applied sciences

MDPI

Article

# Distribution of Harmful Algal Growth-Limiting Bacteria on Artificially Introduced *Ulva* and Natural Macroalgal Beds

**Nobuharu Inaba [1,*], Isamu Kodama [2], Satoshi Nagai [3], Tomotaka Shiraishi [4], Kohei Matsuno [2,5], Atsushi Yamaguchi [2,5] and Ichiro Imai [2]**

1 Civil Engineering Research Institute for Cold Region, Public Works Research Institute, Sapporo City, Hokkaido 062-8602, Japan
2 Faculty/Graduate School of Fisheries Sciences, Hokkaido University, Hakodate City, Hokkaido 041-8611, Japan; k-damakan@fish.hokudai.ac.jp (I.K.); k.matsuno@fish.hokudai.ac.jp (K.M.); a-yama@fish.hokudai.ac.jp (A.Y.); imai1ro@fish.hokudai.ac.jp (I.I.)
3 National Research Institute of Fisheries Science, Japan Fisheries Research and Education Agency, Yokohama City, Kanagawa 236-8648, Japan; snagai@affrc.go.jp
4 Agriculture, Forestry and Fisheries Department, Wakayama Prefecture, Wakayama City, Wakayama 640-8585, Japan; shiraishi_t0002@pref.wakayama.lg.jp
5 Arctic Research Center, Hokkaido University, Sapporo City, Hokkaido 001-0021, Japan
* Correspondence: inaba-n@ceri.go.jp; Tel.: +81-11-841-1695

Received: 8 July 2020; Accepted: 12 August 2020; Published: 14 August 2020


**Abstract:** The intensity and frequency of harmful algal blooms (HABs) have increased, posing a threat to human seafood resources due to massive kills of cultured fish and toxin contamination of bivalves. In recent years, bacteria that inhibit the growth of HAB species were found to be densely populated on the biofilms of some macroalgal species, indicating the possible biological control of HABs by the artificial introduction of macroalgal beds. In this study, an artificially created *Ulva pertusa* bed using mobile floating cages and a natural macroalgal bed were studied to elucidate the distribution of algal growth-limiting bacteria (GLB). The density of GLB affecting fish-killing raphidophyte *Chattonella antiqua*, and two harmful dinoflagellates, were detected between $10^6$ and $10^7$ CFU $g^{-1}$ wet weight on the biofilm of artificially introduced *U. pertusa* and 10 to $10^2$ CFU $mL^{-1}$ from adjacent seawater; however, GLB found from natural macroalgal species targeted all tested HAB species (five species), ranging between $10^5$ and $10^6$ CFU $g^{-1}$ wet weight in density. These findings provide new ecological insights of GLB at macroalgal beds, and concurrently demonstrate the possible biological control of HABs by artificially introduced *Ulva* beds.

**Keywords:** harmful algal bloom; mitigation; macroalgal bed; growth-limiting bacteria

## 1. Introduction

Harmful algal blooms (HABs) are naturally-occurring phenomena in fresh and marine water systems, often causing severe damage to the fishery industry worldwide, through massive kills of commercially cultured fishes and toxin-contamination of shellfish leading to closures of commercial shellfish harvests, and, in some cases, consumption of contaminated shellfish results in human illness and even death [1–3]. Lately, the acceleration of geographic expansion and intensity of HABs on a global scale associated with climate change have been reported [4–7]. Hence, developments of effective countermeasures to control the HAB events are even more urgent and crucial concerns. Mechanical, chemical, genetic, biological, and environmental measures to prevent HAB have been proposed [4], and clay dispersion is the only practical measure confirmed to control HABs formed by *Cochlodinium*

*polykrikoides* in Korean coastal waters [8]. However, the negative impacts of clay on non-target organisms living in the seafloor and water column are also pointed out [9–12]. The control of HABs directly intervenes with surroundings in dynamic ocean environments, demanding an establishment of measures as environmentally friendly as possible. Among all proposed strategies to mitigate HABs, biological controls using naturally living organisms may have the least impact on ecosystems in the present circumstances. Numbers of biological controls using microbes to bivalves have been proposed and tested extensively for several decades [13–20]. In particular, bacteria that kill and/or inhibit the growth of HAB species often associated with bloom termination have received worldwide attention as possible biological control of HABs [21–31].

Although the use of natural pathogenicity could be environmentally friendly and cost-effective to control HABs, introducing potentially invasive bacteria may require a careful manner [32,33]. Recently, a biofilm of seagrass and macroalgae were found to be densely populated by algal growth-limiting bacteria (GLB), including both algicidal and growth-inhibiting bacteria [34–37], indicating their function as an enormous source of GLB for the adjacent seawater [31,38]. The latest microcosm study demonstrated that seawater in the *Zostera marina* bed successfully suppressed artificial *Chattonella* blooms [31], indicating that protection and restoration of seagrass and macroalgal beds prevent the outbreak of HABs through providing the specific microbial communities. However, these holistic approaches may sometimes not be flexible in actual field applications in that they often require specific habitat characteristics such as light, sediment or substrate types, currents, bioturbation, etc. [39,40].

In this study, *Ulva pertusa* beds using mobile floating cages were artificially created to study the distribution of algal growth-limiting bacteria (GLB), seeking flexible and widely applicable technologies to control HABs. A natural macroalgal bed was also studied for comparison.

## 2. Materials and Methods

### 2.1. Installation of Artificial Ulva Bed

Yellowish-green reproductively matured *Ulva pertusa* thalli were provided from Wakayama prefectural fisheries experiment station, Wakayama Prefecture, Japan. After the thallus of *U. pertusa* was gently wiped and dried in the shade for 1–2 h, it was placed into a water tank filled with autoclaved-filtered seawater [41]. Pumice stones were carefully rinsed with autoclaved seawater and gently set to promote attachments of motile zoospore of *U. pertusa* [42]. Then, the pumice stones were placed into mobile floating cages and installed on June 20, 2017, in a nearshore area by an upright seawall at Station 1 (Stn. 1), Kushimoto-cho, Wakayama Prefecture, Japan (Figure 1). The location of installation was carefully selected after confirming the absence of any macroalgal species in the area. However, this may not guarantee attachments of naturally-occurring macroalgal propagules to the tested pumice stone and cages after installation.

### 2.2. Sampling

Samplings were performed on 7 to 8 September 2017, at Stn. 1 (artificial *Ulva* bed) and Stn. 2 (a natural macroalgal bed), Taiji-cho, Wakayama Prefecture, Japan (Figure 1). The weather was mostly cloudy during the sampling on 7 September 2017, with surface seawater temperature and salinity of 27.9 °C, 34.0 PSU at Stn. 1. On 8 September 2017, it was sunny with surface seawater temperature and salinity of 29.2 °C, 34.7 PSU at Stn. 2. The green alga, *U. pertusa*, successfully grown on the pumice stone and floating cage at Stn. 1 (Figure 2) and four dominant macroalgae (one brown alga: *Sargassum dupulicatum*, one red alga: *Gelidium elegans*, two green algae: *Cladophora ohkuboana* and *U. pertusa*) at Stn. 2 were sampled using sterile instruments in a sterile manner. Adjacent surface seawater samples were also collected from both sites. Samplings were conducted at low tide, and all the samples were collected in an autoclaved polycarbonate bottle (500 mL) filled with autoclaved, filtered (0.7 μm GF/F) seawater. All the samples were immediately transported to the laboratory and processed on the same day.

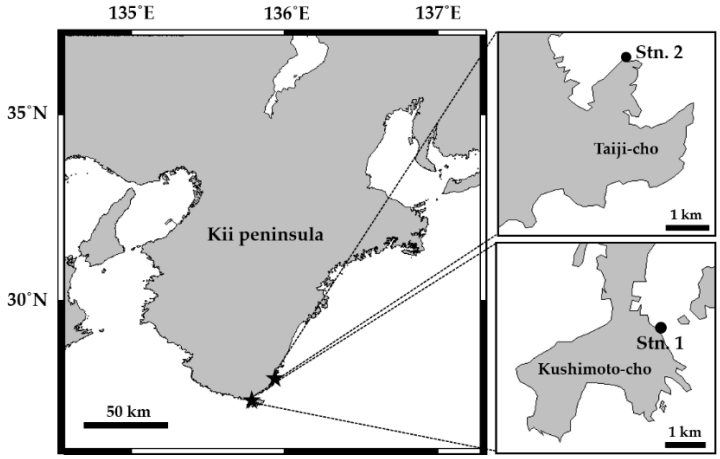


**Figure 1.** Location of the installation of artificial *Ulva* bed (Stn. 1) and the natural macroalgal bed (Stn. 2) at Kushimoto-cho and Taiji-cho, Wakayama Prefecture, Japan.

