# Culturable algalytic bacteria isolated from seaweeds in the Philippines and Japan

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## ABSTRACT

*Bacteria from seaweeds as epibionts have been reported to have major role/s in algal physiology and survival by secreting compounds with bioactivity like inhibitory/other potential harmful/pathogenic effects. Some of these epibionts have been reported to cause mortality against certain species of noxious phytoplankton, i.e. fish-killing and toxic species. In this study, we report a total of 13 algalytic bacteria isolated and genetically identified from surfaces of seaweeds from Japan and the Philippines, mostly belonging to phylum proteobacteria. The effects on the motility and viability of Alexandrium catenella (Whedon and Kofoid) Balech, Chattonella antiqua (Hada) Ono, Skeletonema costatum (Grev.) Cleve, and Symbiodinium sp. were observed for 48 hours. From Japan, Vibrio sp. from Gracilaria sp. and Ulva sp. were most potent. Pseudoalteromonas sp. from a farmed Kappaphycus alvarezii (Doty) Doty in the Philippines was the fastest killing eliciting 100 % mortality on Pyrodinium bahamense var. compressum (Bohm) Steidinger, Tester and Taylor, during the first 24 h of incubation. Results suggest that athecate species are less resilient than the thecate dinoflagellates. Alterierythrobacter sp. and Ruegeria sp. belonging to subphylum α-proteobacteria that have not yet been previously reported to exhibit algalytic activity were also studied.*

**Key words**: *epibionts, algalytic, bacteria, seaweed, Pyrodinium, Kappaphycus*

## INTRODUCTION

Bacteria are attracted to and can form biofilms on surfaces of other organisms such as seaweeds because of the available nutrients from exudates and photosynthates being released by the host organism (*Bryers et al. 1982, Seymour et al. 2009*). Macroalgae lack cell-based immune systems in which bacterial epibionts can act as the protective immune system by releasing anti-microbial chemicals, which may be competitive in function, i.e., antibiotic properties/inhibition of potential pathogenic competitors and/or epiphytes (*Burgess et al. 1999, Evelyn et al. 2001, Kubanek et al. 2003*). Several bacteria causing mortality to a number of harmful microalgae were isolated from different seaweed surfaces, suggesting the possible role of such organisms in preventing formation of microalgal blooms in seaweed beds (*Imai et al. 2006*). *Fukami et al.* (*1991*) isolated *Flavobacterium* spp. capable of inhibiting growth of the fish-killing *Gymnodinium nagasakiense*. This has been followed by increased studies on algalytic bacteria and the growing investigations on their potential application in mitigating and controlling blooms of noxious red tides or harmful algae (*Imai et al. 1995, 1998; Lovejoy et al. 1998, Doucette et al. 1998, Mayali and Doucette 2002, Mayali and Azam 2004; Imai and Kimura 2008*).

In general, bacterial species that can inhibit growth of algae have been suggested as possible biocontrol either by direct or indirect attacks that are accompanied by secretion of active substances (*Lee et al. 2000*). Such interactions and bioactivity have been also used as model systems in search of novel molecules and compounds that may have potential medical or biotechnological applications (*Bernan et al. 1997*). For example, *Jeoung et al.* (*2003*) was able to isolate and characterize bacillamide from *Bacillus* sp. SY-1 that is active against *Cochlodinium polykrikoides*. An extracellular serine protease has also been isolated from an algalytic *Pseudolateromonas* sp. causing mortality to *Skeletonema costatum* (*Lee et al. 2000*). Biosurfactants from *Pseudomonas aeruginosa* were also tested against *Alexandrium minutum*, *Karenia brevis* and *Pseudonitzschia* sp. (*Gustafsson et al. 2009*).

Different algal species could cause different negative effects such as anoxia leading to fish kills which thus greatly affect the fishery/mariculture industry; and various types of poisonings due to consumption of contaminated seafoods (*Azanza and Taylor 2001*). For example, the main causative organism of Paralytic Shellfish Poisoning (PSP) in the Philippines and in the other Southeast Asian countries is the dinoflagellate *Pyrodinium bahamense* var. *compressum* (*Azanza et al. 1997*), thus there is an increasing necessity for the mitigation and management of these blooms. In the Philippines, several types of harmful phytoplankton blooms have caused human toxicity since 1983 (*Azanza 1997*) and fish kills (*Azanza et al. 2005*) hence several ways of

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managing the phenomena have been studied. These include the use of clay (*Padilla et al. 2010*) and possible biocontrol by means of phagotrophic organisms (*Azanza et al. 2010*) and algalytic bacteria from natural seaweed beds and/or culture areas (*Azanza and Fukami 2007*). Seaweed farming has been one of the largest aquaculture industries in both Japan and the Philippines, providing them a huge resource and amount of opportunity in further investigating such organisms and their bioactivity. However, so far, published works on Philippine marine bacteria associated with seaweeds have been limited to the reports of *Largo et al.* (*1995, 1999*), who mentioned that these organisms might play significant roles in the physiology and ecology of their host seaweeds. In Japan, on the other hand, several reports have already been published describing the isolation of such bioactive bacteria, in other sites particularly the Seto Inland Sea but limited reports have been available for studies in the Uranuochi Inlet (*Fukami et. al 1996, Imai and Kimura 2008*). This study then presents results on the isolation and characterization for algalytic activity of culturable bacteria isolated from the epibiota of seaweeds from the two countries, i.e. the Philippines and Japan.

## METHODS

### Sampling sites

**Bolinao, Pangasinan, Philippines**. Seaweed samples were collected on three occasions – December 2006, June 2007 and March 2009 in Patar, Bolinao (**Figure 1**) where natural seaweed beds are located. Salinity range was 33-35 ppt and temperature range was 31-32 ˚C (UP MSI Bolinao Marine station data).

**Danajon Reef, Bohol**. Sampling was done in March 2007 at Danajon Reef, Bohol, Central Visayas (one of the world's few double barrier reefs). Collections were done in three sites; two sites at the "*Eucheuma*" Farm of Marine Colloid, Inc. (MCPI), and another site where there are no seaweed culture beds (**Figure 1**). Salinity range was at 29-33 ppt and temperature was at 30 $^{o}$C.

