

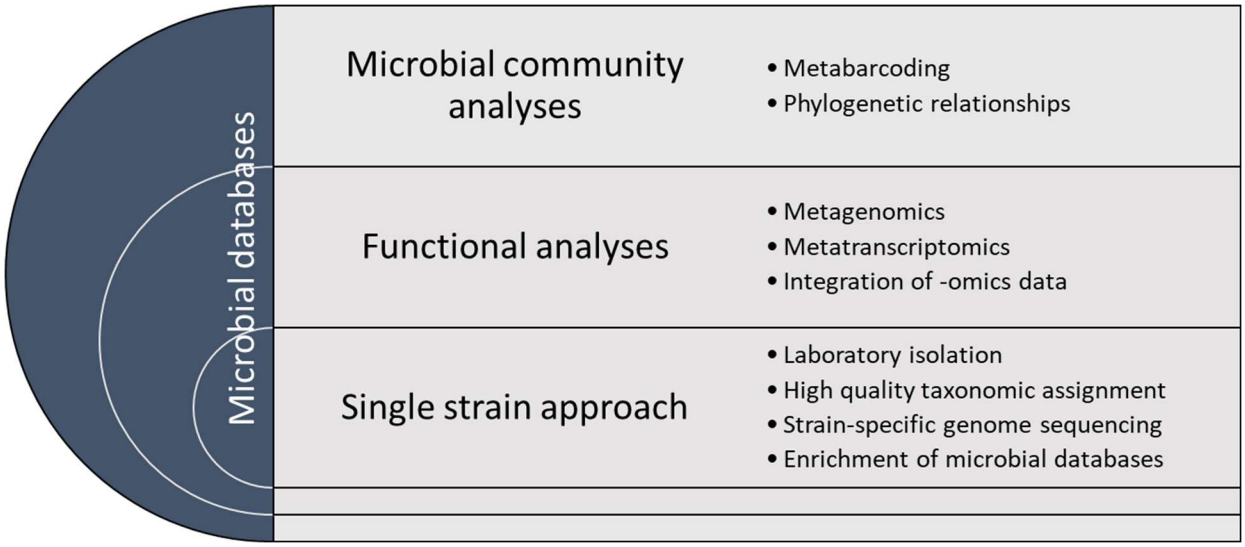


Figure 16. Interrelationships between multiple depths of biome characterization, all which can be unified through microbial database collections. Descriptions (right side) indicate the methodologies available from the respective analyses.

3.4. Single strain approaches

Traditional isolation of microorganisms on selective agar (culture-dependent approaches) results in lower taxonomic diversity than described in culture-independent approaches (232-237). Other isolation approaches include dilution-to-simulation and dilution-to-extinction (238-240), as well as fluorescence-activated cell sorting (FACS) – all of which may be further combined with sequencing technology to provide a deeper level of resolution into the interplay between observed morphology and genetic regulation (241-243).

By restricting the isolate to a pure culture strain in a known nutrient medium, culture-dependent isolation is the most conclusive and powerful technique for morphological and physiological profiling of microbial strains (244-248). Nonetheless, two known obstacles impeding more widespread culturing are identification are (i) inadequate knowledge of nutritional requirements and (ii) inadequate knowledge of obligatory symbiotic relationships (242, 249). By creating artificial selection pressures (through nutrient availability or community composition), certain strains are enriched on the selection medium (250, 251). For some microorganisms with poorly described growth requirements (e.g. marine archaea spp. (252), polyphosphate accumulating organisms (253)), enrichment strategies permit valuable insights into the physiology and metabolic requirements of target organisms (254-257). Thus, despite limitations in the breadth of culture-based studies, these models remain essential for high quality descriptions of a strain. These well-characterized organisms subsequently provide a scaffolding for other techniques that boast broader coverage but a shallower level of analysis (258).

In the context of microbial database collections, data from laboratory isolation and enrichment studies typically involves physiological, morphological, and cultivation parameters on the particular microorganism. Among others, some examples of these bacterial and archaeal metadatabases include BacDive (259), EnsemblBacteria (260), and MorphoCol (261). Users of the graphical user interface typically query individual species of interest, while backend application programming interfaces for some databases allow multiple calling thereby greatly facilitating the accumulation and sorting of database entries (262, 263).

3.5. Metabarcoding analysis

About 15 years of metabarcoding studies have succeeded in providing a cursory survey of all major earth habitats (264-268). The selection of taxonomic marker sequences (*i.e.*, the barcodes) for each clade of interest has been essential to the modern state of sequencing coverage (269-272). Far from being a complete story, major biases persist around the strategies underpinning metabarcoding analysis. The genes targeted for studying a given clade are chosen in order (i) to be variable enough to distinguish different species or strains and (ii) to have sufficiently conserved sequences flanking the gene of interest to design primers. Still, the resolution of the sequenced regions required to discern phylogenetic clades is not uniform for all species (273-276).

Nonetheless, there is growing evidence that using the entire 16S rRNA gene sequence in metabarcoding of bacterial and archaeal sequences is ideal for providing reliable species rank resolution (277). The ability of high-throughput sequencing platforms to easily sequence relevant taxonomic marker sequences has made metabarcoding data the most common datatype in microbial sequence database collections (278-280). Additionally, while metabarcoding typically involves sequencing housekeeping genes, it may also be applied to other genes of interest (281, 282). In doing so, these procedures are able to provide a limited assessment of putative functionality with respect to a single bioprocess for microbial communities under study.

Studies aiming only to describe community composition, metabarcoding remains a cost-effective tool. A recent review by Jovel et al. comparing the effectiveness of 16S rRNA gene sequencing to whole genome sequencing methods concluded that alignment of 16S rRNA gene sequences under a high cut-off threshold for sequence quality can be considered better at capturing phylogenetic diversity down to a genus level, with the greatest confidence in taxonomic assignment reported when using the entire 16S rRNA gene sequence (264, 283, 284). Nonetheless, recent developments in metagenomic analyses have contributed towards higher resolutions in taxonomic assignment as well as more targeted approaches when it comes to functional analyses (285, 286).

3.6. Metagenomic analysis

Unlike metabarcoding, metagenomics (a.k.a. whole genome sequencing; WGS) aims to indiscriminately amplifies all DNA fragments. This results in the vast majority – but not the entirety – of genomes in a sample being amplified. By permitting a relatively unbiased survey of the whole microbial community, resulting genomes may be screened for the presence of metabolic pathways of interest. Screening (mining) genomes for specific sequences associated with particular metabolic profiles can be a powerful tool in discerning potential biogeochemical transformations within the biome, although this does not replace metatranscriptomics, in which pathway activity is measured (287, 288). Nonetheless, such information may help substantiate observed physiochemical shifts in the habitat.

Given the significantly larger datasets than found in metabarcoding studies, WGS data is more laborious to process – requirements which must be weighed against the potential for greater resolution in discerning metabolic pathways (289). Targeted metagenomics – an analysis whereby gene clusters are singled out within a metagenomic dataset for subsequent analysis – can help reduce these demands (290). Targeted metagenomics may be done alone or used as a reference library for bulk metagenomic sequences (291). Similarly, captured metagenomics restricts the dataset to functional genes of interest through hybridization-based oligonucleotide probes in metagenomic libraries (292).

Ultimately, metabarcoding and metagenomic analyses provide theoretical interpretations of biogeochemical processes by suggesting what metabolic pathways may be potentially expressed, based on the presence of identified sequences within the same and data from culture-based studies. The next section will introduce some of the ways in which microbial communities may be investigated from an abiotic perspective as well as the challenges in associating these observations to members of the community composition.

3.7. Microbial community ecology analysis

In contrast to physiological observations of isolated strains in the laboratory or the putative inferences provided by metagenomic analysis, community ecology analysis describes the aggregate potential of a microbial community to interact within its habitat on a physiochemical level (230, 293-296). There are a wide range of pipelines currently available to incorporate ontological analysis with the large sequence datasets generated from metagenomic studies, an area that has been well reviewed (214, 229, 297, 298).

Crucial to understanding microbial community functionality in a habitat is the integration of data from independent sources to genomic sequences. Extraneous clues to microbial functionality may originate from RNA sequences (transcriptomics), indicative of proteins expressed and other regulatory functions, or the direct measurement of compounds within the same (primary or secondary metabolites, proteins, mineral nutrients, etc.). Closed format tools, which include microarray and chip technology, allow for the high-throughput screening of thousands of biomarkers - quantifiable substances indicative of a biological state - within the sample (208). Molecular techniques such as Fluorescence In Situ Hybridization (FISH) and Catalyzed Reporter Deposition FISH (CARD-FISH) contextualize metabolically active communities rendering them a powerful tool for the analysis of biofilms, granules, and other microbial assemblages (299, 300).

Analogous to wide-breadth genomic survey in metagenomics, metatranscriptomics explores the aggregate metabolically active fraction of a biome via direct sequencing of RNA transcripts. Here, total RNA or messenger RNA (mRNA) in a sample is sequenced resulting in a map of active gene expression and regulation (301, 302). The development of poly(A) tailing techniques to stabilize rRNA for reverse transcription provoked a considerable expansion in environmental prokaryotic sequences by overcoming the need for prior sequence knowledge prior to cDNA synthesis (303). This technique has proved widely successful, resulting in thousands to millions of novel taxa being discovered (304). While poly-A tailing vastly improves on primer-based sequencing methods, it nonetheless suffers from its own biases, namely internal poly-A priming and truncated amplification fragments (305). Homopolymeric poly-A stretches as short as 3 A's long have been shown to lead to internal poly-A priming and template switching, as described in detail by Balázs et al. (306). Recently, a study by Roy and Chanfreau (2020) created a bioinformatics pipeline to correct the poly-A identification results from three commonly used read mapping programs (STAR, BWA, and BBMap) (307). A primer-independent high-throughput sequencing (HTS) approach provides an alternative solution to the poly-A tailing obstacle by ligating an RNA oligo (M13) to the 5' end of the target rRNA sequence, followed by reverse transcription with a tailed random-hexamer primer and sequencing (301). PCR-free metatranscriptomics, whereby random hexamer primed reverse transcription is used to target small subunit ribosomal RNA (rRNA), is insensitive to the presence of introns and primer mismatches making it more representative of specifically metabolically active cells, and able to encompass all three domains of life (308, 309).

Other strategies for community analysis specialize in identifying primary or secondary metabolites synthesized by the microbial sample. Metabolomics seeks to identify and quantify all metabolites (compounds ≤ 1500 Da) produced by the metabolically active fraction of the microbial community (299). With the goal of instead focusing exclusively on the proteinic diversity in a sample, metaproteomics provides a temporal-spatial snapshot of the proteins expressed by the metabolically active community (299). By providing a snapshot of the biochemical landscape within a sample, these analyses closely complement both metatranscriptomics and sequence data in what is collectively referred to as meta-omics (figure 17). For more information on the state of meta-omics for community analysis, the reader is referred to recent studies (222, 225, 226, 310-315). Protein, metabolite, or mass spectrometry data each have dedicated collections for samples across wide-ranging biomes (316-321). This creates an initial obstacle when integrating these data with other information on the microbial community structure – stored information about a sample is not uniform. This creates unequal depths of information resolution across multiple samples taken from the same biome.

Mapping biochemical observations back onto the original sequence data is further confounded by several factors. While several strategies exist to segregate the metabolically active microbial community from the total amount of detected genetic sequences (295, 322), it is not possible to achieve a similar separation between the total amount of exudates predicted by the transcriptome and observed through metabolomic or metaproteomic analyses. Ultimately, this is due to a diverse set of challenges: full or partial degradation of exudates before sampling, modification of compounds (e.g., use as reducing equivalents), inadequate sampling resolution, etc. The need for greater possibilities in pooling these diverse datatypes stems from the challenges faced in sampling – challenges which are best addressed by expanding the number of studies contributing to microbial community analysis of a particular biome. In this respect, collecting data from global database collections is the only feasible strategy to collect enough data for a thorough mapping of major biomes.

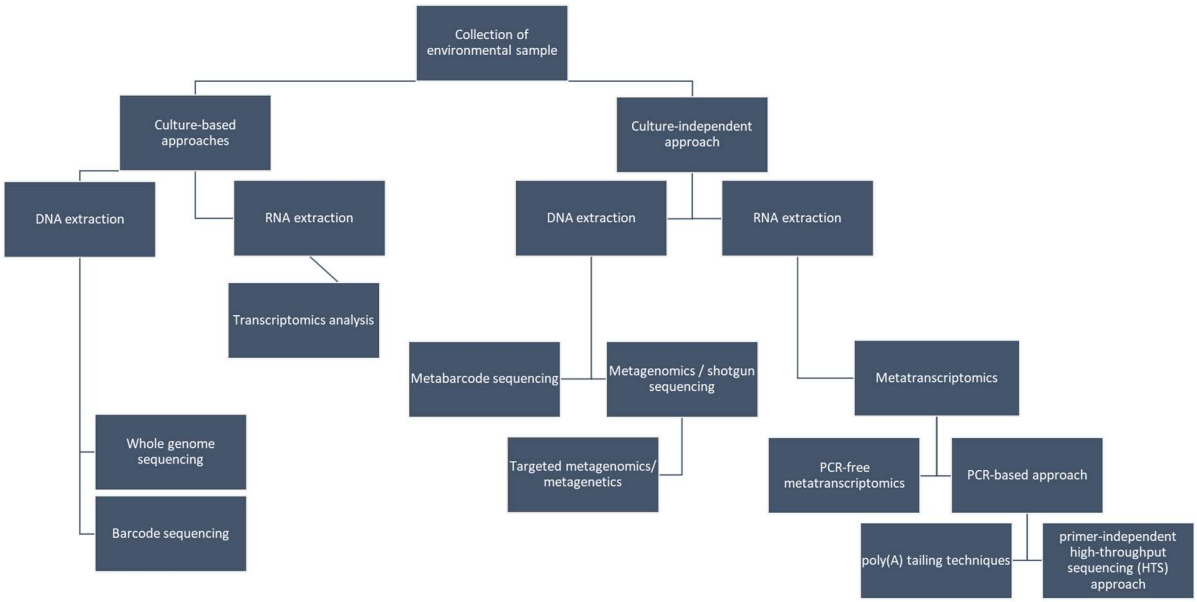


Figure 17. Overview of DNA and RNA based techniques for microbial community sequence analysis.

3.8. Microbial database collections today

The accumulation of data in large taxonomic repositories have opened new possibilities for research into the organization and assembly of microbial communities previously inaccessible due to sparse coverage. Databases for comparative microbiome analyses were first developed to tackle some of the common biases by consolidating studies around the same set of metadata standards (323, 324). Amidst the rapid proliferation of microbiome data, several prominent institutions have set out to create reliable, generic repositories. The most prominent of these databases are summarized in table 2, while a more thorough and regularly updated list can be found in the annual Nucleic Acids Research database issue (325).

Table 2. Examples of public databases for microbial community analysis. Prevalent microbial sequence databases are listed below with indications of their omics integration and functional assignment integration where applicable.

Database name	Data type	Omics approach used	Target Organisms	URL	Reference
Chinese National GenBank (CNGB)	Genomic DNA, omics data	Proteomics focus, storage of data along with experimental conditions and sequence data where applicable.	All organisms	https://db.cngb.org/	(326)
ConsensusPathDB	Metadatabase of molecular functionality databases	Binary and complex protein-protein, genetic, metabolic, signaling, gene regulatory and drug-target interactions, as well as biochemical pathways.	Animal (human, mouse), fungi (yeast)	http://consensuspathdb.org/	(327, 328)
DNA DataBank of Japan (DDBJ)	Whole genomes, omics data	N/A	All organisms	http://www.ddbj.nig.ac.jp	(329-331)
European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL-EBI) ArrayExpress Archive of Functional Genomics Data	Genomic DNA and functional assignments	Accept and archive data generated in experiments that can be characterized as "multi-omics".	All organisms	https://www.ebi.ac.uk/arrayexpress/	(332)
EMBL-EBI BioStudies	Database of biological studies; genomic DNA, protein sequences, functional assignments	Descriptions of biological studies and therein data from within and from outside of the EMBL-EBI database network.	All organisms	https://www.ebi.ac.uk/biostudies/	(333)
EMBL-EBI Omics Discovery Index (Omics DI)	Metadatabase of platforms specialized in omics data	Multiomics, proteomics, metabolomics, transcriptomics, genomics data integration.	All organisms	https://www.omicsdi.org/	(334, 335)

EMBL-EBI Ensembl	Genomic DNA, specifically large genomes	N/A	All organisms	https://www.ensembl.org/index.html	(336)
European Nucleotide Archive (ENA)	Genomic DNA	N/A	All organisms	https://www.ebi.ac.uk/ena/browser/home	(337, 338)
EzBioCloud	16s rRNA gene sequences, whole genome assemblies, metagenomic collections	N/A	Bacteria and Archaeal	https://www.ezbiocloud.net	(339)
Greengenes	16S rRNA gene sequences	N/A	All organisms	https://greengenes.secondgenome.com/	(340)
International Nucleotide Sequence Database Collaboration (INSDC)	Minimally processed sequence data sourced from the DDBJ, NCBI GenBank, and ENA	N/A	All organisms	https://www.insdc.org/	(341, 342)
Joint Genomic Institute Integrated Microbial Genomes (JGI- IMG)	16s rRNA gene sequences, whole genome assemblies, metagenomic collections	Multiomics, proteomics, metabolomics, transcriptomics, genomics data integration.	All organisms	https://img.jgi.doe.gov/index.html	(343)
Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST)	Sequence data and metagenome collections	N/A	All organisms	https://www.mg-rast.org/	(344, 345)
National Center for Biotechnology Information RefSeq (NCBI RefSeq, NCBI BLAST)	Genomic DNA, transcripts, proteins	Proteomics, transcriptomics, genomics data.	All organisms	https://www.ncbi.nlm.nih.gov/refseq/	(346, 347)

NCBI Entrez	Metadatabase of protein sequences and genome DNA (with a focus on protein expression) databases	Proteomics, transcriptomics, genomics data.	All organisms	https://www.ncbi.nlm.nih.gov	(348)
NCBI GenBank	Genomic DNA	N/A	All organisms	https://www.ncbi.nlm.nih.gov/genbank/	(349)
Protist ribosomal reference database (PR2)	18S rRNA sequence database	N/A	Protists	https://pr2-database.org/	(350)
SILVA	(16S/18S, SSU) and large (23S/28S, LSU) subunit ribosomal RNA	N/A	All organisms	https://www.arb-silva.de/	(351)
University of California, Santa Cruz Genome Browser (UCSC Genome Browser)	Genomic DNA	N/A	All organisms with a focus on high-coverage sequences	https://genome.ucsc.edu/	(352)
Ribosomal RNA operon copy number database (rrnDB)	Database designed to catalogue rRNA copy variants in prokaryotes	N/A	Bacteria and Archaea	https://rrndb.umms.med.umich.edu/	(353)
The Microbe Directory (TMD)	Annotated database of microbial sequences, physiology, and morphology.	N/A	Bacteria and Archaea	https://coda.io/@themicrobedirectory/home	(354)

The microbial database collections described in table 2 share a fundamental characteristic – they specialize in specific data types and targeted taxa rather than ecosystems. Recent years have instead seen development of databases centered around a common habitat. Ecosystem-specific databases help address some of the biases prevalent in community analysis by standardizing pipelines and analyses, professionally curating data, and by promoting the dissemination of best practices within a field. While subjected to their own limitations, the emergence of these ES-DB's represents a new step in how

microbial community research is being conducted by prioritizing the production of higher quality sequencing data and facilitating interconnectivity between multi-omics platforms.

3.9. Addressing the limitation of generalized microbial database collections

A fundamental challenge to the collection of microbial community data is the unequal coverage and treatment of parameters across studies. Biases in data collection, processing, and interpretation are not necessarily controllable or resulting from human error. Rather, environmental and technological constraints, as well as the inherent need to accommodate for different sample types and origins hamper reproducibility across studies (figure 18).

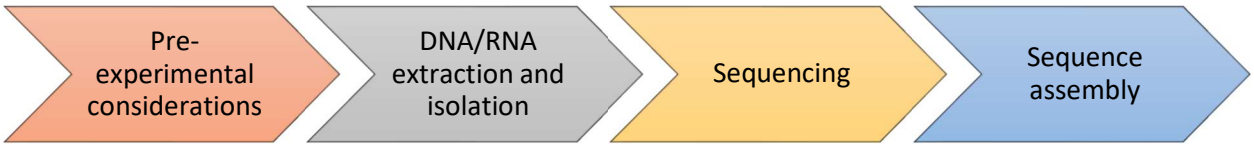


Figure 18. Summary of potential biases implicit in microbial community analysis. Pre-amplification experimental biases, which have a disproportionately costly effect for studies, are highlighted in the figure. Further explanation may be found in table 3.

The inception of "microbiome centers" as part of a knowledge sharing network to streamline the collection and analysis of microbial community data necessarily promotes cross-disciplinary integration (228). They have emerged alongside databases uniquely tailored to concrete, unified research needs surrounding a specific biome, the most prominent of which are summarized in table 3.

Nonetheless, these ecosystem-specific databases are currently limited to individual consortia or lab groups (cf. Human Microbiome Project, MiDAS (355, 356)). Ecosystem-specific databases are an emerging tool in the context of better understanding biomes of interest has not been covered in the literature. The following sections will cover the motivation leading to their emergence, as well as an analysis of their benefits and limitations to community analysis.

3.10. Ecosystem-specific databases as a platform for standardization

Darzi et al. appear to have first proposed the concept of biome-specific microbiome analysis in their 2016 commentary to ISME (357), wherein they provide evidence for the shortcomings of generic approaches in the analysis of microbiomes as well as how these may be addressed through biome-specific databases. A major motivation in the development of ecosystem-specific databases has been the standardization of sampling methods and processing. In turn, this contributes to the generation of higher quality results in terms of accuracy, precision, and reproducibility. Likewise, common standards across studies facilitate the integration of multi-omics tools into community analysis, as well as the ability for multiple studies to be included as additional temporal and spatial snapshots of a sampling region. Furthermore, ES-DB's are always curated by a research group or consortium with experts of the given ecosystem. While the inclusion of metadata greatly improves the quality of microbial databases (210, 358-361), reliably identifying errors within large data sets remains a challenge (362-364).

Improving interconnectivity for studies around the same ecosystem is likewise an integral advantage to ES-DB's. The development the Human Microbiome Project from 2007 – 2019 revealed the strength of manual curation from experts combined with automated assignment tools (365, 366). As a case study for the statistical power of combining studies into aggregate databases through standardized methodologies, the Earth Microbiome Project Consortium collected and analyzed data from 97

microbiome studies, 59 of which were published in peer-reviewed journals (367). Drawing from the conclusions of the EMP and formalized in the 2018 publication by Tripathi et al., wider sampling (more sites) is more effective than deeper sampling (more samples within the same site), down to surprisingly low thresholds (200 sequences / sample) (368). Described otherwise, longitudinal studies of the same ecosystem by different studies is statistically more significant than a singular deep study of the system (369).

Curated databases have allowed for sample pipelines tailored to the microbial communities under study. One such example is the *Actinobacteria* genus *Tetrasphaera*, routinely underestimated in wastewater treatment systems before adaptations to the cell lysis procedure during DNA extraction were implemented in microbial screening (370). These procedural adaptations, concomitant with a push for greater reproducibility across studies investigating wastewater treatment communities has contributed to the formation of the Microbial Database for Activated Sludge (MiDAS 3). MIDAS has since become the most detailed (species-level resolution) ecosystem-specific database for wastewater treatment systems (371, 372). Since then, the MiDAS team has made significant ameliorations to the database, including a field guide for researchers interested in submitting their own data (373).

Table 3. A collection of published ecosystem-specific databases.

