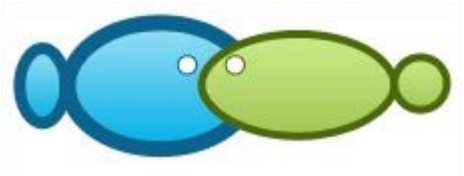

The abundance and diversity of phosphate-solubilizing bacteria in integrated recirculating aquaculture systems as a function of harvesting regime of duckweed (*Lemna minor* L.)

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Abstract. The present study aimed to determine the effects of different harvesting regimes of duckweed (*Lemna minor* Linnaeus) on the abundance and diversity of phosphate-solubilizing bacteria (PSB) in integrated recirculating aquaculture systems (IRAS). The study was conducted in twelve independent IRAS, where four duckweed harvesting regimes of two, four, and six-day harvest intervals tested the abundance and diversity of PSB. After 36 days, ten selected isolates belonging to five different genera were biochemically characterized. These isolates and their respective genera were: PSB1, PSB3, and PSB7 belonging to *Bacillus*, PSB17, and PSB26 to *Pseudomonas*, and PSB15, PSB29, PSB30, PSB32, and PSB35 to *Chryseobacterium*, *Micrococcus*, *Azotobacter*, *Acinetobacter*, and *Achromobacter*, respectively. *In vitro*, *Bacillus cereus* had higher solubilization efficiency and phosphatase activity. Duckweed harvested once every two days produced the highest duckweed biomass, however, harbored fewer bacteria, while six days of harvesting generated the highest duckweed growth index but lower bacterial diversity than the duckweed harvested every four days. The highest Shannon diversity index was obtained with a four-day harvest regime, while the lowest was when the duckweed was not harvested. A positive correlation (R^2 > 0.65) between the specific growth rate (SGR) and biomass of harvested duckweed and the diversity of PSB was found with a four-day harvest regime. Harvesting duckweed every four days provides desirable conditions for the attachment and growth of bacteria, thus increasing the abundance and diversity of PSB. These findings suggest the efficiency of removing excessive phosphorus and the associated PSB by duckweed and increase the productivity of IRAS.
Key Words: barramundi, biofilter, duckweed, integrated recirculating aquaculture system, phosphate-solubilizing bacteria.

Introduction. Aquaculture effluents with a high concentration of phosphorus (P) and nitrogen (N) contribute to eutrophication (Kawasaki et al 2016) and environmental degradation (Herath & Satoh 2015). Although recirculating aquaculture systems (RAS), considerably reduce the effluent load, significant amounts of N and P are still discharged through the protein-skimming mechanism (Martins et al 2010). Excessive P is often found in RAS effluents due to uneaten feed (Verdegem 2013). The removal of P to an acceptable level by various chemical methods is expensive (Jorgensen et al 2012) and requires high chemical dosages (Sun et al 2019). Even though the chemical removal of P rapidly improves the effluent quality (Zhao & Zhang 2017) more than the multi-step biological process, the chemical removal of P produces more sludge than its biological removal (Ruzhitskaya & Gogina 2017).

In any integrated RAS (IRAS), a substantial amount of organic and inorganic matter from uneaten feed and faecal matter accumulates and may subsequently be used by macrophytes. The phosphates could be reduced in an IRAS if the target species is cultivated with plants that utilised phosphates into valuable biomass (Turcois & Papenbrock 2014). The combination of fish culture with phototrophic and herbivorous organisms improves nutrient retention in the system (Turcois & Papenbrock 2014). The roots of various plant species incorporating phosphate-solubilizing bacteria (PSB) play ecophysiological role (Chen et al 2012). The PSB convert insoluble phosphates into

soluble forms (Alori et al 2017) and increase their availability to plants. The plants, in turn, may supply root-borne carbon (C) compounds - mainly sugars - that can be metabolized for bacterial growth (Gomes et al 2017). The microorganisms with phosphate-solubilizing potential rise the soluble phosphate availability and enhance plant growth by increasing the mineralization capacities of organic P and solubilizing precipitated phosphates (Sharma et al 2013), by increasing the supply of other trace elements such as iron, zinc, and by the production of plant growth-promoting regulators (Muszyńska & Labudda 2019).