**Uranouchi Inlet, Southern Japan**. The Uranouchi Inlet (33$^{o}$ 23 to 28 N and 133$^{o}$ 20 to 28 E) is located almost in the middle of Shikoku Island, (Kochi Prefecture), facing the Pacific ocean (**Figure 1**). The shallow semi-enclosed bay, which is about 5 km long, has been utilized for fish farming thereby exerting pressure on water exchange and causing substantial nutrient deposition in the water column, especially the sediment (*Fukami et al. 1991, Fukami et al. 1999*). There were three study sites, i.e., one each in the innermost part of the inlet (Menokuso), the middle part (Mitsumatsu) and the outermost part (Osaki Cape). Fish farming activities are concentrated in the middle part of the bay. Seaweed species sampled from the inlet were *Ulva* sp., *Gelidium* sp., *Gracilaria* sp. and *Polysiphonia* sp. that were collected in June 2007.

### Seaweed sample collection and bacterial isolation

Seaweed samples were collected following the methods of *Imai et al.* (*2006*). Sterile 500 ml bottles with 200 ml filtered seawater were prepared prior to the field collection. Seaweeds were randomly collected in the site and placed inside the collection bottles in 1:2 seawater to seaweed ratio. Samples were kept inside a cooler in black plastic bags while in transport. In the laboratory, samples that were resuspended in the collection bottle were shaken 100 times to make a concentrated bacterial suspension. The solution then was diluted up to $10^{-3}$ prior to spread plating. This was further diluted up to $10^{-2}$ in prepared diluents of sterile seawater. Samples of each diluent (100 µl of the $10^{0}$, $10^{-1}$ and $10^{-2}$) were plated in Marine Agar (MA) plates and incubated at 25±2 $^{o}$C for seven days. Bacterial colony purification was done by re-streaking single isolated colonies in marine agar plates and maintained by monthly sub-culturing in either MA plates or slants.

For the bioactivity assay, bacterial isolates were grown in Marine Broth (Philippines) and FeTY media (Japan) and incubated at for 25±2 $^{o}$C for 24 hours before being added to microalgal cultures.

### Phytoplankton cultures

Cultures of *P. bahamense* var. *compressum* (Böhm) Steidinger, Tester and Taylor, existing in the laboratory of Dr. RV Azanza at the Marine Science Institute, University of the Philippines, Diliman; PbcMZRVA042595 isolated in Masinloc Bay, Zambales on April 25, 1995 were used. The cultures were maintained in 500 ml Erlenmeyer flask under 12 h dark-light condition and 26±2 $^{o}$C air temperature, sub-cultured every 3 weeks in F/2 culture media (*Azanza-Corrales and Hall 1993*).

For the algalytic bacterial isolates from Japan, the following phytoplankton cultures were obtained from those being maintained at Dr. Fukami's laboratory: *Skeletonema costatum* (Grev.) Cleve NIES-323, *Alexandrium catenella* (Whedon & Kofoid) Balech TB-93, *Chattonella antiqua* (Hada) NIES-1 and *Symbiodinium* sp. The cultures of these prey organisms were maintained in fresh SWM media at 22b $^{o}$C, and 14:10 L:D photoperiod.

### Screening of seaweed epibionts for algalytic bacteria

Determination of biocidal effects of the isolates was done using algal assays described by *Lovejoy et al.* (*1998*) and *Imai et al.* (*2006*). In brief, triplicate 1 ml samples of mono-algal cultures of prey organisms listed above were

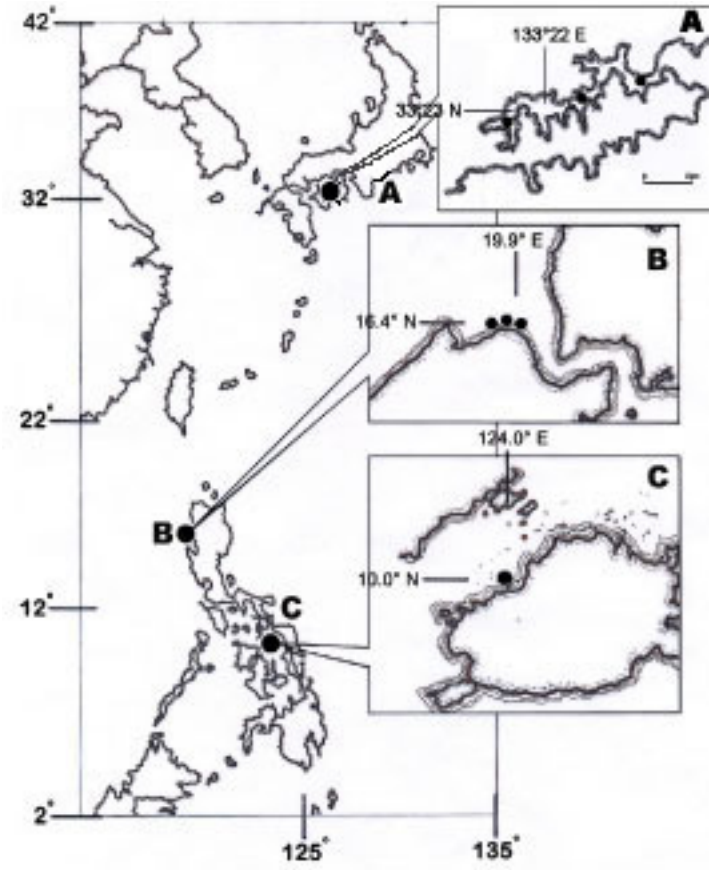


Figure 1. Location of the sampling sites: (A) Uranuochi Inlet, Japan (B) Patar, Bolinao, Pangasinan and (C) Danajon Reef, North of Bohol.

added to 24-well micro plates to which 0.5 ml of diluted seaweed rinses and bacterial cultures in broth were added aseptically and separately. The algal assay plates were monitored every six hours for a period of two days at 25 ºC for Philippine isolates and at 22 ºC for Japanese isolates, and cultures were then monitored daily for a period of 1 week under a Photozoom (Bausch and Lomb) and a Nikon inverted microscope. Index of algalytic effects was based on lysis and death of algal cells. A confirmed algalytic reaction of the isolates was considered when there was a steady increase in algal mortality leading to 100 % mortality within the one-week observation time in at least two of the triplicates. The Most Severe Effect (MSE), particularly loss of motility (cells not motile) and lysis/disintegration (cell membrane disrupted, internal contents in disarray) based on criteria by *Lovejoy et al.* (*1998*) were monitored.

**Identification and characterization of algalytic bacteria**

Identification of isolates was limited to molecular characterization. Isolated bacterial strains that exhibited algalytic activity were identified by PCR amplification of the 16S rDNA gene. DNA extraction was done using QIAgen Blood and Tissue Kit following the manufacturer's protocol. Primers and conditions for the PCR amplification were based on *Azanza et al.* (*2006*). Amplified PCR products were sent to Macrogen Inc., (Korea) for purification and sequencing.

