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Algicidal and growth-inhibiting bacteria associated with seagrass and macroalgae beds in Puget Sound, WA, USA

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ABSTRACT

The algicidal and growth-inhibiting bacteria associated with seagrasses and macroalgae were characterized during the summer of 2012 and 2013 throughout Puget Sound, WA, USA. In 2012, *Heterosigma akashiwo*-killing bacteria were observed in concentrations of 2.8×10^6 CFU g^{-1} wet in the outer organic layer (biofilm) on the common eelgrass (*Zostera marina*) in north Padilla Bay. Bacteria that inhibited the growth of *Alexandrium tamarense* were detected within the biofilm formed on the eelgrass canopy at Dumas Bay and North Bay at densities of $\sim 10^8$ CFU g^{-1} wet weight. Additionally, up to 4100 CFU mL^{-1} of algicidal and growth-inhibiting bacteria affecting both *A. tamarense* and *H. akashiwo* were detected in seawater adjacent to seven different eelgrass beds. In 2013, *H. akashiwo*-killing bacteria were found on *Z. marina* and *Ulva lactuca* with the highest densities of $\sim 10^8$ CFU g^{-1} wet weight at Shallow Bay, Sucia Island. Bacteria that inhibited the growth of *H. akashiwo* and *A. tamarense* were also detected on *Z. marina* and *Z. japonica* at central Padilla Bay. *Heterosigma akashiwo* cysts were detected at a concentration of 3400 cysts g^{-1} wet weight in the sediment from Westcott Bay (northern San Juan Island), a location where eelgrass disappeared in 2002. These findings provide new insights on the ecology of algicidal and growth-inhibiting bacteria, and suggest that seagrass and macroalgae provide an environment that may influence the abundance of harmful algae in this region. This work highlights the importance of protection and restoration of native seagrasses and macroalgae in nearshore environments, in particular those regions where shellfish restoration initiatives are in place to satisfy a growing demand for seafood.

1. Introduction

Harmful algal blooms (HABs) caused by the proliferation of microalgae are a natural, global phenomenon, resulting in damage to aquaculture industries and wild fish populations while also presenting a risk to human health. The fish-killing raphidophyte, *Heterosigma akashiwo*, and species of the neurotoxin-producing dinoflagellate, *Alexandrium*, have been problematic for decades in Puget Sound, WA, USA and the Strait of Georgia, British Columbia, Canada, which comprise the Salish Sea (Lewitus et al., 2012). Blooms of *H. akashiwo* have caused economic damage up to two million US dollars annually to wild and net-penned farmed fish since the first reported bloom in north Puget Sound in 1976 (Rensel, 2007). Mass mortalities of juvenile sockeye salmon along their migration routes to the Bering Sea have been reported in the Salish Sea during years when *H. akashiwo* blooms were severe, suggesting that these blooms may play a role in salmon mortality (Rensel et al., 2010).

Species of the genus *Alexandrium* are known to produce potent neurotoxins that accumulate in filter-feeding shellfish. Consumption of these contaminated shellfish can cause paralytic shellfish poisoning (PSP) which has resulted in human deaths in the Salish Sea region (Trainer et al., 2003). Shellfish beds can be closed for

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several weeks at a time due to these biological hazards impacting subsistence, cultural, recreational and commercial shellfish harvesting (Trainer et al., 2003) and compromising the vitality of the $108 million per year shellfish industry in Washington State (based on 2008 and 2009 data compiled by the Pacific Coast Shellfish Growers Association). Therefore, there is an urgent need to establish mitigation strategies for these harmful algal events.

In the past two decades, the use of bacteria and viruses has been recognized as a potentially promising tool to control HABs (Nagasaki and Yamaguchi, 1998; Imai et al., 1998; Kim et al., 1998), however the field application of these microorganisms as mitigation strategies has not yet been realized. Imai et al. (2009) and Onishi et al. (2014) found high densities of algicidal and growth-inhibiting bacteria from the biofilm associated with eelgrass (*Zostera marina*) that affect several harmful algal species. Empirical data show that *Z. marina* beds have a lower density of phytoplankton, often populated with epiphytic diatoms closely associated with the *Z. marina* canopy (Jacobs and Noten, 1980; Coleman and Burkholder, 1995; Huh et al., 1998). No reasonable explanations have been given for these low abundances of phytoplankton, however the presence of high-density algicidal and growth-inhibiting bacteria on the biofilm of seagrass leaves may play a role. Preserving these bacterial assemblages through restoration of seagrass meadows may create ecosystems that can control HABs (Imai, 2015). The majority of studies on the effects of algicidal and growth-inhibiting bacteria have been performed on seagrass from the coastal waters of Japan (Imai et al., 2009; Onishi et al., 2014), but due to their potential as HAB mitigation, it is essential to investigate these bacterial phenomena in other coastal areas of the world.

In this study, sites in Puget Sound with seagrass or macroalgae containing bacteria having algicidal or growth inhibiting activities against *H. akashiwo* or *Alexandrium* are documented. Algicidal and growth-inhibiting bacteria associated with the eelgrass (*Z. marina*) and the green alga (*Ulva lactuca*) were investigated throughout Puget Sound, WA, USA, during the summer of 2012 and expanded to include other species of seagrass and dominant macroalgae in north Puget Sound in 2013. Enumeration of the cysts of *H. akashiwo* and *Alexandrium* sp. in sediments was carried out at several sites in 2013, including Westcott Bay where *Z. marina* recently has disappeared.

