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An Investigation of the Antifouling Potential of Extracts of the Periostracum of *Mytilus sp.*

Clare Grandison, Andrew Scardino and Simon Ovenden

Maritime Platforms Division
Defence Science and Technology Organisation

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ABSTRACT

This report details the research program investigating the potential antifouling activity of extracts from the periostracum of the marine mussel *Mytilus sp.*. Previous research has suggested that marine mussels utilise multi-faceted defence mechanisms to ensure their fitness does not suffer due to heavy fouling colonisation on their shells. The shell coating known as the periostracum, is indicated as a possible physical and chemical antifouling defence component. In this study, the periostracum was separated from the shell and extracts of the periostracum obtained by serial extraction with three solvents of increasing polarity. The extracts were tested against common marine fouling organisms: diatoms, bryozoans and barnacle larvae. Considerable activity against marine diatoms and bryozoans was shown by dichloromethane and ethyl acetate extracts. Initial Nuclear Magnetic Resonance Spectroscopy (NMR) fingerprinting of the extracts suggests the presence of fatty compounds (potentially triglycerides), hydroxylated groups and aromatics. Further purification by High Performance Liquid Chromatography (HPLC) and NMR analysis would be required to elucidate the structure of the compound responsible for the noted antifouling activity. This would require a significant increase in the amount of mussel periostracum to be acquired.

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*Telephone: (03) 9626 7000
Fax: (03) 9626 7999*

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Executive Summary

Fouling of ship hulls and niche areas represents a significant cost for the maritime industry. The penalties associated with fouling include higher maintenance costs, increased fuel usage due to greater levels of hull friction resistance, lost productivity due to more frequent dry-docking for removal of fouling organisms, decreased operational capability and the cost of compliance with increasingly stringent environmental regulations. These penalties can create a significant burden for maritime operators whether private or public. Fouling can also directly impact on the operational (natural) environment; the translocation of invasive marine pests and the release of environmentally persistent chemical compounds from antifouling coatings are of great concern for long-term marine management.

The 2008 International Maritime Organisation (IMO) global ban on Tributyltin (TBT) antifouling paints, together with growing concerns over potential long-term impacts of biocidal vessel coatings, has encouraged research into novel compounds capable of resisting fouling pressure whilst reducing impacts on the local and extended environment.

This paper presents the findings of a preliminary investigation into the antifouling potential of extracts of the external shell covering (periostracum) of marine mussels against three common fouling organisms. The periostracum is a thin, pliable covering on the outer shell of some mollusc species. It has been observed that mussels with an intact periostracum are able to resist fouling pressures while the shell coating is in good condition. If the periostracum becomes damaged, opportunistic colonisation by fouling organisms can occur. It has been suggested that the periostracum may contain some form of chemical defence which deters fouling.

Extracts were produced from the separated periostracum of marine mussels (*Mytilus sp.*) using solvents of varying polarity. Produced extracts were tested in bioassays using three common fouling organisms (diatoms, bryozoans and barnacle cyprid larvae), to determine if there was any fouling deterrence provided by the periostracum extracts. Extracts were analysed using Nuclear Magnetic Resonance Mass Spectroscopy (NMR).

Results of the bioassays show varying degrees of antifouling activity against all three organisms. It was demonstrated that the greatest activity was contained in extracts produced by the more non-polar solvents, suggesting that the compound responsible is also non-polar. This was supported by NMR analysis which showed the presence of hydroxylated groups and aromatic rings in the extracted compound which are characteristic of a fatty compound, potentially a triglyceride or steroid. A significant increase in the volume of periostracum analysed is required to further elucidate the structure of the compound responsible for the antifouling activity.

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1. Introduction

The term 'fouling' (or 'biofouling') refers to the colonisation of a substrate by an opportunistic organism (or multiple species of colonising organisms), and has been extensively studied and qualified by numerous researchers (Abarzua & Jakubowski, 1995; Callow & Callow, 2002; Wahl, 1989; Yebra *et al.*, 2004).

Previous research has identified discrete stages in the substrate colonisation process, with the initial stages of fouling occurring almost instantaneously on contact with the (typically) marine aquatic environment (Wahl, 1989; Yebra *et al.*, 2004). The rapid formation of this initial biological 'film' over a surface is often quickly followed by colonisation by larger, more conspicuous and persistent organisms (Anderson *et al.*, 2003; Wahl, 1989; Yebra *et al.*, 2004).

Fouling of ship hulls and niche areas represents a significant cost for the maritime industry. Higher maintenance costs, increased fuel requirements due to greater levels of hull drag, lost productivity due to more frequent dry-docking for removal of fouling organisms, and the cost of compliance with environmental regulations create a significant burden for maritime operators, whether private or public (Callow & Callow, 2003; Statz *et al.*, 2006; Yebra *et al.*, 2004). There are also environmental complications of marine fouling. For example, the introduction of alien species into environmentally sensitive areas, and the release of persistent and/or ecotoxic chemical compounds from antifouling coatings into various environmental compartments (Callow & Callow, 2002; Callow & Callow, 2003).

Previously utilised antifouling agents, such as widely used vessel biocidal coatings containing the organotin 'tributyltin' (TBT), although efficacious against fouling, have limitations relating to their toxicity (Anderson *et al.*, 2003; Statz *et al.*, 2006). The toxicity of TBT in the marine environment has been extensively researched, with evidence of oyster deformities, imposex in dog whelks, adverse effects on marine benthic organisms and concentration and accumulation in the marine food chain (Callow & Callow, 2002; Sonak, 2008). As a result of these environmental impacts, TBT is the subject of a relatively recent worldwide ban by the International Maritime Organisation (IMO). As of January 2008, vessels of countries party to the convention and those engaged in activities at ports of convention parties, are no longer permitted to have TBT containing coatings on vessels of any size (IMO, 2001).

TBT self-polishing copolymer coatings have largely been replaced by coatings containing copper-based biocides which are often supplemented with co-biocides such as zinc pyrithione (Callow & Callow, 2002). Although generally less effective for fouling management than TBT containing coatings, copper-based biocides are considered less detrimental to the marine environment (Abarzua & Jakubowski, 1995). Adverse effects to marine biota have been reported at higher levels of copper exposure; however, some particularly sensitive species and life stages have shown to be sensitive to even low levels of copper present in the environment (Nowak & Duda, 1996; Lee & Johnston, 2007; Levy *et al.*, 2007 and Roman *et al.*, 2007).

The restrictions on coatings containing TBT, coupled with growing concerns over the potential environmental effects of copper-based biocides, have led to an increase in the usage and progressive development of silicon based 'foul-release coatings' (Callow & Callow, 2002).

Foul-release coatings have not proven as effective as biocide-based coatings for slow moving vessels or vessels which remain stationary for long periods, as the water 'shear' force required to release the fouling organisms from the substrate is generated only at relatively high speeds (Callow & Callow, 2002; Callow & Callow, 2003).

Due to these limitations with conventional coatings, research into 'biomimetics' – surfaces and compounds inspired by natural systems – capable of resisting fouling pressure whilst reducing impacts on the local and extended environments has grown in importance (Scardino *et al.*, 2009; Ralston & Swain, 2009; Scardino & de Nys, 2011).