For the Japan isolates, total DNA was extracted with the NucleoSpin Tissue DNA Isolation Kit (Machery Nagel) using the manufacturer's protocol. The 16s rDNA gene was amplified using universal eubacterial (forward primer 27F; *Weisberg 1991* and reverse primer 1492R; *Reysenbach et al. 1992*) in a thermocycler (ASTEC, PC707, Program temperature control system) using a sequencing program of 94 ºC (1 min), 35 cycles of 94ºC (10 sec), 54 ºC (20 sec), 68 ºC (1 min 30 sec) with an extension temperature of 68 ºC (9 minutes). The PCR amplified products were cleaned using QIAquick PCR purification kit (Qiagen) and the purified products were sequenced using ABI PRISM™ DNA analyzer (PE Applied Biosystems, Foster City, CA, USA).

The sequences were first edited in Bioedit v.7.4 (*Tamura et al. 2011*, *http://www.mbio.ncsu.edu/BioEdit/bioedit.html*). Edited sequence data were analyzed by comparison with 16s rDNA genes using Basic Local Alignment Search Tool (BLAST) against the NCBI database (*Altschul et al. 1990*). The closest and related sequences were downloaded for phylogenetic analysis. Sequences of bacterial species that were isolated from seaweed rinses that have shown algalytic activity were retrieved from the GenBank and realigned to produce a phylogenetic tree which includes the new algalytic bacterial species from Japan and the Philippines. Alignment was done by ClustalW in BioEdit and a model test was conducted for the dataset using MEGA v.5.0 (*http://megasoftware.net/*). Phylogenetic tree was generated using the Maximum Likelihood with bootstrap support by resampling 1000 times.

**RESULTS**

**Algalytic Activities of Isolated Bacteria**

Only rinses of *Kappaphycus alvarezii* and *Sargassum* spp. were able to show algalytic activity against *P. bahamense* var *compressum* for the preliminary screening; thus, they were the ones only used for other applications such as bacterial isolation. However, bacteria grown from *Sargassum* spp. rinse did not show any algalytic activity against *Pyrodinium* cells. On the other hand, only five bacterial isolates from the *Kappaphycus* rinse were able to exhibit algalytic activity against the same test species, and only two were genetically identified (**Table 1**).

A total of 41 morphologically distinct bacteria were grown from different rinses of seaweeds from the Uranouchi Inlet, however, only 10 have shown biocidal effects against the test organisms. Of the four isolates from *Ulva* rinses, only one showed algalytic activity while two of the nine isolates from *Polysiphonia* sp. showed capacity to elicit algal lysis (**Table 1**). Samples from *Gracilaria* sp. epibionts from Japan had the most number of bacterial isolates with the highest diversity.

Table 1. Number of algicidal bacteria isolated from different seaweed rinses from the Philippines tested against *Pyrodinium bahamense* var. *compressum* and from Uranuochi Inlet, Japan tested on *Alexandrium*, *Chatonella*, *Skeletonema* and *Symbiodinium*.

| Source of Seaweed Rinse | Source of seaweed | Seaweed rinse with algicidal activity | Trials | Number of isolates | Number of isolates with algicidal activity |
|---|---|---|---|---|---|
| *Kappaphycus alvarezii* | Danajon reef, Bohol (seaweed culture site) | + | 1 | 13 | 3 |
| | | | 2 | 12 | 2 |
| *Sargassum* sp. | Patar, Bolinao (natural seaweed site) | + | 1 | 16 | 0 |
| | | | 2 | 5 | 0 |
| *Hydroclathrus* sp. | Patar, Bolinao (natural seaweed site) | - | 1 | 0 | 0 |
| | | | 2 | 0 | 0 |
| *Padina* sp. | Patar, Bolinao (natural seaweed site) | - | 1 | 0 | 0 |
| | | | 2 | 0 | 0 |
| *Enteromorpha* sp. | Patar, Bolinao (natural seaweed site) | - | 1 | 0 | 0 |
| | | | 2 | 0 | 0 |
| *Ulva* sp. | Uranouchi Inlet, Japan | + | 3 | 4 | 1 |
| *Gelidium* sp. | Uranouchi Inlet, Japan | + | 3 | 12 | 2 |
| *Gracilaria* sp. | Uranouchi Inlet, Japan | + | 3 | 16 | 5 |
| *Polysiphonia* sp. | Uranouchi Inlet, Japan | + | 3 | 9 | 2 |

Most of the identified species exhibiting algalytic activity belongs to the genera *Vibrio* or *Pseudoalteromonas* (**Figure 2**). *Pseudoalteromonas* sp., *Vibrio* sp. and *Ruegeria* sp. have all elicited lethal effect to fish-killing prasinophyte *C. antiqua* resulting to 100 % mortality after seven days compared to the other three microalgal species which only showed ≥50 % mortality for the same period of incubation. *Halomonas* sp. from *Gracilaria* sp. on the other hand only caused more than 50 % mortality on the same test organism. Isolates from the rest of the seaweed rinses from Japan exhibited lesser potency than those from *Gracilaria*. Bacteria from *Gelidium* sp., *Ulva* sp., and *Polysiphonia* sp. have only shown loss of motility and death to more than 50 % of the population of the test species.

On the other hand, the *Pseudoalteromonas* sp. isolate from the Philippines showed the fastest rate of algalytic activity on *Pyrodinium* which had its motility and cell integrity significantly affected on the first hour.

**Identification of isolated algalytic bacteria**

The closest 16s rDNA sequence similarity of some isolated bacterial strains to sequences in the NCBI database using the BLAST algorithm is shown in **Table 2**. Majority of the isolates are gram-negative rods belonging to the division Proteobacteria subdivision gamma– *Pseudoalteromonas* sp., *Halomonas* sp. and *Vibrio* sp.

**Phylogenetic diversity of isolated algalytic bacteria**

Molecular identification of the cultivable bacteria isolated from seaweed rinses revealed interesting species that have not been reported previously. Species that were identified in this study have shown 95 % to 100 % similarity when compared to known 16s rDNA sequences in GenBank database and supported by high bootstrap values in the phylogenetic tree (**Figure 3**). Identified putative species from the Philippines and Japan include those related to *Pseudoalteromonas* species, 2 most related to *P. citrea* (Japan 12 and 16) and the other 2 most related to *P. rubra* (Japan 1 and a Philippine isolate). Some were also identified as *Vibrio* sp. (four isolates), *Ruegeria* sp., *Flavobacteriaceae bacterium*, *Alterierythrobater* sp., *Cytophaga* sp. and an unidentified sequence related to *Halomonas* sp.