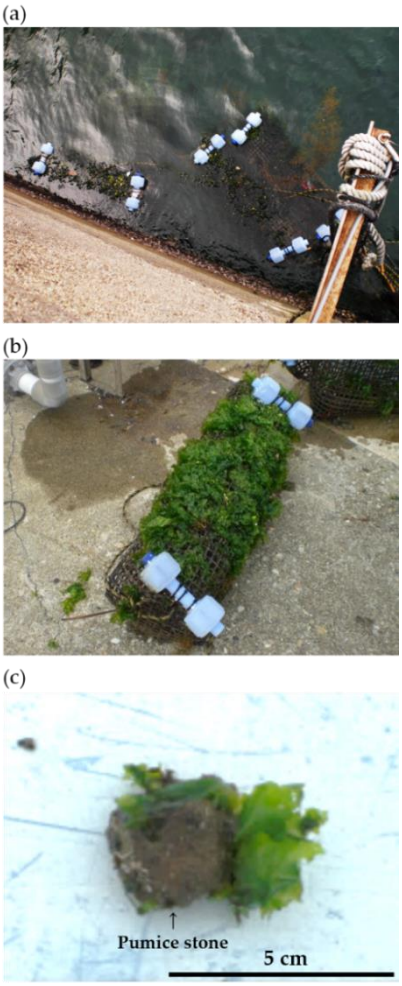


**Figure 2.** Pictures of artificially introduced (**a**) *Ulva* beds using floating cages at Stn. 1; (**b**) a landed floating cage *U. pertusa* is well grown; (**c**) *U. pertusa* grew on the pumice stone.

### *2.3. Sample Processing and Bacterial Culturing*

Macroalgal samples using bottles filled with 200 mL autoclaved filtered seawater were shaken 600 times by hand using a hand tally counter to detach a biofilm formed on the surface of the macroalgae.

Biofilm-suspended samples were serially diluted 10-fold to $10^{-4}$ with autoclaved filtered seawater, and an aliquot (0.1 mL) of each dilution was spread onto a $ST10^{-1}$ marine agar plate (Trypticase peptone 0.5 g, Yeast extract 0.05 g and agar 15 g in 1 L seawater [43]). The adjacent seawater, also collected from both sites, were serially diluted 10-fold to $10^{-4}$, and an aliquot (1 mL) was filtered through autoclaved 3 µm pore size Nucleopore Track-Etch membrane filters (Whatman, GE Healthcare, Parramatta, Australia). The filter was gently placed on a $ST10^{-1}$ marine agar plate to culture the particle-associated bacteria (PAB). An aliquot of 0.1 mL from the filtrate was also used to culture the free-living bacteria (FLB) on the same medium above [44]. All the plates were kept for two weeks in the dark at 20 °C and used for the culturable bacterial enumeration and isolation. For the isolation, colonies were randomly picked by sterilized toothpicks from the agar plate, inoculated onto a 48-well filled with the same bacterial medium and kept at 20 °C in the dark until the following co-culture experiment. Aliquots (10 mL) from all samples were fixed with glutaraldehyde (1% final concentration) and stored at 4 °C for later determination of total bacterial abundance by DAPI staining [45].

### 2.4. Targeted Harmful Algal Bloom (HAB) Species

Two raphidophycean flagellates, *Chattonella antiqua* NIES-1 and *Heterosigma akashiwo* 893, two armored dinoflagellates, *Alexandrium catenella,* and *Heterocapsa circularisquama* and one unarmored dinoflagellate, *Karenia mikimotoi*, were axenically grown and kept in modified SWM-3 medium [46,47]. The cultured temperature was 15 °C for *A. catenella*, 20 °C for *H. akashiwo* and 25 °C for *C. antiqua*, *H. circularisquama* and *K. mikimotoi* under a light intensity of 50–100 µmol photons $m^{-2}$ $s^{-1}$ using a 14:10 h light–dark photocycle in algal growth chambers. Early stationary growth phase cultures were used for following co-culture experiments to test bacterial growth inhibitory activities.

### 2.5. Co-Culture Experiment Using Bacterial Isolates

Cultured bacteria isolated from the biofilm of *U. pertusa* grown on the pumice stone and floating cage (Stn. 1) and four macroalgal species (Stn. 2), as well as PAB and FLB from the surrounding seawater at both sites, had their growth inhibitory properties determined through co-culturing experiments (a single axenic HAB species cultured in the algal medium with a single bacterium) toward five targeted HAB species [37]. Cell densities were diluted with the same algal medium to approximately $10^3$ cells $mL^{-1}$ for *C. antiqua*, *A. catenella*, and *K. mikimotoi* and $10^4$ cells $mL^{-1}$ for *H. akashiwo* and *H. circularisquama*, then 0.8 mL aliquots were incubated for two days in sterilized disposable 48-well microplates to check for any abnormal appearance of cells. Individual bacterial isolates were inoculated into the algal cultures with an approximate density of $10^5$ cells $mL^{-1}$, and the plates were incubated for another two weeks with the conditions described above for the algal cultures. Bacteria-free quadruplicate wells (Controls) were also prepared in each microplate. A Nikon ECLIPSE TE200 inverted microscope was used for daily checks of algal cell mortality and/or any abnormalities of algal cells. The wells with 90% or more of algal cells killed were labeled as algicidal bacteria (AB), and the wells with 90% or more of motility reduced algal cells simultaneously were labeled as growth-inhibiting bacteria (GIB). In this study, the term algal growth-limiting bacteria (GLB) was mainly applied to express a set of bacteria limiting algal growth, including both AB and GIB, to eliminate repeated usage of those terms. The density of AB and GIB was estimated using the following formula: NAG = NV × SAG/NT (NAG is the number of AB or GIB (CFU $mL^{-1}$ or CFU $g^{-1}$ wet weight of macroalgae), NV is the number of viable bacteria (CFU $mL^{-1}$ or CFU $g^{-1}$ wet weight of macroalgae), SAG is the number of bacterial strains that expressed growth inhibitory properties, NT is the number of bacterial strains examined). All of the GLB were isolated aseptically into a 48-well plate after confirming a pure culture by the streak plate method [48].

### 2.6. 16S rRNA Gene Sequencing for Identification

A total of 47 bacterial strains (12 strains from Stn. 1 and 35 strains from Stn. 2) were identified by partial 16S rRNA gene sequencing (500 bp). All strains were cultured in $ST10^{-1}$ liquid medium for two

weeks then centrifuged (8000 rpm) for 5 min to obtain bacterial pellets. Pellets were suspended in phosphate-buffered saline and washed three times to remove the medium. Bacterial DNA was extracted using NucleoSpin Tissue XS (TaKaRa BIO Inc., Japan) and stored at −20 °C. The universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′ [49]) and 519R (5′-GWATTACCGCGGCKGCTG-3′ [50]) were applied to amplify the 16S rRNA gene fragments using Blend Taq®-Plus- (TOYOBO, Japan). The PCR temperature cycling conditions were: initial denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min; final elongation was for 7 min at 72 °C. PCR products were purified using ISOSPIN PCR Product (NIPPON GENE CO., LTD., Japan) before sequencing. Both strands of the PCR products were sequenced using the same primers in a cycle sequencing reaction using a sequencing kit (Big Dye Terminator Cycle version 3.1, Applied Biosystems, USA) using a DNA sequencer (ABI 3130, Applied Biosystems, USA). Nucleotide sequences obtained were aligned using Chromas Pro (ver. 1.7.1). Approximately 500 bp of each consensus sequence were analyzed by A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) sequence similarity search to identify similarity with previously reported bacteria.

## 3. Results

### 3.1. Culturable and Total Bacterial Enumeration

The densities of culturable and total bacteria from seawater and macroalgal biofilms are shown in Table 1 and Figure 3. The densities of culturable free-living bacteria (FLB) at both sites were higher than particle-associated bacteria (PAB) with values of $1.7 \times 10^3$ CFU $mL^{-1}$ at Stn. 1 and $2.3 \times 10^3$ CFU $mL^{-1}$ at Stn. 2, respectively (Figure 3a). The density of culturable *Ulva pertusa* biofilm-associated bacteria on the pumice stone ($2.4 \times 10^8$ CFU $g^{-1}$ wet weight) was about ten times higher than *U. pertusa* collected from the floating cage ($1.9 \times 10^7$ CFU $g^{-1}$ wet weight) (Figure 3b). *Sargassum dupulicatum* associated culturable bacteria showed the lowest density with $7.9 \times 10^6$ CFU $g^{-1}$ wet weight, and the density of *U. pertusa*-associated culturable bacteria was the highest with $4.0 \times 10^7$ CFU $g^{-1}$ wet weight at natural macroalgal bed (Stn. 2, Figure 3b). The density of total bacteria in seawater at Stn. 1 was $2.9 \times 10^6$ cells $mL^{-1}$ (PAB: $2.6 \times 10^6$ cells $mL^{-1}$, FLB: $3.1 \times 10^5$ cells $mL^{-1}$) higher than the density of $6.7 \times 10^5$ cells $mL^{-1}$ (PAB: $3.9 \times 10^5$ cells $mL^{-1}$, FLB: $2.8 \times 10^5$ cells $mL^{-1}$) at Stn.2 (Figure 3c). In contrast to the culturable bacterial densities, the total PAB densities were higher than those of FLB at both sites (Figure 3a,c). The total bacterial density of *U. pertusa* on the pumice stone showed the maximum value of $2.7 \times 10^9$ cells $g^{-1}$ wet weight at Stn. 1 (Figure 3d) as well as culturable bacteria. Total bacterial density of *U. pertusa* was the highest with the density of $1.2 \times 10^9$ cells $g^{-1}$ wet weight at Stn.2 as well, followed by *Cladophora ohkuboana* ($9.9 \times 10^8$ cells $g^{-1}$ wet weight), *Gelidium elegans* ($6.0 \times 10^8$ cells $g^{-1}$ wet weight) and *S. dupulicatum* ($3.9 \times 10^8$ cells $g^{-1}$ wet weight) (Figure 3d). *U. pertusa* grown on the pumice stone appeared to be unhealthy, turning partly black in decay, and resulted in the highest values of both the densities of culturable and total bacteria at Stn. 1.