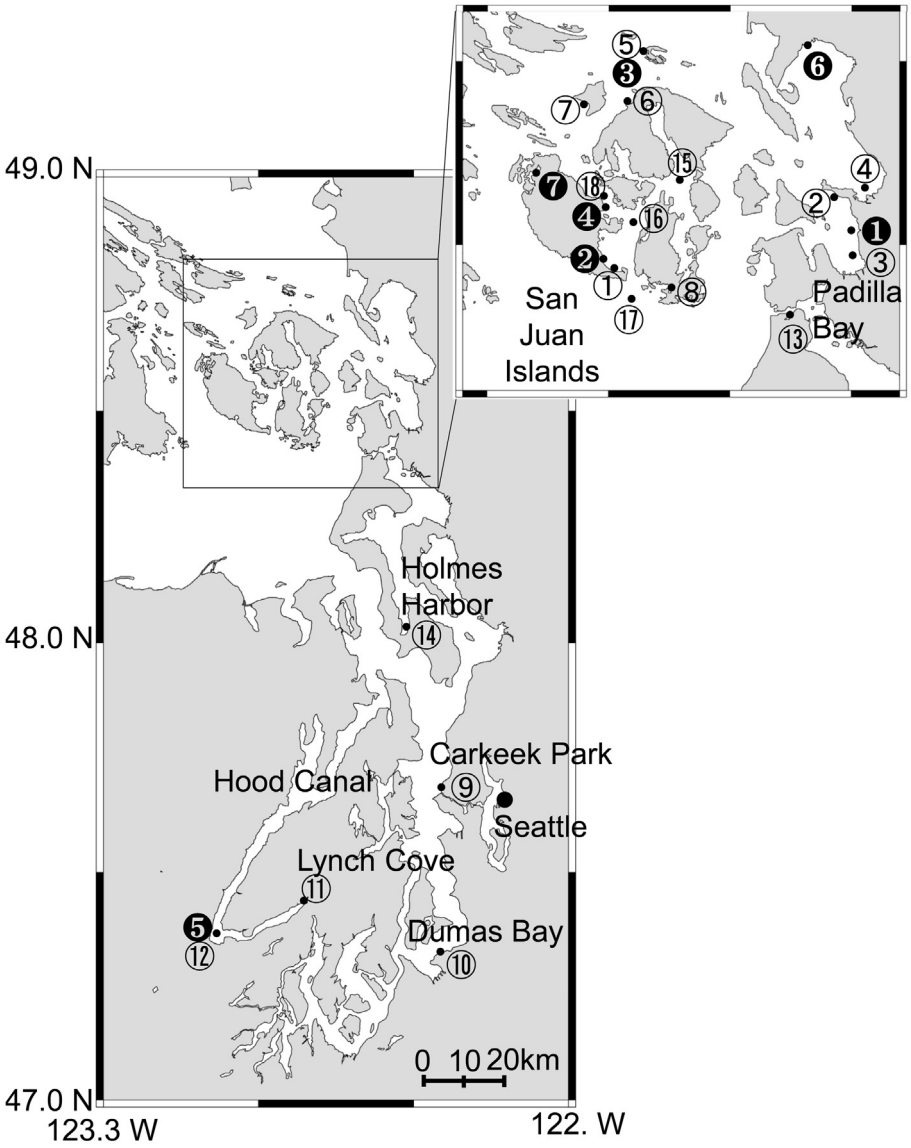

Fig. 1. Sampling locations in Puget Sound, WA, USA in 2012 (○; labeled 1–18) and 2013 (●; labeled 1–7). Site names, species sampled and dates of sampling are listed in Table 1 (2012) and Table 2 (2013).

2. Material and methods

2.1. Sample collection

Seagrass and macroalgae sampling was conducted from 9 June to 5 July 2012 and 1–30 July 2013 in Puget Sound, WA, USA (Fig. 1). In 2012, 14 different seagrass (*Z. marina*) beds, one bull kelp (*Nereocystis luetkeana*), and a green macroalga (*U. lactuca*) bed were sampled (Table 1). In 2013, sampling was performed at 5 different sites (Table 2) and included three seagrass species (*Z. marina*, *Z. japonica* and *Phyllospadix scouleri*) and dominant macroalgae that included two green algae (*U. lactuca* and *Enteromorpha* sp.) and two brown algae (*Fucus distichus* and *Saccharina sessile*).

Seagrasses and macroalgae were collected at low tide from each site using clean scissors by cutting the bottom ~10 cm of the blade and placing it gently into a sterilized polycarbonate sampling bottle (500 mL) filled with autoclaved, filtered (0.7 μm GF/F) seawater (200 mL) for isolation of attached viable bacteria. A seawater sample (500 mL) was collected directly next to a seagrass or macroalgae sample at each site in both 2012 and 2013 for bacterial isolation, phytoplankton counts, and chlorophyll *a* and inorganic nutrient measurements while three offshore water samples were collected for use as controls in 2012 (Table 1). In 2013, sediment samples were collected at six eelgrass beds and at Westcott Bay (Fig. 1, Table 2), a location where eelgrass beds recently have been lost (Wyllie-Echeverria et al., 2003). Samples were placed in a cooler on ice and transported to the laboratory and viable bacteria were separated on the same day.

Table 1
Samples collected in 2012.

No.	Date	Sampling site	Sample type
(1)	2012/6/9	Jackles Lagoon	*Zostera marina* *Ulva lactuca* Seawater
(2)		North Padilla Bay	*Z. marina* Seawater
(3)	2012/6/18	South Padilla Bay	*Z. marina* Seawater
(4)		Samish Bay	*Z. marina* Seawater
(5)		Shallow Bay, Sucia	*Z. marina* Seawater
(6)	2012/6/20	Beach Haven	*Z. marina* Seawater
(7)		North Bay	*Z. marina* Seawater
(8)	2012/6/22	Barlow Bay	*Z. marina* Seawater
(9)	2012/7/2	Carkeek Park	*Z. marina* Seawater
(10)	2012/7/3	Dumas Bay	*Z. marina* Seawater
(11)		Lynch Cove	*Z. marina* Seawater
(12)	2012/7/4	Potlatch State Park	*Z. marina* Seawater
(13)	2012/7/5	Cornet Bay	*Z. marina* Seawater
(14)		Holmes Harbor	*Z. marina* Seawater
(15)	2012/6/11	Offshore1	Seawater
(16)		Offshore2	Seawater
(17)	2012/6/22	Offshore3	Seawater
(18)	2012/6/14	Heaven's Beach	*Nereocystis luetkeana* Seawater

Table 2
Samples collected in 2013.

No.	Date	Sampling site	Sample type
(1)	2013/7/3	Central Padilla Bay	*Z. marina* *Z. japonica* Seawater Sediment
(2)	2013/7/8	Cattle Point	*Z. marina* *U. lactuca* *Phyllospadix scouleri* Seawater
(3)	2013/7/10	Shallow Bay, Sucia	Sediment *Z. marina* *U. lactuca* *Enteromorpha* spp. Seawater
(4)	2013/7/12	Pier at Friday Harbor Lab	Sediment *Z. marina* *Fucus distichus* *Saccharina sessile* Seawater
(5)	2013/7/25	Potlatch State Park	Sediment *Z. marina* Seawater Sediment
(6)	2013/7/9	Bellingham Bay	Sediment
(7)	2013/7/19	Westcott Bay	Sediment

Temperature and salinity were measured in surface waters by a Hydrolab DS5 (OTT Hydromet, Germany). Inorganic nutrients, nitrate plus nitrite (NO_3^- + NO_2^-; hereafter referred to as nitrate), orthophosphate, and silicic acid were analyzed by a Lachat QuikChem 8000 flow injection analysis system using standard colorimetric techniques (Smith and Bogren, 2001; Knepel and Bogren, 2002; Wolters, 2002, respectively).

2.2. Bacterial enumeration and isolation of culturable bacteria

Seagrass and macroalgae samples were vigorously shaken 600 times by hand. The detachment of the biofilm was confirmed microscopically. Samples were serially diluted 10-fold to 10^{-4} with autoclaved seawater and 100 μL of each dilution was spread using a bacterial inoculating loop onto a $ST10^{-1}$ marine agar plate (0.05 g yeast extract, 0.5 g trypticase peptone and 15 g agar in 1 L seawater; Yoshinaga et al., 1997). Seawater samples were also serially diluted 10-fold to 10^{-4} with sterilized seawater and a 1 mL aliquot from each dilution was filtered through a sterile 3.0 μm pore size Whatman Nuclepore filter to separate particle-associated bacteria (PAB) and free-living bacteria (FLB). The filters were placed gently on $ST10^{-1}$ marine agar plates and 100 μL of the filtrates were distributed evenly using a glass spreader on the same medium to culture PAB and FLB, respectively. After incubation for two weeks at 20 °C in the dark, bacterial colonies were visually observed and enumerated to estimate the density of bacteria attached to seagrass and macroalgae as well as PAB and FLB in each water sample (Collins and Lyne, 2004). Colonies on the agar plates were numbered and 36 colonies per each fraction were randomly picked using sterilized toothpicks and inoculated aseptically into separate wells of a 48-well plate containing agar medium using the same incubation conditions as described above. The streak plate method was also used to ensure that a single isolate was obtained (Holt and Krieg, 1994).

Aliquots of the seawater samples (10–15 mL) were collected in sterile centrifuge tubes, glutaraldehyde was added to a 1% final

concentration, and samples were stored at 4 °C. Estimation of total and FLB counts by DAPI staining (Porter and Feig, 1980; Imai, 1987) was performed using a Nikon ECLIPSE80i upright epi-fluorescence microscope with a 100x objective. The number of PAB was calculated by subtracting the difference between the total and FLB in each sample. Microalgae in the samples (1 mL) were counted using a Nikon TE200EF inverted microscope using a Sedgewick-Rafter counting chamber.