Most algalytic species belong to three prominent groups, namely γ-Proteobacteria, α-Proteobacteria and those from the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex. Those from Phylum Proteobacteria dominated majority of the species that have been identified to exhibit algalytic effect with seven species from gamma and only two species from alpha. Furthermore, in terms of number of strains that have been identified in this study, most isolates were from genus *Pseudoalteromonas* and *Vibrio*. Most of the isolates from the Philippines came from *K. alvarezii*, which totaled to five (5) algalytic bacteria. However, only two isolates were processed for molecular identification. The same number of isolates was also found in *Gracilaria* sp. rinses in Japan, all of which were identified genetically.

Interestingly, new species that have not been previously reported were identified in this study to exhibit algalytic activity. The Philippine isolate *Alterierythrobacter* sp. (Euc1) from a natural seaweed bed in Danajon Reef in Bohol and the *Ruegeria* sp. from Japan are addition to the bacterial isolates that have shown activity against dinoflagellate species.

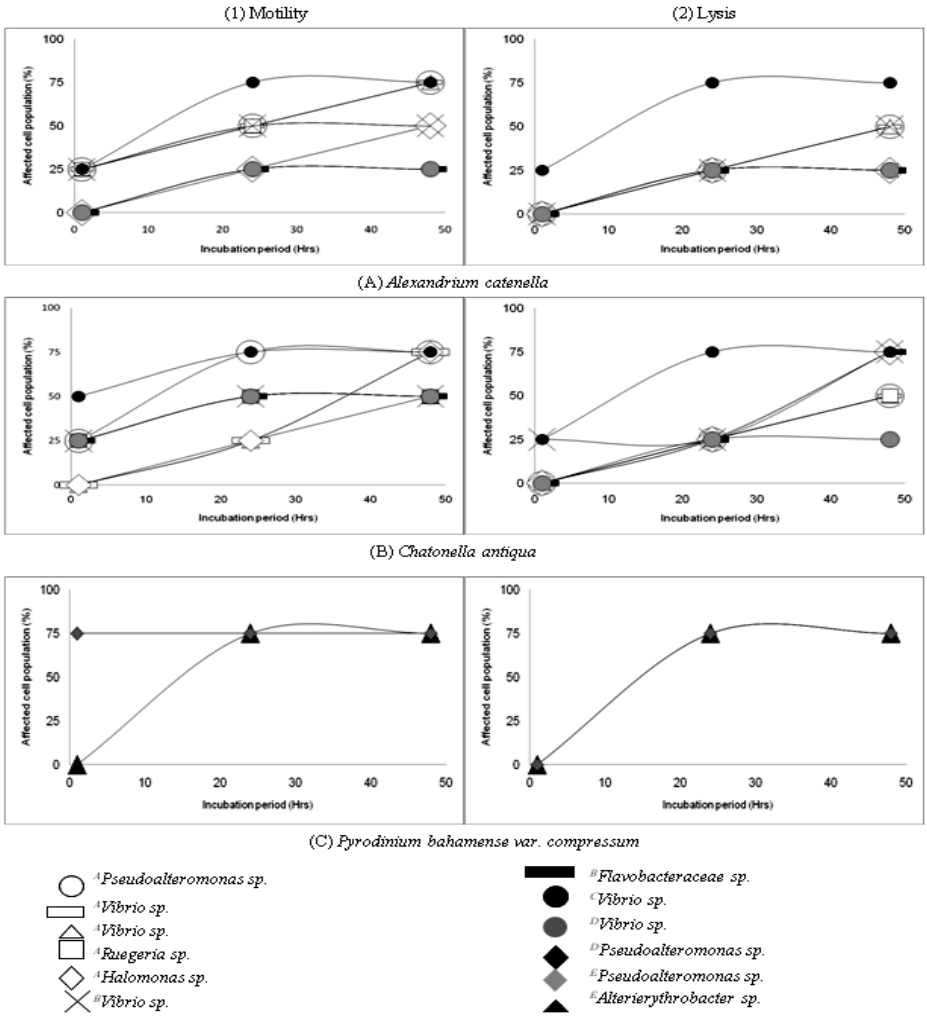


Figure 2. Summary of the algicidal activities of the bacterial species isolated from different seaweed rinses and their effect on the motility and morphology of the test organisms.

Table 2. Genetic affiliation of the 16s rDNA gene of the bacterial isolates from the seaweed rinses from the Philippines and Japan based on NCBI's BLAST that were verified by phylogenetic analysis (see **Figure 3**).

| Isolate no./ code | Origin of isolates | Genetic affiliation | Sequence Similarity[a] (%) | Base pair alignment[b] (Query/Subject) | NCBI Accession No. of Ref. Sequence |
|---|---|---|---|---|---|
| 1 | Uranuochi Inlet, Japan | *Pseudoalteromonas* sp. | 98 % | 688/695 | AB681567.1 |
| 2 | Uranuochi Inlet, Japan | *Vibrio* sp. | 99 % | 734/738 | GU070663.1 |
| 3 | Uranuochi Inlet, Japan | *Vibrio* sp. | 99 % | 620/622 | GU371666.1 |
| 4 | Uranuochi Inlet, Japan | *Cytophaga* sp. | 95 % | 622/653 | DQ395043.1 |
| 5 | Uranuochi Inlet, Japan | *Ruegeria* sp. | 96 % | 655/679 | JX075061.1 |
| 6 | Uranuochi Inlet, Japan | *Vibrio* sp. | 99 % | 626/630 | EU077544.1 |
| 12 | Uranuochi Inlet, Japan | *Pseudoalteromonas* sp. | 97 % | 702/720 | GU361127.1 |
| 13 | Uranuochi Inlet, Japan | *Vibrio* sp. | 98 % | 786/802 | GU070663.1 |
| 14 | Uranuochi Inlet, Japan | *Flavobacteriaceae* bacterium | 100 % | 615/615 | AB106141.1 |
| 15 | Uranuochi Inlet, Japan | Uncultured bacterium | 100 % | 693/693 | HM142471.1 |
| 16 | Uranuochi Inlet, Japan | *Pseudoalteromonas* sp. | 100 % | 676/676 | JX075056.1 |
| Euc2 | Bolinao, Philippines | *Pseudoalteromonas* sp. | 99 % | 687/694 | AB681567.1 |
| DR-R | Danajon Reef, Bohol, Philippines | *Alterierythrobacter* sp. | 100 % | 697/697 | EU440971.1 |

[a]Full name or description of the closest matched sequence.
[b]Maximum percentage of identical nucleotides within the c noted alignment length.

