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Discovery of antimicrobial activities of a marine diatom *Thalassiosira rotula*

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This study investigates the antimicrobial activities of a marine diatom, *Thalassiosira rotula* against microorganisms including three Gram-stain positive and six Gram-stain negative bacteria and one species of yeast. Well-bioassays were used to evaluate the ability to inhibit the growth of bacteria with extracts derived from algae using different extraction techniques of cell wall rupture and organic solvents. The extracts of *T. rotula* inhibited the growth of *Vibrio harveyi, Staphylococcus aureus, Micrococcus luteus* and *Bacillus pumilus*. The freeze/thaw algal paste extract without solvent extraction showed the highest inhibition against *S. aureus* and *B. pumilus*. The hexane:tert-butyl-methyl-ether (H:tBME) extract was the only one inhibiting *M. luteus* and the chloroform:methanol (C:M) extract was the only one inhibiting *Vibrio harveyi*. Sonication with beads for 3 min was the most efficient method of releasing the antibiotic substances. In sequential solvent extraction, the vortexed H:tBME extract had a higher ability against *S. aureus, B. pumilus,* and *M. luteus* than the sonicated H:tBME extract or the C:M extract. The 1:1 combination of sonicated H:tBME and vortexed H:tBME extracts did not exceed that of the separate extract against *S. aureus* or *B. pumilus*. However, the combined sequential C:M and vortexed H:tBME extracts had lower ability against *S. aureus* than the vortexed H:tBME extract alone, indicating the presence of antagonistic compounds in the C:M extract. This study indicates that *T. rotula* possess antimicrobial activities but the release of antibiotics depends on physical or chemical rupture of algal cells and extractive solvents.

Key words: Bacteria, algae, antibiotic, organic solvent, sonication, vortex.

INTRODUCTION

Although antibiotics have become an indispensable part of modern medicine, the inappropriate use of antibiotics has resulted in the emergence of antibiotic resistance to bacterial strains (Mukherjee et al., 2005; Fernandez-Delgado and Suarez, 2009; Wright, 2010). Some antibiotic resistant strains pose serious threats to the status quo of public health, which has strengthened the urgency for searching new and effective antibiotics (Gottlieb and Nimmo, 2011; Tello et al., 2012). In primary industries such as aquaculture, bacterial infections contribute to significant losses of aquatic animals and economic income (Desriac et al., 2010; Beardsley et al., 2011; Gao et al., 2012). This situation is exacerbated by the increasing occurrence of bacteria that are resistant to antibiotics in cultured and wild fish (Kitto et al., 1999). With an escalating number of species for aquaculture and the ever-increasing need to achieve the highest possible productivity in a given system, the incidence of bacterial

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infection among cultured fish has become increasingly common. Successful culturing of many species will therefore require the development of new antibiotics in an attempt to control pathogens such as *Vibrio harveyi*, *Vibrio vulnificus*, *Streptococcus* sp., and those that have become resistant to common antibacterial agents (Wang, 1999). Clearly, it is imperative to discover new antibiotics, not only to combat resistant strains, but to reduce the possibility of further increasing the antibiotic resistance of the bacterial community.

The marine environment is viewed as a new and underexplored source of potentially useful bioactive compounds (Davidson, 1995), particularly given that the microbial, plant and invertebrate diversity in the marine environment far exceeds that of terrestrial habitats (Carte, 1993). The potential for using microalgae to yield novel compounds is strengthened by the fact that microalgae are the foundation of the food chain to support the most of aquatic biomass (Conti and Scardi, 2010). Cconsequently, microalgae may be the primary source for some of the novel molecules found in marine invertebrates.

Microalgae represent a large and underexplored resource of antimicrobial compounds (Guedes et al., 2011). Furthermore, recent advances in algal culture techniques have placed microalgae in a unique position over many other marine organisms, as algae can be cultured under conditions that maximise the production of the desired compound (Chisti, 2007). Thus, the investigation of marine microalgae for their antimicrobial activity is likely to find compounds that can be used in the aquaculture industry. Microalgae are known to produce compounds intracellularly and extracellularly. However, as a large proportion of these compounds is not excreted but remains within the cells (Guedes et al., 2011; Das and Pradhan, 2010), solvent extraction is widely used to extract these secondary metabolites in algae (Zhao et al., 2004). Algal antimicrobials are structurally diverse and it is common for an alga to produce a variety of antibiotic compounds with different spectra of antibiotic activity (Metting and Pyne, 1986). Clearly, one solvent system would most likely be inadequate to extract all chemical classes of antimicrobials produced by an algal species. For instance, Lustigman (1988) extracted the biomass of *Dunaliella salina* in a range of solvents and found that the solvent of butanol extract produced the largest zones and the broadest spectrum of activity, whereas the methanol and chloroform extracts showed no activity. In contrast, Chang (1993) used a variety of solvents to extract *Dunaliella primolecta* and found methanol extracts to be the most active while hexane extracts were inactive. Alternatively, Kellem et al. (1989) examined the antifungal activity of a number of microalgae and found that in some species the methanol extracts were active whereas in others the hexane extracts that were active.

