Short communication

Use of *Bacillus* spp. to enhance phosphorus availability and serve as a plant growth promoter in aquaponics systems

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Abstract

Plant growth promoters (PGP) are microorganisms essential for sustainable food production systems by improving the productivity of crops and mitigating environmental impacts. Microorganisms enhance the P availability to plants by mineralizing organic P and solubilizing precipitated phosphates. This work is focused on the effect of inoculation of a commercial product containing a mixture of *Bacillus* spp. on hydroponically grown lettuce (*Lactuca sativa*) integrated with tilapia (*Orechromis niloticus*) aquaculture in a closed-loop system, in comparison with an untreated control. We determined plant growth and crop quality parameters to assess the efficacy of the beneficial microorganisms. A nutrient dynamics analysis was conducted to evaluate the effect of *Bacillus* inoculation on the changes of nutrient concentration in aquaponics solutions, as well as the phosphorus accumulation in several components (fish, plants, water and solids). We performed a plate-count assay to quantify the number microorganisms present in systems inoculated or not with the commercial *Bacillus* mixture. In general, nutrient dynamics was affected by the inclusion of the *Bacillus* mixture in the water. Systems that received the product showed faster decreases in ammonia concentration and faster increase in nitrite and nitrate concentrations than the control. The untreated aquaponics systems showed lower accumulation of phosphorus in the water than systems receiving the *Bacillus* mixture, which resulted in poor plant growth, low phosphorus accumulation in the leaves and low chlorophyll content. However, the mass balance analysis showed that an external source of phosphorus possibly contributed to the overall P budget in systems receiving the *Bacillus* mixture. The microbial plate count assay demonstrated an active microbiota in aquaponics systems receiving the treatment while untreated systems showed zero microbial counts. The *Bacillus* mixture used in the present study appears to have PGP properties

and to affect P dynamics in aquaponics systems. However, since the product contained traces of phosphorus in its composition, further analysis will be necessary to distinguish whether the advantageous effects promoted by the *Bacillus* occurred as a result of a beneficial microbial activity or a fertilizing effect.

Keywords: *Bacillus*, Aquaponics, Phosphorus, Tilapia, Lettuce

1. Introduction

Integrated aquaculture with soilless vegetable production in a closed-loop system, commonly referred to as aquaponics, has received considerable attention due to the system's capability to raise fish at high density, sustain adequate water quality, minimize water exchange and produce an additional marketable vegetable crop (Danaher et al., 2013). In aquaponics, water from the aquaculture sub-system loaded with fish waste is fed to a biological filtration sub-system mainly responsible for nitrification and solids removal, to then be delivered to a hydroponics subsystem and ultimately utilized by plants as nutrients.

There has been a worldwide trend towards the use of plant-based ingredients in the manufacturing of aquafeeds (Gatlin et al., 2007). Plant-based ingredients (e.g. soybeans, corn and wheat) contain high amounts of phytic acid (or phytate), the main storage form of phosphorus in many plant tissues. However, phytate cannot be utilized by fish as a direct source of phosphorus (Cao et al., 2007; Cho and Bureau, 2001). The addition of the enzyme phytase to liberate free phosphorus from phytate is a necessary step when significant portions of plant-based ingredients are used in aquafeed formulations (Hien et al., 2015). Although a number of plant-produced

62 phytases have been characterized, it has been suggested that the activity of these enzymes in roots
63 is insufficient for effective utilization of phytate by plants (Mudge et al., 2003).

64 Microorganisms known as plant growth promoters (PGP) play an important role in
65 sustainable food production systems by improving the productivity of agricultural crops and
66 mitigating environmental impacts caused by the indiscriminate use of chemical inputs in
67 agriculture (Mangmang et al., 2014). Microorganisms enhance the P availability to plants by
68 mineralizing organic P and solubilizing precipitated phosphates (Khan et al., 2009; Ruzzi and
69 Aroca, 2015). Evidence of naturally occurring rhizospheric phosphorus solubilizing
70 microorganisms dates back to 1903, in which *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B.*
71 *polymyxa*, *B. sircalmous*, *Pseudomonas striata*, and *Enterobacter* are referred to as the most
72 important species (Khan et al., 2009). *Bacillus* is one of the most studied plant growth promoting
73 rhizobacteria (Ahmad et al., 2008), but its use in aquaponics systems has not been described.

