Community Consolidation Dictates Hydroponic Root Microbiome

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Keywords: Rhizosphere, rhizobiome, microbiome, aquaponics, hydroponics

Abstract

Terrestrial plant roots mediate a complex network of prokaryotes and eukaryotes collectively referred to as the rhizobiome. The ability to promote beneficial plant symbionts contributes to increased crop productivity. Despite recognition of the microbial underpinnings, plant cultivation strategies remain primarily focused on plant physiology to determine crop health. The success of plant cultivation in hydroponics indicates that fundamental rhizosphere activity is preserved regardless of the surrounding milieu vis à vis nutrient uptake, abiotic and biotic stress resistance. What has not been explored is how the rhizobiome adapts to soil-less hydroponic or aquaponic cultivation. Microbial dynamics in aquaponic systems are more complex than their hydroponic equivalents insomuch as additional communities are incorporated via the inflow water to plant units from the fish rearing tanks (influenced by the microbiome of the intake water and fish GI tract), influenced in turn by the biofilter community of the recirculating aquaculture system (RAS) (soluble-waste treatment loop).

This study presents results from a series of experiments aimed at analyzing the development of the rhizobiome during cultivation of Batavian lettuce (*Lactuca sativa*) in hydroponic or aquaponic systems coupled to RAS with common carp (*Cyprinus carpio*) or Nile tilapia (*Oreochromis niloticus*). In the first set of experiments, we employed standard water purification techniques (UV, ozone, H2O2) to impede microbial proliferation in hydroponic beds to test the effects of sterilization on microbial diversity and plant growth rates.

A second experiment assessed the capacity of the rhizosphere to resist colonization from foreign microbial communities (upstream RAS microbiome, soil rhizosphere community, probiotic supplementation). These were compared to treatments supplied with sterilized nutrient solution. The similarity of taxonomic profiles across all treatments indicated that the plant roots strongly dictate the rhizobiome regardless of other environmental pressures or microbial influences. Surprisingly, microorganisms corresponding to specific metabolic profiles (e.g. nitrification) were not reflected in the rhizobiome – indicating that nitrifying organisms from the biofilter do not carry over to the rhizosphere but rather are functionally replaced by rhizosphere-specific nitrifiers. Nonindigenous communities in proximity to plants are not capable of displacing the autochthonous rhizobiome in hydroponic nor aquaponic systems.

# Introduction

The region in and around plant roots, commonly referred to as the rhizosphere, is an interspecies nutrient and electron trade zone with stakeholders representing all kingdoms (1-6). The microbial component of the rhizosphere is referred to as the rhizobiome. It is composed of a core component fulfilling essential duties required by the plant at that instant, and a satellite component consisting of strains present at low abundances (7). The core community is stable across all plants and environments, consisting of taxa that are necessarily drawn to the root environment in contrast to bulk soil (8). Recently, a consensus has been reached that plants exert a greater pressure in dictating their rhizobiome than do microorganisms in choosing their hosts (7, 9, 10). Hereby, the rhizosphere manages nutrient uptake needs (1, 11-13), abiotic stress resistance (10, 14, 15), and host defense (16-18). Conceptually, the rhizobiome functions similarly to the animal gut microbiota as an interface with the outside world. The microbial community may directly influence biosynthesis processes in the plant (19, 20), as can other kingdoms present (2, 3). As only 7% of bulk soil microorganisms are enriched in rhizosphere environments (21), the relatively carbon-rich environment of the rhizosphere appears to create a precursory selection pressure. Albeit less diverse, the relatively stable flow of 10-250 mg/g organic acids from the plant into the rhizosphere creates a microbial environment two orders of magnitude more abundant than surrounding soil (22). These root exudates also include amino acids, organic anions, sugars (23-26). The complexity of the dynamic surrounding the formation of these communities has given rise to a plethora of metagenomic studies on the rhizosphere, yet applying this knowledge to predict plant health has remained elusive (7, 10, 12, 27, 28).

Given the discrepancy in rhizosphere diversity across soil-less and soil-based cultivation strategies (figure 1), and given the growing amount of publicly available annotated genomic information (e.g. KEGG, JGI databases), a thorough characterization of the essential core rhizosphere functionality for plants of agricultural importance is an attainable target for this decade. Towards this goal, hydroponics and aquaponics present an attractive environment for this research. Soil-less cultivation shrinks the rhizosphere diversity without affecting plant-mediated nutrient uptake (28, 29). Physiochemical parameters may be more closely monitored as the lack of soil prevents competition from other plants, as well as greatly reduces the risk of soil-borne disease (30, 31). Conversely, these systems are continuously exposed to environmentally persistent microorganisms (water- and airborne colonization). At this level of diversity, inoculation biases are less pronounced in comparison to axenic cultures. Finally, the industrial adoption of hydroponic and aquaponic cultivation systems provide a wide range of comparable parameters regarding plant productivity under specific conditions. Whilst evidence from several studies has indicated improved crop productivity in aquaponic systems (32-36), a consistent empirical explanation is still lacking. For example, recent studies focusing on the diversity of microorganisms in aquaponic systems (37-40) have given rise to many hypotheses as to how the microbial community may lead to increased plant growth rates based on the increased abundance of chelating agents, cofactors, enzymes, or hormones facilitating nutrient bioavailability, either directly or indirectly. Causal factors leading to increased crop production in aquaponics remain speculative (27, 41), although involvement of the microbial community is implicit.

