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Tilapia recirculating aquaculture systems as a source of plant growth promoting bacteria

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ABSTRACT

A recirculating aquaculture system with farmed tilapia is the most popular combination in aquaponics, an integration of aquaculture and hydroponics. Despite nutrient rich fish-rearing water being regarded as a valuable resource for aquaponics, the quality and value of inhabitant microorganisms are certainly

understudied. Our present research illustrates the feasibility of the tilapia-rearing water as a valuable source of beneficial microorganisms called plant growth promoting bacteria (PGPB). Microbial communities were examined with a combination of culture-independent high-throughput 16S rRNA gene sequencing and cultivation methods. Microbial communities determined by high-throughput sequencing indicated the usefulness of Bacteroidetes and Alphaproteobacteria as beneficial microbial indicators to assess the health condition of recirculating aquaculture systems. Siderophore production, ammonia production, and phosphate solubilization assays were used for screening and 41% of isolates were identified as plant growth promoting bacteria. These bacteria were classified as Actinobacteria (eight strains [32% in total], *Dietzia*, *Gordonia*, *Microbacterium*, *Mycobacterium* and *Rhodococcus*), Bacilli (six strains [24%], *Bacillus* and *Paenibacillus*), Flavobacteriia (one strain [4%], *Myroides*), Betaproteobacteria (two strains [8%], *Acidovorax* and *Chromobacterium*) and Gammaproteobacteria (eight strains [32%], *Aeromonas*, *Plesiomonas* and *Pseudomonas*). We found that the tilapia-rearing water naturally contained various lineages of PGPB and could be esteemed as a worthy seed bank of PGPB. Because aquaponics is a difficult system to use pesticides and herbicides, the role of PGPB to prevent plant pathogens and maintain healthy root system may be more important than traditional agricultural settings.

KEY WORDS:
Tilapia, Recirculating aquaculture system (RAS), Aquaponics, Plant growth promoting bacteria (PGPB)

1. INTRODUCTION

Tilapia is are the second most farmed fish in the world and can be cultivated in either freshwater or brackish water (Wang & Lu 2015). The reason for tilapias popularity in aquaculture is its ability to withstand poor water quality, high stocking density, and a large variety of diets (DeLong, Losordo & Rakocy 2009). Nile Tilapia (*Oreochromis niloticus*) is the most common among four tilapia species belongs to the genus *Oreochromis* and accounts for 80 to 90 percent of all commercially farmed tilapia (Popma & Masser 1999; Suresh 2003).

Tilapia farming in a recirculating aquaculture system is the most popular shape in aquaponics, which is a hybrid system of aquaculture and hydroponics growing fish and plants together in one integrated water recirculating system (Love et al. 2015). The fish waste provides an organic food source for the plants, and the plants naturally filter the water for the fish. Aquaponics is considered as an environmentally friendly agricultural method advanced by the concepts of minimal usage of water compared to traditional agricultural systems.

Despite nutrient rich tilapia-rearing water is regarded as a valuable resource for aquaponics, quality and value of microorganisms in a recirculating aquaculture system for aquaponics use is certainly understudied (Bartelme, Oyserman, Blom, Sepulveda-Villet & Newton 2018). We hypothesized that the tilapia-rearing water could be a valuable source of beneficial microorganisms called plant growth promoting bacteria (PGPB) that can promote growth and protect the plant from harmful pathogens (Bartelme et al. 2018, Glick 2012). PGPB are also called plant growth promoting rhizobacteria (PGPR) (Grobelak, Napora & Kacprzak 2015; Timmusk & Wagner 1999) and plant growth promoting microorganisms (PGPM) (Bartelme et al. 2018), and they are used for various crops in sustainable agricultural systems.

Here, we explore the feasibility of the tilapia-rearing water as a valuable source of PGPB. Fish-rearing water samples were collected from Nile Tilapia (*Oreochromis niloticus*) recirculating aquaculture systems in southwest Florida. Water microbial communities were examined with a combination of cultivation methods and culture-independent molecular microbiology techniques (i.e. high-throughput 16S rRNA gene sequencing). Siderophore production, ammonia production, and phosphate solubilization assays were used to identify PGPR in recirculating aquaculture systems. We identified that the tilapia-rearing water naturally contained various lineages of bacteria with inherent plant growth promoting properties that could be used as a natural input to agricultural systems and could be esteemed as a worthy seed bank for future aquaponics.

2. MATERIALS AND METHODS

2.1. Fish culture systems and conditions

Nile Tilapia (*Oreochromis niloticus*) were cultured at three aquaculture farms using year-round integrated recycling aquaculture systems. The general information of RAS and operating conditions are summarized in Table 1. In Infinity Ag Solution, an environmental technology company in Fort Myers reared tilapias in 5000-gallon cylindrical plastic tanks (18927 L) in which pumped up ground water was ozonated, UV treated, degassed, passed through a biofilter, and lastly re-aerated (Fig. 1A). The Imaginarium Science Center (ISC) is growing Nile Tilapia in a 400-gallon (1514 L) tank for environmental education purpose and supplies water to peppers and tomatoes. This aquaponics system is completely enclosed and above ground (Fig. 1B). Southern Fresh Farms (SFF) was raising Nile Tilapia and recycled the water onto all their crops but it was not an aquaponics system (Fig. 1C). Southern Fresh Farms was using 5000-gallon (18927 L) tanks that used ground water treated through biofilters but no UV operation.

2.2. Water chemistry of fish tanks

The water quality parameters such as temperature, pH, dissolved oxygen (DO), total dissolved solids (TDS) and total ammonia nitrogen (TAN-N) were routinely monitored in the experimental period. Temperature, pH, conductivity, total dissolved solids, and DO were measured onsite using waterproof ExStik II meters (Extech, Nashua, NH, USA). Total ammonia nitrogen (TAN), nitrite, nitrate, phosphorus and chemical oxygen demand (COD) were measured using a HACH DR3900 spectrophotometer and their associated test kits (Hach, Loveland, CO, USA). The un-ionized ammonia was calculated from the pH, salinity and temperature. The total suspended solids (TSS) were determined by filtering through a standard GF/F glass fiber filter and drying the residue (Greenberg & Clesceri 1992).