Therefore, different solvents need to be used to determine the true antibiotic potential of an algal species, as the size and polarity of algal metabolites will ultimately determine the metabolites and their solubility in an organic solvent. To recognise the antibiotic potential of an algal species, extracts need to be tested against a broad spectrum of bacteria and fungi. For example, Cooper et al. (1983) reported that aqueous extracts of *Skeletonema constatum* exhibited a broader spectrum of activity when compared to the organic extracts. This implies that there are multiple antibiotics present with different polarities and spectra of activity. Obviously, the broader activity of the aqueous extracts would not be detected if a narrow spectrum of bacteria would be used to test the activity of the extracts. Therefore, it is necessary to extract the algal biomass using a number of solvents which represent a range of polarities (non-polar to polar solvents). Similarly, a suite of test microorganisms should be used if the true antibiotic potential of an algal species is to be recognised.

Various techniques have been used to liberate metabolites from their cells and increase the efficiency of solvent extraction, such as changing ambient salinity to exert osmotic shock before solvent extraction (Viso et al., 1987; Lustigman, 1988), mixing the solvent with algal biomass (Kellam and Walker, 1989), homogenising algal materials (Miura et al., 1993; Wang, 1999) and sonicating algal biomass (Chang et al., 1993; Ohta et al., 1995). Although freeze/thaw extraction is not extensively used in algal extractions, it is a common method to extract bioactive materials that are thermally unstable (Silvia et al., 1998). As raw materials with a small particle size are easy for compound extraction (Silvia et al. 1998), the procedure of cell rupture should be carefully chosen before solvent extraction. Algal cells are diverse in shape, size, cell wall thickness and cell wall composition (Richmond, 2004). Such morphological characteristics may influence the efficiency of a particular extraction technique. Thus, methods of physical rupture to assist penetration of a solvent into the cellular material need to be refined for an algal species of interest if one is to maximise antibiotic yield from the algal biomass.

The aims of this study were to (1) examine the *in vitro* antimicrobial activity of *Thalassiosira rotula* extracts, an algal species commonly used in aquaculture hatchery, against a suite of microorganisms using well bioassays, (2) identify the type of algal extracts with the highest activity, (3) explore extraction techniques in an attempt to increase the antibiotic yield, and (4) identify an appropriate solvent system that would maximise the yield of a particular antimicrobial.

MATERIALS AND METHODS

Experimental organisms

The diatom *T. rotula* was obtained from the Australian National Algae Culture Collection in the Commonwealth Scientific and Industrial Research Organisation (http://www.csiro.au/Organisation-

Table 1. Microorganisms used to test the antimicrobial activity of algal extracts.

Microorganism	Source
Gram-stain positive	
Staphylococcus aureus (Sa)	FMC[1]
Bacillus pumilus (Bp)	FMC
Micrococcus luteus (Ml)	FMC
Gram-stain negative	
Escherichia coli (Ec)	FMC
Enterococcus cloacae (Ecl)	FMC
Gram-stain negative (marine)	
Vibrio natrigens (Vn)	FU[2]
Vibrio harveyi (Vh)	FU
Vibrio fischerii (Vf)	FU
Yeast	
Candida albicans (Ca)	FMC

[1]FMC: Flinders Medical Centre; [2]FU: Flinders University, School of Biological Sciences.

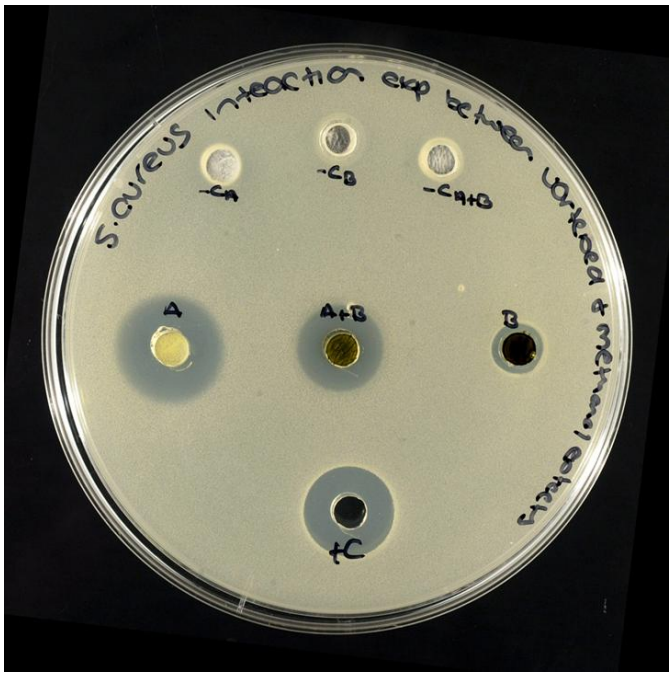

Figure 1. Illustration of well assay to test *T. rotula* extract inhibition on *S. aureus*. The transparent areas are the inhibition zones by -CA (hexane solvent control), -CB (chloroform/methanol control), -CAB (hexane + chloroform/methanol control), A (hexane extract), B (chloroform/methanol extract), AB (hexane + chloroform/ methanol extract), and +C (vancomycin control).