In order to investigate the capacity of plants to optimize their rhizobiome to suit specific growth demands, this study focuses first on determining if the increased microbial load from RAS leads to better utilization of rhizosphere niches by measuring crop harvest parameters in hydroponic, aquaponics, and sterilized aquaponic treatments. We then investigate shifts in taxonomic diversity of the rhizosphere subjected to different microbial sources (hydroponics solution alone, coupling to RAS, probiotic addition, soil-based inoculation) to determine whether the rhizosphere is consolidating around a particular community, or diversifying as a function of different treatments.

The goal of this study was thus to study rhizobiome consolidation dynamics from two perspectives critical to hydroponics/ aquaponics. Firstly, we address effect of sterilization on the rhizobiome on the preservation of exudates and microbial diversity. Secondly, we investigate the robustness of the rhizobiome in soil-less cultivation amidst pressures from foreign microbial communities.

# Methods

Two experiments consisting of three deep water cultivation trials were carried out at the Wageningen UR Greenhouse Horticulture Unit (Bleiswijk, NL). The goals of experiment 1 were to a) reproducibly show an increase in crop harvest yields in an aquaponic system compared to standard hydroponic cultivation, and b) localize the microbial contribution to either the proliferation of microorganisms in the hydroponic unit or to abiotic sources (microbial exudates). Experiment 2 was designed to either discourage microbial growth in the hydroponic community, or actively diversify it by promoting targeted microbial growth through the addition of a potential probiotic.

Across both experiments, all trials had a recirculating aquaculture system (RAS) and a hydroponics (HP) unit which were linked by the treatment conditions (figure 2). Two fish species were used, common carp (*Cyprinus carpio*) (experiment 1) and Nile tilapia (*Oreochromis niloticus*) (experiment 2). Exaudio RZ 79-43 (Rijk Zwaan, NL) Batavian lettuce (*Lactuca sativa*) was grown hydroponically either in hydroponic basins (40 plants/ea.) or boxes (3 plants/ea.) with four replicates per treatment. Boxes were designed only for experiment 2, to create a controlled environment otherwise not possible with basin cultivation (figure 2). Boxes, insomuch as they were self-contained provided better control over microbial exposure to the plants 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. Four microcentrifuge tubes filled with 2% w/v agar-agar (Sigma, NL) were inserted into the sheet. Seeds were placed directly into the agar. Each treatment was conducted in triplicate based on a random spatial distribution. Aeration was supplied with a 2HP blower (Leister Technologies AG) for basins. Due to the few amount of plants per volume nutrient solution in the boxes, it was deemed unnecessary to aerate them. This assumption was validated by DO measurements indicating near similar oxygen levels compared to basins.

For all treatments, seeds were incubated in darkness overnight at 25°C (boxes fully wrapped in aluminum foil + plastic sheet for humidity and contamination control). 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 made weekly, at which time half of the basin volume was displaced by HNS. Supplementation was done as necessary to maintain the following approximate macronutrient composition (mmol/L): 15.0 NO3, 1.5 NH4, 5.0 K, 1.5 Na, 3.0 Ca, 1.5 Mg, 0.1 Si, 0.1 Cl, 1.5 SO4, 0.5 HCO3, 0.5-1.0 P. The following trace elements were also added (µmol/l): 20.0 Fe, 7.0 Mn, 5.0 Zn, 20.0 B, 0.5 Cu, 0.1 Mo. Parameters maintained most rigorously were pH (set to 6-7) and EC (set to 2-2.5). RAS water for crops was taken via the biofilter effluent. In a decanting tank identical to that used to make the HNS, nutrients were added to achieve similar concentrations as the HNS. Any sterilization treatment occurred immediately prior to nutrient addition.