74 Lettuce is the most frequently cultivated plants in commercial aquaponics operations, as
75 reported by 68% of respondents to an international survey (Love et al., 2015), while the most
76 commonly raised aquatic species is tilapia (Love et al., 2015, 2014). This work focused on the
77 effect of inoculation of a commercial product (Sanolife® PRO-W) containing a mixture of *Bacillus*
78 *subtilis* and *Bacillus licheniformis* on hydroponically grown lettuce (*Lactuca sativa*) integrated
79 with tilapia aquaculture (*Orechromis niloticus*) in a closed-loop system, in comparison with an
80 untreated control. We determined plant growth and crop quality parameters to assess the efficacy
81 of the beneficial microorganisms. A nutrient dynamics analysis was conducted to assess the effect
82 of *Bacillus* inoculation on the changes of nutrient concentration in aquaponics solutions, as well
83 as the phosphorus accumulation in several components (fish, plants, water and solids). The study
84 also assessed the microbial activity in the aquaponics systems.

2. Material and Methods

2.1. Aquaponics system design and operation

The experiment was carried out in a free standing steel A-frame greenhouse with a double wall polycarbonate glazing at the Controlled Environment Agriculture Center (CEAC), Tucson, Arizona, USA. The greenhouse is oriented in a north-south configuration. Greenhouse environmental control consisted of evaporative cooling and natural gas heating to maintain temperatures throughout the growing seasons. Environmental parameters (air and water temperature, humidity, and photosynthetic active radiation) were monitored and controlled for optimal plant production. During the experimental period, plants were grown under natural photoperiod conditions and air temperature set point of 25°C. Six replicated experimental aquaponics units, each consisting of a 100-L fish tank, 20-L sump partially filled with 10 L of biofilter medium (Biospheres, Amiracle) and two hydroponics channels were used to conduct the study (Fig. 1). Each individual aquaponics system was treated as an experimental unit and three units per treatment (control and *Bacillus*) comprised the experimental design (n = 3).

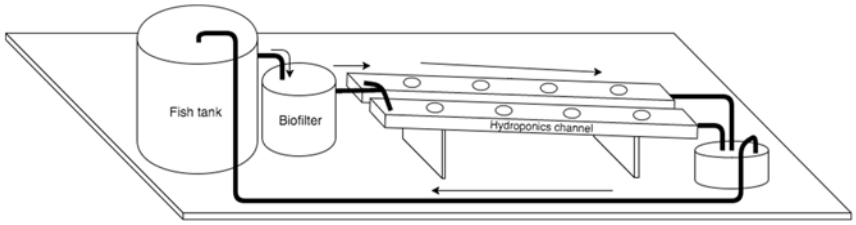

Figure 1. Diagram of an experimental aquaponics unit used in the study.

Potassium was supplemented as potassium sulfate to all aquaponics systems when necessary to maintain a K_2O:N ratio close to 1:1. All systems received the same amount of potassium therefore not all systems were kept under an exact 1:1 K2O:N ratio. We realized that supplementing different amounts of potassium to each system could have caused an interference in plant growth results, since lettuce responds well to potassium fertilization. Magnesium was supplemented by foliar spraying twice a week using a 1.5% solution of magnesium sulfate salt. Micronutrients (Fe, Cu, Zn, Bo, Mn, and Mo) were also supplemented by foliar spraying with a 0.15 g L^{-1} solution of a micronutrient blend product (S.T.E.M, Peters Professional) every two days during the first two weeks and twice a week during the last two weeks after transplanting.

Air temperature, relative humidity, and solar radiation were monitored using a data logger (Campbell Scientific CR23X). Dissolved oxygen in fish tanks was checked on a daily basis using a YSI Pro20 handheld meter and remained constant above 5 mg L^{-1} in all units. The pH of aquaponics nutrient solutions was monitored on a weekly basis using a handheld pH meter (HACH Hq40d multi) and remained constant at 8.1 ± 0.1 throughout the experimental period.

