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A trial to enhance the production yield of a duckweed, *Lemna gibba*, in food factory wastewater utilizing indigenous bacteria

（食品工場排水における土着細菌を活用したウキクサ植物 *Lemna gibba* の生産収率向上の試み）

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ABSTRACT

The Duckweed, Lemnoideae plants, are recently highlighted as one of the useful biomass with multiple uses. The development of effective production of the duckweed biomass using wastewater as cost-free fertilizer is the challenge of this study. I conducted a trial to improve the growth of duckweed *Lemna gibba* in the treated food factory wastewater (WW), namely A-wastewater (A-WW) and K-wastewater (K-WW). These WW contained significantly higher sodium (Na) and phosphate (PO_4), while lower nitrogen (N) by 100-folds compared to a popular duckweed medium, Hoagland. Thus, the duckweed did not grow well in A-WW and K-WW.

Plant growth-promoting bacteria, PGPB, are currently expected to become a new nature-based technology tool for increasing biomass production including the duckweed. **In chapter I**, I call attention to the usage of appropriate PGPB, especially in A-WW and K-WW with uneven nutritional conditions. Active PGPB, *Acinetobacter calcoaceticus* P23 and *Pseudomonas fulva* Ps6, previously obtained from the environmental water in which duckweed naturally grows, did not promote but instead inhibited the growth of *L. gibba* in the WW conditions. Thus, I explored indigenous bacteria from the WW that exhibited growth promotion activity towards *L. gibba*. I demonstrated for the first time that among the indigenous bacteria that naturally grow in the WW unrelated to duckweed habitat, there exist a specific bacterium that can promote the growth of *L. gibba* in both WW conditions, which was *Chryseobacterium* sp. 27AL. Moreover, I determined the key factors that caused the growth inhibition activity of the above-mentioned non-indigenous PGPB strain P23 in the K-WW. It was found that P23 shifted its growth promotion activity on duckweed to growth inhibition in the low N condition. This result suggested that P23 showed nutrient competition with duckweed on the N uptake in the WW condition.

Nevertheless, there is still a challenge remained of the PGPB application in WW, where the biomass yield of *L.gibba* could not fully recover. The WW contained the unbalanced minerals that significantly reduced *L. gibba* growth and also affected PGPB activities. Thus, in **chapter II**, I further identified factors for growth inhibition of *L. gibba* in the WW and demonstrated that modification of WW improved *L. gibba* growth and PGPB growth promotion activities. The result showed that low N and excess PO_4

conditions caused the growth inhibition of *L. gibba*. Supplementation of KNO_3 or NH_4OH and the application of $CaCO_3$ to the WW resulted in the recovery of *L. gibba* growth. Moreover, all the PGPB, including non-indigenous P23, showed higher duckweed growth promotion activities in the mineral-modified WW. Overall, the mineral supplementation within the acceptable level combined with the domestic PGPB application offers a promising way to significantly improve duckweed biomass production in the WW.

On the other hand, during the trial of wild duckweed biomass production in an open-air A-WW tank, Thailand, I observed the overgrowth of microalgae, which dramatically reduced the duckweed growth probably because of their nutrient competition. Therefore, in **chapter III**, I asked if indigenous PGPB for *L. gibba* also have antagonistic activity towards the microalgae. Some eukaryotic microalgae were isolated from wastewater samples, including genera *Chlorella*, *Coelastrella*, *Desmedesmus*, and *Parachlorella*. In addition, *Microcystis aeruginosa*, a prokaryotic microalga well-known to form harmful algae blooms (HABs) by producing microcystin toxin, was also used for examination. I found that some PGPB including 27AL have the ability to inhibit the growth of microalgae, especially *M. aeruginosa*. The effect of PGPB was further explored in the co-culture of duckweed *L. gibba* and *M. aeruginosa*. The result strongly suggested that *Chryseobacterium* 27AL could specifically suppress *M. aeruginosa* and still promoted the growth of duckweed, *L. gibba*, significantly compared to control without bacterial inoculation. This result successfully demonstrated that wonderful PGPB, 27AL, has a dual function of enhancing the duckweed growth while inhibiting duckweed's competitor, microalgae. Overall, this study uncovered the hidden potentials of indigenous PGPB that contribute to the improvement of the duckweed biomass's production efficiency using factory wastewater. To put the strategies developed here into practical use, it is needed to evaluate the feasibility of the scale-up duckweed cultivation system in the actual WW treatment sites for the future.

Keywords

Duckweed biomass, plant growth-promoting bacteria, wastewater, microalgae

ABBREVIATIONS

WW	Wastewater
PGPB	Plant growth-promoting bacteria
PGP	Plant growth promotion
TKN	Total kjeldahl nitrogen
TP	Total phosphorous
IAA	Indole acetic acid
COD	Chemical oxygen demand
CTAB	Cetyltrimethylammonium bromide
DTT	Dithiothreitol
PCI	Phenol chloroform isoamyl alcohol
TE	Tris-EDTA
RT	Room temperature

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INTRODUCTION

Duckweed is the smallest flowering plant that can grow on the surface of still or slow-moving water bodies. Its rapid, asexual reproductive cycle enables it to double its biomass every two or three days under optimal conditions (Stomp, 2005). Studies on duckweed have rapidly expanded from basic biology to bioengineering because of its high value as a useful bioresource (Appenroth et al., 2015). Indeed, duckweed has been shown to display a high starch content (maximum ca. 50% in turion) with low lignin content, making it an advantageous fermentation substrate for both bioethanol and methane production (Xu et al., 2011; Toyama et al., 2018). Additionally, duckweed proteins (maximum ca. 40%) contain the WHO-recommended amino acid ratios and essential amino acids that are important for human and animal nutrition (Goopy and Murray, 2003; Appenroth et al., 2017). Owing to the strong benefits expected in a range of industries, duckweed biomass production has received significant attention in recent times.

Duckweed can be easily cultivated in pond water, sewage effluents, or industrial wastewater (WW) with no land irrigation because of its ability to take up dissolved pollutant minerals as cost-free fertilizer coupled with useful biomass production (Cheng and Stomp, 2009). Mohedano et al. (2012) showed significant performance of duckweed in nutrient-rich swine waste, with the removal of 98.0% of the TKN (Total Kjeldahl Nitrogen) and 98.8% of the TP (Total Phosphorus), and a production of 68 t/ha·year of dry biomass containing 35% crude protein. Duckweed yields of 39.1–105.9 t/ha·year have also been reported from the duckweed cultivation in WW, while the additional treatment of nutrient starvation and salt stress can further improve duckweed starch content up to 31.0–45.8% dry weight (Table 1; Xu et al., 2012). Even though algae have the highest biomass production compared to other crops (Table 1), the harvesting and drying process of algae are still challenging in the actual application (Xu et al., 2012). In some studies, the biomass production of duckweed in WW or natural environment might take a more extended period; thus, recent technologies are introduced to improve the efficiency of the cultivation system. One of the relevant methods is accelerating duckweed growth by inoculating plant growth-promoting bacteria (PGPB) (Yamaga et al., 2010; Toyama et al., 2017; Ishizawa et al., 2020).

Acinetobacter calcoaceticus P23 and *Pseudomonas fulva* Ps6 have been identified as effective growth-promoting bacteria for some duckweed species, including *Lemna aequinoctialis* Welw (Former name: *Lemna aoukikusa*) and *Lemna minor* (Fig. 1; Yamaga et al., 2010; Suzuki et al., 2014; Yamakawa et al., 2018). Notably, the PGPB-reinforced duckweed *L. minor* accelerated biomass production by 1.7–2.4-fold compared to natural duckweed in secondary sewage effluent and river water as well as displayed improved nutrient removal and CO_2 fixation (Fig. 1; Toyama et al., 2017; Ishizawa et al., 2020).

Table 1. Comparison of biomass production of common studied potential energy crops

Potential energy crops	Dry biomass yield (t/ha·year)
Duckweed	39.1–105.9
Algae	90.3–175.2
Switchgrass	5.2–26.0
Bermuda grass	6.1–27.0
Miscanthus	5.0–44.0
Poplar	1.6–14.3
Willow	4.1–21.5

(Xu et al. 2012)

PGPB have long been used to improve the yield of crops with less fertilization and pesticides. In fact, PGPB accelerate the growth of host plants either directly by producing plant growth hormones, facilitating the uptake of minerals, and relieving environmental stresses, or indirectly by acting as biocontrol agents against pathogens (Glick, 2012). However, the interaction between PGPB and the host plant varies depending on the environmental conditions and the plant species (Glick, 2012). For instance, *Azospirillum brasilense* has been reported to increase the shoot and root length of a cordon plant linearly as the nutrients declined, but in nutrient-rich soil, this PGPB exerted no effect on cordon growth (Carrillo-Garcia et al., 2000). Another report showed that an engineered PGPB with IAA-overproduction increased the root weight of blackcurrant cuttings but inhibited root development of sour cherry, probably due to the different sensitivity of the latter to IAA (Dubeikovsky et al., 1993; Glick, 2012). Thus, environmental factors and host plant species should be considered to maximize PGPB activities for practical use.

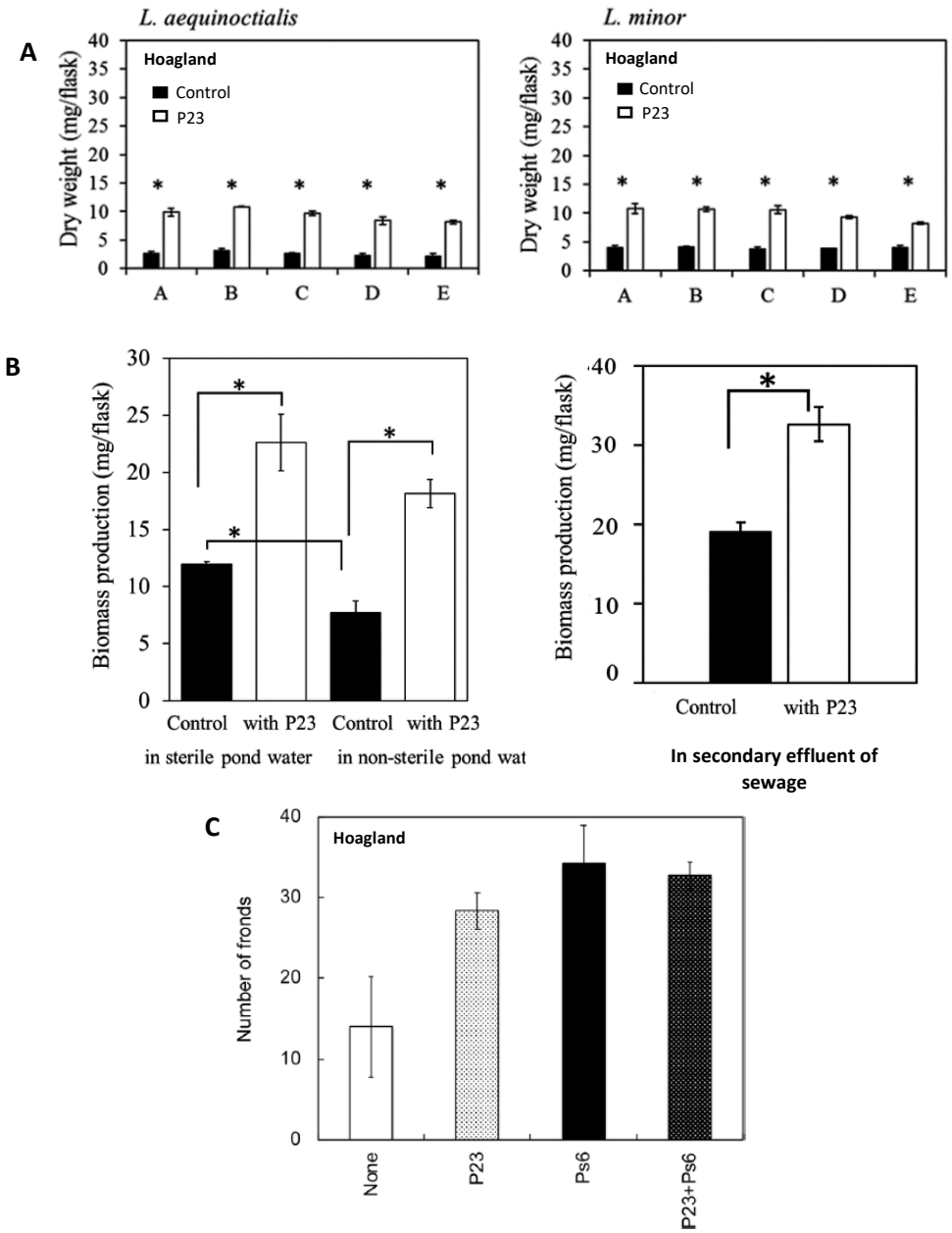


Fig. 1. Effect of PGPB, *A. calcoaceticus* P23 (A, B, C) and *P. fulva* Ps6 (C), on the growth of duckweed in A) different concentration of Hoagland medium (A-E: 1x, 2x, 1x, 1.5x, 2x concentration of Hoagland medium, respectively); B) pond water (left) containing: 1.25 mg/L of N-NH_4 and 0.25 mg/L P-PO_4 and effluent (right) containing: 4.27 mg/L N-NH_4, 0.66 mg/L of N-NO_3, 7.72 mg/L of N-NO_3, and 0.98 mg/L of P-PO_4 (Toyama et al., 2017); C) Hoagland medium (Yamakawa et al., 2018). "Control" and "None" in B and C are the experiment with no P23.

In this study, I conducted a trial to grow duckweed in the food factory WW from Thailand. Before discharge to the river, the treated WW is usually kept for a couple of weeks in a large buffering pond, in which duckweed can be easily cultivated without costly modification of WW treatment facilities (Fig 2). The application of PGPB was

used to enhance the duckweed yield furthermore. Duckweed can be treated first with the bacterial suspension, then the activated duckweed (duckweed with PGPB attached) can be cultivated in the WW. During the growth period of duckweed, it can uptake some nutrients from water thus purifying the WW even more. Some PGPB may lose from the duckweed surface during the cultivation period (Ishizawa et al. 2020), therefore the duckweed needs to be re-activated for another duckweed production cycle. Finally, the duckweed biomass produced in WW can be used for another purpose, such as for the bioenergy production substrate. The plan for the duckweed growth on-site is described in Fig. 2.

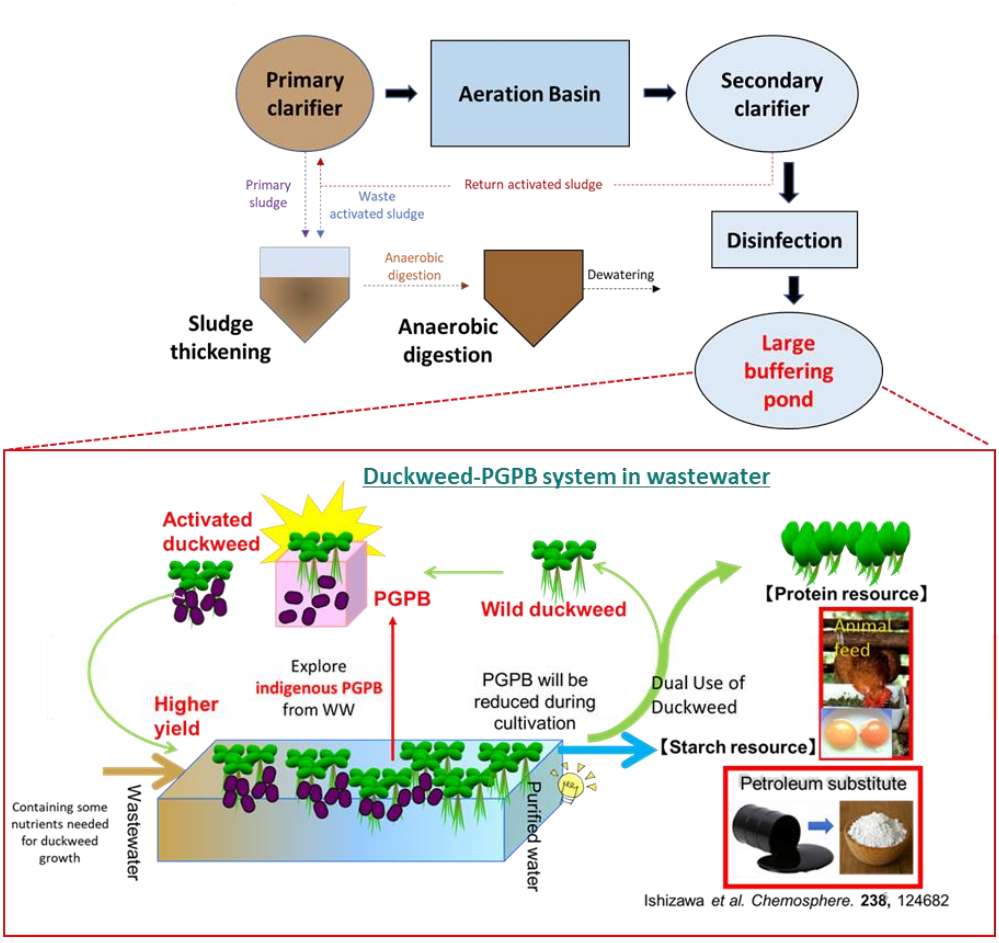


Fig. 2. Model of duckweed cultivation coupled with PGPB application on-site using treated WW kept in large buffering pond.

CHAPTER I

Isolation and evaluation of indigenous bacterial activity on *L. gibba* growth in food factory wastewater (Khairina et al., 2021)

In this chapter, I attempted to enhance duckweed biomass production utilizing *L. gibba* in food factory WW by applying PGPB. Briefly, the effects of PGPB application were initially examined for previously isolated PGPB, i.e., *A. calcoaceticus* P23 and *P.fulva* Ps6. Surprisingly, the results showed that neither P23 nor Ps6 improved the growth of the duckweed *L. gibba* in the treated WW, while P23 showed growth inhibition against *L. gibba*. Then, I wondered if some of the domestic bacteria in the factory WW exhibited growth promotion activity towards *L. gibba* (Fig. 3). I found that bacteria belonging to a special group were selected as PGPB. Moreover, I identified the key factors responsible for the growth inhibition activity of P23 in the treated WW. The findings of this study provide new knowledge for the selection of suitable PGPB for efficient duckweed biomass production using practical food factory WW.

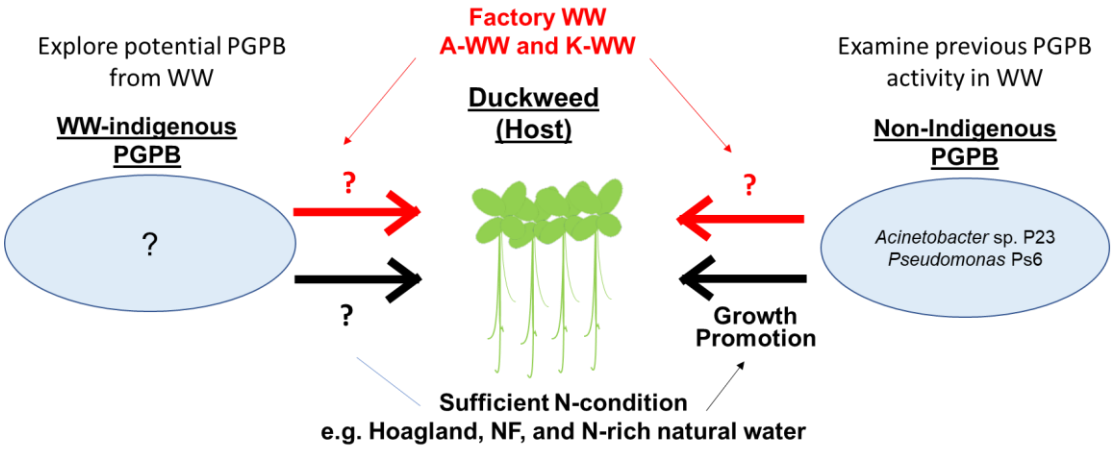


Fig. 3. The graphical abstract of chapter 1: the exploration of functional indigenous PGPB from WW.

1.1. Materials and methods

1.1.1. Plant material

I compared the growth and tolerance of aseptic duckweed strains in the food factory treated WW used in this study. *Lemna gibba* (G3 strain; RDSC serial number: 362; ID: DWC128) was selected as the best candidate among *Landoltia punctata*, *Lemna aequinoctialis*, *Lemna minor*, *Lemna turionifera*, *Spirodela polyrhiza*, *Wolffia microscopica*, *Wolffiella lingulata*, and others (data not shown). Duckweed was cultivated in a plant growth chamber (MLR352, Panasonic Corp., Osaka, Japan) at a temperature of 28 °C, an illuminance of 85 μmol/(m^2·s), a photoperiod of 16 h, and ca. 50% humidity. The sterility of the duckweed stock was routinely confirmed by the absence of bacterial colony formation on R2A agar plates incubated for one week at 30 °C.

1.1.2. Wastewater

Treated WW samples, namely A-wastewater (A-WW) and K-wastewater (K-WW), were collected from the final sedimentation tanks of two food factories. Water samples were sterilized using a membrane filter with a pore size of 0.22 μm (Sartolab, Sartorius AG, Göttingen, Germany) before use for the experiments of gnotobiotic duckweed culture. Unsterilized WW was used to evaluate the effects of indigenous bacterial community and novel PGPB isolates. The anion content of WW was analyzed by ion chromatography (IC-2010, Tosoh Corp., Tokyo, Japan) with a superIC- AZ column (Tosoh) and an eluent of 1.9 mM $NaHCO_3$ + 3.2 mM Na_2CO_3 at a flow rate of 0.8 mL/min and a temperature of 40 °C. Metal elements were analyzed using an ICP emission spectrometer (ICPE-9000, Shimadzu Corp., Kyoto, Japan) at the Hokkaido University Global Facility Center. Ammonium and COD were quantified using the PACKTEST kit (KYORITSU Chemical-Check Lab, Tokyo, Japan) according to the manufacturer's instructions. The pH was measured using the Docu-pH+ pH-meter (Sartorius). The pH of WW was adjusted to 7 in all experiments.

1.1.3. Growth media

Hoagland medium or NF medium was used to grow duckweed aseptically or gnotobiotically. The Hoagland medium contained 0.36 mM KNO_3, 1.68 mM K_2SO_4, 0.99 mM $CaCl_2·2H_2O$, 0.42 mM $MgSO_4·7H_2O$, 0.03 mM $NaH_2PO_4·2H_2O$, 0.012 mM $FeSO_4·7H_2O$, 0.02 mM H_3BO_3, 0.002 mM $MnCl_2·4H_2O$, 0.0003 mM $ZnSO_4·7H_2O$,

0.0001 mM $CuSO_4 \cdot 5H_2O$, and 0.001 mM H_2MoO_4 (Yamaga et al., 2010). The pH was adjusted to 7.0 with ±500 µL of 0.2N NaOH. Hoagland medium has low buffering capacity so the pH adjustment will take a longer time (±20-30 min). The NF medium contained 2.7 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM KH_2PO_4, 5 mM KNO_3, 0.02 mM $FeSO_4 \cdot 7H_2O$, 0.05 mM Na_2-EDTA, 0.02 mM $MnCl_2 \cdot 4H_2O$, 0.05 mM H_3BO_3, 0.001 mM $ZnSO_4 \cdot 7H_2O$, 0.0003 mM $CuSO_4 \cdot 5H_2O$, and 0.0005 mM MoO_3 (Muranaka et al., 2014). The pH was adjusted to 5.0 with KOH.

