May 2nd, 8:30 AM - 10:00 AM

Investigating Algicidal and Growth-inhibiting Bacteria associated with Seagrass and Macroalgae beds in Puget Sound

Nobuharu Inaba
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Investigating Algicidal and Growth-inhibiting Bacteria associated with Seagrass and Macroalgae beds in Puget Sound

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¹Plankton Laboratory, Graduate School of Fisheries Sciences, Hokkaido University
²Laboratory of Marine Environmental Microbiology, Graduate School of Agriculture, Kyoto University.
³Friday Harbor Laboratories, University of Washington
⁴Northwest Fisheries Science Center, National Marine Fisheries Service, NOAA
Introduction

*Heterosigma* red tides and PSP (Paralytic Shellfish Poisoning)

Problems in Puget Sound, WA

- Continuous *Heterosigma* red tides in recent years
  
  Mortality of wild and farmed salmon $2$ million (farmed Atlantic salmon) in 2006 (Rensel 2007)

- Probable mass mortality of juvenile sockeye salmon by *H. akashiwo* in Puget Sound before migrating to Bering Sea (Rensel *et al.*, 2010)

- Frequent occurrences of high levels of PSP has increased since 1957 (WDOH) (Shellfish harvesting closures)

Urgent need to establish mitigation strategy of HABs
Introduction

Using algicidal bacteria as Bio-control of HABs

What is Algicidal Bacteria?

Definition: bacteria which not only compete for nutrients, but actively attack and kill microalgae in order to use their organic matter for growth (Imai 2011)

Algicidal bacteria increase drastically at the end of a red tide (Hallegraeff et al., 2002; Imai 1998b; Kim et al., 1998)

Suggesting

- Algicidal bacteria play important role in decay process of red tide
- Potential use as ecologically friendly counter-measure for HABs
Introduction

Culture studies on algicidal bacteria

Algicidal bacteria from coastal water around the world
- Algicidal range (specific to certain HABs)
- Density to cause algicidal activity
- Killing mechanism (Direct attack or indirect attack)
- Phylogenetic characterization

However,

Very few ecological studies of algicidal bacteria in natural seawater

Where are algicidal bacteria most abundant??

Scale Bar= 50μm
**Introduction**

Seagrass bed as enormous source of algicidal bacteria in Japan

<table>
<thead>
<tr>
<th>HAB species</th>
<th>(CFU / g wet weight)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Heterosigma akashiwo</em></td>
<td>$4.30 \times 10^5$</td>
<td>(Kuroda 2011)</td>
</tr>
<tr>
<td><em>Chattonella antiqua</em></td>
<td>$5.12 \times 10^7$</td>
<td>(Inaba 2013)</td>
</tr>
<tr>
<td><em>Cochlodinium polykrikoides</em></td>
<td>$2.76 \times 10^7$</td>
<td>(Imai <em>et al.</em>, 2009)</td>
</tr>
<tr>
<td><em>Karenia mikimotoi</em></td>
<td>$6.43 \times 10^7$</td>
<td>(ibid.)</td>
</tr>
<tr>
<td><em>Alexandrium tamarense</em></td>
<td>$4.60 \times 10^5$</td>
<td>(Onishi 2010)</td>
</tr>
</tbody>
</table>

*The loss of habitat for algicidal bacteria may have contributed to increased outbreaks of HABs in the coastal zones in recent decades.*

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**Objective**

To investigate algicidal bacteria associated with seagrass and macroalgae beds in Puget Sound: seeking prevention strategies for HAB problems
Material and Methods

Puget Sound, WA, USA

2012 (June - July)

2013 (July)

Sampling

Seagrass

Z. marina

Z. japonica

P. scouleri

Green algae

U. lactuca

Enteromorpha sp.