Structure/National-Facilities/Australian-National-Algae-Culture-Collection/Microalgae-Supply-Service.aspx), Tasmania, Australia, to examine its antimicrobial activity. This alga was grown in natural seawater enriched with F2 media. Algal stocks (100 ml) were maintained and refreshed every two weeks by transferring 10 ml of stock culture into 90 ml of media. Stock cultures of high cell density were used to seed 1 L Schott-bottles filled with 900 ml of media. One-litre cultures were maintained under continuous light at 19°C and aerated at a rate of 100 ml/min. The 1 L cultures were inoculated in 20 L carboys. Algal cultures were subjected to a photoperiod of 12 h light:12 h dark and illuminated by a bank of fluorescent tubes at 2000 lux. Cultures were maintained at 23 ± 1°C and were continuously injected with air containing 5% (v/v) CO_2 at a flow rate of ~600 ml/min.

Nine microorganisms were used to evaluate the antimicrobial activity of the algal extracts (Table 1). These microbes were cryogenically stored at -80°C in 20% glycerol prior to the study. To obtain single colonies, glycerol stocks of *Vibrio* spp. were streaked on to the Hasting agar (Hasting, 1986), and the other micro-organisms were streaked onto the nutrient agar. Agar plates were then incubated at 25°C. After 24 h incubation, plates were removed from the incubator and stored in a fridge at 4°C. These plates were replaced with freshly streaked plates every two weeks.

Testing antimicrobial activity of algal extracts

Using a Sorvall GSA fixed angle rotor in a Sorvall RC-5B refrigerated centrifuge (DuPont Instruments), 1.5 L algal culture was centrifuged (5000 *g*) at 0°C for 10 min. The biomass pellets was then resuspended in the remaining supernatant and transferred to 50 ml centrifuge tubes. This concentrated algal culture was then centrifuged at twice 4500 *g* for 2.5 min. The biomass pellet was used for extraction.

The well bioassay was used to examine the antimicrobial activity of microalgal extracts (Figure 1). Aliquots (10 ml) of the Tryptone soya broth or hasting broth for *Vibrios* were inoculated with a single colony and allowed to grow overnight at 25°C. The optical density (OD) of each overnight culture was measured at 600 nm using a Shimadzu (UV -160 A) spectrophotometer, and adjusted to an OD of 0.1 by the addition of sterile, saline water (0.9% NaCl in water, w/v). Diluted overnight cultures were used to bulk seed the bioassay agar or hasting agar at 2% (v/v) on the agar. Seeded agar was poured on to plates to a depth of 5 mm and once the agar had set, 5 mm wells were punched in to the bioassay plate. To each well, 40 µl of a given solvent extract or solvent control was then added. Plates were then incubated overnight at 25°C for each microorganism. After 18 h, zones of inhibition were measured to the nearest 0.5 mm using a pair of vernier callipers.

Extraction protocols for antibiotic compounds

Upon reaching the stationary phase, 15 L of the well-mixed algal culture was harvested, and divided into ten 1.5 L culture samples and subsequently centrifuged. The fresh weight of each of the 10 algal pellets was then recorded. The volume of one algal pellet was adjusted to 1.5 ml by adding distilled water, and then stored at -18°C. In addition, a 10 ml sample of the supernatant from each 20 L culture was stored at -18°C to test for antimicrobial activity.

The antimicrobial ability of algae was tested on (1) algal free culture media, (2) freeze/thaw algal paste (freeze for 24 h then thaw at room temperature), (3) water as a solvent, (4) hexane:tert-butyl-methyl-ether (H:tBME, 1:1 v/v) as a solvent, and (5) chloroform:methanol (2:1, v/v) as a solvent (Table 2). One solvent was added to algal pellets in triplicate with each pellet receiving 1 ml solvent. To improve the effectiveness of extraction, three physical treatments were used:sonication only, sonication with 1 g

Table 2. Inhibition zone diameters (mm) produced by *Thalassiosira rotula* crude extracts, against nine microorganisms. Zones of inhibition are measured as the means ± SD of the diameter of the zones (well diameter = 5 mm). Different letters in the same column represent significant differences ($P < 0.05$). Abbreviations of test microorganisms refer to Table 1.

Extract	Test microorganism								
	Marine					Terrestrial			
	Vn	Vh	Vf	Ec	Ecl	Sa	Bp	Ml	Ca
Algal free culture media	0	0	0	0	0	0	0	0	0
Freeze/thaw paste of algal biomass	0	0	0	0	0	14.2 ± 0.3^{c}	14.5 ± 0.5^{c}	0	0
Water	0	0	0	0	0	9.7 ± 0.6^{b}	7.8 ± 0.3^{b}	0	0
Hexane: tert-Butyl-Methyl-Ether (H:tBME)	0	0	0	0	0	9.5 ± 0.1^{b}	13.8 ± 0.3^{c}	12.5 ± 0.5	0
Chloroform:methanol (C:M, 2:1)	0	7.8 ± 0.3^{b}	0	0	0	7.3 ± 0.6^{b}	7.5 ± 0.5^{b}	0	0
C:M solvent control	0	5.8 ± 0.3^{a}	0	0	0	6.0 ± 0.1^{a}	6.0 ± 0.1^{a}	0	0
H:tBME solvent control	0	0	0	0	0	0	0	0	0

glass beads (212 to 300 μm diameter); and vortexing. Using the well bioassay, the algal extracts obtained from the different extraction methods were tested for antimicrobial activity against each microorganism (Table 1). Each extract was tested on three separate bioassay plates. Each solvent was also used as a control to measure any activity due to the solvent.