2.3. Harmful algal species used in algicidal and growth inhibition tests

Stationary phase, axenic strains of the fish-killing raphidophycean flagellate, *Heterosigma akashiwo* 893, and the neurotoxin-producing armored dinoflagellate *Alexandrium tamarense* (Anderson et al., 2012) were used for algicidal and growth inhibition tests. Isolates of *H. akashiwo* from Hiroshima Bay, Japan, in 1989, and *A. tamarense*, isolated from Osaka Bay, Japan, in 2007 are part of the culture collection at the Hokkaido University. The cultures were grown and maintained in modified SWM-3 medium (Chen et al., 1969; Imai et al., 1996) at 15 °C for *A. tamarense* and 20 °C for *H. akashiwo* using a light intensity of 50–100 μmol photons m^{-2} s^{-1} and a 14:10 h light-dark photoperiod.

2.4. Density estimation of algicidal and growth-inhibiting bacteria

Bacteria isolated from seagrasses or macroalgae as well as PAB and FLB isolates from seawater were tested using monoxenic co-culture experiments (a single axenic algal species grown in the medium with a single bacterium) to determine algicidal and growth-inhibiting properties toward two harmful algae, *H. akashiwo* and *A. tamarense*. Algal cultures were diluted in modified SWM-3 medium to a cell density of approximately 10^3 cells mL^{-1} and 0.8 mL aliquots were inoculated into sterilized disposable 48-well microplates. After incubation for 1–2 d to confirm the state of healthy swimming algal cells, individual bacterial colonies were picked from $ST10^{-1}$ marine agar medium aseptically using an autoclaved toothpick and inoculated into the algal cultures with approximate density of 10^5 cells mL^{-1}. The plates were incubated for another week using the same conditions as described above for the algal cultures. Quadruplicate wells with no added bacteria were used as bacteria-free controls in each microplate. The proportions of algicidal and growth-inhibiting bacteria normally range from 0-30% of the tested bacteria within a 48-well plate. Therefore within each plate of tested bacterial isolates, over half were controls. Microscopic observation of each well was done daily using a Nikon ECLIPSE TE200 inverted microscope to check for cell mortality or qualitative changes in algal morphology. The wells in which 90% or more of algal cells were killed within one week of incubation were determined to contain algicidal bacteria (AB) and the wells in which reduced motility and/or algae cell deformation occurred were described as containing growth inhibiting bacteria (GIB). The density of AB and GIB was calculated as follows: NAG = NV × SAG/NT (NAG is the density of AB or GIB (CFU mL^{-1} or CFU g^{-1} wet weight), NV is the density of viable bacteria (CFU mL^{-1} or CFU g^{-1} wet weight), SAG is the number of bacterial strains showed algicidal or growth-inhibiting activity, NT is the number of bacterial strains tested: 36 strains per each fraction in this study). Representative morphologies observed during co-culture experi-

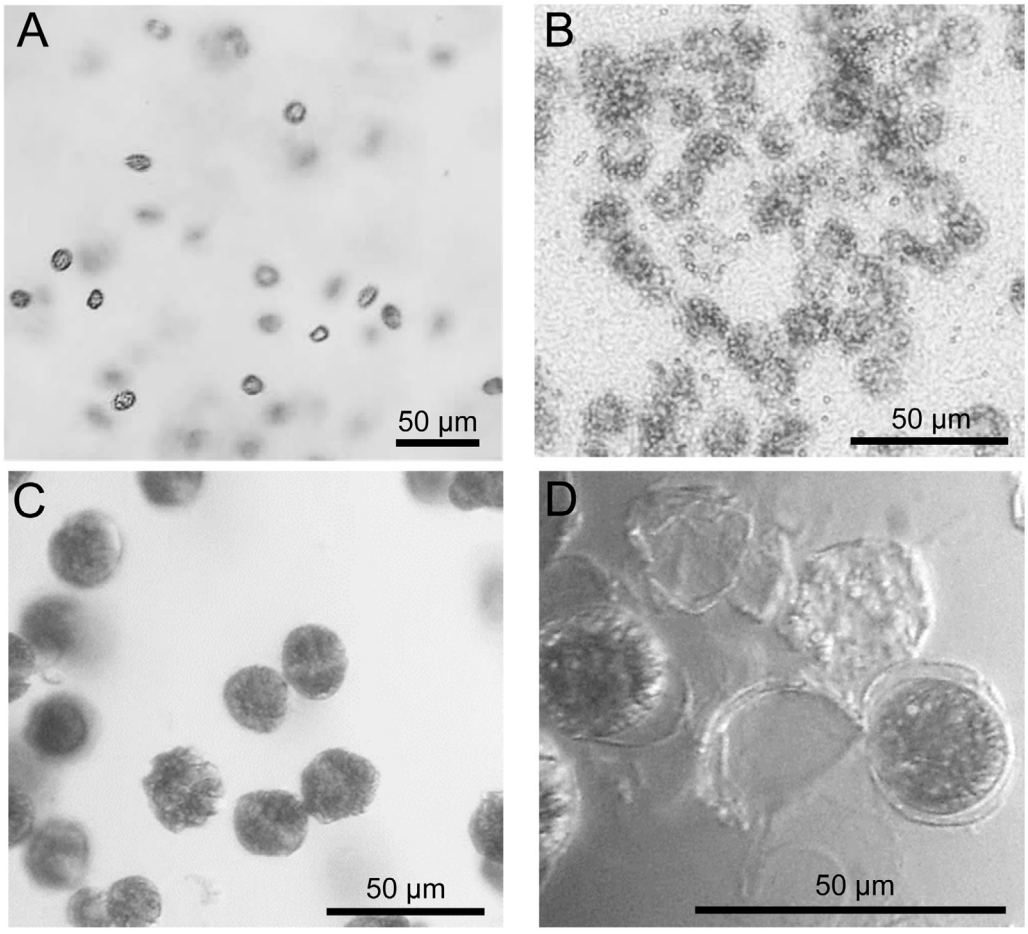

Fig. 2. Representative morphologies observed during co-culture experiments with two harmful algal species after one-week incubation. Bacteria inoculation density was ~10^5 cells mL^{-1}. *H. akashiwo* (A: control, B: killed), *A. tamarense* (C: control, D: killed and inhibited). Scale bar in all panels (A–D) are 50 μm.

ments with the two targeted species (*H. akashiwo* and *A. tamarense*) are shown in Fig. 2.

2.5. DNA extraction and partial 16S ribosomal RNA sequencing

A total of 33 bacterial strains (13 strains of AB and 20 strains of GIB) were cultured in ST10^{-1} liquid medium for 1–2 weeks then 1 mL of each culture was transferred into 2 mL microtubes and centrifuged at 6000 × *g* for 15 min to harvest bacterial pellets. Pellets were suspended in 1 mL of phosphate buffered saline and washed three times with suction to remove the medium. Bacterial DNA was extracted using a boiling method (Valsecchi, 1998) or NucleoSpin Tissue extraction kit (TaKaRa BIO Inc.) and stored at −20 °C until amplification by polymerase chain reaction (PCR). The universal primers 27F (Delong, 1992) and 519R (Stackebrandt and Goodfellow, 1991) were used and the bacterial 16S rRNA gene fragments (approximately 500 bp) were amplified using *Taq* DNA polymerase (New England BioLabs, Inc.) or EX *Taq* (TaKaRa Bio Inc.). The PCR parameters were: an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °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 ethanol precipitation or UltraClean PCR Clean-up kit (MO BIO Laboratories, Inc.) 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) using an Applied Biosystems DNA sequencer (ABI 3130). Resulting nucleotide sequences were aligned using Chromas pro (ver. 1.7.1). A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) sequence similarity search was performed to analyze approximately 500 bp of each consensus sequences in order to identify similarity with previously sequenced bacteria.