Brown algae

S. sessile

F. distichus

N. luetkeana

49.0 N

48.0 N

47.0 N

123.5 W
Materials and Methods

Isolation of bacteria

Seawater sample

*Glutaraldehyde fixation for direct counts (DAPI count)

Particle-associated bacteria (PAB)
Free-living bacteria (FLB)

Z. marina

Shake 500 times (Autoclaved seawater)

Dilution

1/10 …… 1/1000 fold

Hand Pump

Pore diameter (3 μm)

Stationary culture (20°C)

Isolation

1/10 …… 1/10000 fold

Particle-associated bacteria (PAB)
Free-living bacteria (FLB)
Materials and Methods

Co-Culture Experiment

Dilution of algal culture (about $10^3$ cells/mL)

- Algal culture
  - Modified SWM-3

- Dilution of algal culture
  - 0.8 mL
  - Control Culture

- Incubate 1 or 2 days

- Inoculation of bacteria
  - Two wells for each

Detecting
- Algicidal Bacteria
- Growth-Inhibiting Bacteria (GIB)

*Condition of Incubation
(20°C • Light: 14h Dark: 10h • about 100 μmol photons/ m²/ sec)

16S ribosomal RNA Analysis
Results  Density of algicidal bacteria (AB) and Growth-inhibiting bacteria (GIB) detected in 2012

(Target species: *H. akashiwo*)

![Graph showing density of AB and GIB in Padilla Bay and Dumas Bay for *H. akashiwo* and *A. tamarense*]

- **H. akashiwo**
  - Algicidal bacteria (AB): *Z. marina* $2.8 \times 10^6$ CFU/ g wt
  - Seawater: $1.8 \times 10^1$ – $1.6 \times 10^3$ CFU/ mL
  - Growth-inhibiting bacteria (GIB): *U. lactuca* $3.3 \times 10^7$ CFU/ g wt
  - Seawater: $4.2 \times 10^2$ – $2.8 \times 10^3$ CFU/ mL

(Target species: *A. tamarense*)

- **A. tamarense**
  - Algicidal bacteria (AB): *Seawater* $6.3 \times 10^2$ CFU/ mL
  - Growth-inhibiting bacteria (GIB): *Z. marina* $8.3 \times 10^6$ – $1.5 \times 10^7$ CFU/ g wt
  - Seawater: $7.5 \times 10^2$ – $4.1 \times 10^3$ CFU/ mL
Results AB and GIB from different seagrass and macroalgae in 2013
(Target species: *H. akashiwo* 893)

<table>
<thead>
<tr>
<th>Control</th>
<th>Killed cells</th>
<th>AB</th>
<th>GIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z. marina</td>
<td>Z. japonica</td>
<td>Z. marina and U. lactuca</td>
<td>&gt;10^8 CFU/g wt</td>
</tr>
<tr>
<td>U. lactuca</td>
<td>U. lactuca</td>
<td>Z. japonica and U. lactuca</td>
<td>ca. 10^6 - 10^8 CFU/g wt</td>
</tr>
</tbody>
</table>

(Target species: *A. tamarense*)

<table>
<thead>
<tr>
<th>Control</th>
<th>Killed and lost motility</th>
<th>GIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z. marina, Z. japonica, and U. lactuca</td>
<td>Z. marina, Z. japonica, and U. lactuca</td>
<td>ca. 10^5 - 10^8 CFU/g wt</td>
</tr>
</tbody>
</table>
Results Phytoplankton composition at Seagrass Beds in 2013

<table>
<thead>
<tr>
<th>Location</th>
<th>Centric diatoms</th>
<th>Pennate diatoms</th>
<th>Dinoflagellates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Padilla Bay</td>
<td>25%</td>
<td>75%</td>
<td>0%</td>
</tr>
<tr>
<td>Cattle point</td>
<td>25%</td>
<td>50%</td>
<td>25%</td>
</tr>
<tr>
<td>Sucia</td>
<td>50%</td>
<td>25%</td>
<td>25%</td>
</tr>
<tr>
<td>FHL</td>
<td>75%</td>
<td>25%</td>
<td>0%</td>
</tr>
<tr>
<td>Potlatch</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

