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# Changes in Free-Living and Particle-Associated Bacterial Communities Depending on the Growth Phases of Marine Green Algae, *Tetraselmis suecica*

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**Abstract:** Bacteria are remarkably associated with the growth of green algae *Tetraselmis* which are used as a feed source in aquaculture, but *Tetraselmis*-associated bacterial community is characterized insufficiently. Here, as a first step towards characterization of the associated bacteria, we investigated the community composition of free-living (FLB) and particle-associated (PAB) bacteria in each growth phase (lag, exponential, stationary, and death) of *Tetraselmis suecica* P039 culture using pyrosequencing. The percentage of shared operational taxonomic units (OTUs) between FLB and PAB communities was substantially high ( $\geq 92.4\%$ ), but their bacterial community compositions were significantly ( $p = 0.05$ ) different from each other. The PAB community was more variable than the FLB community depending on the growth phase of *T. suecica*. In the PAB community, the proportions of *Marinobacter* and Flavobacteriaceae were considerably varied in accordance with the cell number of *T. suecica*, but there was no clear variation in the FLB community composition. This suggests that the PAB community may have a stronger association with the algal growth than the FLB community. Interestingly, irrespective of the growth phase, *Roseobacter* clade and genus *Muricauda* were predominant in both FLB and PAB communities, indicating that bacterial communities in *T. suecica* culture may positively affect the algae growth and that they are potentially capable of enhancing the *T. suecica* growth.

**Keywords:** *Tetraselmis suecica*; associated bacterial community; free-living bacteria; particle associated bacteria



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## 1. Introduction

Marine green microalga genus *Tetraselmis* is well-known to have a high lipid content and fast growth [1,2], and thus it has been widely used in multiple industries, for example, a source of nutrition for invertebrates in aquaculture [3], feedstock of biofuel production [4], and cosmetic applications [5]. In order to save the cost for algal production, there have been attempts to advance the algal-culture technique which enables gaining a higher biomass of this green algae.

Algal-culture techniques have been developed based on adjusting physiochemical factors (e.g., light intensity, nutrient limitation, temperature, pH, CO<sub>2</sub> concentration, and salinity) which are well known to have an intimate association with algal growth [6,7]. Recently, bacteria which have a symbiotic relationship to algae have been considered as a new factor to advance the algal-culture technique, allowing to gain a higher algal biomass. Bacteria can affect the growth of algae in various ways which ranged from mutualism to parasitism [8–12]. Interestingly, the maximum algal cell density is obviously enhanced when symbiotic bacteria are added to the algal culture, compared to that in the optimum

culture condition (e.g., culture media, temperature, salinity, pH, etc.) without bacteria, for example, the biomass productivity of *T. striata* was enhanced up to two-fold by the addition of two bacterial strains (*Pelgibacter bermudensis* and *Stappia* sp.) [4]. These findings show the possibility that bacteria can be an important factor to advance a culture technique for algae.

Due to the high economic value of *T. suecica*, studies on developing the mass culture technique for this algae have been carried out, and in order to advance this culture technique, bacteria which enhanced the algal growth were isolated more recently [13,14]. Thus, understanding mutualism between *T. suecica* and co-existing bacteria is thought to be important, but there is a significant knowledge gap for this ecological relationship due to a lack of evidence. The two bacterial groups exist in the algal culture. These include free-living (FLB) and particle-associated (PAB) bacteria which are phylogenetically distinct. Based on previous findings, these two groups are closely associated with the growth of algae even though PAB showed stronger species-specific association to the host algae [8,15–17]. In addition, their community compositions are distinctly varied depending on the growth phase of algae due to the change in composition and quantity of dissolve organic matters (DOM) released from the host algae. However, in previous studies, only the FLB composition was identified, and the previous analysis method (terminal restriction fragment length polymorphism) may not be adequate to gain a high resolution for the characterization of bacterial community composition in *T. suecica* culture due to the methodological limitation [13].