Ecosystem-specific database	Target ecosystem(s)	Target organisms	Reference
Biomes of Australian Soil Environments (BASE)	Australian subcontinent, terrestrial systems.	Bacteria, archaea, and general and fungal-specific eukaryotes present in Australian bioregions.	(374)
Dictyopteran gut microbiota reference Database (DictDb)	Dictyopteran gut microbiota.	Bacteria and archaea.	(375)
Earth Microbiome Project (EMP)	Global collection of microbial sequences following standardized protocols.	All organisms, organized by EMP ontology.	(265, 376)
Genome Repository of Oiled Systems (GROS)	Crude oil-associated microbes.	Microbial communities living in and around hydrocarbon oil spills.	(377)
Global Ocean Sampling (GOS)	Open ocean biome.	marine pelagic microbial communities.	(378)
Human Food Project	Diet-acquired human gastrointestinal microbiota.	Bacteria and archaea.	(379)
Human Microbiome Project (HMP)	Microbiome data with focus on human nasal,	Bacteria and archaea.	(380, 381)

	oral, skin, gastrointestinal, and urogenital communities.		
Human Oral Microbiome Database (HOMD)	Human oral microbiome.	Bacteria and archaea.	(382)
Integrative Human Microbiome Project	Human host-microbiome interconnectivity.	Bacteria and archaea.	(356)
MaarjAM	Sequence data associated with the division of arbuscular mycorrhizal fungi Glomeromycota, global distribution	Arbuscular mycorrhizal fungi with biome specific tags.	(383)
Marine databases; MarRef, MarDB, MarCat	MarRef: completely sequenced marine prokaryotic genomes, MarDB: incompletely sequenced prokaryotic genomes, MarCat: catalog of gene and protein sequences from metagenomic studies.	Bacteria and archaea.	(384)
METAgenomics of the Human Intestinal Tract (MetaHIT)	Human intestinal microbiome.	Bacteria and archaea.	(385)
Microbial Database for Activated Sludge (MiDAS)	Activated sludge microbiome.	Bacteria and archaea.	(386)
Oceanic Metagenomics Collection (OMC)	Collection of studies sampling marine environments around the world.	Bacteria and archaea with biome specific tags.	(266, 387)
Rumen and Intestinal Methanogen- DB (RIM-DB)	Ruminant gastrointestinal microbial diversity.	Bacteria and archaea.	(388)
Tara Oceans project	Global eukaryotic plankton sequences.	Plankton and associated prokaryote communities.	(389)
Unified Human Gastrointestinal Genome (UHGG) collection	Human gut microbiome database.	Bacteria and archaea.	(390)

A critical aspect of database management is in the development of internal quality standards. What has been described as the reproducibility crisis, a phenomenon whereby microbiome studies often produce poorly comparable datasets and interpretations thereof, may be addressed through the standardization of methodologies and interconnectivity between researchers (391, 392). A recent review on the critical knowledge gap around sampling and handling in microbiome studies identified 95% of studies as having used subjective sampling methods or inadequately described their methodology (393). Schloss (2018) recently outlined how microbiome studies may improve their integrity and reproducibility through an evaluative rubric (see table 2) (391). Data transparency has likewise been shown to improve community cross-validation (212, 394, 395). The Critical Assessment of Metagenome Interpretation (CAMI), a database in which software is applied against complex reference datasets was designed to facilitate the standardization of bioinformatics processes (396). Others provide more general guidelines and educational tools such as the Statistical Diversity Lab (http://statisticaldiversitylab.com/) (397) and updated resources summarizing best practices in sample preparation for microbiome analyses (210, 398). In contrast to the above protocols that present ways in which standardization may be done, ES-DBs officiate standards in the context of their specific biome.

3.11. A roadmap for ecosystem-specific databases

Environment-specific databases often originate around a persistent knowledge gap that individual studies were not wide or deep enough to elucidate. This is the case of the proposed Drinking Water Microbiome Project (DWMP) which outlines a knowledge gap through a literature comparison indicating a deficit of drinking water microbiome literature compared to other wastewater treatment microbiomes (399). A recent perspective article by de Vrieze (2020) discussed the anaerobic digestion microbiome which would create a more applied database than currently available within the MIDAS infrastructure (355, 400). While many constituents of the core wastewater treatment plant microbiome have been described (401), clear goals and admission criteria (265, 402). Ecosystem-specific databases tend to succeed when they involve experts within the field to provide the necessary experience to judge and curate imported data.

The integration of functional databases with taxonomic collections requires in all cases both top-down and bottom-up engagement as proposed for the DWMP (399). A recent meta-analysis of DNA barcoding databases covering European aquatic habitats highlighted issues in quality control and assurance when integrating diverse databases, resulting in an inconsistent image of taxonomic and subsequently phylogenetic diversity (403). Despite this, interest in greater biome contextualization as well as cross-biome studies appears to be growing. The creation of the Alliance for Freshwater Life, a consortium of researchers studying water quality in natural and anthropogenic environments, demonstrates how properly curated and inclusive databases may develop into policy building and educational platforms beyond their fundamental scientific contribution (404).

Importantly, ecosystem-specific datasets are not limited to environmental studies. In their 2018 article, Kapono et al. recreated the “human environment” as a combination of microbial and chemical data for use in forensics studies (405); nor has the applicability of identifying microbiome-associated biomarkers or keystone species been ignored in health and medicine (398, 406, 407). Similarly, the search for novel genes via bioprospecting depends strongly on accurate genetic annotation and thus may also benefit from more robust reference databases (408, 409).

3.12. Limitations of ES-DB's for omics integration

While ES-DB's appear well disposed to addressing some of the contemporary challenges associated with large microbial community datasets (standardization of sample processing and analysis, data reproducibility, integration of multi-omics technologies from independent studies on the same ecosystem), they are by no means a replacement for universal database collections. As ES-DBs originate out of a deficiency in the organization of the datasets they tend to have clearly delineated scopes. Pinning down an explicit definition for ecosystem-specific databases, thus, remains elusive, as the term currently applies to any database capable of synthesizing multiple datatypes around the study of a single "habitat", be it defined as the human body microbiome or the world's oceans. In essence, the goal of ES-DB's is to ensure that anthropogenic biases (sampling strategies, analysis protocols) are kept to a minimum so that (i) temporal and spatial variability may be better studied across independent studies on the same habitat and (ii) independent research groups specializing in different omics analysis strategies are all able to contribute towards a common knowledge pool. As biomes do not have strict boundaries, ES-DB's may suffer from arbitrary exclusions of relevant data from neighboring biomes. Adding or subtracting biomes into the scope of a particular ES-DB will necessarily lead to a form of the Sorites paradox - pursued to its logical conclusion, adding further biomes would eventually broaden an ES-DB into a generalized microbial collection. Here a grey area emerges when it comes to the border between databases specialized around a common environment across multiple biomes and global databases.

Another crucial limitation to ES-DB's relates to their administration. In order to have professional curation of the dataset, there must be a group of specialists in the field willing and able to provide the service. One way in which the initial entry costs may be lowered could be to establish a standardized meta-structure, applicable to any microbial database collection. Not only would this allow better integration between ES-DB's, but it could decrease the barriers to entry by removing the need for extensive bioinformatics expertise by providing a template for researchers to follow with respect to sample processing and related decision making as well as data organization. Some database organization tools suited towards these aims already exist, although their number will certainly continue increasing. Examples include curative algorithms that could automatically populate ES-DB's from data originating in larger datatype-specific databases but as well organizational databases (*i.e.*, metadatabases) (table 4).

Table 4. A non-exhaustive list of organizational tools for sequence and omics databases; descriptions are derived from database summaries.

Functional database	Purpose	Description	Reference
Functional Ontology Assignments for Metagenomes (FOAM)	Functional analysis	functional gene database designed to screen environmental metagenomic sequence datasets for functionality related to targeted environmental processes.	(410)
EXPath	Functional analysis	Database resource of microarray expression profiles used to infer metabolic pathways for six model plants.	(411)
Ecopath with Ecosim (EWE) (now grouped under EcoBase)	Functional analysis	information repository of EwE models (modeling software for ecological phenomena)	(412)
Genome relative Abundance and Average Size (GAAS)	Functional analysis	Software package for estimations of community composition and average genome length for metagenomes.	(413)
Gulf of Mexico Ecosystem Services Valuation Database (GecoServ) (now called BlueValue)	Ecosystem service evaluation	Worldwide ecosystem valuation information.	(414)
Open access database on climate change effects on littoral and oceanic ecosystems (OCLE)	Ecosystem service evaluation	Ecological-driven database of present and future hazards for European marine life.	(415)
Biofuel Ecophysiological Traits and Yields Database (BETYdb)	Identify bioprocesses	Open-access repository to facilitate the organization, discovery, and exchange of information about plant traits, crop yields, and ecosystem functions.	(416)
jae-f-database	Identify bioprocesses	Global database and 'state of the field' review of research into ecosystem engineering by land animals.	(417)
Genomes OnLine Database (GOLD)	Database organization (metadatabase)	Collection of genome projects and associated metadata.	(418)

Omics DI	Database organization (metadatabase)	Aggregates datasets across multiple public omics data resources.	(419)
ODG	Database organization (metadatabase)	Genomics data integrated with experimental data to create a comparative, multi-dimensional graph database.	(420)

3.13. Conclusion

The establishment of generic repositories for genetic data marked a milestone for the systematization of global microbial diversity cataloguing. Having greatly expanded data accessibility, datatype-specific sequence and omics repositories facilitate novel analyses on data collected from previous studies. Different standards and practices around data collection and processing, however, reduce data robustness and limit the ability for researchers to compare studies. Although no generalizable theory for standardization can be applied across all ecosystems, they are useful when applied across a single ecosystem. Here, we have reviewed how these forces contribute to the emergence of ecosystem-specific databases, a novel strategy to integrate multiple datatypes with important repercussions for data quality and reproducibility.

More widespread implementation of ES-DBs requires more inclusive and accessible bioinformatic infrastructure. While algorithms and pipelines designed to sort and organize existing data are becoming more widespread, resources to facilitate the spontaneous creation of new ES-DBs when applicable are sparse. Concrete standards for data annotation (*i.e.*, tagging) and organization are necessary to facilitate this development, standards which will likewise permit better synthesis of sequence and omics data. By consolidating standards and best practices alongside professional curation of data, higher quality and reproducible datasets will become more commonplace and accessible in the future.

3.14. Contextualization in the thesis

The review paper began as a strategy to coalesce the myriad of terms describing genetic and molecular analyses of microbial communities. Microbiome studies, even when limited to taxonomic and phylogenetic analyses, are subject to diverse and consequential biases (391, 421). Functional analyses of microbial communities may come in all shapes and sizes, sometimes providing information on the community as aggregate and other times only a narrow subset of the population (422-424). Even the term “functional analysis” is misleading – one study may use the term to describe the action of individual community members within their ecosystem, while another will employ the term to describe actions by community members with respect to a particular biogeochemical flow (*e.g.*, nitrogen metabolism) (227, 425, 426). Although I was successful in bringing further attention to the topic through the talks at the 2021 American Aquaponics Association conference, the 2021 Aquaculture America conference, and the 2021 Les Rencontres de l’Aquaponie, there is a lot of momentum required for widespread standardization of protocols, primer sequences, and analytical tools. Simultaneous to the writing of this review, I began looking into strategies to target the single waste product consistently overlooked in aquaculture and aquaponic systems – the fish solids. If the plants are able to control at their own pace colonization of the rhizosphere, then a microbially-mediated remineralization of the fish

solids should not negatively impact plant growth – as long as plant nutritional needs are met and water quality parameters are not degraded. While distribution of fish solids onto agricultural land is common, this is not done in soil-less systems, due to the large amount of carbon contained within, and the impact that has in stimulating heterotrophic bacteria in the water column. This in turn depletes oxygen and stimulates the formation of biofilm, both of which can lead to root rot disease (427). Thus, the goal of the next two chapters was to develop a microbially mediated, economically viable strategy to access the mineral nutrients locked in the fish solids.

4. Improving Plant Health Through Nutrient Remineralization in Aquaponic Systems.

This study was the first in a series designed to revalorize fish solids as a hydroponics nutrient solution. The essential research question here was whether the remineralized nutrients could supplement effluent from a series of fish tanks, to bolster the nutritional demand of a hydroponics crop.

4.1. Abstract

The exploitation of readily bioavailable fish excreta as a source of plant nutrients lies at the cornerstone of aquaponics farming. Research on nutrient cycling in aquaponic systems has devoted considerable attention to the plant uptake of dissolved nutrients in fish excreta, however, the integration of particulate-bound nutrients into downstream hydroponic farming has remained elusive. The high amount of organic carbon present in fish sludge may lead to biofouling (suffocation of roots) if directly incorporated into hydroponic circulation systems, reducing the utility of incorporating fish solids on a large scale. In this study, we implemented a novel treatment system capable of reducing the carbon and nitrogen load of fish solids to produce a liquid fertilizer for a downstream hydroponics unit. Lettuce (*Lactuca sativa*) fertilized with exclusively a commercial nutrient solution, the biofilter effluent (coupled aquaponic system), effluent from the solids treatment system, or the two combined were grown in nutrient flow technique gutters downstream of a recirculating aquaculture system stocked with rainbow trout (*O. mykiss*). While crop yields were lower for the aquaponic treatments compared to lettuce grown in a commercial nutrient solution, plant sap analysis demonstrated a contrasting picture with respect to internal nutrient concentrations, especially micronutrients. Lettuce grown in the commercial hydroponic solution were deficient in several micronutrients (Mg, Ca, Na, Si) nor did they have higher iron concentrations despite the significantly higher EDTA-chelated aqueous iron (460x greater than other treatments) in the nutrient solution. Nutrient uptake in the rhizosphere was not investigated on a molecular level, although stunted rhizosphere growth in the HNS control suggests a weakened capacity for nutrient uptake in comparison to other treatments. Alongside the remineralization of micronutrients, the solids treatment system addressed the common issue of excess carbon leading to biofouling via a total suspended solids reduction of 87.27% ± 9.95 during the coupled RAS-greenhouse cultivation period. Ultimately, these data lead to two important conclusions. First, optimizing nutrient bioavailability is not synonymous to increasing the presence of a nutrient in the water column. Second, estimating ideal nutrient solution concentrations involves both preventing nutrient blocking and improving bioavailability.

4.2. Introduction

In terms of land-use, agricultural production currently occupies half of the world's habitable land (428, 429). A staggering 70% of the global freshwater consumption is currently devoted to agriculture, reaching up to 90% of local supply in some regions (430). The need for high nutrient-use efficiency in existing agricultural systems has also risen in importance due to extreme instances of eutrophication from intensive food production as well as potential phosphorus scarcities (1, 88, 431, 432). These challenges have led to the increase of controlled environment agriculture (CEA), a term that covers protected agriculture (*e.g.*, greenhouse, polytunnels, row covers) and technology-integrated crop management systems (*e.g.*, vertical farming, aquaponics) (433-436). As of 2019, protected agriculture covers 8.83% of all arable land; a figure up from 3.5% in 2016 (436, 437). While CEA platforms are more

efficient cultivation strategies, they must contend with significantly higher infrastructure costs in comparison to traditional soil-based agriculture (438, 439).

Aquaponics is a potentially interesting growing method that can help mitigate some of the additional infrastructure costs of CEA by coupling hydroponic crop production to recirculating aquaculture systems (RAS) (146, 440, 441). In most aquaponics systems, the main aquaculture contribution to hydroponics cultivation is via the biofilter. Biofilters are essential to RAS stability as they remove ammonia that is highly toxic to fish, but they are also the first major N-removal step in coupled aquaponics, with the second being the uptake of nitrogenous species by the crops themselves. The biofilter is simultaneously responsible for the bioconversion of ammonium to nitrate, as well as the reduction of nitrogenous species to nitrogen gas, and removes a significant portion of nitrogen as nitrogen gas (23, 70). The remaining N-fraction leaves the biofilter largely as nitrate with a minority concentration present as nitrite (442). The soluble effluent from a RAS is insufficient to address all plant needs. However, there is significant controversy around the extent to which the nutritional profile should be supplemented with nutrient solution for maximum crop productivity (36, 141, 443).

While hydroponic nutrient supplementation is an easy way to address specific deficiencies, there is an underexplored potential for the remineralization of RAS solid waste as a parallel waste-to-nutrition pipeline to manage agricultural yields. The first solid waste treatment systems in aquaponics were based on either aerobic or anaerobic microbial digesters to increase the solubility of matrix-bound nutrients, with attention mainly devoted to phosphorus and a few plant-relevant micronutrients (56, 444, 445). Hitherto unexplored in aquaponics production system are the wide range of aerobic and anaerobic nutrient remineralization systems currently used in municipal wastewater treatment plants worldwide, such as enhanced biological phosphorus removal (EBPR) (446-448). EBPR has been shown to cheaply and efficiently remineralize the diverse substrate compositions typical of municipal waste (88, 446, 448-452). Typical to EBPR systems is the enrichment of phosphate accumulating organisms (PAO), which play a pivotal role in simultaneous denitrification, carbon catabolism, as well as cyclic phosphorus uptake and release (386, 446). An alternating aerobic-anaerobic environment, typically carried out in a sequential batch reactor (SBR), is essential to the activity of these systems.

While canonical PAOs consist mainly of *Candidatus* Accumulibacter spp., the past decade has shown PAO lifestyles among members of the Actinobacterial genus *Tetrasphaera*; bacteria capable of metabolizing a diverse range of carbon sources (453, 454). Recent studies have furthermore hinted at a relationship between iron and phosphorus in the PAO lifestyle, although the mechanism of action remains unknown (455, 456). Hydrazine reduction, an essential aspect of methanotroph and anammox metabolism, requires considerable amounts of iron and may play a role in the movement of the metal through the EBPR environment (457-459). These biomechanical properties render EBPR systems potentially interesting for aquaponics given that alongside the augmentation of the macronutrient phosphorus, there is an unexplored potential for the remineralization of other plant-relevant nutrients.

The reutilization of one industry's waste products (aquaculture) as a beneficial input to another industrial production process (hydroponics) has made aquaponics into a posterchild for circular economies. The size of an aquaculture system determines the potential scaling of fish to plant production volumes based on waste nutrient availability. It also sets internal limits, without supplementation, on the ability to satisfy plant nutritional needs based on the availability of specific nutrients poorly represented in soluble RAS effluent (e.g. Fe, Mn, Zn, B, Mo, Cu). While fish nutritional

requirements are controlled via the external addition of feed, gauging plant nutritional needs in a coupled aquaponics system is more challenging due to nutrient dynamism across the aquaculture production cycle and across the plant lifespan, not to mention the complex physiochemical influences on nutrient bioavailability (28, 460, 461). Furthermore, the role of the rhizosphere – and its importance in nutrient bioavailability and assimilation - remains poorly understood (116, 119, 138, 140, 195, 462), especially in the hydroponic context (463). The multifactorial increase in both diversity and abundance of the microbial ecosystem in aquaponic systems, as compared to hydroponic counterparts, has previously been discussed as an explanative factor for the discrepancy in fertilizer requirements between the two cultivation systems (34, 141, 442, 464-466). None of the nutrient streams (commercial solution, soluble effluent, remineralized effluent, soluble + remineralized effluent) received additional supplementation over the duration of the study. While it was not expected that this would achieve the maximal yield for any of the aquaponics treatments, it does provide an important perspective into the capacity of the RAS waste streams to supply nutrients to the hydroponics component. If this results in comparable *in situ* nutrient concentrations as determined by plant sap analysis, this may suggest that elevated aqueous nutrient concentrations are alone insufficient at improving agricultural quality and yield.

In these experiments, a novel solids treatment system remineralizing nutrients from fish solids into liquid fertilizer was developed. Unlike other solid waste treatment strategies, EBPR has the potential for extensive C- and N- reduction while remineralizing plant-relevant nutrients, such as P, amidst low biomass production. Lettuce was grown in four parallel circuits, containing an inorganic hydroponic nutrient solution, a traditional coupled aquaponics loop, and two treatments investigating the remineralization capacity of an in-line solids treatment system as an auxiliary source of nutrient to complement standard aquaponics (with and without coupling to a coupled aquaponics loop). Beyond the practical implications of improving resource-use efficiency within the aquaponics context, this project also sought to evaluate agricultural quality as a measure of micronutrient availability.

By simultaneously exploring aquaponic production systems from a fertilizer production as well as waste treatment angles, we test whether microbial remineralized nutrients are able to provide nutrients in a more bioavailable form compared to synthetic hydroponic nutrient solution. In this way, we examine the applicability of aquaponics for low-cost value addition to freshwater RAS for waste reuse. Besides contributing to more efficient, resource-conscious fish and plant production, this study explores the concept of crop quality with respect to micronutrients. Nutrient concentrations in the greenhouse water supply were compared to plant sap analysis data, allowing for a detailed characterization of the capacity for each of the four treatments to satisfy their nutritional demands. This study is the first to assess the capacity of an aquaponics system to target micronutrient bioavailability in downstream agriculture through an in-line solids treatment system. We demonstrate that blanket nutrient excess does not improve nutrient bioavailability and may even diminish plant sap concentrations.

4.3. Materials and Methods

4.3.1. Experimental Design

An experimental aquaponics system was developed at INRAe-PEIMA (Sizun, France) to evaluate the performance of the solids treatment system within a fully functional aquaponics facility. The goal of this experiment was therefore to establish the boundary conditions for the commercial installation of this treatment system. The cultivation system consisted of three separate recirculating aquaculture system

loops operating in parallel in three separate rooms (figure 19). Nutrient solutions were diluted to their final concentration in the four wells within the greenhouse and automatically pumped through eight basins of five parallel nutrient film technique (NFT) gutters (Goponics, France) used to grow the lettuce.

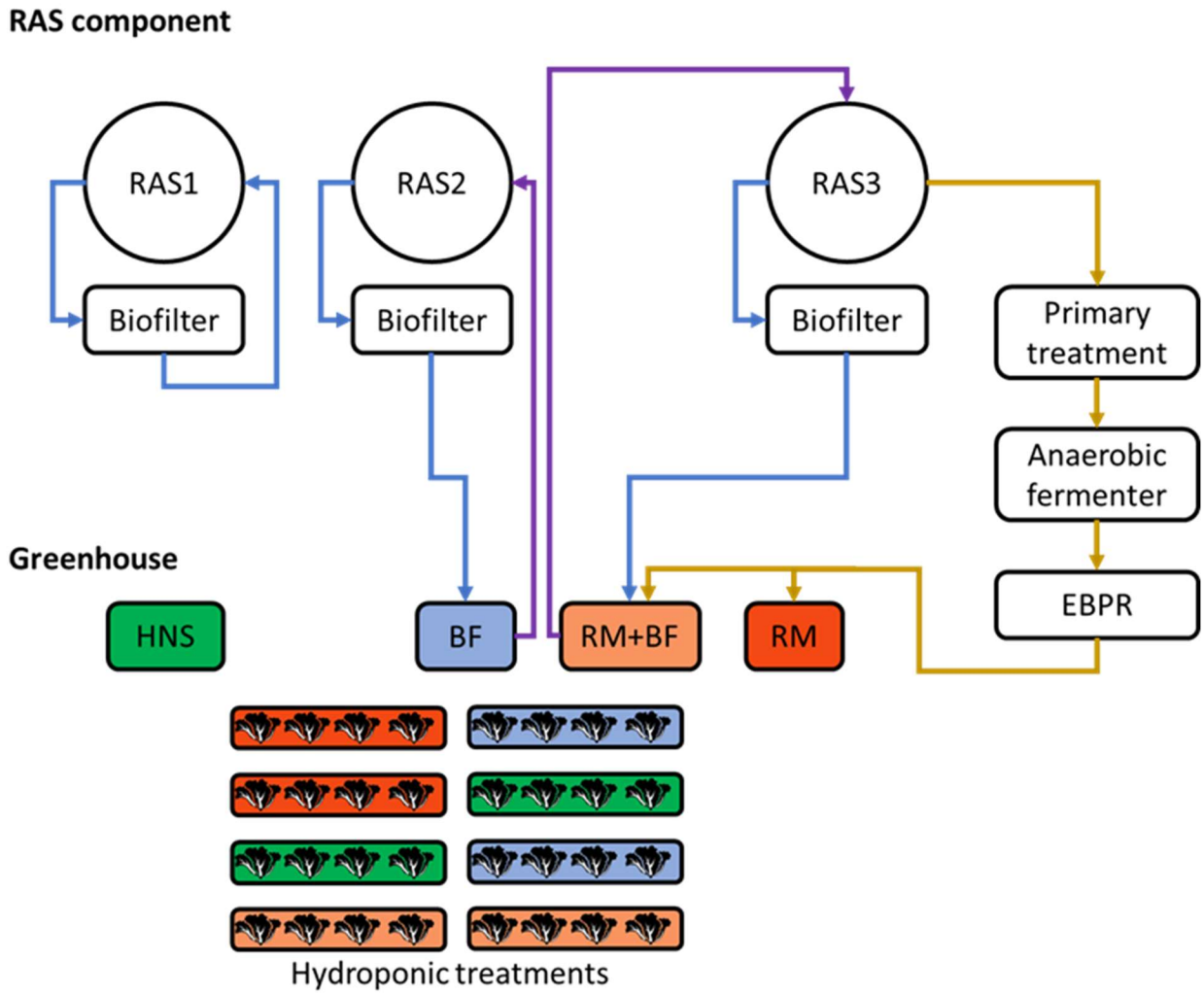


Figure 19. Schematic plan of the three parallel RAS units (left) and greenhouse (right). Blue arrows represent the transfer of wastewater towards the greenhouse, brown arrows represent the transfer of fish solids through the solids treatment pipeline, purple arrows represent the return flows from the greenhouse. Treatments were randomly assigned to their respective gutters.

Of the three RAS units, RAS1 ran independently, RAS2 was linked to the hydroponics treatment BF, and RAS3 was linked to hydroponic treatments RM and RM+BF. RAS2 was thus a traditional coupled aquaponics system, whereby oxidized water exiting the system's biofilter was pumped through the corresponding hydroponics treatment before returning to the fish (BF). The biofilter effluent from RAS3 likewise circulated through the greenhouse, however it was combined with the effluent from the solid waste treatment system (RM+BF). Effluent from the solids treatment system not mixed with biofilter effluent was stored in a separate well (RM). A commercially available hydroponics nutrient solution (Flora series; General Hydroponics, USA) was used as a control group (HNS), manually drained and replaced weekly. Due to the variety of influences on the ultimate *in situ* nutrient concentrations in the plants, no additional nutrient supplementation was done apart from the treatment.

In this study, rainbow trout (*O. mykiss*) were raised from fry on site. Biofilters were set up for RAS two months prior to the addition of fish. An autochthonous lettuce cultivar well-adapted to the temperature and humidity profile of the region (Brittany, France) was chosen for this study and seedlings purchased from Tecnosem (France). Seedlings were transferred to the NFT gutters 3 months after fish cultivation began. The greenhouse unit, while not actively heated, was equipped with a thermometer and an automatic ventilation system that could keep the interior air temperature between 15-25°C throughout the cultivation period, with late-stage temperatures at the lower end of the range. Automatic pumping systems distributed nutrient solutions from the wells to the gutters.