2.2. Diet formulation and manufacturing

A basal diet containing no supplemental inorganic phosphorus (Table 1) was manufactured at the Environmental Research Lab (ERL) in Tucson, Arizona.

126 Table 1.
127 Formulation and chemical composition of basal diet.

Ingredients	Contents
	g kg^{-1}
Soybean meal	538.0
Wheat gluten	80.0
Corn	277.0
Soybean oil	40.0
Calcium carbonate	20.0
DL-methionine	5.0
Mineral and vitamin mix†	40.0
Proximate composition (dry matter basis)	
Crude protein (g kg^{-1})	363.9
Crude lipid (g kg^{-1})	79.0
Ash (g kg^{-1})	20.0
Crude fiber (g kg^{-1})	588.2
Energy (Mcal kg^{-1})	3.77

128 † Mineral and vitamin mix contents per kg of product: Fe 40 mg; Cu 4 mg;
129 Zn 50 mg; Iodine 40 mg; Mn 60 mg; Se 0.4 mg; Co 0.5 mg; vitamin A 2 325
130 000 USP; vitamin D3 65 000 USP; vitamin E 32 500 IU; vitamin K 793.65
131 mg; vitamin C 87 100 mg; vitamin B1 2 600 mg; vitamin B2 3 250 mg;
132 vitamin B6 2 600 mg; vitamin B12 10 000 µg; pantothenic acid 15 600 mg;
133 biotin 40 mg; folic acid 780 mg; niacin 19 500 mg.

134

135 2.3. Fish and lettuce culture conditions

136

137 Tilapia juveniles with an average initial body weight of 70 g were transferred to their
138 respective tanks on 24 December 2015, 15 days before the transplanting of lettuce seedlings for
139 acclimation of the biofilters. The biofilters were inoculated with commercial product including
140 nitrifying bacteria (API QUICK START®) that allows for instant addition of fish in a new system
141 as it immediately starts the natural nitrogen cycle. Four 11-day-old seedlings (Johnny's Seeds, Red
142 Cherokee var., pelleted) were transplanted to each hydroponics channel on 7 January 2016,
143 comprising a total of eight lettuce plants for each aquaponics experimental unit. Plants were grown
144 for 6 weeks and harvested on day 18 February 2016 for biomass determination and tissue analysis.

145 Fish were fed the formulated diets with the aid of automatic feeders at approximately 1.0% of body
146 weight per day, for three weeks. All fish were harvested on day 14 January 2016.
147 Three aquaponics units were randomly assigned to each treatment (*Bacillus* and Control).
148 Aquaponics units assigned to the *Bacillus* treatment received 2.41 grams of a commercial *Bacillus*
149 mixture (Sanolife® PRO-W; 5.0×10^{10} CFU g^{-1}, INVE), twice a week until the end of experiment
150 following manufacturer's instructions (0.02 g of product per litter of water). Product was
151 introduced in the biofilter component of the aquaponics system.
152
153 2.4. Phosphorus content analysis and chlorophyll concentration
154
155 Lettuce samples were collected at the end of the experiment, oven-dried at 65°C for 72
156 hours, and weighed for dry mass determination. Dry samples were finely ground and 10 mg
157 aliquots were transferred to glass vials, to which were added the contents of one potassium
158 persulfate powder pillow (Hach PERMACHEM® Reagents) and 2 mL of sulfuric acid (5.25N,
159 Hach Company, Loveland, Colorado, USA). Vials were transferred to a block digester (HACH
160 COD Reactor) and digested at 150ºC for 4 hours. After complete digestion, vials were let to cool
161 off at room temperature and received 2 mL of NaOH solution (5.0N, Hach Company, Loveland,
162 Colorado, USA). An aliquot (0.1 mL) was pipetted into a different glass vial and the volume was
163 adjusted to 10 mL using demineralized water. Phosphorus concentration in the final sample was
164 determined in a portable colorimeter (Hach DR/850 Colorimeter, Hach Company, Loveland,
165 Colorado, USA) using the Hach Method 8048 (Hach Company, Loveland, Colorado, USA) and
166 the content of phosphorus in the dry sample calculated.