2.3. Sample collection

Water samples were collected once every month for six months. For every pool, water samples were taken in two sterile glass bottles (500 mL), one for the chemical analysis and the other for the microbiological analysis. The samples for chemical analysis were transported to Infinity Ag Solutions within 20 min of collection. The collected bacteria samples were stored in a cooler and transported to Florida Gulf Coast University within 2 hours of collection.

2.4. Bacterial counting

Culturable bacterial numbers (i.e. CFU, colony forming units) were determined on tryptic soy agar (TSA) plates. Each ten microliter of water sample was spread out on the triplicated agar plates and incubated at an average fish tank temperature of 28 ± 2°C for a week. For total cell counting, water samples were fixed with formalin to be 2% (vol/vol) as a final concentration. After cells were stained with 4', 6-diamidino-2-

phenylindole (DAPI), fixed water samples were filtered onto black 0.22-µm isopore membrane filters (GTBP, MilliporeSigma, Burlington, MA, USA). An anti-bleaching agent was used as the mounting medium (AF1; Citifluor Ltd, London, UK). Cells were viewed under UV excitation at 1000× magnification using an Olympus BX-51 epifluorescence microscope. For each filter, more than 10 random fields were viewed to determine cell numbers.

2.5. Identifying plant growth promoting bacteria

Bacterial cultures grown on the TSA plates were purified by repeated streaking and maintained on the TSA or in the tryptic soy broth (TSB). After applying a standard Gram-staining procedure using crystal violet and safranin, isolates were tested for three plant-growth promoting abilities. Bacterial isolates were assayed for siderophores production on the Chrome azurol S agar medium (MilliporeSigma) described by Schwyn & Neilands (1987). Development of a yellow-orange halo around the growth was considered as positive for siderophore production (**Fig. 2A**). Bacterial phosphate solubilization was tested on Pikovskaya's agar plates for phosphate solubilization (Nautiyal 1999). Bacterial strains formed clear zones were identified positive for phosphate solubilization (**Fig. 2B**). Ammonia production was tested by using peptone water (1%) with Nessler's reagent and the color change from brown to yellow was recorded as positive for ammonia production (**Fig. 2C**). All tests were conducted in a temperature-controlled incubator at 28 ± 2°C for 48-72 hours.

2.6. Identification of bacteria with plant growth promoting abilities using 16S rRNA gene analysis

Bacterial DNA was extracted using a Gene Releaser with a standard thermal cycle protocol (65°C for 30 sec, 8°C for 30 sec, 65°C for 90 sec, 97°C for 180 sec, 8°C for 60 sec, 65°C for 180 sec, 97°C for 60 sec, 65°C for 60 sec and 80°C to hold) (Bioventures Inc. Murfreesboro, TN, USA). The PCR amplification of bacterial 16S rRNA gene was carried out using GM3 (AGA GTT TGA TCM TGG C) and GM4 (TAC CTT GTT ACG ACT T) primers (Muyzer, Teske, Wirsen & Jannasch 1995). The 50-µL PCR mixture contained 2.5 µL of the primer set (25 pmol each), 0.25 µL (1.25 U) of *Ex Taq* DNA polymerase (Takara Bio, Otsu, Japan), 5 µL of *Ex Taq* buffer (20 mM $MgCl_2$), 4 µL of deoxyribonucleotide triphosphate mixture, 2 µL of DNA template, and 36 µL of sterilized ultrapure water. The PCR reaction was performed by using a MultiGene OptiMax Thermal Cycler TC9610 (Labnet International, Edison, NJ, USA). The PCR program used for the gene amplification included an initial denaturing step consisting of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, and elongation at 72°C for 30 sec. The final extension step was 72°C for 10 min. After gene amplification, the reaction products were purified using a GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). A sequence primer (515R) was used for Sanger sequencing (GenScript,

Piscataway, NJ, USA). The DNA sequences were identified by RDP classifier (Cole et al., 2013), NCBI BLAST (Altschul, Gish, Miller, Myers & Lipman1990) and EZtaxon (Yoon et al. 2017). The determined partial 16S rRNA gene sequences were deposited in the GenBank under the accession numbers SUB4701988.

2.7. High-throughput sequencing

Water samples were collected three times from three different fish tanks of each aquaculture facility (Tank 1, Tank 2 and Tank 3). Upon return to the laboratory, 250 mL of water samples were passed through 0.2 µm polysulfonecellulose nitrate membrane filters (47mm diameter, ThermoScientific Nalgene Analytical Test Filter Funnels) to collect microbial biomass. A quarter size of filter was cut out and inserted into a FastPrep Lysing Matrix E Tubes (MP Biomedicals) and DNA extraction was carried out using a modified phenol-chloroform extraction method as described previously (Urakawa, Martens-Habbena & Stahl 2010). Nine DNA samples were sequenced using the Illumina MiSeq platform (RTL Genomics, Lubbock, TX, USA). We used 16S rRNA primers (515F GTG CCA GCM GCC GCG GTA A and 806R GGA CTA CHV GGG TWT CTA AT), which cover the hypervariable V4 region. To analyze sequence data, the forward and reverse reads were formatted into FASTQ and merged together using PEAR Illumina paired-end read merger (Zhang, Kobert, Flouri & Stamatakis 2014). The formatted FASTQ files were then converted into FASTA formatted files for further analyses. The obtained reads were run through a RTL-developed quality-trimming algorithm and sorted by length from longest to shortest. USEARCH (Edgar 2010) was used for the prefix dereplication process and clustering of sequences (3% divergence) in which sequences less than 100 bp and singleton clusters were removed. The result of this step was the consensus sequence from each new cluster. Operational taxonomic unit (OTU) selection was performed using the UPARSE-OTU selection algorithm (Edgar 2013) to classify the large number of clusters into OTUs. Chimera checking was performed on the selected OTUs using the UCHIME chimera detection software executed in de novo mode (Edgar, Haas, Clemente, Quince & Knight 2011) and detected chimeric sequences were removed from further analysis. As a final step, each read was mapped to their corresponding non-chimeric cluster using the USEARCH global alignment algorithm (Edgar 2010). Individual reads were further annotated using BLAST and RDP Pipeline (Cole et al. 2013). General statistics of sequence data and clustering analysis were implemented using the PAST ver. 3.14 (Hammer, Harper & Ryan 2001). Additional data analysis and the visualization of data were implemented using Microsoft Excel and SigmaPlot 12.0. The high-throughput 16S rRNA gene sequence data were deposited in the GenBank under Sequence Read Archive accession PRJNA503082.