Bacteria were cultured in either LB medium or R2A medium. The LB medium contained 5 g/L Bacto Yeast extract (BD Difco Laboratories, Franklin Lakes, NJ, USA), 10 g/L Bacto Tryptone (Difco), and 5 g/L NaCl. The pH of the medium was adjusted to 7.2 with NaOH. The R2A medium contained 0.5 g/L each of Bacto Proteose peptone No. 3 (Difco), Bacto Yeast extract, casamino acid, glucose, and soluble starch, 0.3 g/L each of KH_2PO_4 and sodium pyruvate, and 0.05 g/L of $MgSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 7.2 with NaOH. LB and R2A media were solidified by adding 1.5% agar when necessary.

1.1.4. Growth test of *L. gibba* in WW

The growth test was conducted by inoculating aseptic *L. gibba* in a 50 mL of filter-sterilized WW. The growth of *L. gibba* in WW was then compared with the growth in the Hoagland medium. The duckweed growth was measured based on the total number of fronds and dry weight after the cultivation period.

1.1.5. Starch and protein content of duckweed

The starch content was measured based on the standard protocol of Megazyme total starch kit (Megazyme International, Bray, Ireland) using RTS-NaOH procedure as presented in Appendix 7. While protein content was measured based on DC-protein assay BIORAD protocol (Bio-rad Laboratories Inc., California, United States) as described in Appendix 7.

1.1.6. Evaluation of the effect of the indigenous bacterial community from WW on *L. gibba* growth

Ten fronds, leaf-like structures, of aseptic *L. gibba* were cultivated for 14 days in filter-sterilized WW or non-sterilized WW. Duckweed growth was estimated by

counting the total number of fronds and recording the biomass (dry weight) after the cultivation period.

1.1.7. Isolation of bacteria from WW capable of colonizing *L. gibba*

Aseptic *L. gibba* fronds were transferred to flasks containing 50 mL of non-sterilized A-WW or K-WW and cultivated for three days in a plant growth chamber, thereby allowing bacterial adhesion followed by colonization. After cultivation, 10 duckweed fronds were collected, soaked gently with sterilized MiliQ water, and homogenized to release bacteria from *L. gibba* in 1 mL of sterilized phosphate buffer saline using a BioMasher II (Nippi Inc., Tokyo, Japan). The homogenized sample was diluted by a 10^{-1}, 10^{-2}, and 10^{-3} factor using sterilized MilliQ water. The diluted homogenates were spread onto three types of solid media, LB, R2A, and one-fifth-diluted R2A**,** and then incubated at 30 °C for two to three days. All morphologically distinct colony-forming bacteria were isolated and stored at -80 °C in a liquid medium containing 15% glycerol.

1.1.8. Examination of the effect of bacteria on *L. gibba* growth

The effect of bacteria on duckweed growth was examined under two conditions: 1) using a 12-well plate for preliminary selection of PGPB from WW in 4 mL of standard medium (NF medium); or 2) using a 100-mL flask or plant culture dish (SPL Life Sciences, Gyeonggi-do, South Korea) containing 50 mL of WW samples, Hoagland medium, or modified Hoagland medium.

The 12-well-plate method was performed as follows: A bacterial colony was inoculated with an inoculation loop into 4 mL of liquid LB medium in a test tube and shaken for 1–2 days, depending on the growth rate, at 30 °C and ±100 strokes per minute (Personal-11 SD, Taitec, Tokyo, Japan). After growth, bacterial cells were harvested by centrifugation, washed twice, and resuspended with 1 mL of NF medium. Inoculation of duckweed was performed by placing aseptic *L. gibba* on 4 mL of NF medium containing bacterial cells with a final OD_{600} of 0.3 for 24 h. After bacterial inoculation, two fronds of gnotobiotic *L. gibba* were transferred to a new 12-well plate containing 4 mL of NF medium and cultivated in a plant growth chamber. The growth of *L. gibba* was measured by counting the total number of fronds after the cultivation period.

The 100-mL flask or plant culture dish method was performed as follows: A bacterial colony was inoculated with an inoculation loop into 4 mL of liquid LB medium in a test tube and shaken in the water bath for 24h at 30 °C and 100 strokes per minute. Then, 1% of bacterial culture from the test tube was transferred into 20 mL of LB medium in a 100-mL flask and shaken in the water bath for another 24h. Bacterial cells were harvested by centrifugation, washed twice with Hoagland medium, and resuspended in 20 mL of Hoagland medium. Bacterial inoculation was performed by placing aseptic *L. gibba* on 50 mL of Hoagland medium containing bacterial cells at a final OD_{600} of 0.3 for 24 h in a 100-mL flask or plant culture dish. After bacterial inoculation, gnotobiotic *L. gibba* was transferred to 50 mL of fresh bacteria-free WW, Hoagland medium, or modified Hoagland medium. Duckweed growth was estimated by counting the total number of fronds and measuring the biomass (dry weight) after the cultivation period.

1.1.9. Identification of selected PGPB by 16S rRNA sequence analysis

Bacterial DNA was extracted using the InstaGene DNA Purification Matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The DNA was used as a template for PCR amplification using the set of primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′) and the KOD-Plus-Neo DNA polymerase with a standard protocol (Toyobo, Kyoto, Japan). The PCR condition was as follows: initial denaturation (94^{o}C for 2 min), followed by 34 cycles of denaturation (98^{o}C for 10 s), annealing (50^{o}C for 30 s), and extension (68^{o}C for 50 s). Amplicons were purified from the agarose gel with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing cycles consisted of 24 cycles of 96^{o}C for 10 s, 50^{o}C for 5 s, and 60^{o}C for 4 min. The resulting sequences were compared to those included in the GenBank nucleotide sequence database with the NCBI Nucleotide BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for taxonomic identification. The nucleotide sequences of the 16S rRNA genes of stains 27AL and 29AL were deposited in DDBJ/GenBank/EMBL under accession numbers LC567048 and LC567049, respectively.

1.1.10. Quantification of plant-colonizing bacterial cells

Ten duckweed fronds and roots were rinsed twice with sterilized water to remove weakly attached bacteria. The duckweed samples were transferred into 1.5-mL plastic tubes containing 1 mL of sterilized water and homogenized as described above. The homogenized samples were diluted by 10^{-1}-10^{-4} factors, put on LB agar plates (±4 spots of each 10 µL sample), and incubated at 30°C for 24 h. The number of bacterial colonies (countable range 25-250 colonies) was counted, and the average number of plant-colonizing bacterial cells was expressed in colony-forming units (CFU)/plant.

1.1.11. Analysis of general PGP factors produced by bacteria

The production of IAA and related compounds was assessed according to Ishizawa et al. (2017) with modifications. Briefly, bacteria were cultured for 24 h at 30°C in 2 mL of LB medium with or without tryptophan (200 µg/mL), and 1 mL of each culture was centrifuged to recover the culture supernatant. Two hundred µL of Salkowski reagent (29.16 mL of 60% $HClO_4$, 1 mL of 0.5 M $FeCl_2$, and 19.84 mL of MilliQ water) was added to 1 mL of half-diluted supernatant. The mixture was incubated in the dark for 25 min and the absorbance was measured at 530 nm. The relative productivity of IAA and related compounds of these isolates, in the presence or absence of tryptophan, was determined using a standard curve that was constructed using different concentrations of IAA (5–100 µg/mL) from the dilution of IAA stock with MiliQ water (the IAA stock was prepared by suspended 1 mg of IAA in 1 mL of methanol). While siderophore production and phosphate solubilization activity were tested on solid agar media according to Yamakawa et al. (2018).

1.1.12. Examination of bacterial nitrogen metabolism

The pathways of nitrogen metabolism of bacterial strains were retrieved from the KEGG database (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg/pathway.html). The ability of bacteria to utilize each nitrogen compound was tested by cell growth assays in the basal medium. The composition of the basal salt (BS) medium is as follows: 0.41 g/L KH_2PO_4, 0.052 g/L K_2HPO_4, 0.05 g/L Na_2SO_4, 0.5 g/L $CaCl_2$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.005 g/L $FeSO_4 \cdot 7H_2O$, 0.0025 g/L $Na_2MoO_4 \cdot 2H_2O$, and 2 g/L succinic acid. The pH was adjusted to 7.0 with NaOH. BS medium was supplemented with different nitrogen compounds, including 1 g/L of casamino acid (organic nitrogen), 1 g/L of $NaNO_3$, or 1

g/L of NH_4Cl. A single colony was inoculated with an inoculation loop into the BS medium with and without nitrogen, and the bacterial growth was observed after 72 h of shaking at 30°C.

1.1.13. Statistical Analysis

Statistical analysis was conducted using SPSS software ver. 27.0 (IBM, Armonk, NY, USA). All results are reported as mean ± standard deviation (SD) with the values of three sample replicates per experiment. Significance ($P < 0.05$) was calculated using student t-test for experiment within 2 conditions or one-way ANOVA (followed by post-hoc test Tukey HSD if among groups value is significant) for experiment with more than 2 conditions.

1.2. Results and Discussion

1.2.1. Mineral nutrient content and duckweed growth in WW and Hoagland medium

The mineral nutrient content of A-WW and K-WW was compared with that of the popular Hoagland medium (Table 2). A-WW and K-WW had almost similar content of ions and minerals, except for PO_4. Indeed, K-WW contained a significantly higher amount of PO_4 than A-WW and Hoagland medium. However, these WW samples both contained significantly large amounts of Na but a small amount of NO_3 compared to the Hoagland medium. Finally, A-WW and K-WW contained traces of NH_3. With respect to the growth in the Hoagland medium, *L. gibba* growth was significantly reduced by both WW treatments (Fig. 4). In particular, chlorosis (emergence of white color) was observed in K-WW-grown fronds, probably due to excess PO_4 content (3.35 mM) (Appendix 1).

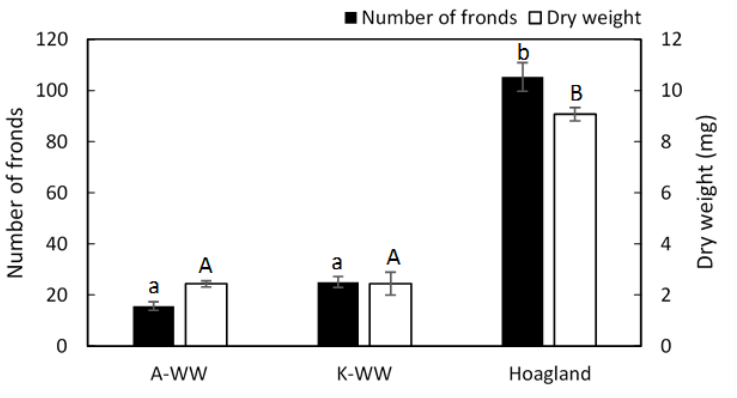


Fig. 4. Comparison of the growth of *L. gibba* in sterilized wastewater and Hoagland medium based on the number of fronds (closed bars) and dry weight (open bars) after 14 days of cultivation. Initial number of fronds was two. Values are mean ± SD (n = 3). Different alphabets between treatments indicates significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

Table 2. Mineral contents of wastewater and Hoagland medium for duckweed

Composition	Content (mM)		
	A-WW	K-WW	Hoagland
B	0.02 [a]	0.02 [a]	0.02 [a]
Ca	0.25 [a]	0.22 [a]	1.00 [b]
Cu	0.0005 [a]	0.0005 [a]	0.0002 [a]
Fe	0.0009 [a]	0.0003 [a]	0.012 [b]
K	0.39 [a]	0.26 [b]	3.72 [c]
Mg	0.16 [a]	0.10 [a]	0.42 [c]
Mn	0.0002 [a]	0.0005 [a]	0.002 [b]
Na*	48.72 [a]	46.11 [a]	0.03 [b]
Zn	0.004 [a]	0.004 [a]	0.0003 [b]
NH_3	0.012 [a]	0.042 [b]	-
Cl	5.35 [a]	11.27 [b]	2.00 [c]
NO_3†	0.006 [a]	0.005 [a]	0.357 [b]
SO_4	10.03 [a]	4.34 [b]	2.11 [c]
PO_4**	0.02 [a]	3.35 [b]	0.03 [a]
COD (mg/L)	20	13-20	-
pH	8.4	8.2	7.0

1. Different alphabets between treatments indicates significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc, n=3)
2. Relative standard deviation was less than 10% for the minerals value higher than 0.1mM, and less than 45% for the mineral values less than 0.1mM
3. *, † the mineral significantly more or less amounts in both wastewaters compared to Hoagland medium, respectively
4. ** the minerals excess only in K-WW

1.2.2. Starch and protein content of duckweed cultivated in WW

Based on the starch and protein analysis, I found that duckweed grown in WW samples has a higher starch content than those grown in standard medium, Hoagland (Fig 5). While protein content was almost similar between duckweed from WW or Hoagland medium. It has been reported that duckweed can accumulate more starch in nutrient starvation and salinity stress (Xu et al., 2012). Thus, I assumed that low N and high salinity (NaCl and Na_2SO4) conditions in A-WW and K-WW triggered the duckweed starch accumulation.

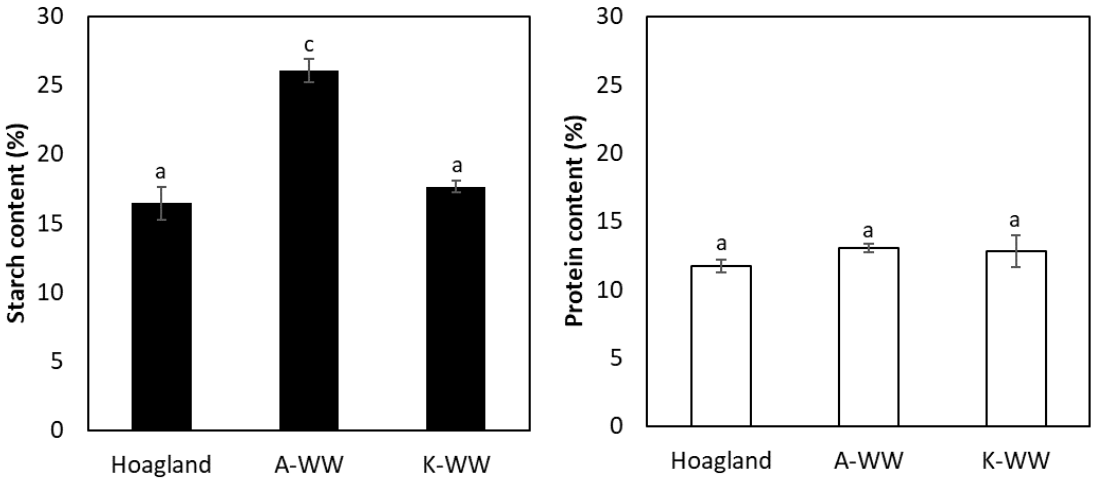


Fig. 5. The starch (left) and protein contents (right) of *L. gibba* in A-WW and K-WW compared to standard growth media for duckweed, Hoagland. The cultivation was conducted for 14 days. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

1.2.3. Examination of the activity of *A. calcoaceticus* P23 and *P. fulva* Ps6 in WW

I tested whether the P23 and Ps6 bacterial strains displayed growth-promoting activities towards *L. gibba* in filter-sterilized A-WW and K-WW. These strains were previously isolated from the surface of duckweed naturally growing in a pond of the Hokkaido University Botanic Garden (Yamaga et al., 2010; Yamakawa et al., 2018). Their activities were different depending on the duckweed species (Appendix 2). P23 could also promote the growth of *Lemna aequinoctialis* to a higher extent than that of *L. minor* (Toyama et al., 2017). Therefore, I first examined the PGP activities of these PGPB towards *L. gibba* in Hoagland medium (Fig. 6A). The growth of *L. gibba* was increased by 1.6- and 1.7-fold by P23 and Ps6, respectively. Next, PGP activities were

examined in A-WW and K-WW (Fig. 6B, 6C). Ps6 showed PGP activity in A-WW (1.25-fold growth increase). However, neither P23 nor Ps6 promoted the growth of *L. gibba* in K-WW; on the contrary, P23 showed a growth inhibition effect on *L. gibba* in both A-WW (0.79-fold) and K-WW (0.75-fold), according to the dry weight.

These results indicated that the PGP activities of P23 and Ps6 are not universal but depend on the plant species, such as *L. minor* or *L. gibba*, and the water conditions, whether optimal medium or nutrient-biased WW. Nevertheless, it is worth noting that P23 showed significant PGP activity in the secondary effluent of a municipal sewage treatment system (Fig. 1; Toyama et al., 2017; Ishizawa et al., 2020). This water contained 4.27–6.01 mg/L NH_4-N, 0.07–0.66 mg/L NO_2-N, 7.72–8.63 mg/L NO_3-N, and 0.98–1.84 mg/L PO_4-P at pH 7.5, but no excess Na, Cl, or SO_4, and thus presented more favorable mineral conditions for duckweed growth than A-WW and K-WW.

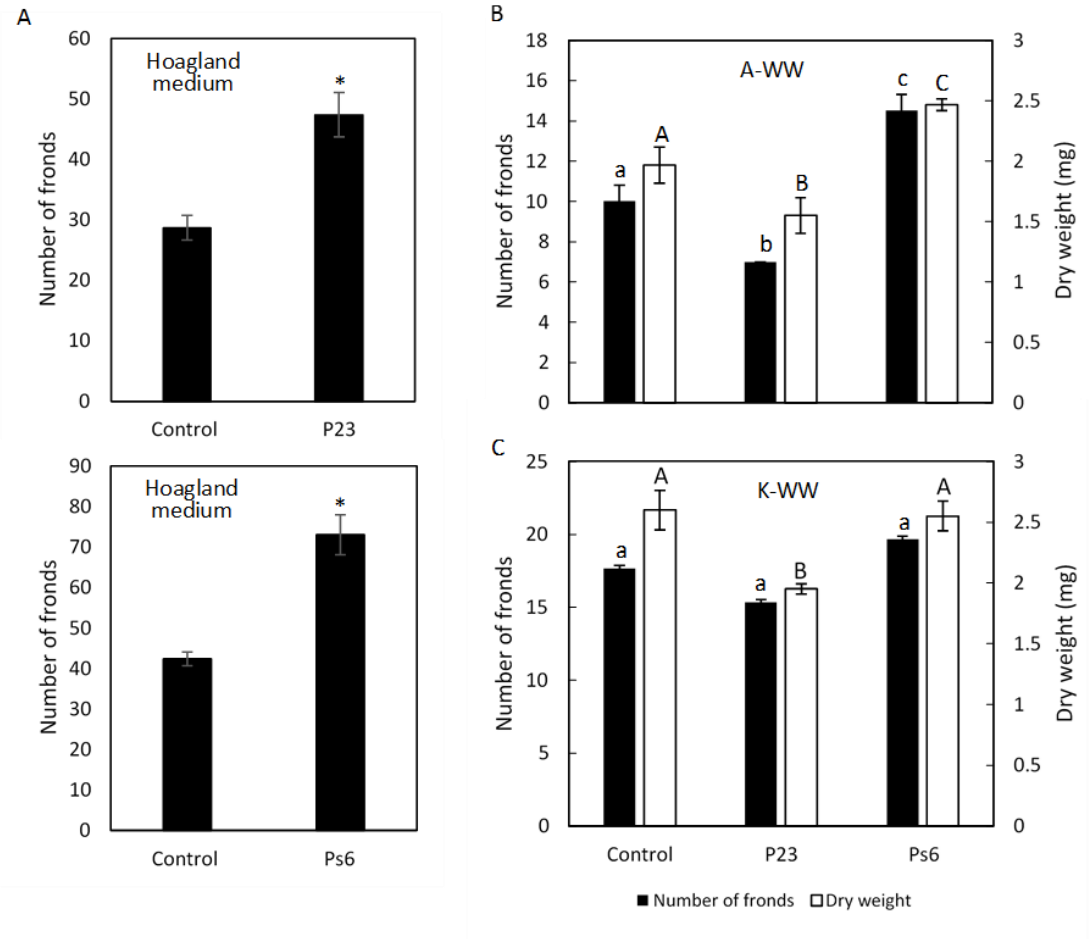


Fig. 6. The effect of PGPB, P23 and Ps6, on the growth of *L. gibba* in sterilized A) Hoagland, B) A-WW, and C) K-WW based on the number of fronds (closed bars) and dry weight (open bars) after 10 days of cultivation. "Control" is aseptic duckweed with no bacteria. Initial number of fronds was two in all experiments. Values are mean ± SD (n = 3). Asterisk (*) in (A) indicate the significant differences between values with and without PGPB (control) (student's t-test, $P < 0.05$). Different alphabets between treatments in (B) and (C) indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

1.2.4. Preliminary examination of PGP activities of indigenous bacteria in A-WW and K-WW

After testing known PGPB, I was interested in seeking novel PGPB from the food factory effluents where A-WW and K-WW had been collected. First, I evaluated how the total microbial community of non-sterile WW affected the growth of *L. gibba*. After 14 days of cultivation, duckweed placed in non-sterilized A-WW and K-WW showed significantly increased dry weight than in sterilized WW by 1.4- and 1.3-fold, respectively (Fig. 7A). Moreover, the fronds of duckweed growing in non-sterilized A-WW were greener and larger (Fig. 7B). These results strongly suggest the possibility that A-WW and K-WW naturally harbor potential PGPB that can promote or restore the growth of duckweed in WW conditions.

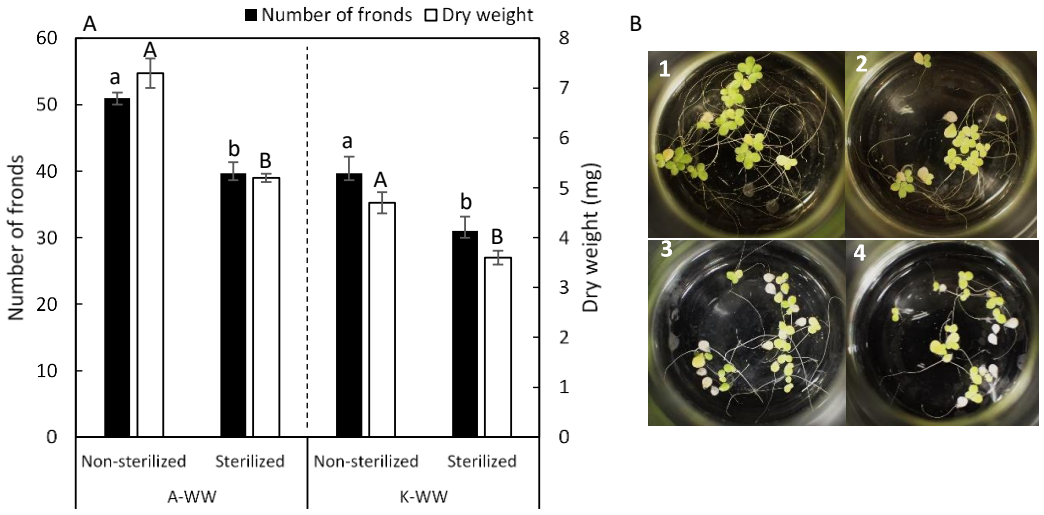


Fig. 7. A) Effect of indigenous microbial community in A-WW and K-WW on *L. gibba* growth based on the number of fronds (closed bars) and dry weight (open bars) after 14 days of cultivation. B) Photo image of *L. gibba* after 14 days of cultivation in 1) non-sterilized A-WW, 2) sterilized A-WW, 3) non-sterilized K-WW, 4) sterilized K-WW. Initial number of fronds was ten. Values are mean ± error (n = 2). Different alphabets indicate the significant differences between values of duckweed growth in sterilized and unsterilized conditions (Student's t-test, $P < 0.05$).