Duckweed (*Lemna minor*) is known as a small, free-floating aquatic plant belonging to the Lemnaceae family and it has been used for wastewater treatments due to its rapid multiplication (Nassar et al 2015; Paolacci et al 2018). A total of 70-80% total phosphate can be removed from wastewaters and orthophosphate removal rates between 83-95% in different types of wastewaters (Ozengin & Elmaci 2007). The use of duckweed species as a biofilter media in RAS decreases a significant amount of orthophosphate and total phosphorus in water and increases the growth of cultivated fish species (Velichkova & Sirakov 2013).

Since PSB are ubiquitous in the whole water column, they play an essential function in regulating the available concentration of inorganic phosphate in the water column for plant uptake (Shardendu et al 2012; Sushanta et al 2012; Cheng et al 2019). Thus, the abundance and diversity of the bacterial communities' act as PSB in RAS are affected by the type of biofilter and the media's surface area per unit volume, as biofilters affect the growth and structure of bacterial communities (Omri et al 2013).

Even though all intensive fish culture systems are a major source of P pollution, relatively few studies have been conducted on the removal of P from these systems (Dauda et al 2019). Previous research demonstrated that the harvesting frequency of duckweed affects the abundance and diversity of heterotrophic bacteria communities, which are involved in nitrogen removal from IRAS (Ardiansyah & Fotedar 2016). Hence, the use of appropriate management strategies including the manipulation of the abundance and diversity of the PSB communities in any IRAS are required to enhance P absorption by rhizoplants (Maitra et al 2015). This can be controlled by the quantity of biofilter media including the amount of duckweed present in real time; therefore, the present study aimed to evaluate the effects of different amounts and different harvest regimes of duckweed in real-time on the composition, abundance, and diversity of phosphate solubilizing, non-pathogenic bacteria that are involved in the removal of phosphates in IRAS.

Material and Method. This experiment was conducted in Curtin Aquatic Research Laboratory (CARL), Curtin University, Perth, Western Australia, from April to May 2017. The experimental setup and design were based on Ardiansyah & Fotedar (2016). As a source of phosphate, 185 mg of di-ammonium phosphate ($(NH_4)_2$ HPO_4) was added to the fish-rearing tanks. Twelve independent recirculating systems were set up, each consisting of three tanks: a fish-rearing tank (400-L circular tank), a biofilter tank (100-L circular tank), and a waste-collection tank (100-L circular plastic drum). Four duckweed harvesting regimes were employed during the 36-day experimental period. The four harvesting regimes were applied randomly by harvesting duckweed at three biofilter tanks at once (in 3 replications) after 2, 4, and 6 days, whereas the remaining three tanks were not harvested (control). Harvested duckweed was replaced with fresh duckweed that had been previously washed. The reduction in water volume caused by evaporation was compensated by adding new freshwater. The observations of duckweed health followed the standard method (Daud et al 2018). The increase in biomass of duckweed on the surface of the water was determined using the growth index (GI) on alternate days. GI of duckweed on the water surface was calculated every second day using the equation GI = Wt/Wo, where Wt = biomass at time t, Wo = biomass at time 0, and t = number of days. While, the increase in biomass of duckweed per unit time of harvesting regime was indicated by specific growth rates (SGR). SGR = (Ln(Wf) - Ln(Wi) x 100)/t, where: Ln(Wf) = the natural logarithm of the final weight, Ln(Wi) = the natural logarithm of the initial weight, and t = time (days).

Isolation. Isolation, enumeration, and evaluation of the bacteria were performed following the procedure described in Ardiansyah & Fotedar (2016). A 10 g fresh weight of

duckweed was randomly collected using 40 mL sample jars containing 10 mL distilled water, and then kept at 4ºC before analysis. The samples were homogenized by centrifugation at 17,000 *g* for five min. A 10 mL sample of the homogenate water was diluted with 90 mL of phosphate-buffered saline (PBS) and homogenized again for two minutes at 1900 *g*. The resulting homogenate was diluted ten times with PBS for 10 min. Then 0.1 mL of the diluted samples were plated onto Pikovskaya agar (PVK) medium in triplicate by the spread plate method (Elias et al 2016) and a conventional pour plate technique. Bacterial colonies were selected according to their morphological appearance and maximum surrounding halo zone as an indicator of phosphate solubilization after 120 h of incubation at 30°C (Zhang et al 2017). Colonies with distinct clear zones and different morphologies were picked and re-streaked onto fresh PVK medium containing 0.5% insoluble tricalcium phosphate and re-incubated at 30ºC for 10 days.