3. RESULTS

3.1. Water quality

Water quality data were collected during August 2015 to March 2016. (**Table 2**). The water temperature ranged between 19.8 to 29.6°C. In Florida, no temperature control is made for intensive tilapia aquaculture and the temperature range reported here was lower than the typical water quality criteria for intensive tilapia aquaculture in the world (29-32°C) (Lukas & Southgate, 2003). The mean pH ranged between 6.4 to 8.9 and it was operated in a standard intensive freshwater fish culture condition (6.0 to 9.0) (Lukas & Southgate, 2003). Colony forming units ranged between 1.0 x 10^4 CFU / mL and 2.6 x 10^5 CFU / mL, which overlapped but slightly higher than the previous studies from earthen brackish water ponds rearing tilapia (10^2 to 10^4 CFU / mL) (Al-Harbi & Uddin 2005; Pakingking, Palma & Usero 2015). Total cell counts ranged between 8.8 x 10^5 cells / mL and 1.3 x 10^7 cells / mL (**Table 2**). Tank 1 water samples were characterized by high phosphorus and nitrate concentrations and low ammonia and nitrite concentrations, suggesting that nitrification was functional (**Table 3**). Water samples from Tank 3 and Tank 4 were characterized by high total ammonia nitrogen, nitrite and low nitrate concentrations. Nitrate and phosphorus concentrations were highly correlated with each other within all recirculating tanks (R^2 = 0.94, p = 0.026). We identified that Tank 1 water had the highest level of nutrients and followed by Tank 2, Tank 4 and Tank 3 in order. These nutrient data consistent with the water management history of these three aquaculture facilities. During the experimental period, Tank 1 had no water change whereas Tank 2 had one complete water change, and Tank 3 and 4 had three partial water changes.

3.2. Plant growth promoting bacteria

From four recirculating tanks, we were able to obtain 61 isolates (**Table 4**). We found that 87.5% of the isolates were Gram-negative, which was similar to the ratio of Gram-negative bacteria found in earthen ponds in the Philippines (84%) (Pakingking et al. 2015) and Saudi Arabia (87%) (Al-Harbi & Uddin 2005). Of these 61 strains, we found that 28 strains (46% of the isolates) were positive for siderophore production (**Table 4**). Siderophores are iron uptake chelating agents and siderophore-producing rhizosphere bacteria can enhance plant growth by increasing the amount of iron near the roots and inhibiting plant pathogens through the competition of available iron (Scher & Baker 1982; Guerinot & Yi 1994; Glick 2012). We also isolated 23 strains positive for phosphate solubilization (38% of the isolates) (**Table 4**). The phosphate solubilization assay determines if the isolated bacteria can solubilize phosphorous into a form that is available for plants (Sharma, Sayyed, Trivedi & Gobi 2013). Phosphorous is an important nutrient for plants but most phosphorus in the soil is found bound and unusable for plants (Glick 2012). Bacteria that can solubilize phosphorous help unlock the phosphorus for plant use and are important for the overall health of the plant (Glick 2012). Ammonia production was confirmed from 12 bacterial strains (20% of the isolates). Overall, 25 isolates (41% of the total isolates)

were identified as PGPB based on phenotypic characterizations. The taxonomic compositions of these isolates were determined by using the RDP classifier and fell into four classes: Actinobacteria (eight strains [32% in total], *Dietzia*, *Gordonia*, *Microbacterium*, *Mycobacterium* and *Rhodococcus*), Bacilli (six strains [24%], *Bacillus* and *Paenibacillus*), Flavobacteriia (one strain [4%], *Myroides*), Betaproteobacteria (two strains [8%], *Acidovorax* and *Chromobacterium*) and Gammaproteobacteria (eight strains [32%], *Aeromonas*, *Plesiomonas* and *Pseudomonas*) (**Fig. 3**). Actinobacteria and Gammaproteobacteria were two most dominant groups. To examine the relationships between these isolates and previously described PGPB, we identified the closest reference species using the EZtaxon (**Table 5**).

3.3. High-throughput sequencing

A total of 339,748 sequences were analyzed including 37,750 sequences as a mean data size of each water sample. The microbiota in three RAS was characterized at phylum and genus level (**Fig. 4** & **Fig. 5**). Overall Proteobacteria was the most dominating phyla of the microbiota (mean ± SE, 42% ± 5.9%, n = 9) and ranged from 8.5% (T1_Oct) to 70% (T3_Aug) (**Fig. 4**). Among the phylum Proteobacteria, Alphaproteobacteria (20% ± 5.8%, n = 9) and Betaproteobacteria (15% ± 3.6%, n = 9) were the most dominated classes and followed by Gammaproteobacteria (6% ± 2.1%, n = 9). Bacteroidetes (21% ± 4.7%, n = 9) was the second-largest phylum and followed by Actinobacteria (14% ± 4.1%, n = 9) and Fusobacteria (14.2% ± 6.8%, n = 9).

4. DISCUSSION

We successfully isolated and identified some beneficial PGPB and the relationships between isolates and previously described PGPB were examined. Three strains were identified as *Bacillus cereus* (99.6-99.8% identity), which elicits significant reductions in the incidence or severity of various diseases on a variety of hosts (Kloepper, Ryu & Zhang 2004). *Bacillus* spp. is common in soil and some strains are considered PGPB because of its ability to inhibit filamentous fungi (Cornea, Grebenisan, Mateescu, Vamanu & Campeanu 2002). Strain IAS_10_E1 was identified as *Paenibacillus dongdonensis* (99.8% identitiy), which is PGPB isolated from rhizospheric soil of *Elymus tsukushiensis* (Son, Kang & Ghim 2014). Strain ISC_2_C1 was identified as *Chromobacterium rhizoryzae* (100% identity), which is PGPB isolated from rice roots (*Oryza sativa*) and showed a good inhibitory effect on a plant pathogenic fungus, *Magnaporthe oryzae* (Zhou et al. 2016). Many species belong to *Bacillus* and *Paenibacillus* have been reported as PGPB (Timmusk & Wagner 1999) and a quarter of isolates belonged to this lineage (**Fig. 3**). Strain IAS_9_C4 was identified as *Myroides xuanwuensis*, which was isolated from forest soil and was able to weather biotite (a silicate mineral) and release silicon, aluminum and iron from the mineral (Zhang, He, Huang & Sheng 2014). Thus, this bacterium can assist the growth of plants by supplying iron and other

minerals. One of the expected roles of PGPB is protection of plants from their pathogens (Glick 2012). Nothing is known about the capability of plant protection of this bacterium, however, *Myroides odoratimimus* has an inhibition rate of 78.9% against a tobacco brown spot pathogen *Alternaria alternate* (You, Zhang, Feng, Wang & Kong 2015).