Sonication time

The effect of sonication time was investigated to identify whether sonication was degrading the antibiotic material. Upon reaching the stationary phase, 13.5 L of well-mixed *T. rotula* culture was harvested and divided into nine 1.5 L aliquots. The 1.5 L culture samples were centrifuged and the wet weight of each algal pellet recorded. A volume of 1 ml of H:tBME was then added to each pellet along with 800 mg of glass beads.

The nine extraction mixtures were divided into three groups with three replicates. Each group was subjected to sonication for 1, 3 and 6 min (100 μA, Qsonica model Q55) on ice, respectively. Once sonicated, the total solvent (H:tBME) volume in each sonicated mixture was brought to 5 ml and allowed to stand for 1 h. Each extraction mixture was then centrifuged for 2.5 min at 4500 *g.* The organic layer from each sample was removed and placed into 10 ml tubes. Extracts were then concentrated under a stream of nitrogen gas to yield 500 μl extracts and stored in eppendorf tubes at -18°C. Extract activities were then examined against *Staphylococcus aureus* and *Bacillus pumilus* using the well bioassay. Each extract was tested once on three separate bioassay plates.

Sequential extractions using different solvents

The screening study suggested that a number of antimicrobials might exist in *T. rotula*. To identify the antimicrobials produced and their solvent affinities, sequential extractions of the algal biomass were performed. Upon reaching the stationary growth phase, 4.5 L of *T. rotula* was harvested and divided into three 1.5 L samples for centrifuging to yield three algal pellets for sequential extraction outlined below.

Vortexed H:tBMe extraction

Algal pellets of *T. rotula* were initially extracted in 5 ml H:tBME, vortexed for 5 min and allowed to stand at room temperature for 1 h. Subsequently, each extraction mixture was centrifuged for 2.5 min at 4500 *g*. The organic extracts from each extracted pellet were then transferred to separate 10 ml centrifuge tubes. Following the removal of the organic extract, each extracted pellet was washed with a further 5 ml H:tBME, vortexed for a total of 4 min and then left to stand for 20 min. Each mixture was centrifuged as above and the organic supernatants transferred to separate, labelled 10 ml tubes.

Sonicated H:tBME extraction

Following the vortexed H:tBME extraction, a volume of 1ml of H:tBME was added to each of the three extracted pellets along with 800 mg of glass beads. Each was then sonicated on ice for 3 min. Once sonicated, the total solvent volume in each mixture was brought to 5 ml and left to stand for 1 h. Subsequently, each mixture was centrifuged and the organic supernatants were placed in individual 10 ml tubes. The sonicated algal material was then washed following the washing procedure implemented in the vortexed H:tBME extraction. Each wash was kept in separate 10 ml tubes.

Chloroform:methanol (C:M) extract

After removal of the organic layer, each algal pellet was dried under nitrogen gas to remove any remaining H:tBME. Once free of the previous solvent system, each pellet was extracted in 5 ml of C:M solvent, vortexed for 5 min, and left to stand for 1 h. The organic supernatant was kept in separate 10 ml tubes. Each extracted pellet was then washed with 5 ml of C:M (2:1 v/v) solvent following the washing procedure implemented in both H:tBME extractions. Using a stream of nitrogen gas, algal pellets were dried of any residual C:M solvents. Once dry the remaining algal material in each extraction tube was extracted and then washed with distilled water. The procedure of extracting and washing was identical to that preformed for the sequential C:M solvent extraction. Sequential extracts were subsequently examined for antimicrobial activity against *B. pumilus*, *S. aureus* and *M. luteus* using the well bioassays. In addition, solvent controls (chloroform:methanol, H:tBME and water) were included on each bioassay plate.