4.3.2. Design of the solids treatment system

The solid waste treatment system involved in the study consisted of a settling basin, an anaerobic fermenter, and a sequential batch reactor (figure 20). Fish solids, passing into the settling basin directly from the RAS drum filter, were first concentrated in the settling basin with excess water evacuated via a lateral pipe that permitted water, but minimal solids, to pass through. A Raspberry Pi microcontroller was utilized to regulate the pumping of the fish solids from the settling basin into the anaerobic fermenter, then into the SBR, and finally from the SBR into the RM and RM+BF wells located in the greenhouse. The microcontroller additionally regulated the aeration through an air compressor and a nitrogen delivery system. The anaerobic digester was kept between 25-35°C by an adjacent water bath, with a pump recirculating water through tubing from the water bath, through the fermenter, and back into the bath in a closed loop. Prior to entering the SBR, the sludge was diluted 1:2 in water originating from the RAS sampling basin. This water, rich in ammonium, was chosen over sourcing from the aquifer to help balance the C:N ratio within the SBR.

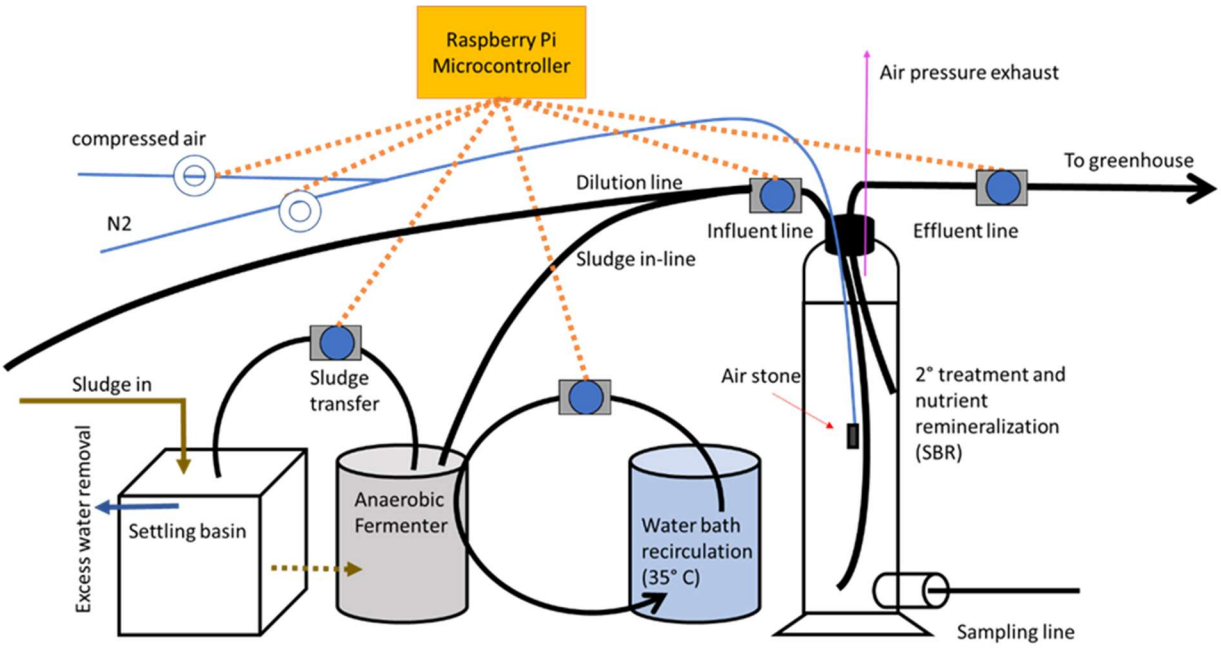


Figure 20. An overview of the solid waste treatment system.

The SBR itself consisted of a 3 L vessel with a main opening at the top, and a secondary lateral opening. That allowed a highly controllable environment where the dissolved oxygen (DO) could be maintained

between 0 and 2 mg O_2/L, and that regulated by the duration that either the compressed air or nitrogen lines were open.

To enrich the PAO proportion in the solid waste treatment pipeline, the SBR followed an alternating aerobic (DO = 2 mg/L) and anaerobic (DO = 0 mg/L) cycle. Due to the physical constraints of accessing the interior of the SBR, the DO, ORP, and pH over the course of the SBR cycle were calibrated externally and not monitored in real time. DO was thus set by measuring the shift during aeration with compressed air, or nitrogen gas, using a portable monitor. ORP proved to be a challenging parameter to measure, and thus was estimated through proxy based on the amount of bioavailable carbon entering the SBR. The SBR cycle was carried out as described in table 5.

Table 5. SBR cycling regime used in this study.

Phase	Action	Duration (seconds)	Description
1	Effluent	100	Evacuation of 1.5 L from the SBR.
2	Influent	100	Import of anaerobic digester sludge diluted in RAS water totaling 1.5 L.
Anoxic Phase			
3	N2	60	Establishment of an anoxic environment.
Anaerobic Phase			
4	Still	1240	Anaerobic fermentation.
Aerobic Phase			
5	Air	600	Aeration of the SBR.
6	Still	300	Aeration turned off to keep DO from surpassing 2 mg/L.
7	Air	900	Aeration of the SBR.
8	Still	900	Shift towards starvation regime to promote P-release in PAOs.

4.3.3. Sampling

Sampling of water quality parameters was done biweekly for each RAS and the hydroponics nutrient solutions across the duration of the respective fish and plant cultivation periods. The pH in each RAS was regulated daily with $NaHCO_3$ to maintain a pH of 7. Similarly, pH in the anaerobic digester was maintained at 7.5. Elsewhere, no modification was carried out as the pH remained stable and within acceptable boundaries. During sampling, ammonia, nitrite, nitrate, phosphorus (total phosphorus and phosphate), chemical oxygen demand (COD), and biological oxygen demand (BOD) were measured (Hach Lange, Germany).

In addition to in situ measurements, samples were assessed for plant relevant nutrients at harvest using commercial technology for greenhouse nutrient monitoring. This allowed a broad survey of nutrients in the RAS and solid waste treatment system (Capinov SAS, France), as well as a of the hydroponics unit including both water quality and plant sap analyses (NovaCrop Control, Netherlands). Lettuce plants were harvested after 8 weeks of hydroponic growth. Fresh and dry shoot and root weights were measured, as well as root length, overall plant health, and total yield.

All data were bioinformatically processed in Microsoft Excel and R. A paired t-test was used to confirm the significance of results wherever stated in the text, with normality and homoscedasticity determined

through Shapiro–Wilk test and Bartlett's test, respectively. All significance tests were performed in Microsoft Excel. A 1-way ANOVA was then run on the harvest parameters to identify divergence across treatments with significant reported at $p < 0.05$. The presence of outliers was then confirmed using the Tukey multiple pairwise-comparisons test.

4.4. Results

4.4.1. Aqueous nutrient concentrations

In situ water quality measurements indicated that the stepwise oxidation of nitrogenous species in the RAS was relatively stable. Total phosphorus and phosphate did not exceed 1 mg/L but did increase following RAS coupling to the HP units, although by the end of the experiment concentrations returned to their original figures (figure 21).

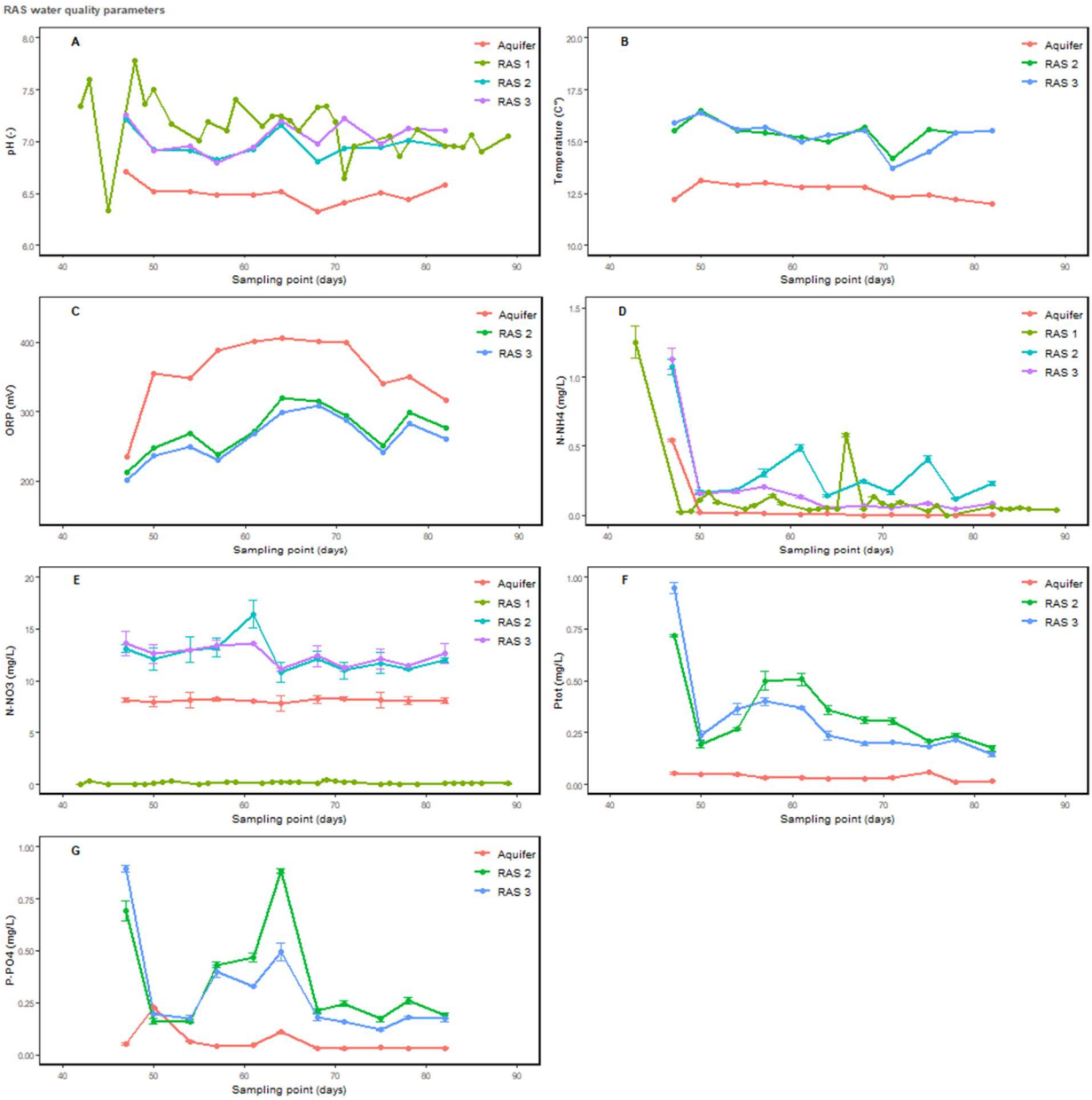


Figure 21. Water quality parameters in the recirculating aquaculture system at INRAe-PEIMA between the coupling of the RAS to the greenhouse (day 45) and the end of the experiment (day 81). RAS1 was operated as a traditional RAS, RAS2 ran as a traditional coupled aquaponics circulation system, RAS3 contained both aqueous and solid waste treatment components.

Results of the water quality tests indicated that many, but not all, essential plant nutrients were available in the water supply (figure 18). Unsurprisingly, virtually all nutrient concentrations in the output from the solid waste treatments (SBR) were elevated compared to the RAS water alone. The notable exception to this rule was Mo. As uneaten feed was directed to the solids treatment system along with excreta, these data imply the nutrient is absent or minimally present in the feed. Charts for P, Fe, NH_4 and NO_3 mirror the shifts expected to occur in the reducing environment. Importantly, as the pH

remained slightly alkaline, we can attribute the liberation of P and Fe to microbial activity and not acidic dissociation.

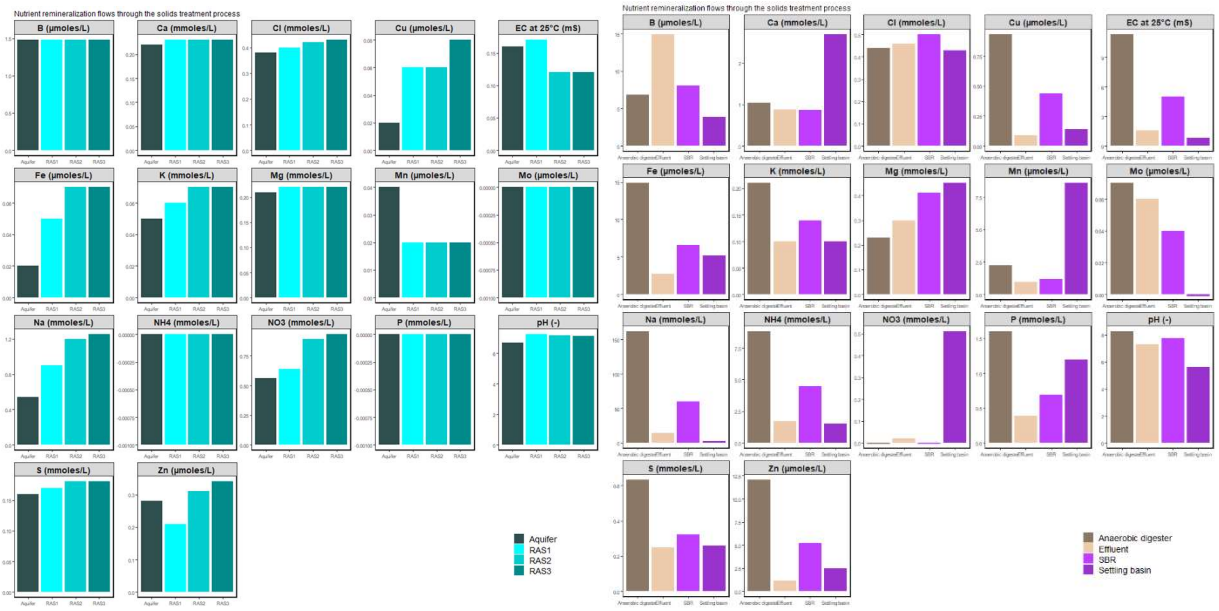


Figure 22. Nutrient load in the RAS (left) and solids treatment pipeline (right) at steady state conditions.

Nutrient composition in the greenhouse wells highlighted the elevated concentrations of virtually all plant-relevant nutrients in the commercial hydroponic nutrient solution (control), with the exceptions being Na, Al, and Si (figure 23). Likewise, the pH across all nutrient solutions remained similar. Due to this high proportion of solutes, the EC of the HNS was proportionally higher. The N-NH4 and N-NO3 concentrations were much higher in the control solution than the coupled aquaponics solution.

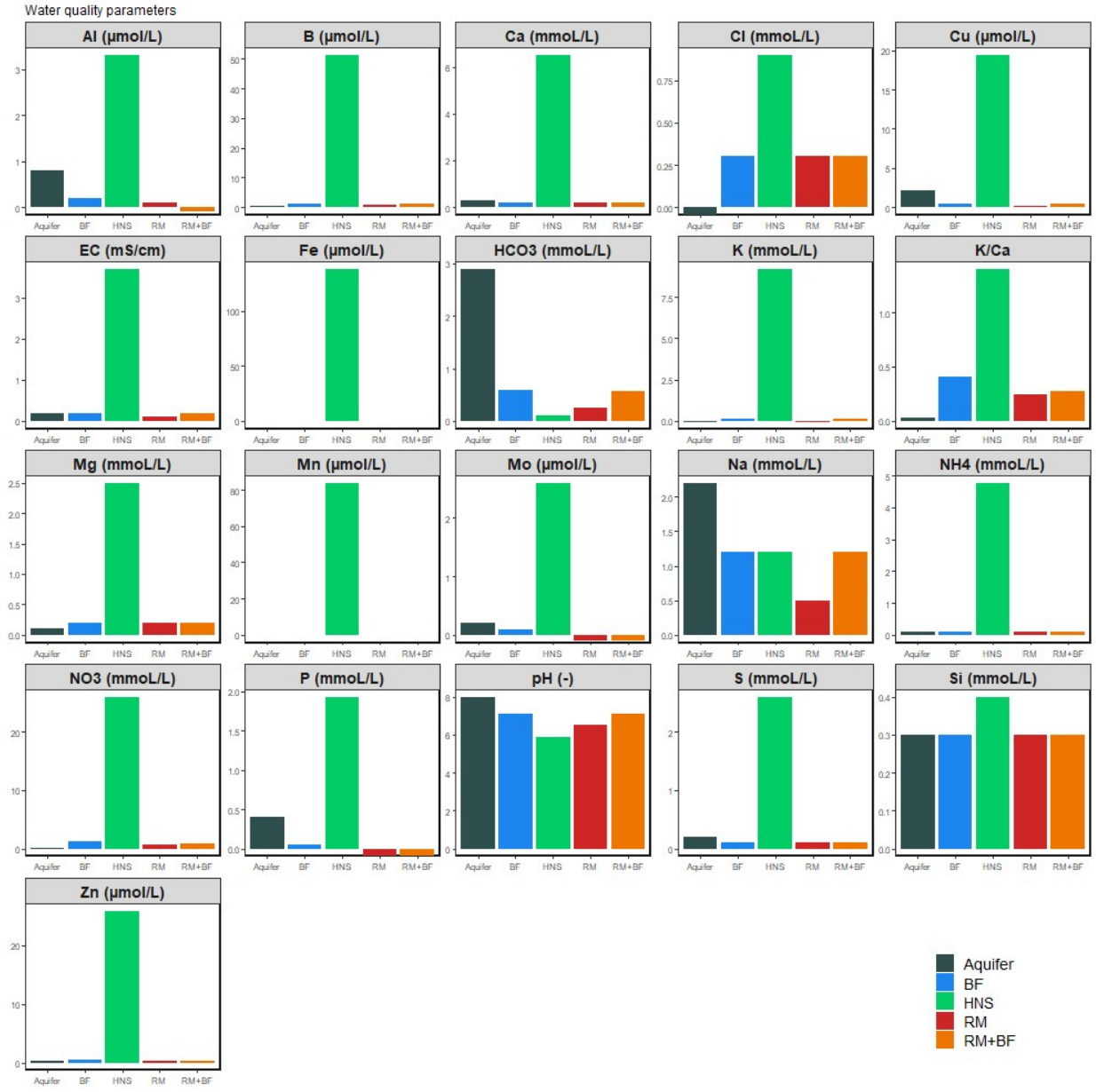


Figure 23. Nutrient loads across greenhouse nutrient solutions, all measurements were taken one week prior to harvest.

4.4.2. EBPR in the aquaponics context

Ultimately, the success of the solids treatment system in adjusting the concentration of several important plant nutrients (figure 22) alongside a drastic reduction in carbon (figure 24) opens a unique niche for sustainable, cost-effective aquaponics cultivation. The adaptation of the enhanced biological phosphorus removal system into the aquaponics system necessitated two fundamental changes in the design. Firstly, the heterogeneity of the fish solids as a C-source did not permit an enrichment of the PAO strains as canonical to EBPR wastewater treatment systems. As the solids treatment system resulted in extensive reductions of the C and N load, this was considered an acceptable trade-off as a high C-load would be unsuitable for use as a liquid fertilizer. Secondly, rather than accumulating phosphorus in the granules as done in EBPR wastewater treatment systems, the operational procedure was modified to promote P-release in the granules immediately prior to effluent evacuation (starvation period following the aerobic phase (table 1)). Figure 24 suggests that most of the phosphorus leaving the system was soluble and accumulating in the downstream nutrient solution wells of the hydroponics unit. With respect to total phosphorus, a significant reduction over the duration of the solids treatment system indicated steady degree of extraction from the solids. Thus, while soluble P leaving the reactor was not significantly higher than the concentration entering the system (disproving the hypothesis), a net conversion of conjugated P to soluble P was evident. What is clear from figure 24, however, is that the contribution of the solids treatment system to the phosphorus demand was low – indicating that this system would need to be scaled up before the nutrient demands from a greenhouse of this size will be met.

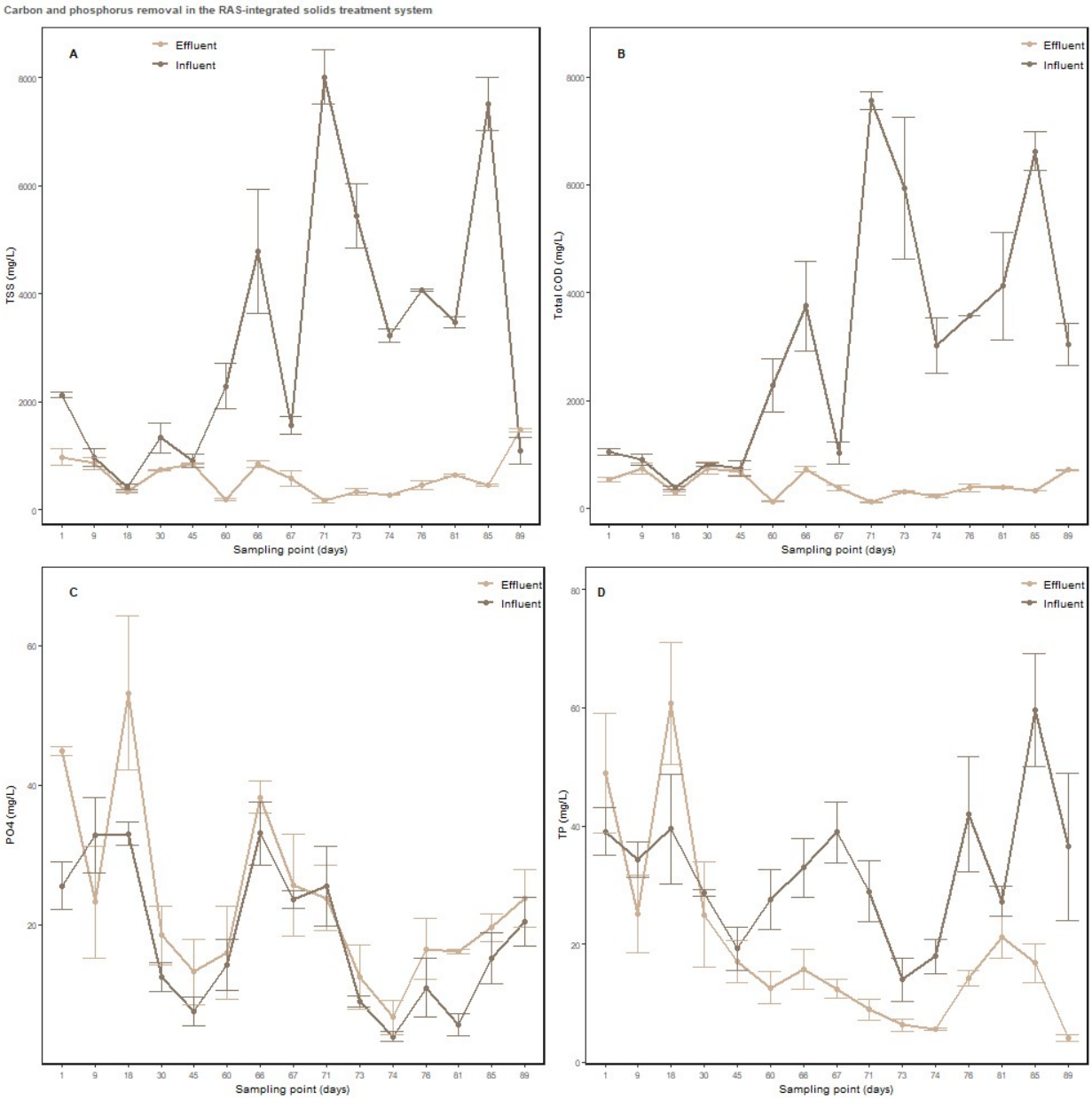


Figure 24. (Left: Total suspended solids and total carbon oxygen demand of sludge prior to entering the sequential batch reactor (SBR). The treatment RM was used to represent accumulation in a downstream hydroponic (HP) unit. (Right: Total (top) and soluble (bottom) phosphorus remineralization in the solids treatment system normalized to total mass transferred. The treatment RM was used to represent accumulation in a downstream hydroponic (HP) unit.

Nonetheless, the high degree of variation in influent COD was initially exacerbated by physical obstacles. These included clumping of the incoming fish solids, reduced flow in the tubing in part due to fish scale and mucous accumulation as well as biofilm growth, a problem that was later solved by diluting the influent sludge and prolonging anaerobic fermentation. Due to this practice, regular wasting of the SBR (removal of accumulated settled solids on the order of ca. 100 mg/ week) is not represented in the graph. These problems are likely irrelevant at greater production capacities where fish solids are more

bioavailable with a decreased proportion of scales and mucous, as well as larger piping diameters to handle greater flows.

4.4.3. Plant nutrient concentrations

The plant sap analysis was chosen as a tool to confirm the successful acquisition of nutrients by the plants from the surrounding aqueous milieu. Old and young leaves were sampled from the plants two weeks before harvest, as per standard NovaCrop Control protocols often used in the hydroponics industry to measure plant health. At harvest, old and young leaves were again sampled along with roots from the same plants to provide a comparative measure of nutrient distribution over time (figures 25-27).