1.2.5. Isolation of PGPB from WW

Isolation of effective bacteria from WW was conducted by first selecting the bacteria that have the ability to adhere to and colonize the surface of duckweed. In fact, many agriculturally useful symbiotic bacteria such as *Rhizobium, Agrobacterium, Pseudomonas, Azospirillum*, and others, have been reported to attach to the plant surface (Wheatley and Poole, 2018). More importantly, the colonization of the host plant during water flow is an essential trait of PGPB of aquatic plants (Yamakawa et al., 2018). Therefore, aseptic *L. gibba* was cultivated in non-sterilized A-WW and K-WW for three days to allow indigenous bacteria to adhere to the surface of duckweed. After cultivation, bacterial strains colonizing duckweed were isolated. After subsequent selection, seven (20AL, 24AL, 25AL, 26AL, 27AL, 28AL, and 29AL) and ten (3KL, 4KL, 5KL, 6KL, 7KL, 15KL, 16KL, 17KL, 18KL, and 19KL) candidate bacterial strains were obtained from the duckweed grown in A-WW and K-WW, respectively, and used for further experiments. Finally, two bacterial strains, namely 27AL and 29AL from A-WW, showed notable PGP activity in NF medium compared to other isolates (Fig. 8A, 8B).

Furthermore, I investigated the PGP ability of strains 27AL and 29AL in A-WW and K-WW conditions. These strains significantly improved duckweed growth, as shown by the increased frond number and dry weight after 10 days compared to the bacteria-free control and P23-inoculated duckweed (Fig. 8C). Therefore, it was clear that the indigenous WW PGPB 27AL and 29AL are more effective than environmental water-derived PGPB in enhancing duckweed biomass production under factory WW conditions.

Based on the 16S rRNA gene sequences, both 27AL and 29AL belong to genus *Chryseobacterium* and the closest species is *Chryseobacterium taichungense*, with identity scores of 98.92% and 98.63%, respectively (Appendix 3) *Chryseobacterium* strains have been reported in a variety of environments, including fresh water, sewage,

and WW (Kämpfer et al., 2003; Bernardet et al., 2006). For example, *C. taichungense* was isolated from a tar-contaminated soil in Taiwan (Shen et al., 2005). Some *Chryseobacterium* strains have also been reported to exert PGP activity. For instance, *Chryseobacterium gleum* alleviated salt stress and enhanced the growth of bread wheat, *Triticum aestivum* L., by producing ACC deaminase, IAA, siderophores, ammonia, HCN, and fungal cell wall hydrolyzing enzymes (Bhise et al., 2017). Moreover, inoculation with *Chryseobacterium palustre* and *Chryseobacterium humi* improved the growth of corn, *Zea mays*, and its biomass production (Marques et al., 2010). It has been suggested that *C. indologenes* AM2 can fix nitrogen upon detection of PCR-amplified DNA fragments using a set of primers for the *nifH* gene (Dhole et al., 2017); however, no *nifH* gene has yet been found in the genome of *C. indologenes*. On the other hand, the examination of general PGP factors of *Chryseobacterium* sp. 27AL and 29AL showed that they both could produce IAA and siderophore compounds (Appendix 4). However, no nitrogen-fixing activity was detected for 27AL and 29AL by acetylene reduction assay (Data not shown). To the best of our knowledge, this is the first report on the isolation of duckweed PGPB from factory WW.

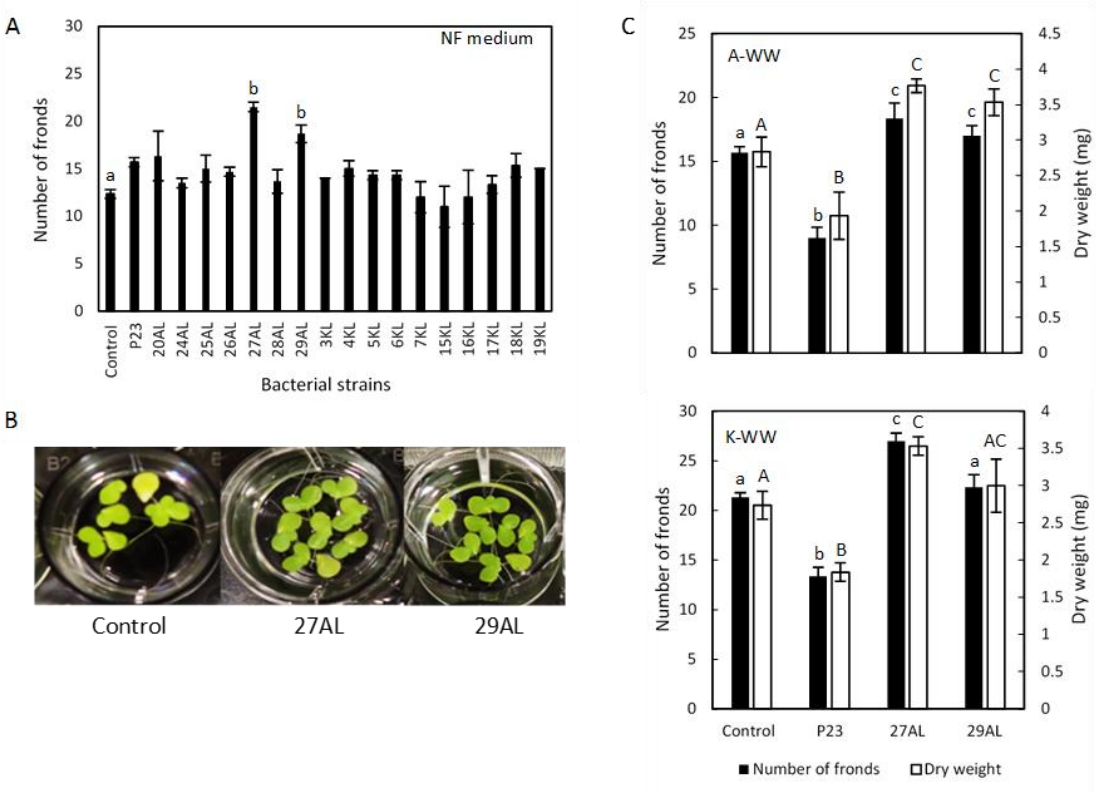


Fig. 8. A) Effect of bacterial strains isolated from WW on *L. gibba* growth compared to previously isolated PGPB (P23) and control (no bacteria) based on the number of fronds after 10 days of cultivation in NF medium. The symbols "AL" and "KL" represent the bacteria isolated from A-WW (A) or K-WW (K) that are capable of adhering on the surface of *L. gibba* (L). B) Photo image of *L. gibba* after 10 days of cultivation in NF medium colonized with the best two PGPB, 27AL and 29AL, and no bacteria control. C) Effects of P23, 27AL, and 29AL in sterilized A-WW and K-WW on the *L. gibba* growth compared to control based on the number of fronds (closed bar) and dry weight (open bar) after 10 days of cultivation. The initial number of fronds was two in all experiments. Values are mean ± SD (n = 3). Different alphabets between treatments in A (only given on the selected bacteria and control) and C indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

1.2.6. Restrictive metabolic pathways of nitrogen in *Chryseobacterium* bacteria

According to the analysis of mineral nutrients described above, both A-WW and K-WW have very low nitrogen contents compared to the plant medium Hoagland, which was assumed to be a growth-limiting factor for duckweed (Table 2). Nitrogen is an essential mineral for the growth and reproduction of living organisms, including duckweed and bacteria (Fang et al., 2007). Consistently, supplementation of ammonium (NH_4) or nitrate (NO_3) to A-WW and K-WW restored duckweed growth (Chapter II, Fig 16). Therefore, I hypothesized that the mutualistic interaction between duckweed and PGPB interfered with the competition for nutrients including nitrogen sources. Indeed, the competition between land plants and soil microorganisms for inorganic and organic nitrogen has been reported even at relatively fertile sites (Kaye and Hart, 1997; Jones et al., 2018).

Therefore, I wondered whether *Chryseobacterium* has some specific trait for avoiding nitrogen competition with a host plant. I then analyzed and compared the nitrogen metabolic pathways retrieved from the KEGG database of three bacterial species, *A. calcoaceticus* CA16, *P. fulva* 12-X, and *Chryseobacterium indologenes* FDAARGOS_337, that are the same or closely related to the PGPB strains P23, Ps6, and the newly isolated 27AL/29AL, respectively. Strains 27AL /29AL shared about 96% identity with the 16S rRNA gene of *C. indologenes* after the BLAST result. Based on the KEGG pathway map (Fig. 9), *Chryseobacterium* has apparently limited nitrogen pathway compared to bacteria of other genera. To confirm this finding, I carefully searched for relevant genes in the *C. indologenes* genome and found putative NarK/NasA family nitrate transporter genes (TLX26322 and TLX26323) and a nitrite reductase gene (TLX26356); however, no nitrate reductase gene was detected. On the

other hand, neither nitrite nor nitrate reduction activities have been reported for most *Chryseobacterium* strains, including *C.* taichungense (Shen et al., 2005). Thus, limited use of nitrogen compounds seems generally shared across the genus *Chryseobacterium*.

To verify this hypothesis, I conducted growth experiments of 27AL in BS medium with different nitrogen sources (Fig. 10). As a control I used *A. calcoaceticus* P23, which is suggested to exploit a broad range of nitrogen sources. This assay revealed that 27AL grew normally in BS medium with casamino acids (organic nitrogen) but failed to grow in media with either NO_3 or NH_4 as sole nitrogen sources. On the other hand, P23 could grow in media containing both organic and inorganic nitrogen compounds. These results strongly suggested that 27AL does not compete with duckweed for inorganic nitrogen sources, which is an advantageous trait for PGPB application under nitrogen-limiting conditions.

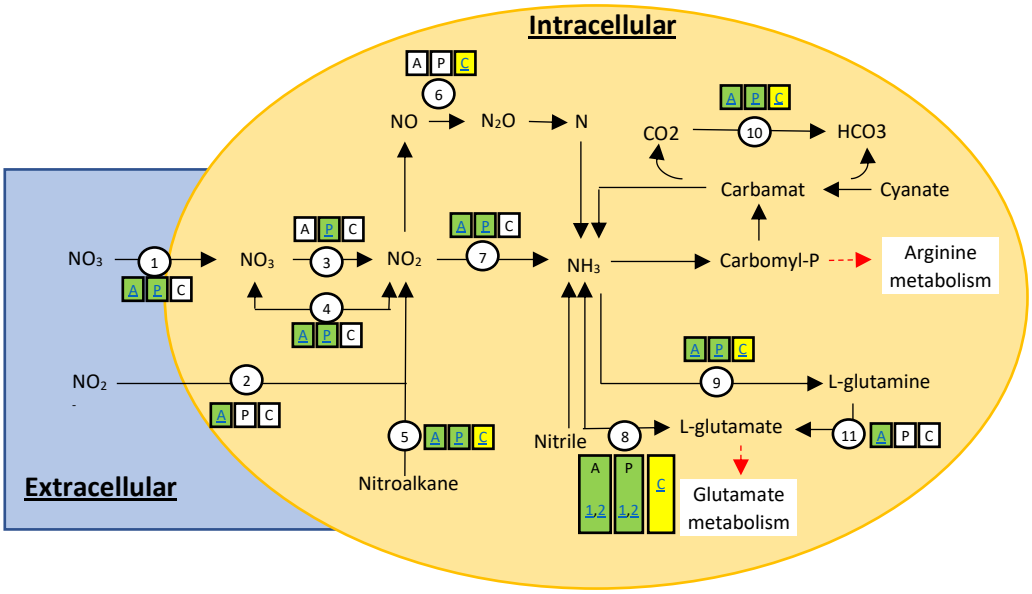
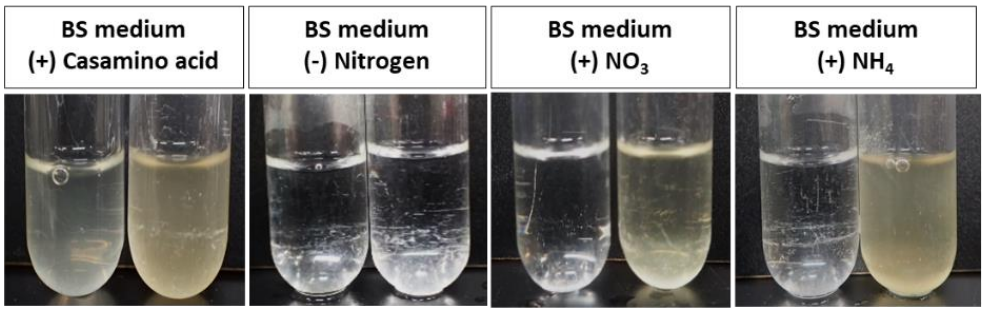


Fig. 9. Nitrogen metabolic pathway retrieved from KEGG database. The green color describes the existence of the gene/enzyme for A) *Acinetobacter calcoaceticus*; P) *Pseudomonas fulva*; while the yellow color describes the existence gene in C) *Chryseobacterium indolegense*, if the bacteria do not have the gene, white color is applied. Numbers 1 to 11 are the genes for: 1) MFS transporter, NNP family, nitrate transporter; 2) MFS transporter, NNP family, nitrite transporter; 3) Nitrate reductase; 4) assimilatory nitrate reductase catalytic; 5) nitronate monooxygenase; 6) nitric-oxide reductase; 7) nitrite reductase; 8) glutamate dehydrogenase; 9) glutamine synthetase; 10) carbonic anhydrase; 11) glutamate synthase, respectively.



Strain	27AL	P23	27AL	P23	27AL	P23	27AL	P23
OD	0.333	1.020	0.002	0.001	0.003	0.250	0.002	0.850

Fig. 10. Growth tests of *Chryseobacterium* sp. 27AL and *Acinetobacter* sp. P23 in BS medium; 1) with casamino acid (1 g/L) as a positive control; 2) no nitrogen (negative control); 3) with $NaNO_3$ (1 g/L); with NH_4Cl (1 g/L). Cultures were shaken for 3 days at 30°C and measured OD_{600}. White lines and spots are scratches of glass test tubes.

1.2.7. Factors affecting PGP behavior of *A. calcoaceticus* P23

P23 exerted no duckweed growth-promoting effect, but rather inhibited the growth of *L. gibba* in A-WW and K-WW conditions as shown in Fig. 6B and 6C. However, as suggested above, nitrogen limitation in WW may be one of the key factors causing this inhibition effect of P23 due to competition with the host plant for nutrients. Therefore, I prepared various Hoagland media with mineral compositions mimicking K-WW in order to identify factors affecting the PGP activity of P23. I chose to mimic K-WW because both PGPB (either P23 or Ps6) could not clearly show PGP activity in this condition. Among the ions and minerals of WW, I focused on NH_4, NO_3, Na, Cl, SO_4, and PO_4 because of their significant excess or depletion compared to the Hoagland medium as well as their biological importance for duckweed growth. Therefore, the Hoagland medium was modified as shown in Appendix 5 and used for duckweed growth experiments (Fig. 11). I found that high PO_4, Na, Cl, or SO_4 conditions did not significantly affect the PGP activities of P23 and the newly isolated 27AL. On the other hand, low nitrogen (NH_4 or NO_3) conditions generally resulted in reduced growth of duckweed by switching the behavior of P23 from duckweed growth promotion to inhibition. In contrast, 27AL promoted duckweed growth under all conditions, including low nitrogen. Moreover, the phenomena of duckweed growth inhibition by P23 and promotion by 27AL were slight but most significantly observed in the combination of low NH_4/NO_3 and high PO_4/Na/Cl/SO_4, which is the condition most similar to K-WW.

Furthermore, I observed the effects of NH_4/NO_3, PO_4, Na, Cl, and SO_4 on bacterial colonization by quantifying average colony-forming units (CFU) per plant in each modified Hoagland medium. Notably, the initial CFU/plant value of P23 (7.3 x 10^5) was more than three times that of 27AL (2.2 x 10^5) suggesting a higher activity of adhesion and growth on duckweed in Hoagland medium. However, the CFU/plant value of P23 after 14 days of growth was dramatically decreased in normal Hoagland medium, from 7.3 x 10^5 to 6 x 10^2. Conversely, the CFU/plant value of P23 after 14 days of growth was highest (7 x 10^4) in the combined low NH_4/NO_3 and high PO_4/Na/Cl/SO_4 condition, in which P23 most severely inhibited duckweed growth (Fig. 11). Understandably, the more P23 cells colonized the duckweed, the more they competed with it for nitrogen. On the other hand, the CFU/plant value of 27AL was not significantly different between normal Hoagland medium (4.3 x 10^3) and low NH_4/NO_3 and high PO_4/Na/Cl/SO_4 conditions (1.1 x 10^3). In summary, these results showed that different mineral conditions could affect the colonization of bacteria on duckweed, with beneficial or detrimental effects depending on nutrient availability in the environment. However, the reason why P23 colonized more in combined low NH_4/NO_3 and high PO_4/Na/Cl/SO_4 conditions is still unclear.

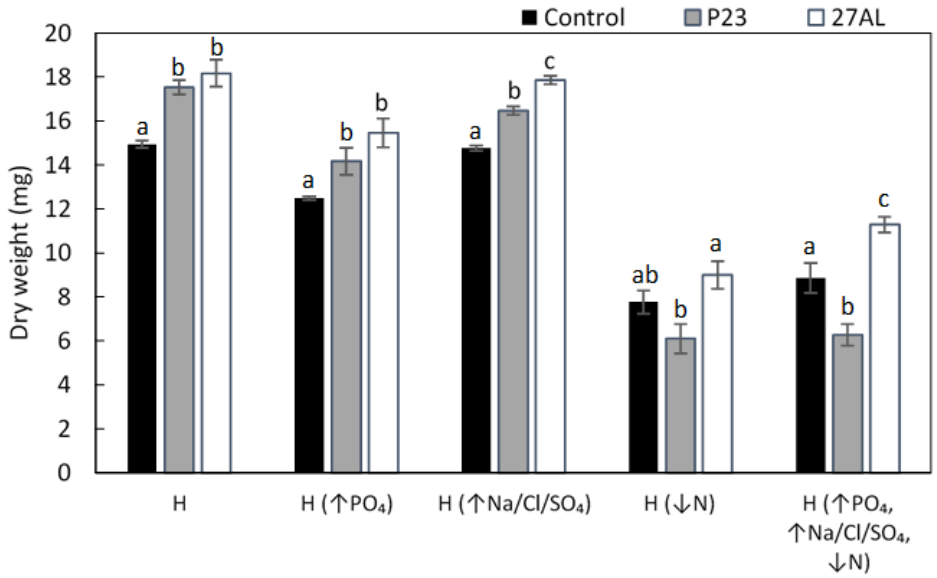


Fig. 11. Effect of different amount of minerals on P23 (grey bars) and 27AL (open bars) activities against *L. gibba* compared to aseptic duckweed control (closed bar) after 14 days of cultivation. The initial number of fronds inoculated was ten. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

Conclusion

PGPB are currently expected to become a new nature-based technology for increasing duckweed biomass production. Here I call attention to the use of PGPB, especially in factory WW with uneven nutritional conditions. Highly active PGPB obtained from the environmental water in which duckweed naturally grows do not have a universal function for plant growth promotion. Nevertheless, I showed for the first time that among the indigenous bacteria that naturally grow in factory WW unrelated to duckweed habitat, there exist bacteria that can promote duckweed growth in the water condition (Fig. 12). To practically produce duckweed biomass in different water environments, it is important to select and utilize PGPB adapted to each environment.

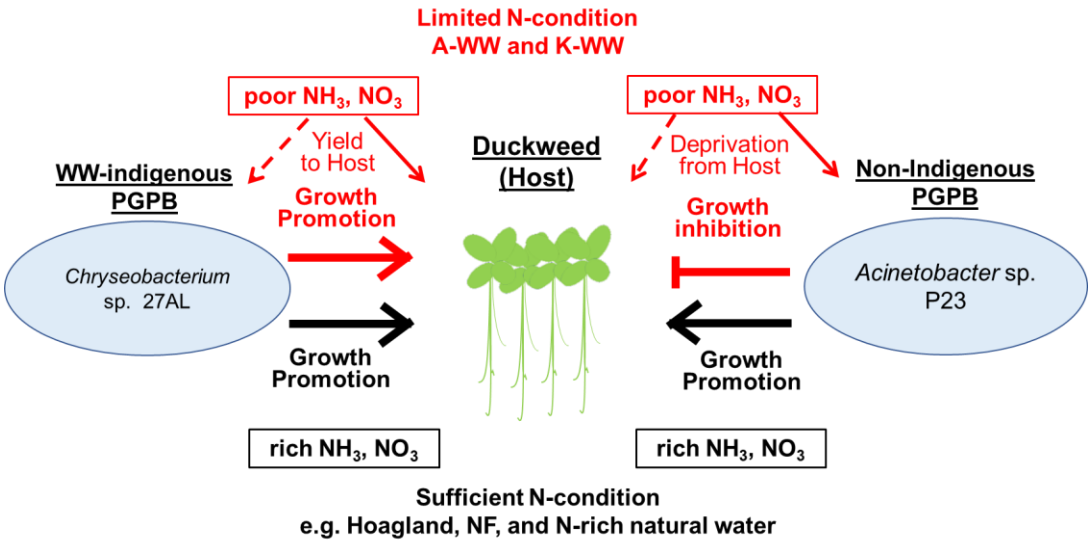


Fig. 12. Effect of different PGPB on duckweed in WW samples (A-WW and K-WW) containing poor nitrogen content. It was showed that indigenous PGPB has a higher potential to improve duckweed yield in WW because of low competition over nitrogen with the duckweed.

CHAPTER II

Mineral modification of wastewater to enhance duckweed biomass production and PGPB activity

This chapter reports a trial for enhancing duckweed biomass production, *L. gibba*, and PGPB activity by mineral modification of WW. In the previous chapter, I have selected the best indigenous PGPB that improved *L. gibba* growth by 1.2-1.3x in A-WW and K-WW, respectively. However, duckweed *L. gibba* biomass in WW was much lower than when cultivated in the optimal medium, Hoagland, by 6x growth reduction. The application of indigenous PGPB could not fully recover *L.gibba* yield so much. Additionally, WW contained the unbalanced minerals that significantly reduced *L. gibba* growth and affected some PGPB activity, suggested in chapter I. To resolve this problem, I conducted the following approaches: 1) Confirmation of the minerals responsible for the growth inhibition of *L. gibba* in WW; 2) Mineral modification of WW that can improve *L. gibba* growth and PGPB activities in WW. Finally, by integrating the application of indigenous PGPB combined with the mineral modification (Fig 13), I expected a high yield of duckweed biomass production in the WW.