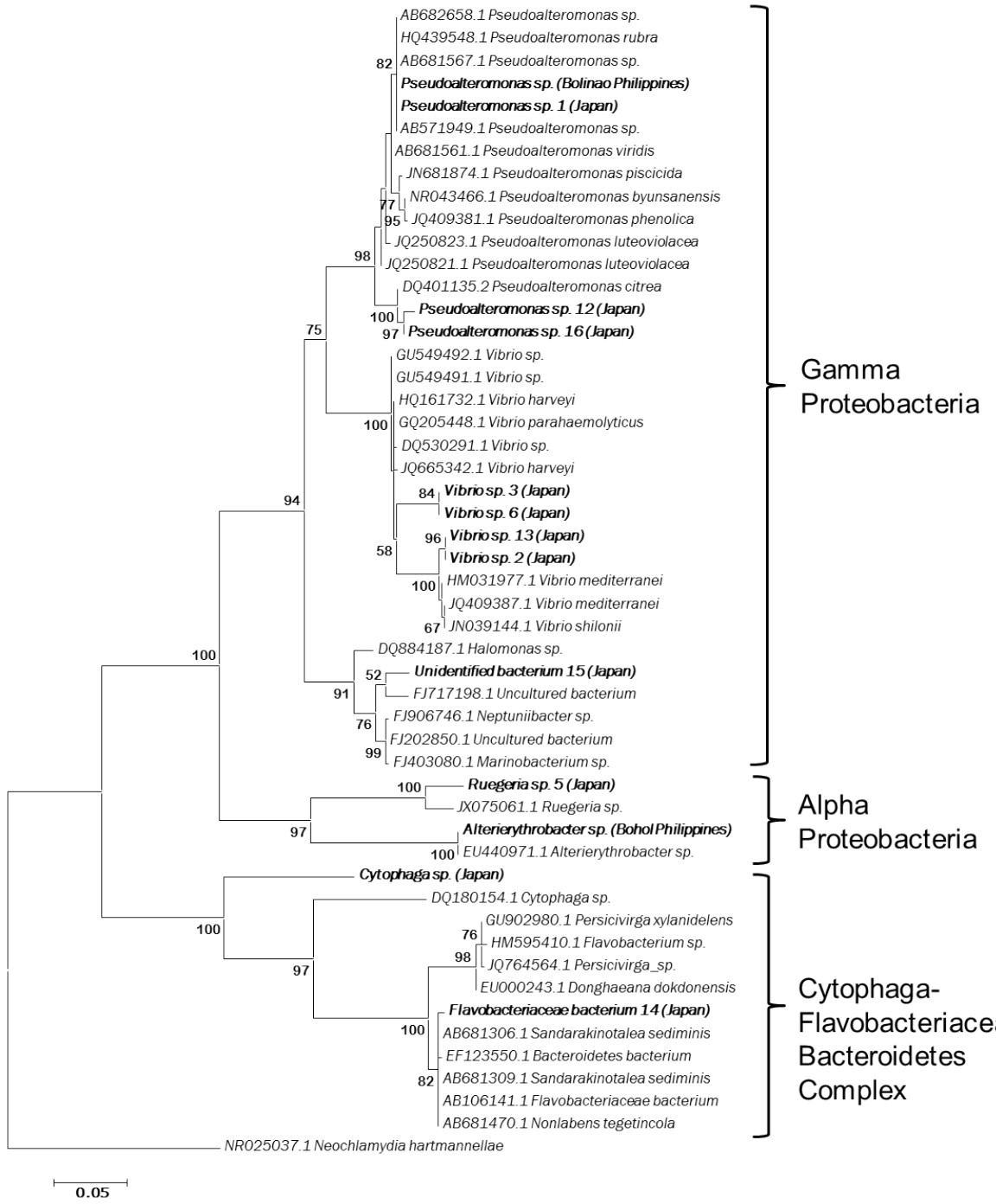


Figure 3. A Maximum Likelihood tree based on the 16s rDNA gene fragments of the bacterial isolates with bootstrap supports generated by resampling 1000 times. Accession numbers precede the identity of the reference sequences.

## DISCUSSION

### Algalytic activity of bacterial isolates and resiliency of algal test species

Same species of bacteria isolated from different seaweeds vary in their algalytic effects against the same test microalgae. For example, *Vibrio* sp. from *Gracilaria* were able to cause 100 % mortality against *C. antiqua* after two days and 50 % loss of motility to *S. costatum* and *Symbiodinium* sp. after the same period. On the other hand, the *Vibrio* isolate from *Polysiphonia* sp. caused 100 % mortality for both *Chattonella* and *Alexandrium* just after 24 hours. The same can be observed with the isolated *Pseudoalteromonas* sp. in which the one from *Gracilaria* had higher biocidal activity compared to the one isolated from *Polysiphonia* sp. Accordingly, differences in the algalytic effects of the same bacterial strain on different phytoplankton species showed specific/varied resistance of the test organisms. For instance, the fastest killing isolate *Pseudoalteromonas* sp. from both the Philippines and Japan had different effects on the different test organisms. In this study, the said species was able to kill *Chattonella* and *Alexandrium* but was ineffective against *Skeletonema* and *Symbiodinum*.

*Fukami et al.* (*1992*) reported that a strain of *Flavobacterium* sp. 5N-3 killed *Gymnodinium nagasakiense* (*Karenia mikimotoi*), however, it had no apparent effects on other red tide plankton species of *Skeletonema costatum*, *Chattonella antiqua* and *Heterosigma akashiwo*. *Kim et al.* (*2009*) also reported the possible specificity of action when they observed that the algalytic *P. haloplanktis* AFMB-08041 was only able to lyse two species of *Prorocentrum*, but it was inefficient against the other dinoflagellates such as *Alexandrium*, *Cochlodinium* and *Gymnodinium* among others. In another study, the same species was found to produce extracellular protease that is effective against *S. costatum*, allowing the bacteria to cause algal lysis by indirect attack (*Lee et al. 2000*). *Lovejoy et al.* (*1998*) also reported the algalytic activity of the same species of bacteria isolated from an estuary against some gymnodinids, rapidophytes and dinoflagellates, but it had no effect on some cyrptomonad, diatom and cyanobacterium species which were attributed to intrinsic characteristics such as hard cell wall, capability to ingest bacterial particles or revert to protective forms. On the other hand, the differences in the rate of algalytic effects on test phytoplankton/algal species as observed *in vitro* may also be attributed to several factors, like initial concentration of prey/test organisms and of the algalytic bacteria (*Fukami et al. 1991, Mayali and Doucette 2002*).