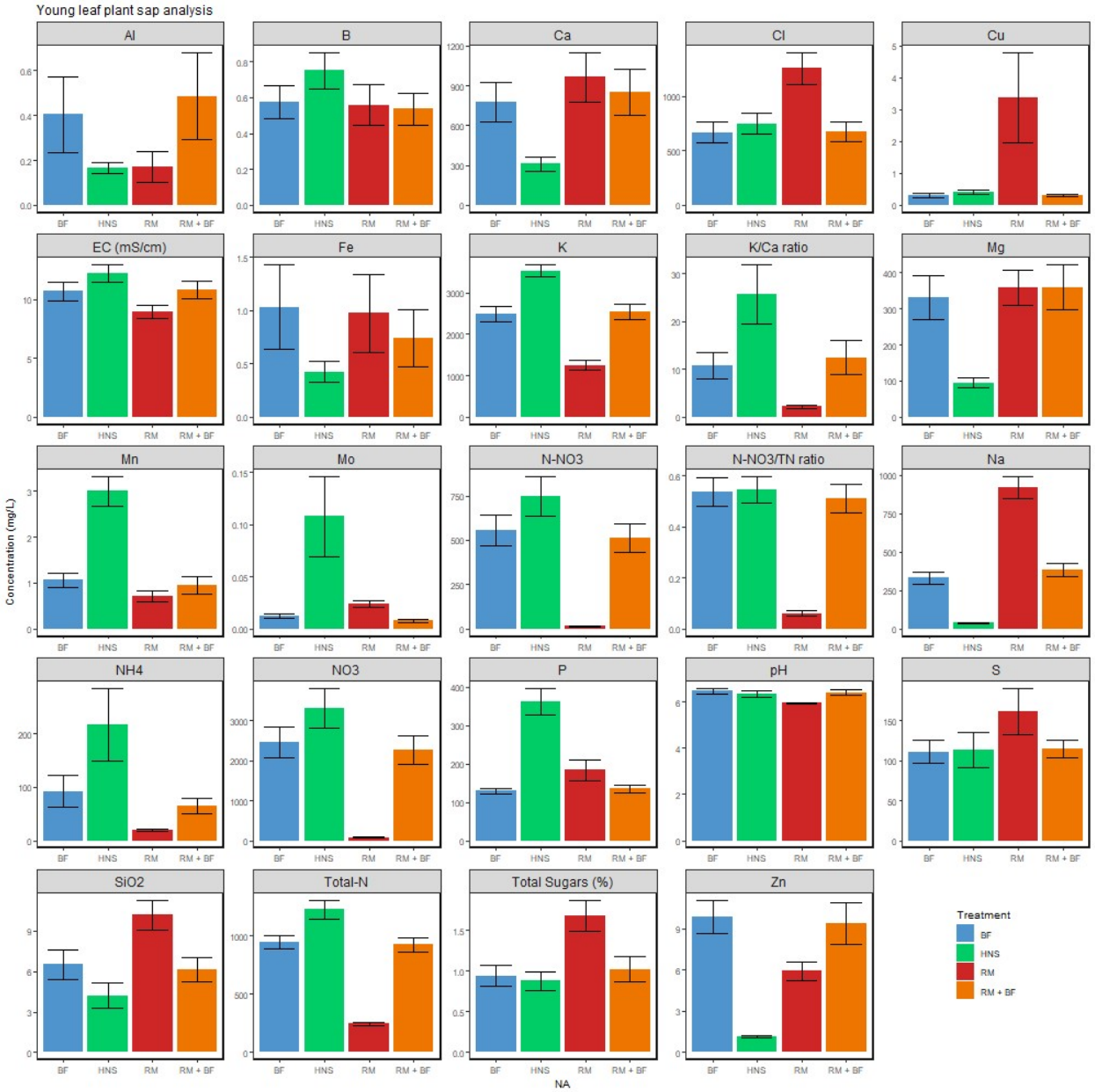


Figure 25. Plant sap analysis for young leaves collected two weeks prior to harvest and at the harvest.

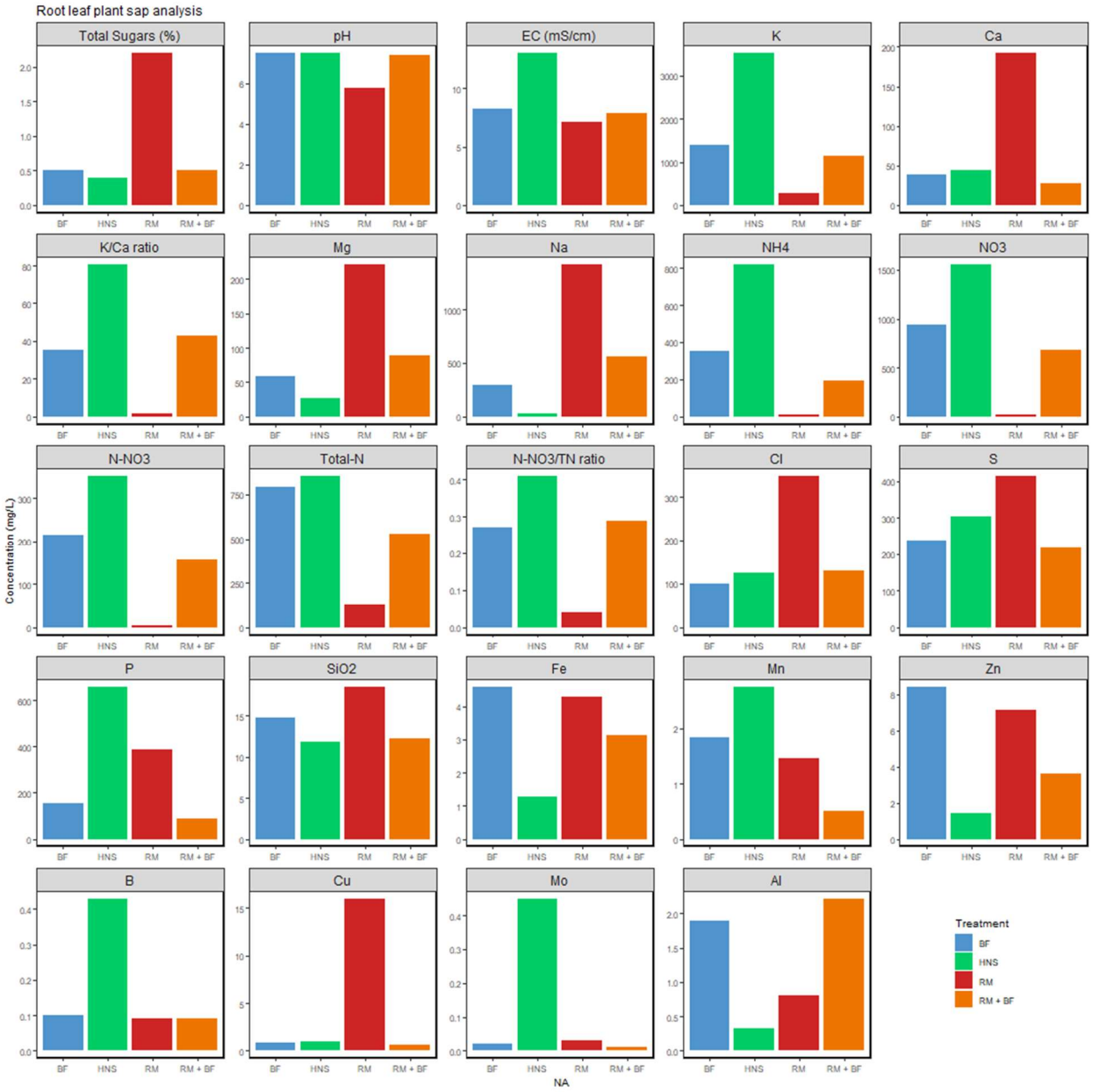


Figure 26. Plant sap analysis for young old leaves collected two weeks prior to harvest and at the harvest.

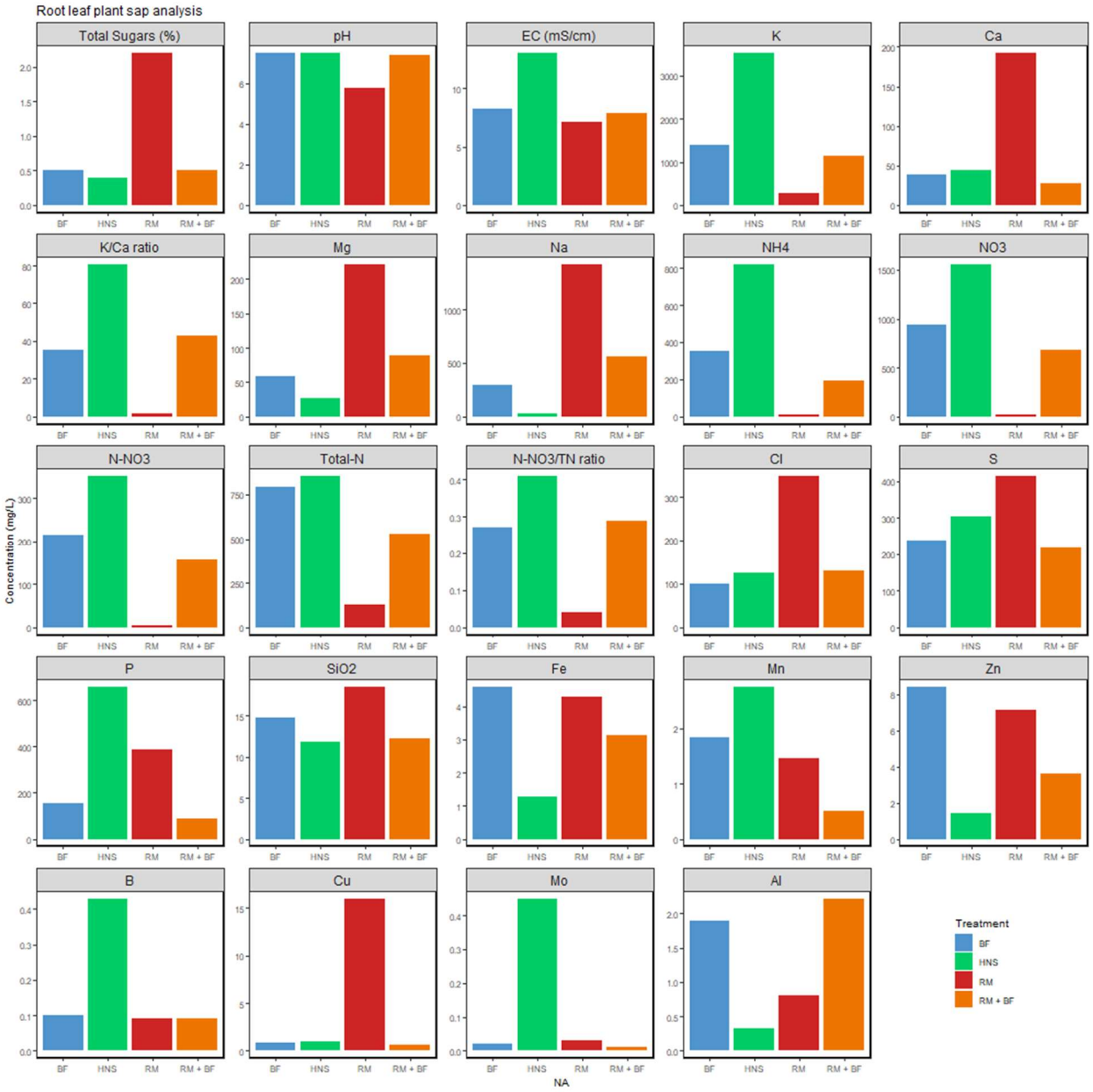


Figure 27. Plat sap analysis of the roots at harvest.

Of the physiochemical parameters, pH levels were constant for all three sample types (young leaves, old leaves, root mass). In terms of EC, all treatments were similar for young leaves, RM was slightly lower than the rest for old leaves, however the HNS root EC was twice that of other treatments. In terms of sugar content, RM was an outlier with the highest percentage while other treatments averaged similarly together.

Plant nutrient concentrations varied drastically across treatments. Of the primary macronutrients, nitrogen (TN, NH4, NO3) and phosphorus were more concentrated in the HNS control group than other treatments in old leaves and roots. N and P concentrations in young leaves were more balanced across all treatments, indicating that aquaponics-fertilized treatments could meet their nutritional needs but

were not in excess of either nutrient. RM tended to be lowest in K, although HNS was significantly higher than other treatments only in the roots. Despite this, RM was the most balanced in terms of K:Ca, while HNS was heavily skewed towards K across all sample types.

For many nutrients, RM and HNS were opposite, with BF and RM+BF treatments falling in between. RM was generally higher in Ca, Mg, Na, Cl, S, SiO_2, Cu and Al although this was not universal for each nutrient at all sample types. The K/Ca ratio, often used as a general monovalent/divalent cation ratio, was most balanced in RM and most skewed towards K in HNS. Besides N and P, HNS had twice as much Fe in young leaves (0.255 ± 0.05 ppm vs. 0.158 ± 0.02 ppm for all other treatments). This was not the case for older leaves where all treatments were similar (averaging 0.158 ± 0.03 ppm) and was the opposite scenario in the roots (HNS = 1.29 ppm, RM = 4.27 ppm, BF = 4.59 ppm, RM + BF = 3.13 ppm). It is thus difficult to correlate iron uptake efficiency to the treatment, however it is clear that the nutrient rich solution did not result in consistently better uptake. In the water quality analysis, Mo was not shown to be present in the RAS and solid waste treatment system but was present in the HNS control.

4.4.4. Harvest

The harvest was carried out after 8 weeks of cultivation as plants were beginning to crowd each other on the rafters. Lettuce heads and roots were weighed at harvest. Crop fresh and dry weight varied significantly across treatments, with the HNS control achieving the highest weight yield (figure 28). Shoot yield varied most considerably across treatments, with the HNS treatment significantly larger than the others at $p < 0.05$ (Table 6). BF and RM+BF formed the next yield category, with the RM treatment trailing behind. While relatively abundant in micronutrients, the RM treatment was specifically deficient in N and K, likely responsible for the stunted growth.

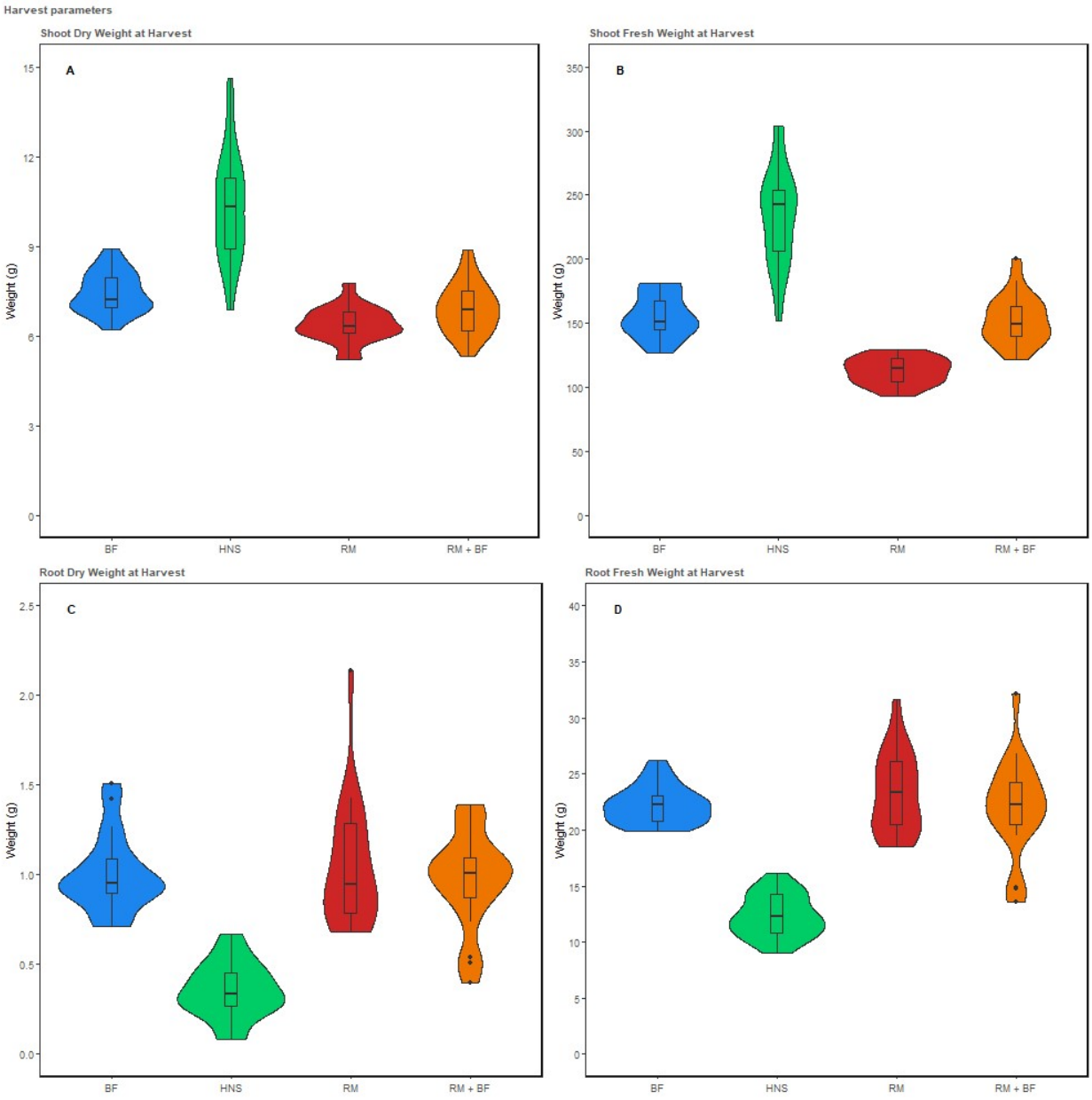


Figure 28. Distribution of harvest weights across treatments. Data are recorded as shoot dry weight (A), shoot fresh weight (B), root dry weight (C), and root wet weight (D).

Root metrics reflected the nutrient saturation of the HNS treatment, with HNS root (fresh and dry) weighing about half of the other treatments. While root length was highly variable within each treatment, HNS similarly had the shortest.

A 1-way ANOVA suggests that all treatments were divergent in both shoot and root weights at $p < 0.05$. Removing the HNS treatment, BF, RM, and RM+BF diverged only in shoot dry and fresh weights but not in root mass. The Tukey multiple pairwise-comparisons test demonstrated that the HNS treatment was indeed the outlier, with the RM+BF and BF treatments being the most similar in harvest parameters (table 6). The ratio between fresh and dry weights across all treatments and within each treatment is

described in table 7. Root lengths were not significantly divergent across any of the treatments as indicated by ANOVA and Tukey multiples tests (figure 28).

Table 6. Tukey multiple pairwise-comparisons indicate that the HNS crop was significantly different from other treatments in both shoot and root weights, although other treatments were more similar for certain metrics.

Multiples	Harvest parameter p-values adjusted to multiples				
	Shoot Dry Weight	Shoot Fresh Weight	Root Dry Weight	Root Fresh Weight	Root length
HNS-BF	**0.00**	**0.00**	**0.00**	**0.00**	0.49
RM-BF	**0.02**	**0.00**	0.95	0.68	0.98
RM + BF - BF	0.43	0.98	0.97	1.00	0.94
RM-HNS	**0.00**	**0.00**	**0.00**	**0.00**	0.62
RM + BF-HNS	**0.00**	**0.00**	**0.00**	**0.00**	0.24
RM + BF-RM	0.43	**0.00**	0.77	0.56	0.74

Table 7. Ratio between fresh and dry weights for shoots and roots at harvest.

Treatment	Samples	Median Fresh/Dry Ratio (%)	Mean Fresh/Dry Ratio (%)
All treatments	Root weight	4.31	4.22
BF		4.29	4.52
HNS		2.73	2.88
RM		4.04	4.49
RM + BF		4.54	4.4
All treatments	Shoot weight	4.71	4.76
BF		4.78	4.8
HNS		4.26	4.44
RM		5.52	5.65
RM + BF		4.61	4.53

Tukey multiple pairwise-comparisons indicate that the HNS crop was significantly different from other treatments in both shoot and root weights, although other treatments were more similar for certain metrics (table 8).

Table 8. Tukey multiple pairwise-comparisons to identify significant differences across harvest parameters for all treatments.

Multiples	Harvest parameter p-values adjusted to multiples				
	Shoot Dry Weight	Shoot Fresh Weight	Root Dry Weight	Root Fresh Weight	Root length
HNS-BF	**0.00**	**0.00**	**0.00**	**0.00**	0.49
RM-BF	**0.02**	**0.00**	0.95	0.68	0.98
RM + BF - BF	0.43	0.98	0.97	1.00	0.94
RM-HNS	**0.00**	**0.00**	**0.00**	**0.00**	0.62
RM + BF-HNS	**0.00**	**0.00**	**0.00**	**0.00**	0.24
RM + BF-RM	0.43	**0.00**	0.77	0.56	0.74

4.4.5. Disease prevalence

With respect to disease, 16 of the 52 HNS lettuce heads had mold growth that developed shortly before harvest. No signs of disease were seen in other treatments. The treatment RM was severely deficient in nitrogen, likely explaining the yellowish coloration of the leaves commonly associated with a nitrogen-deficient state.

4.5. Discussion

Although EBPR is a firmly established strategy for nutrient recuperation from municipal wastewater, it has not yet been investigated in the context of solids treatment for freshwater aquaculture. This study is the first of its kind to assess the suitability of the technology as well as the impact of remineralization on the availability of trace nutrients in the downstream hydroponics unit.

4.5.1. Balancing macronutrient excess with micronutrient deficiencies

Often, the concept of high-output yield (e.g., fast growth, with inherent economic implications) is prioritized over plant health. This view may need to be revised considering the relative abundance of trace nutrients in the lettuce across treatments. The chronic deficiencies present in the HNS control suggest an internal triage response to manage the excesses of other nutrients, a phenomenon known as nutrient lockout (467-469). As reviewed by Marles (2017), several studies have indicated that the nutritional content of vegetables available to consumers has decreased in important micronutrients (e.g. Fe, Zn, Cu, Ca) although the review stresses the lack of consensus surrounding potential causes (470). Nutrient-deficient vegetables is an issue of public health concern (471, 472). Understanding the differences between the hydroponic nutrient solution control and the aquaponics-derived nutrient streams can shed light on possible mechanisms underlying observed discrepancies in bioavailability.

4.5.2. Comparing the commercial nutrient solution to the remineralization/biofilter effluent solution

Comparing aqueous concentrations, it is evident that the control group receiving HNS had access to all relevant macro- and micronutrients at greater quantities in the water supply than was available for other treatments (figure 23). Water originating from the RAS was deficient in Cu, Fe, Mn, and Mo, however all of these elements were recovered from the solids treatment system (figure 22). One of the key findings of this work is the importance of incorporating a solid treatment system into aquaponics systems, that are traditionally reliant primarily on the dissolved nutrient fraction in the water that circulates between the fish and plants. The beneficial impact of integrating solids treatment into aquaponics cultivation were demonstrated by several researchers in the past (56, 444, 473). Sharing fundamental objectives, the solids treatment system discussed in this study improves upon these systems by addressing the primary goal of nutrient remineralization alongside efficient waste treatment (C- and N- removal from bulk sludge to prevent eutrophication) alongside minimal endogenous biomass production. Previous studies, however, restricted their discussion of nutrient remineralization to macronutrients and a small number of micronutrients (155, 444).

With respect to the macronutrient phosphorus, it was assumed that approximately a third of the total system P would be carried downstream as soluble phosphate in the circulating water (25, 106, 474), and that the solids treatment system would further augment this quantity. However, no such trend could be observed in the nutrient solution wells (figure 23). In terms of phosphorus, HNS, RM, and RM+BF had similar concentrations in young leaves. While soluble P was transferred to the greenhouse in the traditional aquaponics setup (BF), it appears that it was insufficient to meet plant needs. Surprisingly,

water concentrations of P were nearly identical across RM, RM+BF, and BF treatments. It seems that the RM and RM+BF treatments were able to adapt to acquire phosphorus more efficiently, or perhaps that the phosphorus supplied by the solid waste treatment system was exceptionally bioavailable. The HNS treatment with 38.6x more P than the other nutrient solutions had significantly higher P accumulation in roots and old leaves in addition to the slight gain in young leaves. Ultimately, based on literature recommendations for lettuce sap P concentration (deficiency below 0.43 % P/DW, sufficiency at 0.55-0.76 % P/DW) we note that all treatments had their demand satiated (475). Zn and Cu blocking, associated with an excess of P, seems to have affected the HNS treatment only in terms of Zn, where it was deficient (< 1 ppm) in older leaves and borderline deficient in young leaves and roots (476-478). No visible signs of Zn deficiency were observable, however (475).

The relationship between pH and nutrient bioavailability has long been a challenge in chemical fertilization as it quickly leads to unideal nutrient solubilities regardless the value. Below a pH of 6, Mn, Zn, Fe become more soluble at the cost of Ca, Mg, and K. The pH of the solids treatment system did not strongly deviate from upstream or downstream components. While acidity in the RAS (pH 7.18 ± 0.04) dropped to 5.61 in the primary treatment, effluent leaving the pipeline had returned above 7, before stabilizing around 6.23 ± 0.5 across all hydroponic well measurements. From this we conclude that acidification was not responsible for the increased solubility of easily-complexed nutrients such as P and especially Fe across the solid waste treatment system.