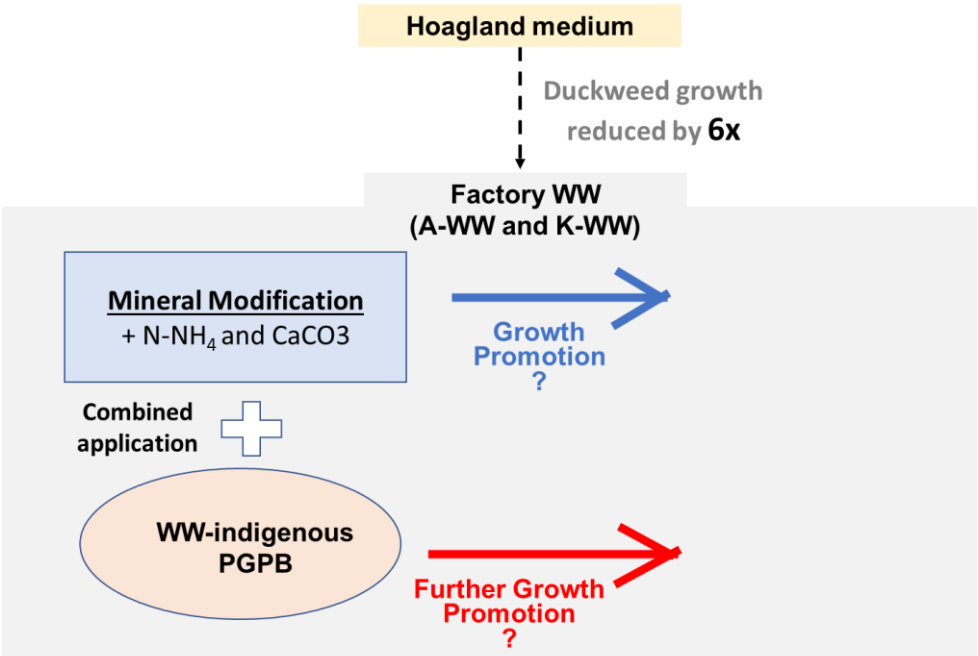


Fig. 13. The graphical abstract of chapter II: Mineral modification of WW for higher duckweed biomass and PGPB activity.

2.1. Materials and methods

2.1.1. Plant material

Plant material and growth condition used in this chapter is referred to chapter I, section 1.1.1.

2.1.2. Wastewater

The WW sample and mineral analysis method used in this chapter is referred to chapter I, section 1.1.2. Additionally, a new batch of WW, namely K-WW (2018), is included in this chapter. While K-WW used in the previous chapter will be noted as K-WW (2017). The pH of WW was adjusted to 7 in all experiments to mimic the initial pH of WW on site. The change of pH of WW samples could gradually happen because of the extended storage.

2.1.3. Growth test of *L. gibba* in WW

The growth test of *L. gibba* in WW was conducted based on the method in chapter 1, section 1.1.4.

2.1.4. Identification of inhibition factors of WW

The analysis of inhibiting factors was performed by comparing the minerals in WW which were significantly higher or lower concentration by 100x than Hoagland medium. The inhibitory test for the suspected minerals was conducted by modifying Hoagland medium with a) NO_3/NH_4, b) PO_4, and c) Na, which were adjusted close to the concentration of WW (Appendix 5). Aseptic *L. gibba* was then inoculated into Hoagland (as control) or modified Hoagland medium (as treatment) and cultivated in the plant incubation chamber. The duckweed growth was measured by counting the total number of fronds and dry weight after the cultivation period.

2.1.5. Mineral modification of WW

The first modification was conducted by supplementing nitrogen with 0.7 mM of N-NO_3 (using KNO_3) or N-NH_4 (using NH_4OH). The sterilized stock of N-NO_3 or N-NH_4 (1000x concentration) was added to the filter-sterilized WW by adjusting the N concentration to 0.7 mM. Then aseptic *L. gibba* was inoculated into 50 mL of N-supplemented WW (as treatment) and non-supplemented WW (as control). The duckweed growth was measured by counting the total number of fronds and dry weight after the cultivation period.

The second modification was conducted to reduce the toxicity effect of excess PO_4. Three methods were tested, including $CaCl_2$, $CaCO_3$, and activated charcoal treatments. About 10 mM of each $CaCl_2$. $7H_2O$ or $CaCO_3$ or 10% (w/v) of activated charcoal was used. The concentration was determined based on the optimization in the preliminary study (Data not shown). For $CaCl_2$ treatment, the sterilized stock (100 mM of $CaCl_2.H_2O$) was added to 50 mL of sterilized WW by adjusting the concentration to 10 mM. For CaCO3 and activated charcoal treatments, WW was first mixed with 10 mM of $CaCO_3$ or 10% (w/v) of activated charcoal and stirred for ±1 h. After the treatment, WW was sterilized using a membrane filter with a pore size of 0.22 μm (Sartolab). The combination treatment with 0.7 mM of $N\text{-}NH_4$ supplementation was also performed for further duckweed growth improvement after $CaCO_3$ or activated charcoal application. Then aseptic *L. gibba* was inoculated into 50 mL of treated WW and non-treated WW (as control). The duckweed growth was measured by counting the total number of fronds and dry weight after the cultivation period.

2.1.6. Plant growth promotion effect of PGPB on *L. gibba* in mineral modified WW

The plant growth promotion test of PGPB was conducted by referring to chapter I, section 1.1.8. Briefly, a bacterial colony was inoculated with an inoculation loop into 4 mL LB medium and then incubated overnight in the water bath at 30 °C with the speed of 100 strokes per minute. Afterwards, 1% of bacterial culture from the first pre-culture was transferred into a 100-mL flask containing 20 mL LB medium and shaken for another 24h. Bacterial cells were harvested by centrifugation, washed twice with Hoagland medium, and resuspended with Hoagland medium. The bacterial inoculation was performed by placing aseptic *L. gibba* on 50 mL of Hoagland medium containing bacterial cells at a final OD_{600} of 0.3 for 24 h in a 100-mL plant culture dish. After bacterial inoculation, gnotobiotic *L. gibba* was soaked twice in Hoagland medium to discard unattached bacteria and subsequently transferred to 50 mL of sterilized modified WW. Duckweed growth was estimated by counting the total number of fronds and measuring the biomass (dry weight) after the cultivation period.

2.1.7. Statistical Analysis

Statistical analysis was conducted using SPSS software ver. 27.0 (IBM, Armonk, NY, USA). All results are reported as mean ± standard deviation (SD) with the values of three sample replicates per experiment. Significance ($P < 0.05$) was

calculated using student t-test for experiment within 2 conditions or one-way ANOVA (followed by post-hoc test Tukey HSD if among groups value is significant) for experiment with more than 2 conditions.

2.2. Results and Discussion

2.2.1. Mineral analysis and *L. gibba* growth in WW

Mineral analysis and duckweed growth test in WW have been performed in the previous chapter; however, a new batch of WW, K-WW (2018), was also used in this chapter (Table 3). The growth of duckweed in a new batch of K-WW (2018) was significantly reduced compared to A-WW, K-WW (2017), and Hoagland medium (Fig. 14). WW has significantly higher or lower minerals of Na, PO_4, and N compared to Hoagland medium. I suspected that those significant excess and limited minerals in WW were the factors inhibiting *L. gibba* growth in WW condition. Thus, in the next experiment, I tried to identify which minerals are responsible for duckweed growth inhibition.

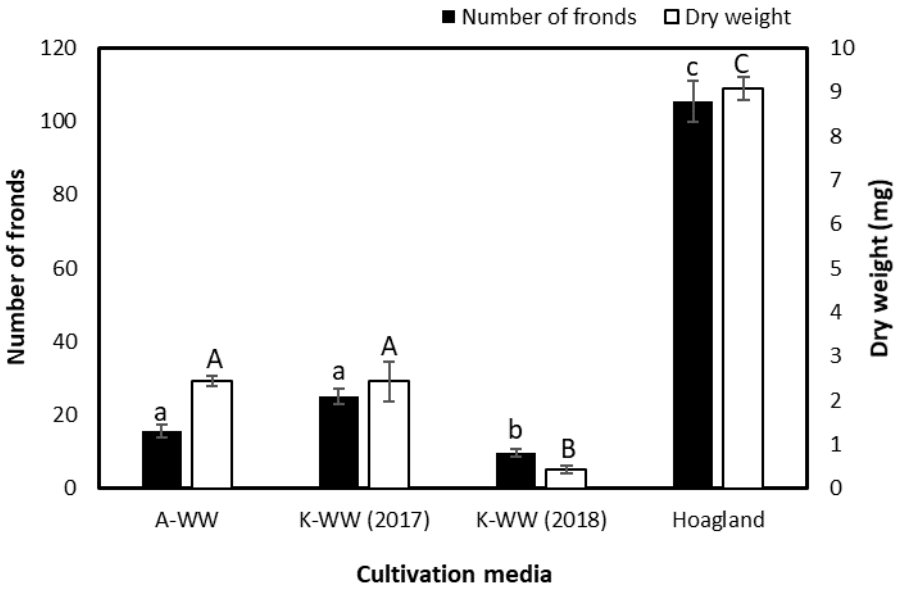


Fig. 14. Comparison of the growth of *L. gibba* in sterilized WW and Hoagland medium based on the number of fronds (closed bars) and dry weight (open bars) after 14 days of cultivation. Initial number of fronds was two. Values are mean ± SD (n = 3). Different alphabets between treatments indicates significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

Table 3. Mineral contents of wastewater and Hoagland medium for duckweed

Composition	Content (mM)			
	A-WW	K-WW (2017)	K-WW (2018)	Hoagland
B	0.02 [a]	0.02 [a]	-	0.02 [a]
Ca	0.25 [a]	0.22 [a]	0.16 [a]	1.00 [b]
Cu	0.0005 [a]	0.0005 [a]	-	0.0002 [a]
Fe	0.0009 [a]	0.0003 [a]	-	0.012 [b]
K	0.39 [a]	0.26 [b]	-	3.72 [c]
Mg	0.16 [a]	0.10 [a]	0.91 [b]	0.42 [c]
Mn	0.0002 [a]	0.0005 [a]	-	0.002 [b]
Na*	48.72 [a]	46.11 [a]	37.10 [b]	0.03 [c]
Zn	0.004 [a]	0.004 [a]	-	0.0003 [b]
NH_3	0.012 [a]	0.042 [b]	0.029 [c]	-
Cl	5.35 [a]	11.27 [b]	16.92 [c]	2.00 [d]
NO_3†	0.006 [a]	0.005 [a]	-	0.357 [b]
SO_4	10.03 [a]	4.34 [b]	15.34 [c]	2.11 [d]
PO_4**	0.02 [a]	3.35 [b]	3.84 [b]	0.03 [a]
COD (mg/L)	20	13-20	13-20	-
pH	8.4	8.2	8.1	7.0

1. Different alphabets between treatments indicates significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc, n=3)
2. Relative standard deviation was less than 10% for the minerals value higher than 0.1mM, and less than 45% for the mineral values less than 0.1mM
3. *, † the mineral significantly more or less amounts in both wastewaters compared to Hoagland medium, respectively
4. ** the minerals excess only in K-WW

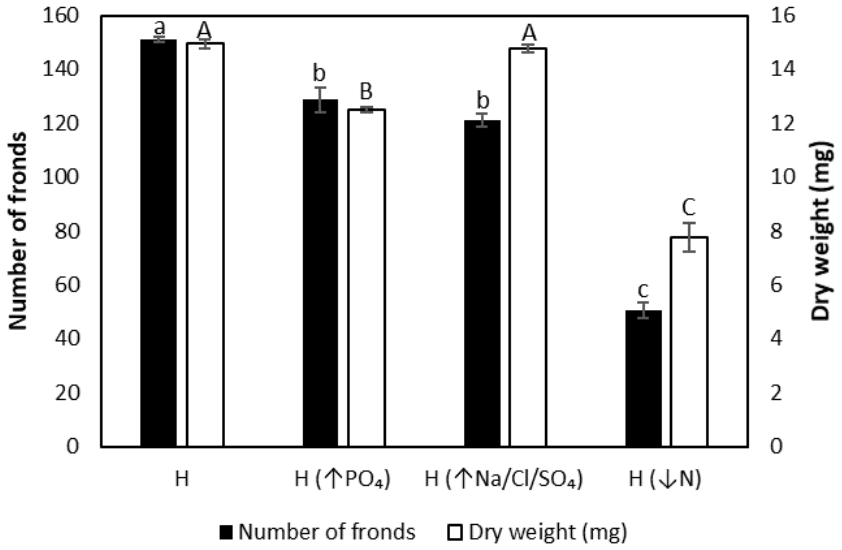
2.2.2. Low nitrogen and excess phosphate conditions were responsible for the growth inhibition of duckweed in the WW

To know which minerals causing the growth inhibition of *L. gibba*, I conducted the duckweed growth test in the Hoagland medium modified with 1) excess Na/Cl/SO_4 (46.11 mM/25.04 mM/13.63 mM, respectively); 2) excess PO_4 (3.35 mM); and 3) low NO_3/NH_3 (0.005 mM/0.04 mM) (Appendix 5), where the concentration was adjusted similar to WW. The result in Fig. 15A and 15B showed the depleted N and excess PO_4 conditions caused the growth inhibition of *L. gibba* in the Hoagland medium. On the other hand, *L. gibba* growth was not significantly reduced in the Hoagland medium with excess Na/Cl/SO_4. Based on Schmid and Landolt (1987), the maximum tolerance of Na/Cl/SO_4 was 217 mM/ 39.9 mM/ 59.3 mM, respectively. It indicated that the modified Hoagland or WW salinity level was still in the range of tolerance level for duckweed. Most plants can survive in the salinity level below 50-100 mM of NaCl, while some other plants (halophytic) can even tolerate higher salinity up to 500 mM of NaCl (Downton, 1984). Nevertheless, high salinity in WW was observed to affect the root expansion in *L. gibba* and *S. polyrhiza* as well as fronds size in *S. polyrhiza* (Appendix 6). Tkuclec et al. (2001) suggested that there might be a connection between growth reduction and osmotic stress caused by high ion concentrations of salt. The plant would spend much energy on osmotic adjustment and stress compounds production, leading to its growth reduction. Overall, I suggested that the salinity up to 23 mM of NaCl and 12 mM of Na_2SO_4 did not severely affect *L. gibba* growth rate, yet it affected the length of the root.

In the excess PO_4 condition, even though the dry weight was not highly reduced, it caused discoloration of fronds (chlorosis) (Fig 15A, 15B). The effect of excess PO_4 on duckweed's chlorosis was also showed in the supplementation of A-WW with a similar amount of PO_4 in K-WW (Appendix 1). It is worth noting that A-WW has a significantly lower amount of PO_4 compared to K-WW. Based on Schmid and Landolt (1987), optimal PO_4 concentration for Lemnaceae ranged between the following limits: *S. polyrrhiza* and *L. minor* of 0.004-0.114 mM, *L. minuscula* of 0.0008-0.114 mM, and *L. gibba* of 0.0008-0.57 mM. The reduction of PO_4 content in K-WW was one of the critical approaches to improving *L. gibba* growth.

Whereas, low nitrogen condition has significantly reduced *L. gibba* biomass in the modified Hoagland medium (Fig. 15A, 15B). Nitrogen is one of the important nutrients that are often associated with the rapid growth and high protein content of duckweed (Leng and Stambolie, 1994). Humphrey et al. (1977) suggested that deficiency of nitrogen reduces duckweed's growth and respiration rate. Previous work by Bergman et al. (2000) and Al-Nozaily (2001) indicated the optimum nitrogen concentrations for Lemnaceae growth ranged from 0.7-2.9 mM of N. While Schmid and Landolt (1987) suggested that the maximum tolerated of nitrogen for Lemnaceae were ranged from 30 mM to 450 mM.

A



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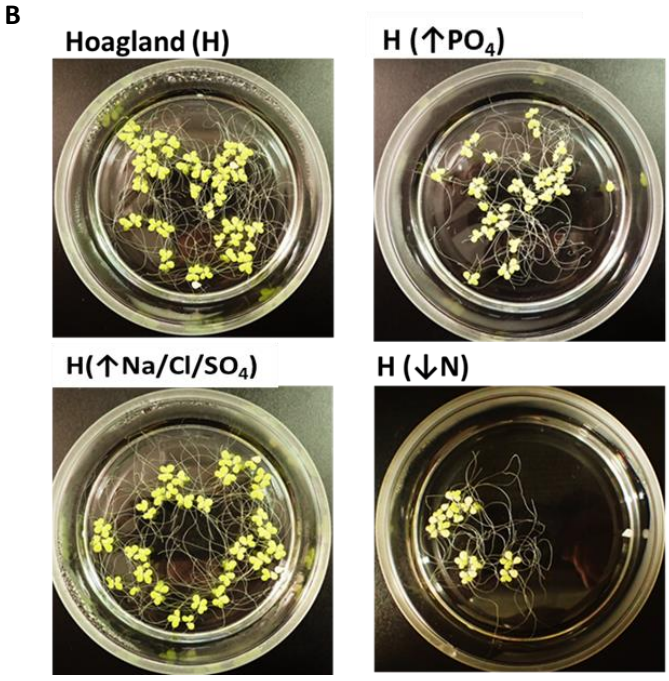

Fig. 15. A) Effect of different amount of minerals on *L. gibba* growth in Hoagland medium based on the number of fronds (closed bar) and dry weight (open bar) after 14 days of cultivation. The initial number of fronds inoculated was 2. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test). B) The image of *L. gibba* growth in different conditions of Hoagland medium after 14 days of cultivation.

2.2.3. Nitrogen supplementation improved duckweed growth in depleted nitrogen WW (A-WW)

Based on the mineral inhibitory test, the WW condition needed to be modified to recover the duckweed biomass. A possible and straightforward method related to the nitrogen limitation issue is by nitrogen supplementation into WW. In the practical experiment on-site, it is highly possible to supplement nitrogen by mixing upstream WW to the final tank of treated WW. The amount of 0.7 mM of nitrogen ($N\text{-}NO_3$ or $N\text{-}NH_4$) was used because the upstream WW can only supply maximum to that level of nitrogen (Data not shown). The result in Fig. 16A and 16B showed that nitrogen supplementation successfully improved duckweed biomass in A-WW by 4-5x, but not so useful in K-WW. Besides, I found that $N\text{-}NH_4$ supplementation was way more effective compared to $N\text{-}NO_3$. Based on Wang and Macko (2011), $N\text{-}NH_4$ was preferred by the plants that live in the wetter and submerged environment. $N\text{-}NH_4$ uptake also has less energy cost because it can directly produce glutamate during the N assimilation. Based on Schmid and Landolt (1987), the maximum tolerated concentration of NH_4 for duckweed is up to 5.87 mM, while the optimum level is ranged from 1.2 to 2.9 mM. It suggested that the addition of 0.7 mM of NH_4 in our experiment is close to the optimal range of nitrogen requirement for duckweed growth. On the other hand, the nitrogen supplementation did not fully recover the condition of duckweed in K-WW. I assumed the excess PO_4 in K-WW might still be a vital inhibition factor even after the N supplementation. Overall, I proposed that the addition of $N\text{-}NH_4$ up to 0.7 mM could produce the high yield of *L. gibba* in A-WW.

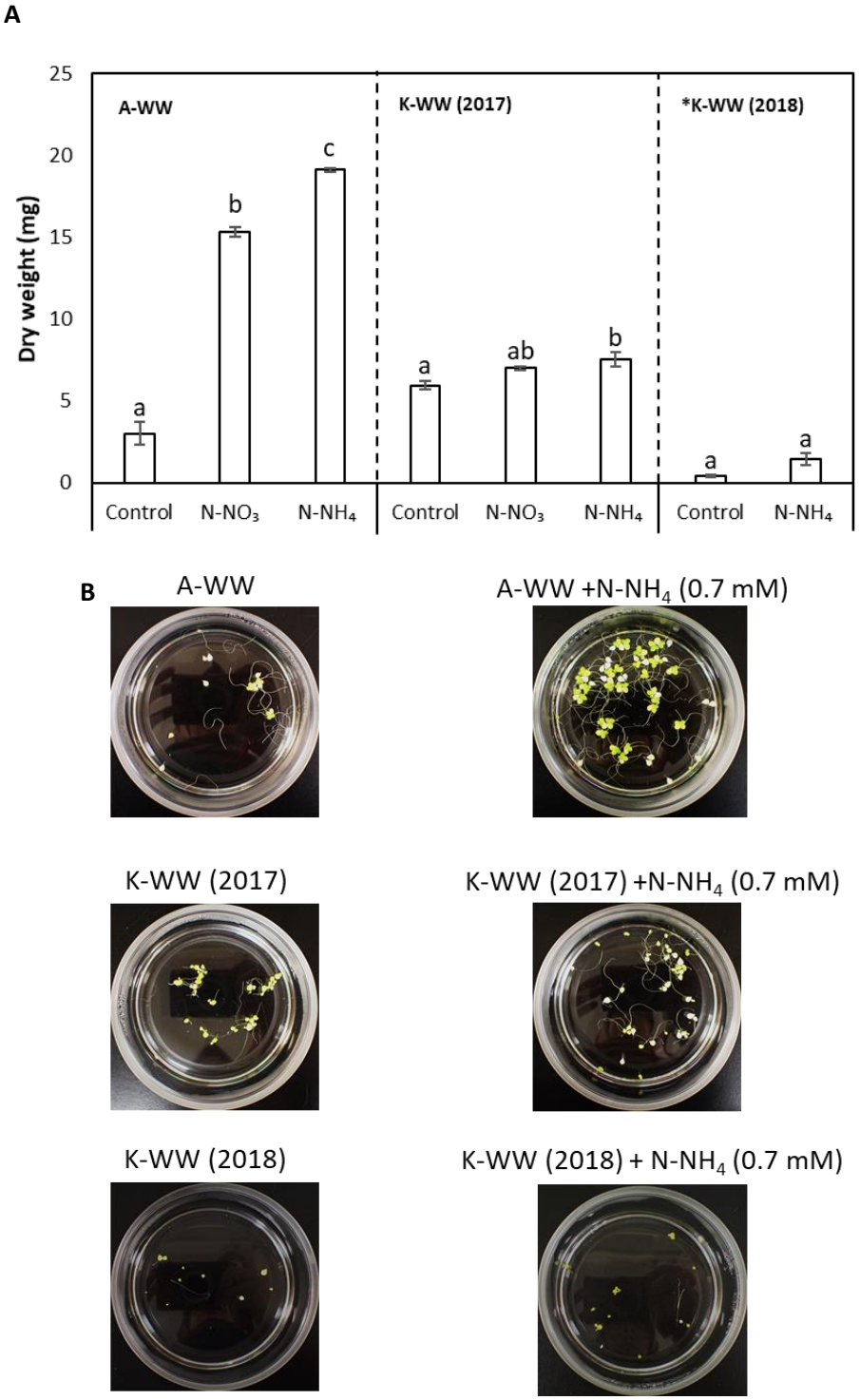

Fig. 16. Effect of $N\text{-}NO_3$/$N\text{-}NH_4$ supplementation on *L. gibba* growth in WW after 20 days of cultivation based on dry weight. *The asterik in K-WW (2018) showed different cultivation length which was only 10 days. The initial number of fronds inoculated was 2. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test). B) The image of duckweed growth in different condition of Hoagland medium after 20 days (A-WW and K-WW 2017) and 10 days (K-WW 2018) of cultivation.