**Table 1.** Summary of culturable and total bacterial densities enumerated from seawater and macroalgal biofilms at Stns. 1 and 2. (FLB: free-living bacteria, PAB: particle-associated bacteria)

| Date | Station | Sample Name | Sample Type | Culturable Bacterial Density (CFU $mL^{-1}$ or $g^{-1}$ Wet Weight) | Total Bacterial Density (Cells $mL^{-1}$ or $g^{-1}$ Wet Weight) |
|---|---|---|---|---|---|
| 2017/9/7 | Stn. 1 | Seawater | FLB | $1.7 \times 10^3$ | $3.1 \times 10^5$ |
| | | | PAB | $1.0 \times 10^3$ | $2.6 \times 10^6$ |
| | | *Ulva pertusa* on pumice stone | Biofilm | $2.4 \times 10^8$ | $2.7 \times 10^9$ |
| | | *U. pertusa* on floating cage | Biofilm | $1.9 \times 10^7$ | $1.7 \times 10^9$ |
| 2017/9/8 | Stn. 2 | Seawater | FLB | $2.3 \times 10^3$ | $2.8 \times 10^5$ |
| | | | PAB | $1.8 \times 10^3$ | $3.9 \times 10^5$ |
| | | *Sargassum dupulicatum* | Biofilm | $7.9 \times 10^6$ | $3.9 \times 10^8$ |
| | | *Gelidium elegans* | Biofilm | $1.5 \times 10^7$ | $6.0 \times 10^8$ |
| | | *U. pertusa* | Biofilm | $4.0 \times 10^7$ | $1.2 \times 10^9$ |
| | | *Cladophora ohkuboana* | Biofilm | $2.0 \times 10^7$ | $9.9 \times 10^8$ |

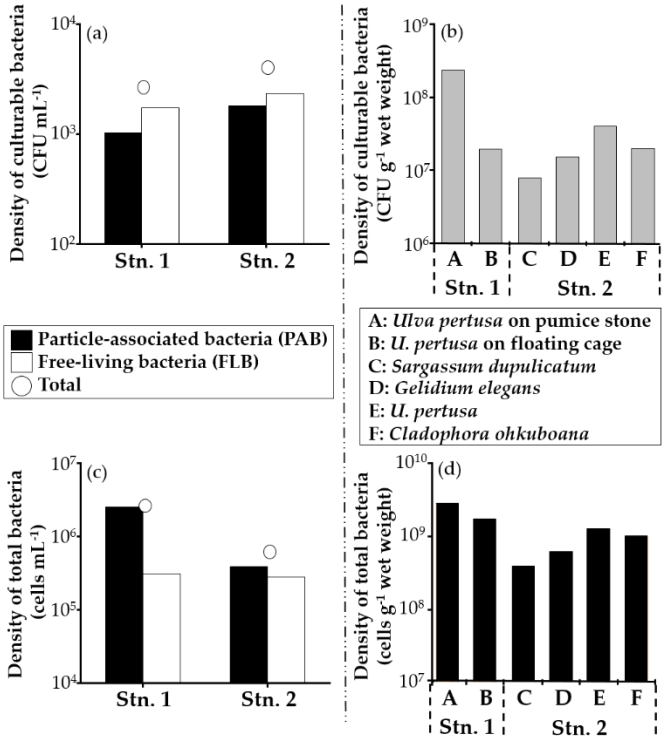


**Figure 3.** The densities of culturable bacteria (**a**): seawater, (**b**): macroalgal biofilms and total bacteria directly counted with DAPI staining (**c**): seawater, (**d**): macroalgal biofilms and at Stns. 1 and 2. Total (○) shown on (**a**) and (**c**) are expressed as the sum of particle-associated bacteria (PAB) and free-living bacteria (FLB). A to F are macroalgal samples used to enumerate the densities.

### *3.2. Density of Growth-Limiting Bacteria*

The densities of growth-limiting bacteria (GLB: the sum of algicidal bacteria (AB) and growth-inhibiting bacteria (GIB)) detected from seawater, and macroalgal samples at Stns.1 and 2, were shown in Table 2 and Figures 4 and 5. GLB targetting at least one HAB species were detected both in seawater and *U. pertusa* samples collected at Stn. 1 (Figure 4). The densities of GLB showed that activities against *Chattonella antiqua* were $3.5 \times 10^2$ CFU mL$^{-1}$ in FLB and $1.9 \times 10^2$ CFU mL$^{-1}$ in PAB (Figure 4a,b). GLB against *Heterocapsa circularisquama* and *Karenia mikimotoi* were detected only in PAB with densities of $1.9 \times 10^2$ CFU mL$^{-1}$ and 93 CFU mL$^{-1}$, respectively (Figure 4b). AB targeted *C. antiqua,* and *H. circularisquama* were detected from *U. pertusa* on the pumice stone with the same density of $5.9 \times 10^6$ CFU g$^{-1}$ wet weight (Figure 4c). GIB targeted *H. circularisquama* was detected from *U. pertusa* on the floating cage at a concentration of $1.5 \times 10^6$ CFU g$^{-1}$ wet weight (Figure 4d). There were no GLB negatively affecting the growth of *Alexandrium catenella* and *Heterosigma akashiwo* at Stn. 1. GLB targeting at least one HAB species were also detected from all samples collected at Stn. 2 (Figure 5). The density of GLB showed that the activity against *H. circularisquama* was detected in FLB with a density of 69 CFU mL$^{-1}$ (Figure 5a). GLB isolated from PAB at Stn. 2 had a wide range of activities affecting four HAB species except for *A. catenella* with densities ranging between $1.1 \times 10^2$ CFU mL$^{-1}$ and $7.9 \times 10^2$ CFU mL$^{-1}$ (Figure 5b). GLB affecting the growth of *A. catenella*, *H. circularisquama*, and *K. mikimotoi* were found from the *S. dupulicatum* biofilm with densities of $4.0 \times 10^5$ CFU g$^{-1}$ wet weight, $2.4 \times 10^6$ CFU g$^{-1}$ wet weight and $4.0 \times 10^5$ CFU g$^{-1}$ wet weight, respectively (Figure 5c). GLB affecting *C. antiqua,* and *H. circularisquama* were detected from *G. elegans* biofilm with densities of $7.6 \times 10^5$ CFU g$^{-1}$ wet weight and $1.5 \times 10^6$ CFU g$^{-1}$ wet weight, respectively (Figure 5d). GLB isolated from *C. ohkuboana* targetted four HAB species, except *K. mikimotoi*, with densities of $1.0 \times 10^6$ CFU g$^{-1}$ wet weight for *A. catenella*, $5.0 \times 10^5$ CFU g$^{-1}$ wet weight for *C. antiqua*, $5.0 \times 10^5$ CFU g$^{-1}$ wet weight for *H. akashiwo*, and $4.0 \times 10^6$ CFU g$^{-1}$ wet weight for *H. circularisquama*,

respectively (Figure 5e). GLB were found in *U. pertusa* with densities of $3.0 \times 10^6$ CFU $g^{-1}$ wet weight for *A. catenella*, $6.0 \times 10^6$ CFU $g^{-1}$ wet weight for *H. circularisquama* and $2.0 \times 10^6$ CFU $g^{-1}$ wet weight for *K. mikimotoi*, respectively (Figure 5f). GLB negatively affecting the growth of all five HAB species were detected at the natural macroalgal bed (Stn. 2).