- **Centric diatoms**
  - *Cylindrotheca closterium* 20μm
  - *Odontella spp.* 25μm
  - *Detonula pumila* 20μm

- **Pennate diatoms**
  - *Licmophora spp.* 20μm

- **Dinoflagellates**
  - *Dinophysis spp.* 20μm
  - *Amphiprora spp.* 50μm
Results

Investigating *H. akashiwo* and *Alexandrium* Cyst at eelgrass beds in 2013

* Westcott Bay (Seagrass bed recently disappeared)

<table>
<thead>
<tr>
<th>sampling station</th>
<th>cells/g wet sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Westcott Bay</td>
<td>3400</td>
</tr>
<tr>
<td>Sucia</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Padilla Bay</td>
<td>&lt;200</td>
</tr>
<tr>
<td>North Berlinham Bay</td>
<td>&lt;200</td>
</tr>
<tr>
<td>FHL</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Potlatch</td>
<td>&lt;200</td>
</tr>
</tbody>
</table>

Cyst of *H. akashiwo* detected only from Westcott Bay where eelgrass bed recently disappeared (3400 cells/g wet sediment)
Summary

- First report on algicidal and growth-inhibiting bacteria associated with seagrass and macroalgae in Puget Sound
- Algicidal and growth-inhibiting bacteria were detected high density from *Z. marina*, *Z. japonica* and *U. lactuca*
- Bacteria isolated from eelgrass at Padilla Bay showed algicidal or growth-inhibiting activity against all tested harmful algae
- Very few harmful algal species observed in the seawater at eelgrass beds
- *Heterosigma* cyst were only detected from Westcott Bay where eelgrasses recently disappeared
Preservation and restoration of seagrass and macroalage beds to create potential environments for the prevention of harmful algal blooms.
Thank you very much for listening!!

Acknowledgement

Funding from E.S. Morse institute
(Research exchange program between FHL, UW and marine labs in Japan)

Friday Harbor Labs, UW
Marco Hatch (Northwest Indian College)

Nobuharu Inaba, Hokkaido University
n_inaba84@fish.hokudai.ac.jp
## Results

### List of phytoplankton

<table>
<thead>
<tr>
<th>Phytoplankton</th>
<th>Padilla Bay</th>
<th>Cattle point</th>
<th>Sucia</th>
<th>FHL</th>
<th>Potlatch</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cerataulina pelagica</em></td>
<td></td>
<td><em>Pseudo-nitzschia</em> spp.</td>
<td><em>Odontella</em> spp.</td>
<td><em>Cylindrotheca / Nitzschia</em></td>
<td><em>Navicula</em> spp.</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em></td>
<td></td>
<td></td>
<td></td>
<td><em>Pseudo-nitzschia</em> spp.</td>
<td><em>Bacillaria</em> spp.</td>
</tr>
<tr>
<td><em>Melosira</em> spp.</td>
<td></td>
<td></td>
<td></td>
<td><em>Diploneis</em> spp.</td>
<td><em>Cylindrotheca / Nitzschia</em></td>
</tr>
<tr>
<td><em>Paralia sulcata</em></td>
<td></td>
<td></td>
<td></td>
<td><em>Cymbella</em> spp.</td>
<td><em>Rhizosolenia</em> spp.</td>
</tr>
<tr>
<td><em>Pseudo-nitzschia</em> spp.</td>
<td></td>
<td></td>
<td></td>
<td><em>Licmophora</em> spp.</td>
<td><em>Paralia sulcata</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Cocconeis</em> spp.</td>
</tr>
</tbody>
</table>
Puget Sound 2014