It has been observed that some marine species such as mussels are able to resist fouling when in good physiological condition (Abarzua & Jakubowski, 1995; Wahl, 1998; Scardino *et al.*, 2003; Scardino & de Nys, 2004; Bers *et al.*, 2006a). Mussels have a tough, yet pliable, proteinaceous shell covering secreted by the mantle, which is known as the 'periostracum' (Harper & Skelton, 1993, Scardino *et al.*, 2003). Scardino *et al.* (2003) and Bers *et al.* (2006b) noted that fouling organisms showed some preference toward compromised areas of the periostracum for initial attachment and that mussels with an intact periostracum showed a greater resistance to fouling pressure. Several studies have recorded the influence of a physical fouling deterrent in the form of a microtopography on mussel shells (Wahl *et al.*, 1998; Bers & Wahl, 2004; Scardino & de Nys, 2004). Bers *et al.* (2006b) suggested that in conjunction with the recognised physical antifouling defences of marine mussels (such as 'sweeping' of the shell by the foot), there appears also to be a chemical element to the defence. Bers *et al.* (2006b) were able to demonstrate some antifouling activity toward a variety of common fouling organisms from the extracts taken from the periostracum of the marine blue mussel, *Mytilus edulis*; however, the periostracum was not separated from the rest of the mussel shell, and as a result, it is unclear if the antifouling effects are surface-bound.

The aim of this study was to investigate antifouling activity of extracts of *Mytilus sp.* periostracum when separated from the shell, against various common fouling species (including marine diatoms, bryozoan larvae and barnacle cyprids) and to attempt to isolate and identify extracts responsible for the antifouling activity.

2. Method

2.1 Periostracum removal

Mussels were collected from the DSTO test site at Williamstown (Booth Pier, BAE Systems - 37°51'41.40"S, 144°54'38.06"E) and Werribee (Werribee South Jetty - 37°58'23.22"S, 144°41'13.65"E) intertidal sites and frozen for storage.

Whole samples were submersed in a vinegar and seawater 'pickling' solution to aid removal of the periostracum. A 1:2 vinegar:seawater mixture (approximately 2% acetic acid), was found to be most effective to loosen the periostracum, without degrading the underlying shell. Specimens were gently cleaned of associated detritus (mud, debris, byssal threads etc) and

any obvious microfouling films, prior to submersion in the vinegar solution. Samples were retained in the pickling solution for approximately 24 hours, after which time the periostracum was peeled from the shell with forceps and stored in seawater. Mussels with obvious existing fouling, severely damaged shells or noticeably abraded periostracum were rejected. The periostracum peels were washed twice with HPLC grade water (ASTM Type 1) to remove excess associated salts and acetic acid and then frozen to -20°C prior to freeze drying. Freeze dried samples were then ground to a fine powder using an electric food grade grinder.

2.2 Bioassays

Solvent extractions were performed on the ground periostracum using three solvents: dichloromethane (DCM), ethyl acetate (EA) and methanol (MeOH). Serial extractions were performed, utilising the solvents in order of increasing polarity (DCM/EA/MeOH). The solvents were mixed with the ground periostracum samples by sonification, and allowed to extract for approximately 24 hours. Methanol extractions were performed twice. Extracts were filtered and the extract sample concentrated *in vacuo* using a BUCHI R-210 Rotary Evaporator. Samples were transferred to stoppered vials, dried under nitrogen and weighed. Samples were reconstituted to known concentrations using appropriate solvent to use for bioassays (Figure 1, Step 1). A series of bioassays was performed using three common fouling organisms: marine diatoms (*Amphora sp.* and *Haslea sp.*), bryozoans (*Bugula neritina*, *B. dentata* and *B. flabellata*) and barnacle (*Amphibalanus reticulatus*) cyprid larvae. All assays were performed in Schott flat-bottomed 40 mmx12 mm lidded dishes.

2.2.1 Diatom assay

A number of diatom-based bioassays were conducted to determine the antifouling bioactivity of the periostracum extracts. The bioassays guided the fractionation of the extracts to isolate the most active extract fractions. The complete assay protocol is shown in Figure 1. Assays were performed using cultured *Amphora* and *Haslea sp.* diatom cells from the culture collection held at the School of Botany of the University of Melbourne, Australia. Diatom cells were originally collected from the DSTO test site at BAE Systems in Williamstown Victoria. Cells were isolated and maintained in 200 mL conical flasks supplemented with K-medium plus silicates (K+Si) (Anderson, 2005). Cultures were maintained at 16°C under Sylvania 58W Luxline Plus® and Gro-Lux® fluorescent lamps with a 12 hour light:dark cycle. Prior to testing, cells were concentrated in solution using a rotary centrifuge. After preparation of the test assay plates, the plates were refrigerated at 16°C and exposed to a 12 hour light:dark cycle under Sylvania 58W Luxline Plus® and Gro-Lux® fluorescent lamps for between 24 and 48 hours. UV fluorescence microscopy was used to determine mortality using a Leica DM2500 Light Microscope with UV filter (Red/GFP) under 10x objective. Mortality was determined by the colour of fluorescence recorded – red reflectance indicating a living organism and green reflectance indicating the diatom is dead. Red reflectance (red emission) of the living cell is caused by the excitation of chlorophyll *a* and *b* contained in chloroplasts in a viable cell. Dead cells have damaged or absent chloroplasts which no longer contain chlorophyll and hence no longer fluoresce (Rost, 1995; Hense *et al.*, 2008). Images were recorded using a Leica DC300F microscope mounted camera with Leica Image Manager 50 image processing software.