The present study aims to elucidate bacterial taxa which were associated with the growth of *T. suecica*. To address this, we investigated the community composition of both FLB and PAB and a variation in the two bacterial groups depending on the growth phase of *T. suecica* with the next generation sequencing (NGS) approach, allowing a high taxonomic resolution.

## 2. Materials and Methods

### 2.1. Algal Culture and Cell Growth Analysis

A culture of *T. suecica* P039 (cell size: Average 16.2  $\mu\text{m}$  length and 10.6  $\mu\text{m}$  width in 33 cells) which was isolated from coastal water in Deukryang Bay, Korea, was obtained from the Korea Marine Microalgae Culture Center (Pukyong National University, Busan, Korea). It has been maintained in an autoclaved f/2 medium [18] at 20 °C in a 12:12 h light:dark cycle with a photon flux density of approximately 65  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The growth stage of *T. suecica* was determined based on the growth curve (Figure S1).

Cell growth curves were analyzed at every 2-day intervals using cell numbers measured with the Sedgwick-Rafter counting chamber. At the same time, bacterial cells were counted by using the 4',6-diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich, Darmstadt, Germany) staining method [19]. Briefly, all glass wares and reagents were sterilized with 10% nitric acid treatments and filtration with a 0.22  $\mu\text{m}$  nucleopore membrane (Millipore, Cork, Ireland), respectively. As for bacteria counting, 20 mL of cultures was preserved with 1 mL of formaldehyde (37% formaldehyde), and then stored at 4 °C and a dark condition. For DAPI staining, we mixed 1 mL of the preserved cultures and 100  $\mu\text{L}$  of DAPI (0.1  $\mu\text{g mL}^{-1}$ ), and incubated it for 5 min. Then, the cells were filtered through the pore size 0.2  $\mu\text{m}$  black filter (Isopore membrane; Millipore, Bedford, MA, USA) using a hand pump with less than 178 mmHg in pressure. The filters were mounted on an objective slide, and observed using a fluorescent microscope (Axioskop, Carl Zeiss, Oberkochen, Germany).

### 2.2. Sample Collection and DNA Extraction

To investigate a change in the bacteria community composition depending on the growth stages, the *T. suecica* culture was harvested in lag (day 2), exponential (day 8), stationary (day 20), and decline (day 32) growth phases. In addition, we used the size-fractionated filtration method for collecting the separate bacteria of both PAB and FLB

from the culture. One hundred mL of *T. suecica* culture were filtered sequentially through a 10 µm (PAB) and 0.2 µm (FLB) pore size membrane filter (diameter: 47 mm; Millipore, Cork, Ireland). Each loaded filter was immediately placed in a 2-mL microtube (Axygen Sciences, CA) that contained 800 µL extraction buffer (100 mM Tris-HCl, 100 mM Na<sub>2</sub>-EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB) and was then stored at −80 °C until the DNA was extracted.

The DNA was extracted using a modified CTAB protocol [20]. Briefly, 2 mL of microtubes containing membrane filters and the extraction buffer (100 mM Tris-HCl, 100 mM Na<sub>2</sub>-EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB) were subjected to three cycles of immersion in liquid N<sub>2</sub> until completely frozen and then thawed in a 65 °C water bath. After 8 µL of proteinase K was added (10 mg mL<sup>−1</sup> in TE buffer), the samples were incubated at 37 °C for 30 min. Then, after the addition of 80 µL of 20% sodium dodecyl sulfate (SDS), the samples were incubated at 65 °C for 2 h, shaken gently with an equal volume of chloroform-isoamyl alcohol (24:1), and then centrifuged at 10,000 × *g* for 5 min. The aqueous phase was transferred to a new 2 mL tube containing 88.8 µL of 3 M sodium acetate (pH 5.1), and 587 µL of isopropanol (≥99%) was added. Following centrifugation at 14,000 × *g* for 20 min, the supernatant was decanted, 1 mL of cold 70% ethanol was added, and the samples were centrifuged at 14,000 × *g* for 15 min. The pellets were air dried at room temperature before being dissolved in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8).