Two fish per experimental unit were collected and individually ground using a blender. The ground-fish paste was weighed and oven-dried at 65ºC for 72 hours. Fish dry samples were finely ground and 10 mg aliquots were processed as described for lettuce dry samples. Before the beginning of the experiment, six fish with similar weight and from the same batch of animals used in the experiment were processed as described above to determine the initial phosphorus content in the carcass. All fish used in the P content analysis had their scales removed before being put in the blender to facilitate the grinding process. Pellets from the feed used in the experiment were finely ground, and 10 mg aliquots were processed as described for lettuce dry samples. Solids that accumulated over the trial period were collected at the end of the experiment from each aquaponics biofilter. A 0.5 L aliquot of the original solids sample was homogenized for two minutes in a blender. A 0.1 mL aliquot was pipetted from the blended sample, and the volume adjusted to 5 mL using demineralized water and processed using Hach Total Phosphorus Method 8190. The chlorophyll concentration index (CCI %) was measured on three different leaves per plant using a portable meter (Apogee CCM-200) prior to harvesting.

2.5. Nutrient concentration dynamics

On a weekly basis, water samples were taken from each aquaponics sump to determine dissolved concentration of ammonia, nitrite, nitrate, orthophosphate, potassium, and total dissolved solids (TDS). Ammonia concentration was determined using the Hach Ammonia Low-Range Standard Method 10023. Nitrite concentration was determined using the Hach Nitrate High-Range Standard Method 8192. Nitrate concentration was determined using the Hach Nitrate High-Range Standard Method 8039. Orthophosphate concentration was determined using the Hach

method 8048. Potassium concentration was determined using a portable K^+ meter (LAQUAtwin K^+; Horiba, Kyoto, Japan). Total dissolved solids concentration was determined with a portable meter (Hach Company, Loveland, Colorado, USA).

2.7. Phosphorus budget analysis

The total phosphorus input, output, uptake and accumulation in the culture system during the rearing cycle were assessed at the beginning and end of the trial. Phosphorus input and output in the form of water was calculated by multiplying the P concentration by the total volume of water in the aquaponics systems. Phosphorus input in the form of water represents nutrient contained in water on the day plants were transplanted. The water source was the same for all aquaponics systems and therefore contained approximately the similar concentration of dissolved phosphorus in all experimental units. Phosphorus output in the form of water represents phosphorus present in the water not utilized by plants on the harvest-day. Solids samples were taken from each aquaponics biofilter (that also functioned as a solids collector), and the phosphorus present in samples was multiplied by the volume of solids (15 L).

The total phosphorus input in the form of feed was calculated by multiplying the total amount of feed supplied by the phosphorus content of feed (decimal fraction). The total phosphorus input and output in the form of fish biomass was calculated by multiplying the P concentration in tilapia carcass (decimal fraction) and bythe total tilapia dry biomass. The total phosphorus output in the form of plant biomass was calculated by multiplying the P concentration in lettuce dry tissue (decimal fraction) by the total lettuce dry biomass.

The total amounts of phosphorus present in each component described above were divided by the total phosphorus input in the form of fish feed and were expressed as a percentage of total phosphorus input as feed. The phosphorus recovery fraction was determined by dividing the total sum of P outputs by total sum of P inputs.

2.8. Microbial plate count assay

Water samples from the aquaponics biofilters were aseptically collected, and immediately processed for the microbial plate count assay. 0.1 ml of each sample was suspended in 0.9 mL of sterile saline water (0.85% NaCl), and serial dilutions were performed until the 10^{-6} dilution. Subsequently, 0.1 mL of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were seeded on Tryptone Soy Agar (TSA) plates and incubated at 30°C for 24 h. In addition, 0.1 mL of the original undiluted water sample was seeded on the agar plates to serve as the positive control and also plates not inoculated were incubated as negative control to ensure that plates were not contaminated during their preparation. Each analysis was conducted in triplicate. Following incubation, the number of colony forming units (CFU mL^{-1}) was recorded when colonies were visibly distinct and countable.