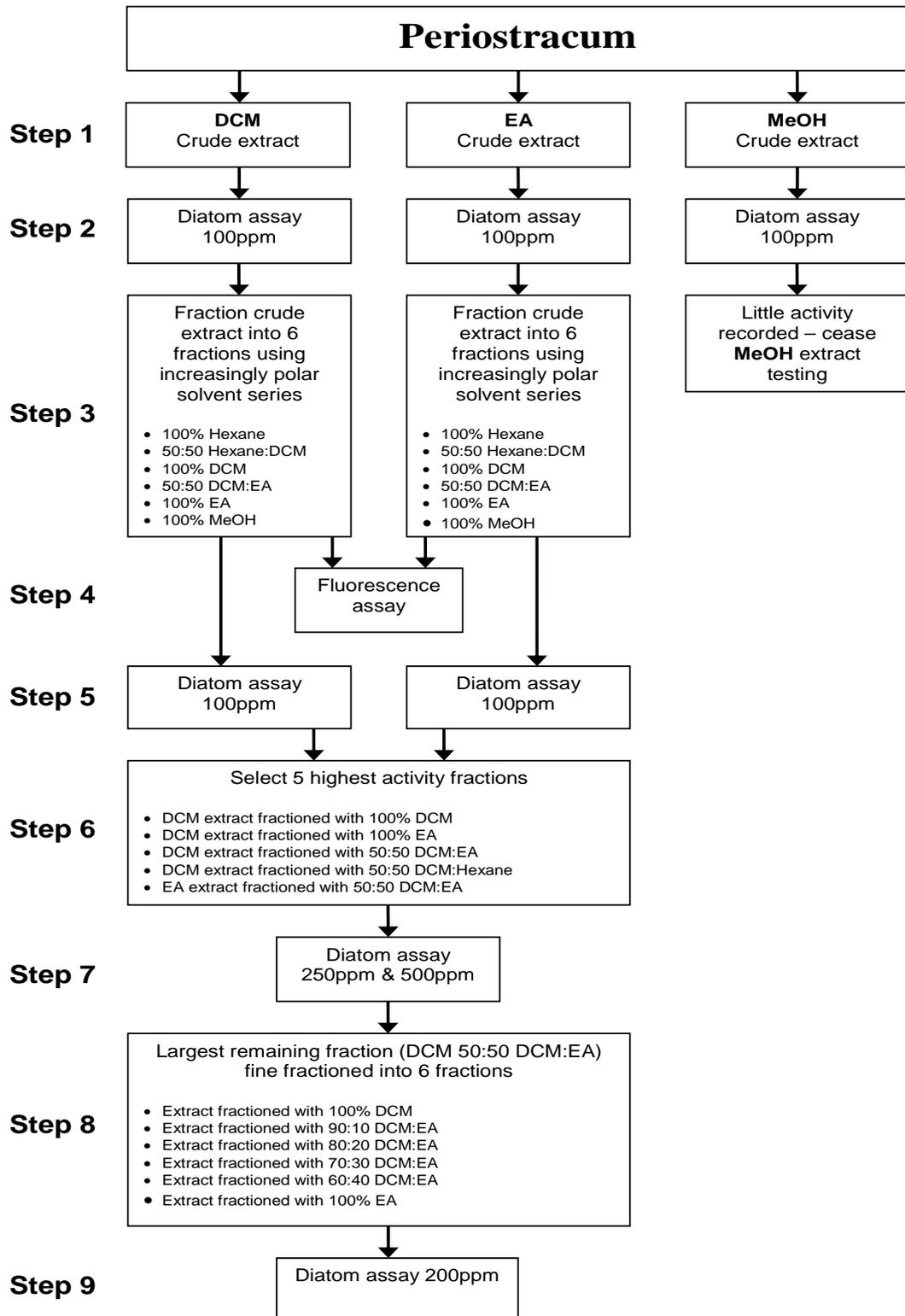


Figure 1 Diatom bioassay protocol DCM=Dichloromethane, EA=Ethyl acetate, MeOH=Methanol

Crude periostracum extracts were made up to a known concentration of 10000 ppm in solvent prior to introduction to the test assay plates. Crude (non-fractionated) extracts of the mussel periostracum were tested in triplicate at a concentration of 100 ppm in a total volume of 1 mL (Figure 1, Step 2). Solvent and seawater controls were also performed in triplicate. All solvents and extracts were allowed to coat the base of the test plates and evaporate to dryness prior to the addition of cell culture and growth medium.

DCM and EA crude extracts were fractionated through a normal phase silica column (Figure 1, Step 3) and the resultant fractions tested for both fluorescence (with no diatom cells present) (Figure 1, Step 4) and activity at 100 ppm (with cells present) (Figure 1, Step 5). The fractionation used an increasingly polar solvent series to produce the six fractions:

100% HEX→50:50 HEX:DCM→100% DCM→50:50 DCM:EA→100%EA→100% MeOH.

Fluorescence assay plates contained only the fractionated extract (enough 10000 ppm stock solution to coat the bottom of the dish) and seawater to a total volume of 1 mL. Fraction activity assay plates were also made to a total volume of 1 mL. Fraction activity assay plates contained 10 µL of extract fraction, 200 µL diatom cell culture and 790 µL of K+Si growth media. Due to the small amount of sample available for testing, only single extract fraction plates were performed. Five extract fractions showing promising activity were selected for further testing at different concentrations (Figure 1, Step 6). The five fractions selected were:

- DCM extract fractionated with 100% DCM
- DCM extract fractionated with 100% EA
- DCM extract fractionated with 50:50 DCM:EA
- DCM extract fractionated with 50:50 DCM:HEX
- EA extract fractionated with 50:50 DCM:EA

The five selected fractions were tested in a total volume of 2 mL at 250 ppm and 500 ppm on single plates only (no replicates) (Figure 1, Step 7). All assay plates contained 200 µL of diatom culture. Duplicate solvent controls at 250 ppm and 500 ppm were also performed, as well as an appropriate negative seawater control in triplicate.

The largest remaining fraction sample (DCM 50:50 DCM:EA) was fine fractionated into a further six fractions (Figure 1, Step 8). The fractions were again produced using an increasingly polar solvent series:

100% DCM→90:10 DCM:EA→80:20 DCM:EA→70:30 DCM:EA→60:40 DCM:EA→100% EA

The six resultant fractions were tested for activity in a total volume of 2 mL at 200 ppm extract concentration (Figure 1, Step 9). All assay plates contained 200 µL diatom culture in the 2 mL total volume. The extracts were performed in duplicate. Seawater and solvent controls were performed concurrently.

To determine any lethal effects of the extraction and fractionation solvents (DCM, EA, MeOH and HEX) a number of procedural control assays were conducted. A 'baseline' bioassay was conducted to establish the effects of the extraction solvents on the diatom cells. Each solvent

was tested in duplicate at 20, 50 and 100 ppm in seawater. Hexane solvent controls were conducted at two concentrations of 50 ppm and 100 ppm. All plates were supplemented with K+Si growth medium to a total volume of 2 mL. All plates had approximately 10 'drops' of concentrated diatom cell culture added. Negative controls were performed in triplicate. All bioassays conducted had concurrent seawater controls performed in duplicate.

2.2.2 Bryozoan assay

The efficacy of periostracum extract at deterring settlement was tested using bryozoan larvae. Mature colonies of bryozoans (*Bugula sp.*) were collected from the DSTO test platform at BAE Systems, Williamstown. Colonies were transferred to the laboratory and held in a closed, dark container with constant air supply for 24 hours prior to being induced to spawn by exposure to a strong, direct light source. If the bryozoans failed to spawn after approximately 40 minutes, colonies were returned to a darkened container and the process repeated after 24 hours. Following successful spawning, larvae were collected and retained in fresh seawater until the bioassays were conducted.

Only a small number of viable larvae were collected during spawning likely due to the extreme weather conditions experienced by the adult colonies immediately prior to field collection, which may have affected the physiological condition of the specimens. Due to the small number of viable larvae available for testing, the two most promising extract fractions (as determined by the diatom assays), were selected for testing: DCM extract fractionated with 100% DCM and DCM extract fractionated with 100% EA. All plates were made up to a total volume of 2 mL. The two extract fractions were tested on single plates at 100 and 250 ppm. Extracts were introduced to the plates and the liquid 'smeared' across the base of the plate and allowed to evaporate to dryness prior to the addition of larvae. Plates were returned to a dark environment and retained for approximately 48 hours. Plates were then assessed for larvae settlement and metamorphosis. Negative seawater controls were performed in duplicate and solvent controls were performed at 100, 250 and 500 ppm for DCM and EA.

2.2.3 Barnacle assay

Crude (non-fractionated) DCM, EA and MeOH extracts were sent to the School of Marine and Tropical Biology at James Cook University (Townsville) to be tested in a barnacle cyprid attachment assay.