2.6. Estimating density of cysts in the sediment

Surface sediments (0–1 cm depth) were collected in 2013 at six different seagrass beds during low tide in north Puget Sound including Westcott Bay (sediment sample sites are shown in Table 2). Sediments were scooped gently from the surface of the intertidal zone using a clean tablespoon. Cysts that possessed germinability were estimated using the most probable number method (MPN; Imai and Itakura, 1991; Anderson et al., 1995). One gram of sediment sample was suspended in modified SWM-3 medium to obtain a concentration of 0.1 g wet weight mL^{-1}. Germanium dioxide (1 mg L^{-1} final concentration) was added to the culture medium to inhibit the growth of diatoms (Lewin, 1966). The sample was diluted serially (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) using modified SWM-3, then 1 mL aliquots of each dilution were dispensed into wells of disposable 48 microplates and incubated at 20 °C for *Heterosigma* and 15 °C for *Alexandrium*, using a light intensity of approximately 50 μmol photons m^{-2} s^{-1} and a 14:10 h light-dark photoperiod.

The appearance of *Heterosigma* and *Alexandrium* vegetative cells in each well was confirmed using an inverted microscope (Nikon ECLIPSE TE200) after 6–8 d incubation. Wells containing vegetative cells were scored as positive and the MPN of germinable *Heterosigma* and *Alexandrium* cysts in the sediment was estimated in accordance with a statistical table (Throndsen, 1978; Itoh and Imai, 1987).

3. Results

3.1. Environmental parameters

Temperature at the sampling sites ranged between 12.0–20.0 °C in 2012 and 13.8–25.3 °C in 2013. Salinity measurements

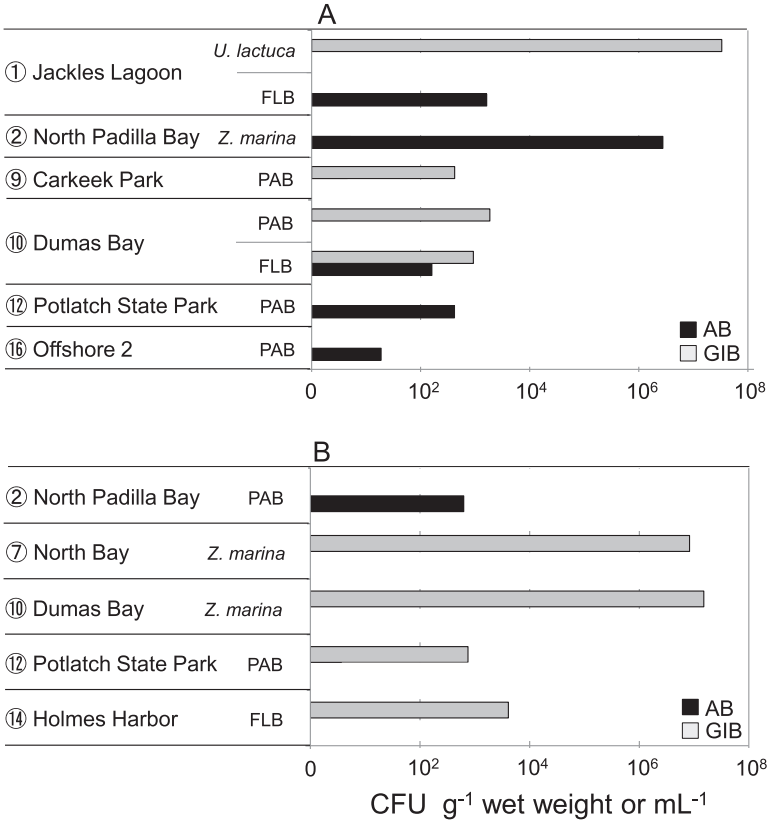

Fig. 3. Density of AB and GIB isolated from *Z. marina*, *U. lactuca* and seawater (labeled FLB or PAB) at each site (Fig. 1, numbered open circles and Table 1) against *H. akashiwo* (A) and *A. tamarense* (B) in 2012.

ranged between 14.0–30.2 in 2012 and 12.0–31.1 in 2013. The lower salinities observed at Carkeek Park in 2012 were due to freshwater input from nearby Pipers Creek and at Potlatch State Park due to high precipitation in the area prior to sampling in 2013. In 2012, chlorophyll *a* ranged between 0.1–32.5 $\mu g\ L^{-1}$ and in 2013, from 1.7–11.0 $\mu g\ L^{-1}$. High chlorophyll *a* was observed at Holmes Harbor (32.5 $\mu g\ L^{-1}$) and Dumas Bay (13.7 $\mu g\ L^{-1}$) in 2012, and at Cattle Point (11.0 $\mu g\ L^{-1}$), Shallow Bay, Sucia Island (5.2 $\mu g\ L^{-1}$) in 2013. Concentrations of inorganic nutrients varied between 0.8–113.5 μM (SiO_2), 0.2–21.2 μM ($NO_3^- + NO_2^-$) and 0.0–2.2 μM (PO_4) in 2012 and 32.8–101.0 μM (SiO_2), 1.2–10.0 μM ($NO_3^- + NO_2^-$) and 0.0–0.2 μM (PO_4) in 2013.

3.2. Distribution of algicidal and growth-inhibiting bacteria at seagrass beds

Distributions of algicidal and growth-inhibiting bacteria (AB and GIB) against *H. akashiwo* and *A. tamarense* in Puget Sound in 2012 are shown in Fig. 3(A and B). Algicidal bacteria (AB) and growth-inhibiting bacteria (GIB) were distributed extensively throughout Puget Sound. The highest density (2.77×10^6 CFU g^{-1} 1 wet leaf) of *H. akashiwo*-killing bacteria was detected from the biofilm of *Z. marina* collected at North Padilla Bay (Fig. 3 A). Likewise, AB that killed *A. tamarense* (6.30×10^2 CFU mL^{-1}) were also isolated from seawater collected adjacent to the Padilla Bay site (Fig. 3B). The highest density of GIB that affected *A. tamarense* (1.50×10^7 CFU g^{-1} wet leaf) was detected on the biofilm of *Z. marina* while AB (1.60×10^2 CFU mL^{-1}) and GIB (2.78×10^3 CFU mL^{-1}) that affected *H. akashiwo* also were detected from the adjacent seawater at Dumas Bay (Fig. 3). Potlatch State Park (Fig. 3) was one of the rare sites where AB (4.16×10^2 CFU mL^{-1}) and GIB (7.50×10^2 CFU mL^{-1}) that affected both *H. akashiwo* and *A. tamarense* were isolated. Notably, a relatively high percentage (17%) of total viable bacteria isolated from Potlatch State Park showed strong algicidal activity (mortality of *H. akashiwo* was observed within three days). The density of GIB that affected *H. akashiwo* was the highest (3.32×10^7 CFU g^{-1} wet weight) on green alga, *U. lactuca* collected from Jackles Lagoon (Fig. 3A). No AB or GIB were found on *Nereocystis luetkeana* or associated with its surrounding seawater.