### 2.3. Pyrosequencing

Metagenomic sequencing was performed using the 454 GS FLX Titanium Sequencer System (Roche, Basel, Switzerland). Briefly, target rDNA retrieved from the cultured samples was amplified using the polymerase chain reaction (PCR) performed with two universal bacterial primers: 27F, 5'-GAG TTT GAT CMT GGC TCA G-3' and 518R, 5'-TTA CCG CGG CTG CTG G-3'. Each primer was tagged using multiplex identifier (MID) adaptors according to the manufacturer's instructions (Roche, Mannheim, Germany), which allowed for the automatic sorting of the pyrosequencing-derived sequencing reads based on the MID adaptors. In addition, MID-linked 27F and 518R were linked to the pyrosequencing primers 5'-CGT ATC GCC TCC CTC GCG CCA TCA G-3' and 5'-CTA TGC GCC TTG CCA GCC CGC TCAG-3', respectively, according to the manufacturer's instructions (Roche, Mannheim, Germany).

Metagenomic PCR was performed with 20 µL reaction mixtures containing 2 µL 10× Ex Taq buffer (TaKaRa, Kyoto, Japan), 2 µL of a dNTP mixture (4 mM), 1 µL of each primer (10 pM), 0.2 µL Ex Taq polymerase (2.5 U), and 0.1 µg of the environmental DNA template. PCR cycling was performed in an iCycler (Bio-Rad, Hercules, CA, USA) at 94 °C for 5 min, followed by 35 cycles at 94 °C for 20 s, 52 °C for 40 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The resulting PCR products were electrophoresed in 1.0% agarose gel, stained with ethidium bromide, and viewed under ultraviolet transillumination.

Prior to pyrosequencing, amplified PCR products were individually purified using a Dual PCR Purification Kit (Bionics, Seoul, Korea) and, subsequently, equal volumes of each purified PCR product were mixed together. Pyrosequencing of the MID-tagged PCR amplicons was performed using a 454 GS FLX Titanium system (Roche, Mannheim, Germany) with a commercial service at Macrogen, Inc. (Seoul, Korea).

### 2.4. Pyrosequencing Data Analysis

After each sequencing procedure had been completed, a quality check was performed to remove short sequence reads (e.g., less than 150 bp), low-quality sequences, sequence artifacts and chloroplast sequences, and any non-bacterial ribosome sequences and chimeras [21,22]. Using the basic local alignment search tool (BLAST), all of the sequence reads were compared to the Silva rRNA database [23]. Sequence reads which were similar with an E-value of less than 0.01 were admitted as partial 16S rDNA sequences.

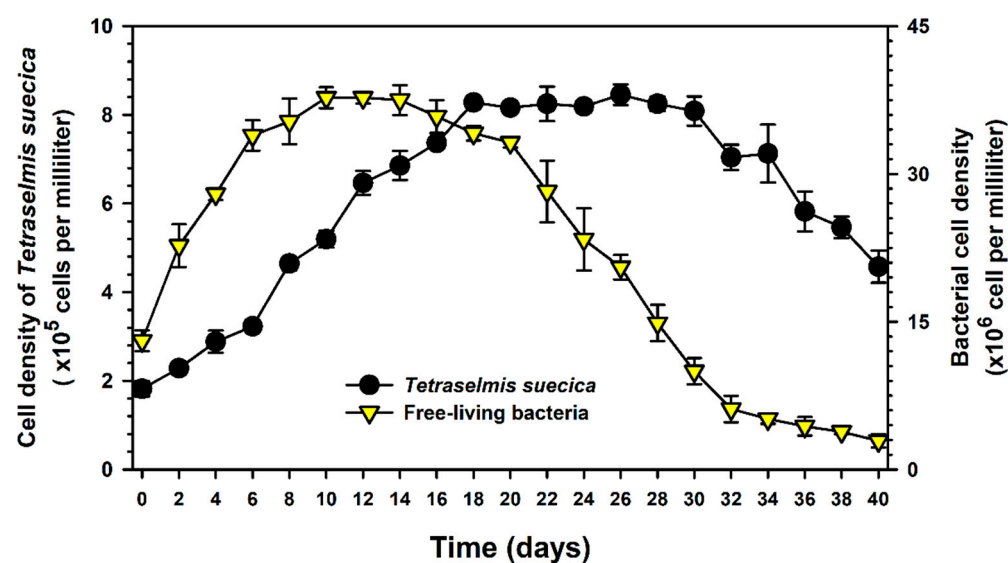
The taxonomy of the sequence with the highest similarity was assigned to the sequence read (genus and class level). To analyze operational taxonomic units (OTUs), the CD-HIT-OTU software was used for clustering [24] and the Mothur software was used for Shannon-Weaver diversity and Chao richness estimation [25]. All data from the 454 pyrosequences were deposited into the Genbank database.