It has been widely documented that cultured bacteria normally occupy minor components in microbial communities and more than 99% of microbes are unculturable (Amann, Ludwig & Schleifer 1995). In this study, 1.2 ± 0.7% (mean ± SD, n = 23) of bacteria were culturable based on the estimate of CFU on TSA agar and total cell counts determined by fluorescence microscopy. Thus, we may anticipate incongruity between culture-based and culture-independent microbial community analysis. For example, Firmicutes formed 24% of isolates, however, the relative abundance of Firmicutes was moderate (2.5% ± 0.6%, n = 9) in the high-throughput sequencing analysis. The dominance of Gammaproteobacteria among cultured species (32%) did not match to the data of high-throughput sequencing. (0.6-21%) (**Fig. 3 and 4**).

The dominance of Proteobacteria and Bacteroidetes was congruent to previous high-throughput sequencing analyses from commercial scale RAS for Atlantic Salmon (Rud et al. 2017) and experimental RAS for Convict Grouper (*Epinephelus septemfasciatus*) (Lee et al., 2016), despite water conditions (e.g. salinity and temperature) of the RAS were quite different from our study. Occasionally, Proteobacteria and Bacteroidetes were inversely proportional to each other (Lee, Lee, Kim, Myeong & Kim 2016; Ruan, Guo, Ye, Liu & Zhu 2015), however, it was not the case in this study. The microbiota of Tank 1 was clearly distinguishable from those of Tank 2 and Tank 3 at the phylum level (**Fig. 4**). The microbiota of Tank 2 and Tank 3 occasionally overlapped each other, despite these two systems were so different; one was typical RAS with moderate operation size while the other was a small-scale aquaponics (**Fig. 4**). The largest difference among microbiota was found in the population of Fusobacteria, which is occasionally found in RAS (Fan et al. 2016; Giatsis et al. 2015). There were extremely higher percentages of Fusobacteria in Tank 1 (33 to 51%) while Fusobacteria formed a small population in the Tank 2 and Tank 3 (0.12 to 1.5%). Negative correlations of the relative abundance against Fusobacteria were found in Actinobacteria (R^2 = 0.48, p = 0.039, n = 9) and Betaproteobacteria (R^2 = 0.52, p = 0.028, n = 9). Within the Fusobacteria, 95% to 99% of sequences were classified as *Cetobacterium*, an anaerobic Gram-negative bacterium highly associated with animal gut including fish and sea mammals (Foster et al. 1995; Roeselers et al. 2011). We also found some sequences associated with Verrucomicrobia, which includes facultative and obligate anaerobes commonly found in soil, water and digestive tracts of animals including human (Dubourg et al. 2013; Navarrete et al. 2015). The abundance of Verrucomicrobia in Tank 1 (4 to 12%) was moderately higher than that of Tank 2 (0.3 to 0.5%) and Tank 3 (0.3 to 5%) although no significant difference was found (ANOVA, p = 0.053, n = 3). The recent human gut

microbiota study has documented the high-level of colonization of Verrucomicrobia following broad-spectrum antibiotic treatment (Dubourg et al. 2013). The genus level identification of major sequences revealed that they belong to the genus *Akkermansia*, human intestine associated anaerobic bacteria (Derrien, Vaughan, Plugge & de Vos 2004) (**Fig. 5**). Because Tank 1 maintained moderate to high DO concentrations (8.3 to 10 mg/L), which were not capable growth condition for anaerobes, the dominance of members of *Cetobacterium* and *Akkermansia* was considered as fecal origin from reared tilapia. The higher percentage of these bacteria attributed to a constant resuspension of fish waste caused by the shape of circular tank and convectively generated upward water flow in Tank 1. This tank had a main drain (127 mm in diameter) in the center of the tank to generate upward water flow in the tank to minimize the volume of dead water (**Fig. 1A**). The low frequent water exchange history may also attribute to the formation of this unique microbial flora. The resident bacteria of the tilapia's rearing environment directly mirrors the composition of the tilapia's gut microbiota (Del'Duca, Cesar & Abreu 2015; Giatsis et al. 2015; Pakingking et al. 2015). Conversely, our data showed that gut microbiota could potentially influence microbial community of fish-rearing water. It should be noted that no negative impact on the tilapia growth and system operation were documented in this aquaculture facility.

Nitrifying microorganisms play an essential role in the nitrogen cycle of RAS because the accumulation of ammonia and nitrite is critical in this system (van Rijn 2013). The high-throughput sequencing data were used to identify nitrifying microbial communities. The initial automatic pipeline analysis showed that the sequences belonged to Nitrosomonadaceae (*Nitrosomonas* and *Nitrosospira*) and they formed seven OTUs and less than 0.3% of total communities: Three *Nitrosomonas*, two *Nitrosospira* and two *Nitrosococcus*. However, following nucleotide BLAST analysis with an exclude uncultured/environmental sample sequences option confirmed that only three OTUs were confidently associated with ammonia oxidizers. These OTU sequences were closest to the following cultured ammonia oxidizer species: *Nitrosomonas oligotropha* (96% identity), *Nitrosomonas ureae* (98% identity) and *Nitrosospira multiformis* (95% identity). No ammonia-oxidizing archaea were detected in this study. Our results indicate that monitoring of fish-rearing water is not suitable for the tracking of nitrifying microorganisms in RAS because these microorganisms mainly inhabit biofilters (Urakawa, Tajima, Numata & Tsuneda 2008; van Rijn 2013).