Nutrient solutions were added to the treatments weekly. In basins, plants were grown either in HNS or nutrient supplemented biofilter effluent (HNS-BF) as summarized by table 1. Here, we refer to HNS from two full crop cycle as mature HNS (HNS m), in the sense that we assume it to have formed a stable microbiome at that point. Three types of sterilization were chosen: UV exposure, H2O2 addition, and ozonation. For the UV treatment, a UV light (5W lamp, capacity: 2500 L) was applied for 48 h. Ozonation was done on site at the Aquatic Research Facility. Hydrogen peroxide was applied at 35% v/v (equivalent to 150 ppm) to the aquaculture effluent 5 days prior to nutrient supplementation. Finally, an enrichment culture of *Bacillus subtilis* (5x1011CFU/g stock, applied to achieve a final concentration of 2 mg/L) was added to the HNS once when the main root stem was ca. 2 cm long. *B. subtilis* is widely used in commercial aquaculture facilities for both fish and crustaceans (42, 43), as well as in hydroponics (44-46). A one-way ANOVA test was used to determine the differences in crop growth (crop fresh weight and root dry weight), with post-hoc Tukey test performed to assess significant differences between treatments.

In experiment 2, a first crop cycle was carried out exclusively in basins with half of the ten basins consisting of lettuce grown on HNS and the other half on nutrient supplemented biofilter effluent. After eight weeks, the crop was harvest and new seedlings were planted, keeping the original HNS/HNS-BF layout. Box trials were set up alongside the second basin crop cycle. The second crop cycle was terminated after six weeks.

Treatments were done with three technical replicates and supplied with nutrient solutions as described in table 1.To make sterilized HNS or HNS-BF, freshly made nutrient stock solutions were filter sterilized (0.22 µm). The probiotic effect of *B. amyloliquefaciens* (5x1011CFU/g stock, applied to achieve a final concentration of 2 mg/L) was added to sterilized HNS and to unsterilized HNS-BF. Unlike the aforementioned *B. subtilis, B. amyloliquefaciens* has been developed as a probiotic in hydroponics but not RAS (47-49). As a plant growth promoting probiotic not present in RAS, there is minimal risk (in comparison to *B. subtilis*) that OTUs detected during 16s sequencing corresponding to this bacterium could originate in the RAS rather than the applied formulation. Soil inoculum was sourced from a 2-month-old Batavian lettuce plant growth locally in potting soil. Care was taken to remove specifically soil with a high density of roots so that the rhizobiome would be transplanted into the hydroponics environment. Approximately 50 mg of soil was added directly to the boxes.

Water samples during all three trials were analyzed weekly for nutrient concentrations, pH, and EC (Groen Agro Control, NL). Any nutrient deficiencies were corrected on a weekly basis. Dissolved oxygen (DO) was kept saturated for both experiments. During the first experiment, temperature (°C) in the hydroponics unit fluctuated between 14-24°C (trial 1) and 17-29°C (trial 2). During the second experiment it was controlled at 16°C. Broad spectrum lighting was maintained at 200 µmol/s/m2 for 16 h/day for all trials, although supplementation was not done for trial 2 (due to summer conditions providing adequate irradiation). Crops were harvested after 6 weeks in the basins. Fresh and dry weight (oven-dried, 80°C for 48 h) of both the harvestable crop and the roots were calculated. Community DNA was isolated from each component of the RAS and hydroponic components for subsequent metagenomic analysis. As a control, mature HNS and HNS-BF nutrient solutions originating from the same stock solutions as the basins were used in the boxes.

Pikovskaya agar was used to screen for phosphate solubilizing bacteria (50). Siderophore activity was screened for using Schwyn and Neilands universal siderophore detection medium (51). In short, root samples from HNS treatments (ca. 5 mg) were suspended in phosphate buffered saline solution and vigorously vortexed for 10 min. The solution was then plated across three plates. Strong phosphate-solubilizing or siderophore-producing colonies were detected by a color change in the medium resulting in a halo. These colonies were streaked on new plates, individual colony forming units were then plated again to ensure purity. DNA from the resulting isolate was extracted, the identity of the isolates was confirmed by amplifying the V3-V4 region of the 16s rRNA gene and comparing the sequenced results to the NCBI database.

For metagenomic profiling, DNA was isolated from the roots of each technical replicate using the DNeasy PowerSoil Kit (Qiagen, Germany). For basins, root samples were taken randomly from three plants. For boxes, all plant roots in an individual box were combined for the DNA extraction. Purified DNA was sequenced using universal 16s rDNA bacterial primers (BaseClear B.V., NL). Sequenced OTUs were taxonomically identified by comparison with NCBI databases. Taxonomically identified OTUs were then screened against the KEGG reference database using a python script, wherein KEGG entries with annotated genomes were identified and key terms were pulled that were associated with specific genes of interest. The resulting three datasets (OTUs without KEGG annotated genomes, OTUs with KEGG annotated genomes and therein associated matrix of pulled genes of interest) were subsequently processed using an R script.