The density of AB and GIB in 2013, isolated from the three different seagrass species, *Z. marina*, *Z. japonica* and *Phyllospadix scouleri*, two dominant green algae, *U. lactuca* and *Enteromorpha* sp., and the two brown algae, *Fucus distichus* and *Saccharina sessile*, is shown in Fig. 4. A very high density of *H. akashiwo*-killing bacteria was detected on *Z. marina* (1.08×10^8 CFU g^{-1} wet leaf) and *U. lactuca* (1.38×10^8 CFU g^{-1} wet leaf) at Shallow Bay, Sucia Island (Fig. 4A). Bacteria that inhibited *A. tamarense* growth were also found in seawater adjacent to seagrass beds in Shallow Bay (FLB: 2.48×10^2 CFU mL^{-1}, PAB: 2.58×10^2 CFU mL^{-1}). Several GIB that acted against both tested harmful species were isolated from *Z. japonica* from central Padilla Bay and *U. lactuca* from Cattle Point, Sun Juan Island. Bacterial densities varied between 3.8×10^7–2.8×10^8 CFU g^{-1} wet weight for *Z. japonica* and

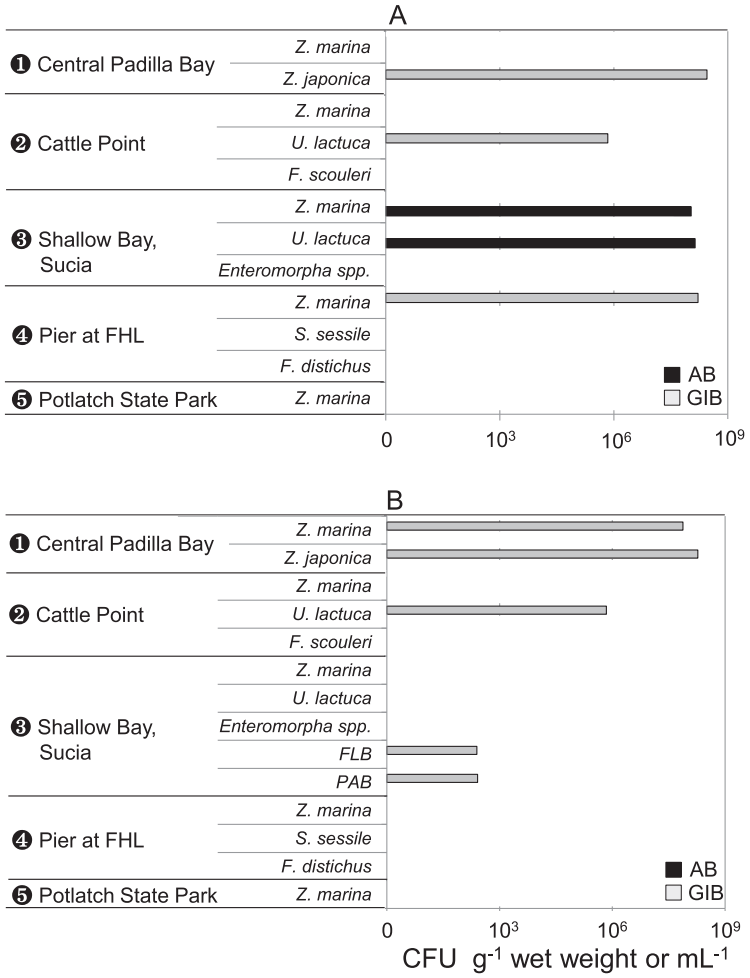

Fig. 4. Density of AB and GIB isolated from different seagrasses or macroalgae, and seawater (labeled FLB or PAB) at each site (Fig. 1, numbered filled circles; and Table 2) against *H. akashiwo* (A) and *A. tamarense* (B) in 2013.

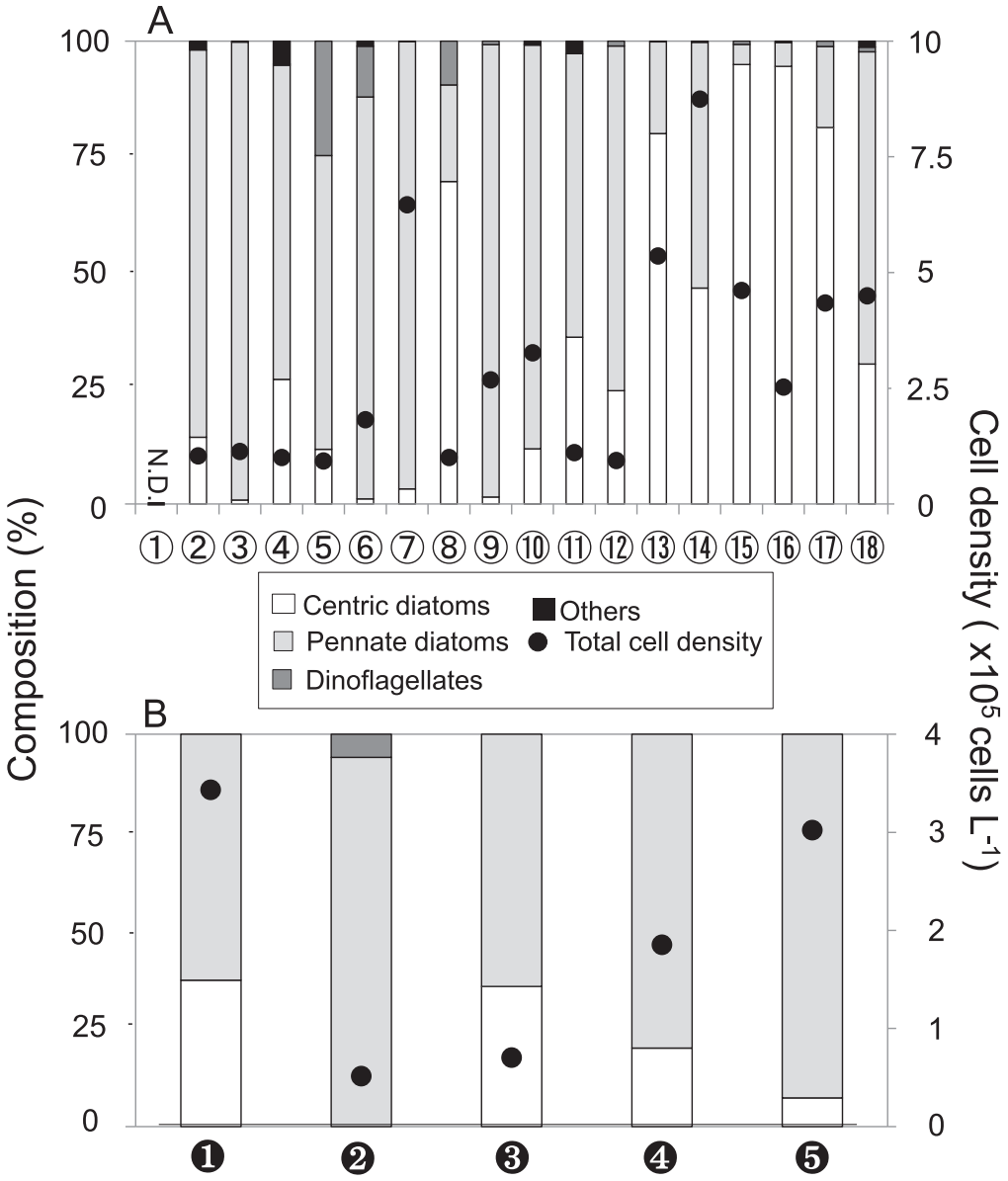

Fig. 5. Phytoplankton composition and abundance at all sampling sites in 2012 (A) and 2013 (B). Note that this exclude sites 6 and 7 for which sediment samples were collected only in 2013.

7.0×10^5 CFU g^{-1} wet leaf for *U. lactuca*. This is the first report confirming the attachment of algal GIB from biofilm on *Z. japonica*.

3.3. Composition and abundance of phytoplankton

The abundance and composition of phytoplankton at each sampling site in 2012 and 2013 are shown in Fig. 5(A and B). The highest densities were observed at Holmes Harbor (8.7×10^5 cells L^{-1}) in 2012 and central Padilla Bay (3.4×10^5 cells L^{-1}) in 2013, otherwise, relatively low abundances of phytoplankton were observed at most of seagrass sampling sites (0.9–8.7×10^5 cells L^{-1} in 2012 and 0.5–3.4×10^5 cells L^{-1} in 2013). More than 60% of the phytoplankton at seagrass sites was composed of pennate diatom genera such as *Diploneis*, *Cymbella*, *Licmophora*, *Navicula* and *Nitzschia*, except at Barlow Bay and Cornet Bay. Centric diatoms were also observed (Fig. 5A and B), including the genera *Rhizosolenia*, *Skeletonema*, *Melosira* and *Detonula*. The dinoflagellate *Protoperidinium* (0.2×10^5 cells L^{-1}), was detected at Shallow Bay, Sucia Island, in 2012 and *Dinophysis rotundata* was observed at Cattle Point, Sun Juan Island, in 2013. Few dinoflagellates were found in both years.