It seems that among the test organisms, the unarmored species like *Chattonella* were more prone to algalytic effects than the others. *Chattonella* was the least resilient against all the Japan isolates. The same observations were reported by *Imai and Kimura* (*2008*) when 6 different bacterial strains with direct and indirect modes of attack elicited 100 % mortality against *C. antiqua*. Possibly, the non-athecate characteristic of the rapidophyte makes it more exposed and vulnerable to both bacteria attaching to its surface and the compounds being released in the surrounding area. On the other hand, the armored *A. catenella* has shown more resiliency but *S. costatum* and *Symbiodinium* sp. were the most resistant for all the treatments used, though, algalytic effects on *Alexandrium* spp. have already been reported by *Lovejoy et al.* (*1998*).

Tests against *Pyrodinium* have shown 50-100 % mortality for all the tested algalytic bacteria. Studies have indicated that dinoflagellates undergo pellicle encystment when it experience harsh conditions including bacterial attack (*Nagasaki et al. 2000; Tarutani et al. 2001; Onda et al. In Prep.*). In this study, bacterial presence resulted to ecdysis in the *Pyrodinium*. However, the temporary encasement had not been enough in protecting itself against complete lysis caused by the algalytic bacteria. The inefficiency of encystment might have been limited by time, such that the rate of bacterial attack is faster than the time needed by the algae to fully develop into temporary cysts, or that the compounds being secreted by the algalytic bacteria are strong enough to degrade even the cyst wall.

Interestingly, the rinses from the *Sargassum* sp. have shown 100 % *Pyrodinium* cell death but no algalytic bacteria were isolated from it. Such activity could have been possibly due to the seaweed's by-products as they are known to produce some macromolecules that have anti-fouling or antibacterial activities. The same defense mechanism was observed in the Australian seaweed *Delisea pulchra* which appears to be capable of controlling the colonization of bacteria by producing compounds that interfere in the colonization trait of the bacteria (*Steinberg et al. 1998*). Another possibility is the presence of unculturable algalytic bacterial species since only 1 % of the bacterial population present in an mL of seawater can actually be cultured and isolated (*Alavi et al. 2001*).

**Genetic diversity**

In this study, a total of 12 algalytic bacteria have been isolated from the different seaweed epibionts belonging to four different major groups. Many of the previously reported algalytic bacteria from seaweeds were almost the same with those that have been isolated from Japan and the Philippines. In the study by *Imai et al.* (*2006*), algalytic bacteria from the surface of *Ulva* sp. and *Gelidium* sp. belonged to the genera *Alteromonas*, *Pseudoalteromonas*, *Vibrio*, *Cytophaga*, *Cellulophaga* and *Octadecabacter*, and the family Rhodobacteraceae. Such strains were observed to cause lysis of a wide number of species belonging to Bacillariophyceae, Raphidophyceae and Dinoflagellates (*Imai et al. 1995, Mayali and Azam 2004*).

The two most abundant species in terms of strains isolated were from *Vibrio* and *Pseudoalteromonas*, which were also from the same subphylum. *Pseudoalteromonas* belongs to subphylum gammaproteobacteria that is being considered as one of the most abundant and flexible species in terms of habitat. Studies have shown that related species such as *Pseudoalteromonas rubra* and *P. auranta* are capable of antibiotic production (*Vijaya and Veera 2007*), thus, they may play a symbiotic role in warding off potential pathogenic bacteria in the surface of the seaweed. Our isolates are most related to *P. rubra* and *P. citrea*. Interestingly, several clades of *Pseudoalteromonas* and its relatives were shown to possess different genes capable of degrading seaweed carbohydrates such as k-carrageenases, ß-porphyrases and ß-agarases (*Hehemann et al. 2010*), which could also possibly explain why they are abundant in the seaweed beds.

Reports showed that some strains of marine bacterium *Vibrio* sp. were found to produce alginate lyase, the enzyme that is active in degrading alginate or alginic acid. Alginate is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively, and is the main anionic polysaccharide structure of most brown and red algae (*Wang*

*et al. 2006*). The same bacterial species was also found to promote "ice-ice disease" resulting to the whitening and decay of seaweed tissues in *Kappaphycus alvarezii* (*Largo et al. 1995; Largo et al. 1999*). It is interesting to note that *Vibrio* sp. isolates from this study were from rinses of red algae *Gelidium* sp., *Gracilaria* sp. and green *Ulva* sp. The same bacteria were also abundant in the surfaces of *Ulva pertusa* and *Porphyra yezoensis* collected from the seashore of Jiaozhou Bay, Qingdao, China (*Duan et al. 1995*). Furthermore, some strains of *Vibrio* sp. isolated from the seawater collected in Sagami Bay in Kanagawa Prefecture, Japan exhibited the production of agarase, the enzyme which degrades agar that is found in the cell wall of most agarophytes. In addition, a novel endotype beta-agarase enzyme was also isolated from the bacterium *Vibrio* sp. JT0107 and decomposes the alginic acid component of *Laminaria* sp. and *Undaria pinnatifida* (*Sugano et al. 1993*). Such studies suggest that *Vibrio* species may actually be a naturally occurring parasite compared to the more symbiotic *Pseudolateromonas*, since their infective activity have also been reported in other species such as humans (*Schmidt et al. 1979*), corals (*Banin et al. 2000*) and others. Thus, it could be expected then that the *Vibrio* would be absent in a healthy macroalga, as what has been observed in this study.

Another significant finding of this research is the isolation of two other bacterial species that have not been previously reported to exhibit algalytic activity. Both *Alterierythrobacter* sp. and *Ruegeria* sp. have shown high algalytic activity against the test dinoflagellate species. Both species belong to subphylum alphaproteobacteria which are mostly symbiotic and very abundant in the environment. Some studies reported that a soil isolate of *Alterierythrobacter* is capable of hydrocarbon degradation such as hexadecane (*Shibata and Robert 2009*) but their presence has not yet been fully explored in biological systems such as seaweed surfaces. The isolation of *Ruegeria* that has algalytic properties is also unusual primarily because many studies have shown that such species is commonly associated or a symbiont (mutualist/commensalist) to a number of dinoflagellates such as *Gymnodinium catenatum*, *Pfiesteria* sp., *Scrippsiella* sp., *Prorocentrum lima*, *Alexandrium* sp. and *A. tamarense* (*Alavi et al. 2003, Green et al. 2004, Hold et al. 2001, Lafay et al. 1995, Jasti et al. 2005*). Most species under this group have been known to be phototrophs, a special character of the said subphylum, allowing them to live independently without the need of degrading substrates/ preying on hosts. Thus, the report of algalytic activity in these species which make them in a way parasitic (*Imai et al. 1993*) is unusual, since most of the species belonging to alphaproteobacteria are symbiotic or mutualistic.