To examine the similarities and differences in the community composition (at the OTU level) among the samples (FLB and PAB communities depending on the growth phase of *T. suecica*), PRIMER ver. 7 [26] and R studio ver. 5.3 [27] were used to generate the heatmap and dendrogram for the hierarchical cluster analysis, together with similarity profiles (SIMPROF,  $p = 0.05$ ). All the analyses were performed based on the Bray-Curtis dissimilarity index.

### 3. Results and Discussion

#### 3.1. Cell Growth of *Tetraselmis suecica* and Bacteria in Cultures

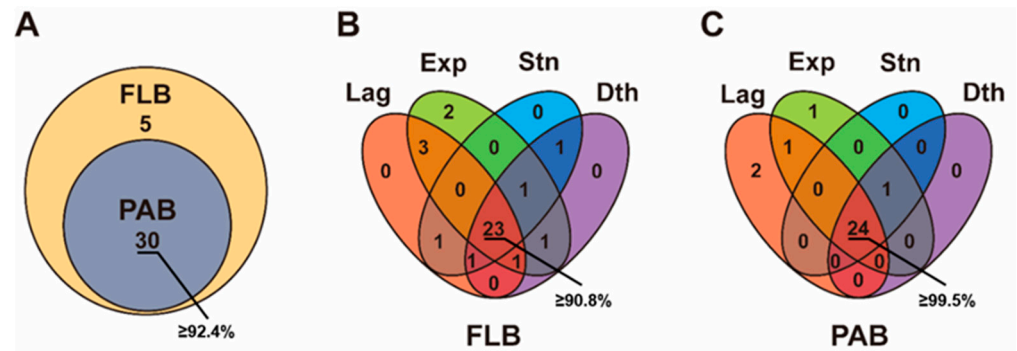
The green algae *T. suecica* and FLB in the culture appeared as a common, typical S-shaped growth curve (Figure 1), but the growth of FLB was faster than that of *T. suecica*. The highest density ( $3,774,000 \text{ cells mL}^{-1}$ ) of bacterial cells was shown at day 10, whereas *T. suecica* reached the maximum cell density ( $827,830 \text{ cells mL}^{-1}$ ) at day 18. Community compositions of FLB and PAB were investigated using the samples which were collected at day 2, 8, 20, and 32, referring to the lag, exponential, stationary, and death growth phase of *T. suecica*.



**Figure 1.** The growth curves of *Tetraselmis suecica* and the associated bacteria. Arrows represent each sampling point. Abbreviations: FLB: Free-living bacteria; PAB: Particle-associated bacteria; Exp: Exponential; Stn: Stationary; Dth: Death.

#### 3.2. Pyrosequencing Data and Comparison of FLB and PAB in *Tetraselmis suecica*

The pyrosequencing analysis generated 3742–9409 nucleotide fragments of FLBs and 8078–13,725 nucleotide fragments of PAB. All of the samples reached saturation in the rarefaction curve indicating that a sufficient amount of sequences was identified by the pyrosequencing used in this study (Figure S1). The number of bacterial OTUs ( $\geq 97\%$  similarity) in FLB and PAB were 35 and 30, respectively, and all bacterial OTUs in PAB were present in FLB (Figure 2A).