Evaluation of phosphate solubilizing efficiency. Analysis of the PSB trait was made by determining the solubilization efficiency (%) of the selected isolates following the method of Pande et al (2017). The selected colonies were then removed from the plates and purified in triplicate on LB agar.

Enumeration. Enumeration of the PSB communities was determined using the total plate count method; the colonies were counted, and the concentration of bacteria present was expressed as CFU per mL for the attached bacteria, as previously reported by Blanco-Vargas et al (2020).

Phosphatase activity. Phosphatase activity was determined using 0.1 M disodium p-nitrophenyl as a substrate. For the assay, 3 mL of PSB culture on the PVK medium, 1 mL universal buffer (5x) adjusted to pH 6.5 (Behera et al 2017), and 0.5 mL of the substrate were pipetted into a 20 mL reagent vial and incubated at 30°C for 90 min. The reaction was stopped by cooling at 2°C for 15 min. Then, 20 mL of 0.5 N NaOH was added, and the mixture was transferred to a 50 mL volumetric flask, where the volume was increased to 50 mL with distilled water. The p-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Schoebitz et al 2013). Controls were made in the same way using 20 µg mL^{-1} disodium p-nitrophenyl.

Quantitative estimation of indole acetic acid (IAA). The procedures were performed according to Chaiharn & Lumyong (2011). The PSB isolates were inoculated in Czapek's dox broth and incubated at 28±2°C for 72 h. The culture was centrifuged at 3000 rpm for 15 min, and 2 mL of the supernatant was mixed with 1 mL of Salkowsky's reagent. The optical density was determined using a spectrophotometer at 535 nm.

Putative PSB identification. The putative PSB was identified by the biochemical method described in Bergey's Manual of Determinative Bacteriology (Garrity et al 2005). A variety of biochemical tests including Gram stain, catalase, oxidase, hydrogen sulfide production, gelatinase test, nitrate reduction, starch hydrolysis, citrate utilization, ornithine decarboxylase, indole, tyrosine hydrolysis, arginine dihydrolase, methyl-red and Voges-Proskauer, urease, glucose, fructose, lactose, maltose, mannose, sucrose, xylose, TSI agar test, and motility test, were conducted according to the standard procedure (Aditi et al 2017). API 20E system was used to confirm the identification of the PSB.

Bacteria diversity index. The bacteria diversity index assumes that an individual bacterium is randomly sampled from a large population so that all species are represented in the sample. The Shannon diversity index was used to determine the species diversity with the following equation: $H = - [\Sigma Pi * Ln(Pi)]$, where H = Diversity index, Pi = the number of each species in the sample/total number of samples, and Ln(Pi) = the natural logarithm of this proportion.

Data analysis. A one-way analysis of variance (ANOVA) was used to examine the significance of any differences between mean concentrations of data obtained, followed by the Tukey HSD test, while the relationship between the growth of duckweed and bacterial abundance and diversity was analyzed using regression analysis. Values of R^2 <

0.65 were considered to have poor correlations, whereas R^2 > 0.65 indicated good correlations. A $p < 0.05$ was considered significant for all analyses.

Results and Discussion. Forty-seven isolates of the putative PSB were screened from duckweed in the biofilter tank. Among the isolates, ten morphologically different bacterial isolates were observed (Table 1). Different harvest regimes caused significant differences ($p < 0.05$) in the bacterial abundance. The harvesting regime significantly impacted the abundance of six types of PSB (PSB1, PSB3, PSB15, PSB17, PSB26, and PSB35). Duckweed harvested every 2 days had the lowest abundance of the putative PSB, while the highest PSB abundance was found in duckweed harvested every 4 days. However, no significant difference was found in PSB abundance between duckweed harvested every 4 days and those harvested every 6 days.