3.4. *Heterosigma and* Alexandrium *cysts in the sediments*

Cysts of *H. akashiwo* and *Alexandrium* were not detected (<200 cells g^{-1} wet sediment) in 2013 at sampling sites where seagrasses were thriving, including Padilla Bay, Shallow Bay, and Potlatch State Park. In contrast, 3400 cells g^{-1} wet sediment of *H. akashiwo* cysts were detected from Westcott Bay (site 7 in 2013, north of San Juan Island; Fig. 1 and Table 2) where eelgrass disappeared in 2002 (Wyllie-Echeverria et al., 2003) and has not returned (Gaeckle et al., 2011). However, no *Alexandrium* cysts were detected in Westcott Bay.

3.5. Identification of algicidal bacteria (AB) and growth-inhibiting bacteria (GIB)

A total of 33 strains of AB and GIB were analyzed by partial 16S ribosomal RNA sequencing. The genus and class of AB and GIB classified for each of the host organisms are shown in Fig. 6 and for different seawater fractions (FLB and PAB) in Fig. 7. These AB and GIB were classified as α, β, and γ within the phylum Proteobacteria,

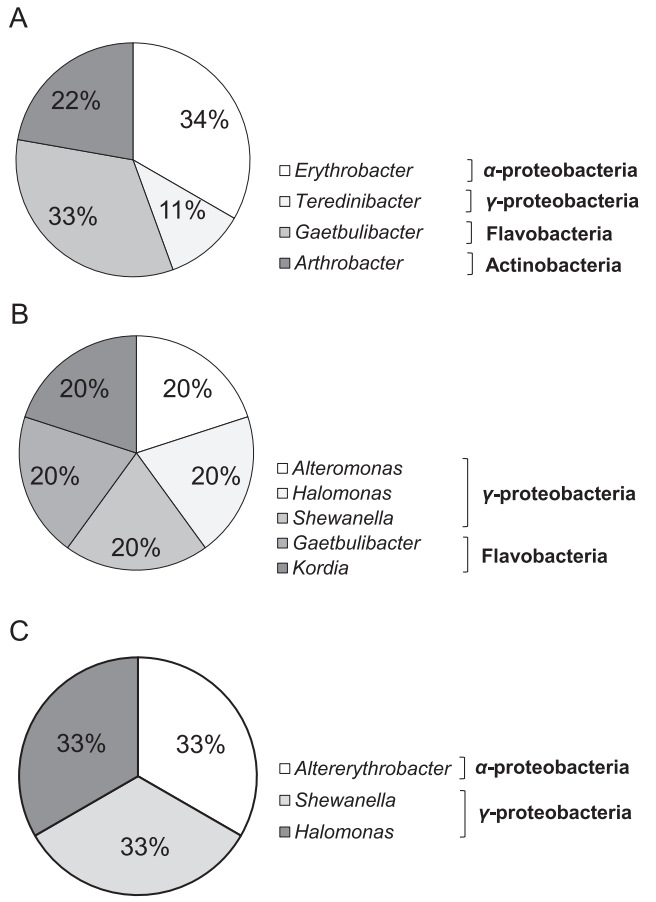

Fig. 6. Proportion of AB or GIB in 2012 and 2013 associated with different host organisms, *Z. marina* (A), *Z. japonica* (B) and *U. lactuca* (C).

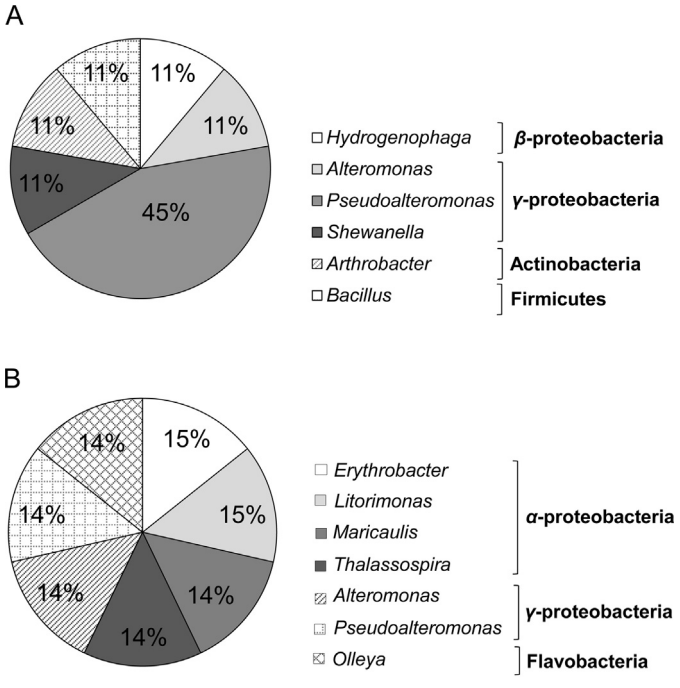

Fig. 7. Proportion of AB or GIB classified as free-living bacteria, FLB (A) and particle-associated bacteria, PAB (B).

the class Flavobacteria from the phylum Bacteroidetes, and the classes Actinobacteria and Bacillus from the phylum Firmicutes. The AB and GIB detected on *Z. marina* biofilm was composed of 34% *Erythrobacter* (α-proteobacteria) while this genus composed 15% of PAB in seawater. *Alteromonas* and *Shewanella* (γ-proteobacteria), and *Arthrobacter* (Actinobacteria) were also detected from *Z. marina*, *Z. japonica*, *U. lactuca* and seawater at several seagrass meadows. β-Proteobacteria (*Hydrogenophaga* sp.), with killing behavior against *H. akashiwo*, was isolated from the FLB fraction in Dumas Bay in 2012. To our knowledge, this is the first documentation of β-proteobacteria with algicidal ability.

4. Discussion

Marine heterotrophic bacteria often are classified into two different groups: particle-associated bacteria (PAB) and free-living bacteria (FLB), to describe their association (or lack of association) with particulate organic matter (Bidle and Fletcher, 1995). These two types of bacteria are reported to vary in distribution, speciation (Bidle and Fletcher, 1995) and in type of hydrolytic protease activity (Bidle and Azam, 1999). In Japan, higher densities and frequencies of AB and GIB have been observed in nearshore waters compared to offshore environments (Inaba et al., 2014). Quantitative studies of AB and GIB have revealed a higher proportion in the particle-associated form compared to the free-living form (Park et al., 2010; Inaba et al., 2014). Likewise, in Puget Sound, the majority of bacteria isolated from seawater having activity against *H. akashiwo* and *A. tamarense* were in the particle-associated form (Fig. 3). Intense exoenzyme activity on marine aggregates (Smith et al., 1992) and higher ectoproteolytic activity in PAB compared to FLB demonstrate the important role of PAB in carbon cycling in the ocean (Bidle and Azam, 1999).

Imai et al. (2009) and Onishi et al. (2014) detected high densities of AB attached to the biofilm growing on seagrass (*Zostera marina*) leaves from several Japanese nearshore coastal areas. In Puget Sound, *Erythrobacter* (α-proteobacteria), *Alteromonas* (γ-proteobacteria), *Shewanella* (γ-proteobacteria) and *Arthrobacter* (Actinobacteria) with algicidal or growth-inhibiting activity were detected on the biofilm of two seagrass species (*Z. marina* and *Z. japonica*), one green alga (*U. lactuca*) and also from the adjacent seawater (Figs. 6 and 7). Previous studies have shown that *Alteromonas* and *Shewanella*, isolated from biofilm of *Z. japonica* and *U. lactuca*, and seawater surrounding the seagrass canopy, are common AB or GIB genera in coastal seawater globally (Mayali and Azam, 2004; Hare et al., 2005). The present study focused on characterization of AB and GIB from nearshore regions of Puget Sound with activity against harmful algal species isolated from coastal waters of Japan. Future studies should test the activity of bacteria isolated from Puget Sound against target algal strains originating from the same region to characterize bacterial activity against native algae populations.