**Figure 2.** Bacterial operational taxonomic units (OTUs) Venn diagram. (A): Venn diagram enumerates shared bacterial OTUs between free-living (FLB) and particle-associated (PAB); (B,C): Venn diagrams enumerate FLB and PAB OTUs, shared by and exclusive to lag, exponential (Exp), stationary (Stn), and death (Dth) growth phases of *T. suecica*. The percentage number (lower right corner of each Venn-diagram) indicates the proportion of shared bacterial OTUs.

Based on alpha diversity indices (Shannon and Simpson), the level of bacterial diversity in FLB was clearly higher than that in PAB (Table 1). The averages of diversity indices of FLB were 3.39 (Shannon) and 0.84 (Simpson), but those of PAB were 1.99 (Shannon) and 0.57 (Simpson), respectively. Together with these findings, the bacterial community in FLB was more diverse than that in PAB.

**Table 1.** Number of sequence reads and alpha diversity indices (Shannon and Simpson) PAB and FLB samples, which were isolated from each growth phase of *T. suecica* culture (P-039).

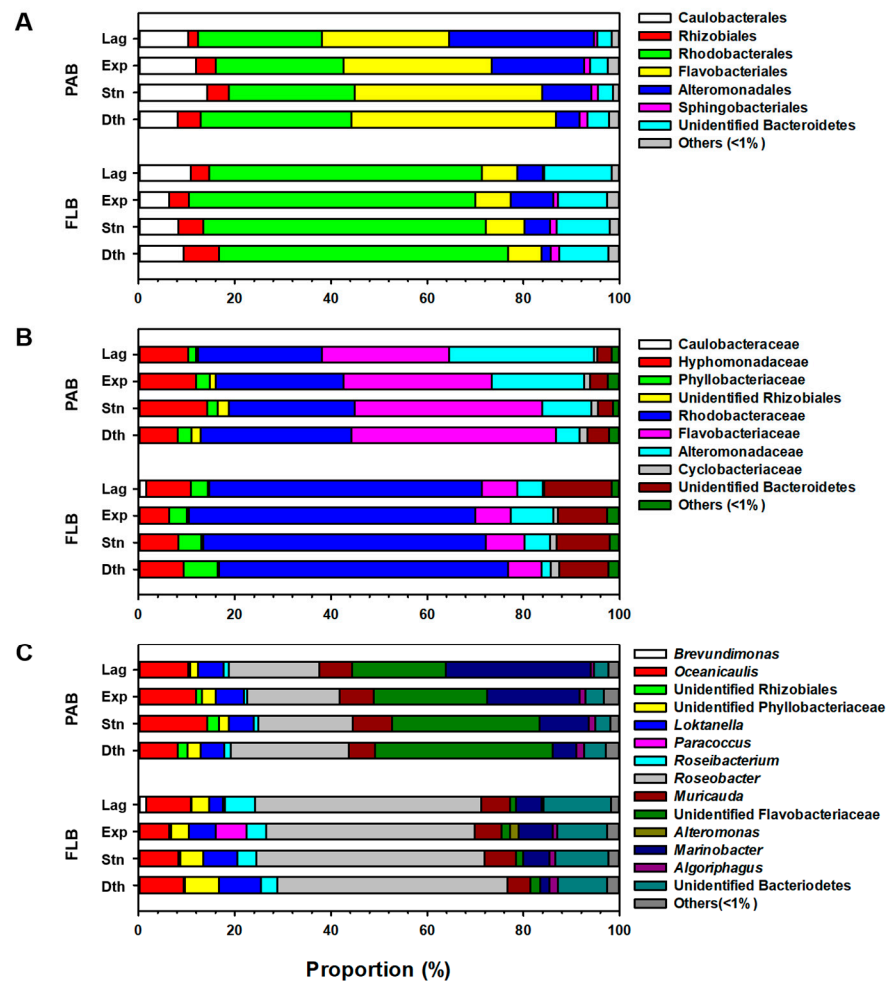
Samples		Read Count	Diversity Indices	
			Shannon	Simpson
Particle-associated	Lag	10,683	2.023	0.569
	Exponential	13,725	1.908	0.543
	Stationary	9433	1.961	0.565
	Death	8078	2.066	0.594
Free-living	Lag	7233	3.105	0.792
	Exponential	3742	3.540	0.850
	Stationary	9409	3.418	0.844
	Death	7854	3.490	0.863