Antibiotic activities of combined extract from different solvents

To determine the presence of antagonistic compounds, we

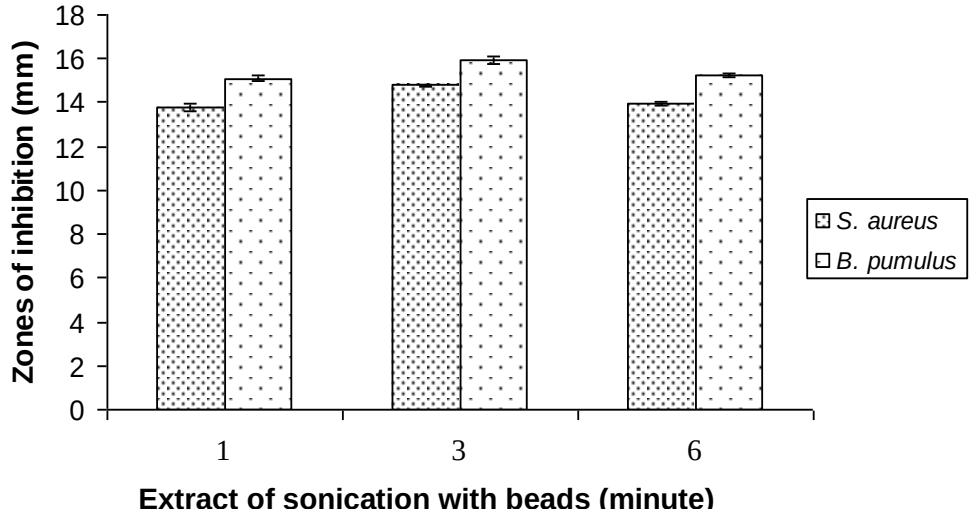

Figure 2. Effect of sonication time on *T. rotula* H:tBME extract activity. Activity in the well bioassay was measured as mean zones (±SE) of inhibition in triplicate for *T. rotula* H:tBME extracts against *S. aureus* and *B. pumilus*. Extract biomass was subjected to sonication of 1, 3 or 6 min.

combined several of the sequential extracts to examine if their combined activity differed from their individual activity. The vortexed H:tBME extract and the sonicated H:tBME extract were each diluted to 50% of their original concentration by the addition of H:tBME (1:1). A third extract was then formed by mixing the vortexed H:tBME extract with of the sonicated H:tBME extract (1:1). These extracts were then bioassayed in triplicate against *B. pumilus* and *S. aureus*. The methodology outlined above was then repeated using the vortexed H:tBME extract and the sequential C:M extract. Extracts and their respective solvent control were bioassayed against *S. aureus*.

Statistical analysis

Single factor analysis of variance was conducted using statistical software (SPSS, 18.0) to determine whether extract methods were significantly different from that of the solvent control. All zones of inhibition were converted to logarithmic values before being analysed to improve data normal distribution. Multiple comparisons were then preformed using the Bonferroni post hoc test when the main effect of treatments was significant. Linear regression was performed to determine the relationship between extract concentrations and the zone size of inhibition. Differences between the means were considered significant at the level of $P < 0.05$.

RESULTS

Antimicrobial activities of algal extracts

Antimicrobial activity was evaluated by measuring the diameter of the zone of inhibition in the well bioassay. The algal cell free culture media did not inhibit the growth of any microorganisms, but the freeze/thaw paste, water, H:tBME and C:M extracts were active against *S. aureus* and *B. pumilus* (Table 2). The freeze/thaw paste extract exhibited the highest antimicrobial activity of any algal extract, inhibiting *S. aureus* and *B. pumilus* to a similar degree. The activity of the H:tBME extract against *B. pumilus* was similar to that of the freeze/thaw paste. However, the activity of the H:tBME extract against *S. aureus* was significantly lower ($P = 0.001$) than that of the freeze/thaw paste extract. Comparing all extracts, the H:tBME extract exhibited the highest activity against *M. luteus* and the C:M extract was active against *V. harveyi* ($P = 0.001$) when compared to the C:M solvent control.

The cell free supernatant was inactive against any microorganisms, while the freeze/thaw paste and the H:tBME extract were consistently the most active extracts against *B. pumilus*. The C:M and water extracts were the only extracts active against *V. harveyi*. The activity of the freeze/thaw paste was usually higher than that of the best solvent extract. *S. aureus* and *B. pumilus* were the most sensitive microbes, while *Escherichia coli*, *Escherichia cloacea* and the yeast *C. albicans* were not inhibited by any of the algal extracts. The C:M solvent extract could inhibit the growth of *V. harveyi*, *S. aureus* and *B. pumilus*compared to the C:M control ($P<0.001$).

Effects of cell rupture methods on antimicrobial activities

Sonicating for 1, 3 or 6 min had some effect on the activity of the H:tBME extracts in the well assay. Multiple comparisons indicated that the 3 min sonication treatment had significantly higher activity against *B. pumilus* than both the 1 min and 6 min sonication treatments ($P = 0.002$ and $P = 0.008$, respectively, Figure 2). Extracts sonicated for 3 min were more active against