2.9. Statistical analysis

Results were expressed as mean ± S.D. and group mean differences were analyzed using Student's T-TEST for all data except for the phosphorus budget and nutrient concentration dynamics analyses. Statistical significance was assumed at levels > 95% ($p < 0.05$). A two-way repeated measures ANOVA was run to determine the effect of different treatments on changes of nutrient concentrations over the weeks The same statistical procedure was used to determine the

effect of different treatments on the accumulation of phosphorus in the several pools (fish, plants, water and solids) The Bonferroni procedure was used for subsequent pairwise comparisons if the ANOVA was significant. The statistical software package SPSS (IBM Corp, 2016) was used for all statistical procedures.

3. Results

3.1. Lettuce growth and phosphorus content

Plants showed slow growth during the first 21 days after being transplanted to aquaponics systems. Part of the slow initial growth was attributed to the low light intensity and short days. In addition, in all aquaponics systems in both treatments, plants showed signs of severe chlorosis in new leaves, which was flagged initially as a possible nutrient deficiency or toxicity, and might have also contributed to slowing down the growth. Plants from the *Bacillus*-treated systems showed faster recovery than plants from untreated systems. By the end of the trial plants that received a *Bacillus* mixture in the water accumulated more dry mass, more phosphorus and more chlorophyll (Table 2), and were visually taller than the control (Fig. 2):

Table 2.
Growth, P content and chlorophyll index of lettuce grown in aquaponics systems

Treatment	[1]Dry mass (g)	[2]P content (%)	[1]Chlorophyll index
Control	1.30±0.92	0.25±0.03	5.27±1.31
Bacillus	4.09±0.87	0.54±0.02	7.27±1.33
p-values	.019	.0001	<.0001

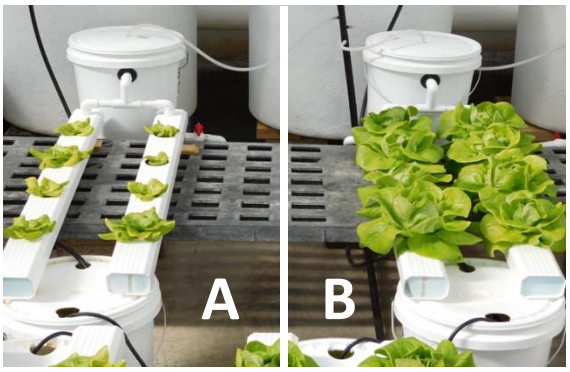

Fig. 2. Lettuce biomass accumulation in aquaponics systems. "A" represents the control and "B" represents aquaponics systems inoculated with a commercial *Bacillus* mixture.

3.2. Nutrient concentration dynamics

The effect of inoculating with a *Bacillus* mixture in aquaponics systems on the nutrient dynamics was determined based on nutrient changes over the course of several weeks. Not all weeks were considered for the statistical analysis. To reduce the number of factors in the analysis, four specific weeks were chosen with constant intervals depending on the nutrient analyzed. For ammonia, nitrite and nitrate, weeks 1, 2, 3 and 4 were selected because major changes occurred within this time frame. Also after week four, ammonia and nitrite concentrations tended to be too low in both treatments to be analyzed. For orthophosphate, weeks 1, 3, 5 and 7 were selected for the statistical analysis due to major concentration changes within this period. All nutrient concentration results and statistical parameters are expressed in Fig. 3.