**Table 2.** Summary of growth-limiting bacteria (GLB) densities against five different harmful algal bloom (HAB) species estimated through co-culture experiments from seawater and macroalgal biofilms at Stns. 1 and 2.

| Date | Station | Sample Name | Sample Type | The Density of GLB against Five Different HAB Species (CFU $mL^{-1}$ or $g^{-1}$ Wet Weight) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | *Alexandrium catenella* | *Chattonella antiqua* | *Heterosigma akashiwo* | *Heterocapsa circularisquama* | *Karenia mikimotoi* |
| 2017/9/7 | Stn. 1 | Seawater | FLB | — | $3.5 \times 10^2$ | — | — | — |
| | | | PAB | — | $1.9 \times 10^2$ | — | $1.9 \times 10^2$ | 93 |
| | | *Ulva pertusa* on pumice stone | Biofilm | — | $5.9 \times 10^6$ | — | $5.9 \times 10^6$ | — |
| | | *U. pertusa* on floating cage | Biofilm | — | — | — | $1.5 \times 10^6$ | — |
| 2017/9/8 | Stn. 2 | Seawater | FLB | — | — | — | 69 | — |
| | | | PAB | — | $3.4 \times 10^2$ | $1.1 \times 10^2$ | $7.9 \times 10^2$ | $2.3 \times 10^2$ |
| | | *Sargassum dupulicatum* | Biofilm | $4.0 \times 10^5$ | — | — | $2.4 \times 10^6$ | $4.0 \times 10^5$ |
| | | *Gelidium elegans* | Biofilm | — | $7.6 \times 10^5$ | — | $1.5 \times 10^6$ | — |
| | | *Cladophora ohkuboana* | Biofilm | $1.0 \times 10^6$ | $5.0 \times 10^5$ | $5.0 \times 10^5$ | $4.0 \times 10^6$ | — |
| | | *U. pertusa* | Biofilm | $3.0 \times 10^6$ | — | — | $6.0 \times 10^6$ | $2.0 \times 10^6$ |

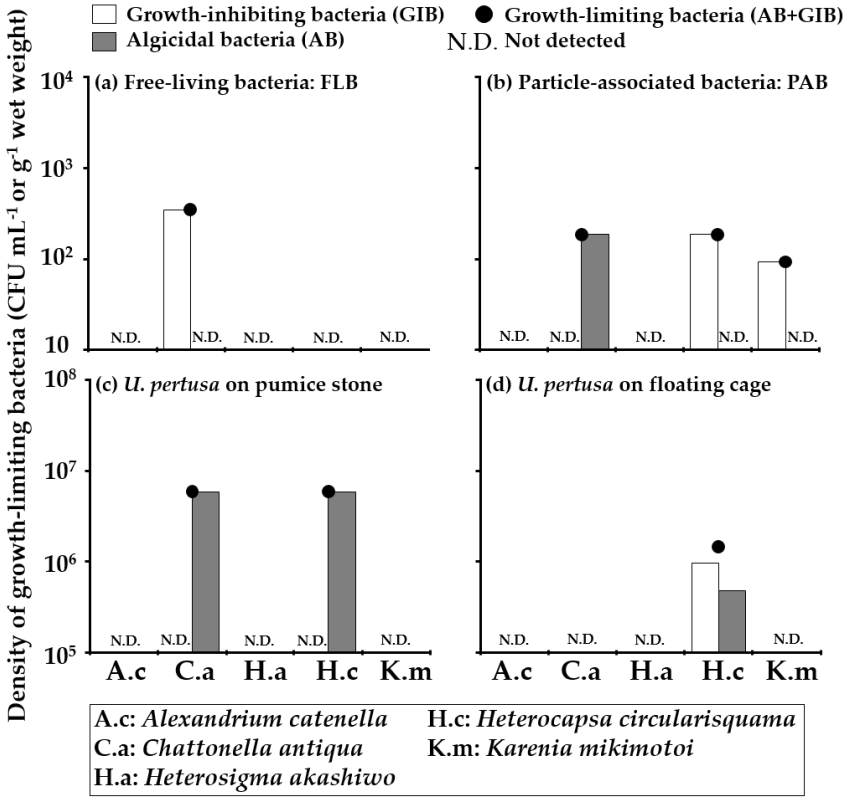


**Figure 4.** The densities of growth-limiting bacteria detected from seawater (**a**): FLB, (**b**): PAB and *U. pertusa* biofilms (**c**): on pumice stone, (**d**): on floating cage collected at Stn.1. The densities of growth-limiting bacteria (•) are expressed as the sum of algicidal bacteria (AB: ■) and growth-inhibiting bacteria (GIB: □). The tested HAB species and their abbreviations are displayed at the bottom.

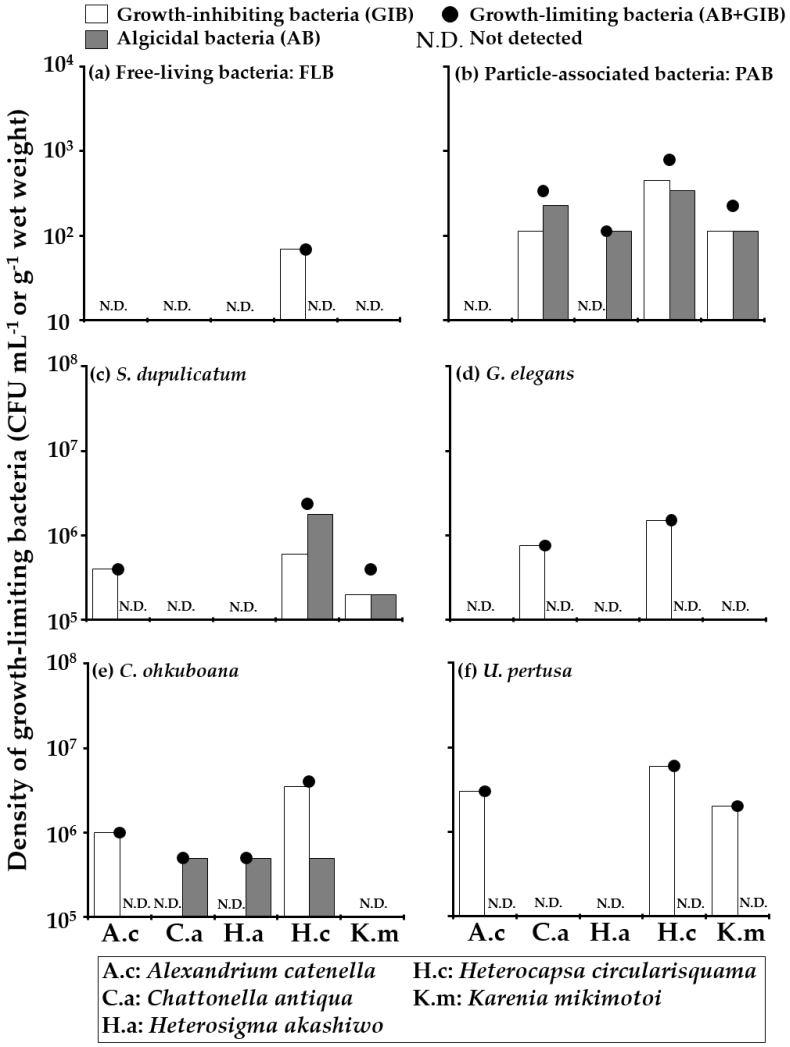


**Figure 5.** The densities of growth-limiting bacteria detected from seawater (**a**): FLB, (**b**): PAB and four macroalgal species (**c**): *S. dupulicatum*, (**d**): *G. elegans*, (**e**): *C. ohkuboana* and (**f**): *U. pertusa* collected at Stn.2. The densities of growth-limiting bacteria (•) are expressed as the sum of algicidal bacteria (AB: ■) and growth-inhibiting bacteria (GIB: □). The tested HAB species and their abbreviations are displayed at the bottom.

### 3.3. Composition of Growth-Limiting Bacteria

The genus and class of GLB identified from different seawater fractions (FLB and PAB), and each of the host macroalgae at both sites are shown in Figure 6. All of the GLB isolated from seawater and macroalgal biofilms were comprised of the classes *Alphaproteobacteria*, *Gammaproteobacteria*, and *Flavobacteria* at Stns. 1 and 2. Seven GLB isolated from seawater at Stn. 1 were classified as the genera of *Nautella* (*Alphaproteobacteria*) in FLB, *Alteromonas*, *Pseudoalteromonas* (*Gammaproteobacteria*) and *Tenacibaculum* (*Flavobacteria*) in PAB (Figure 6a). In total, five GLB isolated from *U. pertusa* grown on the pumice stone and the floating cage at Stn. 1 were found to belong to the genera of *Pseudoalteromonas*, *Vibrio* (*Gammaproteobacteria*) and *Aquimarina* (*Flavobacteria*), respectively (Figure 6b,c). The eight GLB detected in seawater at Stn. 2 were one *Winogradskyella* (*Flavobacteria*) as FLB, *Roseobacter* (*Alphaproteobacteria*), *Alteromonas*, *Pseudoalteromonas* and *Vibrio* (*Gammaproteobacteria*) and *Aquimarina*, *Winogradskyella* (*Flavobacteria*) as PAB (Figure 6d). The twenty-seven GLB strains isolated from four macroalgal biofilms consisted of two genera from *Alphaproteobacteria*, *Phaeobacter*, and *Roseovarius*, and three genera from *Gammaproteobacteria*, *Alteromonas*, *Pseudoalteromonas*, and *Vibrio* plus three genera

of *Flavobacteria*, *Aquimarina*, *Dokdonia* and *Winogradskyella* (Figure 6e–h). About 67% of GLB found from four macroalgal biofilms at the natural bed (Stn. 2) were the bacteria classified into the class *Flavobacteria*. *Aquimarina* was the only GLB found from all four macroalgae at the natural bed (Stn. 2).