The proportion of shared OTUs between FLB and PAB communities was remarkably high. It was 100% of PAB and  $\geq 92.4\%$  of FLB (Figure 2A). According to previous findings, this proportion is determined by the concentration of particles [28–30], and the proportion of shared bacterial OTUs between FLB and PAB communities was relatively high in certain marine environments where a higher concentration of particles existed [31–34]. In this study, we used the culture which contained a high concentration of cells. Therefore, this might result in a high proportion of shared bacterial OTUs between the two bacterial communities in this study. Interestingly, we have obtained similar results (i.e., high proportion of shared bacterial OTUs) in previous studies where dinoflagellate cultures were used [28,35].

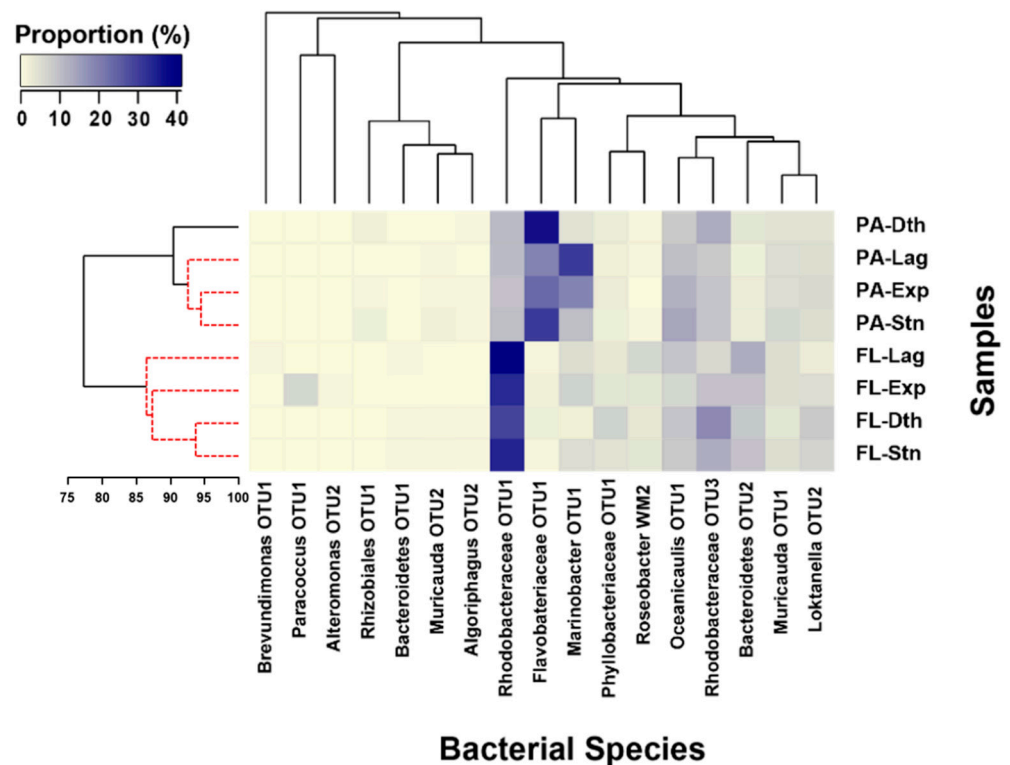
### 3.3. Difference between FLB and PAB Communities in *Tetraselmis suecica* Culture