Cyprid larvae of the barnacle *Amphibalanus reticulatus* were mass reared from brood stock collected from intertidal settlement plates immersed at the Townsville Yacht Club (19°15'31.80"S, 146°49'19.40"E). Adult brood stocks were maintained at 24°C under a 12 hour light:dark cycle and fed *Chaetoceros muelleri* (CSIRO culture code CS-176), *Isochrysis sp.* (CSIRO culture code CS-177) and *Artemia salina* larvae. 48 hours prior to inducing spawning, brood stock was dark adapted then induced to spawn using a water temperature change (+2°C), light 'shock' and the addition of *Chaetoceros muelleri* into the water. Spawned larvae were collected over a three hour period and transferred to filtered seawater (FSW). Collected larvae were cultured for 6 days in 2-3L Erlenmeyer flasks under filtered aeration at 24°C under a 12 hour light:dark cycle. The culture was maintained at 1 nauplii mL⁻¹ contained in 34 ppt FSW with 250 000 cells mL⁻¹ *Chaetoceros muelleri* (Thiyagarajan, 2002). The culture was

supplemented with antibiotics Erythromycin (10 ppm, Sigma Aldrich code E5389) and Streptomycin (10 ppm, Sigma Aldrich code S6501). Water was changed daily. When all nauplii reached cyprid stage, cyprids were filtered from solution and maintained in fresh seawater at 4°C in darkened conditions until required for settlement assays.

All crude extracts were tested at 100 and 250 ppm. Extracts were introduced to the plates and the liquid 'smeared' across the base of the plate and allowed to evaporate to dryness prior to the addition of larvae. Assays were assessed for settlement, and settled individuals enumerated after 72 and 116 hours. Seawater and solvent controls were performed concurrently, with solvent controls performed at 100 and 250 ppm for DCM, EA and MeOH.

2.3 Bioassay guided fractionation

Results obtained from initial diatom bioassays of crude extracts suggest activity occurs contained in the more non-polar extracts (DCM and EA extracts). The results of these initial assays were used to guide the fractionation of the crude extracts by normal phase silica column using an increasingly polar solvent series of Hexane (HEX), DCM, and EA.

Initial fractions were produced by introducing the crude extract into the silica column and flushing with approximately 5mL of increasingly polar solvent mixtures, as seen below:

100% HEX → 50:50 HEX:DCM → 100% DCM → 50:50 DCM:EA → 100% EA

These fractions were used in bioassays and were fingerprinted using ¹H NMR.

Further fractioning was performed on the combined DCM 50:50 DCM:EA extract by introducing the 50:50 DCM:EA fraction into the silica column and flushing with approximately 5mL of increasingly polar solvent mixture, as seen below:

100% DCM → 90:10 DCM:EA → 80:20 DCM:EA → 70:30 DCM:EA → 60:40 DCM:EA → 100% EA

2.4 Characterisation/identification of crude extracts

2.4.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

All NMR experiments were conducted in deuterated NMR solvents supplied from Cambridge Isotope Laboratories Inc.. DCM and EA extracts and fractions were suspended in approximately 600 µL of *d*-chloroform, while MeOH extracts were suspended in approximately 600 µL deuterated methanol (*d*₄-methanol). Resuspended material was filtered prior to ¹H-NMR analysis. NMR spectroscopy was performed using a Bruker Avance 500 MHz NMR spectrometer using the standard ¹H NMR pulse sequence (zg30) and acquisition parameters.

3. Results

3.1 Diatom assays

A baseline assay was conducted to establish the effects of DCM, EA and MeOH on cell mortality. Solvent toxicity (demonstrated by the absence of red fluorescence which indicates the death of the organism) was not observed or was minimal for the three solvents (DCM, EA and MeOH) at 20 or 50 ppm, and was not observed at 100 ppm for methanol. Both EA and DCM appeared to adversely affect growth and survival of diatoms at 100 ppm. Figures 2-4 shown recorded fluorescence of the assay plates at the conclusion of the assay period. Red cells indicate living organisms, while green cells indicate dead organisms. As illustrated in Figure 2 and 3 respectively, DCM and EA showed no toxicity at 20 ppm or 50 ppm; however, did cause mortality at 100 ppm. As shown in Figure 3, mortality as a result of MeOH toxicity was not observed.

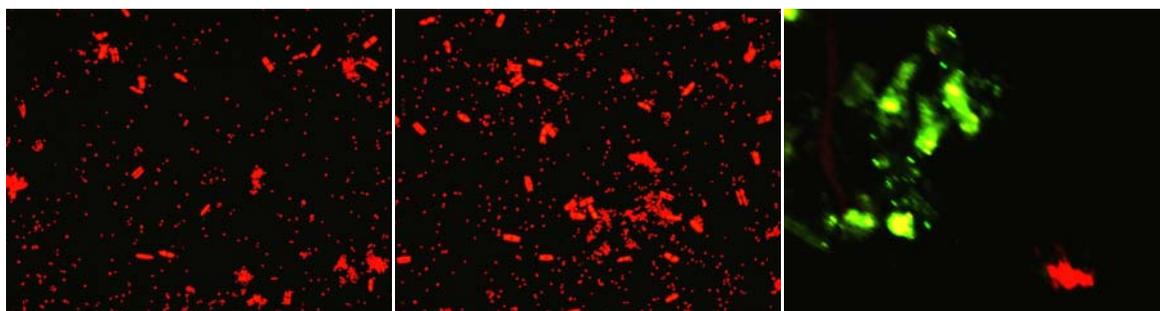


Figure 2 DCM solvent toxicity assay. Red cells indicate living organisms. L-R: DCM 20 ppm, DCM 50 ppm, DCM 100 ppm

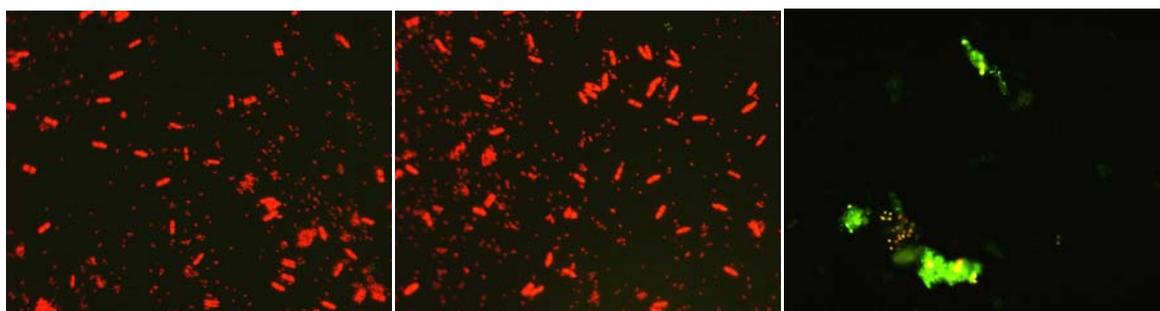


Figure 3 EA solvent toxicity assay. L-R: EA 20 ppm, EA 50 ppm, EA 100 ppm

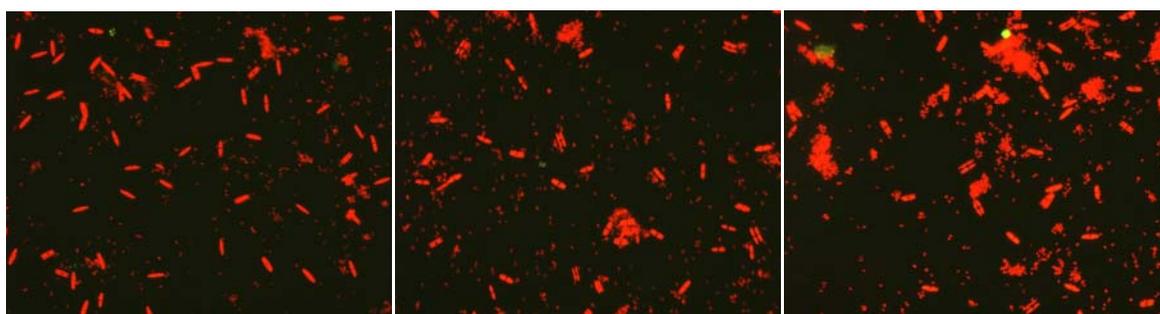


Figure 4 MeOH solvent toxicity assay. L-R: MeOH 20 ppm, MeOH 50 ppm, MeOH 100 ppm