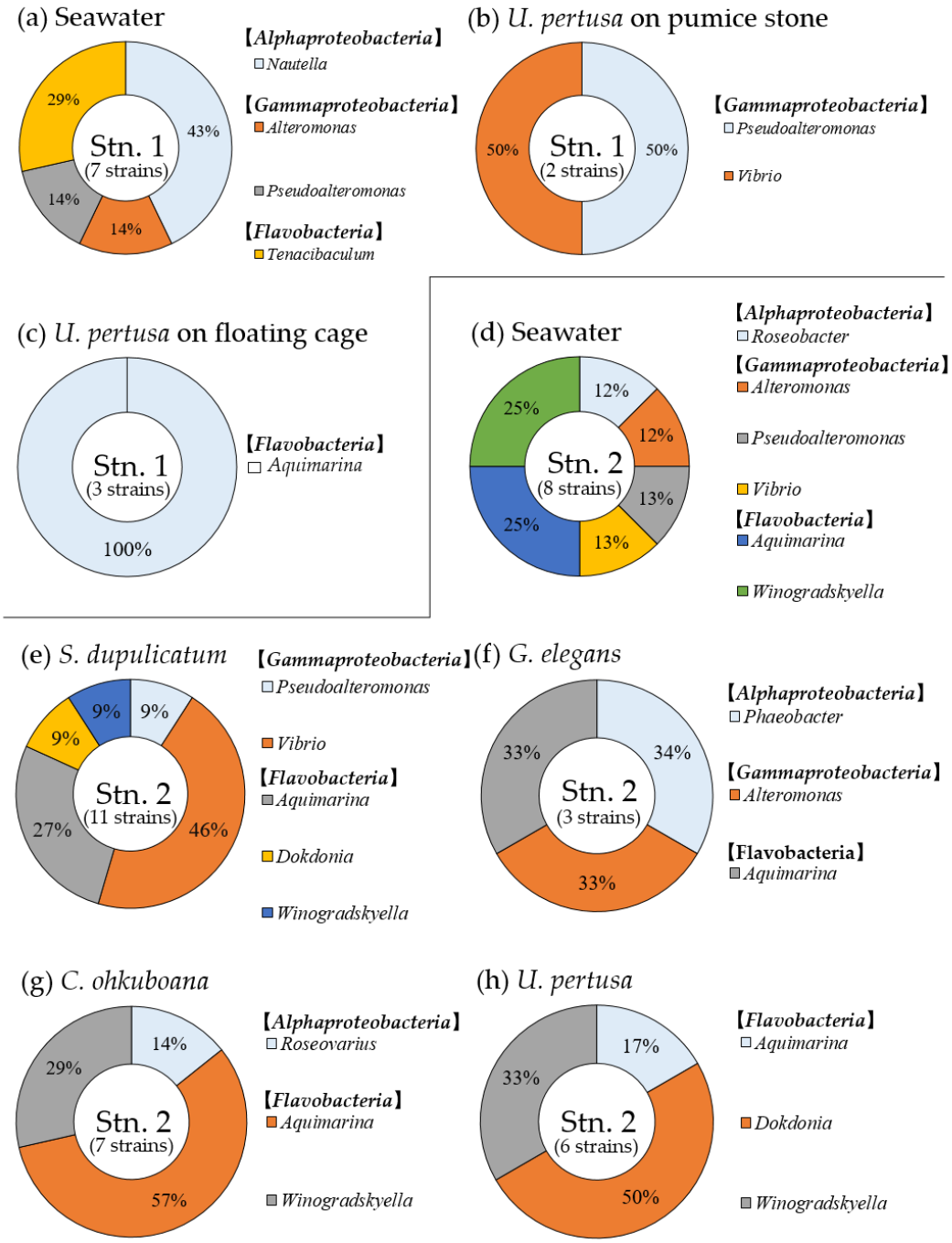


**Figure 6.** The composition of growth-limiting bacteria (class and genus) isolated from seawater and the different host macroalgae at Stn. 1 and 2. (Stn. 1: surface seawater (**a**), *Ulva pertusa* on pumice stone (**b**), *U. pertusa* on floating cage (**c**), Stn. 2: surface seawater (**d**), *Sargassum dupulicatum* (**e**), *Gelidium elegans* (**f**), *Cladophora ohkuboana* (**g**), *U. pertusa* (**h**)).The number in parenthesis shows the number of strains used for 16S rRNA gene sequencing for identification.

## 4. Discussion

Recent findings of growth-limiting bacteria densely living in a biofilm of seagrass and macroalgae have offered an insight into possible future HAB control by harnessing ecosystem services [51]. Meanwhile, the loss of seagrass and macroalgal beds due to human impacts have been accelerated over the last half-century [52–54]. Such deterioration of coastal vegetation coincided with the increasing occurrences of HAB [51,55]. The increasing toxic blooms of the dinoflagellate *A. minutum* accompanied by the large-scale decline of seagrass beds around the Mediterranean coast is one of the specific cases [56]. Inaba et al. [37] documented that *H. akashiwo* cysts were found only from the sediments where eelgrass had disappeared and studied sites where the abundant growth-limiting bacteria detected rarely experienced blooms of paralytic shellfish toxin-producing dinoflagellate *Alexandrium* in Puget Sound, WA, USA. On the positive side, the efforts to protect and restore seagrass and macroalgal beds are underway worldwide [57,58]; for instance, vigorous restoration activities of seagrass bed in Hinase, Okayama, Japan increased the natural bed more than twenty times since 1985 [57]. The efforts will enhance the resistance of coastal areas against HAB events in the long run. However, this holistic approach to prevent HABs may not be as applicable as direct controls, such as the use of flocculants [59]. Thus, it is crucial to develop an environmentally friendly measure that can be widely applicable and cost-effective.

*Ulva* can be attractive candidate organisms revealed to harbor GLB [34,37], growing fast compared to other macroalgae or seagrass [60,61], relatively easy to culture [62], restoring water quality through the uptake of excess nutrients [63,64], releasing allelochemicals negatively affecting the growth of HAB species [65,66], and having commercial importance in that they are frequently consumed under the name "Aonori" in Asia [67,68]. Several *Ulva* species have already been tested to be organisms composing large scale integrated multi-trophic aquaculture (IMTA) systems [67,69]. In the present study, harmful algal growth-limiting bacteria (GLB) were found in the order of $10^6$ CFU per 1 g of *U. pertusa* grown on the pumice stone and the mobile floating cage at Stn. 1, showing the equivalent value to the natural growing macroalgal species at Stn. 2 as well as previous studies estimating the densities of GLB on *Ulva* sp. and *Gelidium* sp. in Osaka Bay, Japan [34] and on *U. lactuca* in Puget Sound, USA [37]. To our best knowledge, this is the first report to reveal that algal growth-limiting bacteria can be harbored within a few months on the newly introduced *Ulva* thallus, suggesting the possible future field application to control HABs by introducing *Ulva* beds as a source of GLB.

Previous studies have shown that a higher proportion of GLB was often associated with particles [28,44]. In the study in Puget Sound, most isolated bacteria from seawater having more extensive activity ranges were in the particle-associated form [37]. In the present study, the density of PAB (Particle-associated bacteria) as GLB was higher than FLB (Free-living bacteria) at the natural macroalgal bed (Stn. 2), killing most of the tested HAB species except *A. catenella* (Figure 5b). Moreover, 88% of GLB detected from seawater at the natural macroalgal bed was the same bacterial genera as GLB detected from macroalgal biofilms (Figure 6). It is known that macroalgal beds are one of the essential sources releasing particulate and dissolved organic matter (POM and DOM) to the surrounding environments [70–72]. The POM derived from the macroalgae at Stn. 2 may have increased substrate availability for specific bacterial communities with high exoenzymatic activities [73–75] that resulted in frequent detection of GLB as PAB forms in the water. This could be reasonably explained by highly dense GLB harbored on a macroalgal surface being provided synchronously with macroalgal-derived POM to the surrounding water.

Many bacteria previously reported as algicidal and/or growth-inhibiting bacteria were among the *Cytophaga/Flavobacterium/Bacteroidetes* (CFB) group [27]. They are also critical players for the degradation of organic matter in aquatic environments [76]. Ecologically important roles toward macroalgae of the CFB group have been demonstrated, such as controlling morphogenesis [77,78], promotion and/or inhibition of spore germination, and colonization [79,80], close association with diseases [81], etc. In the present study, more than half of GLB were identified as bacteria belonging to the class *Flavobacteria*. Notably, the genus *Aquimarina* within the class *Flavobacteria* were found

from all of the macroalgae and 25% of GLB in seawater samples at Stn. 2 (Figure 6). With regard to the growth-limiting activity range, all GLB isolated from seawater and *Ulva* samples at Stn. 1 were found to exhibit specific activities in that 92% of GLB negatively affected the growth of only one HAB species (*C. antiqua* or *H. circularisquama*) (Figure 7a); however, GLB isolated from natural macroalgae bed showed a much broader range of activities on tested HAB species (Figure 7b). About 90% of GLB affecting the growth of more than one HAB species were found to be *Flavobacteria*, which consisted of *Aquimarina* and *Dokdonia* (Figure 7b). *Aquimarina* isolated from red alga on the coast of China is shown to exhibit diverse agarase activities, enhancing the degradation of host algal tissue [82]. These results suggest the multiple roles of the members of *Flavobacteria* diffusing around macroalgal beds. The physiological and biochemical characteristics of macroalgae predetermine the composition of their biofilm microbial communities [83], and the host species-specific microbial association in aquatic angiosperms is known [84]. It is worth noting that natural macroalgal beds with higher diversity may offer various inhabiting substrates for diverse GLB, functioning effectively as bloom control for multiple harmful algal species (Figure 7b).