The important role of AB and GIB as regulators of phytoplankton abundance and succession in the marine environment has been described previously (Imai et al., 1998; Doucette et al., 1998, 1999; Lovejoy et al., 1998; Mayali and Azam, 2004). Most studies characterize the taxonomy of these organisms and describe their killing and growth-inhibiting activity while only speculating on their ecological role, which is poorly understood. The further characterization of ecosystems with notably high and recurrent densities of AB and GIB will provide insight into the most favorable habitats, substrates, and ecological roles of these organisms. In Puget Sound in 2012, a high density of GIB against *H. akashiwo* was detected on the green alga, *U. lactuca*, collected from Jackles Lagoon in south San Juan Island, and AB against *H. akashiwo* were isolated from nearby seawater (Fig. 3A). In 2013, GIB against both algae were isolated from *U. lactuca* at Cattle Point and nearby Jackles

Table 3
Summary of published studies on algicidal or growth-inhibiting bacteria targeting HAB species.

Year	Sampling site	Source	Target HAB species	Maximum density of AB or GIB	Method	Remark	Reference
Coastal water							
1990–1991	Tanabe Bay, Japan	Seawater	*Karenia mikimotoi*	9.3×10^4 MPN mL^{-1}	MPN	*K. mikimotoi* bloom	Yoshinaga et al. (1995)
1992	Hiroshima Bay, Japan	Seawater	*H. akashiwo* *Chattonella antiqua*	7.7×10^3 MPN mL^{-1} 1.2 MPN mL^{-1}	MPN	*H. akashiwo* bloom	Imai et al. (1998)
1994–1995	Hiroshima Bay, Japan	Seawater	*H. akashiwo* *C. antiqua*	2.6×10^2 MPN mL^{-1} 5.6 MPN mL^{-1}	MPN	*H. akashiwo* bloom	Kim et al. (1998)
1997	Huon Estuary, Australia	Seawater	*Gymnodinium catenatum* *H. akashiwo* *C. marina* *G. sanguineum*	16.8 CFU mL^{-1} 0.8 CFU mL^{-1} 0.8 CFU mL^{-1} 16.8 CFU mL^{-1}	Bacterial isolation and co-culture	No bloom event	Lovejoy et al. (1998)
1997–1998	Northern Harima-Nada, Japan	Seawater	*Chattonella* spp. and diatom	1350 cells mL^{-1}	Immunofluorescence assay to detect *Cytophaga* sp. J18/M01	Small bloom of *Chattonella* spp.	Imai et al. (2001)
2002–2004	Kiawah Island Ponds, USA	Brackish water	*H. akashiwo* *Fibrocapsa. japonica* *C. subsalsa*	51 MPN mL^{-1} 112 MPN mL^{-1} 131 MPN mL^{-1}	MPN	Brackish detention ponds	Liu et al. (2008)
2005	Harima-Nada, Japan	Seawater	*H. akashiwo* *C. antiqua* *F. japonica* *Heterocapsa circularisquama*	20 MPN mL^{-1} 140 MPN mL^{-1} 34 MPN mL^{-1} 4 MPN mL^{-1}	MPN	Diatom bloom	Park et al. (2010)
2011	Yatsushiro Sea, Japan	Seawater (nearshore)	*C. antiqua*	1.6×10^4 CFU mL^{-1}	Bacterial isolation and co-culture	No bloom event	Inaba et al. (2014)
Macroalgae and seagrass beds in coastal Japan							
1999	Osaka Bay	*Ulva* sp.	*H. akashiwo* *F. japonica* *K. mikimotoi*	1.4×10^3 MPN g^{-1} 1.2×10^4 MPN g^{-1} 7×10^4 MPN g^{-1}	MPN	Green alga	Imai et al. (2002)
		Gelidium sp.	*H. akashiwo* *F. japonica* *K. mikimotoi*	2.6×10^5 MPN g^{-1} 1.3×10^6 MPN g^{-1} 4.9×10^5 MPN g^{-1}	MPN	Red alga	
		Seawater	*H. akashiwo* *F. japonica* *K. mikimotoi*	1.6×10^2 MPN mL^{-1} 4.3×10^2 MPN mL^{-1} 4.3×10^3 MPN mL^{-1}	MPN	Macroalgae bed	
2001	Tanabe Bay	Seawater	*C. antiqua* *K. mikimotoi*	10 MPN mL^{-1} 2.1×10^3 MPN mL^{-1}	MPN	Aquaculture pond with *Ulva pertusa*	Imai et al. (2013)
		Seawater (offshore)	*C. antiqua* *K. mikimotoi*	10 MPN mL^{-1} 4.6×10^2 MPN mL^{-1}	MPN	No bloom event	
	Osaka Bay	Seawater	*C. antiqua* *K. mikimotoi*	10 MPN mL^{-1} 1.1×10^4 MPN mL^{-1}	MPN	*Ulva* bed	
		U. pertusa	*C. antiqua* *K. mikimotoi*	1.65×10^4 MPN mL^{-1} 1.38×10^6 MPN mL^{-1}	MPN		
2002–2003	Shimo-Haya Bay	Seawater	*H. akashiwo* *C. antiqua* *F. japonica* *K. mikimotoi*	10^3 MPN mL^{-1} 10^2 MPN mL^{-1} 1.4×10^2 MPN mL^{-1} 2.2×10^4 MPN mL^{-1}	MPN	Aquaculture pond with *Ulva pertusa*	Imai et al. (2012)
2002–2003	Shimo-Haya Bay	*U. pertusa*	*H. akashiwo* *C. antiqua* *F. japonica* *K. mikimotoi*	10^2 MPN g^{-1} 10^3 MPN g^{-1} 1.9×10^5 MPN g^{-1} 1.1×10^6 MPN g^{-1}	MPN	Aquaculture pond with *Ulva pertusa*	
2006	Osaka Bay	Seawater	*H. akashiwo* *C. antiqua* *K. mikimotoi*	2.4×10^3 CFU mL^{-1} 4.8×10^3 CFU mL^{-1} 2.4×10^3 CFU mL^{-1}	Bacterial isolation and co-culture	*Z. marina* bed	Imai et al. (2009)
2006	Osaka Bay	*Z. marina*	*H. akashiwo* *C. antiqua* *K. mikimotoi*	N.D. 9.19×10^6 CFU g^{-1} 6.4×10^7 CFU g^{-1}	Bacterial isolation and co-culture		
Macroalgae and seagrass beds in Puget Sound, USA							
2012–2013	Puget Sound	Seawater	*H. akashiwo* *A. tamarense*	1.8×10^3 CFU mL^{-1} 4.1×10^3 CFU mL^{-1}	Bacterial isolation and co-culture	(10) Dumas Bay (14) Holmes Harbor	This study
		Z. marina	*H. akashiwo* *A. tamarense*	1.6×10^8 CFU g^{-1} 7.5×10^7 CFU g^{-1}		(4) Pier at FHL (1) Central Padilla Bay	
		Z. japonica	*H. akashiwo* *A. tamarense*	2.8×10^8 CFU g^{-1} 1.8×10^8 CFU mL^{-1}		(1) Central Padilla Bay (1) Central Padilla Bay	
		U. lactuca	*H. akashiwo* *A. tamarense*	1.3×10^8 CFU g^{-1} 6.9×10^5 CFU g^{-1}		(3) Shallow Bay, Sucia (2) Cattle point	

N.D. = Not detected

Lagoon (Fig. 1), sites far from centers of urban development. About 100–10,000× higher densities of AB and GIB were observed in the biofilm of *Z. marina*, *Z. japonica* and *U. lactuca* in Puget Sound compared to densities published for the nearshore waters of Japan (Inaba et al., 2014; Table 3), illustrating the importance of these bacteria to the Puget Sound ecosystem. However, the studies in Japan are the most comprehensive in that they were conducted during both bloom and non-bloom events, and included both macroalgae and seagrass beds. A comprehensive comparison of AB and GIB isolated from coastal waters of Japan, USA, and Australia, including their maximum densities and activity against selected HAB species, is shown in Table 3.