The overapplication of nitrogen (esp. nitrate) remains the most common detrimental impact of fertilizer misuse on crop health (479). Excess nitrate in plants leads to consistent disease symptoms such as excess intracellular moisture uptake, cell elongation, decreased total sugars content, and a weakening of the cell wall (480). The commercial HNS was highly charged in both ammonium and nitrate (47x and 30x more concentrated than other treatments, respectively). While this disparity directly translated into higher ammonia and nitrate concentration in all plant sap samples, it led to only a slightly higher (1.7x) total nitrogen concentration, leaving N-NO3/TN ratios to be similar to other treatments. Total nitrogen, calculated as the sum of inorganic and organic sources, is indicative of internal protein concentrations (about 85% of TN consists of protein (475)). Nitrogen needs were satisfied in all treatments except for RM, which displayed clear signs of N-deficiency both visually as described in the literature (30, 475) and as well indicated plant sap analysis (figure 7).

Nitrate reductase requires Mo as a cofactor in the conversion of nitrates to amino acids, although it is difficult to assign a target threshold at which point this need is met. Barring potential blocking from other nutrients, this need appears to be satisfied when Mo nutrient solution concentrations exceed 0.06 mg/L as the case in across all treatments within this study. There was no significant difference in Mo concentrations in young leaves, however HNS contained higher concentrations in old leaves and root samples) (481). None of the treatments appear to have suffered from Mo deficiency (<0.01 ppm).

Iron is also required along with Mo for healthy nitrogenase activity among other essential enzymatic functions (30). While the hydroponic nutrient solution was 460x higher in Fe than other nutrient solutions in our treatments, the extra supplementation resulted in only a 1.6x increase (from 0.1575 ± 0.02 to 0.255 ± 0.05 ppm Fe) in young leaves, no significant difference in old leaves, and a decreased concentration in the roots compared to other treatments. It is important to note that we are unable to comment on the speciation (and thus the bioavailability) of iron. However, as the HNS control was prepared weekly and contained EDTA-chelated iron, we can at least maintain that iron was soluble and

flowing through the roots. The reduction of iron from insoluble Fe^{III} to Fe^{II} occurs most commonly under anaerobic conditions. Recorded ORP values in the 200-400 mV range suggest that if this occurs, it is done in rhizospheric microenvironments or through the action of siderophore producing microorganisms (475, 482).

Magnesium is required for the production of chlorophyll at a Mg:N ratio of 1:4, and deficiencies can lead to nitrate hyperaccumulation (483, 484). Despite the HNS having the highest concentration of Mg (14x greater than found in other treatments), young HNS leaves were deficient for Mg (< 100 ppm), although this was not the case for older leaves. K concentrations in the HNS treatment reflected the priority given to this nutrient by commercial fertilizers (92x increase in the nutrient solution). RM plants, which had the lowest K concentration in young and old leaves, simultaneously had the highest total sugar percentage of all treatments, and thus were likely not symptomatic of K deficiency as described elsewhere (485, 486). The total sugars percentage is a widely used measure of plant health in terms of biotic and abiotic stress resistance. No treatments were considered deficient in total sugars (< 0.5%), however there was much variability across treatments and sample types.

The counterbalance of K against Mg, Ca, and Na is a well-established example of nutrient blocking (475, 480, 483). The HNS treatment was richest in K across all sample types (young and old leaves, roots) while containing the least amount of Mg, Ca, and Na compared to all other treatments (figures 8,9). This contrasts heavily to the available concentrations of K (<0.1 mmol/L in other treatments, 9.2 mmol/L for HNS), Mg (14x more concentrated in the HNS than other treatments), and Ca (29x more concentrated in the HNS than other treatments), although Na was in a similar range (0.5 – 2.2 mmol) across all treatments. A relative low concentration of Ca in the HNS treatment was observed across all sample types, despite an abundance (29x greater than other treatments) in the water supply, however this was not below recommended values for lettuce (475).

Chloride levels were similar across the HNS, BF, RM+BF treatments for sample type (although varied significantly across sample type). RM Cl concentrations (6.9 mg Cl/ g DW), likely elevated as a reaction to nitrate deficiency, were well below toxicity thresholds (>23.0 mg Cl/ g DW) (487, 488). Sulfur was not deficient for any of the treatments, although the HNS treatment had the least across all sample types. While a known relationship between S and N concentrations has already been established, it is not well understood how N-excess impacts S metabolism; synergistic effects with P and K uptake have been suggested (475). Boron aluminum, and copper were not deficient for any treatment. Silicon, widely associated with disease suppression (489), was deficient in HNS young leaves, with low values reported for BF and RM + BF young leaves. No deficiency was seen in other sample types.

Of all the nutrients, Mn was definitively deficient (<1.2 ppm) in all treatments except for the HNS for young leaves, and at the limit of deficiency for RM lettuce in their old leaves and roots. As Mn deficiency is associated with retarded growth, this may have played a role in the yield discrepancy (490). However, none of the common indications of Mn deficiency were visible across any of the treatments (475).

4.5.3. Nutrient concentration comparison between the three alternative liquid fertilizer solutions

As the entire cultivation system (RAS-greenhouse coupling, with solids treatment) was in continuous operation, the hydroponic well nutrient concentrations (figure 23) were considered representative of the concentrations that the plants were exposed to in the respective treatments. From this, we were able to determine the extent to which plants were able to satisfy their nutritional needs at these concentrations.

Condensing the above analysis across study results reveals a surprising set of trends. Firstly, all treatments were below the recommended threshold for iron, according to suggestions for instance by commercial companies routinely assessing hydroponic crop health (*e.g.*, NovaCrop Control, Netherlands) who were used for the analysis and conduct testing for the well-established Dutch hydroponics industry. The topic of iron supplementation in aquaponics was reviewed recently by Kasozi et al. (2019), who highlighted the lack of consensus around optimal concentrations for vegetal and fruit-bearing plants (115). Nonetheless, it remains perplexing that the EDTA-chelated, highly concentrated, commercial iron solution was not capable of increasing vegetal iron concentrations. In the aquaponic systems, however, the story of iron is more complicated. On the one hand, iron was not detected in any of the three RAS, nor the aquifer environment (figure 22). The anaerobic fermenter had elevated iron concentrations at levels that would have been sufficient for plant needs (0.83 mg/L), however these concentrations were not maintained in the effluent nor nutrient solution wells. Ultimately, the similarity of all four treatments in the plant sap concentrations suggests that much work needs to be done in understand iron solubilization dynamics, whether the iron needs of plants are being satisfied, and how the rhizosphere can be better recruited to fulfill this demand.

RM was manly deficient in two macronutrients, K and N, as well as the micronutrient B. The BF lettuce alone were deficient notably in P, but as well B as per NovaCrop Control guidelines. All of these requirements were met in the RM+BF treatment, suggesting that the proposed solid waste treatment system has significant potential to address plant nutritional needs. The HNS control suffered from some unique deficiencies, namely of Mg and Ca in young leaves, as well as Na and Si in both young and old leaves. On the other side of the spectrum, all other treatments suffered from Mn deficiency. Thus, while iron supplementation remains an open question, Mn must definitely be supplied in aquaponics given the insufficient access to the micronutrient through the fish feed. Likely, there is an ideal nutrient supplementation level greater than the baseline concentrations established here. Whether this demand will be satiated by an expanded solids treatment system alone will need to be established in future studies.

4.5.4. Sizing up the solids treatment system to match aquaponic needs

This study investigated whether an in-line, EBPR-inspired solids treatment system could improve nutrient remineralization while removing excess carbon and nitrogen from the system. These trends were demonstrable; however, it is likewise obvious that the efforts were insufficient to satiate all plant micro-nutrient needs.

On average, the solids treatment system resulted in a 12x removal of total COD between the anaerobic fermenter (influent) and the hydroponics unit (effluent). Although this value does not account for solids removed from the system for SRT control, it is a considerable reduction. Considering the 450 kg tons of fish in the system producing ca. 45 kg dry weight solids with a theoretical average P of 23.9 mg/g dry

weight (41), the solids treatment system encountered a theoretical P load of 1.08 kg. This resulted in ca. 44 mg P was provided to the greenhouse daily from the ca. 1.85 g of sludge from the anaerobic digester passing through the SBR daily. While a P uptake requirement for the plants in not possible to define here, scaling up the SBR three-fold would at least provide a daily discharge of around 150 ppm, a reasonable target concentration for plant P demand.

4.5.5. Yields comparison

Across all treatments, an average of ca. 95% of the total weight (shoot and root) consisted of water. A notable exception to this rule were the HNS roots, which were ca. 97% water (table 8). While from a mass yield perspective it is not desirable to increase the relative amount of root mass compared to marketable vegetal biomass, the essential role of the rhizosphere in plant nutrient acquisition and stress tolerance cannot be neglected. Rhizophagy has been identified as a principal mechanism for nutrient acquisition and microbial shepherding by plants, a topic that is well reviewed in current literature (491-493). In addition to nutrient uptake, endophytic microorganisms are now understood to be crucial to several fundamental plant functions (growth and development, oxidative stress reduction, disease, and predation prevention) (494-496). Plants that naturally grow in soil-less environments (e.g., bare rock) are particularly reliant on a diverse and well-developed endophytic community, which may suggest similar patterns in hydroponic cultivation systems (497-499). Ignoring the role of the rhizosphere is ignoring a fundamental plant organ (116, 119, 125, 136, 168, 462, 500). In this context, the differences in root length and mass between the control and other treatments suggest an underexplored contribution of the rhizosphere to nutrient uptake in hydroponic cultivation.

The impact of microbially-suppressing agrochemicals strongly diminishes and shifts the rhizosphere community, with effects on both the effective bioavailability of nutrients and the rhizospheric reserves available to plants (482, 501-503). An inhibited exchange of organic acids and nutrients between plants and their rhizosphere has been shown to engender drastic effects on nutrient-recycling and secondary metabolites (impacting taste, antioxidant capacity, etc. of the crop). These changes have been described in soil systems although contextualization in the hydroponic context is lacking (116, 119, 482, 504, 505). The onset of mold in nearly a third of the HNS treatment lettuce, but not in other treatments, suggests that even while the plants obtained a better mass yield, they were potentially compromised in other aspects. Whether this could be linked to nutrient deficiencies (e.g. Si deficiency in young leaves has been linked to increased disease susceptibility (506)) or whether it is the result of a diminished rhizosphere community, was not confirmed in this experiment, but is worthy of further investigation.

4.6. Conclusion

Fundamentally, the challenge of closed environment agriculture is one of resource-use optimization. The exploitation of readily available, soluble aquaculture effluent expanded our conception of nutrient transfer in the hydroponic environment to include the role of microorganisms and the rhizosphere. Nutrient remineralization has not been adopted unanimously, mainly due to the challenges and carbon reduction and the additional costs associated with existing waste revalorization systems. This study contributes to the field by presenting a novel strategy for solids treatment to this base inspired from EBPR processes found in municipal wastewater treatment plants. This system permits simultaneous waste treatment (C- and N- reduction) with low residual biomass generation and a diverse trace nutrient spectrum for downstream hydroponics cultivation. To gauge the impact of the nutrient streams on

agricultural yield and quality, we did not supplement for deficient nutrients. This strategy provided a unique perspective into the ability of the hydroponic crops to take up aqueous nutrients.

For this investigation, the micronutrient profiles of the remineralized effluent, traditional coupled aquaponics, and a commercial hydroponic nutrient solution were measured. Nutrient concentrations diverged significantly between the aquaculture-derived treatments and the commercial solution, which eclipsed other treatments for virtually every measured element in the water column. In contrast, plant sap analysis did not reflect a universally higher nutrient content in lettuce grown under excessive nutrient conditions.

Lettuce grown in the commercial HNS likewise experienced deficiencies of Mg and Ca (young leaves) as well as Na and Si (both young and old leaves). Uptake of certain elements (Cu, Fe, Mg, S, Zn) was greater across aquaponic treatments than initially predicted, however, Mn was not detectable in the aquaponic treatments. B and P were especially low in the standard aquaponics treatment (fertilization with soluble RAS nutrients only). Together, this suggests that the solids treatment system in parallel to RAS soluble effluent may be advantageous for aquaponic facilities seeking to maximize the benefits of the fish solids for plant nutrition. Nonetheless, iron remains the most capricious element to provide for plants. The evidence that neither the commercial solution, nor aquaponic treatments was completely successful in increasing iron uptake, suggests a need for future studies to determine minimal “optimal” concentrations for plants, including the repercussions of mineral nutrient deficiencies on crop yield and nutritional quality.

4.7. Contextualization in the thesis

This study demonstrated that nutrient remineralization could achieve the dual goals of solids treatment and fertilizer production to complement RAS soluble effluent and meet the nutritional demands of plants. Such a strategy could be advantageous for aquaponic facilities seeking to maximize the benefits of fish solids for plant nutrition. Importantly, this study did not have a well-developed anaerobic digestion system, yet was still able to render many nutrients (most importantly – iron) more bioavailable to the lettuce than the hydroponic nutrient solution. While further optimization of the hydroponics cultivation was considered to be outside the scope of this thesis, this study provides preliminary data into the potential for the production of a liquid fertilizer from fish solids. This embodies the main goal of this dissertation, which is to utilize microbial processes to close the circularity loop in an economically viable manner. Evident, however, was the need to better understand the fundamental parameters driving the remineralization process. The fifth chapter in this thesis targets the potential for biomethane production from the aquaculture solids as a revalorization strategy alongside the liquid fertilizer production.

5. Simultaneous Biomethane Production and Solids Waste Treatment in Aquaculture

5.1. Abstract

The rapid expansion of the aquaculture industry has brought about a heightened focus on the waste produced by high intensity fish farming. In closed-containment, recirculating aquaculture systems (RAS), fish solids are mechanically separated and/or coagulated before being disposed as waste. Subsequent revalorization is typically limited to the direct dispersal of aquaculture solids onto agricultural fields. Here, we developed a novel, continuous flow, low-cost solids waste treatment system for freshwater and saline RAS. Rotating drum filter backwash was collected as the primary feedstock for anaerobic digestion. A laboratory scale set up was used to monitor the conversion of the solids into a methane-rich (60-80% purity) biogas stream. Iron supplementation (ferric iron at 100 mg/L and 1000 mg/L) improved salt tolerance of the methanogenic community, leading to higher methane yields in a supplemented ($FeCl_3$ at 1000 mg/L) saline treatment than the saline control. The application of iron additionally improves pH stability and volatile fatty acid utilization. The methane yield ranged from 0.1-0.4 NL CH_4/ g VS across the three freshwater treatments and the iron-supplemented saline treatment, however, it was significantly lower for the saltwater control: ranging between 0.08-0.25 NL CH_4/ g VS. These values correspond to a percentage yield of 57% - 86% of the total biomethane potential. Overall, implementing anaerobic digestion for RAS waste valorization may generate significant amounts of biomethane to be used in electricity and heating for large-scale aquaculture facilities, while even for smaller facilities it may off-set costs and mitigate environmental impacts of the waste streams.

5.2. Introduction

In 2018, world salmon aquaculture production reached 2.2 million tons, corresponding to an estimated nutrient loss of 889 kilotons of carbon, 1.13 million tons of nitrogen, and 20.6 kilotons of phosphorus into coastal waters (12, 17). This discharge is related to metabolic processes (the excretion of carbon-rich mucous, exhaled ammonia, and urea), uneaten feed (partial digestion of the carbon source, nitrogen and phosphorus in other forms, such as proteins) as well as all microbially-mediated derivatives of the decomposition process (18).

Aquaculture waste streams can be divided into two broad categories, *i.e.*, dissolved and suspended fractions. Treatment of the dissolved fraction focuses on the simultaneous removal and neutralization of nitrogenous species, resulting in the formation of nitrate (19-23), although the removal of dissolved organic carbon occurs simultaneously (507, 508). Other mineral nutrients (509) are also carried downstream to varying degrees, depending on their solubility at the neutral pH typical of the upstream water source and their complexation in the fish solids or feed (24-28). Under pressure from both regulatory agencies and the public, solids waste management is an increasingly important issue for the further development of the global aquaculture industry (14-16). The development of closed containment systems for land and coastal cultivation facilities, referred to as recirculating aquaculture systems (RAS), has been indicative of this paradigm shift towards increased water and nutrient-use sustainability (12, 13). In RAS, suspended solids 60–200 μm are removed from the circulating loop through the use of a rotating drum filter (40, 510). These solids are recoverable – in contrast to the relatively more open net-pen or flow-through raceways.

Solids management in closed aquaculture systems is essential due to the deleterious direct and indirect effects of suspended solids on finfish health (41, 49, 50, 510). There are many types of solids collection systems available for freshwater aquaculture facilities, each with unique advantages and disadvantages (41, 51). Solids are taken out of the circulating system through diverse, and often facility-specific, collection designs (drum filter, swirl separator, or radial flow settler) at which point there are two terminal options for fish solids: neutralization (*e.g.*, biological degradation and disposal of residual sludge where, if allowed, it is often redirected towards municipal waste treatment streams), or revalorization as part of other bioprocesses (52, 510). The inherent financial costs associated with the collection and removal of fish solids is increasingly incentivizing aquaculturists to explore sludge revalorization, including reselling dewatered wastes as organic fertilizers (44, 45, 511). Anaerobic biorefineries have been shown to integrate well with aquaculture systems, although their current stage of development suggests that considerable time and innovation is still needed before they become economically viable at a commercial scale (53-56).

These refineries prioritize biogas production through anaerobic digestion as a crude mixture of methane and CO_2 from carbon-rich waste streams. When the methane fraction is purified to remove potential contaminants (nitrogenous species, oxygen, and H_2S), the resulting stream is referred to as biomethane. Varying in size and complexity, combined heat and power (CHP) systems combust biogas or biomethane with the aim of generating heat and electricity (512). Recent years have seen the biogas market grow considerably in scale to meet increasing energy demands, while also better achieving sustainability and climate goals (513-516). The potential applications of anaerobic digestion in recirculating aquaculture has been recently reviewed (517), however, data is limited for aquaculture compared to other agricultural resources, such as livestock farming (514, 515, 518).

The development of cost-effective waste treatment solutions in closed containment systems is critical for the aquaculture industry to reduce discharge, especially as nutrient pollution contributes to eutrophication of local water bodies, which is being increasingly regulated (519). In this context we quantified the stability of biogas production from fish solids over an extended period of time (95 days) with the goal of assessing the capacity of this technology to alleviate waste treatment costs for recirculating aquaculture systems. We furthermore addressed the role of iron in maximizing the biomethane potential. Iron, known to be an essential nutrient for methanogenesis (400, 520), was supplemented to the aquaculture solids as ferric chloride at a low and high concentration as part of an initial investigation into the iron requirements of the anaerobic community. The choice in concentrations allowed for the dichotomy between a control treatment where iron is a limiting reactant for biological and chemical processes, a situation where iron is sufficient for biological processes only (low iron concentration; 100 mg/L) and a situation where iron is not limiting for biological nor chemical reactions (high iron concentration, 1000 mg/L). Both freshwater and saline (12 g/L) environments were explored in this study to broaden the applicability of the technology to include a wide range of fish-production types. Iron supplementation under saline conditions was explored in a deficiency/excess duality (1000 mg/L addition). The multiplicity of treatments was then contextualized at scales relevant for aquaculture farms, creating a framework for the implications from this study for environmentally and economically sustainable aquaculture solids treatment.

5.3. Materials and Methods

5.3.1. Inoculum and feedstock

Aquaculture solids were collected from a rainbow trout (*O. mykiss*) recirculating aquaculture system at the Brussel Integrated Greenhouse (BIGH), Belgium. Sludge was collected from the backwash coming from a 10 L rotating drum filter (0.85 µm mesh), corresponding to flow rate of 0.24 m^3/h. The sludge was allowed to settle in the collection containers for at least 24 h, resulting in ca. 2% w/v sludge. Only the settled solids were used in the experiment, which were stored at 4°C until use. An anaerobic inoculum was obtained from a full-scale mesophilic digester provided by Innolab (Belgium) and was used to jumpstart methanogenic activity (Table 9). The inoculum was diluted with tap water to a final concentration of 10 g COD/L.

Table 9. Initial characterization of the settled aquaculture solids and anaerobic inoculum used in this study. FW = fresh weight.

Parameter	Unit	Settled aquaculture solids	Anaerobic inoculum
Chemical Oxygen Demand (COD)	g COD kg^{-1} FW	56.63 ± 1.14	85.31 ± 1.98
Total solids (TS)	g TS kg^{-1} FW	36.64 ± 1.34	59.69 ± 0.84
Volatile solids (VS)	g VS kg^{-1} FW	21.34 ± 1.23	42.37 ± 0.77
VS/TS	%	58.26 ± 1.05	70.98 ± 0.56
COD: VS ratio	-	2.65 ± 0.16	2.01 ± 0.06
Total Kjeldahl Nitrogen (TKN)	g N kg^{-1} FW	8.90 ± 0.38	1.52 ± 0.05
Volatile fatty acids (VFA)	mg COD kg^{-1} FW	1340 ± 131	281 ± 92

5.3.2. Reactor set-up and operation

Anaerobic digestion was carried out in Schott bottles (1 L) filled to 80% with sludge. These Schott bottles were homogenized by gently stirring before sampling but were not otherwise mixed during the experiment. Biogas collection columns were set up for each Schott bottle (tubing connection) to allow for biogas capture and quantification. Thrice weekly, two 5 mL syringes were used to collect biogas for each treatment directly from the column, whereupon samples were immediately processed (see section 2.3). In this way, sampling represents the average headspace composition produced between any two feeding points. An acid salt bath (HCl solution at pH ≈ 3) stained with methyl orange prevents CO_2 dissolution and escape from the column headspace (figure 29).

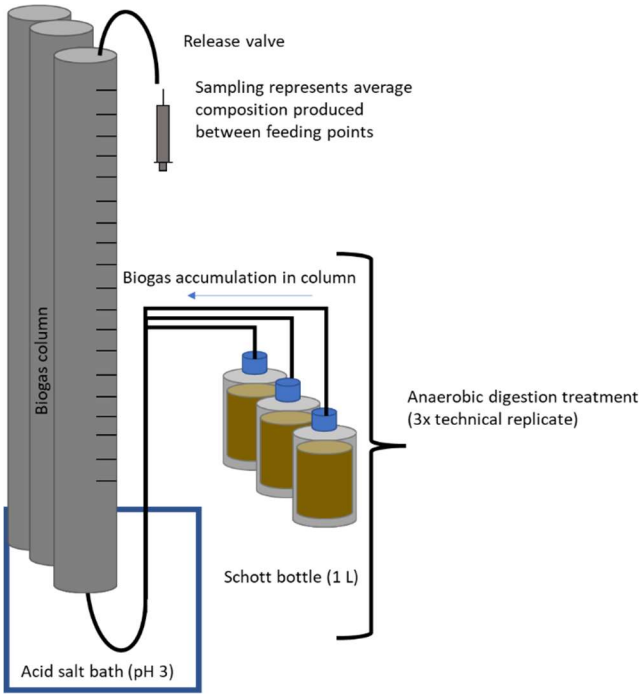


Figure 29. Graphical representation of the experimental set-up. Biological triplicates of each treatment were each linked to a biogas column. A low pH water bath ensured stable CO2 concentrations in the headspace; columns were sampled at each feeding point to create an average headspace sample.

Treatments included three freshwater and two simulated saline water treatments consisting of 12 g/kg salt mix (Instant Ocean, USA) added to the aquaculture solids at each feeding. Each treatment was performed in triple biological replicates, and all treatments were kept in a temperature-controlled room (28° C). The three freshwater treatments included a control (no iron addition), a low iron (100 mg/L) and a high iron (1000 mg/L) treatment, with ferrous chloride added from a stock solution during feeding. The two saline treatments were divided between a control (no iron addition) and high iron (1000 mg/L), likewise added at each feeding.

The anaerobic digesters were operated as a continuous stirred-tank reactor with manual shaking in which hydraulics retention time is always the same as the solids retention time as there is no separation of the liquid from the solids. Here, we will refer only to the SRT. The SRT was slowly reduced from 80 days to 20 days over a two-week period with the effect of gradually increasing the organic loading rate (OLR) while allowing for the microbial community in the inoculum to adapt to the aquaculture solids feedstock (Table 10).

Table 10. Description of the adjustment protocol to acclimate the inoculum to the aquaculture solids feedstock.

Period (d)	Target SRT (d)	Organic loading rate (OLR) (g COD/L digester * d)	Success criteria
0 – 7	80	0.57	No significant signs of instability.
8 – 14	40	1.13	The pH should be > 7 without adjustment.
15 – 25	20	2.27	The pH should stabilize to within 0.2 units.
25 – 95	20	2.27	The pH should be >7, biogas production will determine which treatment is more successful.

Feeding consisted of manually replacing digestate with new substrate (aquaculture solids) as per the volume exchange rate (SRT × interval of days between feeding). To do this, Schott bottles were shaken to homogenize the digestate, then briefly opened to remove digestate and add new feedstock. This was carried out thrice weekly at which time digester pH was measured and biogas potential was assessed (quantification of biogas volume and composition). Once weekly, samples were taken for total and volatile solids measurements, as well as nutrient and volatile fatty acid analysis.