High-throughput sequencing approaches allow us to analyze microbial communities in a different depth of taxonomic rank (from phylum to species). Lines of evidences suggested that RAS microbial community compositions are consistent despite the differences of operational conditions. Nearly all reports documented that Proteobacteria and Bacteroidetes are two most abundant taxonomic groups at the phylum level (Lee et al. 2016; Ruan et al. 2015). The dominance of these two phyla are also common in various aquatic environments (Garcia, Ketover, Loh, Parsons & Urakawa 2015; Vaz-Moreira, Egas,

Nunes & Manaia 2011; Urakawa & Bernhard 2017). A major difference of microbial communities found in RAS and various aquatic systems is the abundance of the phylum Cyanobacteria, which is minor in RAS but formed a substantial population in tilapia-rearing ponds (Fan et al. 2016). Generally, microbial community analysis at phylum level critically limit the resolution of high-throughput sequence analysis (Fan et al. 2016). In this study, we focused on the microbial population analysis of Alphaproteobacteria, Bacteroidetes and Verrucomicrobia at the genus level (**Fig. 5**). These genus level identifications of microbial community successfully discriminate microbial flora of each water sample and this concept is outlined by Urakawa & Bernhard (2017). We propose the usefulness of Bacteroidetes and Alphaproteobacteria as beneficial and universal microbial indicators to assess the health condition of RAS. These two groups were abundant in both freshwater and saltwater RAS. Bacteroidetes were originally classified as *Cytophaga-Flexibacter-Bacteroides* (CFB) group and their importance of degradation of high-molecule organic matters are well recognized (Kirchman 2002). The majority of species belonging to the *Cytophaga* and *Flexibacter* groups are aerobic while species belong to *Bacteroides* are anaerobes. Thus, monitoring of population structures and succession of Bacteroidetes may be beneficial for the water management of aquaculture to assess the redox value and degradation process of organic matters.

Despite, the sequences belonged to the genus *Rhizobium* were found in all aquaculture facilities, their relative abundance was less than 0.2%. No sequences were identified as *Frankia* spp. in the high-throughput sequencing data. These results suggested that representative nodule-forming rhizobacteria are less abundant in tilapia-rearing water although other PGPB were abundant in the same water. The potential importance and richness of *Rhizobium* were reported from rhizospheres of constructed floating wetland plant roots despite the low abundance of *Rhizobium* in ambient water (Urakawa, Dettmar & Thomas 2017). Because aquaponics is a difficult system in which to use pesticides and herbicides, the role of PGPB to prevent plant pathogens and maintain healthy root system may be more important than traditional agricultural settings.

5. CONCLUSION

This study shed light on the potential value of the water that houses tilapia. Microbial communities found in tilapia-rearing RAS had inherent plant growth promoting properties that could be used as a natural input to agriculture. Our research revealed that nutrient rich tilapia-rearing water used in RAS could be utilized as an isolation source of PGPB and ideal for aquaponics because plants can directly receive a benefit from circulating water that naturally embraces PGPB. Although the results here have demonstrated the usefulness of tilapia effluent, future work is required to design an integrated system to efficiently produce large quantities of PGPB by rearing tilapia in RAS. In the future, PGPB production

may reduce the use of chemical fertilizer in agriculture and horticulture, and being a beneficial component for tilapia aquaculture and aquaponics.

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CONFLICT OF INTEREST

These authors declare no conflict of interest.

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Figure legends

Fig. 1.

Examined recirculating aquaculture systems: Infinity Ag Solutions recirculating tanks (a), Imaginarium Science Center aquaponics system (b) and a rectangular tank of Southern Fresh Farms (c).

Fig. 2.

Phenotypic tests for the identification of plant growth promoting bacteria showing a positive colony of siderophore production (a), phosphate solubilization (b) and the negative (three left test tubes) and positive (three right test tubes) results of ammonia production (c).

Fig. 3.

Taxonomic composition and relative abundance of plant growth promoting bacteria isolated from tilapia-rearing water in three recirculating aquaculture systems.

Fig. 4.

Taxonomic affiliation of high-throughput sequencing reads showing microbial composition at the phylum level. Relative abundance of each microbial group (a) and Ward's method of hierarchical clustering of nine samples (three sampling from each tank, Tank 1, 2 and 3). (a) Proteobacteria are shown at the class level. Some major taxonomic groups are labeled to increase the readability of the figure: Ac, Actinobacteria; Al, Alphaproteobacteria; Ba, Bacteroidetes; Be, Betaproteobacteria; Fu, Fusobacteria; Ga, Gammoproteobacteria; Ve, Verrucomicrobia. (b) The numbers near the nodes show bootstrap percentages.

Fig. 5.

Taxonomic composition and relative abundance at the genus level in three bacterial lineages, (a) Alphaproteobacteria, (b) Bacteroidetes and (c) Verrucomicrobia. The total numbers of sequence counts in each sample are normalized to be 10,000 in total to remove the bias of different number of sequence reads in each sample.

Table 1. Recirculating aquaculture systems and operated conditions

Location[1]	Tank	Area of tanks (m^2)	Water volume (L)	Water depth (cm)	Feed (kg/day)	Feed type	Average weight of fish (kg)	Type of biofilter
IAG	1	54.72	20063	121.92	1.4	Tilapia ultimate growout pellet	0.68	Drum filter
ISC	2	1.86	1699	91.44	0.03	Wardley pellet formula	0.31	Submerged bed filter
SFF	3	15.04	19860	91.44	0.711	Purina aqua pro 400	1.1	Submerged bed filter
SFF	4	15.04	19860	121.92	0.711	Purina aqua pro 400	0.59	Submerged bed filter

[1] IAG: Infinity Ag Solutions, ISC: Imaginarium Science Center, SFF: Southern Fresh Farms.