2.2.4. Nitrogen supplementation improved PGPB activity in nitrogen depleted WW (A-WW)

I successfully showed the significant improvement of *L. gibba* growth in nitrogen supplemented A-WW, specifically using 0.7 mM of N-NH_4, in the previous result. Then I wondered if PGPB activity could also be enhanced when the higher amount of nitrogen available in WW. I have confirmed that nitrogen limitation could shift PGPB behavior, *A. calcoaceticus* P23, from giving growth promotion effect to causing the inhibitory effect on *L. gibba* (chapter I). The competition of nitrogen between PGPB and duckweed host could happen because both organisms require nitrogen for growing. Thus, I expected the sufficient nitrogen availability in WW could recover the growth promotion activity of PGPB on duckweed.

In this experiment, I tested the same PGPB collections used in chapter I, namely *A. calcoaceticus* P23 (non-indigenous PGPB) and *Chryseobacterium* sp. 27AL (WW-indigenous PGPB). Parallelly, I selected some indigenous PGPB with high growth promotion on *L. gibba* in N-NH_4 supplemented WW, which were 5KL, 7KL, and 17K affiliated with *Pseudomonas* sp., *Aeromonas* sp., and *Pannonibacter* sp., respectively (Appendix 3). The activity of P23 and 27AL were compared with the newly selected indigenous PGPB in the A-WW supplemented by 0.7 mM of N-NH_4. It is worth noting that the new PGPB were not so effective in the non-supplemented WW (Table 4). The result in Fig. 17 and Table 4 showed that most PGPB activities improved in N-NH_4 supplemented A-WW compared to their activity in the original WW. The result indicated that nitrogen supplementation could improve not only duckweed growth but also PGPB activity. It is a common practice to integrate the PGPB application with fertilizer to improve crop production efficiency. Based on Antonella di Benedetto et al. (2017), the interaction between PGPB and plant can act differently depending on the microorganisms and environmental conditions. Some studies also suggested that the benefits of PGPB were observed only when organic or chemical fertilizer was included (Antonella di Benedetto et al., 2017). It demonstrated the importance of sufficient nutrients to maintain the mutual relationship between the PGPB and the host plant.

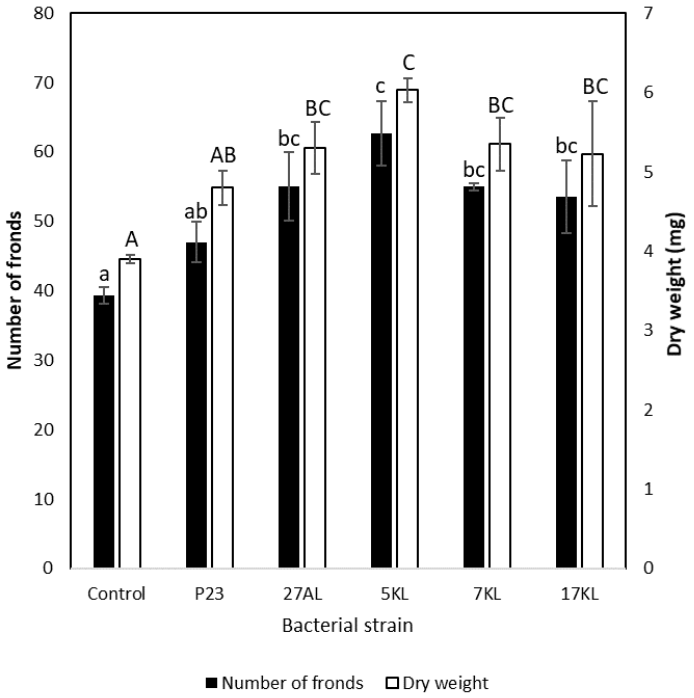

Fig. 17. Effect of indigenous PGPB from WW (27AL, 5KL, 7KL, 17KL) and non-indigenous PGPB (P23) on *L. gibba* growth compared to control (no bacteria) based on the number of fronds (closed bar) and dry weight (open bar) after 10 days of cultivation in A-WW supplemented by 0.7 mM of N-NH_4. The initial number of fronds inoculated was 2. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

Table 4. The effect of N-NH_4 supplementation of A-WW on PGPB activity on *L. gibba*

Treatment	*Growth promotion effect of PGPB on *L. gibba* (x-fold) based on the dry weight	
	non-supplemented A-WW	N-NH_4 supplemented A-WW
P23	0.7x	1.2x
27AL	1.2x	1.4x
5KL	1x	1.5x
7KL	1.4x	1.4x
17KL	1.1x	1.3x

*Growth promotion effect (x-fold) was calculated by divided the dry weight of *L. gibba* inoculated by PGPB with control (without PGPB)
N-NH_4 (0.7 mM) was used.

2.2.5. Nitrogen supplementation and $CaCO_3$ application improved duckweed growth in depleted nitrogen and excess phosphate WW (K-WW)

As I confirmed in the previous experiment, nitrogen supplementation did not fully recover *L. gibba* growth and condition in K-WW. I suspected that excess PO_4 in K-WW was still the main problem. Thus, to reduce the toxicity of excess PO_4, some approaches were performed, including $CaCl_2$, $CaCO_3$, and activated charcoal treatments (da silva and Nzihou, 2016; Ouakouak et al., 2017; Füredi-Milhofer et al., 1975). After the $CaCl_2$ treatment, I observed a significant reduction of PO_4 content in both K-WW (2017) and K-WW (2018) (Fig. 18). However, the improvement of *L. gibba* condition could only be observed in K-WW (2017) but not in K-WW (2018). Based on the mineral analysis, K-WW (2018) has a higher amount of Cl and SO_4 compared to K-WW (2017) (Table 5). It was suggested that the addition of $CaCl_2$ made the salinity level of K-WW (2018) intensified. The Cl concentration increased to 38.6 mM in K-WW (2018), which was almost close to the maximum tolerance of Cl for duckweed (39.92 mM) (Schmid and Landolt, 1986). Finding the other method that does not increase the salinity level in the K-WW is crucial. Thus, in the next trial, I performed $CaCO_3$ and activated charcoal treatments.

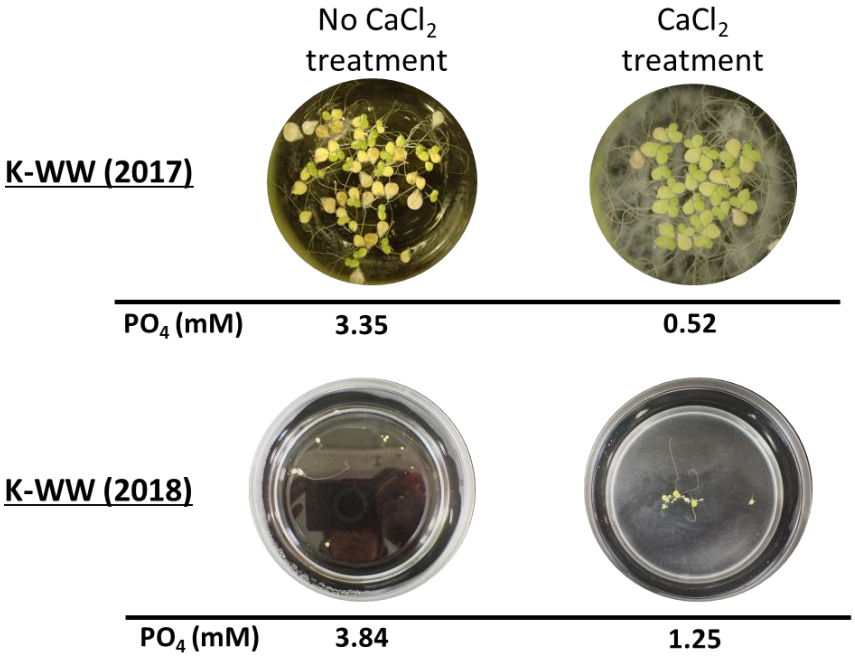

Fig. 18. The image of $CaCl_2$ treatment using 10 mM of $CaCl_2.2H_2O$ on *L. gibba* growth compared to control (no $CaCl_2$ addition) in K-WW (2017) and K-WW (2018) after 10 days of cultivation.

Table 5. The mineral change of K-WW after $CaCl_2$ addition

ineral	Mineral content (mM)			
	K-WW (2017)	K-WW (2018)	K-WW (2017) + 10 mM of $CaCl_2.2H_2O$	K-WW (2018) + 10 mM of $CaCl_2.2H_2O$
Na	46.1	37.1	46.1	37.1
Ca	8.85	6.47	10.22	10.16
Cl	11.27	16.92	32	38.6
SO_4	4.34	15.34	4.5	15.34
PO_4	3.35	3.84	0.52	1.25

In our next trial, I observed a significant improvement of *L. gibba* condition after $CaCO_3$ application in K-WW (2018). While the combination of $CaCO_3$ with N-NH_4 supplementation gave the highest biomass of *L. gibba* (Fig. 19). Interestingly, based on the mineral analysis, $CaCO_3$ treatment in K-WW (2018) did not reduce PO_4 content but only supplement calcium into the WW (Table 6). This suggested that the addition of calcium might reduce the toxicity effect of excess PO_4 in K-WW. Based on Thor (2019), calcium is essential for plant growth and development either in normal or stress conditions. It acts as an important factor for cell wall and membrane stability and as a second messenger in many physiological processes, including the response of plants to abiotic or biotic stresses. Thus, I suggested that calcium supplementation may strengthen the duckweed response to the stress of excess PO_4 in K-WW. Additionally, it was also shown that the addition of Na2CO3 to Hoagland medium did not increase *L. gibba* growth (Ohnuki, 2021 Master Thesis). This result suggested that CO_3^{2-} does not benefit *L. gibba* growth.

On the other hand, activated charcoal treatment increased magnesium and potassium concentration while reducing COD (chemical oxygen demand) and half of the PO_4 concentration (Table 6). However, *L. gibba* growth did not significantly improve even after PO_4 reduction by activated charcoal (Fig. 19). It might be caused by the high absorption of organic compounds that may be needed for duckweed nutrients. In this experiment, I could not test the effect of $CaCO_3$ and activated charcoal in K-WW (2017) because the sample has been exhausted.

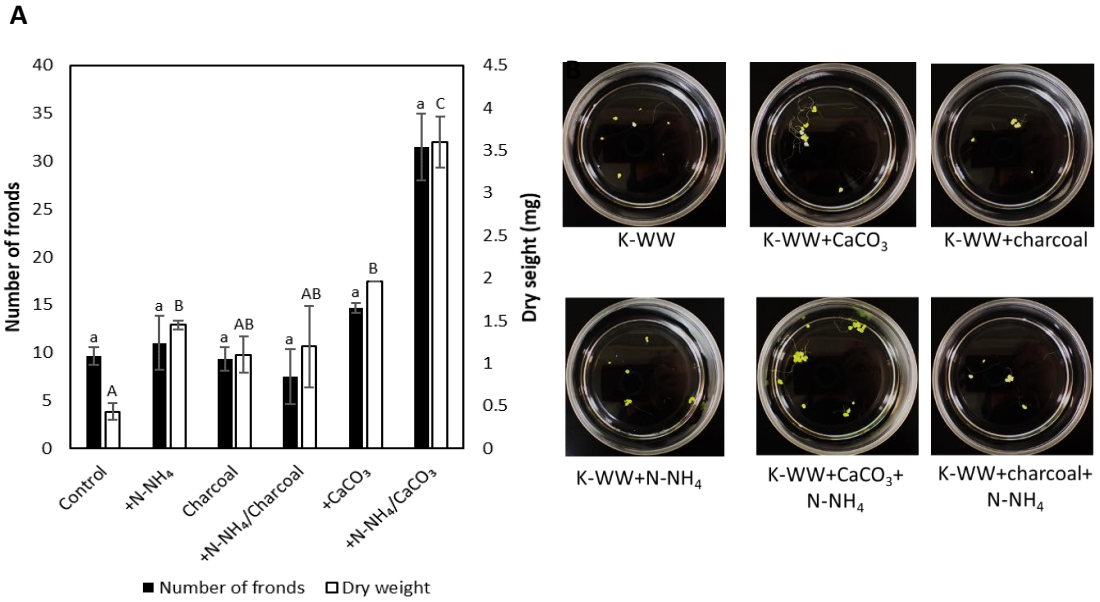

Fig. 19. Effect of $CaCO_3$ (10 mM), activated charcoal (10% w/v), $N\text{-}NH_4$ (0.7 mM), or treatment combination on *L. gibba* growth in K-WW (2018) after 10 days of cultivation based on the number of fronds (closed bars) and dry weight (open bars). The initial number of fronds inoculated was 2. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test). B) The image of duckweed growth in K-WW (2018) with different treamments after 10 days of cultivation.

Table 6. Comparison of mineral change in K-WW (2018) after $CaCO_3$ and activated charcoal treatment

Minerals	Mineral content (mM)		
	K-WW	K-WW + activated charcoal	K-WW +$CaCO_3$
Mg*	0.01	1.57	0.04
Ca†	0.20	0.24	0.58
K*	nd	1.48	nd
Na	39.93	47.98	43.87
NH_4	0.05	0.03	0.02
PO_4*	3.76	1.73	3.81
SO_4	14.49	16.36	16.51
Cl	15.79	20.72	18.77
NO_3	nd	nd	nd
COD* (mg/L)	13-20	5	13-20
pH	7.9	8.1	7.9

* the mineral change after the addition of activated charcoal (10% w/v)
† the mineral change after the addition of $CaCO_3$ (10 mM)

2.2.6. Nitrogen supplementation and $CaCO_3$ application improved PGPB activity in depleted nitrogen and excess phosphate WW (K-WW)

I successfully demonstrated the effectiveness of $CaCO_3$ combined with nitrogen supplementation in K-WW. In the next trial, I was interested in observing PGPB activity in modified K-WW. In this experiment, I used non-indigenous PGPB, P23 and Ps6 strains, and indigenous PGPB, 27AL, 5KL, 7KL, and 17KL strains, which have been described in the previous section 2.2.4 and chapter I. The result was showed in Fig. 20 and Table 7 where most PGPB activity on *L. gibba* improved after WW modification. The improvement of PGPB activity could happen because of the recovery of host condition and essential nutrition in K-WW. Overall, the $CaCO_3$ and nitrogen supplementations could improve the growth of duckweed and PGPB activity in K-WW.

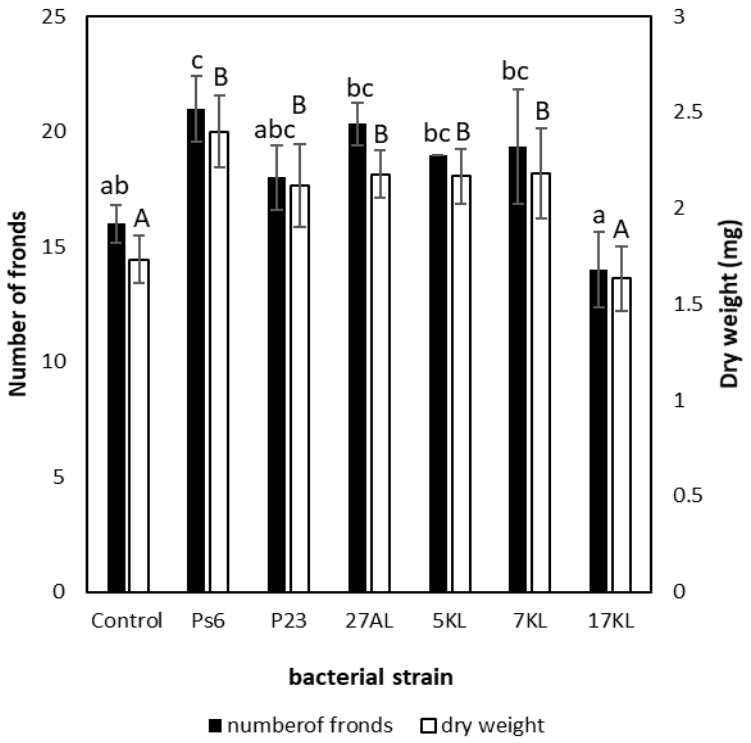

Fig. 20. Effect of indigenous PGPB from WW (27AL, 5KL, 7KL, 17KL) and non-indigenous PGPB (P23 and Ps6) on *L. gibba* growth compared to control (no bacteria) based on the number of fronds (closed bar) and dry weight (open bar) after 10 days of cultivation in K-WW (2018) with $CaCO_3$ (10 mM) and N-NH_4 (0.7 mM) combination. The initial number of fronds inoculated was 2. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

Table 7. The growth promotion effect of PGPB on *L. gibba* growth in K-WW (2018) treated with $CaCO_3$ and $N\text{-}NH_4$ combination

Treatment	*Growth promotion effect of PGPB on *L. gibba* (x-fold) based on the dry weight	
	non-treated K-WW (2018)	treated K-WW (2018)
P23	0.8x	1.2x
Ps6	1x	1.4x
27AL	1.3x	1.3x
5KL	1.1x	1.3x
7KL	1.1x	1.3x
17KL	0.8x	0.9x

*Growth promotion effect (x-fold) was calculated by divided the dry weight of *L. gibba* inoculated by PGPB with control (without PGPB)
Treated K-WW (2018) was K-WW treated by $CaCO_3$ (10 mM) and $N\text{-}NH_4$ (0.7 mM) supplmentation

Conclusion

I examined the effects of mineral supplementation to produce *L. gibba* in the food-factory effluent containing depleted nitrogen and excess phosphate. Overall, the result demonstrated that the optimum supplementation and the utilization of domestic PGPB offer a promising way to improve duckweed biomass production in the WW significantly (Fig 21). To test the efficiency of this strategy in practical use, it is needed to scale up the cultivation of *L. gibba* in the open-air environment with the actual condition of the WW in the factory.

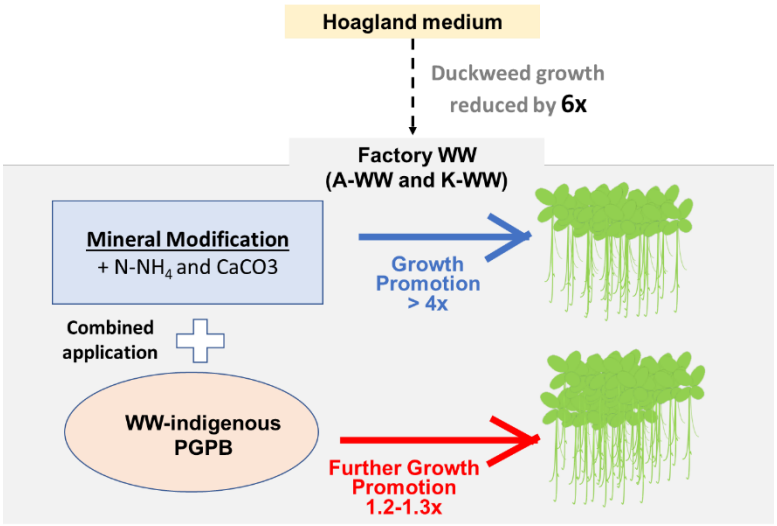

Fig. 21. The effect of PGPB and mineral modification on *L. gibba* growth in food-factory WW. For the on-site plan, the supplementation can be conducted by mixing the upstream WW containing higher nitrogen with the treated WW in the final sedimentation tank.

CHAPTER III

Potential of PGPB to inhibit the growth of microalgae as duckweed competitors

In this chapter, I describe the potential of indigenous PGPB to inhibit the growth of microalgae. In the trial to cultivate the wild duckweed in Thailand using unsterilized A-WW, I observed the overgrowth of microalgae on the pond, reducing duckweed growth dramatically. I found the same phenomenon when cultivating the duckweed in the new batch of unsterilized A-WW (Fig. 23) on a lab trial. The reduction of duckweed in the existence of microalgae could have happened because of 2 factors: 1) nutrient competition; or 2) toxin production by microalgae. Therefore, I am interested to see if there is a possibility that the selected indigenous PGPB for *L. gibba* have antagonistic activity towards microalgae. This chapter includes the isolation and purification of microalgae from WW samples, molecular identification of microalgae, and algicidal activity test of some PGPB on microalgae. Besides, I also tested algicidal activity against *Microcystis aeruginosa*, a prokaryotic microalga that belongs to the cyanobacteria group, which can form harmful algae blooms (HABs) by producing various toxins. *M. aeruginosa* has been reported to inhibit duckweed growth (*Lemna*) by producing microcystin toxin (Fig. 24; Saqrane et al., 2007). Overall, this study's finding can lead us to select appropriate PGPB which has a dual function to enhance the duckweed growth while inhibiting the growth of duckweed's competitor, microalgae (Fig. 22).

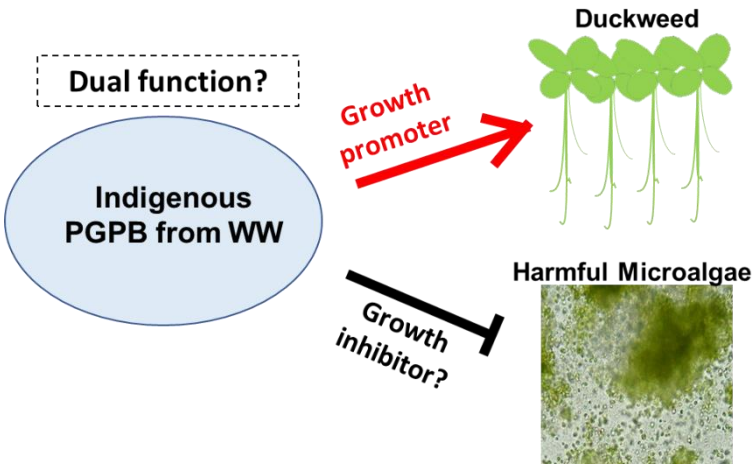

Fig. 22. Graphical abstract of Chapter III: Exploration of PGPB that have potential to inhibit microalgae growth.

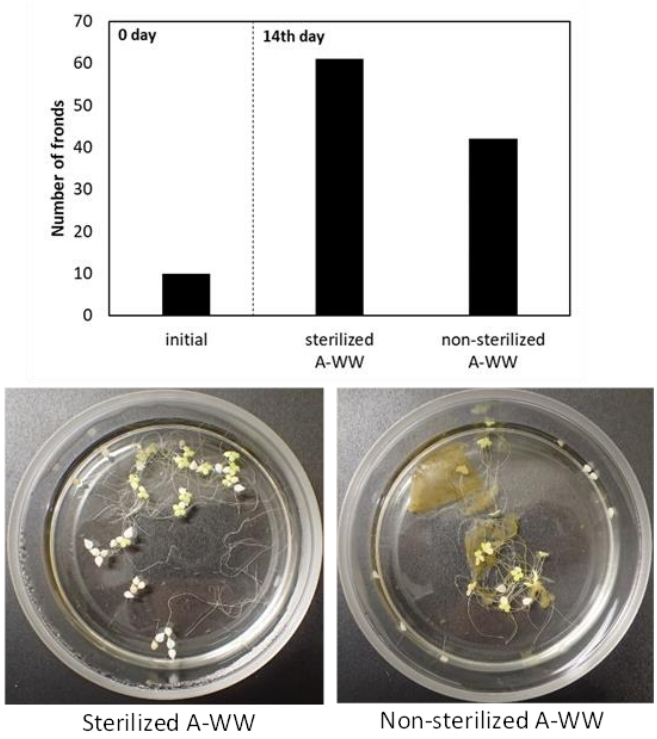

Fig. 23. The effect of microalgae grown in non-sterilized A-WW (new batch 2018) on *L. gibba* growth after 12 days of cultivation based on the number of fronds. The initial number of fronds inoculated was 10.