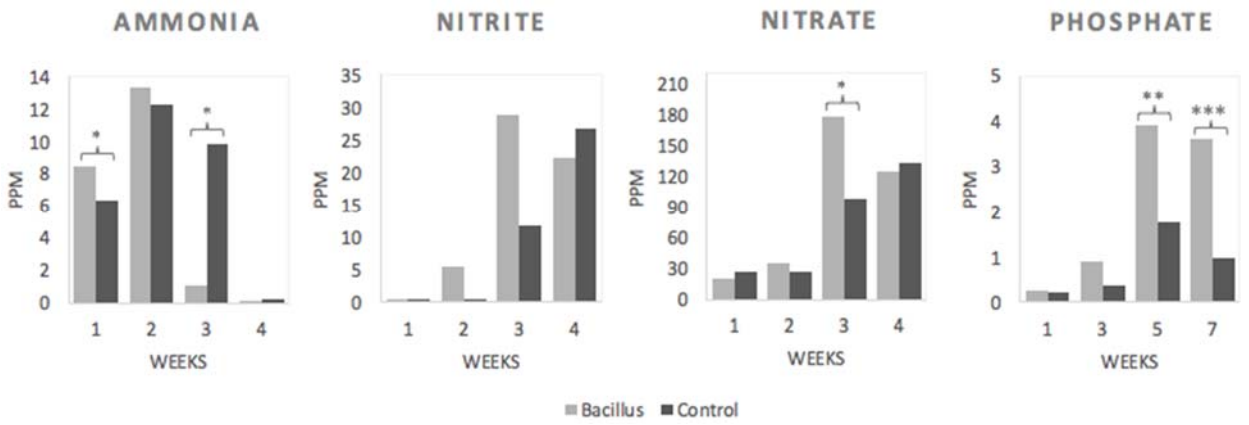

Fig. 3. Nutrient concentration changes in aquaponics systems receiving diets with and without *Bacillus* inoculation. Asterisks represent simple effects of treatment within each week based on the linearly independent pairwise comparisons among the estimated marginal means. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The repeated measures ANOVA showed that *Bacillus* affected the concentration dynamics of nitrogen and phosphorus in the aquaponics solutions ($p < 0.05$). Changes in the concentration of reactive phosphate changes were highly affected by the inclusion of *Bacillus* in the water ($p < 0.001$). *Bacillus*-treated systems showed an increase in reactive phosphate in the water, but differences were only statistically different after the third week of trial. Ammonia concentrations were affected by the inclusion of *Bacillus* in the water ($p = 0.007$). Systems inoculated with *Bacillus* showed a more rapid decrease in the concentration of ammonia in the water compared to the control. *Bacillus*-treated systems had almost no ammonia in the water at week 3, but it took one extra week for the untreated systems to lower ammonia concentration at the same concentration. The faster decrease of ammonia concentration in *Bacillus*-treated systems than the control was matched by the faster increase of nitrite and nitrate concentrations. Due to high data variability within treatments, nitrite concentration changes over the weeks showed no effect of *Bacillus* inoculation ($p > 0.05$),. Nitrate concentrations were affected by the inclusion of *Bacillus* in the water ($p = 0.038$), so that at week 3 there was a statistically significant spike in

nitrate concentration in *Bacillus*-treated systems, that correlates with the decrease in ammonia and increase in nitrite in the same week.

3.3. Phosphorus budget analysis

The overall phosphorus budget, which is composed of the phosphorus accumulated in all measured output components, is shown in Table 3. The inoculation of *Bacillus* affected the distribution of nutrients in the different aquaponics outputs ($p < .001$). In the lettuce pool, *Bacillus*-treated systems showed higher P accumulation than the control. In the water pool, the control accumulated less phosphorus than *Bacillus*-treated systems. For both plants and solids pools, the treatments had no effect on the P accumulation.

Table 3.

Phosphorus budget of all measured pools in aquaponics systems with/without *Bacillus* inoculation

Treatment	P input (g)	P outputs (g)				
	Feed	[1]Fish	Plants	[1]Water	Solids	[2]Unaccounted
Control	0.39	0.25±0.03	0.02±0.01	-0.04±0.01	0.14±0.01	0.01±0.00
Bacillus	0.39	0.16±0.03	0.17±0.03	0.03±0.01	0.19±0.01	-0.17±0.05
		----------[3]Pairwise comparisons----------				
p-values		NS	.002	<.0001	.043	.005

[1]Values considered only the net P accumulation over the trial period (subtracted the initial P).
[2]Amount of P not recovered from the total P input in the form of feed.
[3]Simple effects of treatment (*Bacillus* vs. Control) within each P pool based on the linearly independent pairwise comparisons among the estimated marginal means. NS = not significant.

3.4. Microbial plate count

Systems that received the commercial *Bacillus* mixture showed significantly higher microbial counts than the control (Table 4). In fact, TSA plates seeded with water samples from

317 the control showed no measurable (or detectable) bacterial growth, even at the positive control
318 plates seeded with the original water samples without dilution.