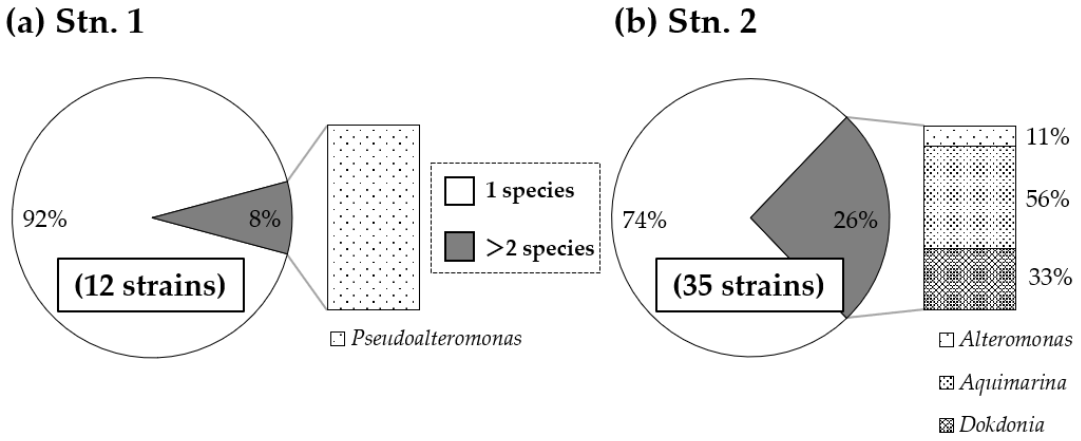


**Figure 7.** The growth-limiting activity ranges of GLB against all five harmful algal species (*A. catenella*, *C. antiqua*, *H. akashiwo*, *H. circularisquama*, and *K. mikimotoi*) isolated from (**a**) Stn. 1 and (**b**) Stn. 2. It includes all GLB strains isolated from seawater and macroalgal samples at each site. Total strains tested are shown in parenthesis in the lower center of the pie chart.

## 5. Conclusions

The present study illustrated that GLB with equivalent value as naturally growing macroalgae could be harbored on the surface of artificially introduced *U. pertusa* for less than three months, providing novel insight into the potential use of the artificial introduction of *U. pertusa* beds as biological control of HABs. Results from the natural macroalgal bed suggested that higher macroalgal diversity provide diverse inhabiting substrates for GLB, functioning effectively as bloom control for multiple harmful algal species. It should be also emphasized that the protection and restoration of natural macroalgal beds may serve as not only critical habitats for numerous marine and estuary organisms but also one of the original hotspots of GLB, contributing to mitigating HABs in coastal environments. Further investigations to understand the diffusive behavior of GLB are needed to achieve the practical application.

**Author Contributions:** All authors contributed the project. N.I. wrote the manuscript. I.K. supported the sampling and performed the algal and part of the molecular analyses. S.N. supported molecular analyses. T.S. supported sampling and zoospore preparation. N.I., K.M., A.Y. and I.I. conceived and designed the experiments. All co-authors participated in discussions and review of the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported financially in part by a Grant-in-Aid for Scientific Research grants 18K14506 (Early Career Scientists) from the Japanese Society for the Promotion of Science (JSPS) and a Grant-in-Aid

(Technological developments for characterization of harmful plankton in the seawater: No. 16808839) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

**Acknowledgments:** We would like to show sincere thanks to the members of Fisheries Engineering Research Team, Civil Engineering Research Institute for Cold Region (CERI), Japan for administrative support, especially, Sawako Shirai, technical assistant. We also express our gratitude to Masaru Okamoto, Shinkotec co., ltd for the suggestions on pumice stone.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Trainer, V.L.; Eberhart, B.-T.L.; Wekell, J.C.; Adams, N.G.; Hanson, L.; Cox, F.; Dowell, J. Paralytic shellfish toxins in Puget Sound, Washington State. *J. Shellfish Res.* **2003**, *22*, 213–223.
2. Trainer, V.L.; Bates, S.S.; Lundholm, N.; Thessen, A.E.; Cochlan, W.P.; Adams, N.G.; Trick, C.G. *Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring and impacts on ecosystem health. *Harmful Algae* **2012**, *14*, 271–300. [CrossRef]
3. Imai, I.; Yamaguchi, M. Life cycle, physiology, ecology and red tide occurrences of the fish-killing raphidophyte *Chattonella*. *Harmful Algae* **2012**, *14*, 46–70. [CrossRef]
4. Anderson, D.M.; Cembella, A.D.; Hallegraeff, G.M. Progress in understanding harmful algal blooms: Paradigm shifts and new technologies for research, monitoring, and management. *Annu. Rev. Mar. Sci.* **2012**, *4*, 143–176. [CrossRef]
5. Wells, M.L.; Trainer, V.L.; Smayda, T.J.; Karlson, B.S.O.; Trick, C.G.; Kudela, R.M.; Ishikawa, A.; Bernard, S.; Wulff, A.; Anderson, D.M.; et al. Harmful algal blooms and climate change: Learning from the past and present to forecast the future. *Harmful Algae* **2015**, *49*, 68–93. [CrossRef]
6. Gobler, C.J.; Doherty, O.M.; Hattenrath-Lehmann, T.K.; Griffith, A.W.; Kang, Y.; Litaker, R.W. Ocean warming since 1982 has expanded the niche of toxic algal blooms in the North Atlantic and North Pacific oceans. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 4975–4980. [CrossRef]
7. Gobler, C.J. Climate change and harmful algal blooms: Insights and perspective. *Harmful Algae* **2020**, *91*, 101731. [CrossRef]
8. Seger, A.; Park, T.G.; Hallegraeff, G. Assessment of the efficacy of clay flocculation in Korean fish farm waters: *Cochlodinium* cell removal and mitigation of ichthyotoxicity. *Harmful Algae* **2017**, *61*, 46–55. [CrossRef]
9. Archambault, M.-C.; Bricelj, V.M.; Grant, J.; Anderson, D.M. Effects of clay, used to control harmful algal blooms, on juvenile *Mercenaria mercenaria*. *J. Shellfish Res.* **2002**, *21*, 395–396.
10. Shumway, S.E.; Frank, D.M.; Ewart, L.M.; Ward, J.E. Effect of yellow loess on clearance rate in seven species of benthic, filter-feeding invertebrates. *Aquac. Res.* **2003**, *34*, 1391–1402. [CrossRef]
11. Sengco, M.R.; Anderson, D.M. Controlling harmful algal blooms through clay flocculation. *J. Eukaryot. Microbiol.* **2004**, *51*, 169–172. [CrossRef] [PubMed]
12. Seo, K.S.; Lee, C.K.; Park, Y.T.; Lee, Y. Effect of yellow clay on respiration and phytoplankton uptake of bivalves. *Fish. Sci.* **2008**, *74*, 120–127. [CrossRef]
13. Shirota, A. Red tide problem and countermeasures. *Int. J. Aquat. Fish. Technol.* **1989**, *1*, 195–223.
14. Matsuyama, Y.; Miyamoto, M.; Kotani, Y. Grazing impacts of the heterotrophic dinoflagellate *Polykrikos kofoidii* on a bloom of *Gymnodinium catenatum*. *Aquat. Microb. Ecol.* **1999**, *17*, 91–98. [CrossRef]
15. Sakata, T. Control of harmful microalgae by microorganisms. In *Mechanisms, Prediction, and Mitigation of Harmful Algal Blooms in Japan*; Ishida, Y., Honjo, T., Fukuyo, Y., Imai, I., Eds.; The Japan Fisheries Resource Conservation Association: Tokyo, Japan, 2000; pp. 215–235. (In Japanese)
16. Jeong, H.J.; Kim, J.S.; Yoo, Y.D.; Kim, S.T.; Kim, T.H.; Park, M.G.; Lee, C.H.; Seong, K.A.; Rang, N.S.; Shim, J.H. Feeding by the heterotrophic dinoflagellate *Oxyrrhis marina* on the red-tide raphidophyte *Heterosigma akashiwo*: A potential biological method to control red tides using mass-cultured grazers. *J. Eukaryot. Microbiol.* **2003**, *50*, 274–282. [CrossRef]
17. Brussaard, C.P.D. Viral control of phytoplankton populations—A review. *J. Eukaryot. Microbiol.* **2004**, *51*, 125–138. [CrossRef]
18. Park, M.G.; Yih, W.; Coats, D.W. Parasites and phytoplankton, with special emphasis on dinoflagellate infections. *J. Eukaryot. Microbiol.* **2004**, *51*, 145–155. [CrossRef]