In R, each dataset was subdivided into six data frames related to the taxonomic level. The first two datasets (OTUs alone and OTUs found in the KEGG reference database) were processed through a pipeline to determine the statistical significance of the treatments. To visualize the dataset, species frequency, species occurrence, the relative frequency of occurrence, and the richness of species across treatments were plotted. The following diversity indices were then calculated: species richness, Shannon entropy, diversity index and evenness, Simpson diversity index and evenness, and Pielou evenness. Different transformations were then applied to the data frames to determine which strategy best normalized the data to a linear distribution without warping. These standardization techniques included square root and logarithmic scaling, standardization by species maxima (Chord transformation), standardization by treatments (Hellinger transformation), and double standardization by species and treatments (Chi-squared, Wisconsin transformations).

As no clear break with outliers was visible, the dataset was run twice without modification and with a maximum threshold of 1000 hits for any individual OTU present, summed across all treatments. Once normalized, the data was sorted into dendrograms using two agglomerative clustering methods: nearest and furthest neighbor sorting. The nodal distance between objects in the dendrogram (cophenetic distance) was calculated for all object pairs (cophenetic correlation). The method giving the highest cophenetic score was determined to be the best clustering model for the calculated distance matrices. Next, the degree of membership of each object to its cluster (silhouette width), the optimal number of clusters (silhouette optimal) was calculated for the original distance matrices. Binary matrices were computed from the dendrogram to determine the Mantel optimal number of clusters. These data were used to compute a reordered dendrogram considered to be the most robust clustering arrangement. This clustering arrangement was used as the backbone to create heatmaps of the species distribution across sites and as well the distribution of genes of interest across sites. Multiple factor analysis (MFA) was then used to generate heatmaps of the OTU distribution across treatments for each taxonomic rank.

Corresponding intensities for the gene prevalence search results were plotted as heatmaps (z axis) against OTU assignment (y axis) and treatments (x axis). As the gene prevalence is linked to the OTU assignment only, OTU/treatment combinations where the OTU was not present were first nullified. The gene prevalence was then plotted on the remaining OTU/treatment combinations. As 16s sequencing is relative in abundance, OTU abundance was considered in terms of presence/absence when making the plots.

# Results

Results from experiment 1 indicated no significant different in crop head weight between treatments. Nutrient supplemented biofilter effluent treatments or those inoculated with the *B. subtilis* enrichment culture had marginally greater root weights than the mature HNS treatment. H2O2 and ozone sterilization appear to have marginally impacted root growth, whereas this trend was not observed with UV sterilization (figure 3). To corroborate the results and additionally determine if the crop head weight was due to increased water content or biomass, crop heads were dried post initial weighing. No significant difference in the wet crop head weight was observed. Amongst the dry crop head weights, the nutrient supplemented biofilter effluent (HNS BF) was greater than other treatments. No significant difference was observed across dry root weights. Due to weekly supplementation, nutrients remained stable across both trials. It appears that given equal nutrient concentrations, hydroponically-grown plants did not receive any advantage from the RAS effluent. It furthermore appears that sterilization of the RAS effluent did not significantly impact crop head weight.

In experiment 1, we aimed to assess whether growth outcomes could be explained by a consolidation of the microbial community around a homogenous rhizosphere microbiome. Unlike previous trials in which plants were grown exclusively in basins, this trial employed the use of smaller boxes to allow a greater control over the microbial environment surrounding the plants. Sterilization treatments could thus be done with minimal cross-over between treatments by applying nutrient solutions separately for each box (likewise reducing contamination across technical replicates).

Extracted DNA passed all quality checks prior to sequencing. The Chao1 nonparametric richness estimator was chosen for this dataset due to the low abundance of most OTUs. Because 16s sequencing does not return true abundances, the number of OTUs detected in each sample (hits) is considered to be relative and thus valid only within this dataset. In total, 4232 individual OTUs were retrieved at the species rank, representing 896,948 hits across all treatments of which there were a total of 72,670 unclassifiable OTUs, meaning 8.1% of all hits were unassigned. The entire dataset was then screened against the KEGG database to identify annotated genomes. 1193 OTUs were matched against the KEGG database. 229 OTUs were matched but did not have annotated genomes and were rejected. To validate the representability of the 964 matched, annotated genomes, both KEGG-matched and unmatched OTU datasets were processed in R.

Species richness (Shannon diversity index) is higher in the outlier-removed, KEGG matching dataset compared to the unprocessed dataset (2.63 vs. 1.60). The Simpson diversity index increased along the same margin (2.27 vs. 1.57 for the outlier-removed and unprocessed dataset, respectively. As species richness would only have decreased through data processing, this indicates a greater evenness across samples following data processing.

The cluster structure for both datasets was indistinguishable to five clusters corresponding to the following controls: soil, water source, and RAS or biofilter effluent, with all other treatments grouping in a fifth cluster. Interestingly, the hydroponic nutrient stock solution clustered with the hydroponic treatments. Clustering decisions were chosen based on a consensus between elbow, gap, Mantel, silhouette and NbClust (R package, bootstrapped 500 times) before being visually evaluated. Based on silhouette partitioning of the predicted clusters, phylogenetic diagrams were calculated based on Ward’s method and plotted (52-54).