5.3.3. Analytical techniques

Total and volatile solids were measured using a drying oven (100 °C) and a muffle oven (550 °C) using standard methods (521). Kjeldahl nitrogen was likewise measured using standard methods (521). The COD was measured using the Hach LCK 514 (Hach-Lange, Germany). Volatile fatty acid (VFA) composition was measured by gas chromatography (GC-2014, Shimadzu®, The Netherlands) with a DB-FFAP 123-3232 column (30m x 0.32 mm x 0.25 µm; Agilent, Belgium) and a flame ionization detector (FID) calibrated for VFA concentration range of 30 to 1000 mg/L using a nitrogen gas carrier (522). The COD-adjusted volatile fatty acid values were calculated by multiplying the measured acid concentration by the ratio of the required oxygen for combustion to acid molecular weight (e.g., 1.07 for acetic acid).

A 2 mL syringe was used for CH_4 and CO_2 analysis (two syringes per treatment), with sampling taken from a gas sampling tube (Lenz, Germany). The gas phase composition was analyzed with a Compact GC (Global Analyser Solutions, Breda, Netherlands), equipped with a Molsieve 5A pre-column and Porabond column (CH_4, O_2, H_2, and N_2) as well as a Rt-Q-bond pre-column and column (CO_2, N_2O, and H_2S). Concentrations of gases were determined by means of a thermal conductivity detector, with detection limits for each gas range from 0.05% v/v to 100% v/v. Anion and cation concentrations were measured using ion chromatography (Metrohm, Switzerland) using a Metrosep A Supp 5- 150/4.0 (61006520) column. Detection limits for ions ranged between 0.05 to 100 mg ion/L.

The sludge volume index was calculated based on the height of settled sludge inside the Schott bottle observed immediately prior to feeding. As 800 mL of sludge was present per liter digester, index values were adjusted for one liter of sludge.

5.3.4. Biogas estimation

From the GC results, the percentage CH_4 as part of the headspace gas composition was calculated by:

$$\%CH_4 = 100 * \frac{CH_4}{CO_2 + CH_4}$$

The volume of CH_4 produced per liter reactor at standard temperature and pressure was calculated by:

$$Volume_{CH_4} = \%CH_4 * Volume_{biogas,daily} \frac{273\ K}{301\ K}$$

The CH_4 yield was calculated from the volume of CH_4 produced per liter reactor divided by the volume of feed sludge added (L) multiplied by its VS or COD (g/L sludge) content. This results in the methane yield were related to the initial COD of the sludge (L CH_4 / g COD) or to volatile solids (L CH_4 / g VS).

$$CH_{4_{yield,VS}} = \frac{Volume_{CH_4}}{\frac{g\ VS}{L\ sludge} * Volume_{feed}} \qquad CH_{4_{yield,COD}} = \frac{Volume_{CH_4}}{\frac{g\ COD}{L\ sludge} * Volume_{feed}}$$

Based on these yield products, the annual energy and electricity production was estimated assuming a CHP electricity conversion efficiency of 40% and a methane to electricity conversion of 1 m^3 CH_4 = 10 kWh and calculating the MJ energy produced as 3.6 MJ = 1 kWh (513, 523). The electricity production is then calculated by:

$$Electricity\ yield\ \frac{KWh}{L\ sludge} = CH_{4_{yield,VS}} * \frac{10\ kWh}{1\ m^3 CH_4} * 40\%\ efficiency * \frac{g\ VS}{L\ sludge}$$

The economic sustainability was worked out from the minimal biogas production volume as outlined by Cucchiella et al. (2019) (524), and typical rainbow trout farm feed conversion yields of 1.1 – 1.36 kg Feed/kg biomass, plugged into a calculator derived from the above formulae.

5.3.5. Data analysis

Data analysis was performed in Excel and R version 4.0.3, with figures generated entirely in R. Relevant libraries used include: ggplot2 (525), ggpubr (526), dplyr (527), tidyverse (528), tidyr (529), cowplot (530), grid (531), and gridExtra (532). A paired t-test was used to confirm the significance of results wherever stated in the text, with normality and homoscedasticity determined through Shapiro–Wilk test and Bartlett's test, respectively. All significance tests were performed in Microsoft Excel.

5.4. Results

5.4.1. Yields and energy production rates

The primary focus of this study was to determine the capacity for long-term biomethane production from aquaculture solids under optimized conditions for methanogenesis. This included an inoculum from a working anaerobic digester provided the starter community, a solids retention time based on the influent carbon and nitrogen loading rates to align with literature values for maximal methanogen growth (533, 534). The temperature set point of 28°C provides a preferential environment for methanogenesis over aceto- and acidogenesis while minimizing heating costs. In this study, we evaluate the capacity of iron supplementation to additionally bolster methanogenesis of the aquaculture solids.

The yield of methane produced per volume of incoming solids is the primary indicator of this performance, here displayed in terms of volatile solids (Figure 30A) and chemical oxygen demand

(Figure 30B). The saltwater treatments performed worse than freshwater treatments, with the saltwater control lagging furthest behind ($p < .001$). With the exception of a dip around day 70, a yield in the range of 0.3 – 0.4 NL CH_4 per g VS was typical for all other treatments over the experimental duration. Taken per gram COD the yield was less, around 0.2 – 0.3 NL CH_4/ g COD, corresponding to a percentage yield of 57% - 86% of the total biomethane potential (Figure 31A, B). The percentage yield was calculated as the realized BMP as a percentage of the theoretical BMP based on the volatile solids. In other words, it is a reflection on the efficiency with which the feedstock can be converted into methane.

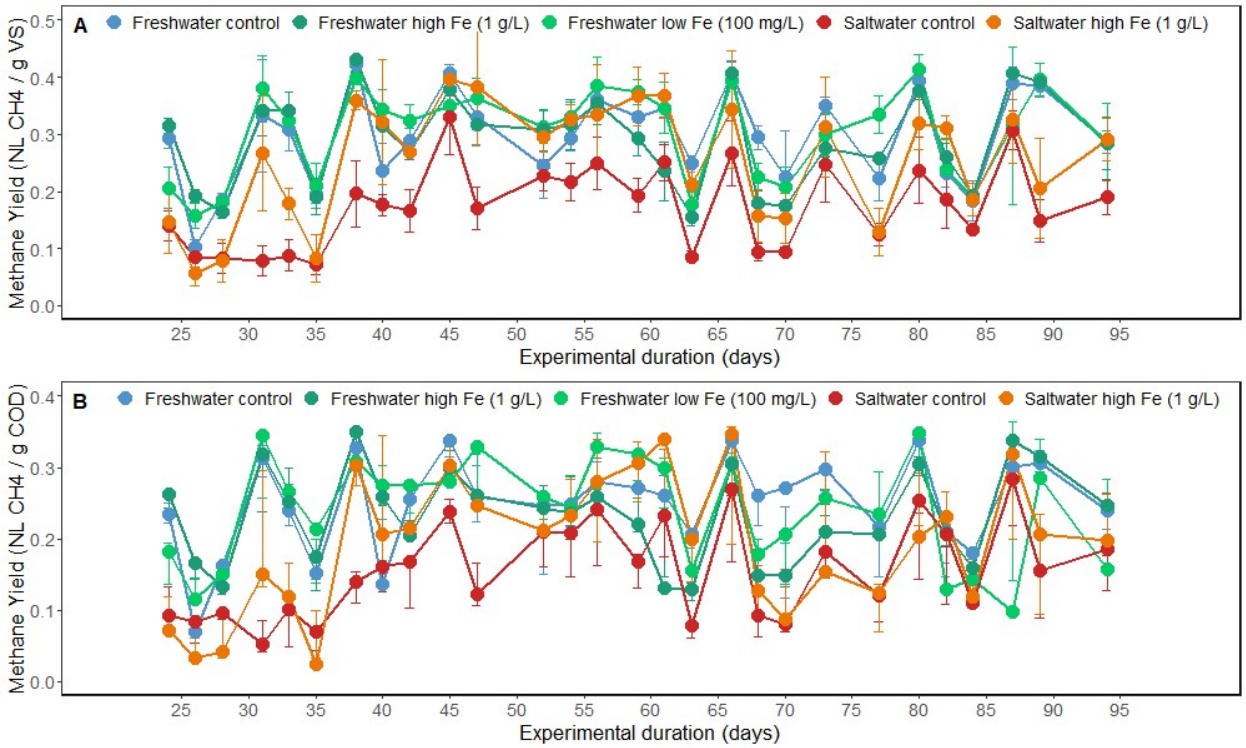


Figure 30. Methane yields per liter sludge, based on volatile solids (VS) (A) and chemical oxygen demand (COD) (B).

The percentage of methane as a component of the biogas was consistent across all treatments. Gas chromatography analyses suggest that CH_4, and CO_2 are the main gas components, with no H_2S or N_2O detected. Most treatments fluctuated between 70-80% methane purity over the duration of the experiment (Figure 31A). The saltwater control achieved a lower methane production rate than other treatments ($p < .001$). Nonetheless, rate differences across treatments favored freshwater treatments and the iron supplemented saline treatment compared to the saline control treatment ($p < .001$ for each comparison, respectively) (figure 31B).

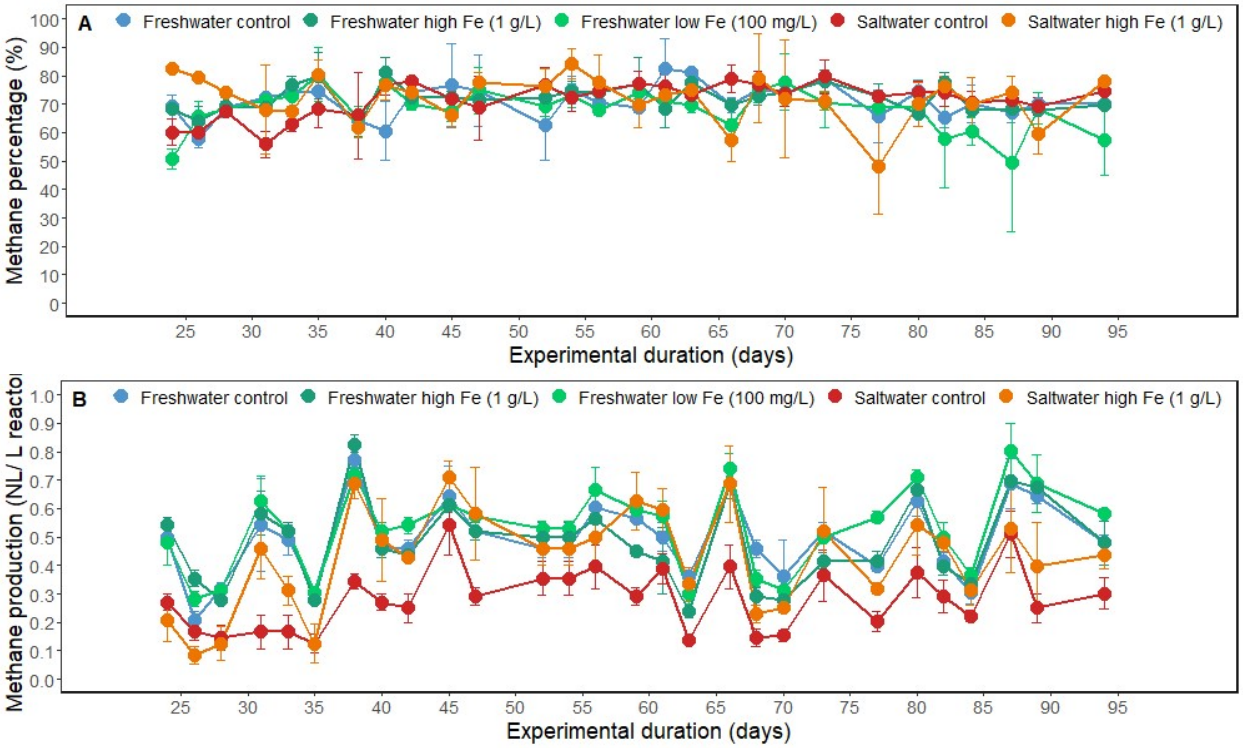


Figure 31. (A) Methane purity in the biogas across treatments. (B) Volume of methane produced across treatments, normalized per liter reactor at STP.

Methane production rates translate proportionally into electricity and energy production rates (Figure 32A, B). With the exception of the saltwater control performing worse than other treatments ($p < .001$), a range of 0.02-0.035 kWh/ L reactor of electricity and 0.05-0.12 MJ/ L reactor was typical for most of the experimental duration.

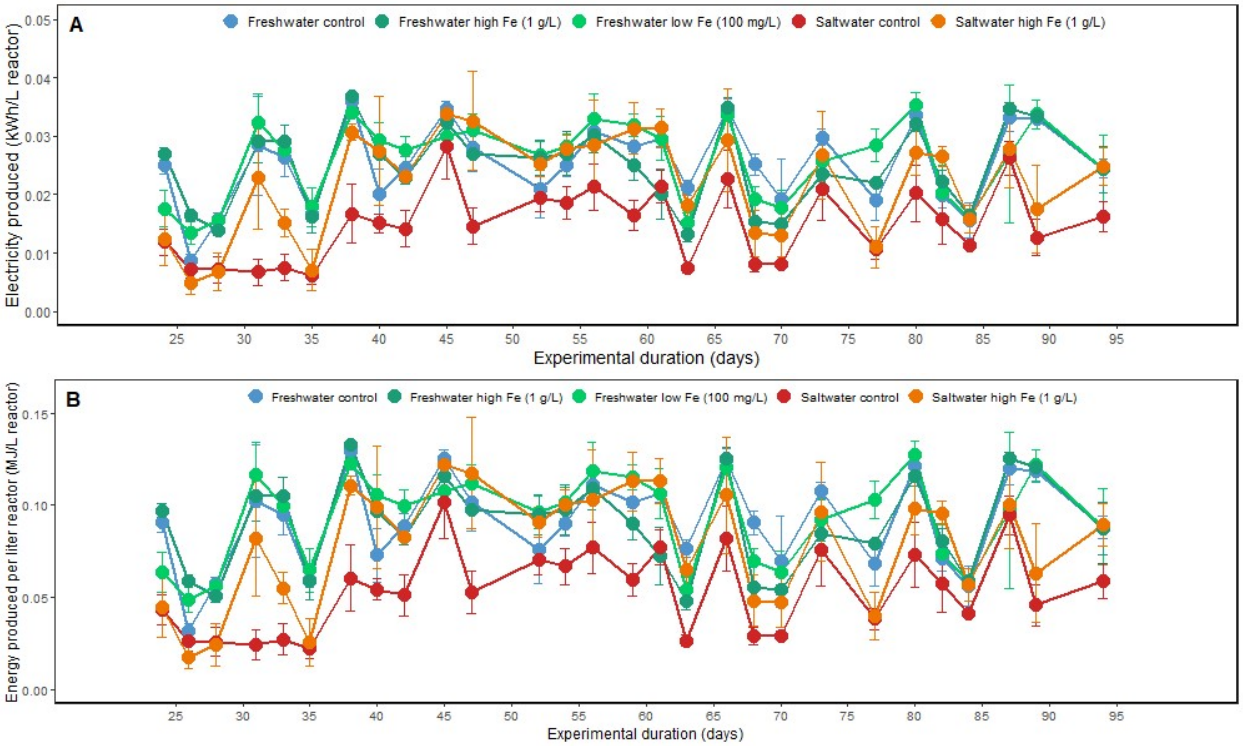


Figure 32. Estimated electricity production per liter reactor (A) and energy yield per liter reactor (B).

Backyard farms will likely not warrant investing in their biomethane potential through the purchase of a combined heat and power system. However, even small industrial scale aquaculture facilities (1 000 tons annually) could produce 88.14 kWh per day, assuming an electricity generation of 0.0275 kWh/ L reactor and a feed conversion ratio of 1.1 for rainbow trout weighing on average 3.5 kg and growing at 16° C.

5.4.2. Long-term stability of the anaerobic digester

The pH fluctuated over the duration of the study (Figure 33), however, each treatment remained within a range of ± 0.5. The freshwater treatments maintained significantly ($p < .001$) higher pH values (near pH = 7), compared to saline water treatments, suggesting the presence of an environment conducive to methanogenesis. Saltwater treatments regularly skirted along the lower tolerable range for methanogenesis (ca. pH 6.5), however, this did not result in a reduction in biogas production compared to other treatments.

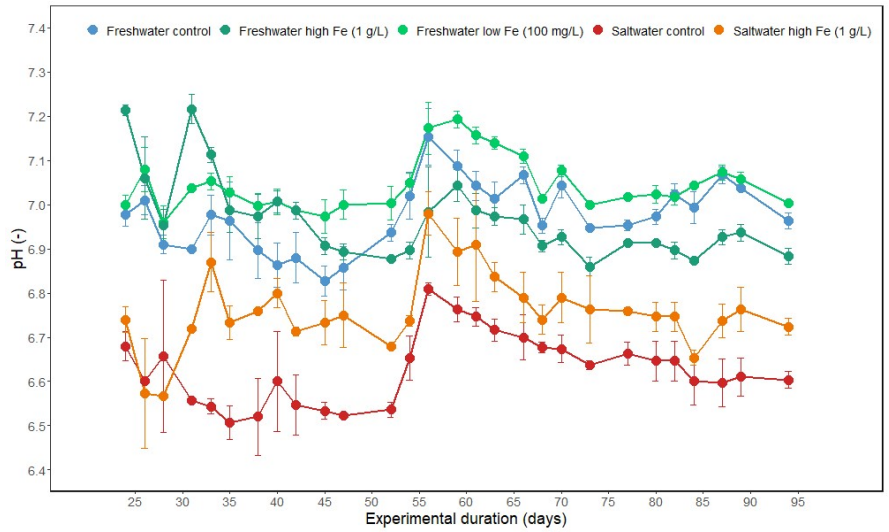


Figure 33. Consistency of pH across treatments.

The volatile fatty acid accumulation was highest in the saltwater control treatment, with longer chains (≥C3) accumulating significantly ($p < .001$) more than in other treatments. The methanogenesis efficiency was similar across treatments with minimal accumulation of VFAs > C5 (Figure 34A). Iron supplementation apparently fortified pH under saline conditions, however, the effect likely requires only low (≤ 100 mg/L) iron concentrations to meet methanogen demand, as no significant difference ($p = 0.104$) was observed between the two freshwater iron treatments. The ratio of acetate to total VFA ratio over time (figure 34B) indicates the acetate utilization efficiency by the microbial community. Despite a higher total VFA load compared to other treatments, the saline control did not deviate significantly ($p = 0.284$).

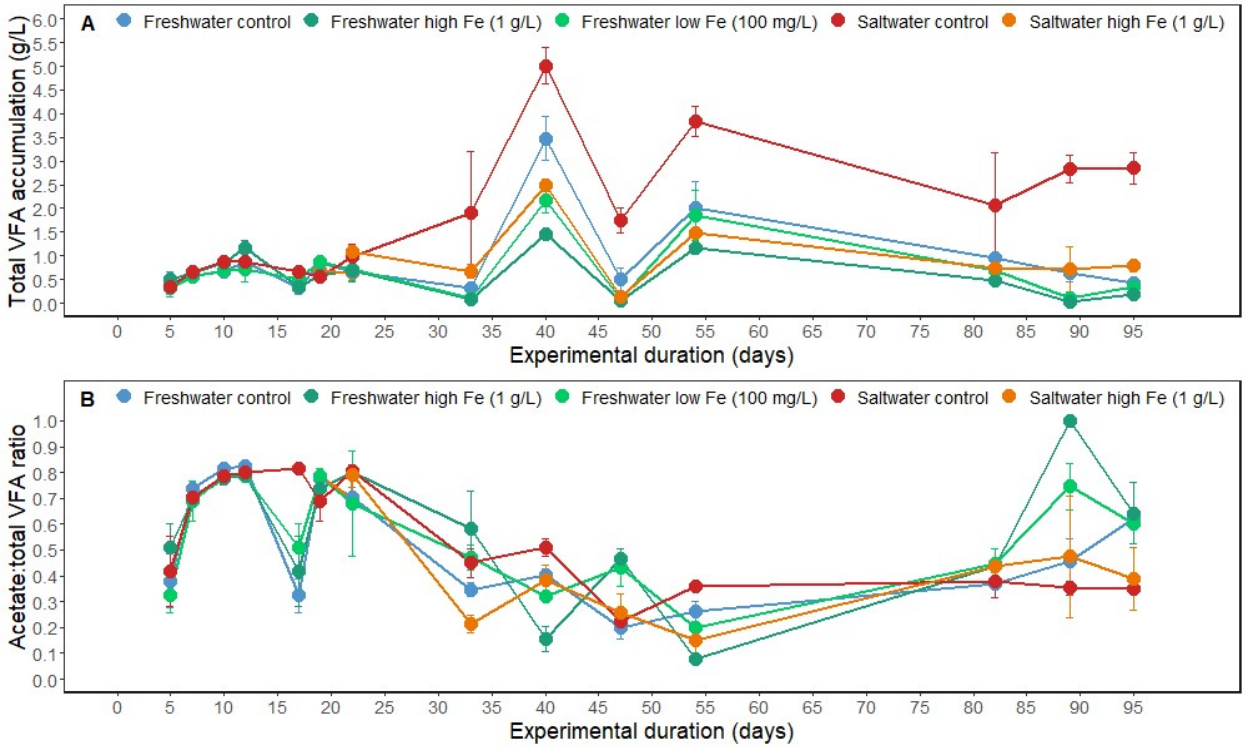


Figure 34. Total COD-adjusted volatile fatty acid accumulation over the experimental duration (A). Ratio of acetate to total VFA (B).

The IC anion and cation analyses revealed similar patterns across treatments, with the clearest distinctive factor being the presence of the sea salt mixture (Na^+, Cl^-, Mg^{2+}, Ca^{2+}). Of the nitrogenous compounds, ammonium was initially high, but decreased to a stable concentration at ca. 250- 500 mg/L after 20 days of operation. This is likely due to a shift in the feedstock composition compared to the initial inoculum. Other ions were detected at stable concentrations in the digestate for the entire duration of the experiment: sulphate 3.3 ± 2.1 mg/L, phosphate 58.5 ± 13.2 mg/L, and potassium 227.0 ± 37.1 mg/L (figure 35).

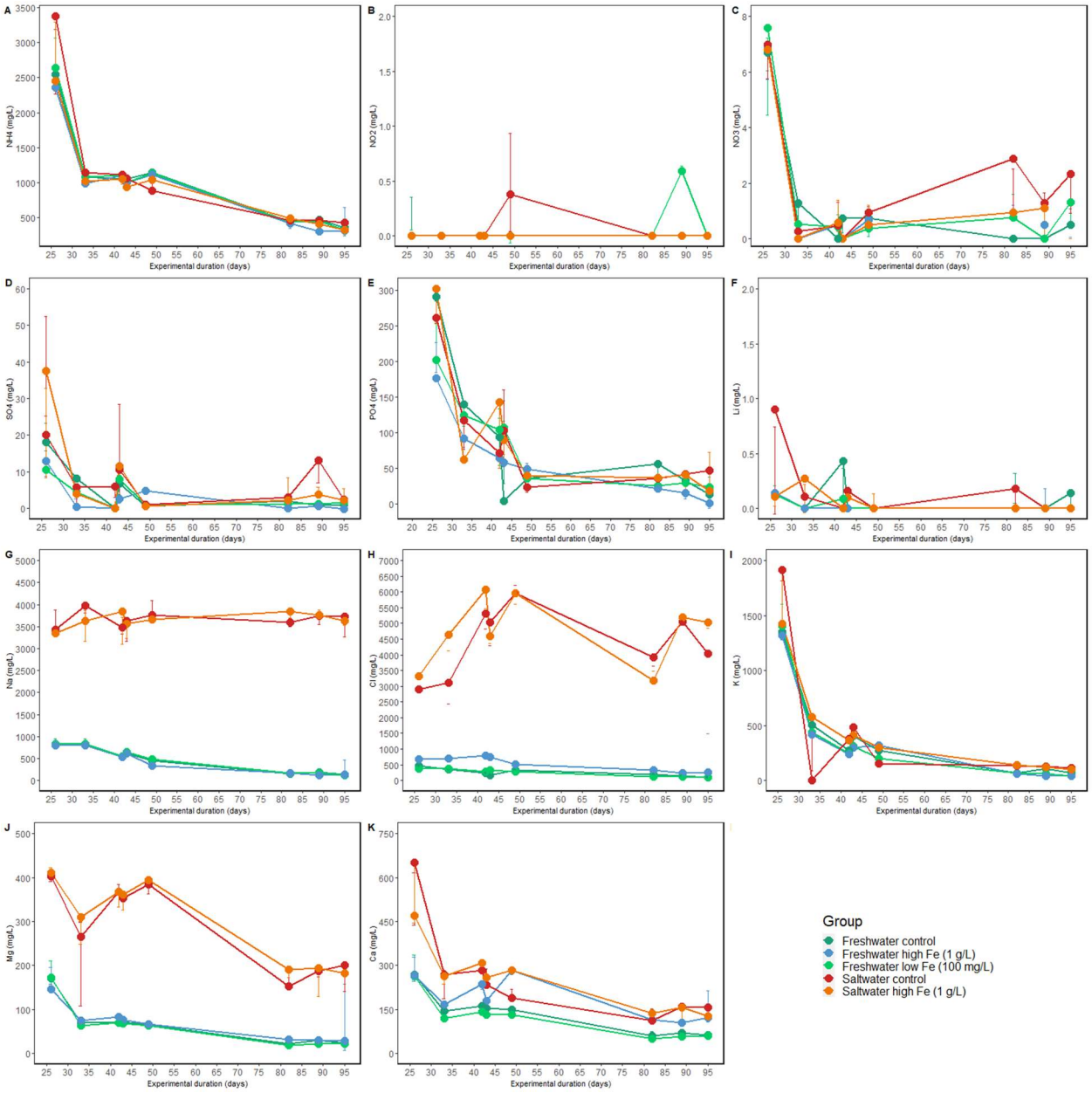


Figure 35. IC results for anions and cations measured across treatments in this study.