Images recorded from fluorescence microscopy examination of the assay plates on completion of the assay period were assessed visually and a judgement made as to the level of mortality of the cells in the particular assay plate determined by a decrease in red fluorescence of live cells and corresponding rise of green fluorescence of dead cells. The high levels of background fluorescence meant that pixel counts to quantify cell death were unable to be performed and a qualitative visual assessment was made instead. The observed effects (mortality of cells at the conclusion of the assay) were rated on a qualitative scale ranging from (-) to (+++):

- (-) rating represents a very poor performance of the tested extract with less than 10% cell mortality estimated from the observed images
- (+) indicates an estimated mortality rate of between 10% and 50% of cells present
- (++) indicates an estimated 50% to 90% mortality of cells present
- (+++) indicates a very strong effect observed, with greater than 90% of cells present being dead

Table 1 summarises the results of the series of diatom assays performed using crude and fractionated periostracum extracts showing the relative strength of the observed activity assessed by changes in fluorescence of the diatom cells at the completion of the assay. Results from bioassays demonstrate that the greatest relative activity occurred equally in the DCM fractionated with 100% DCM (particularly at 250 and 500 ppm); DCM fractionated with 100% EA (particularly at 500 ppm); DCM fractionated with 50:50 DCM:EA fine fractionated with 90:10 DCM:EA; DCM fractionated with 50:50 DCM:EA fine fractionated with 80:20 DCM:EA; DCM fractionated with 50:50 DCM:EA fine fractionated with 70:30 DCM:EA and DCM fractionated with 50:50 DCM:EA fine fractionated with 60:40 DCM:EA.

Table 1 Summary of results of diatom assay testing of various periostracum extracts

Solvent/extract	Solvent/Fraction	Corresponding figure	Relative strength of observed activity
DCM	Baseline (procedural control)	Figure 2	- (20 ppm) - (50 ppm) + (100 ppm)
EA	Baseline (procedural control)	Figure 3	- (20 ppm) - (50 ppm) + (100 ppm)
MeOH	Baseline (procedural control)	Figure 4	- (20 ppm) - (50 ppm) - (100 ppm)
DCM	Crude	Figure 5	++
EA	Crude	Figure 5	++
MeOH	Crude	Figure 5	- (poor/no observed activity)
DCM crude	100% HEX	Figure 8	- (poor/no observed activity)
DCM crude	50:50 HEX:DCM	Figure 9; 13	- (200 ppm) + (250 ppm) ++ (500 ppm)
DCM crude	100% DCM	Figure 8; 11	++ (200 ppm) +++ (250 ppm) +++ (500 ppm)
DCM crude	50:50 DCM:EA	Figure 8; 12	++ (200 ppm) ++ (250 ppm) ++ (500 ppm)
DCM crude	100% EA	Figure 8	+ (200 ppm) ++ (250 ppm) +++ (500 ppm)
DCM crude	100% MeOH	Figure 9	- (poor/no observed activity)
EA crude	50:50 DCM:EA	Figure 10; 13; 14	++ (200 ppm) ++ (250 ppm) ++ (500 ppm)
EA crude	100% MeOH	Figure 10	- (poor/no observed activity)
DCM crude fractionated with 50:50 DCM:EA	Fine fractionated with 100% DCM	Figure 15	- (poor/no observed activity)
DCM crude fractionated with 50:50 DCM:EA	Fine fractionated with 90:10 DCM:EA	Figure 15	+++
DCM crude fractionated with 50:50 DCM:EA	Fine fractionated with 80:20 DCM:EA	Figure 15	+++
DCM crude fractionated with 50:50 DCM:EA	Fine fractionated with 70:30 DCM:EA	Figure 16	+++
DCM crude fractionated with 50:50 DCM:EA	Fine fractionated with 60:40 DCM:EA	Figure 16	+++
DCM crude fractionated with 50:50 DCM:EA	Fine fractionated with 100% EA	Figure 16	+

Key: (-) < than 10% cell mortality, (+) 10% to 50% cell mortality, (++) 50% to 90% cell mortality, (+++) >90% cell mortality

3.1.1 Crude periostracum extract assay

DCM and EA extracts caused increased mortality (and therefore had increased activity) in comparison to MeOH extracts; however, high levels of 'background' fluorescence from the periostracum extracts made it difficult to definitively assess the activity of the crude extracts. MeOH extracts showed neither increased mortality nor increased background fluorescence. In Figure 5, high levels of green background fluorescence are evident in the images of the crude DCM and EA extracts. As this is the same colour recorded when the diatom cells are no longer viable, the colour could not be definitively attributed to dead cells (and hence activity in the periostracum extracts) or background fluorescence.

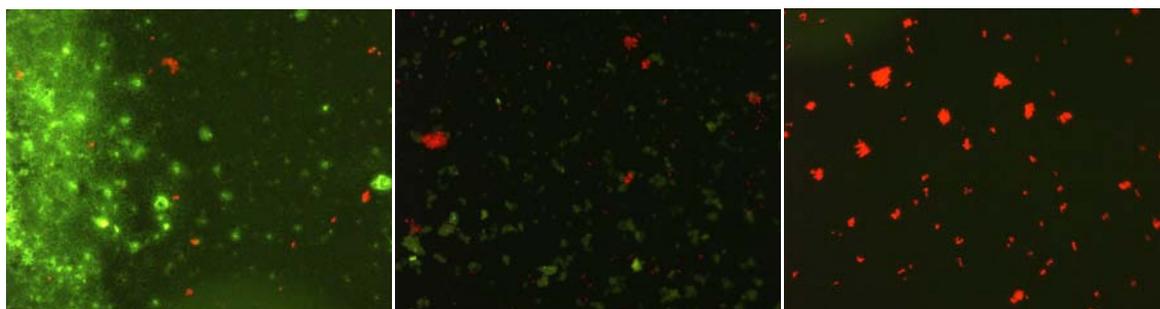


Figure 5 Crude DCM, EA and MeOH periostracum extract assay. L-R: DCM extract 100ppm, EA extract 100ppm, MeOH extract 100ppm

3.1.2 Periostracum extract fraction fluorescence assay

An assay was performed to evaluate the level and colours of fluorescence that were directly attributable to the periostracum extract alone, rather than the response of the diatoms to the introduction of a toxicant. The assay was performed with only periostracum extract fractions with no addition of diatom cells. High levels of background fluorescence were recorded, with a variety of colours present in the different fractions. Figure 6 shows the background fluorescence recorded from periostracum extracts produced by extraction with 100% DCM, DCM extract fractioned with 100% EA and DCM extract fractioned with 50:50 DCM:HEX. Figure 7 shows the background fluorescence recorded for the DCM extract fractioned with 50:50 DCM:EA, EA extract fractioned with 100% MeOH and the EA extract fractioned with 50:50 DCM:EA. The cause of the fluorescence is likely to be an artefact from extraction, as DCM and EA are known to have a UV absorbance. Whilst the auto-fluorescence increased the complexity of the assessment, differentiation of the diatom cells from the extract auto-fluorescence was possible, enabling a determination of cell mortality.