To visualize the distribution of OTUs across treatments, heat maps based on the above dendrograms were plotted. At higher taxonomic ranks, clustering becomes distorted due to the lack of input diversity. Conversely, at lower taxonomic ranks the data is unreadable due to the quantity of hits displayed. Due to the segregation used in generating the data (presence in KEGG database or not, removal of highly abundant (> 1000 hits) OTUs), the global clustering structure was assessed for each taxonomic rank and data subset. Importantly, global trends in the data were preserved regardless of taxonomic rank or subset. While only a single subset/taxonomic rank combination is presented here, other taxonomic ranks may be found in the supplementary materials. A link to the raw data is included in the Data Availability section.

Ultimately, 22% of the uniquely identified OTUs had annotated genomes in the KEGG database. The distribution of gene and metabolic pathways amongst annotated genomes were plotted against OTUs and treatments which allowed us to create a metric for total metabolic capacity (done for nitrogen, phosphorous, iron, and sulfur) as well as the abundance of specific genes encoding ammonia, nitrite, and nitrate. These were chosen as part of an exploratory project to evaluate the KEGG database as a tool for predicting gene expression capabilities based on 16s sequencing data. The subset for nitrogen metabolism is described in figure 9; plots for other metabolic profiles of interest are provided in the supplementary materials.

In addition to the described bioinformatics approach, we also plated water column and root samples on two selective agars to screen for strong phosphate-solubilizing or siderophore producing bacteria. Surprisingly, of the nine strains sequenced (table 2), all but two were of the genus *Erwinia,* the remaining two being unidentified *Enterobacter* species. Both genera fall into the same order of Enterobacterales.

[Figures 4-9 go here]

# Discussion

**Consolidation of the rhizobiome**

This study presents the first concentrated metagenomic analysis of the microbial rhizosphere community in the hydroponic component of an aquaponic system. Whereas previous studies have employed sequencing techniques to characterize other compartments of aquaponic systems in detail (e.g. RAS tanks) or global microbial diversity across aquaponic systems (55-59), none have directly evaluated the effect of active modifications to the hydroponic segment on the crop rhizobiome. Directly comparing yields between aquaponics and hydroponics has proven to be inconsistent and poorly reproducible (27, 41). As both aquaponic and hydroponic systems strive to maximize crop productivity through the same conventional means (greenhouse design, cultivar selection, etc.), they distinguish themselves primarily in their aqueous milieu, which although do not differ in availability of essential nutrients (these are normally supplemented to a level where they are not limiting), may differ in terms of microbial abundance and diversity. Thus, our study focused on the community development at the main interface between the aqueous milieu and the plant – the rhizosphere. This research is furthermore relevant in relation to sterilization practices in aquaponics (e.g. with the goal of protecting crops from unwanted colonizers), an area that remains controversial. Some studies advocate for continuous cycling of water between RAS and HP components (coupled aquaponics) (60, 61), while others have advocated for a discrete separation (decoupled aquaponics) with no return of microorganisms from the HP to the RAS (32, 62-66). We thus sought to determine whether sterilization (reducing microbial proliferation across units) succeeds in limiting mainly adverse rhizosphere colonizers or rather all members of the community indiscriminately.

A diversity of factors influencing rhizobiome composition, resulting from both the plant (genotype, life stage) and the environment (water source, nutrient profile) (7, 67), suggests a dynamic system. However, our results instead show a universal consolidation of the rhizobiome around a relatively consistent and narrow taxonomic profile (figures 4, 5, 6). This likely represents the combined core and satellite microbiome, as described previously by Yeoh et al. (2017) (8) and Compant et al. (2019) (7). As recently shown by Bartelme et al. (2019) (59), facility conditions strongly dictate the microbial populations present in RAS and aquaponic systems. Our results suggest that a similar facility-specific microbiome has formed in the hydroponic basins and boxes. Considering the RAS component as a pseudo-stable influence on the hydroponic microbial community, our results suggest that the unique microorganisms present due to the fish or biofilter do not directly shape the rhizobiome composition. Studies on the rhizobiome in other systems likewises indicate a similar pattern of consolidation. For instance, Schreiter et al. (2014) observed that the lettuce rhizobiome was consistent across varying soil types (68), 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 (29). This trend appears to be a hallmark of all terrestrial plants (9, 10, 69, 70). Thus, it appears that selection pressures exerted by the plant to consolidate the rhizobiome around a particular profile are a fundamental aspect of plant physiology, preserved regardless the environment surrounding the rhizosphere. 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.