19. Salomon, P.S.; Imai, I. Pathogens of harmful microalgae. In *Ecology of Harmful Algae, Ecological Studies Vol 189*; Granéli, E., Turner, J.T., Eds.; Springer: Berlin, Germany, 2006; pp. 271–282.
20. Imai, I.; Kakumu, A.; Ohara, S.; Yuki, T.; Koike, K.; Hagiwara, E.; Ogawa, K.; Yoneyama, H. Feasibility studies on sediment perturbation as control strategies for *Chattonella* red tides. *Bull. Fish. Sci. Hokkaido Univ.* **2017**, *67*, 57–66. [CrossRef]
21. Imai, I.; Kim, M.C.; Nagasaki, K.; Itakura, S.; Ishida, Y. Relationships between dynamics of red tide-causing raphidophycean flagellates and algicidal micro-organisms in the coastal sea of Japan. *Phycol. Res.* **1998**, *46*, 139–146. [CrossRef]
22. Imai, I.; Sunahara, T.; Nishikawa, T.; Hori, Y.; Kondo, R.; Hiroishi, S. Fluctuations of the red tide flagellates *Chattonella* spp. (Raphidophyceae) and the algicidal bacterium *Cytophaga* sp. in the Seto Inland Sea, Japan. *Mar. Biol.* **2001**, *138*, 1043–1049. [CrossRef]
23. Lovejoy, C.; Bowman, J.P.; Hallegraeff, G.M. Algicidal effects of a novel marine *Pseudoalteromonas* isolate (class proteobacteria gamma subdivision) on harmful algal bloom species of the genera *Chattonella*, *Gymnodinium* and *Heterosigma*. *Appl. Environ. Microbiol.* **1998**, *64*, 2806–2813. [CrossRef] [PubMed]
24. Doucette, G.J.; Kodama, M.; Franca, S.; Gallacher, S. Bacterial interactions with harmful algal bloom species: Bloom ecology, toxigenesis and cytology. In *Physiological Ecology of Harmful Algal Blooms, NATO ASI Series*; Anderson, D.M., Cembella, A.D., Hallegraeff, G.M., Eds.; Springer: Berlin, Germany, 1998; Volume G41, pp. 619–647.
25. Doucette, G.J.; McGovern, E.R.; Babinchak, J.A. Algicidal bacteria active against *Gymnodinium breve* (Dinophyceae). I. Bacterial isolation and characterization of killing activity. *J. Phycol.* **1999**, *35*, 1447–1454. [CrossRef]
26. Skerratt, J.H.; Bowman, J.P.; Hallegraeff, G.; James, S.; Nichols, P.D. Algicidal bacteria associated with blooms of a toxic dinoflagellate in a temperate Australian estuary. *Mar. Ecol. Prog. Ser.* **2002**, *244*, 1–15. [CrossRef]
27. Mayali, X.; Azam, F. Algicidal bacteria in the sea and their impact on algal blooms. *J. Eukaryot. Microbiol.* **2004**, *51*, 139–144. [CrossRef]
28. Park, J.H.; Yoshinaga, I.; Nishikawa, T.; Imai, I. Algicidal bacteria in particle-associated form and in free-living form during a diatom bloom in the Seto Inland Sea, Japan. *Aquat. Microb. Ecol.* **2010**, *60*, 151–161. [CrossRef]
29. Paul, C.; Pohnert, G. Interactions of the algicidal bacterium *Kordia algicida* with diatoms: Regulated protease excretion for specific algal lysis. *PLoS ONE* **2011**, *6*, e21032. [CrossRef]
30. Pokrzywinski, K.L.; Place, A.R.; Warner, M.E.; Coyne, K.J. Investigation of the algicidal exudate produced by *Shewanella* sp. IRI-160 and its effect on dinoflagellates. *Harmful Algae* **2012**, *19*, 23–29. [CrossRef]
31. Inaba, N.; Trainer, V.L.; Nagai, S.; Kojima, S.; Sakami, T.; Takagi, S.; Imai, I. Dynamics of seagrass bed microbial communities used to control artificial *Chattonella* blooms: A microcosm study. *Harmful Algae* **2019**, *84*, 139–150. [CrossRef] [PubMed]
32. Sanders, N.J.; Gotelli, N.J.; Heller, N.E.; Gordon, D.M. Community disassembly by an invasive species. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 2474–2477. [CrossRef] [PubMed]
33. Secord, D. Biological control of marine invasive species: Cautionary tales and land-based lessons. *Biol. Invasions* **2003**, *5*, 117–131. [CrossRef]
34. Imai, I.; Fujimaru, D.; Nishigaki, T. Co-culture of fish with macroalgae and associated bacteria: A possible mitigation strategy for noxious red tides in enclosed coastal sea. *Fish. Sci.* **2002**, *68* (Suppl. S1), 493–496. [CrossRef]
35. Imai, I.; Yamamoto, T.; Ishii, K.I.; Yamamoto, K. Promising prevention strategies for harmful red tides by seagrass beds as enormous sources of algicidal bacteria. In Proceedings of the 5th World Fisheries Congress, Tokyo, Japan, 13 December 2009. 6C_0995_133.
36. Onishi, Y.; Mohri, Y.; Tuji, A.; Ohgi, K.; Yamaguchi, A.; Imai, I. The seagrass *Zostera marina* harbors growth-inhibiting bacteria against the toxic dinoflagellate *Alexandrium tamarense*. *Fish. Sci.* **2014**, *80*, 353–362. [CrossRef]
37. Inaba, N.; Trainer, V.L.; Onishi, Y.; Ishii, K.; Wyllie-Echeverria, S.; Imai, I. Algicidal and growth-inhibiting bacteria associated with seagrass and macroalgae beds in Puget Sounds, WA, USA. *Harmful Algae* **2017**, *62*, 136–147. [CrossRef] [PubMed]
38. Sakami, T.; Sakamoto, S.; Takagi, S.; Inaba, N.; Imai, I. Distribution of three algicidal *Alteromonas* sp. strains in seagrass beds and surrounding areas in the Seto Inland Sea, Japan. *Fish. Sci.* **2017**, *83*, 113–121. [CrossRef]

39. Green, E.P.; Short, F.T. *World Atlas of Seagrasses*; University of California Press: Berkeley, CA, USA, 2003; p. 324.
40. Hurd, C.L.; Harrison, P.J.; Bischof, K.; Lobban, C.S. *Seaweed Ecology and Physiology*, 2nd ed.; Cambridge University Press: Cambridge, UK, 2014; p. 551. [CrossRef]
41. Yamada, Y.; Saito, E. *On Some Culture Experiments with the Swarmers of Certain Species belonging to the Ulvaceae*; Scientific Papers of the Institute of Algological Research; Faculty of Science, Hokkaido Imperial University: Hokkaido, Japan, 1938; Volume 2, pp. 35–51.
42. Mori, S.; Hidaka, K.; Ushirokawa, T. Aratana Kaiso No Saibyokishitsu No Kento. *Bull. Fukuoka Fish. Mar. Technol. Res. Cent.* **2017**, *27*, 19–26. (In Japanese)
43. Yoshinaga, I.; Kawai, T.; Ishida, Y. Analysis of algicidal ranges of the bacteria killing the marine dinoflagellates *Gymnodinium mikimotoi* isolated from Tanabe Bay, Wakayama Pref., Japan. *Fish. Sci.* **1997**, *63*, 94–98. [CrossRef]
44. Inaba, N.; Watanabe, T.; Sakami, T.; Nishi, H.; Tahara, Y.; Imai, I. Temporal and spatial distribution of algicidal and growth-inhibiting bacteria in the coastal sea of southwest Japan. *J. Plankton Res.* **2014**, *36*, 388–397. [CrossRef]
45. Porter, K.G.; Feig, Y.S. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **1980**, *25*, 943–948. [CrossRef]
46. Chen, L.C.M.; Edelstein, T.; McLachlan, J. *Bonnemaisonia hamifera* Hariot in nature and in culture. *J. Phycol.* **1969**, *5*, 211–220. [CrossRef]
47. Imai, I.; Itakura, S.; Matsuyama, Y.; Yamaguchi, M. Selenium requirement for growth of a novel red tide flagellate *Chattonella verruculosa* (Raphidophyceae) in culture. *Fish. Sci.* **1996**, *62*, 834–835. [CrossRef]
48. Holt, J.G.; Krieg, N.R. Enrichment and isolation. In *Methods for General and Molecular Bacteriology*; Gerhardt, P., Murray, R.G.E., Hood, W.A., Krieg, N.R., Eds.; ASM Press: Washington, DC, USA, 1994; pp. 179–215.
49. Delong, E.F. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5685–5689. [CrossRef] [PubMed]
50. Lane, D.J. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*; Stackebrandt, E., Goodfellow, M., Eds.; Jon Wiley & Sons: Chichester, UK, 1991; pp. 115–176.
51. Imai, I. Interactions between harmful algae and algicidal bacteria associated with seaweeds and seagrasses. In *Marine Protists*; Ohtsuka, S., Suzuki, N., Horiguchi, T., Eds.; Springer: Tokyo, Japan, 2015; pp. 597–619.
52. Middelboe, A.L.; Sand-Jensen, K. Long-term changes in macroalgal communities in a Danish estuary. *Phycologia* **2000**, *39*, 245–257. [CrossRef]
53. Waycott, M.; Duarte, C.M.; Carruthers, T.J.B.; Orth, R.J.; Dennison, W.C.; Olyarnik, S.; Calladine, A.; Fourqurean, J.W.; Heck, K.L., Jr.; Hughes, A.R.; et al. Accelerating loss of seagrass across the globe threatens coastal ecosystems. *Proc. Natl. Acad. Sci. USA* **2019**, *106*, 12377–12381. [CrossRef] [PubMed]
54. Filbee-Dexter, K.; Wernberg, T. Rise of turfs: A new battlefront for globally declining kelp forests. *Bioscience* **2018**, *68*, 64–76. [CrossRef]
55. Kemp, W.M.; Boynton, W.R.; Adolf, J.E.; Boesch, D.F.; Boicourt, W.C.; Brush, G.; Cornwell, J.C.; Fisher, T.R.; Glibert, P.M.; Hagy, J.D.; et al. Eutrophication of Chesapeake Bay: Historical trends and ecological interactions. *Mar. Ecol. Prog. Ser.* **2005**, *303*, 1–29. [CrossRef]
56. Abdenadher, M.; Hamza, A.; Fekih, W.; Hannachi, I.; Bellaaj, A.Z.; Bradai, M.N.; Aleya, L. Factors determining the dynamics of toxic blooms of *Alexandrium minutum* during a 10-year study along the shallow southwestern Mediterranean coasts. *Estuar. Coast. Shelf. Sci.* **2012**, *106*, 102–111. [CrossRef]
57. Tanaka, T.; Furukawa, K.; Kuwae, T.; Imai, I. Report of the 32nd Joint symposium of Laiaison council of Academic societies on coastal environment "Road of *Zostera* bed restoration in Japanese coastal zones" -Past processes and the future prospects. *Nippon Suisan Gakkaishi* **2017**, *83*, 1042–1053. [CrossRef]
58. Tamburello, L.; Papa, L.; Guarnieri, G.; Basconi, L.; Zampardi, S.; Scipione, M.B.; Terlizzi, A.; Zupo, V.; Fraschetti, S. Are we ready for scaling up restoration actions? An insight from Mediterranean macroalgal canopies. *PLoS ONE* **2019**, *14*, e0224477. [CrossRef]
59. Anderson, C.R.; Sellner, K.G.; Anderson, D.M. Bloom prevention and control. In *Harmful Algal Blooms (HABs) and Desalination: A Guide to Impacts, Monitoring, and Management*; Anderson, D.M., Boerlage, S.F.E., Dixon, M.B., Eds.; Intergovernmental Oceanographic Commission of UNESCO: Paris, France, 2017; pp. 205–222.
60. Pedersen, M.F.; Borum, J. Nutrient control of algal growth in estuarine waters: Nutrient limitation and the importance of nitrogen requirements and nitrogen storage among phytoplankton and species of macroalgae. *Mar. Ecol. Prog. Ser.* **1996**, *142*, 261–272. [CrossRef]