In our Puget Sound study, it is interesting that no AB and GIB were detected on three brown algae, *N. luetkeana*, *F. distichus*, *S. sessile*; the green alga, *Enteromorpha*, and one seagrass species, *P. scouleri*. This lack of activity associated with certain substrates supports the observation that physiological and biochemical properties of macroalgae predetermine the composition of the adhering microbial communities (Beleneva and Zhukova, 2006). Lachnit et al. (2009) compared bacterial communities associated with four different macroalgae in two different habitats and demonstrated that bacterial communities derived from macroalgae of the same species but originating from different habitats were more similar than bacteria from different species of macroalgae inhabiting the same ecological niche. Such host species-specific microbial selections are also observed in aquatic angiosperms (Crump and Koch, 2008). These results motivate further investigation to describe the mechanism of selective interactions of AB and GIB with specific seagrass and macroalgae species.

A high density of *H. akashiwo*-killing bacteria was detected in 2013 on the biofilm associated with *Z. marina* leaves (Fig. 4A) collected from Shallow Bay, Sucia, at the northern end of the sampling sites (Fig. 1). The causative bacterium was identified as *Erythrobacter* sp. (α-proteobacteria). This shallow semi-enclosed bay, which has a relatively low water turbulence and a small tidal range, provided a suitable environment for the formation of a complex and thick biofilm (Dobretsov, 2010) enabling dense bacterial attachment on *Z. marina*. Chlorophyll *a* was also relatively high at this site. AB and GIB were detected in seawater at other sites and times when chlorophyll *a* was relatively high, such as Holmes Harbor and Dumas Bay in 2012, and Cattle Point in 2013, suggesting that live or decaying phytoplankton associated with seagrass biofilm (e.g. epiphytic diatoms) provided a favorable environment for AB and GIB (Inaba et al., 2014). It is also likely that elevated chlorophyll *a* provided a source of organic material (dissolved and particulate) to support the growth of heterotrophic bacteria including AB and GIB.

Algicidal bacteria appear to target harmful dinoflagellates preferentially over many diatom species. In both 2012 and 2013, the composition of phytoplankton in seawater within the seagrass canopy at most of the sites in Puget Sound was greater than 60% pennate diatoms (Fig. 5). Pennate diatoms, such as *Nitzschia* and *Navicula*, which were also observed in the samples, showed strong resistance against tested algicidal bacteria that possess a wide range of killing activity against dinoflagellates, raphidophytes, and centric diatoms (Kuroda, 2012). Kawamura and Hirano (1992) described colonization of seagrass by epiphytic diatoms, resulting in protection of these cells from changing physiochemical conditions and grazing pressures. Centric diatoms, such as *Rhizosolenia* and *Detonula*, were often observed in the seawater samples at seagrass sites. Interestingly, Inaba et al. (2014) showed that *Rhizosolenia* was most abundant in seawater when the density of AB that killed the harmful raphidophyte, *Chattonella antiqua*, was highest. These data suggest that defense mechanisms allow certain phytoplankton, including epiphytic diatoms, to be resistant to the effects of some bacteria, thereby allowing them to live in a biofilm that has high densities of AB and GIB. Paul and Pohnert (2013) demonstrated the release of a protease by *Chaetoceros didymus* in response to lytic enzymes from AB, suggesting a possible active defense mechanism used by diatoms against AB.

Our study demonstrates the distribution of AB and GIB throughout five different basins in Puget Sound, WA (Figs. 3 and 4). Padilla Bay was the only site where AB or GIB, isolated from *Z. marina* or *Z. japonica*, showed activity against both tested algae in both years when densities of AB and GIB exceeded 1–10×10^6 CFU g^{-1}. Padilla Bay supports the largest contiguous stand of eelgrass in the continental United States (Bulthuis, 1995; Gaeckle et al., 2008; Wyllie-Echeverria and Ackerman, 2003) providing more than 3×10^7 m^2 of both subtidal and intertidal benthic habitat for *Z. marina* and *Z. japonica*. Outbreaks of harmful algal blooms caused by *Alexandrium* spp. that cause PSP have been rare in Padilla Bay during the last decade (Moore et al., 2009) and southern Hood Canal has only recently experienced problems with paralytic shellfish toxins (Washington State Department of Health database, 2015). Although shellfish harvesting at Hoodsport, a site <10 km to the north of Potlatch State Park, was closed due to high concentrations of paralytic shellfish toxins (105 μg/100 g shellfish) in varnish clam in August 2015, shellfish samples collected from Potlatch State Park and analyzed for paralytic shellfish toxins in summer 2015 did not contain quantifiable concentrations of toxin, supporting the possibility that seagrass at this site continue to harbor bacteria (see Potlach State Park data from 2012 in Fig. 3) that have growth-limiting activity toward a newly introduced population of toxic cells.

Phytoplankton seed populations, called resting stage cells or cysts, live in the sediment until conditions become favorable for them to germinate. These seed populations influence the phytoplankton community in the water column (Itakura et al., 1997) and also play a crucial role in the initiation of algal blooms (e.g., Imai and Itakura, 1999). *Heterosigma akashiwo* and *Alexandrium* spp. have cyst stages in their life cycles and the presence or absence of cysts in the sediments provides a historical record of HABs (e.g., Horner et al., 2011). If *Z. marina* beds provide unfavorable conditions for these harmful organisms to survive, very few or no cysts would be expected to exist in the sediment. In the present study, *H. akashiwo* cysts were observed in the sediment collected from Westcott Bay, where eelgrass disappeared in 2002 (Wyllie-Echeverria et al., 2003). The absence of harmful cysts of *H. akashiwo* and *Alexandrium*, in Padilla Bay, Shallow Bay, and Potlatch State Park (Table 2, Fig. 1), sites with healthy eelgrass populations, support the hypothesis that the abundance of AB and GIB at locations where HABs historically have not been documented, create an unfavorable environment for harmful phytoplankton.

Stands of *Z. marina* are variable in Puget Sound and declining populations have been documented at some sites in the San Juan Archipelago, central Puget Sound, and Hood Canal (Gaeckle et al., 2008; Wyllie-Echeverria et al., 2010). Sites supporting seagrasses and macroalgae populations serve as critical habitats for marine and estuarine animals, providing nursery, migratory grounds, and food resources (Kenworthy et al., 2006; Lembi and Waaland, 1988). In the present study, high densities of AB and GIB associated with seagrasses and macroalgae were confirmed for the first time in Puget Sound, WA, USA. In particular, it is believed that this is the first report showing the attachment of GIB on the biofilm of *Z. japonica* and the identification of β-proteobacteria with algicidal ability. These unusual associations shed light on the importance of seagrasses and macroalgae as a nursery for growth-limiting bacteria that provide an ecological pathway to influence the abundance of harmful algae in this region. In the future, controlled manipulative field experiments (e.g. mesocosm studies) are

needed to test the effects of seagrass or seaweed removal on HAB cysts and vegetative cell survival.

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