**Assessing rhizobiome resilience to foreign influences**

The clustering model reveals several significant trends in microbial population dynamics. If the RAS were able to exert a significant pressure on the rhizobiome composition we would see hydroponic treatments supplied with nutrient-supplemented biofilter effluent clustering separately from those provided HNS alone - this was not observed. Pioneering bacteria originating from seeds are known to shape the next generation rhizobiome (16, 67, 71, 72). If the seed microbiome was strongly influencing rhizobiome development, we would expect the HNS stock solution to be divergent from the treatments. Our data show that the HNS remains consistently clustered with the hydroponic treatments and always as a separate cluster from the other controls (water source, RAS, biofilter effluent). This suggests an alternative story, insomuch as the nutrient profile is exerting a strong selection pressure on the pelagic hydroponic community. The fact that this community is maintained in the sterilized treatments makes it probable that they are derived from environmentally persistent bacteria at the facility, and are not defined by the specific water source. Further evidence for this hypothesis can be found when reviewing the soil-inoculated treatments. Community diversity was poorly retained when soil-based lettuce roots were used to inoculate sterile HNS. What was rather unexpected was that these treatments gravitated towards the same global consensus as the other hydroponic treatments, despite filter sterilization of the HNS and no direct contact between treatments. This suggests that the HNS creates a selection pressure for environmentally-persistent, oligotrophic microorganisms able to thrive in relatively high nutrient environments (compared to the facility water source). Phylogenetic diagrams place the HNS stock near the edge of the HNS treatment cluster (figure 6). Thus, although the majority of the HNS stock microbiome is transferred to the hydroponics component, several taxa undergo major shifts in abundance during this transition. The basal hydroponic community is thus likely environmentally inoculated, is water source independent, and is capable of interacting with the rhizosphere to an extensive degree. Figures 7 and 8 show the shifts in taxa across treatments at the class and genus level.

**Elucidating rhizobiome functionality**

To elucidate the mechanisms by which coupling the hydroponics component to the RAS brings about a shift in the rhizobiome we first define the term "functionality" with respect to the role of microorganisms to plant health. It is tempting to interpret the aforementioned basal hydroponics microbiome as the core hydroponic-rhizobiome. This, however, leads to several issues. There is no consensus on which taxonomic rank is sufficient to declare an OTU assignment as definitively having a certain functional role in the rhizosphere. Framed otherwise, we ask whether is it sufficient to consider the entire genus of *Bacillus* as plant growth promoting, or all strains of the species *Bacillus subtilis*, or only certain strains? The region conserved among all known strains of a species, the pan-genome, can be quite low (40% for *Escherichia coli*) (73), creating diverse phenotypes at the subspecies level (74-76), in particular due to the frequency of homologous recombinant events (77). Thus, it is likely that the phylogenetic plasticity of microorganisms ultimately prevents any clear link between taxonomy and function. This is especially pertinent as the genomic resolution required to distinguish pathogens from plant growth promoters is impossible without whole genome phylogeny and likely as well *a priori* knowledge of potential pathogenicity (72, 75, 78, 79). Furthermore, some bacteria may be beneficial under certain conditions, yet can become pathogens when environmental conditions change (80). A detailed discussion on the phenotypic plasticity of symbionts has been covered by Pérez-Brocal et al. (2013) (79); case studies for certain common microorganisms can be found in Dobrindt et al. (2013) (75). In our study, by plating organisms on selective media for phosphate-solubilization and siderophore activity, we were able to isolate several members of the *Erwinia* genus; a clade known to have versatile profiles ranging from plant growth promotion, rhizosphere opportunism, to obligate pathogenesis (81-85). That the strongest siderophore producing and phosphate solubilizing bacteria were all of the same order (Enterobacterales, table 2) is quite interesting, and is a topic that should be explored further. Evidently, functionality implies a state of transient symbiosis in which specific needs of the plant are accomplished by means of microbial interactions.

Other strategies are required to reveal specific functional contribution of organisms in the rhizosphere to plant health. Due to the combination of plant promiscuity in selecting its rhizobiome constituency and genotypic plasticity among microorganisms, we suggest the following two strategies as tools to assess rhizosphere dynamics: predicative functional assignment and transcriptomic validation.