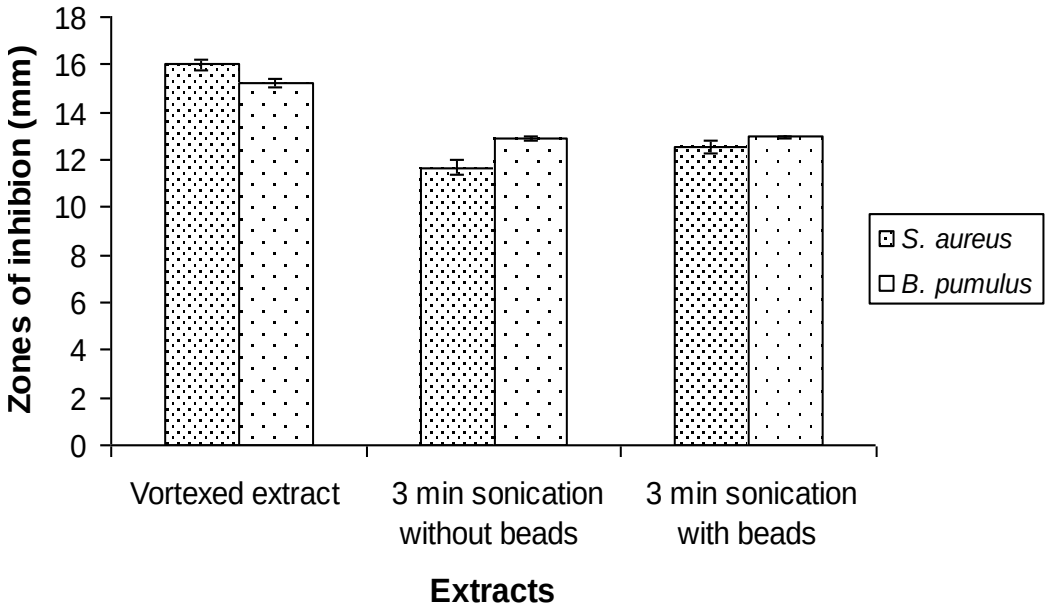

Figure 3. Effect of vortexing and sonication with or without beads on the activity of *T. rotula* H:tBME extracts. Activity in the well bioassay was measured as mean zones (±SE) of inhibition in triplicate for *T. rotula* H:tBME extracts against *S. aureus* and *B. pumilus*. Treatments included vortexing the biomass in H:tBME, and sonicating the algal biomass for 3 min with and without beads before extracting in H:tBME.

S. aureus than those sonicated for 1 min (P = 0.022), although the 3 min sonication appeared slightly more active against *S. aureus* than the 6 min sonication treatment (P = 0.112).

Since the 3 min sonication treatment showed best extraction results, the 3 min sonication treatment was used as a reference to compare the activity of vortexed extracts and sonicated extracts with and without glass beads. This treatment was chosen as it previously displayed the highest activity, and would act as a control for other extraction methods.

Sonicating *T. rotula* biomass with or without beads did not significantly alter the H:tBME extract's ability to inhibit the *B. pumilus* (P = 0.99), but the use of beads in sonicating did significantly improve the extracts ability to inhibit *S. aureus* (P = 0.023, Figure 3). Of all the extraction treatments using the well bioassay, the vortexed H:TBME extraction treatment exhibited the highest antimicrobial activity against both *S. aureus* and *B. pumilus* (P < 0.05, Figure 3).

To ensure the observed zones of inhibition increased as a function of extract concentration, the *T. rotula* extracts of increasing concentration were bioassayed against the most sensitive microbe *B. pumilus*. Linear regression was then performed to determine the relationship between extract concentration and zones of inhibition (Figure 4). Based on the trend, the activity of *T. rotula* extracts showed an increase with extract concentrations (R^2 = 0.95, P < 0.001).

Activities of single sequential solvent extracts

Multiple solvent systems detected the presence of more than one antimicrobial substance. To gain a perspective into the number of antimicrobials produced by *T. rotula* and identify the most appropriate solvents to extract them, sequential extractions of the biomass were performed (Table 3).

The vortexed H:tBME extract for *T. rotula* exhibited the highest activity against *S. aureus*, *B. pumilus* and *M. luteus* (P < 0.05, Table 3). Sequential C:M extracts showed activity against all microbes. Its activities against these microorganisms were lower than those of the vortexed H:tBME extractions, but similar to those of the sonicated H:tBME extract. The extraction controls of the water and H:tBME did not show any antimicrobial activity, but the C:M solvent inhibited *B. pumilus*.

Activities of combined sequential extracts

The sonicated H:tBME extract was combined with the vortexed H:tBME extract (v:v=1:1). The combined extract activity was then compared to the activity of the individual extracts which had been diluted by 50% (Table 4).

The vortexed H:tBME (½ conc) extract and the combined extract had a higher activity than the sonicated H:tBME (½ conc) extract (P< 0.05). When tested against *B. pumilus*, the combined extract exhibited the same

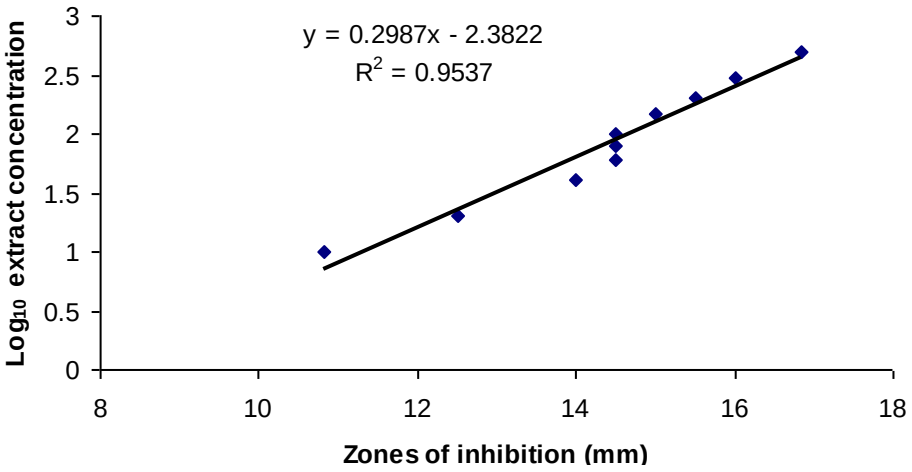

Figure 4. Relationship between extract concentration and zones of inhibition produced by *T. rotula* H:tBME extracts against *B. pumilus*.