・アマモ場海水にH.a人工赤潮添加によるマイクロコズム実験
・Padilla Bayにて経時的な殺藻・増殖阻害細菌、植物プランクトン組成の調査
（*二者培養試験に用いる細菌数を多くする、アマモ数個体を用い検証）
・NOAAのH.akashiwo有菌株から優占細菌株を分離し、増殖速度を蛍光値で測定
Heterosigma-associated bacteriaの藻類株に対する影響を評価。
増殖及び増殖阻害細菌といった細菌との3者培養を通して現場海水中で起きている現象をより現実的に再現
・アマモ場の規模と殺藻細菌の供給量の推定
・殺藻及び増殖阻害細菌の遺伝子解析
・アマモ場の泥（アマモ消失エリアの調査）
・強力な殺藻細菌の分離（詳細な性状解析）

今後
・人工デトライタス添加による殺藻能の検証
・殺藻細菌のクオラムセンシング機構解明（AI-1及びAI-2両方を検証）
Cyst detection of *H. akashiwo* and *Alexandrium* by MPN

1. **Mixing**
   
2. **Suspend into sterile filtered seawater**

3. **Serial dilutions**
   (Modified SWM-3 + GeO₂ 1 mg/L)

4. **Incubation**
   (14 days, 20°C, 14 h Light:10 h Dark, 50 µmol photons m⁻² s⁻¹)

5. **Identification**
   (Inverted light microscope)
   Vegetative cells = Positive
   → Count the number of positive wells (each dilution)

6. **Calculation MPN cells cm⁻³ wet sediment**
   By statistical tables
   (Throndsen, 1978; Itoh and Imai, 1987).
Results (2012)
Algicidal bacteria (AB) and Growth-inhibiting bacteria (GIB) detected from seawater at eelgrass beds and offshore

(Target species: *H. akashiwo* 893)

<table>
<thead>
<tr>
<th>Eelgrass Beds</th>
<th>Offshore</th>
</tr>
</thead>
<tbody>
<tr>
<td>① 6/9 Jackles Lagoon</td>
<td>①5 6/11 ES10</td>
</tr>
<tr>
<td>② 6/18 North Padilla Bay</td>
<td>②6 6/22 JPN001</td>
</tr>
<tr>
<td>③ Samish Bay</td>
<td>③ 7/2 South Padilla Bay</td>
</tr>
<tr>
<td>④ South Padilla Bay</td>
<td>④ 7/3 Dumas Bay</td>
</tr>
<tr>
<td>⑤ 6/20 Shallow Bay, Sucia</td>
<td>⑤ 7/4 Potlatch State Park</td>
</tr>
<tr>
<td>⑥ Beach Heaven</td>
<td>⑥ 7/5 Cornet Bay</td>
</tr>
<tr>
<td>⑦ North Bay</td>
<td>⑦ Holmes Harbor</td>
</tr>
<tr>
<td>⑧ 6/22 Barlow Bay</td>
<td>⑧ 6/22 Offshore</td>
</tr>
<tr>
<td>⑨ 7/2 South Carkeek</td>
<td>⑨7 6/22 Offshore</td>
</tr>
<tr>
<td>⑩ 7/3 Dumas Bay</td>
<td>⑩7 6/22 Offshore</td>
</tr>
<tr>
<td>⑪ Lynch Cove</td>
<td>⑪7 6/22 Offshore</td>
</tr>
<tr>
<td>⑫ 7/4 Potlatch State Park</td>
<td>⑫7 6/22 Offshore</td>
</tr>
<tr>
<td>⑬ 7/5 Cornet Bay</td>
<td>⑬7 6/22 Offshore</td>
</tr>
<tr>
<td>⑭ Holmes Harbor</td>
<td>⑭7 6/22 Offshore</td>
</tr>
</tbody>
</table>

*AB* and *GIB* detected from seawater at eelgrass beds and offshore.