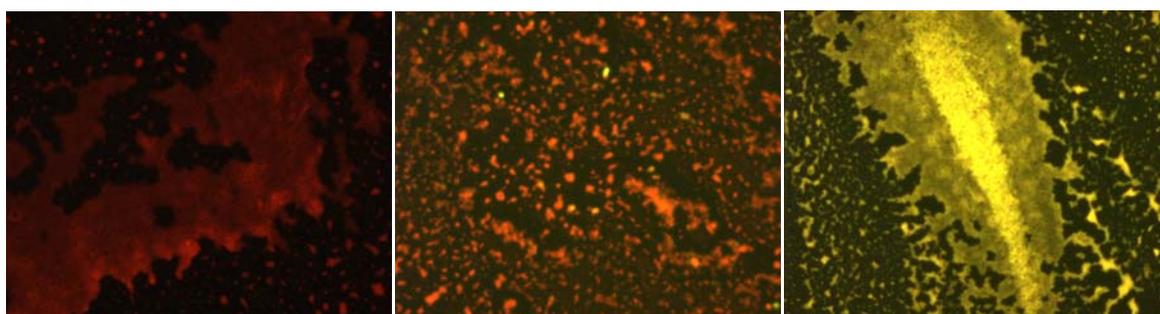


Figure 6 *Periostracum* extract fraction fluorescence assay. L-R: DCM extract fractioned with 100% DCM, DCM extract fractioned with 100% EA, DCM extract fractioned with 50:50 DCM:HEX

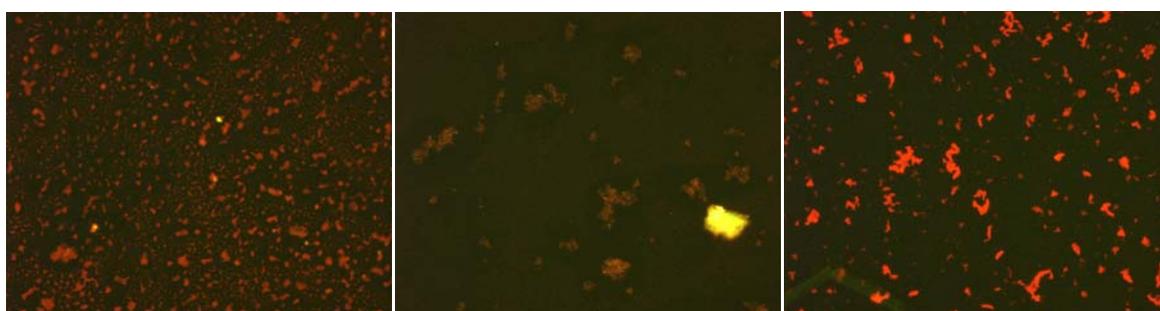


Figure 7 *Periostracum* extract fraction fluorescence assay. L-R: DCM extract fractioned with 50:50 DCM:EA, EA extract fractioned with 100% MeOH, EA extract fractioned with 50:50 DCM:EA

3.1.3 *Periostracum* extract initial fraction activity assay

Biocidal activity indicated by green colouring from dead diatom cells and a lack of viable (red) cells was noted in the following fractions: DCM extract fractioned with 100% DCM (Figure 8), DCM extract fractioned with 100% EA (Figure 8), DCM extract fractioned with 50:50 DCM:EA (Figure 9), DCM extract fractioned with 50:50 DCM:HEX (Figure 9) and EA extract fractioned with 50:50 DCM:EA (Figure 10). Little biocidal activity was observed in the DCM extract fractioned with 100% HEX (Figure 8), DCM extract fractioned with 100% MeOH (Figure 9) and the EA extract fractioned with 100% MeOH (Figure 10).

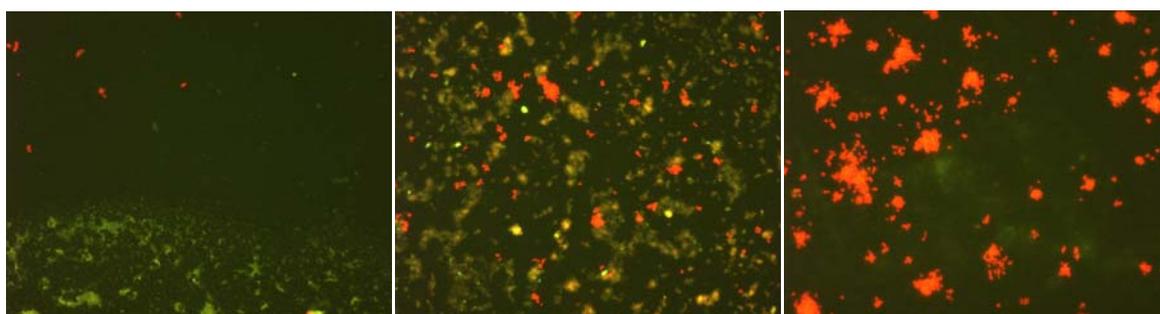


Figure 8 *Periostracum* extract initial fraction activity assay. L-R: DCM extract fractionated with 100% DCM, DCM extract fractionated with 100% EA, DCM extract fractionated with 100% HEX

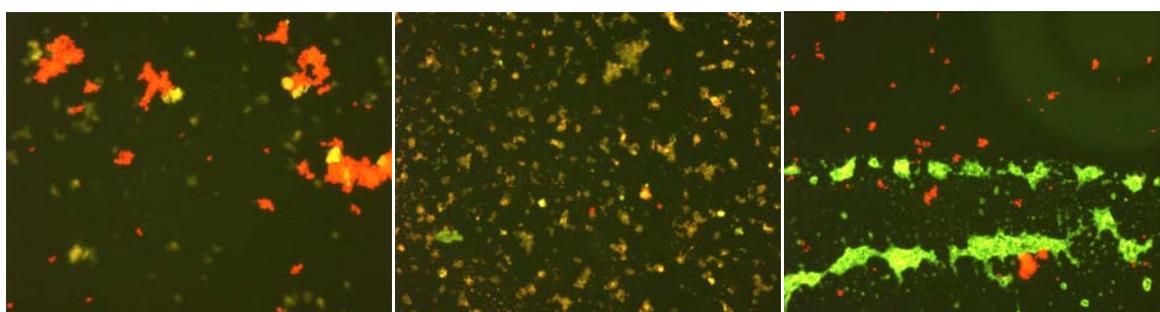


Figure 9 *Periostracum* extract initial fraction activity assay. L-R: DCM extract fractionated with 100% MeOH, DCM extract fractionated with 50:50 DCM:EA, DCM extract fractionated with 50:50 DCM:HEX

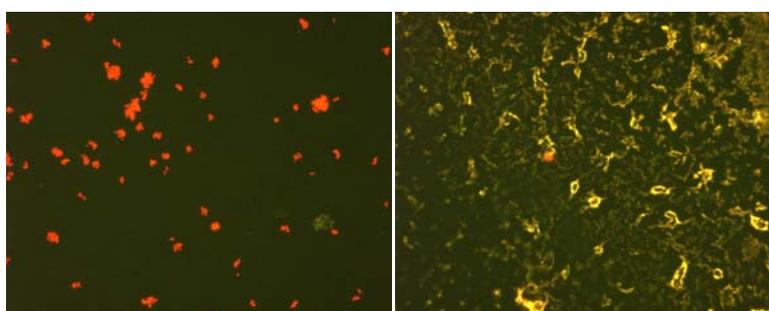


Figure 10 *Periostracum* extract initial fraction activity assay. L-R: EA extract fractionated with 100% MeOH, EA extract fractionated with 50:50 DCM:EA