Table 2. Water quality collected during the experimental period

	pH	Temp (°C)	DO (mg/L)	Conductivity (µS/cm)	Total dissolved solid (mg/L)	Total suspended solids (mg/L)	Chemical oxygen demand (mg/L)	Cultured cells (x 10^4) (CFU/mL)	Total cells (x 10^6) (cells/mL)
Tank 1 (IAG)	8.90 (±0.20) 8.69-9.23	26.4 (±2.9) 21.2-29.6	9.05 (±0.67) 8.34-9.87	877 (±39) 825-930	616 (±29) 578-656	2.9 (±1.7) 1.0-6.0	59.2 (±15.4) 46-86	5.15 (±5.14) 1.04-13.6	4.28 (±2.84) 0.88-8.12

Tank 2	6.38	25.1	7.36	659	294	5.3	14.3	5.46	6.10
(ISC)	(±0.78)	(±2.9)	(±1.74)	(±556)	(±255)	(±3.57)	(±10.1)	(±2.15)	(±0.59)
	5.47-7.58	19.8-27.6	4.61-8.87	113-1557	78-781	0.5-10	8-26	2.65-7.86	5.39-6.71
Tank 3	7.83	25.5	6.38	1436	987	4.01	112.4	15.0	7.90
(SFF)	(±0.38)	(±1.2)	(±0.63)	(±138)	(±79)	(±2.2)	(±11.6)	(±8.03)	(±1.86)
	7.46-8.45	23.4-26.7	5.57-7.24	1226-1625	854-1070	2.5-8.5	99-128	1.82-26.2	5.83-9.98
Tank 4	8.06	25.4	8.09	1479	1023	6.0	105.4	8.32	10.4
(SFF)	(±0.36)	(±1.0)	(±0.84)	(±68)	(±52)	(±1.9)	(±14.0)	(±4.35)	(±2.25)
	7.49-8.38	23.4-26.1	6.54-8.82	1394-1563	968-1091	3.3-8.6	89-122	2.04-14.0	6.72-13.4

Data indicate the mean (top), standard deviation (middle) and range (bottom) (n = 6).

Table 3. Nitrogen and phosphorus concentrations during the experimental period

	Total ammonia nitrogen (mg-N/L)	Unionized ammonia (mg-N/L)	Nitrite (mg-N/L)	Nitrate (mg-N/L)	Phosphate (mg-P/L)
Tank 1	0.214	0.079	0.127	14.99	8.5
(IAG)	(±0.142)	(±0.050)	(±0.093)	(±3.43)	(±3.1)
	0.025-0.340	0.015-0.128	0.055-0.27	11.38-19.21	4.8-13.2
Tank 2	0.683	0.0077	0.086	7.87	3.8
(ISC)	(±0.792)	(±0.018)	(±0.090)	(±4.00)	(±3.0)
	0.018-1.83	0.00003-0.045	0.010-0.237	1.65-12.60	0.9-8.9
Tank 3	1.222	0.071	0.379	1.91	1.2
(SFF)	(±0.987)	(±0.090)	(±0.132)	(±1.46)	(±0.9)
	0.348-2.99	0.009-0.251	0.247-0.560	0.55-4.14	0.3-2.3
Tank 4	0.760	0.070	0.417	4.00	3.4
(SFF)	(±0.482)	(±0.062)	(±0.301)	(±3.20)	(±2.2)
	0.087-1.31	0.007-0.163	0.014-0.878	0.92-8.43	0.7-5.8

Data are shown as the mean, standard deviation and range (n = 6).

Table 4. Characterization of plant growth-promoting bacteria

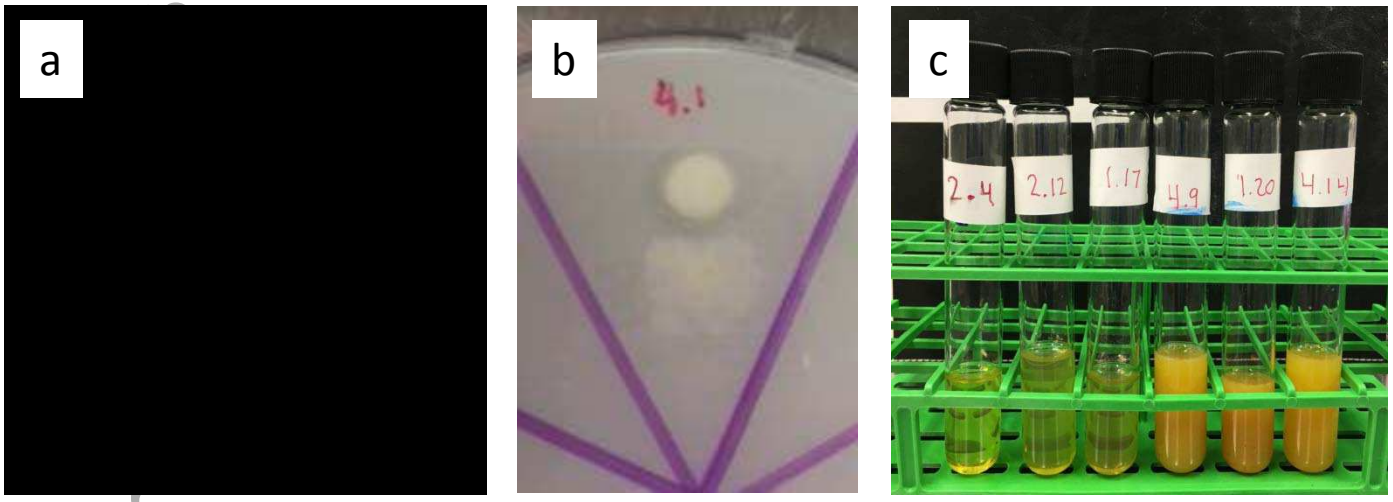
Location	Number of bacteria examined	Positive for siderophore production	Positive for NH_3 production	Positive for phosphate solubilization	All tests were negative
Tank 1 (IAG)	15	9 (60)	3 (20)	7 (47)	3 (20)
Tank 2 (ISC)	13	4 (31)	1 (8)	5 (38)	5 (38)
Tank 3 (SFF)	14	7 (50)	3 (21)	3 (21)	6 (43)
Tank 4 (SFF)	19	8 (42)	5 (26)	8 (42)	3 (16)
Total	61	28 (46)	12 (20)	23 (38)	17 (28)

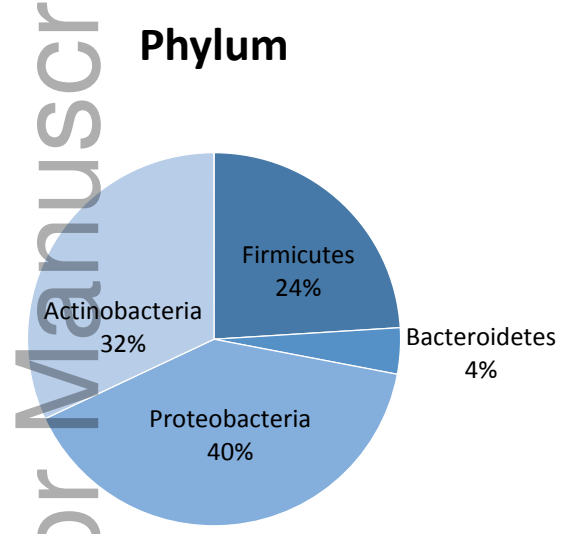
Data are the number and percentage of cultures.