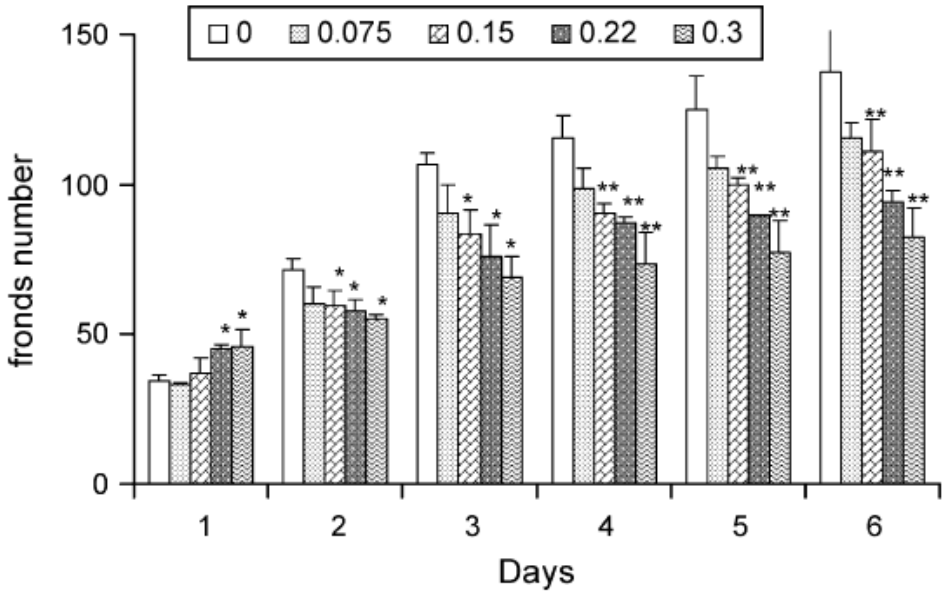

Fig. 24. Effect of microcystins/MC (µg/mL) from *M. aeruginosa* on the growth of *L. gibba* exposed to various concentrations of MC extract during 6 days. Data are mean ± S.D. (n = 3), vertical bars show standard deviation. Significant differences between controls and treatments are indicated by * $p < 0.05$ and **$p < 0.01$; t-test was used for comparisons). (Saqrane et al., 2007)

3.1. Materials and Methods

3.1.1. Plant and microalgae

The plant material and its growth condition are described in chapter I, section 1.1.1. While for the microalgae samples, two group of microalgae were used: 1) Prokaryotic microalga **(***Microcystis aeruginosa* NIES-843 from National Institute for Environmental Studies (NIES)); 2) Eukaryotic microalgae isolated from WW samples (section 3.1.3).

3.1.2. Growth condition and media of microalgae

Microalgae were cultivated in a plant growth chamber (MLR352, Panasonic Corp., Osaka, Japan) at 28 °C, an illuminance of 85 μmol/(m^2·s), a photoperiod of 16 h, and ca. 50% humidity. For the culture stock, 5% of WW-isolated microalgae and 10% of *M. aeruginosa* were inoculated in the liquid MA medium and re-cultured every 10 days. For the isolation of the microalgae from WW, MA and CM agar media (1.5%) were used.

MA medium contained of: 0.05 g/L of $Ca(NO_3)_2.4H_2O$, 0.1 g/L of KNO_3, 0.05 g/L of $NaNO_3$, 0.04 g/L of Na_2SO_4, 0.05 g/L of $MgCl_2.6H_2O$, 0.1 g/L of β-Na_2glycerolphosphate.$5H_2O$, 0.005 g/L of $Na_2EDTA.2H_2O$, 0.0005 g/L of $FeCl_3.6H_2O$, 0.005 g/L of $MnCl_2.4H_2O$, 0.0005 g/L of $ZnCl_2$, 0.005 g/L of $CoCL_2.6H_2O$, 0.0008 g/L of $Na_2MoO_4.2H_2O$, 0.02 g/L of H_3BO_3, 0.5 g/L of bicine. pH was adjusted to 8.6 by using 1N NaOH.

CM medium contained of: 1 g/L of $(NH_4)_2HPO_4$, 1 g/L of KH_2PO_4, 0.2 g/L of $MgSO_4.7H_2O$, 0.02 g/L of $CaCl_2.2H_2O$, 3 g/L of $Fe_2(SO_4).7H_2O$, 1.8 g/L of $MnCL_2.4H_2O$, 1.5 g/L of $CoSO_4.7H_2O$, 0.4 g/L of $ZnSO_4.7H_2O$, 0.2 g/L of $Na_2MoO_4.2H_2O$, 0.02 g/L of $CuSO_4.5H_2O$, 0.1 g/L of Thiamine hydrochloride, and 0.0005 g/L of cyanocobalamine. The final pH will be ±6.8 for this medium.

3.1.3. Isolation and purification of microalgae from WW samples

Two WW samples were used for the isolation of microalgae, which were; 1) Facultative anaerobic pond (Malaysia); 2) KP-WW (Japan). I tried to isolate microalgae from A-WW too; however, the microalgae cannot be recovered because of the prolonged storage of WW samples in a cold temperature room at 4^oC.

The isolation of microalgae was conducted by diluting the WW samples first by10^{-1}-10^{-4} dilution rate. The diluted samples of 100 µL were spread on MA and CM agar plates (1.5% agar) and incubated in a plant incubation chamber for seven days. The single microalgae colony characterized by the green colour was picked and grown in 4 mL of liquid MA medium. The purity of microalgae was observed in the microscope (Olympus BX-50, Olympus co., Tokyo, Japan) and the selection of microalgae was conducted based on their distinct morphology.

3.1.3. Elimination of bacteria from microalgae culture

The elimination of bacteria from microalgae culture was conducted by filtration and followed by antibiotic treatment. The filtration was performed using sterilized filter paper (3µm pore size) and filter holder (Advantec, Advantec Toyo Kaisha, Osaka, Japan). The microalgae estimated to have a size range of 5-20 µm (Lamminen et al., 2019) will be trapped on the membrane filter while bacteria with less than 3 µm of size will pass through the filter membrane. At first, 1 mL of microalgae culture (7 days pre-culture in MA liquid medium) was taken using 1 mL syringe and injected into the filter set attached on the top of the falcon tube. After that, 1 mL of sterilized water was injected twice on the filter set to wash the microalgae culture on the filter paper. After subsequent washing steps, the filter paper containing microalgae was put on the 24 well-plate filled with 1mL of sterilized water. The microalgae cells were released from filter paper using the micropipette tip, and 250 µL of the filtrate was inoculated into 2 mL of fresh MA medium added by 0-10% of the antibiotic stock. The antibiotic stock contained of: 500 mg/L of ampicillin, 200 mg/L of kanamycin, 125 mg/L of tetracycline, 20 mg/L of chloramphenicol, and 2.5 mg/L of streptomycin (Jones et al., 1973, with modification). Microalgae were incubated in a plant incubation chamber for 1-5 days; for each day, 50 µL of culture was moved in a 2 mL of fresh MA medium without antibiotic. The purity of microalgae culture including *M. aeruginosa* were confirmed regularly by no bacterial colony formation on R2A and LB agar media as well as microscope observation (Olympus BX-50, Olympus co., Tokyo, Japan).

3.1.4. Identification of microalgae

DNA isolation of microalgae (Kim et al., 2012, with modification) was conducted by firstly harvesting 5-6 mL of microalgae cells and washing the cell pellets twice using sterilized Mili-Q water with centrifugation at 5000 rpm for 10 minutes. The

microalgae pellet was then resuspended with 1 mL of sterilized TE buffer (or Mili-Q water) and moved to the screw-capped tube (the tube for multi beads shocker). Then, three cycles of freezing at -80^{o}C for 5 min and thawing in the water with room temperature (RT) for 5 min were conducted to break the cells partially. About 1 gr of 0.5 mm beads (Yasuikikai co., Osaka, Japan) that has been previously sterilized in the oven at 180^{o}C for 30 min and cooled down at RT was added to the frozen sample. The sample was then beaten at 2700 rpm, 30 sec on, 30 sec off, for 5 cycles using a multi beads shocker (MB755U(S), Yasuikikai co., Osaka, Japan) for the physical cell disruption. Then, 600 µL of CTAB buffer mixed with DTT (Appendix 8) was added into the homogenized sample and subsequently incubated at RT for 20 min and then at 65^{o}C heat block for 30 min; the sample was vortex every 10 min. Microalgae contained a very complex cell wall so that CTAB as a chemical treatment was needed to ensure a complete cell disruption. Next, 2-5 µL of RNAse was mixed into the sample and incubated for another 30 min at a temperature of 37^{o}C. The aqueous phase (±700 µL) was then taken and moved into a fresh 1.5 mL microtube. The transferred sample was then mixed with 1 volume of PCI (phenol:chloroform: isoamyl alcohol) for the separation of DNA from other protein and organic compounds and centrifuged at 10.000 rpm for 5 min. After the centrifugation, the most upper part layer of the sample was collected with an end-cut blue tip to the fresh 1.5 mL microtube. The PCI step was conducted at least three cycles until the interphase layer became clear. For the DNA precipitation, the sample from the previous step was mixed with 0.1 volume of 3M NaOAc (pH 5.2), 20 µL of glycogen, and 1-2 volume of 100% ethanol and centrifuged at 12.000 rpm for 20 min. Subsequently, the DNA pellet was mixed with 1 mL of 70% ethanol for washing the DNA from the remaining salt and then centrifuged at 12.000 rpm for 10 min. Afterwards, the pellet was dried by vacuum for ±30 min and resuspended with 60 µL of TE buffer or sterilized Mili-Q water. The DNA isolation step is described in Appendix 9.

The DNA was used as a template for PCR amplification using the set of primers (A) 18S rRNA univ F (5'-CCTGGTTGATCCTGCCAG-3′) and 18SrRNA univ R (5′-TTGATCCTTCTGCAGGTTCA-3′) with a target size of ±1500 bp (Chaidir et al. 2016) and another set of primers (B) 18S rRNA ss3 (5'-GATCCTTCCGCAGGTTCACCTACGGAAACC-3′) and 18S rRNA ss5 (5'-GGTGATCCTGCCAGTAGTCATATGCTTG-3′) with a target size of ±1800 bp (Khaw et al. 2020) based on the standard

protocol of the KOD-Plus-Neo DNA polymerase KIT (Toyobo). The conditions used for PCR amplification were as follows: initial denaturation at 95^{o}C for 5 min and then 34 cycles of denaturation at 95^{o}C for 1 min, primer annealing at 55-60^{o}C for 1 min, and chain extension for 1 min at 72^{o}C, followed by a final extension at 72^{o}C for 5 min. The target amplicons were purified from agarose gel with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing cycles consisted of 24 cycles of 96^{o}C for 10s, 50^{o}C for 5s, and 60^{o}C for 4 min. The resulting sequences were compared to those included in the GenBank nucleotide sequence database with the NCBI Nucleotide BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for taxonomic identification.

3.1.6. The effect of *M. aeruginosa* on *L. gibba* growth

I confirmed how *M. aeruginosa* affects *L. gibba* growth by using MA medium instead of Hoagland because *M.aeruginosa* could not grow in Hoagland medium. Briefly, *M. aeruginosa* cells (7 days-age culture) were harvested and re-suspended with a new fresh MA medium. *M. aeruginosa* was then inoculated into a new 50 mL MA medium in a 100-mL flask by adjusting the cell concentration with OD_{680} of 0.006, 0.03, 0.06, or 0.12. It is worth noting that *M. aeruginosa* cannot grow well in plant culture disk, probably because of the lack of air that can diffuse in the plant culture disk. Then the aseptic duckweed was inoculated into the MA medium containing different concentrations of *M. aeruginosa.* The duckweed growth was estimated by counting the total number of fronds and measuring the biomass (dry weight) after the cultivation period.

3.1.5. Preliminary test of algicidal activity of PGPB against microalgae isolated from WW and *M. aeruginosa*

The PGPB used in this experiment consisting of WW-indigenous PGPB (27AL, 5KL, 7KL, and 17 KL) and non-indigenous PGPB (P23, Ps6, and MRB10) that have been proved to promote the growth of duckweed, specifically *L. gibba* and *L. minor*. Each bacterial strain has been described in chapter 1, chapter II, and appendix 3, except for MRB10. Bacterial strain MRB10 is affiliated to *Bacillus subtilis* that has been confirmed its promotion activity on *L. minor* in Hoagland medium condition.

The algicidal activity test on the isolated microalgae from WW was conducted by halo formation assay using an overlaid agar medium (Luo et al. 2013). The overlaid agar medium consisted of the bottom layer containing 10-15 mL of MA medium (with 1.5% agar) and the top layer containing the mixture of 3 mL of MA medium (with 1% agar) and 2 mL of microalgae culture (7 days-age culture). For the top layer, the autoclaved MA agar medium was kept first at 60^{o}C using heat-block before mixing with microalgae culture. After overlaid agar medium preparation, 15 µL of the bacterial culture that has been washed and resuspended with LB medium was put on the top layer of the overlaid agar medium. Then, the halo formation around the bacterial colony caused by microalgae growth inhibition was observed after 7 days of incubation in the plant incubation chamber.

The algicidal activity against *M. aeruginosa* could not be conducted using an overlaid agar medium because it could not grow on the agar medium even though some modifications have been performed. Thus, the algicidal activity against *M. aeruginosa* was conducted in the liquid system in MA medium (Guo et al., 2015, with modification). Briefly, 10% of *M. aeruginosa* (7 days-age culture) was inoculated into fresh MA medium. Then about 10% of bacterial culture (pre-culture overnight in LB medium, washed, and resuspended with MA medium) was inoculated into *M. aeruginosa* containing MA medium. It is worth noting that washing the bacterial culture with MA medium was necessary because the contamination of the LB medium (±10%) could inhibit the growth of *M. aeruginosa* in the MA medium dramatically. The mixture was then incubated for another 10 days, and the inhibition effect was observed evey 5 days based on the cell number from microscope observation.

3.1.7. The algicidal activity of PGPB in the co-culture of *M.aeruginosa* and *L. gibba* (suspension test)

The algicidal activity of PGPB, specifically 27AL and MRB10, in the co-culture of *M. aeruginosa* and *L. gibba* condition was conducted using MA medium. For the bacteria preparation, a bacterial colony was inoculated with an inoculation loop into 4 mL of liquid LB medium in a test tube and shaken in the water bath for 24h at 30 °C and 100 strokes per minute. Then, 1% (200 µL) of bacterial culture from the test tube was transferred into 20 mL of LB medium in a 100-mL flask and shaken in the water bath for another 24h. Bacterial cells were harvested by centrifugation, washed twice

with MA medium, and resuspended in 20 mL of MA medium. While *M. aeruginosa* was prepared by harvesting the cells of 7 days-age culture and re-suspending it with a new fresh MA medium. Aseptic *L. gibba* was then inoculated into 50 mL of MA medium containing *M. aeruginosa* with final OD_{680} of 0.05 and bacterial cells with different concentrations ranged from OD_{600} of 0.05-0.5. Duckweed growth was estimated by counting the total number of fronds and measuring the biomass (dry weight) after the cultivation period. While *M. aeruginosa* growth was observed based on the number of cells from the microscope observation.

3.2. Result and Discussion

3.2.1. Isolation of microalgae from water samples

Isolation of microalgae from water samples resulting in a total of 157 colonies. Twelve samples with a size of ±5 µm were selected based on their distinctive morphology and purity (Fig. 25). Some selected strains might have similar morphology; but, they were selected more than once because of their high abundance in the water samples. Additionally, whether they are a similar strain or not are needed to be re-confirmed by molecular identification. The elimination of bacteria from microalgae was conducted for the selected samples. The result showed that the addition of 5-10% antibiotic stock with the length of exposure for 48-72 h was the most effective method to eliminate bacteria without killing the microalgae (Table 8). The sterilization of microalgae was checked by spreading algae culture on LB and R2A media and also by microscope observation.

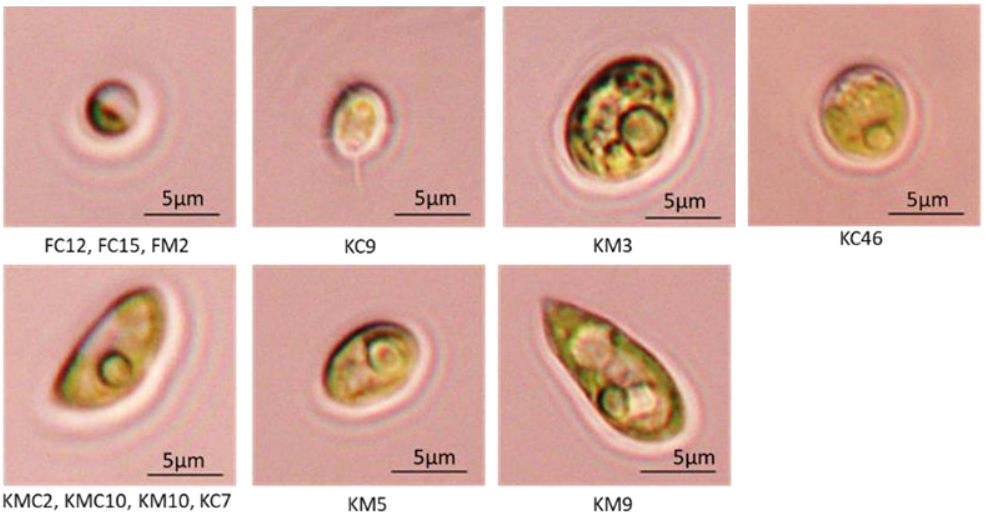

Fig. 25. Microscopic photographs (magnification 10x40) of different microalgae isolated from some water samples. The first K and F symbols of strains code were referred to the WW samples in which the microalgae were isolated, KP-WW or facultative anaerobic pond, respectively.

Table 8. The efficiency of bacteria elimination from microalgae using antibiotic

Length of antibiotic treatment	Algae	Contamination check (LB/R2A)						Sub-culture in new MA without antibiotic after antibiotic treatment
		1	2	3	4	5	6	
		0%	0.5%	1%	5%	7.5%	10%	
24 h	FM2	+	+	+	+	+	+	All cultures grow
	KM9	+	+	+	-	-	+	All cultures grow
	KM5	+	+	+	+	+	-	All cultures grow
	KM10	+	+	+	+	-	-	All cultures grow
	KC7	+	+	+	-	-	-	All cultures grow
	KM3	+	+	-	-	-	-	All cultures grow
	FC12	+	+	+	+	+	+	All cultures grow
	FC15	+	+	+	-	-	-	All cultures grow
	KMC2	+	+	+	+	+	-	All cultures grow
	KC9	+	+	+	+	+	+	All cultures grow
	KC46	+	+	+	+	+	-	All cultures grow
	KMC10	+	+	+	-	-	-	All cultures grow
48 h	FM2	+	+	+	-	-	-	All cultures grow
	KM9	+	+	+	-	-	-	All cultures grow
	KM5	+	+	+	-	-	-	All cultures grow
	KM10	+	+	+	+	-	-	All cultures grow
	KC7	+	+	+	+	-	-	All cultures grow
	KM3	+	+	+	-	-	-	All cultures grow
	FM2	+	+	-	-	-	-	All cultures grow
	KM9	+	+	+	-	-	-	All cultures grow
	KM5	+	-	-	-	-	-	All cultures grow
	KM10	+	-	-	-	-	-	All cultures grow
	KC7	+	+	+	-	-	-	All cultures grow
	KM3	+	-	-	-	-	-	All cultures grow
72 h	FM2	+	+	+	+	-	-	All cultures grow
	KM9	+	+	+	-	-	-	All cultures grow
	KM5	+	+	+	-	-	-	All cultures grow
	KM10	+	+	+	-	-	-	All cultures grow
	KC7	+	+	+	-	-	-	All cultures grow
	KM3	+	+	+	-	-	-	All cultures grow
	FM2	+	+	-	-	-	-	All cultures grow
	KM9	+	+	+	-	-	-	All cultures grow
	KM5	+	-	-	-	-	-	All cultures grow
	KM10	+	-	-	-	-	-	All cultures grow

	KC7	+	+	+	-	-	-	All cultures grow
	KM3	+	-	-	-	-	-	All cultures grow
After 5 days	FM2	+	+	+	-	-	-	All cultures grow
	KM9	+	+	+	-	-	-	Only cultures of treatment 1-5% grow
	KM5	+	+	+	-	-	-	All cultures grow
	KM10	+	+	+	-	-	-	Only cultures of treatment 1-5% grow
	KC7	+	+	+	-	-	-	All cultures grow
	KM3	+	+	+	-	-	-	All cultures grow
	FM2	+	+	-	-	-	-	All cultures grow
	KM9	+	+	+	-	-	-	Only cultures of treatment 1-5% grow
	KM5	+	-	-	-	-	-	All cultures grow
	KM10	+	-	-	-	-	-	Only cultures of treatment 1-5% grow
	KC7	+	+	+	-	-	-	All cultures grow
	KM3	+	-	-	-	-	-	All cultures grow

Treatments 1-6 consist of the use of 0-10% of antibiotic stocks
+ indicates there is still contamination in microalgae cultures
- indicates there is no contamination in microalgae cultures

3.2.2. Identification of microalgae

After the elimination of bacteria from microalgae, the identification of microalgae was conducted. The primer used for each microalga is presented in Table 9. Some microalgae were successfully identified consisting of *Chlorella*, *Coelastrella*, *Desmedesmus*, and *Parachlorella* genus. Most of the isolated microalgae belonged to the eukaryotic group. No prokaryotic group was found because the isolation media or the growth condition might not be suitable for their growth. Many studies reported using eukaryotic microalgae and duckweed combination for wastewater treatment (Bouali et al., 2012; Li et al., 2020). However, the effect of eukaryotic microalgae on duckweed growth was rarely reported. Roijackers et al. (2004) have reported that *Lemna* growth was reduced significantly when co-culture with the algae mixture, specifically in the low nitrogen condition (0.1-1 mg/L of N). However, it was not clear what types of algae they used explicitly. Nevertheless, the result suggested that nutrient competition between duckweeds and algae seems likely to be strong.

Table 9. Identification of microalgae isolated from some water samples.