3.1.4 *Periostracum* extract selected five fraction activity assay

The five fractions with the greatest biocidal activity as determined by initial fraction assays (DCM extract fractionated with 100% DCM, DCM extract fractionated with 100% EA, DCM extract fractionated with 50:50 DCM:EA, DCM extract fractionated with 50:50 DCM:HEX and EA extract fractionated with 50:50 DCM:EA), were assayed again at 250 and 500 ppm (Figures 11-14). Auto-fluorescence of the extract was again present; however, strong biocidal activity was noted particularly in the DCM extract fractionated with 100% DCM (Figure 11), with universal mortality at both concentrations. DCM extract fractionated with 100% EA (Figure 11) also

showed promising biocidal activity; however, the activity was variable in effect at lower concentrations. All other extract fractions (Figures 12-14) showed some activity, particularly at higher concentrations, but auto-fluorescence was again high, and results difficult to quantify.

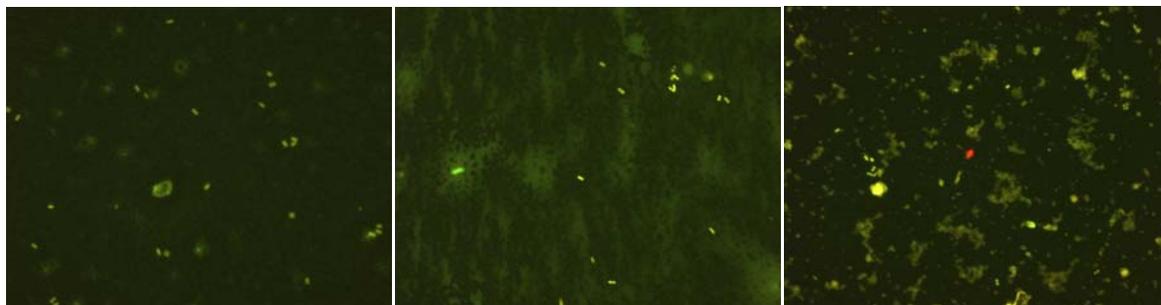


Figure 11 *Periostracum* extract selected five fraction activity assay. L-R: DCM extract fractioned with 100% DCM at 250 ppm, DCM extract fractioned with 100% DCM at 500 ppm, DCM extract fractioned with 100% EA at 250 ppm

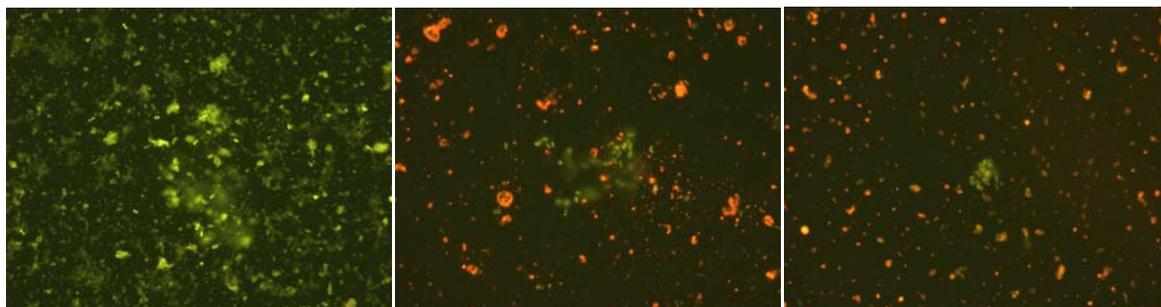


Figure 12 *Periostracum* extract selected five fraction activity assay. L-R: DCM extract fractioned with 100% EA at 500 ppm, DCM extract fractioned with 50:50 DCM:EA at 250 ppm, DCM extract fractioned with 50:50 DCM:EA at 500 ppm

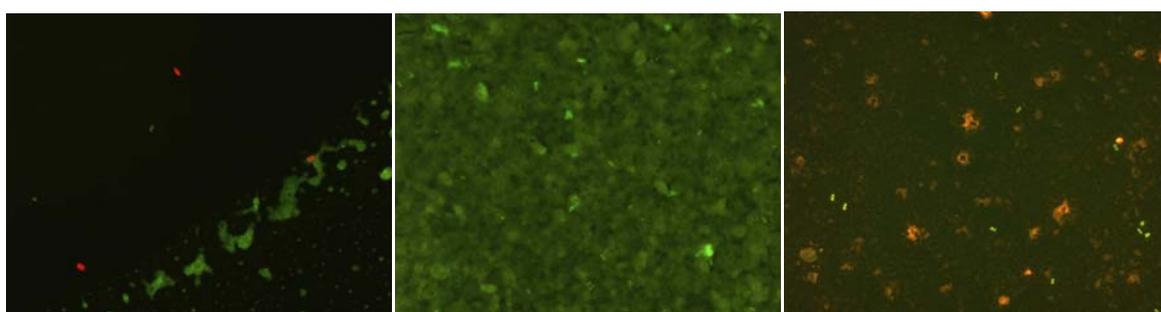


Figure 13 *Periostracum* extract selected five fraction activity assay. L-R: DCM extract fractioned with 50:50 DCM:HEX at 250 ppm, DCM extract fractioned with 50:50 DCM:HEX at 500 ppm, EA extract fractioned with 50:50 DCM:EA at 250 ppm

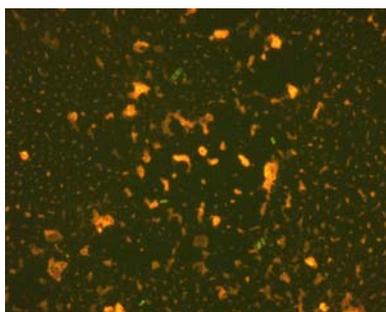


Figure 14 *Periostracum* extract selected five fraction activity assay. EA extract fractioned with 50:50 DCM:EA at 500 ppm

3.1.5 50:50 DCM:EA fine fraction assay

The combined DCM crude extract fractioned with 50:50 DCM:EA was fine fractioned and tested at 200 ppm in a diatom bioassay. Biocidal activity was poor for the 50:50 DCM:EA fractioned with 100% DCM (Figure 15); however strong activity was noted for 50:50 DCM:EA fractioned with 90:10 DCM:EA (Figure 15), 50:50 DCM:EA fractioned with 80:20 DCM:EA (Figure 15), 50:50 DCM:EA fractioned with 70:30 DCM:EA (Figure 16) and 50:50 DCM:EA fractioned with 40:60 DCM:EA (Figure 16). Activity was observed for 50:50 DCM:EA fractioned with 100% EA (Figure 16), however, cells appeared to be in various stages of physiological decline, though not all appeared to be dead. Biocidal activity for the 50:50 DCM:EA extract fractioned with 100% EA (Figure 16), was unusual compared to other tested fractions, as the mortality did not appear to be as localised to the test region of the plate (i.e. biocidal effects were observed occurring outside the extract 'smear' zone) which had not been previously recorded. The reason for this effect is unclear, but may be due to the active compound leaching out of the 'smear zone' into the surrounding liquid.