61. Pedersen, M.F.; Borum, J.; Fotel, F.L. Phosphorus dynamics and limitation of fast- and slow-growing temperate seaweeds in Oslofjord, Norway. *Mar. Ecol. Prog. Ser.* **2010**, *399*, 103–115. [CrossRef]
62. Hirata, H.; Kohirata, E.; Guo, F.; Xu, B.T.; Danakusumah, E. Culture of the sterile *Ulva* sp. (Chlorophyceae) in a mariculture farm. *Suisan Zoshoku* **1993**, *41*, 541–545.
63. Neori, A.; Krom, M.D.; Ellner, S.P.; Boyd, C.E.; Popper, D.; Rabinovitch, R.; Davison, P.J.; Dvir, O.; Zuber, D.; Ucko, M.; et al. Seaweed biofilters as regulators of water quality in integrated fish-seaweed culture units. *Aquaculture* **1996**, *141*, 183–199. [CrossRef]
64. Neori, A.; Chopin, T.; Troell, M.; Buschmann, A.H.; Kraemer, G.P.; Halling, C.; Shpigel, M.; Yarish, C. Integrated aquaculture: Rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. *Aquaculture* **2004**, *231*, 361–391. [CrossRef]
65. Wang, Y.; Yu, Z.; Song, X.; Tang, X.; Zhang, S. Effects of macroalgae *Ulva pertusa* (Chlorophyta) and *Gracilaria lemaneiformis* (Rhodophyta) on growth of four species of bloom-forming dinoflagellates. *Aquat. Bot.* **2007**, *86*, 139–147. [CrossRef]
66. Tang, Y.Z.; Gobler, C.J. The green macroalga, *Ulva lactuca*, inhibits the growth of seven common harmful algal bloom species via allelopathy. *Harmful Algae* **2011**, *10*, 480–488. [CrossRef]
67. Bolton, J.J.; Robertson-Andersson, D.V.; Shuuluka, D.; Kandjengo, L. Growing *Ulva* (Chlorophyta) in integrated systems as a commercial crop for abalone feed in South Africa: A SWOT analysis. *J. Appl. Phycol.* **2008**, *21*, 575–583. [CrossRef]
68. Kawashima, Y.; Akasaki, T.; Matsumoto, Y.; Yamazaki, Y.; Shimada, S. Species identification of imported and Japanese commercial green algal products based on phylogenetic analyses using the nrITS2 and 5S rDNA spacer regions. *Fish. Sci.* **2013**, *79*, 521–529. [CrossRef]
69. Msuya, F.E.; Kyewalyanga, M.S.; Salum, D. The performance of the seaweed *Ulva reticulata* as a biofilter in a low-tech, low-cost, gravity generated water regime in Zanzibar, Tanzania. *Aquaculture* **2006**, *254*, 284–292. [CrossRef]
70. Duggins, D.O.; Eckman, J.E.; Sewell, A.T. Ecology of understory kelp environments. II. Effects of kelps on recruitment of benthic invertebrates. *J. Exp. Mar. Biol. Ecol.* **1990**, *143*, 27–45. [CrossRef]
71. Leclerc, J.C.; Riera, P.; Leroux, C.; Lévêque, L.; Davoult, D. Temporal variation in organic matter supply in kelp forests: Linking structure to trophic functioning. *Mar. Ecol. Prog. Ser.* **2013**, *494*, 87–105. [CrossRef]
72. Campbell, I.; Macleod, A.; Sahlmann, C.; Neves, L.; Funderud, J.; Øverland, M.; Hughes, A.D.; Stanley, M.S. The environmental risks associated with the development of seaweed farming in Europe—prioritizing key knowledge gaps. *Front. Mar. Sci.* **2019**, *6*, 107. [CrossRef]
73. Smith, D.C.; Simon, M.; Alldredge, A.L.; Azam, F. Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. *Nature* **1992**, *359*, 139–142. [CrossRef]
74. Bidle, K.D.; Azam, F. Accelerated dissolution of diatom silica by marine bacterial assemblages. *Nature* **1999**, *397*, 508–512. [CrossRef]
75. Teeling, H.; Fuchs, B.; Becher, D.; Klockow, C.; Gardebrecht, A.; Bennke, C.; Kassabgy, M.; Huang, S.; Mann, A.; Waldmann, J.; et al. Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **2012**, *336*, 608–611. [CrossRef] [PubMed]
76. Kirchman, D. The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol. Ecol.* **2002**, *39*, 91–100. [CrossRef]
77. Matsuo, Y.; Imagawa, H.; Nishizawa, M.; Shizuri, Y. Isolation of an algal morphogenesis inducer from a marine bacterium. *Science* **2005**, *307*, 1598. [CrossRef] [PubMed]
78. Marshall, K.; Joint, I.; Callow, M.E. Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. *Microb. Ecol.* **2006**, *52*, 302–310. [CrossRef] [PubMed]
79. Patel, P.; Callow, M.E.; Joint, I.; Callow, J.A. Specificity in the settlement-modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha*. *Environ. Microbiol.* **2003**, *5*, 338–349. [CrossRef]
80. Matsuo, Y.; Suzuki, M.; Kasai, H.; Shizuri, Y.; Harayama, S. Isolation and phylogenetic characterization of bacteria capable of inducing differentiation in the green alga *Monostroma oxyspermum*. *Environ. Microbiol.* **2003**, *5*, 25–35. [CrossRef]
81. Zozaya-Valdes, E.; Egan, S.; Thomas, T. A comprehensive analysis of the microbial communities of healthy and diseased marine macroalgae and the detection of known and potential bacterial pathogens. *Front. Microbiol.* **2015**, *6*, 146. [CrossRef]

82. Lin, B.; Lu, G.; Zheng, Y.; Xie, W.; Li, S.; Hu, Z. *Aquimarina agarilytica* sp. nov., agarolytic species isolated from a red alga. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 869–873. [CrossRef] [PubMed]
83. Beleneva, I.A.; Zhukova, N.V. Bacterial communities of some brown and red algae from Peter the Great Bay, the Sea of Japan. *Microbiology* **2006**, *75*, 348–357. [CrossRef]
84. Crump, B.C.; Koch, E.K. Attached bacterial populations shared by four species of aquatic angiosperms. *Appl. Environ. Microbiol.* **2008**, *74*, 5948–5957. [CrossRef] [PubMed]