## CONCLUSION AND RECOMMENDATION

With regards the potential use of the isolated bacterial species and/or their bioactive compounds against harmful microalgae, further studies are needed before algalytic bacteria could be implemented as a management measure for HABs or any other environmental problems.

## REFERENCES

Alavi, M., T. Miller, K. Erlandson, R. Schneider and R. Belas. 2001. Bacterial community associated with Pfiesteria-like dinoflagellate culture. Environ. Microbiol. 3:380-396.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Azanza-Corrales R. and S. Hall. 1993. Isolation and culture of *Pyrodinium bahamense* var. *compressum* from the Philippines. In: Smayda, YJ, Shimizu, Y (Eds.) Toxic Marine Phytoplankton Blooms in the Sea. Elsevier Science Publishers, Amsterdam, pp 725–730.

Azanza R.V. 1997. Contributions to the understanding of the bloom dynamics of *Pyrodinium bahamense* var *compressum*: A red tide causative organism. Science Diliman. 9(1&2):1-6.

Azanza, R. V. and F. J. R. Max Taylor. 2001. Are *Pyrodinium* blooms in the Southeast Asian region recurring & spreading? A view at the end of the Millennium. Ambio. 30(6):356-364.

Azanza, R.V., Y. Fukuyo, L.G. Yap and H. Takayama. 2005. *Prorocentrum minimum* bloom and its possible link to a massive fish kill in Bolinao, Pangasinan, Northern Philippines. Harmful Algae. 4(3): 519-524.

Azanza, M. P. V., R. V. Azanza, V. M. D. Vargas, C. T. Hedreyda. 2006. Bacterial endosymbionts of *Pyrodinium bahamense* var. *compressum*. Microbial. Ecol. 52(4):756-764.

Azanza R. V. and K. Fukami. 2007. Can seaweed monocultures and polycultures help alleviate HABs/red tides? Presented in 19th International Seaweed Symposium in Kobe, Japan, 26-31 March, 2007.

Azanza R. V., L.J. Cruz, F. A. Carino, A. G. Blanco and V. M. Butardo. 2010. Paralytic shelfish toxin concentration and cell-density changes in *Pyrodinium bahamense – Noctiluca scintillans* feeding experiments. Toxicon, 55:1017-1023.

Banin E, Israely T, Kushmaro A, Loya Y and Rosenberg E. 2000. Penetration of the coral-bleaching bacterium *Vibrio shiloi* into *Oculina patagonica*. Appl. Environ. Microbiol. 66(7):3031-3036.

Bernan, V. S., M. Greenstein, and W. M. Maiese. 1997. Marine microorganisms as a source of new natural products. Adv. Appl. Microbiol. 43: 57 – 89.

Bryers J. D. and W. G. Characklis. 1982. Processes governing primary biofilm formation. Bioeng. 24:2451-2476.

Burgess J.G., M. Elizabeth, M. Bregu, A. Meams-Sprugg and K.G. Boyd. 1999. Microbial antagonism: a neglected avenue of natural products research. J. Biotechnology. 70:27-32.

Doucette, G.J., Kodama, M., Franca, S., Gallacher, S. 1998. Bacterial interactions with harmful algal bloom species: Bloom ecology, toxigenesis, and cytology. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. NATO ASI Series vol. G41. Springer-Verlag, Berlin, pp. 619–647.

Duan D., L. Xu, X. Fei and H. Xu. 1995. Marine organisms attached to seaweed surfaces in Jiaozhou Bay, China. World J. Micro. and Biotech. 11(3):351-352.

Evelyn, A., Y. Liming, K. G. Boyd, P. C. Wright, and J. G. Burgess. 2001. The symbiotic role of marine microbes on living surfaces. Hydrobiologia. 461: 37 – 40.

Fukami, K., T. Nishijima, H. Murata, S. Doi, Y. Hata. 1991. Distribution of bacteria influential on the development and decay of *Gymnodinim nagasakiense* red tide and their effects on alga growth. Nippon Suisan Gakkaishi. 57:2321-2326.

Fukami K, Sakaguchui K, Kanou M, Nishijima T. 1996. Effect of bacterial assemblages on the succession of blooming phytoplankton from *Skeletonema costatum* to Heterosigma akashiwo. In: Yasumoto T, Oshima Y, Fukuyo Y (eds), Harmful and Toxic Algal Blooms, pp.335-338. UNESCO: Paris.

Fukami, K., A. Yuzawa, T. Nishijima, and Y. Hata. 1992. Isolation and properties of a bacterium inhibiting the growth of *Gymnodinium nagasakiense*. Nippon Suisan Gakkaishi, 58: 1073-1077.

Green, D. H., S. I. Blackburn, L. E. Llewellyn, A. P. Negri and C. J. S. Bolch. 2004. Phylognetic and Functional diversity of the bacteria associated with *Gymnodinium catenatum*: Evidence from the ssu rDNA of cultivable and non-cultivable bacteria, pp. 408-410. In Steidinger, K. A., J. H. Landsberg, C. R. Tomas, and G. A. Vargo (Eds.). Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. Harmful Algae 2002, October 21-25 2002, St Petersburg, Florida.

Gustafsson S., M. Hultberg, R. I. Figueroa, and K. Rengefors. 2009. On the control of HAB species using low biosurfactant concentrations. Harmful Algae. 8:857-863.

Hehemann JH, Correc G, Barbeyron T, Helbert W, Czjzek M and Michel G. 2010. Transfer of carbohydrate0active enzymes from marine bacteria to Japanese gut microbiota. Nature. 464:908-912.

Hold, G. L., E. A. Smith, M. S. Rappe, E. W. Maas, R. B. Moore, C. Stroempl, J. R. Stephen, J. I. Prosser, H. Birkbeck and S. Gallacher. 2001. Characterization of bacterial communities associated with toxic and non-toxic dinoflagellates: *Alexandrium* spp. and Scrippsiella trochoidea. FEMS Microbiol. Ecol. 37: 161–173.

Imai, I., Y. Ishida, and Y. Hata. 1993. Killing of marine phytoplankton by a gliding bacterium *Cytophaga* sp., isolated from the coastal sea of Japan. Mar. Biol. 116: 527-532.