Table 1

The abundance of PSB attached to the duckweed in the biofilter tank

Putative PSB bacteria	*Harvest regimes*			
(cfu x 10^3/mL)	*6 days*	*4 days*	*2 days*	*No harvesting*
PSB1	5.23 ± 0.21^{b}	5.34 ± 0.26^{b}	4.76 ± 0.34^{a}	4.70 ± 0.32^{a}
PSB3	3.74 ± 0.03^{b}	3.85 ± 0.06^{b}	3.29 ± 0.03^{a}	3.37 ± 0.01^{a}
PSB7	1.79 ± 0.08^{a}	1.75 ± 0.09^{a}	1.64 ± 0.05^{a}	1.59 ± 0.05^{a}
PSB15	4.73 ± 0.12^{b}	4.85 ± 0.19^{b}	4.12 ± 0.13^{a}	4.24 ± 0.15^{a}
PSB17	5.17 ± 0.11^{b}	5.09 ± 0.26^{b}	4.53 ± 0.26^{a}	4.98 ± 0.08^{b}
PSB26	4.60 ± 0.14^{b}	4.52 ± 0.17^{b}	3.85 ± 0.48^{a}	3.97 ± 0.03^{a}
PSB29	1.84 ± 0.21^{a}	1.79 ± 0.27^{a}	1.65 ± 0.35^{a}	1.71 ± 0.46^{a}
PSB30	2.16 ± 0.13^{a}	2.01 ± 0.16^{a}	1.87 ± 0.10^{a}	1.95 ± 0.10^{a}
PSB32	2.89 ± 0.02^{a}	2.94 ± 0.04^{a}	2.75 ± 0.02^{a}	2.80 ± 0.08^{a}
PSB35	3.47 ± 0.08^{b}	3.52 ± 0.17^{b}	3.05 ± 0.07^{a}	3.13 ± 0.05^{a}
Total	35.62 ± 0.43^{b}	35.66 ± 0.44^{b}	31.51 ± 0.37^{a}	32.44 ± 0.39^{a}

Note: values in the same row with the same superscript letter are not significantly different ($p > 0.05$).

Harvesting duckweed every four days resulted in a higher Shannon diversity index value (H = 2.2910) than the two-day harvest (2.2365), the six-day harvest (2.2745), and the unharvested (control) (H = 2.2257) (Table 2). PSB abundance and duckweed biomass harvest were poorly correlated ($R^2 < 0.65$) with the harvest regime but at the four-day harvest, a significantly stronger correlation between the bacterial diversity and the harvested duckweed biomass was observed. Similarly, the SGR harvest of duckweed was significantly correlated with the abundance and diversity of PSB at the four-day harvest regime ($R^2 > 0.65$; $p < 0.05$), whereas the SGR and biomass harvest of the duckweed harvested every six days had a poor relation to the abundance and diversity of PSB ($R^2 < 0.65$).

Table 2

Correlation between SGR and total biomass harvest of duckweed with abundance and diversity of PSB over the 36-day trial

Parameters	*Harvest regimes (days)*			
	6	*4*	*2*	*0*
$R^2_{\text{(biomass vs abundance)}}$	0.5971 ± 0.02^{a}	0.6192 ± 0.06^{b}	0.5941 ± 0.04^{a}	0.6185 ± 0.00^{b}
$R^2_{\text{(biomass vs diversity)}}$	0.6141 ± 0.01^{a}	0.6502 ± 0.01^{b}	0.6193 ± 0.05^{a}	0.6835 ± 0.03^{b}
$R^2_{\text{(SGR vs abundance)}}$	0.5852 ± 0.06^{a}	0.6984 ± 0.06^{b}	0.6248 ± 0.03^{a}	0.6894 ± 0.03^{b}
$R^2_{\text{(SGR vs diversity)}}$	0.6242 ± 0.01^{a}	0.6623 ± 0.01^{b}	0.6193 ± 0.05^{a}	0.6835 ± 0.03^{b}
Shannon diversity index (H)	2.2745 ± 0.01^{bc}	2.2910 ± 0.01^{c}	2.2365 ± 0.01^{b}	2.2257 ± 0.00^{a}
GI	3.15 ± 0.02^{c}	3.09 ± 0.03^{bc}	3.15 ± 0.04^{a}	3.04 ± 0.03^{ab}
Total biomass harvest	674.45 ± 0.54^{b}	1003.45 ± 1.29^{c}	1880.2 ± 0.67^{d}	99.75 ± 0.11^{a}
SGR	8.14 ± 0.00^{b}	9.31 ± 0.00^{c}	11.05 ± 0.00^{d}	3.04 ± 0.00^{a}

Values in the same row with the same superscript letter are not significantly different ($p > 0.05$).