Table 3. Antimicrobial activity in sequential solvent extracts of *Thalssiosira rotula* as exhibited in well bioassays. Zones of inhibition include well diameter (5 mm).

Extracting order	Extract	Microorganism[a]		
		Bp	Sa	Ml
1	Vortexed H:tBME extract	17.4 ± 0.5[c]	18.8 ± 0.3[c]	14.7 ± 0.1[c]
2	Sonicated H:tBME extract	9.6 ± 0.1[b]	7.5 ± 0.5[b]	9.1 ± 0.5[b]
3	C:M extract	7.7 ± 0.3[b]	7.3 ± 0.3[b]	7.7 ± 0.5[b]
	C:M solvent control	6.0 ± 0.1[a]	0	0
	Water extract control	0	0	0
	H:tBME control	0	0	0

Different letters in the same column represent significant differences (P< 0.05). Abbreviations of the microorganisms and solvent refer to Table 1.

Table 4. Mean zones of inhibition produced against *Staphylococcus aureus* and *Bacillus pumilus* when the *T. rotula* vortexed H:tBME extract was combined with the sonicated H:tBME extract. Activity was compared to the zones of inhibition (mm) produced by the individual extracts.

Extract	*S. aureus*	*B. pumilus*
Vortexed H:tBME extract	16.5 ± 0.1[b]	15.5 ± 0.1[b]
Sonicated H:tBME extract	6.3 ± 0.3[a]	7.5 ± 0.1[a]
Combined H:tBME extract	15.7 ± 0.3[b]	15.5 ± 0.1[b]
H:tBME solvent control	0	0

Different letters in the same column represent significant differences (P < 0.05).

degree of activity as that of the vortexed H:tBME (½ conc) extract alone. Against *S. aureus*, zones of inhibition appeared to be smaller in the combined extract when compared to the vortexed H:tBME (½ conc) extract. This difference, however, was not significant (P = 0.206).

Similarly, the vortexed H:tBME extract was combined with the sequential C:M extract (v:v=1:1),and the antimicrobial activities are reported in Table 5. The vortexed H:tBME (½ conc) extract and the combined extract were significantly more active than the sequential C:M (½ conc) extract as depicted by their zones of inhibition against *S. aureus*. In addition, the results clearly showed

Table 5. Mean zones of inhibition produced against *Staphylococcus aureus* when the *T. rotula* vortexed H:tBME extract was combined with the sequential C:M extract. Activity was compared to the zones of inhibition (mm) produced by the individual extracts. Different letters in the same column represent significant differences ($P < 0.05$).

Extract	*S. aureus*
Combined extracts of vortexed H:tBME and sequential C:M	13.5 ± 0.5^{b}
Vortexed H:tBME extract	17.5 ± 0.1^{c}
Sequential C:M extract	8.0 ± 0.5^{a}

that the vortexed H:tBME extract had a signi-ficantly reduced activity against *S. aureus,* when com-bined with the C:M extract ($P = 0.001$).

DISCUSSION

Screening microalgae for antimicrobial activity

This study demonstrates that *T. rotula* had the highest antimicrobial activity against *S. aureus, B. pumilus,* and *M. luteus* and was active against *V. harveyi*, but had no antibiotic activity on *V. natrigens, V. fischerii, E. coli, E. cloacae* and a yeast *C. albicans.* A variety of solvents for the extraction of algal biomass have been reported with hexane, methanol, chloroform, benzene and iso-propanol being the most frequently used (Fastner et al., 1998; Herrero et al., 2005; Kang and Sim, 2007). In comparison, this study shows that the most non-polar solvent system (that is, H:tBME) consistently extracted the compounds with the highest activity against Gram-positive microbes. Thus, the antimicrobial compounds active against *Vibrio* appear to have a higher polarity than those active against the Gram-positive microbes since the *Vibrio* was only inhibited in the polar solvent of chloroform and methanol.

The presence of antibacterial activity in natural sea-water is well known (Taskin et al., 2007; Ibtissam et al., 2009; Mandeel et al., 2010; Salem et al., 2011). Lustigman (1988) found that in addition to the algal biomass having activity, the culture filtrate of *Dunaliella salina* was also active against Gram positive, Gram negative and marine bacteria. In addition, Trick et al. (1984) also reported that the antibiotic β-diketone was released into the culture filtrate by *Prorocentrum minimum*. However, similar to Kellem and Walker (1989), this study observed no antimicrobial activity in the culture supernatant. This may have been because the culture filtrate was too dilute, or because of physiological, environmental and experimental conditions.