Interestingly, there was a clear difference in the bacterial community composition between FLB and PAB communities even though a proportion of shared bacterial OTUs was remarkably high. The genus *Roseobacter*, belonging to the class Rhodobacteraceae (56.7–60.1%), was most dominant in the FLB community (Figure 3). The second most dominant groups were genera *Oceanicaulis* (family Hyphomonadaceae, 6.3–9.3%), *Loktanella* (Rhodobacteraceae, 2.9–8.6%), *Muricauda* (Flavobacteriaceae, 4.8–6.5%), *Roseibacterium* (Rhodobacteraceae, 3.5–6.3%), and *Marinobacter* (Alteromonadaceae, 1.9–7.1%). Whereas,

in the PAB community, the family Flavobacteriaceae (19.5–36.9%, unidentified at the genus level) and the genus *Marinobacter* (10.2–30.1%) were predominant groups, with *Oceanicaulis* (8.2–14.3%), *Loktanella* (4.8–5.8%), and *Muricauda* (5.5–8.1%) the second most dominant genera (Figure 3). These two bacterial communities at the OTU level were significantly distinct (77.5% of similarity level,  $p = 0.05$ ) in the cluster analysis and the SIMPROF test (Figure 4). Organic matters released from phytoplankton play an important role in the determination of abundance and community composition of heterotrophic bacteria in aquatic environments [8]. There are two types of organic matters which originated from phytoplankton [8], one is the low molecular weight (LMW) molecule (e.g., amino acids, organic acids, and carbohydrate, etc.) and the other is the high molecular weight (HMW) macromolecule (e.g., polysaccharides, proteins, nucleic acids, and lipids, etc.). The environments where FLB and PAB communities distribute are clearly distinct in terms of the ratio between LMW and HMW. Thus, this may lead to a significant difference in the community composition between FLB and PAB. For example, the most dominant taxa in FLB and PAB were Rhodobacteraceae (genus *Roseobacter*) and Flavobacteriaceae, respectively. Based on previous findings, the *Roseobacter* clade is capable of utilizing various LMW compounds that originated from phytoplankton as a carbon source [36–38]. Whereas, Flavobacteriaceae prefer to use HMW compounds and can convert these into LMW compounds [39].



**Figure 3.** Composition of the FLB and PAB communities in lag, exponential (Exp), stationary (Stn), and death (Dth) growth phases of *T. suecica*. (A): Order; (B): Family; and (C): Genus level.



**Figure 4.** Heatmap for a comparison of the bacterial community composition among the samples at the species level. The values in the heat map represent the log-transformed relative abundance ( $\text{Log } X + 1$ ) of each bacterial OTU, and similarity matrices were generated using the Bray–Curtis method. In the cluster analysis on Y-axis, the red dot-lines indicate where divisions are not statistically significant, as judged by similarity profiles (SIMPROF) carried out at  $p = 0.05$ .

#### 3.4. Variation in Bacterial Community Composition Depending on the Growth Stage of *Tetraselmis suecica*

The number of bacterial OTUs observed in the lag, exponential, stationary, and death growth phases of *T. suecica* was 29, 31, 27, and 28, respectively for FLB (Figure 3B) and 27, 27, 25, 25, respectively for PAB (Figure 2C). There was no significant difference in bacterial diversity depending on the growth phase of host green algae (Table 1). The range of the Shannon index was 3.105–3.540 (FLB) and 1.908–2.066 (PAB), and the range of the Simpson index was 0.792–0.863 (FLB) and 0.543–0.594 (PAB). Whereas, depending on the growth phase of *T. suecica*, the community composition of FLB and PAB was varied, and, interestingly, the PAB community was more variable than the FLB community (Figures 3 and 4). In the hierarchical cluster analysis, the PAB community composition in the death phase of *T. suecica* was significantly ( $p = 0.05$ ) distinct to that in the other growth phases (lag, exponential, and stationary phases) at the OTU level, where there was no significantly different composition of FLB communities in each growth phase of green algae (Figure 4). To our knowledge, PAB has a stronger species-specific association to the growth and physiological condition of host algae [15–17,28,35], since the microhabitat for PAB is provided by algae [40,41].