319 Table 4.
320 Microbial plate count in aquaponics systems treated or not with *Bacillus* spp. mixture

Treatment	Plate count (10^5 CFU mL^{-1})
Control	0.0±0.0
Bacillus	6.0±0.3
p-values	<.0001

321

322 4. Discussion

323

324 The high levels of ammonia and nitrite during the first three weeks of experiment
325 dramatically affected the performance of fish in all aquaponics systems and forced the premature
326 removal of all animals 21 days after the beginning of the trial. The imposed premature harvest
327 hampered any possibility of assessing the effect of *Bacillus* on performance and P accumulation
328 in Nile tilapia in our study and therefore fish growth data were not shown.

329 Plants also appeared to be negatively affected by the high concentration of nitrite in the
330 water. According to Oke (1966) a small concentration of nitrite in nutrient solution can have
331 adverse effects on plant growth.. During the first three weeks of experiment, plants showed slow
332 growth and barely doubled in size. At 21 days after transplanting, all plants from both treatments
333 began to recover and reestablish normal growth. Plants in the control persisted with mild chlorosis
334 signs for a longer period than plants grown in *Bacillus*-treated systems. The nutrient concentration
335 dynamics analysis showed that *Bacillus* accelerated the conversion of nitrite to nitrate in
336 comparison to the control, which might have explained the faster recovery of *Bacillus*-treated
337 plants than the ones grown in untreated systems.

Systems treated with *Bacillus* showed a significant increase in the orthophosphate (PO_4) concentrations in the aquaponics nutrient solution. It is worth noting that after the third week of experiment, the feeding procedure was ceased in all systems since all fish had to be removed. Therefore, the increase in orthophosphate concentration after week 3 presumably occurred due to mineralization of an existing source of phosphorus in the system. Since *Bacillus* sp. is known as a phosphatase-producer, it possibly contributed to organic phosphorus mineralization in the studied aquaponics systems inoculated with the commercial *Bacillus* mixture. Even the untreated systems showed a slight increase in the concentration of dissolved orthophosphate in solution after the third week of experiment, but not as prominent as the increase in *Bacillus*-treated systems. It is possible that an indigenous microbial community introduced by fish possess the ability to mineralize unavailable forms of phosphorus in aquaponics nutrient solutions. All systems components (hydroponics channels, fish tanks, biofilters) were sanitized before the experiment with bleach, and subsequently all biofilters were inoculated with a commercial product containing nitrifying autotrophic bacteria, but not *Bacillus* sp. Therefore the likely source of microorganisms other than the commercial *Bacillus* inoculants was the fish introduced in the systems.

The concentration dynamics of ammonia and nitrate were affected by the inclusion of the *Bacillus* mixture in aquaponics systems. The nutrient concentration dynamics analysis showed that *Bacillus* accelerated the conversion of nitrite to nitrate in comparison to the control. *Bacillus*-treated systems showed a faster decrease in the ammonia concentration in nutrient solution than the control. *Bacillus* sp. uses glutamine as its most preferred source of nitrogen, but in the absence of glutamine, alternative nitrogen sources such as ammonia can be used (Detsch and Stülke, 2003). Under high external ammonia concentrations, *Bacillus* sp. cells acquire ammonia via diffusion to be used in the nitrogen metabolism for the synthesis of glutamate (Gunka and Commichau, 2012).

Therefore it is possible that *Bacillus* contributed to lower the concentration of ammonia in the nutrient solution through direct uptake.