The current study has demonstrated for the first time, the strong antibacterial activity in the marine diatom *T. rotula*. In the past, a *Thalassiosira* sp. was examined by Visoet al. (1987) and found to be active against *S. aureus* and *M. luteus*, but inactive against *E. coli* and *C. albicans*. Furthermore, *T. nana* and *T. decipiens* were reported to be active against a number of soil bacteria (Kitto et al., 1999). Thus, it appears that the genus *Thalassiosira* has the ability to produce antimicrobial secondary metabolites.

Antimicrobial activity is evaluated on the ability of an algal extract to inhibit certain microbes. It is therefore conceivable that under a different set of test microbes, the activity of the algal extract could have been signify-cantly different. For instance, Ohta et al. (1994) investigated the activity of *Chlorococcum* HS-101 against seven strains of methicillin resistant *S. aureus* and found the zone sizes of inhibition ranged from 17.7 ± 1.5 to 28.3 ± 1.7 mm. Viso et al. (1987) found that extracts con-taining eicosapentaenic acids from the diatom *Asterionella japonica* exhibited strong antibiotic activity after a short period of illumination. Similarly, Miura et al. (1993) observed that extracts of *Chlorella* were inactive against *B. subtilis* in the dark, but became active when illuminated. Thus, it is possible that the alga in the current study may have produced light sensitive antimicrobials, but their production would not have been recognised because the bioassay plates were incubated in dark.

Effects of cell rupture methods on antimicrobial substance release

Numerous techniques have been used to release antimicrobial compounds into the extracting solvents (Vlachos et al., 1996; Herrero et al., 2005; Shanmughapriya et al., 2008). This study found that sonicating with glass beads (240 to 300 μm) was the most efficient way to release the antimicrobial com-pounds from the biomass of *T. rotula*. However, in the well bioassay the vortexed extract exhibited an activity significantly higher than that of the sonicated extracts, suggesting that the method of physical rupture of algal cells may interfere with the effectiveness of antibiotic property in algae.

Zones of inhibition were reduced when algal pellets were sonicated for 6 min. This indicates that sonicating beyond 3 min may degrade some of the antibiotic mate-rial. Sonication can generate very high local temperature

around the probe, which could degrade the antibiotic material if it would be heat sensitive. This scenario is possible considering that antibiotics produced by *D. primolecta* are degraded when temperatures exceed 40°C (Chang et al., 1993) and that the antibiotic material of *D. salina* is degraded at temperatures exceeding 25°C (Lustigman, 1988). Thus, it is possible that the decrease in antibiotic activity may result from degradation of antibiotic material due to overheating in the 6 min sonication procedure.

This may partially explain why the algal paste in the screening experiment had an activity higher than that of the H:tBME solvent extraction. These antibiotic compounds seem to be relatively polar, and would therefore have a low affinity for the H:tBME which is a relatively nonpolar solvent system. Thus, to extract out more of these polar compounds, a more polar solvent system should be used and the extraction time should be increased. Although sonication could effectively break cell walls, it may degrade the antibiotic activity of the extract. Therefore, increasing the extraction time in a particular solvent system should be considered during extraction.

Sequential solvent extraction of algal biomass

It is commonly observed that a microalgal species produces multiple antibiotic substances, each with different spectra of activity (Miura et al., 1993; Ohta et al., 1995). *T. rotula* was no exception, exhibiting the ability to produce a diverse array of antimicrobial compounds, that is, some are only active against *S. aureus*, while others display broader spectra of antibacterial activity. In the sequential solvent extraction, the H:tBME solvent extracted much more antibiotic compounds than the C:M solvent extracts from the *B. pumilus*, *S. aureus* and *M. luteus*, suggesting the use of C:M solvent is not appropriate.

In the sequential extraction with physical rupture on algal cells, the vortexed H:tBME extract showed similar ability of inhibition against *S. aureus* and *B. pumilus* as the combined extracts of vortexed and sonicated H:tBME solvent, suggesting that the one step of vortexed extraction is adequate and time efficient.

It is well known in the clinical application of antibiotics that the presence of multiple antibiotics together commonly results in decreased activity (Krogstad and J, 1986). In this study, the combined extracts of vortexed H:tBME and sequential C:M reduced the inhibitory ability against *S. aureus*compared to the vortexed H:tBME extract alone, suggesting the presence of antagonistic compounds in the C:M extract. This study did not identify the property of antibiotic compounds and the mechanism of compounds. However, future research is required to determine the identity of the antagonistic interactions between antibiotics.

Conclusion

This study reports the presence of antibacterial compounds in *T. rotula* which inhibit the growth of *V. harveyi, S. aureus, Bacillus pumilus* and *M. luteus*. Vortexed H:tBME was the most effective extract method, but the use of glass beads for algal cell rupture could aid the release of antibiotic substances. However, the time of sonication may need to be adjusted for an individual species of interest. In a screening situation, 1 to 3 min sonication with beads would appear to be sufficient to release the majority of the antibiotic material from the biomass, particularly when performing sequential extractions. The presence of antagonists in the extracts of *T. rotula* suggests that the use of a crude algal extract without separation may underestimate the true antibiotic potential.

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