Total solids were highest for saline treatments (Figure 36A). Volatile solids remained similar across all treatments (Figure 36B), as similarly reflected in the TS/VS ratio (Figure 36C). Important to note is the high variability during the start-up period (days 0 – 25), which is typical in anaerobic digesters as the microbial community adapts to the increasing SRT. Total and volatile solids were taken from shaken digesters, meaning they comprised both settled and soluble particles. While a constant TS/VS ratio suggests the microbial activity was maintained at the same rate throughout the study, looking at the VS as a percentage of TS suggests that an accumulation of undigested solids occurred over time. While at the beginning of the study this value was greater than 50%, the average dropped below 50% towards the end of the study, however the high standard deviation limits our capacity to draw definitive conclusions from these data (figure 36D).

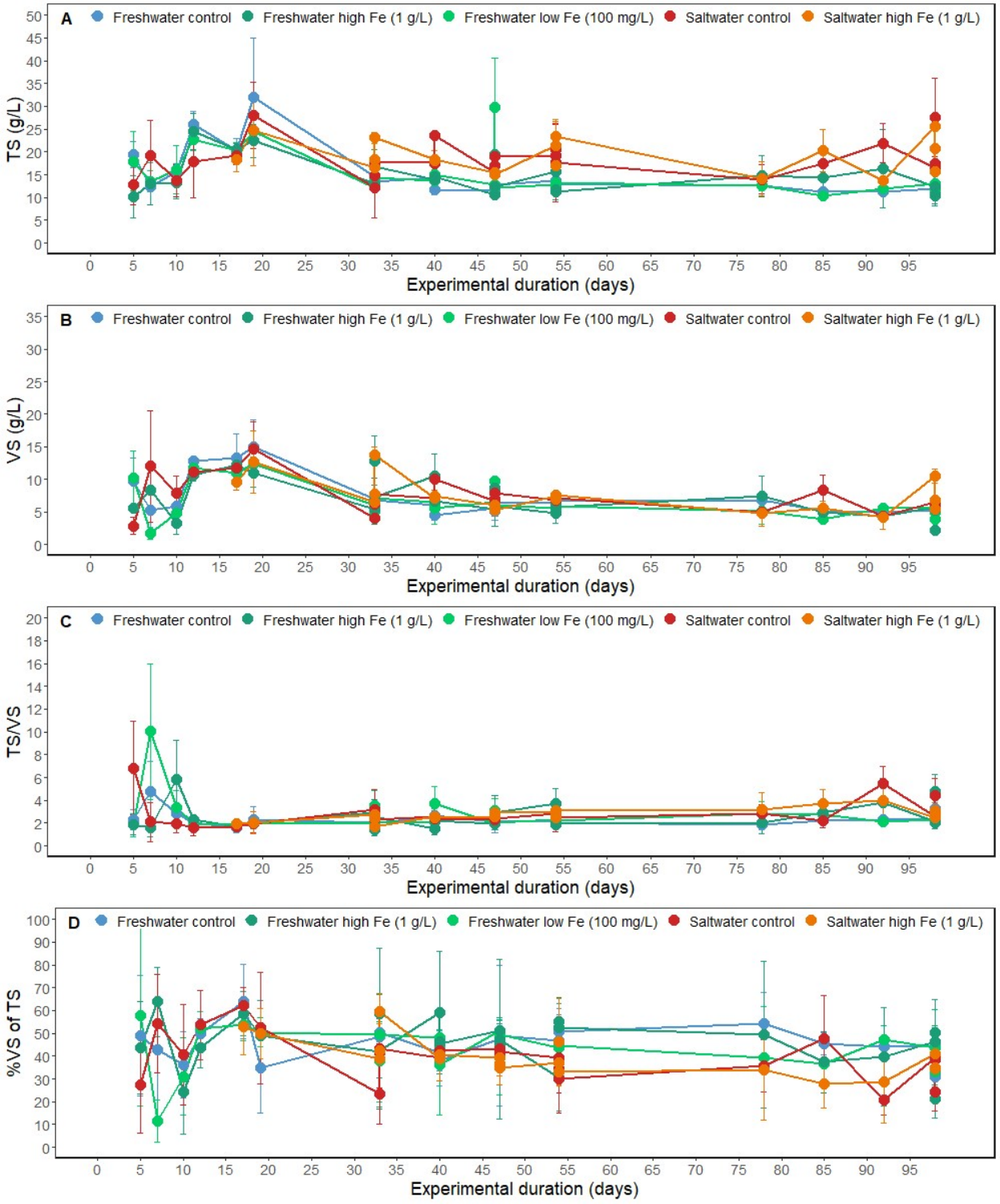


Figure 36. Evolution of total solids (TS) (A), volatile solids (VS) (B), as well as their ratio (C) over time, and (D) the percentage of VS as a portion of TS.

While the sludge volume index (SVI) was only measured during the last stage of experimental period, the divergences were consistent across 25 days of observation (Figure 37). It was hypothesized that a higher SVI would be observed in saline treatments owing to the higher ionic stabilization of the solids

and floc and decreased microbial activity, however this was not the case (p=.132 between freshwater and saline controls). While low iron supplementation significantly reduced the SVI (p<.001 between the 100 mg/L iron treatment and the control), it did not appear to reduce the SVI significantly at the higher concentration (1000 mg/L Fe addition) under freshwater (p=.367) nor saline conditions (p=.063).

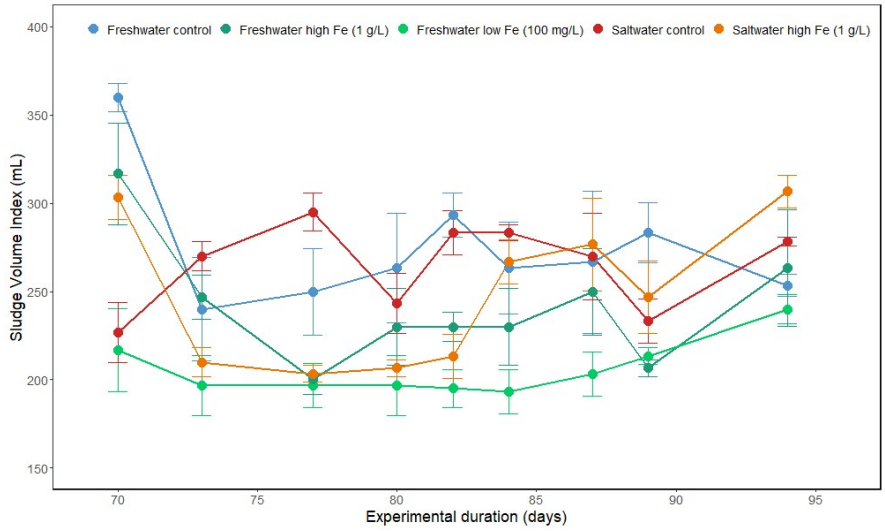


Figure 37. Sludge volume index measurements across treatments.

5.5. Discussion

5.5.1. Both saline and freshwater anaerobic digestion of solid aquaculture waste results in stable biogas production

In this study, we demonstrated the feasibility for long-term biogas production in simulated freshwater and saltwater anaerobic digestion systems using rainbow trout solids as the feedstock. The novelty of the approach in this study is its ability to incentivize responsible solids management through the potential for electricity generation, applicable in any aquaculture farm where fish solids are selectively removed from the water column. Using pH as the most immediate signal for reactor health, it is evident that freshwater conditions were less stressful on the methanogenic community, which is further supported by the volatile fatty acid profile in which fewer C3 and longer VFAs are present. This suggests that under saline conditions, the methanogenic community was partially inhibited from converting acetate (C2) into methane. The consistency of volatile solids measurements indicated a similar amount of organic matter across treatments, indicative of the metabolically active fraction of the microbial community. With the exception of some outliers, the TS:VS ratio remained between 1.5 – 3, resulting in a %VS of TS between 40-50%. As the %VS relates to the degree of microbial activity in the digester, it is worth noting the consistency of these results with municipal wastewater treatment systems (534, 535). Furthermore, while regular variations were noticeable across the experimental duration, these trends affected all treatments simultaneously. The fed-batch model used in this study can create a feast-famine alternation across the three days between each feeding, possibly explaining the observed fluctuations.

5.5.2. The contribution of biogas to the economic and sustainable picture depends on the scale of the aquaculture farm

One of the key goals of this work was to gauge the practicality of biogas collection for aquaculture farms. The lab-scale reactor experiments performed in this study enable the estimation of the electrical and heat potential from fish solids. While previous studies on the biomethane potential (BMP) from saline aquaculture solids achieved similar yields as observed here (0.279-0.3 NL/g VS compared to 0.2-0.4 NL/g VS in this study) (536, 537), the novelty added through the current investigation is in determining the long-term stability of the anaerobic digestion microbial community and potential energy yield. Methane yields fluctuated for each treatment ranged generally between 0.2-0.4 L CH_4/ kg VS with a variation of ≈ 0.2 NL CH_4 / g VS. These yields are comparable to other agricultural waste streams, such as cow or sheep manure (538). Importantly, methane purity was generally higher than literature values: 60-80% CH_4 in this study, compared to 65% for cow manure (538) and 60% previously reported for freshwater aquaculture solids (539). We attribute this observation to a few factors: a relatively optimized anaerobic digestion design compared to previous studies on BMP generation from aquaculture solids (temperature, pH, iron addition, the use of the inoculum from a BMP anaerobic digester, ideal retention time and volumes based on feedstock characteristics) and as well a homogenous, nitrogen-rich feedstock lacking inhibitive products (as may occur in wastewater treatment). Considering that modern CHP systems run at around 40% electrical efficiency and around 45% heat efficiency (513, 523), these results suggest that establishing a biogas-generating waste treatment system could address operational and maintenance costs of an aquaculture facility (540).

The reduced biogas volume produced by the saline control treatment emphasizes the importance of iron supplementation, at least for saline aquaculture systems. Methanogens use iron as an electronic shuttle, allowing them to prevent interference from the high environmental ionic load created by the saline environment. Typically, iron-based coagulants result in a denser sludge than other common coagulants (*e.g.*, aluminum) (541), corroborated by the sludge volume index results observed in this study. One study investigating the use of inorganic coagulants ($FeCl_3$ and polymeric aluminum sulfate) for pretreatment prior to BMP from brackish aquaculture solids found an improved yield in the iron but not aluminum treatments (542). Aluminum exposure has likewise been associated an increased risk of Alzheimer's disease, limiting the downstream applications for aluminum-treated solids (543, 544). As such, $FeCl_3$ is both a safer and more effective coagulant to augment sludge density and settling efficiency.

Investing in the CHP system represents a critical consideration vis à vis the return-on-investment for aquaculture facilities. For large facilities, solids disposal is as great a concern as is reducing operational costs such as electricity and heat. The process of collecting biomethane from organic waste has become widespread over the past two decades for large-scale agricultural and intensive animal husbandry facilities within the European Union (516, 545), with biogas deployment schemes elsewhere around the world growing at a slower rate (546). A recent case study in Italy determined that for a biogas plant to be profitable, a minimum production level of 200 kWh is necessary (524). The US energy market is significantly more privatized and as such, there are wider price fluctuations both geographically and temporarily. Recent federal incentives for biogas (US Federal Energy Regulatory Commission Order 2222 (FERC-2222)) make the operation more attractive, especially for large facilities (547). The ability to accumulate biogas before combustion could allow facilities to time their electricity generation with peak demand hours, however the economics of this process will need to be worked out for a given operation.

Naturally, further capital investments and technical innovation in the sector have the potential to make biogas production at lower volumes more profitable in the near future (514). To produce 200 kWh daily, we estimate needing a rainbow trout farm size producing 42 T annually based on the electricity yields and methane production rates from this study. For smaller aquaculture farms (50 - 150 kWh; equivalently 10 - 32 T production), the advent of smaller scale electricity generation units, such as micro combined heat and power (mCHP) systems, may provide a more practical solutions (548, 549). The smallest of facilities may opt for makeshift options, such as the Mini Methane Generator Project (550). Ultimately, most energy in CHP goes towards heat generation, with possible outputs including pressurized steam, hot air, and hot water.

Aquaculture solids contain a majority fraction of mineral nutrients compared to those dissolved in the water column (41). While optimizing the remineralization of nutrients was not a priority in this study, several trends could be observed. Firstly, virtually all nitrogenous species were reduced to ammonia. In the reducing environment of the anaerobic system, proteinic nitrogen is liberated during the decomposition of organic matter. Ammonium toxicity would not be a concern as the pH never exceeded 8, however ammonia concentrations in the digester were low (stabilizing around 500 mg/L reactor) compared to other anaerobic digestion feedstocks (551). Simultaneously, mineral nutrients are liberated from the colloidal matrices within the aquaculture solids. While this was outside the scope of this study, there is clearly a possibility for further treatment (*i.e.*, aeration of the reactor digestate), which could allow the effluent stream to be applied to hydroponic plant cultivation as we have investigated previously (186).

One element of concern in anaerobic digestion is sulfur, due to its propensity to form a noxious gas. In our study, H_2S was not detected in the reactor headspace nor were sulfate concentrations in the digestate exceptionally high (remaining below 10 mg/L reactor). Sulfate levels were higher in the saline treatments owing to the contribution of the Instant Ocean salt mix. Previous studies claimed that the use of an inoculum derived from anaerobic processes improved methane yield while reducing H_2S production when added to aquaculture solids (552), however, our results suggest that the H_2S is instead precipitated chemically by cations in the digestate. No significant difference was detected in the soluble sulfate concentrations between iron versus control treatments. Future work on the solids treatment system described in this study will need to review the potential for H_2S production, as there is evidence from the literature that it is likely produced as a byproduct during this process (553, 554).

5.5.3. Iron addition stabilizes biogas production under saline conditions

High salinity typical to full strength seawater (35 g/L) has been previously claimed to be a cause of low methane yields from aquaculture solids (555). While full-strength seawater was not investigated in this study, our results demonstrate that methane production from saline water at 12 g/L is similar to freshwater yields. Saline anaerobic digestion of the aquaculture solids remains stable well after the effect of the inoculum would have diminished, suggesting that the methanogenic community of the inoculum successfully colonized the new digester conditions. Crucially, the addition of iron to the saltwater sludge seems to have alleviated salt stress compared to the control, as evidenced by the pH stability. Under freshwater conditions, low iron supplementation (100 mg/L) as well as high (1000 mg/L) iron supplementation significantly improved methane yield compared to the freshwater control ($p<.001$ for each comparison, respectively). However, methane production rates were not higher in the iron supplemented freshwater sludge at low ($p =.195$) nor high ($p =.790$) concentrations.

Although it was hypothesized that the addition of iron would help coagulate the aquaculture solids under saline conditions, the total solids concentration was not significantly lower in the iron-supplemented saline treatment compared to the saltwater control (p=.995), nor was the sludge volume index significantly decreased (p=.063). However, the saline control treatment yielded significantly (p <.001) less methane than the iron supplemented saline treatment. These discrepancies are visible in the volume of biogas produced (production rate), although they are not reflected in the percentage of methane in the biogas (p=.422 between saltwater control and iron-supplemented saltwater treatments). Biogas purity was likewise similar between freshwater and saltwater control treatments (p =.481), suggesting that the methanogenic community was able to maintain its niche under the higher ionic conditions. These observations suggest that the salinity of the sludge does not exclude its use for biogas production. A stressed methanogenic community in the saline control treatment was likewise portrayed through the volatile fatty acid profile. While other treatments did not have a significant accumulation of VFAs longer than C3, the saltwater control had consistently higher VFA concentrations up to C8. The fact that this backlog was alleviated through iron supplementation further lends credence to the notion that iron may improve the resilience of the methanogen community to exogenous perturbations. A previous study investigating the use of $FeCl_3$ as a coagulant for a brackish aquaculture solids digestion system indicated an inhibitory effect when using 6 g/L $FeCl_3$ (542) – a concentration much higher than those used in this study. This discrepancy might explain why our study did not observe any inhibitory effect. Rather, we encourage further research to explore lower $FeCl_3$ concentrations to determine the minimal effective concentration (*i.e.*, whether enough iron should be present to satisfy the biological demand of methanogens or is a higher concentration needed to chemical precipitate inhibitory elements such as sulfur).

5.5.4. Limitations and future outlook

The use of biological replicates allows for a better investigation into the variability of the microbial communities as they adapt to the feedstock. The biogas yield and production calculations incorporate measurable inputs from all three biological replicates with the result that the standard deviations in this study were wide. Biological variation is always present; however, we believe that many of these variations will be resolved at larger scales as aquaculture solids entering in an active facility – solids entering the treatment system – will be consistently fresh, and an automated pumping system will regularize the handling process. The effect of variable temperature – even if the digester itself is maintained at 28°C – may create seasonal variations in the microbial community as described elsewhere (556, 557). Nonetheless, trends are visible for treatment groups (freshwater vs. saltwater) in a way consistent across all parameters. To simulate a saline sludge input, Instant Ocean was added to the incoming aquaculture solids to achieve a concentration of 12 g/L. However, this resulted in the digester reaching a sodium concentration of between 3.5 and 4 g/L (figure 35) over the course of the experiment due to the low volume exchange rate. While biogas yield was similar for the freshwater and brackish water treatments in this study, the effect of salinity will require further study. Namely, the digestate salinity should be raised to higher concentrations (e.g., 6, 12, and 35 g/L) to map out the influence in BMP. Section 4.2 describes the complex interplay between sulfur, biomethane production, and iron requiring further research - especially under saline conditions where sulfur concentrations are considerably higher.

Bringing this experiment to the next technology readiness level will require pilot and industrial scale studies, as well as measurements over a longer period. There are several tiers of aquaculture facilities as

described in section 4.2, and, likely, the profitability model differs tremendously based on size and usage (aquaculture vs. aquaponic farms).

5.6. Conclusion

The investigation into the biogas potential from aquaculture solids reveals promising results both in terms of biogas yields and long-term process stability. Advantages of this approach are the low operational costs, the stability of the biogas production, and the possibility to recuperate investment/operational costs through electricity generation. Globally, this study indicates a high consistency in biomethane composition (%CH_4) across treatments, suggesting that while the methanogenic community may be suppressed under saline conditions or in the absence of sufficient iron, it is not outcompeted. Iron supplementation was found to be useful under saline, but not freshwater conditions. However, the effect appears to improve the rate of methane production, but not the yield. Hence, it is possible to change the paradigm of waste treatment from a costly burden into a cost-alleviating activity with direct implications for industrial stakeholders in aquaculture.

5.7. Contextualization in the thesis

This study presented for the first time an in-depth investigation into the possibility for biogas production from fish solids under optimized, yet industrially relevant conditions, including inoculum addition, iron supplementation, a conscientious targeted hydraulic retention time, and VFA analysis. This deeper level of analysis sets this study apart from its predecessors in the aquaculture field (539, 558, 559). Beyond demonstrating the feasibility of biomethane production, this study laid out a framework for the implementation of such as system, including a preliminary economic analysis. As such, chapters 4 and 5 describe a new direction for solids treatment – one in which the most expensive waste product from aquaculture facilities is revalorized as a liquid fertilizer and energy generation platform. While the biomethane potential is not such that a farm will likely be supplying electricity to the local power grid, it can be sufficient to partially cover on-site heating and CO_2 requirements (if greenhouse production is present).

6. Thesis Discussion

This thesis presents an in-depth analysis of the flow of microorganisms and nutrients in aquaponic systems, with an applied focus on revalorization of fish solids into economically interesting outputs. it is the goal of this to work to provide scenarios win which the investment costs implicit in developing waste treatment infrastructure may be offset through revalorized products (biogas, liquid fertilizer). In the following sections, these processes will be reviewed individually with the goal of tying together the main themes of this dissertation.

6.1. Opportunities to direct microbial communities to augment nutrient transfer

The flow of microbial communities is important in terms of determining the potential for specific metabolic lifestyles. As demonstrated in chapter 2, microbial presence does not guarantee colonization – it merely allows for those microorganisms to occupy open niches. The two mechanisms of microbial community assembly are referred to as stochastic (*e.g.*, dispersal) and deterministic (*e.g.*, selection) processes (560). In all aquaponic facilities, water from aquaculture tanks flows into hydroponic beds, although the final percentage may vary from 30 - 100% of the flow. A return flow exists in coupled aquaponic systems only. While stochastic forces ensure that the hydroponics bed is constantly bathed in microbial immigration, deterministic forces appear to restrict microorganisms to well-defined niches. Thus, with respect to nutrient transfer, two aspects of the global architecture that can be manipulated are compartmentalization (niche creation) and maturation.

6.1.1. Compartmentalization to promote specialization

Similar to wastewater treatment systems, compartmentalization allows for separate microbial communities to form. Recirculating aquaculture systems already exploit the compartmentalization through the use of biofilters for ammonia neutralization. Based on chapter 5, nutrient remineralization requires at least three compartments to allow for both biomethane production and nutrient remineralization, which is a more complex system than the basic aerobic digestion systems commonly employed in small-scale aquaponic farms. A liter of aquaculture solids in our study contained 56.63 ± 1.14 g COD kg^{-1} FW and 8.90 ± 0.38 g N kg^{-1} FW (table 9) which is lower than reported in literature (336 g COD kg^{-1} FW, 35.2 g N kg^{-1} FW, 27.6 g P kg^{-1} FW (41)), likely due to discrepancies in the sample collection strategy. In aerobic digestion, heterotrophic bacteria are dominant. Their respiration removes the majority of carbon from the system – around 50%. Typically, aerobic digestion require C:N ratios in the 25:1 to 35:1 by weight range (561). In our study, this ratio was around 6:1, and in the literature reference around 9:1. Under these conditions, it is expected that as the high proteinic fraction of the solids decomposes, large amounts of ammonia will be released which can pose a health risk at larger scales. Considering additional costs related to air supply, mixing requirements, and sediment removal, aerobic digestion was concluded to likely lack economic viability at industrial scales.

Anaerobic digestion on the other hand, has the potential for biomethane production and nutrient remineralization as we described in chapters 4 and 5. With an emphasis on economic sustainability, our system was constructed with a minimal number of compartments. Prior to anaerobic digestion, a settling basin greatly increases the sludge density. In our study, solids were collected directly from the drum filter, resulting in a lower density – and consequently less biomethane production per liter sludge. The anaerobic digestion process itself can be optimized in future, although there is likely a system-specific cost-benefit tradeoff between improving the biological efficiency of the process and refraining from adding additional infrastructure (reducing the economic viability of the system).

6.1.2. Long-term resilience through maturation

The second deterministic driver that may augment the efficiency of nutrient transfer is maturation. Specifically, the nurture of slow growing organisms has a disproportionate impact on biochemical processes. Keystone microbial taxa often play this role through their exceptionally high connectivity to other taxa, making them crucial to the stability and resilience of microbial environments (562). Similar to multicellular keystone organisms, these microbial taxa either perform key functions themselves or orchestrate functions that stabilize the facility environment. For example, archaea were long thought to be inconsequential to biochemical flows, due to their relatively low abundance in aquatic systems (2-5% of total nucleic acids (563)). More recently, the role of archaea in nitrogen metabolism has been firmly established (564-571). This role is significant enough to attribute archaea as a global driver of oceanic carbon cycling (carbon fixation/ methane production) and nitrogen cycling (ammonia oxidation, reduction of N_2O to N_2) (572-574). Practically, keystone taxa may be identified through bioinformatic techniques previously shown to be effective in the analysis of environmental aquatic samples and aquaculture-specific samples (562, 575).

Microorganisms that stimulate or enrich beneficial commensal communities may be considered probiotic strains. A probiotic may play an auxiliary role in promoting the growth or resilience of a keystone taxon, or themselves be represented as keystone taxa in a specific system. One study identified a strain of *Lactobacillus paracasei* as a keystone species in the human gut, due to its capacity to produce certain extracellular enzymes important in the degradation of inulin (576). Prebiotics, substances which are not directly digested by the host organism but rather stimulate the symbiotic microbial community, likely also play an important role in the stability of the keystone species. Conversely, keystone pathogens exist, with *Porphyromonas gingivalis*, a keystone bacterium in the development of periodontal disease being a well-studied example (577, 578). Examples such as the *Lactobacilli* and *P. gingivalis* keystone taxa/probiotics suggest that the concept of "keystone" on a microbial level may be described as the initiators or drivers of community-driving metabolic pathways. The outcomes of such pathways may be metabolites useful for other bacteria beneficial to the host, or metabolites which lead to further dysbiosis and disease. While the results of chapter 2 suggest that probiotic addition with the intention of impacting the plants must consider plant-specific preferences, the broader utilization of probiotics remains enticing and worth further study.