Imai, I., Y. Ishida, K. Sakaguchi, and Y. Hata. 1995. Algicidal marine bacteria isolated from northern Hiroshima Bay, Japan. Fish Sci. 61:624-632.

Imai, I., Kim, M.C., Nagasaki, K., Itakura, S., Ishida, Y. 1998. Relationships between dynamics of red tide-causing raphidophycean flagellates and algicidal micro-organisms in the coastal sea of Japan. Phycol. Res. 46, 139–146.

Imai, I., D. Fujimaru, T. Nishigaki, M. Kurosaki, and H. Sugita. 2006. Algicidal bacteria isolated from the surface of seaweeds from the coast of Osaka Bay in the Seto Inland Sea, Japan. African J. Mar. Sci. 28 (2): 319-323.

Imai I and Kimura S. 2008. Resistance of the fish-killing dinoflagellate *Cochlodinium polykrikoides* against algicidal bacteria isolated from the coastal sea of Japan. Harmful Algae. 7:360-367.

Jasti, S., M. E. Sieracki, N. J. Poulton, M. W. Giewat and J. N. Rooney-Varga. 2005. Phylogenetic diversity and specificity of bacteria closely associated with *Alexandrium* spp. and other phytoplankton. Appl. Environ. Microbiol. 71: 3483-3494.

Jeong, S.Y., Ishida, K., Ito, Y., Okada, S., Murakami, M. 2003. Bacillamide, a novel algicide from the marine bacterium, *Bacillus* sp. SY-1, against the harmful dinoflagellate *Cochlodinium polykrikoides*. Tetrahedron Lett. 44, 8005–8007.

Kim JD, Kim JY, Park JK and Lee CG. 2009. Selective control of the *Prorocentrum minimum* harmful algal blooms by a novel algal-lytic bacterium Pseudoalteromonas haloplanktis AFMB-008041. Mar Biotechnol. 11:463-472.

Kubanek J, Jensen PR, Keifer PA, Sullards MC, Collins DO and Fenical W. 2003. Seaweed resistance to microbial attack: a targeted chemical defense against marine fungi. PNAS. 100(12):6916-6921.

Lafay, B., R. Ruimy and C. Rausch De Traubenberg. 1995. Roseobacter algicola sp. nov., a new marine bacterium isolated from the phycosphere of the toxin-producing dinolagellate *Prorocentrum lima*. Int. J. Syst. Bacteriol. 45: 290-296.

Largo, D. B., K. Fukami, and T. Nishijima. 1995. Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Euchema denticulatum* (Solieriaceae, Gigartinales, Rhodophyta). J. Appl. Phycol. 1995, 7(6):545-554.

Largo DB, Fukami K and Nishijima T. 1999. Time-dependent attachment mechanism of bacterial pathogen during ice-ice infection in *Kappaphycus alvarezii*

(Gigartinales, Rhodophyta). J Appl Phycol. 11:129-136.

Lee SO, Kato J, Takiguchi N, Kuroda A, Ikeda T, Mitsutani A and Ohtake H. 2000. Involvement of an extracellular protease in algicidal activity of the marine bacterium *Pseudoalteromonas* sp. strain A28. Appl. Environ. Microbiol. 66(10):4334-4339.

Lovejoy, C., J. P. Bouman, and G. Hallegraeff. 1998. Algicidal effects of a novel marine *Pseudoaltermonas isolate* (Cl Proteobacteria, Gamma Subdivision) on harmful algal bloom genera *Chattonella*, *Gymnodinium*, and *Heterosigma*. Appl. Environ. Micobiol. 64(8): 2806-2813.

Mayali, X. and F. Azam. 2004. Algicidal bacteria in the sea and their impact on algal blooms. J. Eukaryotic Microbiol. 51: 139-144.

Mayali, X., and G. J. Doucette. 2002. Microbial community interactions and population dynamics of an algicidal bacterium active against *Karenia brevis* (Dinophyceae). Harmful Algae. 1:277-293.

Nagasaki, K., M. Yamaguchi, and I. Imai. 2002. Algicidal activity of a killer bacterium against the harmful red tide dinoflagellate *Heterocapsa circularisquama* isolated from Ago Bay, Japan. Nippon-Suisan-Gokkaish. 66:666-673.

Padilla, L.V., M. L. San Diego-McGlone, and R.V. Azanza. 2010. Exploring the potential of clay in mitigating *Pyrodinium bahamense* var. *compressum* and other harmful algal species in the Philippines. J. Appl. Phycol. Published online 08 April 2010.

Reysenbach, A.L., L. J. Giver, G. S. Wickham and N. R. Pace. 1992. Differential amplification of rRNA genes by Polymerase chain reaction. Appl. Environ. Microbiol. 58(10):3417-3418.

Schmidt U, Chmel H and Cobbs C. 1979. Vibrio alginolyticus infections in humans. J. Microbiol. 10(5):666-668.

Seymour JR, Ahmed T and Stocker R. 2009. Bacterial Chemotaxis towards the extracellular products of the toxic phytoplankton *Heterosigma akashiwo*. J Plankton Res. 31(12):1557-1561.

Shibata AK and Robert FM. 2009. Shifts in alkane-degrading bacteria genotypes during bioremediation of a vegetated coastal soil. World J Microbiol Biotechnol. 25:1667-1675.

Steinberg PD, Schneider R and Kjelleberg S. 1997. Chemical defenses of seaweeds against microbial colonization. Biodegradation. 8:221-220.

Sugano, Y., I. Terada, M. Arita, M. Noma, and T. Matsumoto. 1993. Purification and characterization of a new agarase from a marine bacterium, *Vibrio* sp. strain JT0107. Appl. Environ. Microbiol, 59(5):1549-1554.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar. 2011. MEGA5: evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731-2739.

Tarutani, K., K. Nagasaki, S. Itakura, and M. Yamaguchi. 2001. Isolation of a virus infecting the novel shellfish-killing dinoflagellate *Heterocapsa circularisquama*. Aquat. Microb. Ecol. 23:103–111.

Vijaya, B. and R.A. Veera. 2007. Interference with cell-to-cell signaling: A potential therapeutic Approach against *Vibrio* species. Wang, J., H. Mou, X. Jiang, and H. Guan. 2006. Characterization of a novel β-agarase from a marine *Alteromonas* sp. SY37-12 and its degrading products. Appl. Micro. and Biotech. 71(6):833-839.

Weisberg, W.G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697-703.