The increase in orthophosphate concentration in aquaponics systems treated with *Bacillus* was reflected in the enhanced growth and phosphorus accumulation in plants. Plants grown in *Bacillus*-treated systems accumulated approximately four times as much dry mass and twice as much phosphorus as the control. Plants grown in untreated systems showed lower chlorophyll content than *Bacillus*-treated systems. It is likely that the poor performance and quality of plants grown in untreated systems were related to the low phosphate availability demonstrated by the phosphate dynamics analysis. Phosphate is one of the key substrates in energy metabolism and biosynthesis of nucleic acids and membranes, therefore plays an important role in photosynthesis, respiration, and regulation of a number of enzymes in plants. When phosphate concentrations in solution culture are as low as recorded in the untreated aquaponics systems, the P uptake by plants is dramatically affected. Plants need specialized transporters in the roots to extract phosphorus from solutions with low P concentrations to transport inorganic P across root cell membranes against an intracellular concentration gradient that can be 1000-fold higher than the external solution (Schachtman, 1998). Most plants need a phosphate concentration of 20 to 30 μM (1.9-2.8 mg L^{-1}) for adequate growth in solution culture (Asher and Loneragan, 1967). According to the nutrient concentration dynamics analysis for the untreated systems in our study, the concentrations of orthophosphate never exceeded 2.0 mg L^{-1}, and remained lower than 1.0 mg L^{-1} most of the time, which are slightly below the 1.9-2.8 mg L^{-1} threshold discussed above.

The P budget analysis showed that systems treated with *Bacillus* accumulated more phosphorus in plants and dissolved in the water than the control. The amount of unaccounted P in *Bacillus*-inoculated systems also exceeded the total phosphorus provided in the fish feed. We

hypothesized that the fish feed was not the only source of phosphorus in systems receiving the *Bacillus* treatment. We traced possible sources of P that could have contributed to affect overall P budget. We performed a separate analysis on the *Bacillus* product and found that it contained approximately 0.60% of total P and 0.43% of reactive P. Considering the low microbial recovery determined by the microbial plate count assay, in which only 4.3% of all bacteria provided by the product was viable, it is possible that *Bacillus* has a short living period in the system and therefore becomes a phosphorus source after cells die. Thus, our nutrient budget analysis was hindered and we were not able to determine how much of the increase in concentration of phosphorus in the water resulted from P mineralization from the feed or the product.

Nevertheless, we still believe there is evidence that the microbial activity was the main source of the positive effects observed in systems treated with *Bacillus* mixture. The plate assay demonstrated an active microbiota in aquaponics systems treated with the *Bacillus* mixture, while plates seeded with water samples from untreated systems showed no detectable microbial growth. During the first three weeks of trial there were no changes in the concentration of dissolved orthophosphate in all aquaponics systems, treated or not with *Bacillus*, even though the commercial product used in the study contained traces of phosphorus. If the product was a direct source of phosphorus, we would expect a linear increase in the concentration of P in the water right after the first week of experiment. Thus, the surplus of orthophosphate dissolved in the water of *Bacillus*-treated systems was likely due to microbial activity. We did not run a thorough analysis to determine that the microbial growth on TSA plates were in fact the two strains present in the commercial product. However, based on previous plate cultures of the commercial product performed in our laboratory and comparison of the morphology of bacterial colonies and cultural

406 characteristics, we determined that the colonies isolated from the aquaponics systems were the
407 same present in the commercial *Bacillus* mixture.

408 The study highlights the importance of understanding nutrient dynamics for plants grown
409 in aquaponics system. When plant-based diets were fed to fish in aquaponics systems, low amounts
410 of dissolved orthophosphate were produced in the nutrient solution, which constrained growth and
411 quality of lettuce. The addition of a commercial mixture of *Bacillus* spp. enhanced plant growth,
412 increased the P accumulation in plant tissues, and increased the chlorophyll content in the leaves.
413 Also systems that received the *Bacillus* mixture demonstrated higher concentrations of dissolved
414 orthophosphate in the water than untreated systems. However, because we detected traces of
415 phosphorus in the commercial product used in our experiment, we were not able to affirm that the
416 benefits promoted by the *Bacillus* mixture were a result of microbial activity, or the inherent P
417 contribution of the product, or a combination of both. We recommend that in future studies using
418 such microbial preparations in aquaponics systems, researchers isolate the fertilizer effect by
419 simply adding a control treatment in which the product is sterilized. We also recommend
420 acclimating biofilters for a period of at least three weeks before the introduction of fish and plants
421 in the system to avoid any toxicity effect caused by the accumulation of ammonia and nitrite.
422 *Bacillus* affected the dynamics of ammonia, nitrite and nitrate in aquaponics and appears to speed
423 up the nitrification process, but the mechanisms involved still need to be elucidated.

424
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