Harvest of duckweed biomass and PSB abundance were poorly correlated ($R^2 < 0.65$) to harvest regime. Significantly stronger correlations between duckweed biomass at harvest and PSB diversity were observed at the 4-day harvest regime. A 4-day harvest regime may provide the desirable duckweed biomass on the water surface and optimal availability of organic substrates as carbon and energy sources for the bacteria (Ishizawa

et al 2017). Likewise, the diversity of PSB was significantly correlated (R^2 > 0.65) with SGR of duckweed at the 4-day harvest regime, and the unharvested (control). Furthermore, harvesting duckweed every two and six days resulted in a poor relationship between SGR and biomass harvest with the abundance and diversity of PSB. Different harvest regimes also had a significant influence on the GI of duckweed. Duckweed harvested every two and six days had the highest GI of 3.15, whereas those unharvested control had the lowest GI of 3.04 (Table 2). However, higher GI of the 2 and six-day harvest regime do not have stronger relationship with the abundance and diversity of PSB communities.

Phosphate solubilization efficiency, ***IAA***, ***and phosphatase activity***. The results of the solubilization efficiency of the selected PSB are shown in Table 3. Among the ten bacterial isolates, the highest efficiency was observed for PSB1 (E = 83.33%), followed by PSB17 (E = 75.00%), and then PSB15 (E = 62.50%), whereas PSB29 (E = 33.33%) and PSB32 (E = 34.47) had comparatively lower efficiencies. All putative PSB isolates produced IAA.

Table 3

The phosphate solubilizing efficiency and phosphatase activity recorded for the putative PSB attached to duckweed in the biofilter tank

Putative PSB	*Phosphate solubilization efficiency (E, %)*	*IAA production (µg L^{-1})*	*Phosphatase activity (µmol $g^{-1}h^{-1}$)*	*R^2 (Phosphate solubilization efficiency vs Phosphatase activity)*
PSB1	83.33±0.01	7.35±0.01	33.68±0.01	0.921
PSB3	53.59±0.01	7.45±0.02	28.92±0.01	0.921
PSB7	43.93±0.01	7.00±0.04	23.92±0.02	0.921
PSB15	62.50±0.02	7.60±0.01	30.85±0.02	0.921
PSB17	75.00±0.01	7.23±0.01	31.25±0.03	0.921
PSB26	60.83±0.01	7.00±0.02	29.84±0.03	0.921
PSB29	33.33±0.02	7.08±0.02	17.65±0.01	0.921
PSB30	44.82±0.05	6.55±0.03	18.85±0.01	0.921
PSB32	34.47±0.02	7.08±0.02	15.84±0.06	0.921
PSB35	50.00±0.01	6.07±0.01	25.32±0.01	0.921

Data are shown as the mean±SE.

The highest IAA (7.60 µg L^{-1}) was produced by PSB15, followed by PSB3 (7.45 µg L^{-1}), while the lowest IAA (6.07 µg L^{-1}) was produced by PSB35. Furthermore, the maximum phosphatase activity was observed in PSB1 (33.68 µmol g^{-1} h^{-1}), followed by PSB17 (31.25 µmol g^{-1} h^{-1}), while the minimum phosphatase activity was observed in PSB32 (15.84 µmol g^{-1} h^{-1}). The remaining bacterial isolates secreted phosphatase enzyme in the range of 17.65-30.85 µmol g^{-1} h^{-1}. The correlation coefficient values (R^2 = 0.921) showed a significant relationship ($p < 0.05$) between the phosphate solubilization efficiency of all PSB bacteria and the ability of these bacteria to produce phosphatase enzymes.

Morphology and biochemical characteristics of PSB. Ten bacterial isolates were determined by colony morphology, gram staining, and pigmentation (Table 4). Gram-negative bacteria dominated the bacterial communities. Specifically, 40.41% of gram-negative bacilli isolates were identified as *Pseudomonas* and *Chryseobacterium*; 29.79% of isolates of gram-positive bacilli belonged to *Bacillus*; 23.41% of isolates of gram-negative ovoid, cocci, or coccobacilli were identified as *Azotobacter*, *Acinetobacter*, and *Achromobacter*, and 6.39% of isolates of gram-positive cocci were identified as *Micrococcus* (Table 4). Non-pigmented isolates belonged to *Bacillus*, *Achromobacter*, and *Acinetobacter*, whereas non-motile isolates were characterized as *Chryseobacterium*, *Acinetobacter*, *Azotobater*, and *Micrococcus*.