Sample	sequence length	primer name	query cover	Percent identity	Affiliation
FC12	596	18S rRNA univ	100%	98.32%	*Chlorella sorokiniana*
FC15	469	18S rRNA univ	97%	98.72%	*Chlorella sorokiniana*
FM2	569	18S rRNA univ	100%	98.95%	*Chlorella sorokiniana*
KC7	546	18S rRNA univ	100%	99.82%	*Coelastrella saipanensis*
KM5	520	18S rRNA univ	100%	97.33%	*Coelastrella* sp.
KM10	640	18S rRNA univ	100%	99.53%	*Coelastrella sp.* *Asterarcys sp.* *Pseudospongiococcum sp.* *Scenedesmus sp.*
KMC2	506	18S rRNA univ	100%	97.84%	*Coelastrella sp.* *Asterarcys sp.* *Pseodospongiococcum sp.* *Scenedesmus sp.*
KC9	406	18S rRNA ss3-ss5	96%	97.96%	*Desmodesmus sp. UMT-B20*
KC46	545	18S rRNA ss3-ss5	99%	99.45%	*Parachlorella hussii ACOI*
KM3	640	18S rRNA ss3-ss5	100%	99.86%	*Coelastrella oocystiformis*
KM9	640	18S rRNA ss3-ss5	100%	99.06%	*Coelastrella sp.* *Asterarcys sp.* *Pseudospongiococcum sp.* *Scenedesmus sp.*
KMC10	565	18S rRNA ss3-ss5	100%	100%	*Coelastrella saipanensis*

3.2.3. The effect of *M. aeruginosa* on the growth of *L. gibba*

The effect of *M. aeruginosa* on the growth of *L. gibba* was tested in MA medium. It was shown that the concentration of *M. aeruginosa* with OD_{680} higher than 0.03 showed a strong inhibition effect on duckweed growth by 4x (Fig 26A). The fronds were also smaller and less green than the control without *M. aeruginosa* (Fig 26B). This result was consistent with Jang et al. (2007), where they reported the growth inhibition of *Lemna* caused by *M. aeruginosa*. The inhibition effect may be caused by the microcystin produced by *M. aeruginosa* as suggested by Saqrane et al., (2007).

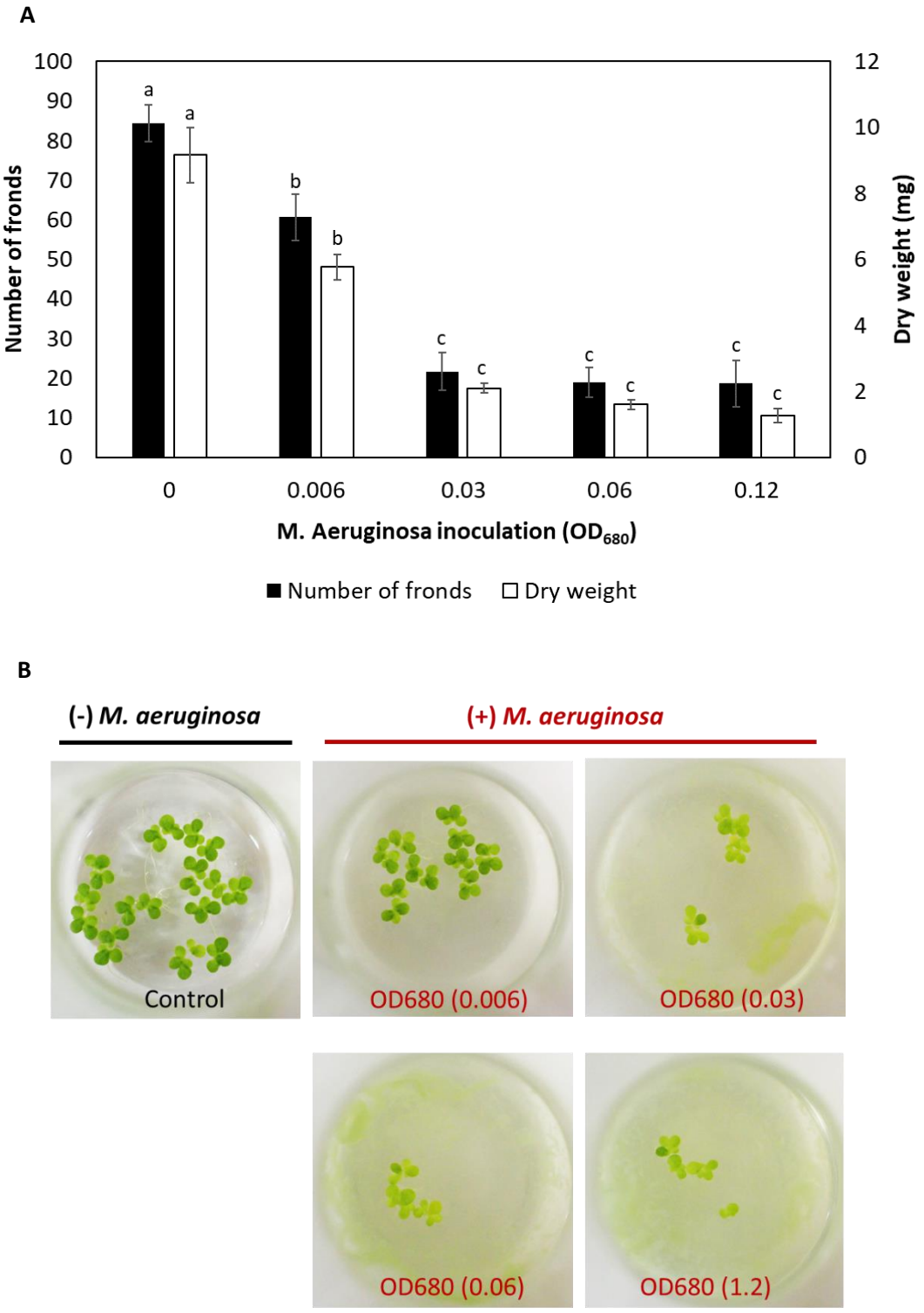

Fig. 26. A) The effect of different concentration of *M. aeruginosa* on *L. gibba* growth in MA medium based on the number of fronds (closed bars) and dry weight (open bars) after 10 days of cultivation. Initial number of *L.gibba* fronds is 2. Values are mean ± error (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test). B) Photo image of *L. gibba* after 10 days of cultivation with different concentration of *M. aeruginosa.*

3.2.4. Preliminary test of algicidal activity of PGPB against microalgae isolated from WW and *M. aeruginosa*

In this experiment, the algicidal activity of indigenous PGPB (27AL, 5KL, 7KL, and 17KL) and non-indigenous PGPB (P23, Ps6, and MRB10) were tested. The result showed that most PGPB did not have algicidal activity on microalgae isolated from WW, except for MRB10 (Table 10). On the other hand, some PGPB successfully inhibited the growth of harmful algae, *M. aeruginosa*, which were 27AL, 17KL, and MRB10 (Fig. 27). Among them, 27AL and MRB10 were selected for the algicidal activity test in the co-culture of *M. aeruginosa* and *L. gibba* in the MA medium.

Table 10. Algicidal effect of several PGPB on microalgae from WW

Algae	Algicidal activity (Halo formation)						
	Indigenous PGPB				Non-indigenous PGPB		
	27AL (OD 0.6)	5KL (OD 0.3)	7KL (OD 0.6)	17KL (OD 0.6)	Ps6 (OD 0.2)	P23 (OD 0.7)	MRB10 (OD 0.6)
FC12	-	-	-	-	-	-	-
FC15	-	-	-	-	-	-	-
FM2	-	-	-	-	-	-	-
KC7	-	-	-	-	-	-	-
KC9	-	-	-	-	-	-	-
KC46	-	-	-	-	-	-	-
KM3	-	-	+	-	-	-	+
KM5	-	-	-	-	-	+	+
KM9	-	-	+	-	-	-	+
KM10	-	-	-	-	-	-	+
KMC2	-	-	-	-	-	-	-
KMC10	-	-	-	-	-	-	-

+ means the bacteria have algicidal activity indicated by the halo formation
- means the bacteria have no algicidal activity indicated by no halo formation
Bacterial concentration was measured at an absorbance of OD_{600}

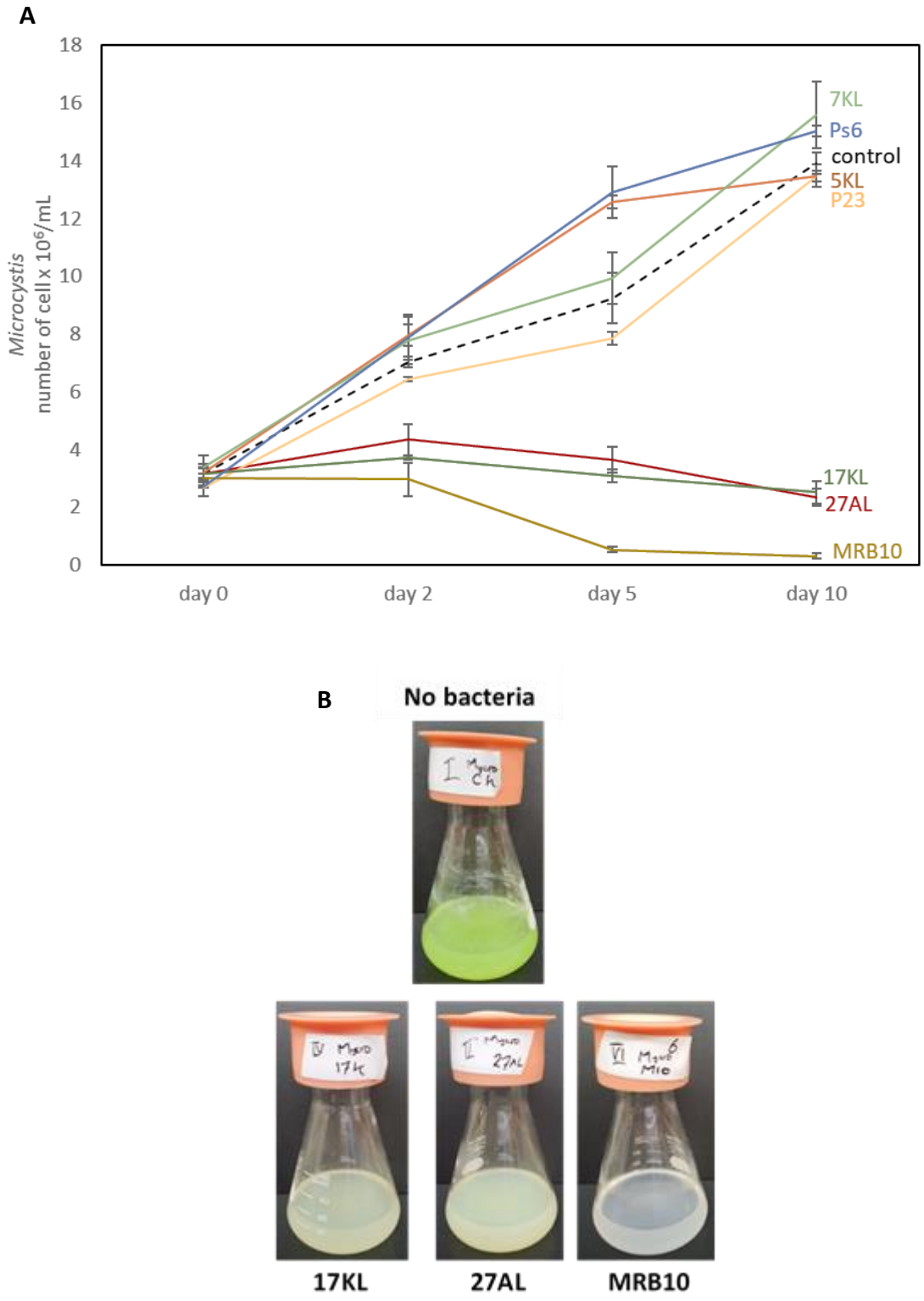

Fig. 27. A) The algicidal activity of several PGPB on *M. aeruginosa* in MA liquid medium after 10 days of cultivation based on the microscope observation of *M. aeruginosa* cell number. The bacterial OD_{600} was as follows: 27AL (0.5) ; 5KL (0.4); 7KL (0.4); 17KL (0.5); P23 (0.6); Ps6 (0.3); MRB10 (0.4). Values are mean ± SD (n = 3). B) Photo image of M. aeruginosa culture after 10 days of cultivation with different PGPB.

Chryseobacterium, *Pannonibacter*, and *Bacillus* groups have been reported for their algicidal activity against prokaryotic microalgae (cyanobacteria). *Chryseobacterium* produced potent protein compounds, diketopiperazines, that increased intracellular reactive oxygen species (ROS) levels while decreasing the activities of antioxidases, effective quantum yield, and electron transport rate of *M. aeruginosa* (Guo et al. 2015). In comparison, *Bacillus* was the most reported cyanobactericidal bacteria where 62.9% of them can efficiently kill *M. aeruginosa*. *Bacillus subtilis* produced surfactin to inhibit the growth of cyanobacteria (Ahn et al. 2003). Others reported that *B. subtilis* can disintegrate cyanobacteria colonies, making them susceptible to environmental pressures (Bi et al. 2019). On the other hand, *Pannonibacter* sp. was reported to inhibit cyanobacteria by direct contact (Chen et al. 2017). *Acinetobacter* sp. has also been investigated for its algicidal activity on *M. aeruginosa* by the production of organic compounds, 4-hydroxyphenethylamine (Yi et al., 2015) . Overall, the result showed the potential of some PGPB on *M. aeruginosa.*

3.2.5. The algicidal activity of PGPB in the co-culture of *M.aeruginosa* and *L. gibba* (suspension test)

To confirm the dual function of PGPB as a duckweed growth promoter and microalgae growth inhibitor, the effectiveness of the algicidal activity of selected PGPB (27AL and MRB10) was tested in the co-culture of *L. gibba* and microalga (*M. aeruginosa*). In this experiment, I only tried *M. aeruginosa* as the most reported harmful microalga for other organisms in the aquatic environment. For *Chryseobacterium* 27AL, it showed that initial bacterial inoculation of OD_{600} (0.3) or higher has the most effective inhibition effect on *M. aeruginosa* and promotion effect on *L. gibba* by 4x compared to control without bacteria. While MRB10 has a very strong activity on *M. aeruginosa* even in the lowest initial bacterial concentration (OD_{600} = 0.05). However, MRB10 did not promote but inhibited *L. gibba* growth compared to control with no bacteria. The inhibition of MRB10 on duckweed growth in co-culture condition with *M. aeruginosa* was still unclear. There may be two possibilities, including nutrient competition or the algicidal compounds produced by MRB10 is not specific to *M. aeruginosa.* It is best to know the mechanism of algicidal activity in 27AL and MRB10 to plan a suitable application in the field.

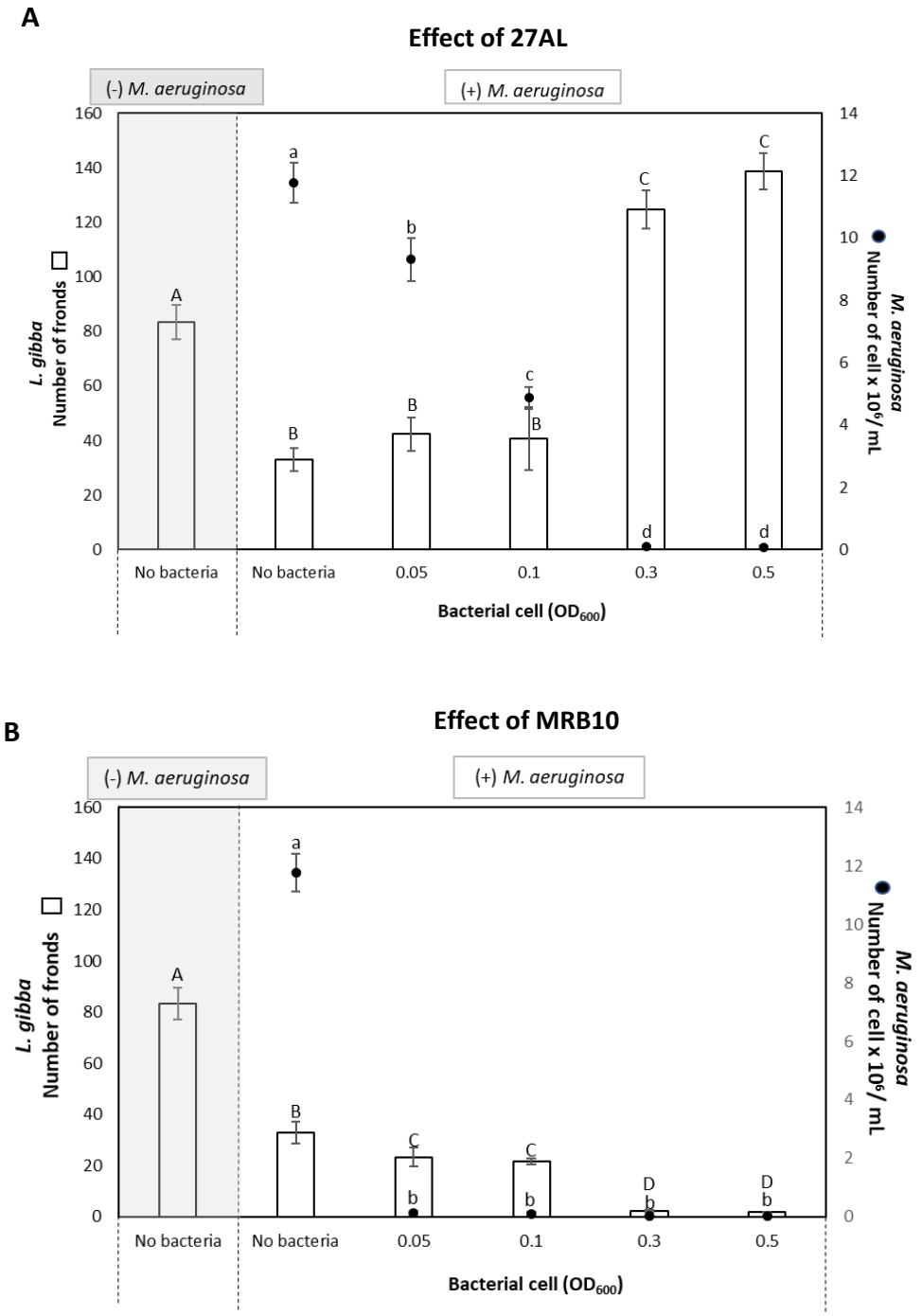

Fig. 28. The effect of different concentration of A) 27AL and B) MRB10 on the growth of *L. gibba* (open bar) and *M. aeruginosa* (closed circle) in MA medium after 10 days of cultivation. L. gibba growth was evaluated by the number of fronds while *M. aeruginosa* growth was evaluated by the number of the cells. Initial number of *L. gibba* fronds is 2 and initial concentration of *M. aeruginosa* was OD_{680} (0.05). Values are mean ± error (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

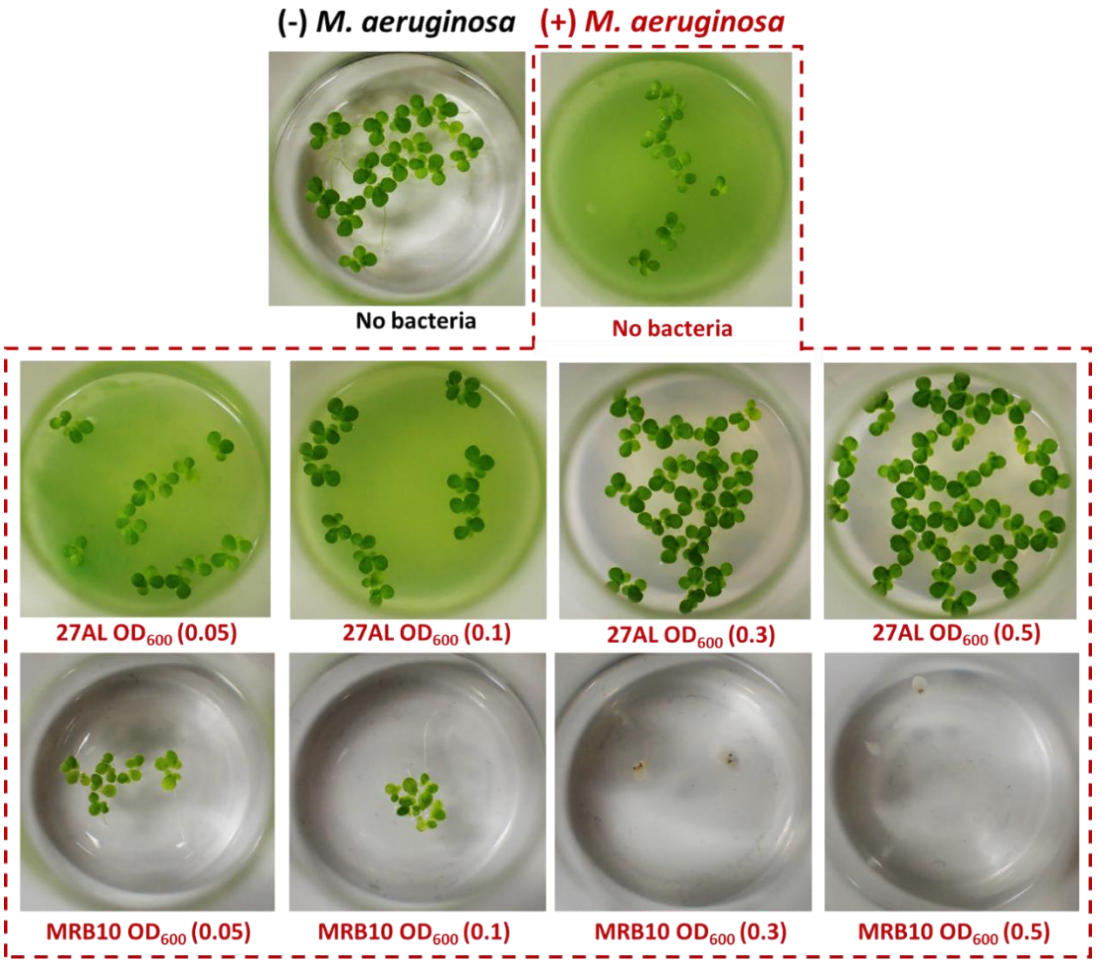

Fig. 29. Photo image of 27AL and MRB10 effect on the growth of *L. gibba* and *M. aeruginosa* after 10 days of cultivation.

Conclusion

The overgrowth of microalgae should impede duckweed biomass production in open pond conditions. The reduction of duckweed in the existence of microalgae could have happened via nutrient competition or toxin production. In this study, I found that duckweed PGPB, 27AL, also functions to inhibit microalgae growth. Strain 27AL may produce some active compounds or attack *M. aeruginosa* by cell-cell contact. The growth of *L. gibba* was significantly increased by 27AL even in the presence of harmful prokaryotic microalgae, *M. aeruginosa.* I successfully demonstrated the high potentials of indigenous WW-bacteria for effective production of the duckweed biomass on site. It remains to verify the stability of the enhanced growth of *L.gibba* in the treated WW open pond reservoir.