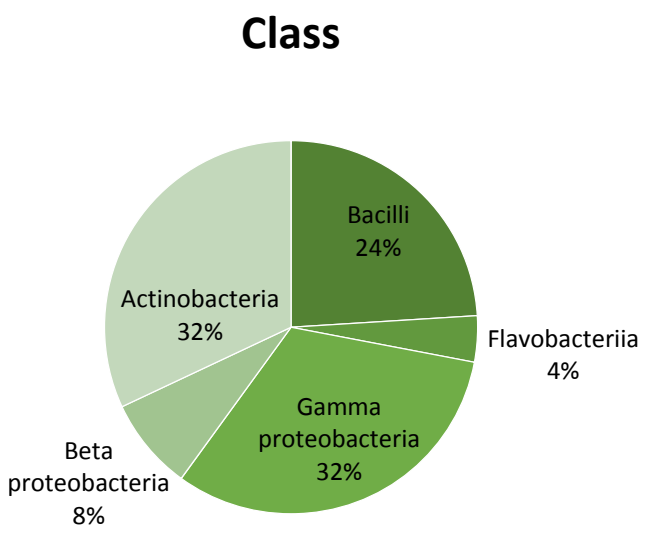
Table 5. Taxonomic identification of plant growth-promoting bacteria using 16S rRNA gene sequences

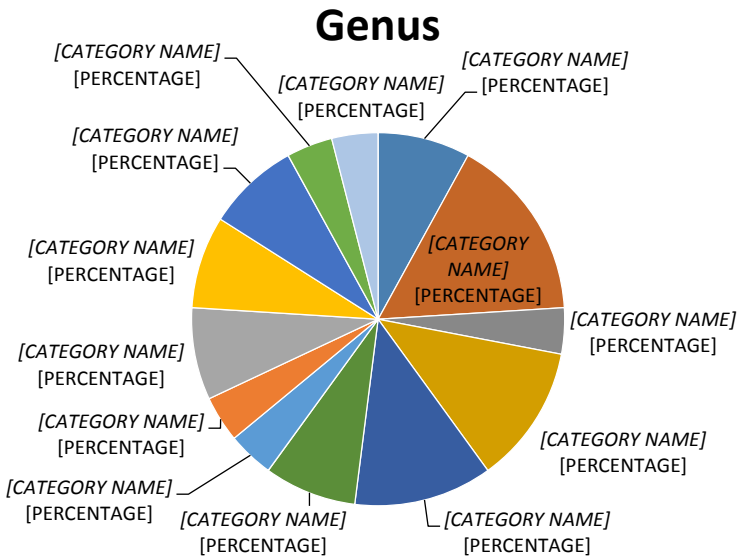
Isolate	Class	Top-hit taxon	Top hit type strain	Diff./total nucleotide	Similarity (%)
SFF_19L_D3	Actinobacteria	Dietzia timorensis	ID05-A0528(T)	0/433	100
IAS_6_A8	Actinobacteria	Mycobacterium farcinogenes	DSM 43637(T)	0/433	100
IAS_5_A3	Actinobacteria	Gordonia hongkongensis	HKU50(T)	0/437	100
SFF_4R_ C5	Actinobacteria	Gordonia polyisoprenivorans	NBRC 16320(T)	7/436	98.39
IAS_4_C8	Actinobacteria	Rhodococcus jostii	DSM 44719(T)	4/432	99.07
ISC_6_D5	Actinobacteria	Rhodococcus jostii	DSM 44719(T)	2/433	99.54
IAS_9_C4	Actinobacteria	Microbacterium diaminobutyricum	RZ63(T)	10/445	97.75
ISC_12_D8	Actinobacteria	Microbacterium sediminicola	YM10-847(T)	10/458	97.82
SFF_17L_G	Flavobacteriia	Myroides xuanwuensis	DSM 27251(T)	3/469	99.36
IAS_20_A4	Bacilli	Bacillus wiedmannii	FSL W8-0169(T)	8/490	98.37
ISC_1_B7	Bacilli	Bacillus cereus	ATCC 14579(T)	2/482	99.59
ISC_5_D4	Bacilli	Bacillus cereus	ATCC 14579(T)	1/482	99.79
SFF_7R_B3	Bacilli	Bacillus cereus	ATCC 14579(T)	1/481	99.79
IAS_10_E	Bacilli	Paenibacillus dongdonensis	KUDC0114(T)	1/448	99.78
ISC_10_C3	Bacilli	Paenibacillus xylanilyticus	XIL14(T)	2/488	99.59
ISC_3_A1	Betaproteobacteria	Acidovorax delafieldii	DSM 64(T)	0/443	100
ISC_2_C1	Betaproteobacteria	Chromobacterium rhizoryzae	LAM1188(T)	0/129	100
IAS_1_A2	Gammaproteobacteria	Aeromonas veronii	CECT 4257(T)	0/464	100
ISC_15_F	Gammaproteobacteria	Aeromonas veronii	CECT 4257(T)	1/462	99.78
SFF_21L_B6	Gammaproteobacteria	Aeromonas veronii	CECT 4257(T)	0/462	100
IAS_13_C2	Gammaproteobacteria	Plesiomonas shigelloides	NCTC 10360(T)	0/457	100
SFF_21R_B5	Gammaproteobacteria	Plesiomonas shigelloides	NCTC 10360(T)	1/474	99.79
SFF_9R_B2	Gammaproteobacteria	Plesiomonas shigelloides	NCTC 10360(T)	2/460	99.57
IAS_15_A7	Gammaproteobacteria	Pseudomonas alcaligenes	NBRC 14159(T)	9/475	98.11