We screened taxonomically assigned OTUs against the KEGG database. For each OTU, the sum of all genes surrounding a particular function of interest were calculated. The presence of OTUs across treatments was plotted, with the sum of genes incorporated as an intensity scale (figure 9). The goal of developing this tool was to more easily screen gathered taxonomic data for genes of interest that could be found in the KEGG database. This does not mean that the genes are being expressed, rather that they are likely present in the genome of the assigned OTU. Furthermore, this technique assumes the affinity of proteins transcribed from analogous genes assigned a particular function to be the same. This tool does not replace transcriptomic studies, but complements them (i.e. providing sequence information to aid in designing primers). Although likely best applied to identify the presence of specific genes of interest (e.g. enzymes, metabolites), we tested the method on our dataset for several generic metabolic pathways: nitrogen metabolism (subset into nitrite, nitrate, and ammonia related genes), phosphorous, iron, and sulfur metabolism.

there was no visible correlation between the presence of nitrogen metabolizing OTUs in the RAS or biofilter effluent and the hydroponic beds (figure 9). Thus, although nitrogen metabolizing OTUs dominate both the RAS and biofilter, those specific organisms do not appear to become integrated into the rhizobiome. Although a subset of OTUs do install themselves in the rhizosphere, the majority appear to originate elsewhere. As plants release considerable amounts of nitrogen (86) via exudates, these results may reflect changes in the concentrations of nitrogenous species proximal to the rhizosphere. It has been observed that bacterial communities in soil environments are sensitive to increased nitrogenous loads - reductions of up to 50% of total biomass and shifts in relative taxonomic abundances, with oligotrophic taxa particularly diminished (87-91). How these two selection pressures on the nitrogen metabolizing community of the rhizosphere counterbalance is unknown. Heat maps of other metabolic profiles chosen in this study are included in the supplementary materials.

In an 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 (36). While the identity of those exudates remains unknown, our results further support their hypothesis by showing that the RAS microbiota does not play a significant role in shaping hydroponic rhizobiomes.

**Re-interpreting the effect of sterilization in hydroponic/aquaponic systems**

We investigated if the method of sterilization impacts the integrity of exudates in the effluent. In terms of oxidation-reduction potential, ozone (2.07) is greater than H2O2 (1.78). UV-C radiation, can interact directly with organic molecules, can precipitate many trace metals, as well as generate and dissociate ozone (92). Conceptually, it was considered that from most to least destructive, UV would be followed by ozone and then H2O2 in terms of potentially detrimental effects on plant growth. Crop head and root weight measurements did not show consistent differences between two harvest during experiment 1 (figure 3). It does not appear that the method of sterilization significantly impacted the harvest outcome, although the high variability suggests a precarity in the ability of the plant to resist stressors.

We then sought to investigate whether sterilization might serve as a preventative measure to curb latent opportunistic pathogens or simply heterotrophic bacteria that would reduce nutrient availability, and whether this would be reflected in differences of the rhizobiome composition. The singular role of sterilization is to add an additional diversity-reducing selection pressure alongside the hydroponic environment (pelagic, biofilm, and rhizosphere components), selecting for microorganisms that can thrive under the facility-specific conditions (temperature range, salinity, etc.). Of the three microenvironments, only the water-root interface is relevant to crop productivity, and thus we are only concerned about taxa that may diminish production via proliferation in the rhizosphere. One of the main mechanisms employed by plants in shaping their rhizobiome is commonly referred to as the ‘cry-for-help’ hypothesis (72), a phenomenon whereby plant selects for microorganisms capable of alleviating specific stressors (nutrient deficiency, abiotic stress, etc.). The goal of sterilization must take this into account, and may thus be framed as “a mechanism by which the movement of latent, waterborne pathogens are reduced to benign numbers”. Unfortunately, this technique also results in a reduction in the movement of beneficial bacteria.

Sterilization is widely used in hydroponics (93-97) to discourage the proliferation of pathogens during nutrient recirculation (98, 99). Aquaponics, conversely, does not use sterilization between components (e.g. RAS water is not sterilized prior to entering the hydroponics unit). Instead, most facilities in coupled aquaponics constantly recirculate the nutrient solution between the RAS and hydroponics components. In decoupled systems, a similar process occurs, but unidirectionally between the RAS and plants.

In coupled systems, a lack of knowledge surrounding the establishment and maturation of communities on a system level means that we cannot predict how long the nutrient solution should recirculate between RAS and HP components before a stable, resilient, plant growth promoting community forms. Given the slow growth requirements of k-strategists (e.g. anammox (100-102), archaea (56, 103)), system-wide population maturation likely is on the order of months or years (104). It would not be unreasonable to expect successive waves of colonization to mark this period, as is similarly observed within the rhizobiome during root growth (105-107). This type of system would be most adept to handling stresses by increase the amount of candidate microbes for the rhizosphere as plant needs change, and limiting the spread of r-strategists through competition. If this hypothesis is proven, then circulation in aquaponics may play a more centralized role than currently recognized in plant rhizobiome resilience.