Ten species of putative PSB were characterized by both biochemical tests and confirmed with the API 20E (Table 4). All isolates were characterized as the same species by both conventional biochemical tests and API 20E. Out of these ten PSB isolates, as shown in Table 4, *Bacillus* was represented by three species, PSB1, PSB3, and PSB7, *Pseudomonas* was represented by 2 species, PSB17 and PSB26, whereas *Chryseobacterium*, *Micrococcus*, *Azotobacter*, *Acinetobacter*, and *Achromobacter* had only one species each, PSB15, PSB29, PSB30, PSB32, and PSB35 respectively.

Table 4

The biochemical characteristics of PSB isolated from the biofilter tanks

Test	*The PSB isolate*									
	PSB1	*PSB3*	*PSB7*	*PSB15*	*PSB17*	*PSB26*	*PSB29*	*PSB30*	*PSB32*	*PSB35*
Colony morphology	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Cocci	Ovoid	Cocci	Coccobacilli
No. clones (%)	19.15	6.39	4.25	12.76	14.89	12.76	6.39	4.45	6.39	10.54
Pigmentation	Yellowish-green	Translucent	White	Yellow	Yellowish-green	Brownish-yellow	Yellowish-orange	Yellow	White	White
Spore	+	-	+	-	-	-	-	-	-	-
Motility	-	+	+	-	+	+	-	+	-	-
Gram stain	+	+	+	-	-	-	+	-	-	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	-	-	+	-	+	+	+	-	+
H_2S production	-	-	-	-	+	-	-	-	-	-
Gelatinase	+	-	+	+	+	-	+	+	-	-
Nitrate reduction	-	+	+	+	-	+	+	+	-	+
Starch hydrolysis	+	+	+	+	+	-	-	+	-	-
Citrate utilization	-	-	+	+	+	+	+	+	+	+
Ornithine	-	-	-	-	-	-	-	+	-	-
Indole	-	-	-	+	+	-	-	+	-	-
Tyrosine hydrolysis	+	-	-	-	+	-	-	-	+	+
Arginine dihydrolase	-	+	-	-	+	+	-	+	-	+
Methyl Red	-	-	+	-	+	+	-	-	-	+
Voges-Proskauer	-	-	+	+	+	-	-	-	+	-
Urease	+	-	-	-	+	+	-	+	+	-
Glucose	+	+	+	+	+	+	+	+	-	-
Fructose	+	+	+	+	+	+	-	+	-	+
Lactose	-	-	-	-	+	-	-	-	-	-
Maltose	+	+	+	+	+	-	-	+	-	-
Mannose	+	+	+	+	+	+	-	-	-	-
Sucrose	+	+	+	-	+	-	-	+	-	-
Xylose	-	-	+	-	-	+	+	+	-	+

Where: PSB1 = *Bacillus cereus*; PSB3 = *Bacillus licheniformis*; PSB7 = *Bacillus subtilis*; PSB15 = *Chryseobacterium indologenes*; PSB17 = *Pseudomonas fluorescens*; PSB26 = *Pseudomonas mendocina*; PSB29 = *Micrococcus luteus*; PSB30 = *Azotobacter vinelandii*; PSB32 = *Acinetobacter calcoaceticus*; PSB35 = *Achromobacter xylosoxidans*.

Abundance and diversity of PSB. Different harvest regimes significantly affected the abundance of PSB (Table 1), in which six of ten PSB communities, *Bacillus cereus, Bacillus licheniformis, Chrysobacterium indologenes*, *Pseudomonas fluorescens*, *Pseudomonas mendocina* and *Achromobacter xylosoxidans* were significantly affected ($p < 0.05$) by different harvest regimes. Duckweed harvested with the shortest interval of 2 days had lower abundance than those with longer harvest intervals of 4 and 6 days. Similar results had been previously observed in the abundance of the heterotrophic bacteria associated with the different harvesting regimes in barramundi (*Lates calcarifer*) RAS (Ardiansyah & Fotedar 2016). Therefore, the four-day harvest regime may provide the desirable duckweed biomass and optimal availability of organic substrates as carbon and energy sources for the PSB.

Only ten isolates from forty-seven showed the formation of halo zones around the growing colonies on PVK media plates. Halo zones are formed because of the transformation of glucose into organic acids, in which glucose is the carbon source used in the test of PSB isolated from the biofilter to dissolve the bonded phosphor marked by halo zones (Pande et al 2017). However, the production of organic acids was not determined in the present study; thus, the authors cannot confirm whether all PSB isolates are capable of producing organic acids.