Herren and McMahon (2018) identified 33 keystone taxa out of 7,081 microorganisms from large-scale metagenomic datasets (562). Similar strategies were used in clinical settings to identify keystone taxa of the chicken GI tract (579). Importantly, these strategies rely on a combined phylogenetic and taxonomic assignment tool (TaxAss), which bears similarities to other equally advanced techniques, such as the Microbial Database for Activated Sludge (MiDAS), a manually curated repository for activated sludge communities established by researchers at Aalborg University, and, thus, also to AutoTax, the tool supplying the initial automated assignment in this database (580, 581). The only keystone analysis to-date on an aquatic system was done by Herren and McMahon in 2018 (562). No such study has been carried out on commercial aquaculture or aquaponic facilities, despite empirically observed stability arising in facilities operating over long periods of time (cf. biofilters from the aquaculture department in Wageningen University and Research operating continuously since the 1970's, Lethbridge College aquaponics facility operating continuously for 20+ years; personal communications with Ep Eding and Nicholas Savidov, respectively). Despite these observations, the overlap between keystone species and probiotics is poorly understood. One path forward may be to compare lists of common/popular

probiotics, previous microbial taxonomic studies (34, 582), common databases (GenBank, MAR databases, etc.) alongside collections of keystone species from aquaculture or aquaponic facilities. The diversity of tools described in chapter 3 highlights efforts being done to address this gap. As covered in the chapter, practical biases in the treatment of samples and data must be addressed when targeting keystone taxa. Returning to the archaea example, differences in the 16S rRNA genes between bacteria and archaea contribute to a poor coverage of archaeal communities in many metagenomic studies (583, 584). Future studies will need to be aware of these pitfalls in order to elucidate the practical impact of keystone taxa for aquaculture and aquaponic facilities.

6.1.3. Two paths for nitrogen treatment

The ultimate goal of processing anaerobic digestate is dependent on the intended use of the subsequent nitrogen. Ammonia and nitrate forms of nitrogen are crucial for plant growth; however, discharging them directly into the environment may lead to eutrophication of local water bodies. Therefore, two relevant scenarios to consider in this system are an effluent rich in soluble nitrogen and an effluent with minimal nitrogen concentrations.

If the objective of the treatment system is to increase the effluent's soluble nitrogen content for hydroponic plant cultivation, then a nitrifying reactor is likely the most suitable option. During the anaerobic digestion process, the readily bioavailable carbon is mostly consumed, resulting in the digestate consisting mainly of non-readily bioavailable carbon. Consequently, the bioavailable carbon-to-nitrogen ratio is quite low. With a short hydraulic retention time and an aerating environment, we hypothesize that the resulting effluent can serve as a nitrate-rich liquid fertilizer. Chapter 4 presented a preliminary assessment of the quality of such a liquid fertilizer, with much room for process optimization. However, some fraction of the solids will precipitate, and empirical data are needed to predict whether this affects the system's ability to solubilize mineral nutrients locked in the fish solids. Further research is necessary to characterize the effluent when solids treatment operates under optimized conditions. At a minimum, measuring the nutrient composition of the input sludge, precipitated solids, and plant-relevant nutrients will enable better evaluation of the economic potential of such a fertilizer production system.

Conversely to aquaponic facilities, aquaculture facilities will prefer a waste stream devoid of nitrogen as their goal is to discharge the effluent or return it to the main recirculation loop. Previous studies have shown that a C:N ratio of 3-5 is sufficient to support the metabolism of heterotrophic denitrifiers (585, 586). While processing anaerobic digestate from aquaculture solids has not yet been explored, a cyclical nitritation – denitritation reactor was used by Peng et al. (2017) to treat anaerobic digestate from piggery slurry, emulating the C:N ratio of the digestate from fish solids (587). These studies demonstrate a promising horizon for complete neutralization of the anaerobic digestate from aquaculture facilities. This strategy would be especially useful for saltwater RAS, which cannot use their effluent for plant cultivation, yet would negatively impact the local environment were they to discharge a high-nitrogen effluent. With the potential of significant biomethane production from large aquaculture facilities, this could provide a realistic incentive for companies to treat their waste stream.

6.1.4. The value of phosphorus capture

Phosphorous is another nutrient that may be specifically manipulated through a compartmentalized microbial community. Enhanced biological phosphorus removal (EBPR) allows the biological treatment of soluble waste to be expanded to insoluble particles, as developed for P removal in activated sludge

systems at wastewater treatment plants (588). The EBPR has been shown to cheaply and efficiently handle feedstocks typical for municipal waste low in readily bioavailable carbon (88, 446, 448-452). The EBPR process is centered around the cultivation of phosphate accumulating organisms (PAOs) – heterotrophic bacteria capable of accumulating phosphorus in excess of their own metabolic requirements. By definition, PAOs are not grouped into a taxonomically succinct clade, but are instead different types of bacteria that have developed mutations in the *pho* regulon network allowing for their unique lifestyle (589, 590), although the *pho* regulon itself is considered a master regulator for many bacteria as it directly regulates N metabolism and some aspects of C metabolism (589).

Exploiting this important metabolic node has the potential to improve fish sludge treatment, however, operating a PAO enriching sequential batch reactor over a 34 day period did not result in a stable reactor (figure 38). In the experiment, aquaculture solids were added as the feed source with acetate measured as a proxy for bioavailable SCFAs for the PAOs. An inoculum was derived from granular sludge and mainstream sludge derived from a local wastewater treatment plant (West Point Treatment Plant, Seattle, USA). Initially, PAO feed was provided by a synthetic solution of 185 mg/L acetate-COD, 40 mg/L NH4-N and 20 mg/L PO4-P. Based on rough estimates for equivalent COD provided by Marques et al. (2019) (454), an additional 0.53 g/L of casein hydrolysate was supplemented. After two weeks, 80 L of fish solids were collected from a nearby freshwater aquaculture facility growing rainbow trout (Riverence, USA). These solids were stored in a large container at room temperature over the duration of the experiment (1 month), and the amount fed to the SBR was calculated based on targeting similar acetate-COD and NH4-N concentrations as were present in the sythnetic solution. The pH was set to 7.5, with automatic regulation controlled by the addition of either 1 M NaOH or 1 M HCl. Aeration was controlled by a gas pump recirculating compressed air. Maximal dissolved oxygen (DO) was set to 2.0 mg/L during the aerobic phase; N2 gas was used to achieve a DO of 0 for the anaerobic phase. It is visible that the readily bioavailable carbon was being consumed (figure 38A). Ammonia was partially taken up in the anaerobic phase and then largely oxidized into nitrate, some of which was released in the effluent (figure 38C, D). As seen in figure 38B, there was no measurable decrease of phosphate over the course of the SBR cycle. It was later determined through metabarcoding that the PAO community diminished over time, likely due to the presence of other carbon sources acting as a substrate for their competitors. For reference, a schematic of the expected increase and decrease for relevant SBR parameters is depicted in figure 38F.

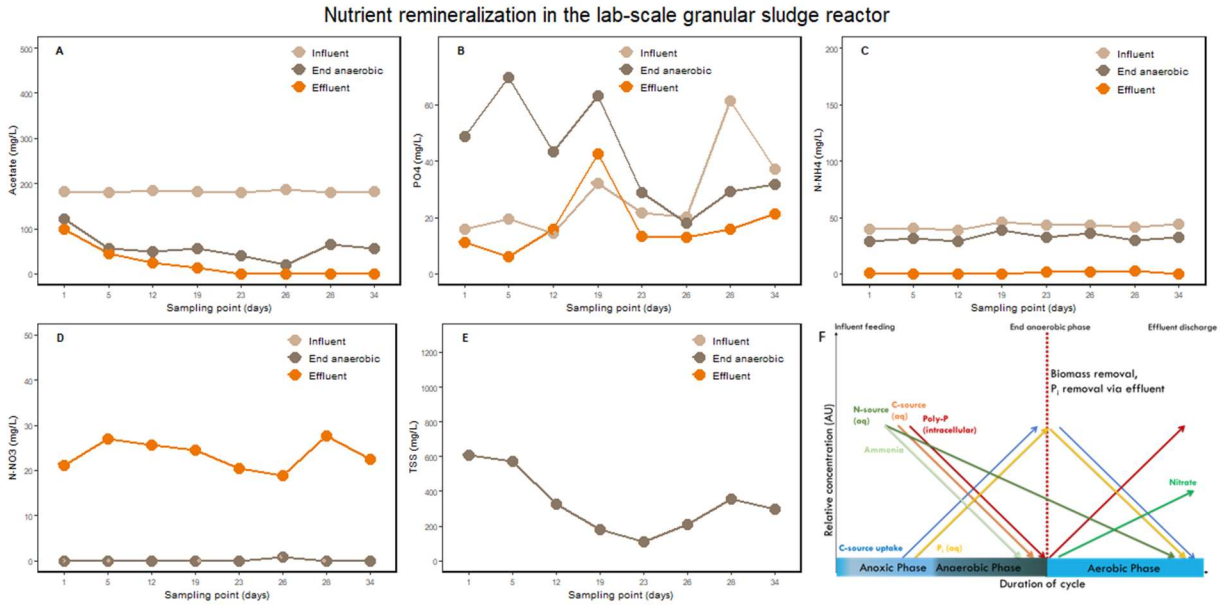


Figure 38. Operational parameters of the PAO-SBR fed aquaculture solids without prior anaerobic digestion demonstrating fluctuations of the A) feed (acetate), B) phosphate, C) Total Kjeldahl nitrogen, D) ammonia, and E) nitrate. Graph F displays the theoretical fluctuations expected over an SBR cycle.

The straight-forward explanation for the failure of the aquaculture-fed EBPR comes down to the C:N:P ratio. The target COD concentration in artificial PAO nutrient solutions is usually around 400 mg/L split between acetate and propionate, ammonia is 40 mg/L, and the phosphate concentration is 20 mg/L (ratio of 10:1:0.5). Superficially, the aquaculture solids appear to match this ratio well (ratio of 10:1:0.8, from (41)), although practically there is still a considerable fraction of carbon that is not readily bioavailable. This led to excessive growth of heterotrophic bacteria and eventually compromising the PAO granules. Were this cultivation strategy repeated, it should be done using the anaerobic digestate as the feedstock. Potentially, the pH of the anaerobic digester may be lowered to discourage biomethane production, increasing the preferred substrate for PAOs - short chain fatty acids. This strategy would need to be fine-tuned to balance these benefits against the resulting preferential environment for glycogen accumulating organisms, which would be in competition with PAOs (591).

As seen by acidifying the aquaculture sludge matrix (figure 39), most of the phosphorus is not naturally soluble at a neutral pH. Phosphorus solubility is generally not affected by chemical, mechanical, thermal, and biological pretreatment prior to anaerobic fermentation (112). A large farm releasing these solids into the environment would be carrying with it a constant stream of 3 g/L phosphorus - greatly exceed background concentrations. Addressing the phosphorus question is essential for the expanding aquaculture industry from the perspective of pollution mitigation as well as for the aquaponics industry from the perspective of rendering inaccessible nutrients more bioavailable.

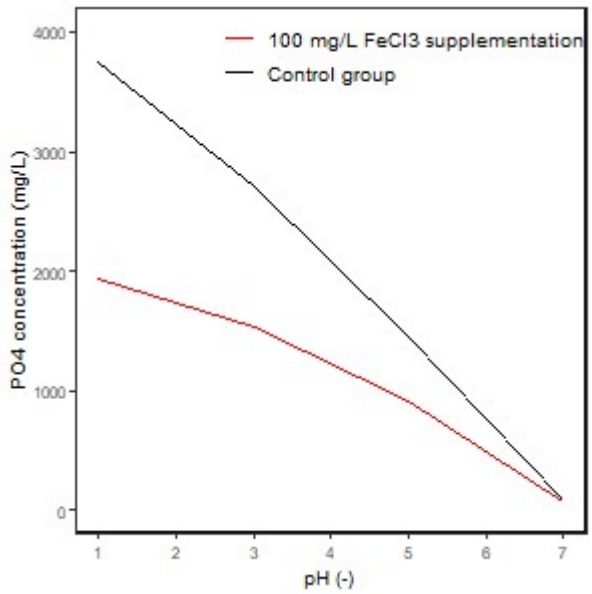


Figure 39. Increased solubilization of phosphorus at lower pH and in the absence of iron. AD control is anaerobic digestate without Fe supplementation, AD Fe is anaerobic digestate including iron at 100 mg/L.

6.1.5. Potential hazards related to sulfur

It is additionally worth mentioning sulfur and its relationship to nutrient transfer. For freshwater systems, sulfur is present at minute concentrations (0 - 630 mg/L in rivers, from 0 - 250 mg/L in lakes, 0 - 230 mg/L in groundwater). This stands in contrast to seawater (averaging around 2.7 g/L) (592). These different magnitudes of sulfur have significant implications for anaerobic digestion and biogas production, as sulfur-reducing bacteria can convert sulfate (SO_2^{-4}) and elemental sulfur (S^0) to hydrogen sulfide (H_2S), which may be released as part of the biogas. H_2S is both flammable and highly toxic with concentrations above 100 ppm considered to be immediately dangerous (593). One effective strategy for H_2S from flue gas is biological desulfurization (594). The process is based on the oxidation of H_2S by injection of a small amount of air (2–5%) into the raw biogas. For this kind of desulfurization, *Sulfobacter oxydans* bacteria or other sulfur-oxidizing bacteria must be present to convert H_2S into elementary sulfur and sulfurous acid. For the desulfurization inside the digester, *S. oxydans* is naturally present but less efficient given the anoxic conditions. Air can be directly added in the headspace of the digester so long as enough surface area is present, however, this reduces the relative methane concentration. An alternative solution is to wash the gas with water in a subsequential biological desulfurization of the aqueous phase. As with most solutions, this brings about further challenges, due to the additional treatment infrastructure. Our study (chapter 5) observed that sulfur appeared to precipitate as a black material, which we can assume was iron sulfide. This may be one of several applications that iron addition may play in facilitating nutrient transfer and the efficiency of the waste treatment process, which we explore in section 6.3.1.

6.2. Economic incentives for solids treatment

This dissertation demonstrated the added value possible for aquaculture wastewater through biogas and liquid fertilizer production. With respect to biogas production, sludge density and volume will determine the profitability threshold. For example, a small aquaculture farm producing 50 tons of salmonids annually will require around 65 T feed annually, resulting in around 180 L solids produced

daily. Based on our study (chapter 5), this will lead to 1.86 m^3 CH_4 / daily, which can be utilized for a total of 67 KJ/ daily. In a typical combined heat and power system this is 18.6 kWh electricity/ daily and 44.10 MJ heat/ daily. However, a facility of this size would require around 59.40 GJ natural gas daily to meet their heating needs. As such, the most likely application for biogas production – regardless of whether considering an aquaculture or aquaponics facility – will probably be to heat a boiler on site rather than to generate electricity.

For aquaponics facilities, the solids treatment system is likely most interesting for its nutrient supplementation (in the form of CO_2 from burning the biogas and as a micronutrient-rich liquid fertilizer). The anaerobic digestion slurry produced (digestate) is an improved fertilizer in terms of both its availability to plants (595) and its rheology (596). Among macronutrients, nitrogen was not reliably supplied to the greenhouse during our preliminary study, however, implementing the improved anaerobic digestion parameters from Chapter 5 would likely go a long way in addressing these shortcomings. Chapter 4 demonstrated that virtually all micronutrients were bioavailable to the plants receiving the remineralized effluent, as demonstrated by plant sap analysis on the leaves and roots (196). Evidently, micronutrient in the fish solids were supplied to the hydroponics crop at adequate concentrations to sustain growth, although some deficiency in potassium and manganese were noticed in the treatment receiving exclusively the remineralized effluent. Reexamining the effluent composition in a system with a more optimized the anaerobic digestion step will help evaluate the true suitability of the solids for use as a liquid fertilizer.

The question of nutrient transfer from an aquaculture perspective is one of nutrient "elimination". While still in a nascent stage of development, a strategy for combined anaerobic digestion coupled with a nitrogen and phosphorus removal (or even restricting processing of the digestate to nitrogen removal alone) would greatly reduce discharge costs. Discharge limits depend on the total suspended solids, total nitrogen load, and total phosphorus load of the effluent. Future studies will need to model effluent nutrient profiles under different management strategies (*e.g.*, temperature, HRT) and scalability to better map out profitability scenarios.

6.2.1. Iron application

Iron plays a disproportionately important role among nutrients in anaerobic digestion, owing to its use as a coagulant and ability to enhance stability of biogas production in the anaerobic (chapter 5), as well as a strategy to limit H_2S production (section 6.1.5) in the treatment of solids in saltwater aquaculture facilities.

Coagulation is the process of adding chemicals to untreated water to destabilize the particles (floc) within the water. Sludge settling as a volume-reducing step is particularly suited as a pre-treatment to microbial digestion of the solids by separating suspended solids from the background water column. Coagulation is a technique already in regular use in aquaculture, albeit with the goal of off-site evacuation (597-599). Previous studies focusing on coagulation for RAS have resoundingly favored the use of geotextile bags, however, the regular use acrylamide-based coagulants in the aquaculture industry poses certain health and environmental concerns (597, 600, 601). With respect to saltwater coagulation, the most recent work in the field of aquaculture was done by Guerdat et al. (2013) (600). At the time of the study, it was not economically viable to attempt any recuperation of fish solids from marine aquaculture sites, but recent developments in both solids treatment strategies and open pen designs (*e.g.*, FishGLOBE (FishGLOBE AS, Norway)) as well as closed recirculating systems (*e.g.*, Glitne

(Sogn Aqua AS, Norway)) have created opportunities to revisit this topic (41, 602). The ideal saltwater coagulant should have a high cationic strength that is able to outcompete sodium ions for the negatively charged function groups (hydroxyl, carboxyl, phosphate terminal groups) on the floc surfaces. Inorganic coagulants, while effective for the flocculation of saltwater particles, require 5-10x higher dosages compared to freshwater systems (603). Research on the effect of saltwater intrusion into freshwater settling basins indicates a displacement effect by sodium ions of divalent cations from the floc structure, significant above 1 mEq/L, inhibiting a major process in floc stabilization referred to as divalent cation bridging (DCB) theory (604). In terms of inducing flocculation, Ca^{2+} was shown to be strongest, followed by $Mg^{2+} > K^{+} > Na^{+}$ in descending order (605). Besides DCB, extracellular polymeric substances (EPS) matrices produced by the flocs undergo significant changes, ultimately increasing the sludge volume index (SVI) (605). To induce flocculation effectively, the trends induced by increased monovalent cations must be reversed or neutralized.

Several metal-based coagulants are used in saltwater coagulation as reviewed elsewhere (598, 606-608). Ferrous sulfate ($FeSO_4$), zinc sulfate ($ZnSO_4$), and ferric chloride ($FeCl_3$) are the most interesting, due to their lesser toxicity potential for aquatic animals than other commonly used coagulants, such as aluminum sulfate and alum salts (609, 610). Despite our demonstration of the value of Fe addition to reactors in the form of $FeCl_3$, the idea of adding a coagulant is generally disliked in the aquaculture industry due to the constant costs and small potential profit margin in solids treatment. With the primary objective of achieving an economically viable waste treatment solution unfulfilled, we therefore propose to explore in future the use of a regenerative coagulation strategy, such as magnetic iron nanoparticles. For instance, the magnetic iron nanoparticles magnetite (Fe_3O_4), has been shown to effectively precipitate COD (611). Given that most of the high-value fish produced in European aquaculture come from saltwater systems, further research into this aspect could be valuable in adapting the technology explored here for the most significant aquaculture market segments.

6.2.2. Anaerobic digestion as a platform for other downstream outputs

The strength of anaerobic digestion as a tool to decompose organic wastes lies in its versatility on both ends. In terms of input, the technology may be adapted to virtually any type of organic waste. It may be possible for facilities to combine multiple organic waste streams together in their waste treatment process. This strategy is commonly done with livestock manure to improve biogas production (513, 612). Although chapter 5 focused on biogas production, there are other possible outputs for anaerobic digestion, albeit these often come with additional processing costs. This could provide an alternative to liquid fertilizer production in situations where the market demand is lacking, or the salinity exceeds plant tolerance. Here, digestion is arrested prior to methanogenesis and the organic acids are further processed (613, 614).

Currently, the recovery of volatile fatty acids (VFAs) from anaerobic digesters remains challenging although exploiting the increasing hydrophobicity of longer SCFAs (C7 and greater) appears to be the most viable option (615, 616). The selective cultivation of chain elongation communities which take up short SCFAs (*e.g.,* acetate) as a substrate is perhaps the most promising strategy for this purpose (617, 618). Another output of interest could be the production of polyhydroxyalkanoates (PHAs) from SCFAs in the digester. PHAs have a multitude of uses as reviewed elsewhere, although the high cost of production strangles industry expansion (619, 620). Halophilic PHA producers in conjunction with halophilic anaerobic digestion may be a promising revalorization strategy for saline anaerobic digestion. The *Bacillus megaterium* strain uyuni S29 was shown to be a strong PHA producer at high saline

concentrations (41 wt% at 45 g/L NaCl) (621) which may be found in desalination brine. Some decoupled aquaponic designs use desalination to allow for the return of clean water to the RAS (181), although no commercial facilities have adopted this technique due to the high costs of desalination (Hydroccitanie (France), Regen Aquaculture (USA), personal communication). Another interesting vector for PHA production could be with anaerophilic purple phototrophic bacteria (PPB). A recent study demonstrated significant COD, TAN and sulfur species concentrations considerably by a PPB culture, with about half of the influent N and P incorporated as biomass (622). The dependence of PPB on infrared light may be exploited as an enrichment strategy, a technique currently being explored for the adaptation of these organisms to wastewater treatment as described extensively in a recent review (623). The PPB research follows similar trends to algal research: the goal is to exploit unique growth conditions for the conversion of wastewater into biomass or high-value secondary metabolites. The ability of PHA producing bacteria to grow in freshwater and hypersaline conditions makes them interesting for all types of aquaculture solids treatment (622, 624, 625).

A recent publication demonstrated the capacity for some *Streptomyces* species to produce polycyclopropanated high energy biofuels with an energy density 30% greater than jet fuel (626). While this study grew *Streptomyces* using sucrose as a carbon source, it has been shown elsewhere that volatile fatty acids can influence secondary metabolite production in *Streptomyces gilvosporeus* (627) and Streptomyces *ambofaciens* (628). Further research should examine the potential for relevant *Streptomyces* species to use SCFAs as a carbon source either alone or in co-culture with symbionts to revalorize anaerobic digestate into high-value products. While the studies summarized above are interesting for their optimistic outlook on this novel bio-industry, they tend to disguise challenges in downstream processing and scaling that needs to be addressed in future research before this type of production will be economically feasible.

7. Thesis Conclusion

The goal of this thesis was to assess the potential of microbial community management for waste treatment and nutrient remineralization in aquaponic food production systems as a CEA model, prioritizing economically viable and circular solutions. The projects in this thesis involved tracking the colonization of microorganisms downstream from the RAS, establishing a systematic framework for their study through the use of ecosystem-specific databases, as well as assessing nutrient remineralization biogas production from aquaculture solids. The thesis emphasizes the importance of economically sustainable waste treatment solutions and presents experimental results, followed by a discussion of practical and economic scalability. The main findings are that optimizing solid waste treatment systems can provide aquaculture facilities with an effective method to recover some of the treatment costs through the production of biogas and liquid fertilizer. Burning biogas can provide three resources, depending on the scale: heat, electricity, and CO2 generation. While electricity production is likely only economic at large scales or in facilities that supplement the fish solids with other organic waste streams, heat is universally useful for any building, and CO2 supplementation is a common production-boosting technique in greenhouses. Aquaponic facilities may thus capitalize on three to four outputs from the solids treatment system.

Scientifically, this dissertation approaches the challenge of waste revalorization first from the perspective of screening opportunities for microbial intervention and secondly from the perspective of novel niche creation through compartmentalization. Chapter 2 investigated the possibility of upstream microbial communities to impact the downstream hydroponic beds. This study complemented existing evidence from the literature regarding the dynamics of rhizosphere colonization, namely that the plant exerts a greater influence over microbial colonization than do environmental parameters. Such findings suggest that bio-based nutrient remineralization systems should be safe with respect to plant growth, and the increased microbial flow to downstream CEA should not pose any particular stress on the crop. Unfortunately, this also suggests that probiotics are likely ineffective at shaping the community or at least will need to be designed to better accommodate plant preferences. The metabarcoding approach used in the project revealed an important disparity in the amount of data available on microbial communities, namely that it is difficult to screen large datasets of putative taxonomies against other databases specializing in other relevant data (e.g., -omics data). Chapter 3 thus sought to properly investigate the issue and identify solutions. The best path forward was determined to be the advancement of environmentally specific databases. This was presented in Chapter 3 as a review paper.

Acknowledging these intrinsic barriers to a microbial approach, the second half of the dissertation shifted the focus towards niche creation. Chapter 4 presents a study comparing plant growth through the remineralization of nutrients from the aquaculture solids with industrial hydroponic nutrient solutions. Here, anaerobic digestion was used to decompose organic matter, which was then diluted in a hydroponic sump. The diluted digestate in the oxygenated sump environment created a suitable environment for remineralization, which we demonstrated in the study to be highly bioavailable for the plants. Finally, chapter 5 optimized the anaerobic digestion process to prioritize biogas production. Taken together, these studies demonstrate the vast potential of solids treatment to provide added benefits for both aquaculture and aquaponic farms while addressing environmental concerns linked to the discharge of nutrient-rich biochemical streams. Developed and scaled further to meet industrial demand, this technology will allow aquaculture and aquaponics to become a poster child of 21st-century industry – productive, efficient, and circular.

8. References

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