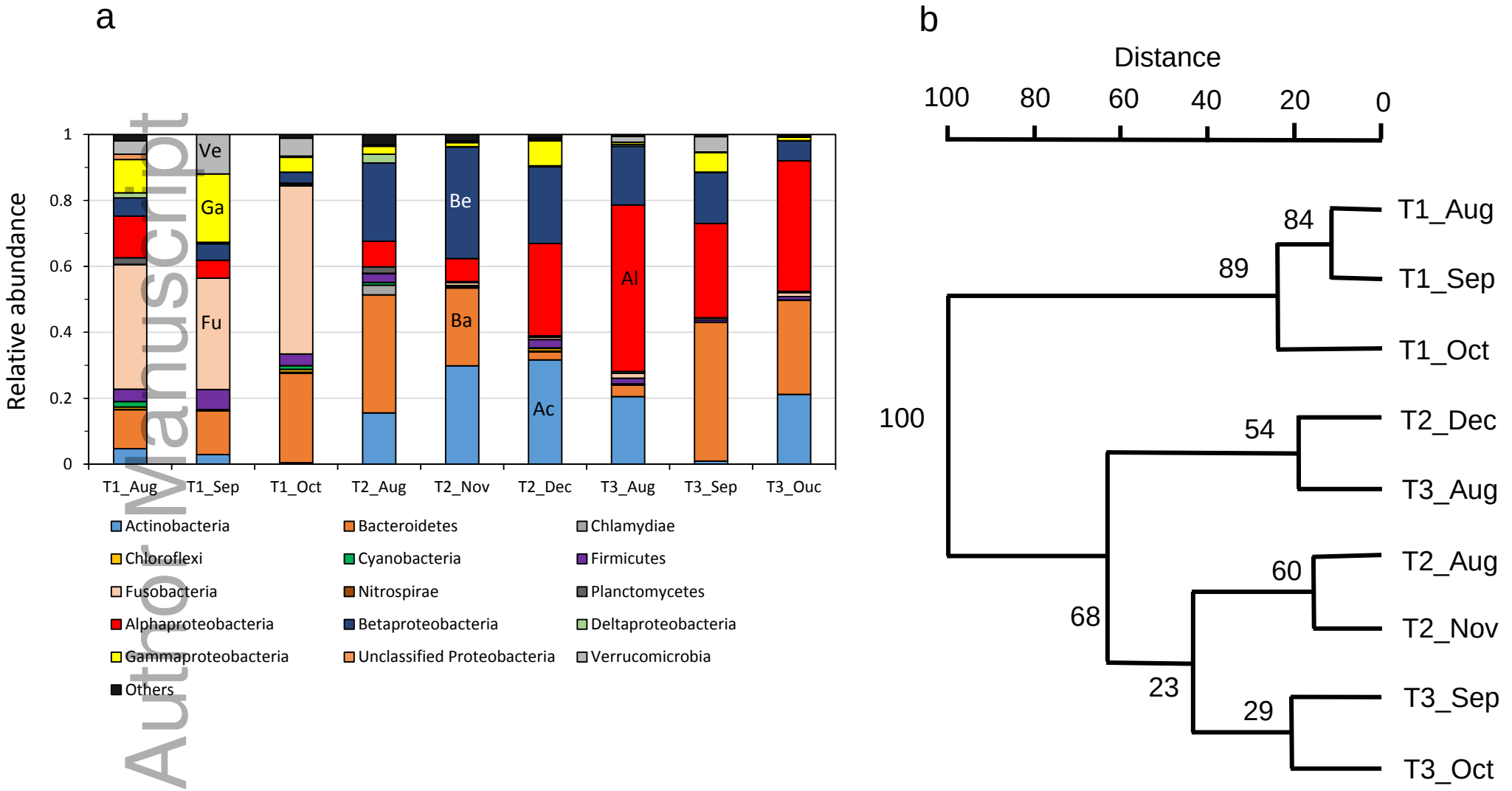
SFF_23L_D6	Gammaproteobacteria	Pseudomonas peli	R-20805(T)	4/468	99.15

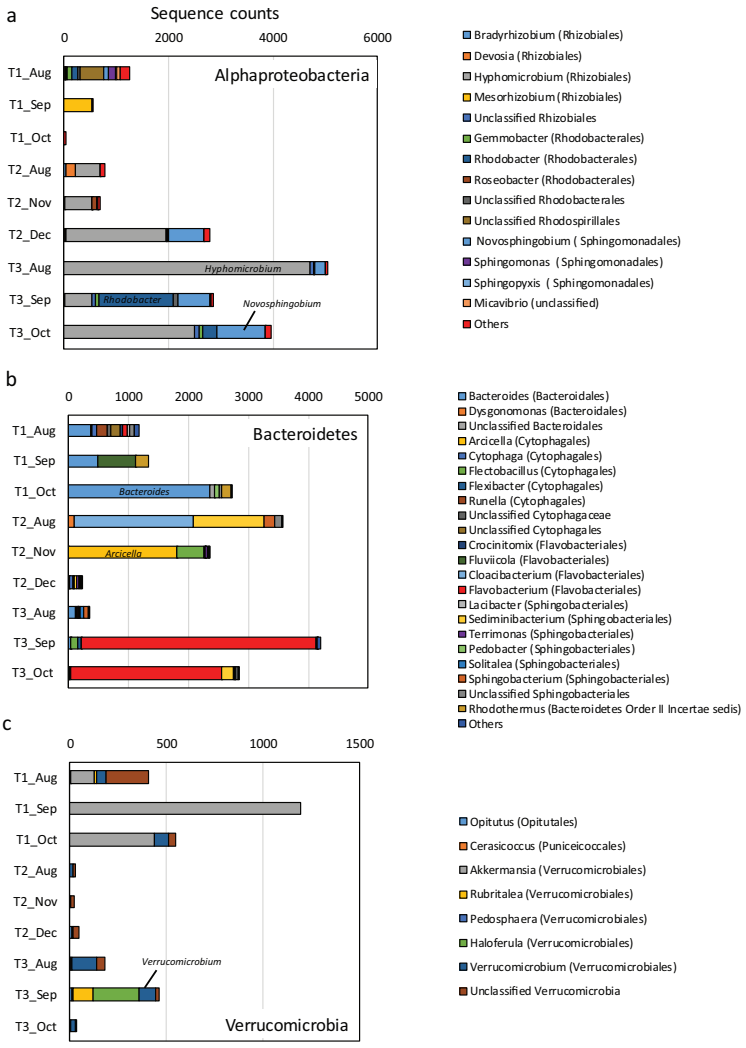












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