There is increasing evidence that PSB improves plant growth due to the biosynthesis of plant growth substances rather than to their action in releasing available P. In the present study, all PSB isolated from duckweed were capable of producing IAA. Majda & Robert (2018) stated that IAA is the biologically active form of auxin that stimulates radical system growth and increases the uptake of nutrients by plants. Thus, it has prominent effects on plant growth and development (Egamberdieva et al 2017). The results indicated that all PSB isolates can secrete physiologically active auxins. Shahab et al (2009) reported that PSB isolated from mung bean (*Vigna radiata*) excreted phytohormones, including auxins. Other researchers found similar results; that isolated bacterial species from rice fields have the potential to produce IAA (Etesami et al 2015). These results were also similar to the findings of Bal et al (2013); where the PSB isolated from the rhizophore of Chinese cabbage (*Brassica rapa*) were found to solubilize P in the media, and besides this, they were able to produce IAA. However, the capacity to synthesize IAA is widespread among the PSB. Production of IAA greatly varies among different species and is influenced by culture conditions, growth stage, and substrate availability (Mohite 2013).

Phosphatase enzyme activity. Phosphatase enzyme activity showed that all the selected PSB isolates solubilized inorganic phosphates into various degrees in the PVK culture medium by producing phosphatase enzymes. The PSB have been shown to increase the solubility of the P-insoluble compound through the secretion of extracellular enzymes such as organic acid, phytase, and phosphatase enzymes (Khan et al 2014). These enzymes are found in all organisms but only bacteria, fungi, and some algae can secrete them outside their cells. However, the PSB are more effective in P solubilization than fungi (Nacoon et al 2020). Among the whole microbial population in the aquatic environment, PSB constituted 1-50%, while phosphorus-solubilizing fungi (PSF) constituted only 0.1-0.5% in P solubilization potential (Ingle & Padole 2017).

Furthermore, during the conversion process, P is partially assimilated by PSB but the amount made soluble and released is more than the requirement of the PSB. The excess amount released is made available to plants. During this conversion process, extracellular enzymes convert calcium phosphate to di- or monobasic phosphates and then become biologically available to plants (Kalayu 2019).

The stronger correlation between phosphate solubilizing efficiency and phosphatase enzyme activity may indicate an increase in the availability of P in the medium, which is assumed to be facilitated through increased phosphatase enzyme activity (Singh & Prakash 2012; Behera et al 2017). The results suggest that all selected PSB can produce phosphatase enzymes to convert insoluble phosphate into the soluble form, which leads to increased available phosphate for plants (Kalayu 2019). Microorganisms increase P availability to the macrophytes by mineralizing organic P and

by solubilizing precipitated phosphates (Alori et al 2017). Additionally, Behera et al (2017) found a positive correlation between phosphate solubilizing activity and phosphatase enzyme activity. These previous findings are in agreement with the present results, which showed that the increase in inorganic phosphate levels was due to phosphate solubilization, and did not repress phosphatase production by the PSB isolates found in the study.

Morphology and biochemical characteristics of PSB. The results found in this study are in line with those reported by Maitra et al (2015) that *Pseudomonas, Chryseobacterium*, *Aeromonas*, and *Acinetobacter* are common inhabitants of various aquatic environments. Furthermore, Maitra et al (2015) and Adeleke & Babalola (2021) reported that the bacterial genera with the capacity to solubilize insoluble inorganic phosphate compounds amongst aquatic environments are *Bacillus*, *Enterobacter*, *Agrobacterium*, *Pseudomonas*, *Acinetobacter*, *Achromobacter*, *Micrococcus*, *Chryseobacterium*, and *Aerobacter*.

The most common bacterial genera in this study were *Bacillus*. The majority of the *Bacillus* species showed high phosphatase activity but the proportion varied with species. The *Bacillus* consisted of obligate aerobes (*B. subtilis*) or facultative anaerobes (*B. cereus* and *B. licheniformis*). The genus is known to have more characteristics promoting plant growth: synthesis of phytohormones such as IAA (auxins); nitrogen fixation; antifungal activity by siderophore production; antibiotics; enzymes; and phosphorus solubilization (Nabti et al 2013).

Sharma et al (2013) reported that the genus *Pseudomonas* was a suitable genus of bacteria in dissolving bonded P from any source, and was dominant in the mineralization of organic phosphorus. This type of bacteria can also be found in extreme saline and alkaline water habitats (Poli et al 2017). In this study, *Pseudomonas* consisted of *P. fluorescens* and *P. mendocina*. Bacteria from the genera *Bacillus* and *Pseudomonas* are among the most powerful phosphate solubilizers (Kalayu 2019).