Finally, future rhizobiome maturation studies should prioritize active growth regions. Root tips are the site of growth (similar to hyphal tips in filamentous fungi), and thus the main point of active community interaction between plants and microorganisms (23). As such, they display a markedly different community, becoming less diverse further away from the tip (10, 108). Root border cells – often considered to be the white blood cells analogue for plants in the rhizosphere – may persist outside of the roots for weeks or months and are known to interact with both pathogenic and nonpathogenic members of the microbiome (109-111). These unique cells, marked by highly dynamic transcriptomic profiles (111-113), can produce up to >90% of carbon-based root exudates (110, 112, 114). RNA-seq has been shown to be an effective tool in rhizosphere studies when assessing transcriptional dynamics (115-117). It is not known how root border cell expression or translocation differs between soil and soil-less culture. Nonetheless, given their important to terrestrial rhizobiome management, they likely play a non-negligible role in shaping analogous aquaponic and hydroponic microbial communities.

**Microalgae – the rhizobiome storage chest?**

A related, currently overlooked factor in this dynamic is the role of microalgae in relation to the rhizobiome. Microalgae are known to interact with heterotrophic bacteria through the excretion of organic carbon (118, 119). Furthermore, their presence is ubiquitous in water-based plant cultivation. Whether algal exudates can provide safe havens for rhizobacteria “on standby” remains to be determined. If the algal-bacteria relationship surrounding the transfer of carbon in exchange for nutrients or stress-alleviation indeed mirrors rhizosphere-bacteria relationships to a significant degree, it may explain the observed similarity between the HNS stock and hydroponics treatments.

# Conclusion

Enhancing rhizobiome resilience and health has previously been described as a bifurcated approach to increase crop yields while simultaneously improving plant survivability (72). The complexity of the rhizosphere environment as well the large datasets involved in metagenomic studies make discerning trends difficult. Our study confirms that rhizosphere consolidation around a relatively stable community composition occurs in both aquaponics and hydroponics. Given similar observations of rhizobiome consolidation in aeroponics and soil environments, this is likely a fundamental process for rhizosphere management by plants. Important for the field of aquaponics, this indicates that the bacterial community in roots is not directly modulated by the RAS or biofilter. Other phenomena, such as the presence of exudates and organic acids may play a role determining growth rates of plants in aquaponic systems, although no direct benefit appears to occur as a result of changes to the microbiome. Our data suggests that the hydroponic environment does not hinder rhizosphere development, insomuch as the plant remains dominant in shaping its rhizobiome. How exudate profiles and border cell adaptations to soil-less cultivation change in soil-less cultivation is still missing from this picture.

The fundamental trend that does appear to occur in hydroponic environments is a multi-step reduction in diversity, first limited by the operational parameters (e.g. facility conditions, use of sterilization steps) and then by the plants themselves, wherein they exert a highly selective pressure on microorganisms that enter the rhizosphere.

We re-iterate that taxonomic comparison is assumptive, as are conclusions about the interactions based on singular timepoints. The dynamic nature of microbial structure means that microbiome functionality assessments must be prioritized over cataloguing. Measuring and assessing crop health in terms of macroscopic plant features and water quality parameters are well established fields. As such, future studies would do well to focus on the functionality that certain taxa are providing in the system specifically in response to stressors in the rhizosphere (e.g. abiotic, pathogenic stresses). It is well established that this occurs at the root tip, and as our data demonstrates, it is a signal initiatedby the plant. This is likely accomplished by modulating the quantity and diversity of root exudates as a function of stressors on the plant, in a manner likewise dependent on genotype and life stage. Bacteria that thrive in this environment do not necessarily reflect the community of the RAS and biofilter. This was demonstrated by mapping metabolic profiles (nitrogen, phosphorous, iron, sulfur) across treatments, indicating a divergence between the communities responsible for nutrient cycling in the RAS, biofilter, and hydroponic rhizobiomes. Future studies should couple predicative genotyping with transcriptomic analyses of the root tips. Linking the transcription of genes or pathways of known importance to shifts in the rhizobiome will inform crop management through a deeper understanding of which stresses are actively working on the plant at a particular timepoint.

# Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# Author Contributions

Alyssa Joyce and Victor Lobanov conceived the presented idea. Victor Lobanov carried out the experiments with input from Johanna A. Bac-Molenaar and Alexander Boedijn. Victor Lobanov wrote the paper with input from all authors.

# Funding

We would like to acknowledge funding from FORMAS Joyce 2017-00242 and the WUR Wimek mobility program.

# Acknowledgments

We would like to acknowledge the contributions of employees at WUR Bleiswijk for their assistance with the hydroponic cultivation, in particular Jan Janse, Marcel van de Graaf, and Trudy van Twist. We thank Peter Clift for his integral assistance in implementing the data analysis pipeline in python. Joe Pate likewise provided constructive insight in the interpretation of the data.

# Data Availability Statement

The datasets analyzed for this study, as well as an electronic version of the figures in this publication can be found in the repository “FiM2020Rhizobiome” at: <https://github.com/vonabol1/FiM2020Rhizobiome>.

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