Rhodobacteraceae OTU1, belonging to the *Roseobacter* clade, was generally predominant in both FLB and PAB communities even though its proportion in FLB was higher than that in PAB. This suggests that this bacterial OTU may be intimately associated with *T. suecica*. The *Roseobacter* clade can provide growth-promoting compounds (e.g., vitamin B<sub>12</sub>) to various algal species, including *Tetraselmis* [12,42,43]. Based on a recent study [13], the growth yield of *T. suecica* F and M33 was increased when three isolates from the *Roseobacter* clade were co-cultivated. Interestingly, the bacterial isolate, belonging to genus *Muricauda*, has also shown the capability to enhance the growth of *T. suecica* F and M33 [13].

This genus was one of the dominant taxa in both FLB and PAB communities in our study. Given these findings, dominant bacterial groups of PAB and FLB communities established in *T. suecica* culture may positively contribute to the growth of host green algae. However, the actual impact of these bacterial taxa on the growth of *T. suecica* is thought to be unclear. For example, a cell number of *T. suecica* was largely decreased in the death phase, but the proportion of these bacterial taxa was not significantly decreased (Figures 1 and 3). To our knowledge, inorganic nutrients which are essential to algal growth were generally depleted in the death phase of algae due to the consumption by the algae. Thus, it is more likely that the decrease in *T. suecica* cells in the death phase may be caused by the depletion of inorganic nutrients even though growth promoting bacteria (e.g., *Roseobacter* clade and *Muricauda*) were present.

In the PAB community, the proportion of *Marinobacter* was gradually decreased in accordance with the increase in cell number of *T. suecica*. Its proportion in the lag and exponential growth phase was clearly higher than that in the stationary and death phase, in which the algal growth rate was relatively low (Figures 1 and 3). Several clades of this bacterial genus can secrete a siderophore called vibrioferrin, allowing the promotion of the algal assimilation of iron [44]. Therefore, a lower proportion of *Marinobacter* in the stationary and death phases may adversely affect the growth of *T. suecica*.

Flavobacteriaceae can primarily consume HMW compounds and convert them into LMW compounds [39]. Thus, an abundance of this bacteria is generally elevated when a number of HMW compounds were released from the algae, as a result of cell lysis [45–48]. Similarly, in this study, the proportion of Flavobacteriaceae in the PAB community gradually increased in accordance with an increase in the cell number of *T. suecica* (Figures 1 and 3), resulting in an elevation of the HMW compound concentration.

### 3.5. Conclusions and Remarks

The present study is the first to characterize FLB and PAB communities in *T. suecica* culture using the NGS approach. Based on our findings, FLB and PAB communities were significantly distinct ( $p = 0.05$ ), the PAB community was more affected depending on the growth phases of *T. suecica* than FLB community. In addition, bacterial taxa (e.g., *Roseobacter* clade and *Muricauda*) which are capable of enhancing the growth of host green algae were dominant in both FLB and PAB communities, irrespective of growth phase of *T. suecica*. These findings suggest that bacterial community in *T. suecica* culture may positively affect the growth of host algae. However, to evaluate the actual impact of bacterial communities on the growth of *T. suecica*, further extensive studies, such as growth promoting mechanism of bacteria in algal culture, are needed.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/2077-1312/9/2/171/s1>. Figure S1: OTU counts (A) and rarefaction (B) of each growth phase of *Tetraselmis suecica*. Abbreviations: FLB: Free-living bacteria; PAB: Particle-associated bacteria; Exp: Exponential; Stn: Stationary; Dth: Death.

**Author Contributions:** Conceptualization, B.S.P. and J.-S.K.; methodology, W.-J.C. and R.G.; investigation, W.-J.C. and H.K.; data curation, B.S.P.; writing—original draft preparation, B.S.P.; writing—review and editing, B.S.P., W.-J.C., R.G., H.K. and J.-S.K.; supervision, J.-S.K.; funding acquisition, B.S.P. and J.-S.K. All authors have read and agreed to the published version of the manuscript.

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