A considerable number of bacteria from different genera are capable of solubilizing phosphate including *Chryseobacterium*, which has been isolated from a wide range of habitats such as soil, plant roots, sludge, fish, sewage, fresh water, freshwater sediment, marine water, and marine sediment. The genus *Chryseobacterium* was first created for five species formerly classified as members of the genus *Flavobacterium* (Song et al 2021). Various *Chryseobacterium* species have been shown to solubilize phosphate by releasing organic acids that mobilize P, and by releasing phosphatase to release phosphate groups bound to organic matter (Kalayu 2019).

P availability depends on the degree of solubilization by various organic and inorganic acids produced by microorganisms, and the genus *Micrococcus* is one of the most important microorganisms that produce a substantial number of phosphates. *Micrococcus* inhabits a wide range of environments including soil, water, and dust, and can sustain well in environments with high salt concentration and little water. This genus has been shown to possess multiple plant growth traits like P-solubilisation, and IAA, and siderophore production (Afzal et al 2019).

Various bacterial genera are involved in various biotic activities important to nutrient turnover dynamics (Ahemad & Kibret 2014). Thus, there is ongoing research to discover various rhizobacteria that possess novel traits such as phosphate solubilization (Ahemad & Khan 2012) capacities. Among the bacterial genera with this capability is *A. xylosoxidans* (Ahemad & Kibret 2014). This species shows a considerable level of nitrogenase activity, IAA production, and P solubilization ability. In addition, this species may also increase copper uptake by plants and increase their shoot length, root length, and fresh weight, and dry weight of plants (Yang et al 2013).

Azotobacter is a genus of gamma-proteobacterium belonging to the family Pseudomonadaceae. It is an obligately aerobic, free-living Gram-negative bacterium that is broadly dispersed in various environments including water, soil, and sediments (Dangi 2015). *Azotobacter* has favorable effects on plant yields due to its capability in fixing nitrogen and solubilizing phosphates (Nosrati et al 2014). *A. vinelandii* produces metabolically dormant cysts, which are formed under unfavorable environmental

conditions; thus, it is suitable for use in diverse environments (Garcia et al 2014). Several *Azotobacter* species isolated from wheat rhizophore has shown the ability to solubilize tricalcium phosphate, Mussoorie rock phosphate, and also to produce IAA. Additionally, the use of *Azotobacter* may increase seed yield, plant height, and microbial population in soil (Peng et al 2013).

A. calcoaceticus has demonstrated the potential capability of P solubilization through the production of organic acids, and can provide plant growth-promoting factors such as the production of IAA and siderophores. This species also exhibits resistance to lead (Pb) and antibiotics. The use of *A. calcoaceticus* effectively increases the available Pb in the rhizosphere soil and promotes the growth of the host plant, which leads to an increase in Pb uptake (Ren et al 2013).

All the putative PSB from the present study have been known to play a fundamental role in the phosphate solubilization process in a diverse range of ecosystems (Anand et al 2016). Our research suggests that duckweed biofilters in IRAS are suitable substrates for the attachment, survival, and growth of PSB.

A short two-day harvest regime reduced the PSB abundance in biofilter tanks, whereas a six-day harvest regime, or no harvest, decreased duckweed biomass due to chlorosis and disconnection of their fronds. The results showed that a four-day harvest regime maintains the optimum biomass of duckweed. Furthermore, the different harvesting regimes influenced the abundance and diversity of PSB in IRAS. The selected PSB isolates should be further evaluated in larger scale *in vivo* trials regarding the mechanism and efficiency of P solubilization, and their potential application in managing sustainable aquaculture systems.

Conclusions. Duckweed in the biofilter of RAS is a suitable substrate for the attachment, survival, and growth of phosphate solubilizing bacteria. A short 2-day harvesting regime reduced the abundance of phosphate solubilizing bacteria in the biofilter tank, whereas the 6-day or no-harvesting regime decreased duckweed biomass. The results suggested that the 4-day harvesting regime maintained optimum duckweed biomass in the biofilter tank. Harvesting duckweed every four days provides desirable conditions for the attachment and growth of bacteria, thus increasing the abundance and diversity of PSB. These findings suggest the efficiency of removing excessive phosphorus and the associated PSB by duckweed and increase the productivity of IRAS.

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