<table>
<thead>
<tr>
<th>AB</th>
<th>GIB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.8 x 10^1 – 1.6 x 10^3 CFU/mL</strong></td>
<td><strong>4.2 x 10^2 – 2.8 x 10^3 CFU/mL</strong></td>
</tr>
</tbody>
</table>

*AB* and *GIB* detected from seawater at eelgrass beds and offshore.

<table>
<thead>
<tr>
<th>AB</th>
<th>GIB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6.3 x 10^2 CFU/mL</strong></td>
<td><strong>7.5 x 10^2 – 4.1 x 10^3 CFU/mL</strong></td>
</tr>
</tbody>
</table>
## Results

GIB detected from seagrass and macroalgae

(Target species: *K. mikimotoi*)

<table>
<thead>
<tr>
<th>Location</th>
<th>Date</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Padilla Bay</td>
<td>7/3</td>
<td><em>Zostera marina</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Z. japonica</em></td>
</tr>
<tr>
<td>Cattle Point</td>
<td>7/8</td>
<td><em>Z. marina</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>U. lactuca</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Phyllospadix scouleri</em></td>
</tr>
<tr>
<td>Shallow Bay, Sucia</td>
<td>7/10</td>
<td><em>Z. marina</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>U. lactuca</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Enteromorpha spp.</em></td>
</tr>
<tr>
<td>Pier at FHL</td>
<td>7/12</td>
<td><em>Z. marina</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Fucus distichus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Saccharina sessile</em></td>
</tr>
<tr>
<td>Potlatch state park</td>
<td>7/25</td>
<td><em>Z. marina</em></td>
</tr>
</tbody>
</table>

### GIB

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Z. marina</em></td>
<td>3.8 x 10⁷ CFU/g wt</td>
</tr>
<tr>
<td><em>Z. japonica</em></td>
<td>4.7 x 10⁷ CFU/g wt</td>
</tr>
<tr>
<td>Seawater (PAB)</td>
<td>4.3 x 10³ CFU/mL⁻¹</td>
</tr>
<tr>
<td><em>U. lactuca</em></td>
<td>7.0 x 10⁵ CFU/g wt</td>
</tr>
</tbody>
</table>
Results

GIB detected from seagrass and macroalgae

(Target species: *A. tamarense*)

<table>
<thead>
<tr>
<th></th>
<th>CFU / g wet weight or mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z. marina</td>
<td>$10^8$</td>
</tr>
<tr>
<td>Z. japonica</td>
<td>$10^8$</td>
</tr>
<tr>
<td>Z. marina</td>
<td>$10^6$</td>
</tr>
<tr>
<td>U. lactuca</td>
<td>$10^4$</td>
</tr>
<tr>
<td>P. scouleri</td>
<td>$10^2$</td>
</tr>
<tr>
<td>Z. marina</td>
<td>$10^6$</td>
</tr>
<tr>
<td>U. lactuca</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Enteromorpha spp.</td>
<td>$10^2$</td>
</tr>
<tr>
<td>FLB</td>
<td>$10^2$</td>
</tr>
<tr>
<td>PAB</td>
<td>$10^2$</td>
</tr>
<tr>
<td>Z. marina</td>
<td>$10^6$</td>
</tr>
<tr>
<td>S. sessile</td>
<td>$10^6$</td>
</tr>
<tr>
<td>F. distichus</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Z. marina</td>
<td>$10^6$</td>
</tr>
</tbody>
</table>

Killed and lost motility

Control
## Material and Methods

### Sampled species

<table>
<thead>
<tr>
<th>Seagrass</th>
<th>Green algae</th>
<th>Brown algae</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Z. marina</em></td>
<td><em>U. lactuca</em></td>
<td><em>S. sessile</em></td>
</tr>
<tr>
<td><em>Z. japonica</em></td>
<td><em>Enteromorpha sp.</em></td>
<td><em>F. distichus</em></td>
</tr>
<tr>
<td><em>P. scouleri</em></td>
<td></td>
<td><em>N. luetkeana</em></td>
</tr>
</tbody>
</table>