SUMMARY

In chapter I, I have successfully isolated an indigenous WW bacterium, *Chryseobacterium* sp. 27AL, that has a growth promotion effect on *L. gibba* in A-WW and K-WW by 1.2-1.3x, respectively. While non-indigenous PGPB, *A. calcoaceticus* P23 and *P. fulva* Ps6 did not have apparent PGP activity by 0.7-1x. Based on the KEGG pathway map, *Chryseobacterium* has a limited N (nitrogen) pathway compared to *A. calcoaceticus*. Neither nitrite nor nitrate reduction activities has been reported for most *Chryseobacterium* strains. The growth test of *Chryseobacterium* sp. 27AL in minimal medium with casamino acid, nitrate, or ammonium confirmed that this strain preferred organic nitrogen over the inorganic nitrogen sources. In contrast, *A. calcoaceticus* P23 could utilize both organic and inorganic N sources. These result strongly suggested that P23 has higher competition with duckweed for the nitrogen sources, which is not an advantageous trait for PGPB application under nitrogen-limiting conditions.

In chapter II, I identified the inhibition factors of *L. gibba* growth in WW. The result showed that depleted nitrogen and excess phosphate conditions caused the growth inhibition of *L. gibba*. Two modifications were tested to improve duckweed growth in A-WW and K-WW, which were: 1) Supplementation of N-NH_4 (0.7 mM); 2) Application of $CaCO_3$ (10 mM). The mineral modifications surprisingly improved *L. gibba* growth by 4-5x and enhanced most PGPB growth promotion activity in WW.

In chapter III, I observed the potential of PGPB against the microalgae from WW and *M. aeruginosa*. I found that most of the microalgae isolated from WW belonged to the eukaryotic group. The algicidal activity test showed that most PGPB has less algicidal activity on eukaryotic microalgae. However, some worked effectively against *M. aeruginosa,* for example; indigenous PGPB *Chryseobacterium* 27AL and *Pannonibacter* 17Kl and non-indigenous PGPB, *Bacillus* MRB10. When examining the PGPB effect on *L. gibba* and *M. aeruginosa* co-culture, I found that *Chryseobacterium* 27AL showed a dual function as a duckweed growth promoter and microalgae (*M. aeruginosa*) growth inhibitor. Overall, this study uncovered the hidden potentials of indigenous PGPB that contribute to the improvement of the duckweed biomass's production efficiency using factory wastewater.

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APPENDICES

Appendix 1: Effect of high PO_4 concentration on *L. gibba*

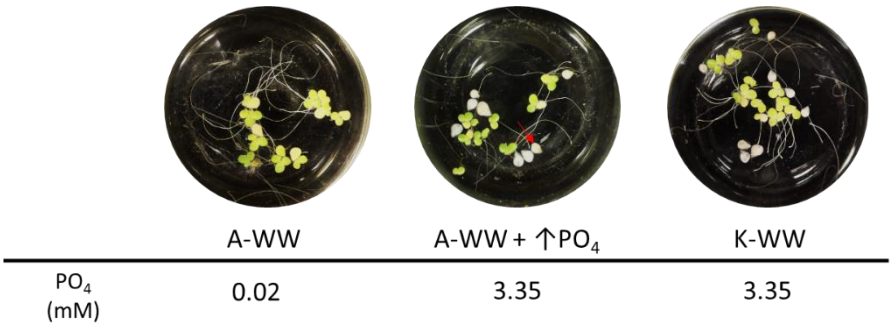


Fig. S1. Effects of different PO_4 concentration on *L. gibba*. Experiments were conducted by growing *L. gibba* for 10 days in A-WW, K-WW, and modified A-WW that was adjusted PO_4 concentration similar to K-WW. Change of the fronds color from green to white (chlorosis) was observed upon growing under high PO_4 (3.35 mM) condition.

Appendix 2. The effect of *A. calcoaceticus* P23 and *P.fulva* Ps6 on different duckweed species

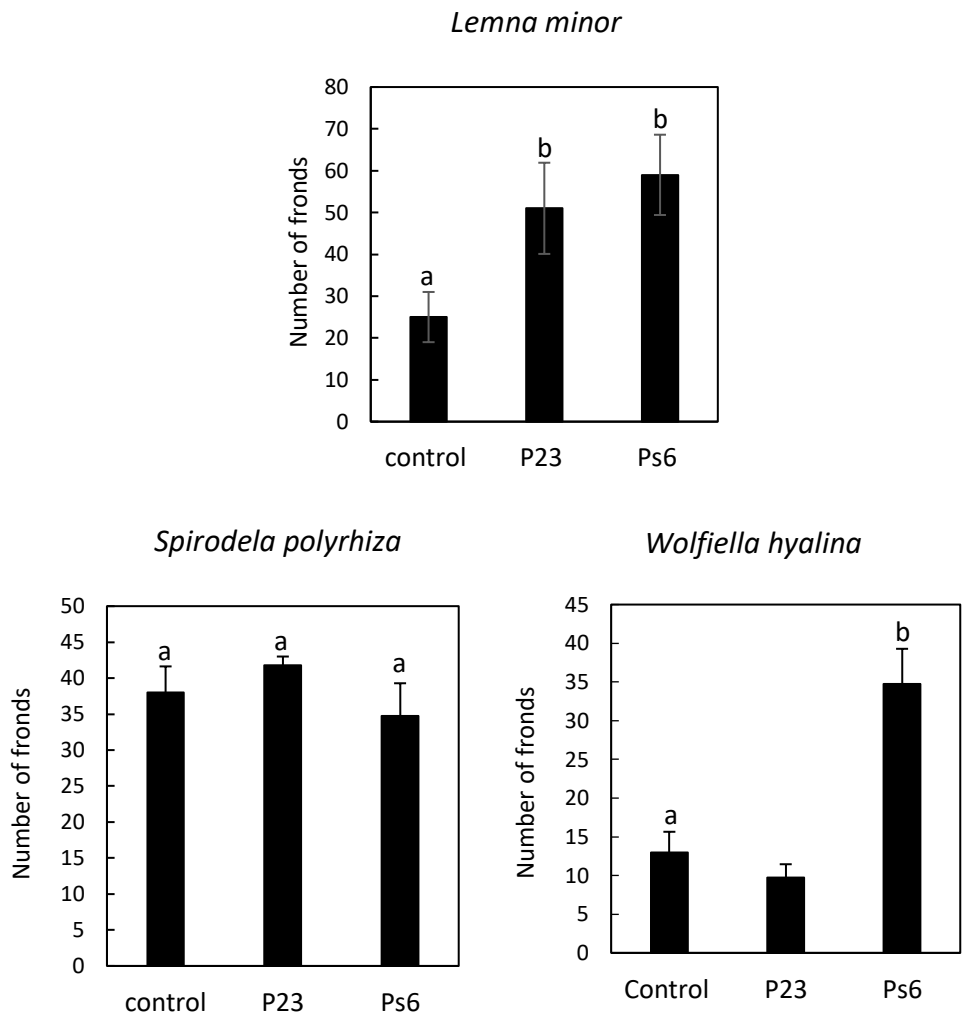


Fig. S2. Effects of previously isolated PGPB (P23 and Ps6) on the growth of different duckweed species after 10 days in Hoagland medium. The initial number of fronds inoculated was two in all experiments. Values are mean ± SD (n = 3). The results show that the duckweed growth-promoting activity of each PGPB is various depending on the duckweed species. Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

Appendix 3. Sequence data of PGPB isolated from WW

Table S1. Sequence data of PGPB isolated from WW

Isolate	Gene back accession number	Sequence length	Query cover	Percent identity	Affiliation	Note
27AL	LC567048	1362	99%	98.92%	*Chryseobacterium taichungensis*	PGPB in A-WW and K-WW
29AL	LC567049	1376	99%	98.63%	*Chryseobacterium taichungensis*	PGPB in A-WW and K-WW
5KL	-	889	100%	100%	*Pseudomonas guguanensis*	PGPB in N supplemented A-WW and K-WW condition
7KL	-	883	100%	100%	*Aeromonas ceviae*	PGPB in N supplemented A-WW and K-WW condition
17KL	-	972	100%	100%	*Pannonibacter phragmitetus*	PGPB in N supplemented A-WW condition

Appendix 4. General plant growth promotion factors of PGPB isolated from WW compared to *A. calcoaceticus* P23 and *P.fulva* Ps6

Table S1. Production of general PGP factors of some PGPB

Bacterial strain	IAA	Phosphate solubilization	Siderophore
P23	–	+	+
Ps6	+	+	–
27AL	+ (81.73 µg /mL)	–	+
29AL	+ (83.76 µg /mL)	–	+
5 KL	+ (68.55 µg /mL)	+	+
7 KL	+ (68.69 µg /mL)	+	+
17 KL	+ (64.49 µg /mL)	+	+

IAA production: The value in parenthesis is the amount of IAA produced in LB medium supplemented with tryptophan.
Phosphate solubilization: Formation of clear zone around the colony on Pikovskaya agar plate.
Siderophore production: Formation of orange color zone around the colony on CAS agar plate.

Appendix 5. Mineral content of Hoagland medium after mineral modification

Table S2. Modification of Hoagland medium to examine the effect of significantly large and small amount of minerals on the PGPB activities for duckweed

Minerals	Treatment (mineral content, mM)					K-WW
	Hoagland: **H**	[1]Hoagland with high PO_4: **H(↑PO_4^-)**	[2]Hoagland with high Na/Cl/SO_4: **H(↑Na/Cl/SO_4)**	[3]Hoagland with low N: **H(↓N)**	[4]Hoagland with high PO_4, high Na/Cl/SO_4, low N: **H(↑PO_4↑Na/Cl/SO_4↓N)**	
Na	0.03	3.35†	46.11	0.03	49.46†	46.11
SO_4	2.11	2.11	13.63†	2.11	13.63†	4.34
Cl	2.00	2.00	25.04†	2.00	25.04†	11.27
NO_3	0.36	0.36	0.36	0.005	0.005	0.005
PO_4	0.03	3.35	0.03	0.03	3.35	3.35
NH_3	-	-	-	0.04	0.04	0.04

Underlined values indicate the minerals modified to Hoagland medium.
†Values changed due to the unavoidable effect of counterions.
[1] Addition of 3.32 mM of $NaH_2PO_4.2H_2O$
[2] Addition of 23 mM NaCl and 11.52 mM Na_2SO_4
[3] Adjustment of N with 0.04 NH_4Cl and 0.005 KNO_3
[4] Combination of all 1-3

Appendix 6. The effect of different salinity concentration on duckweed morphology

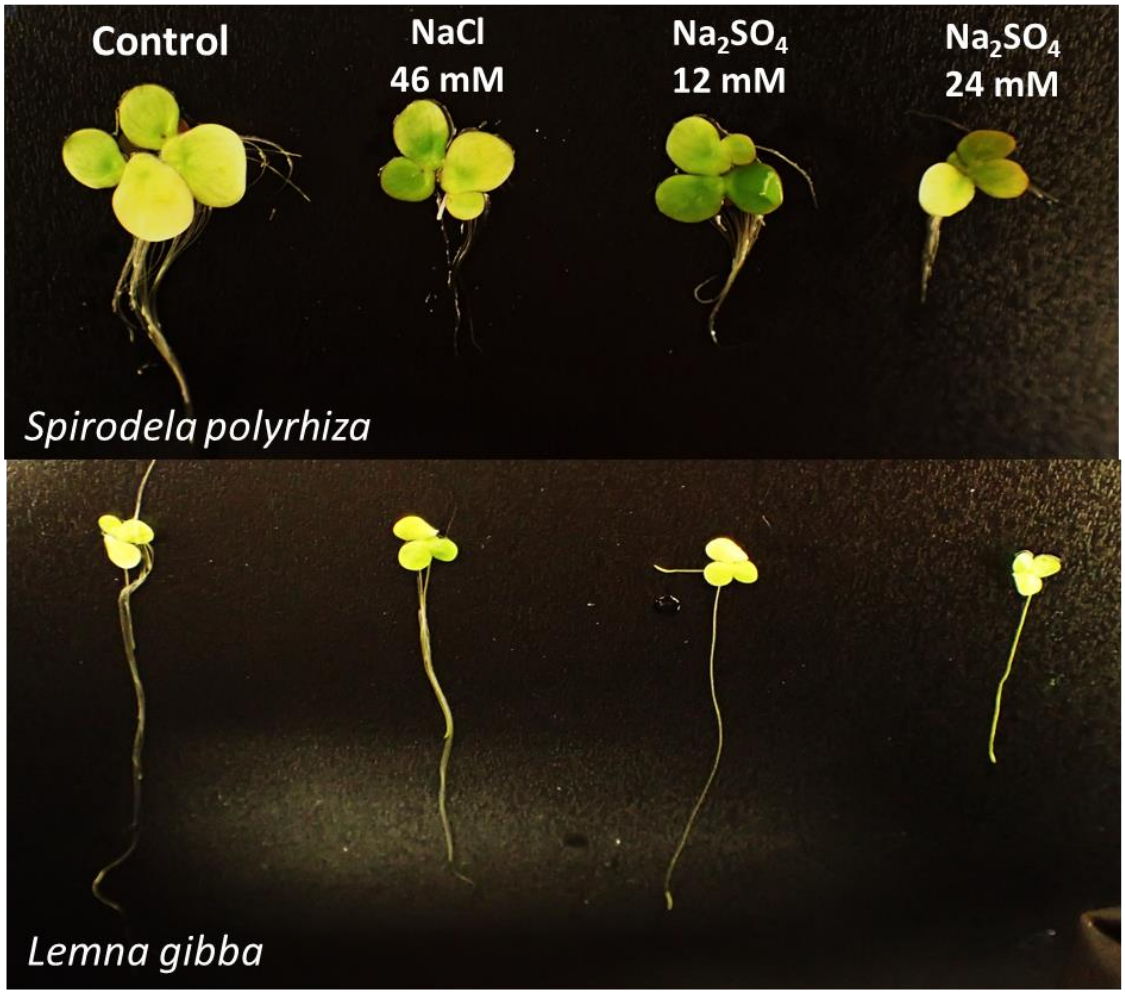


Fig. S3. The effect of different concentrations of NaCl or Na_2SO_4 on the growth of *Spirodela polyrhiza* and *Lemna gibba* in Hoagland medium after 20 days of cultivation.

Appendix 7. Protocol for starch and protein measurement on duckweed

1. Starch measurement

Sample preparation

a. Measure the total fresh weight of the duckweed sample
b. Freeze-dry the duckweed sample for ±2-3 days
c. Measure the total dry weight of duckweed samples
d. Calculate %moisture of duckweed with the equation (a)
e. Mill the dried duckweed by mortar and pestle until it became a powder-like form
f. Transfer the sample to a falcon tube and store at room temperature for the starch assay

$$\%\text{Moisture} = \frac{\text{Total fresh weight (mg)} - \text{Total dry weight(mg)}}{\text{Total dry weight (mg)}} \text{ x } 100\% \quad \textbf{(a)}$$

Starch assay

a. Accurately weigh 25-100 mg of dried duckweed (equivalent to 300~1000 fresh fronds) that has been prepared from the protocol above and transferred into the glass test tube with a cap
   Note: If the starch content of some samples were compared, use the same amount of dry weight for the starch measurement
b. Add 0.2 mL of 80% ethanol and vortex for 10s
c. Add 2 mL of cold 1.7 M NaOH solution, vortex, and incubated on ice for 15 min (vortex every 2 min)
d. Add 8 mL of sodium acetate buffer (600 mM, pH 3.8) plus $CaCl_2$ (5 mM) and vortex (if the lumps form in the bottom of the tube, invert the tube several time and then vortex again until the sample mixed well with the solution)
e. Add 0.1 mL of undiluted thermostable α-amylase (cut the edge of the yellow tip before pipetting since the solution is viscous) and mixed it well until no lumps form
f. Add 0.1 mL of amyloglucosidase (cut the edge of the yellow tip before pipetting since the solution is viscous) and mixed it well until no lumps form
g. Incubate the sample in the water bath at 50^{o}C for 30 min
h. Transfer 2 mL of the sample into a 2 mL microtube and centrifuged at 13.000 rpm for 5 min
i. Transfer 0.1 mL of supernatant into a new glass test tube.
   Note: for the sample that have possible starch content more than 10%, further dilution of supernatant is needed before using it for the next step.
j. Prepare the blank and standard as follows:
   Blank: 0.1 mL of sodium acetate buffer (100 mM, pH 5) plus CaCL2 (5 mM)
   Standard: 0.1 mL of standard glucose

Transfer the blank and standard into the glass test tube

k. Add 3 mL of GOPOD into the sample, Blank, and standard tubes then incubate in the water bath at 50°C for 20 min

l. The absorbance of the sample was then measured against a blank reagent at 510 nm.

m. Then calculate the starch content by calculation (b).

Note: If the %moisture of the duckweed sample is known, then the calculation is continued to the equation (c). Starch % w/w (as is) is the calculation result of equation (b) and the moisture content is the calculation result of equation (a)

$$\text{Starch, \%} = \Delta A \times F \times \frac{EV}{0.1} \times D \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta A \times F \times EV \times \frac{D}{W} \times 0.90$$

(b)

Starch % w/w (dry wt. basis):

$$= \text{Starch \% w/w (as is)} \times \frac{100}{100 - \text{moisture content (\% w/w)}}$$

(c)

ΔA = absorbance of sample solution read against reagent blank, less the absorbance of the sample blank read against the reagent blank (only where a sample blank is determined).

F = factor to convert absorbance values to mg glucose (100 mg glucose divided by the GOPOD absorbance value obtained for 100 mg of glucose).

EV = sample extraction volume [10.4 mL].

0.1 = volume of sample analyzed.

D = further dilution of sample solution (either undiluted, or diluted 5-fold or 11-fold)

1/1000 = conversion from mg to mg.

100/W = conversion to 100 mg sample; W = sample weight in mg.

162/180 = factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch.

Note: 1.7 M NaOH solution, sodium acetate buffer (600 mM, pH 3.8) plus $CaCl_2$ (5 mM), and sodium acetate buffer (100 mM, pH 5) plus CaCL2 (5 mM) are not provided in the kit. Be sure to prepare before the measurement.

For GOPOD reagent, please prepare the solution following the megazyme kit protocol before use.

2. Protein measurement

Sample preparation

a. Freeze-dry the duckweed sample for ±2-3 days

b. Mill the dried duckweed by mortar and pestle until it became a powder-like form

Protein extraction (Aposcience)

a. Weigh 25-100 mg of dried duckweed that has been prepared from the above protocol and put in a 1.5 mL of microtube
   Note: If the protein content of some samples were compared, use the same amount of dry weight for the protein measurement
b. Add 1 mL of cooled extraction reagent to the sample and incubate in ice for 30 min
c. Centrifuge the sample at 4^{o}C for 20 min at 12.000-15.000 x g
d. Transfer 200 µl of the supernatant into the new cold 1.5 mL microtube and store the sample in -20^{o}C for the protein assay.

Protein Assay (DC protein assay Bio-Rad kit)

a. Preparation of working reagent
   Add 20 µl of reagent S to each ml of reagent A that will be needed for the run to make reagent A'.
   Note: If precipitate forms, warm the solution at 50^{o}C and vortex

b. Prepare 3 - 5 dilutions of a protein standard (BSA/Bovine Serum Albumin) containing from 0.2 mg/ml to about 1.5 mg/ml protein. A standard curve should be prepared each time the assay is performed.

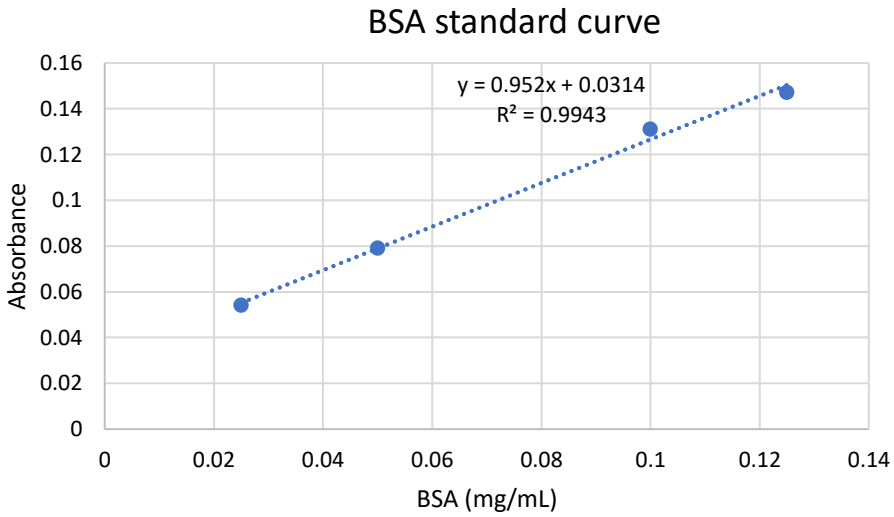
c. Pipet 100 µl of standards and samples into clean, dry test tubes.

d. Add 500 µl of reagent A' (from step a) into each test tube. Vortex.

e. Add 4.0 ml reagent B into each test tube and vortex immediately.

f. After 15 minutes, absorbances can be read at 750 nm. The absorbances will be stable for at least 1 hour.

g. Calculate %protein with the equation (d)



$$\%\text{Protein (w/w)} = \frac{\text{P x D x V1}}{\text{V2 x W}} \text{ x } 100\% \quad \textbf{(d)}$$

P = Protein content of sample after the absorbance conversion with standard curve equation (mg/L)
D = Dilution factor
V1 = Sample extraction volume (1 mL) from protein extraction step
V2 = Sample analyzed volume (0.1 mL) from protein assay step
W = Weight of sample (mg)

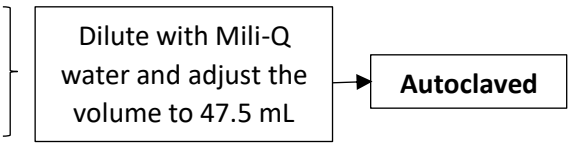
Appendix 8: Protocol for making CTAB buffer (+DTT) for DNA isolation of microalgae

CTAB buffer (+DTT) contained of:

1. 2% CTAB (Cetrimonium bromide)
2. 100 mM Tris. HCl pH 8
3. 1.4 M NaCl
4. 20 mM EDTA pH 8
5. 50 mM DTT (dithiothreitol)

Preparation for making 50 mL CTAB buffer (+DDT) is described as follows:

1. 1 gr of CTAB
2. 5 mL of 1M Tris.HCl pH 8 stock
3. 2 mL of 0.5M EDTA pH 8 stock
4. 4.07 gr of NaCl



5. 2.5 mL of filter-sterilized 1 M DTT is added into autoclaved CTAB buffer (DTT cannot be autoclaved)

Note: When mixing reagents 1 to 4, it may not completely be solubilized, and the colour will be a white turbid, but it will be solubilized after autoclaved.
Before using CTAB buffer (+DTT) for DNA extraction, warm the solution first in 60^{o}C until the solution become transparent.

Appendix 9: DNA isolation of microalgae protocol (Kim et al., 2012, with modification)

LIST OF PUBLICATION

Khairina, Y., Jog, R., Boonmak, C., Toyama, T., Oyama, T., Morikawa, M., 2021. Indigenous bacteria, an excellent reservoir of functional plant growth promoters for enhancing duckweed biomass yield on site. Chemosphere. https://doi.org/10.1016/j.chemosphere.2020.129247