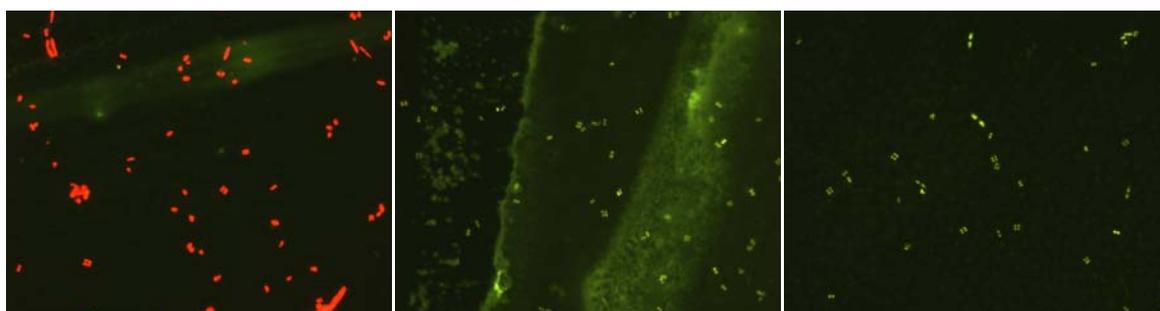


Figure 15 50:50 DCM:EA fine fraction assay. L-R: 50:50 DCM:EA fractioned with 100% DCM, 50:50 DCM:EA fractioned with 90:10 DCM:EA, 50:50 DCM:EA fractioned with 80:20 DCM:EA

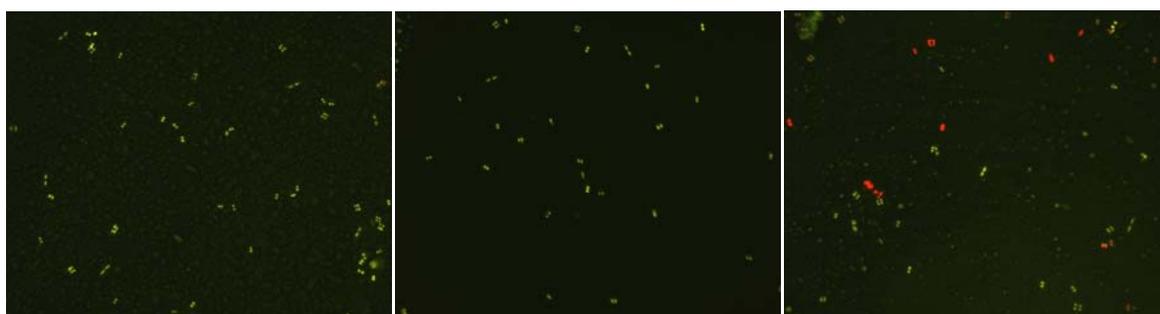


Figure 16 50:50 DCM:EA fine fraction assay. L-R: 50:50 DCM:EA fractionated with 70:30 DCM:EA, 50:50 DCM:EA fractionated with 40:60 DCM:EA, 50:50 DCM:EA fractionated with 100% EA

3.2 Bryozoan assay

The bryozoan larvae assay was small in scale, as very few larvae were able to be harvested due to low organism spawning levels. Consequently, only two extract fractions were selected for testing: DCM extract fractionated with 100% EA and DCM extract fractionated with 100% DCM. The tested extracts showed some antifouling effect, with metamorphosis and settlement adversely affected by both the EA and DCM fractions. Activity was most pronounced at higher concentrations for the EA fraction (250 ppm), whilst the DCM fraction showed inhibition of metamorphosis and settlement at both test concentrations (100 ppm and 250 ppm). Table 2 lists the results of the bryozoan assay and shows the numbers of larvae recorded in four particular states at the conclusion of the assay.

Table 2 Results of bryozoan assay

Plate	S&M	NS&M	S&NM	NS&NM
Seawater Control (SW)1	2	3		
Seawater Control (SW)2	5			
DCM 100 ppm	1	1	1	3
DCM 250 ppm	2	2	2	
DCM 500 ppm	2	2		1
EA 100 ppm	1		3	2
EA 250 ppm	4		3	
EA 500 ppm	1	5		
DCM 100% EA 100 ppm	5	1		
DCM 100% EA 250 ppm			6	
DCM 100% DCM 100 ppm			7	
DCM 100% DCM 250 ppm			5	5

(S) Settled larvae; (M) Metamorphosed larvae; (NS) Non-settled larvae; (NM) Non-metamorphosed larvae. SW 1 and 2 plates refer to concurrent seawater controls.

3.3 Barnacle cyprid attachment assay

Figures 17 and 18 show that antifouling performance of the tested crude extracts was variable when tested in a barnacle cyprid attachment assay.

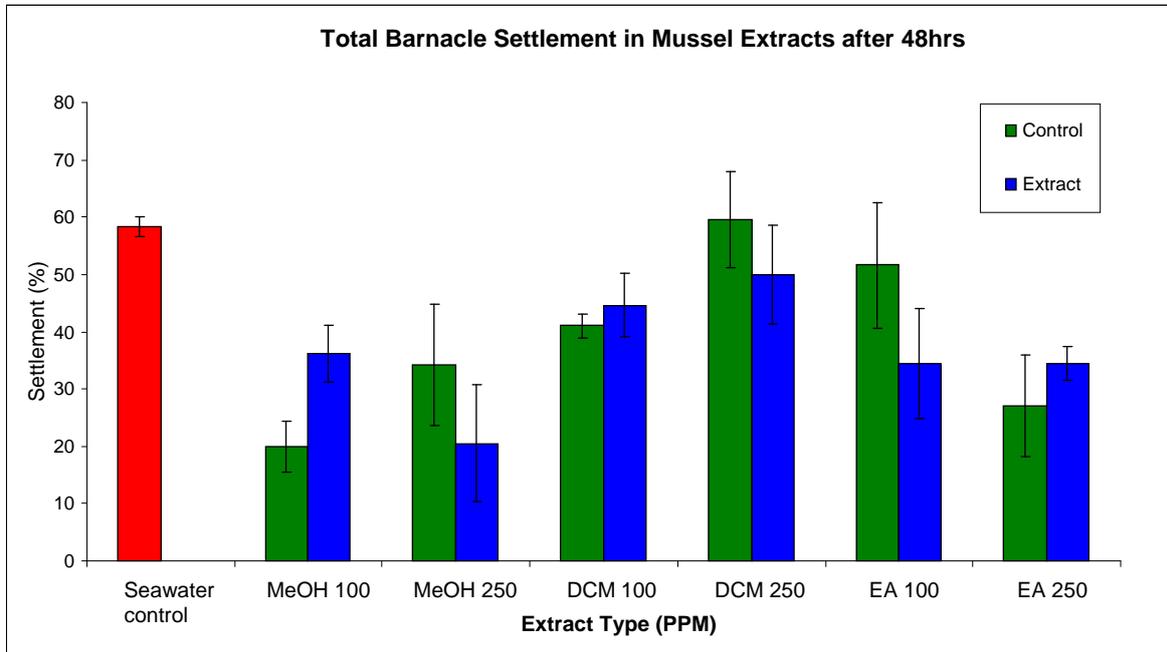


Figure 17 Graph of barnacle percentage settlement after 48 hours for different extract fractions

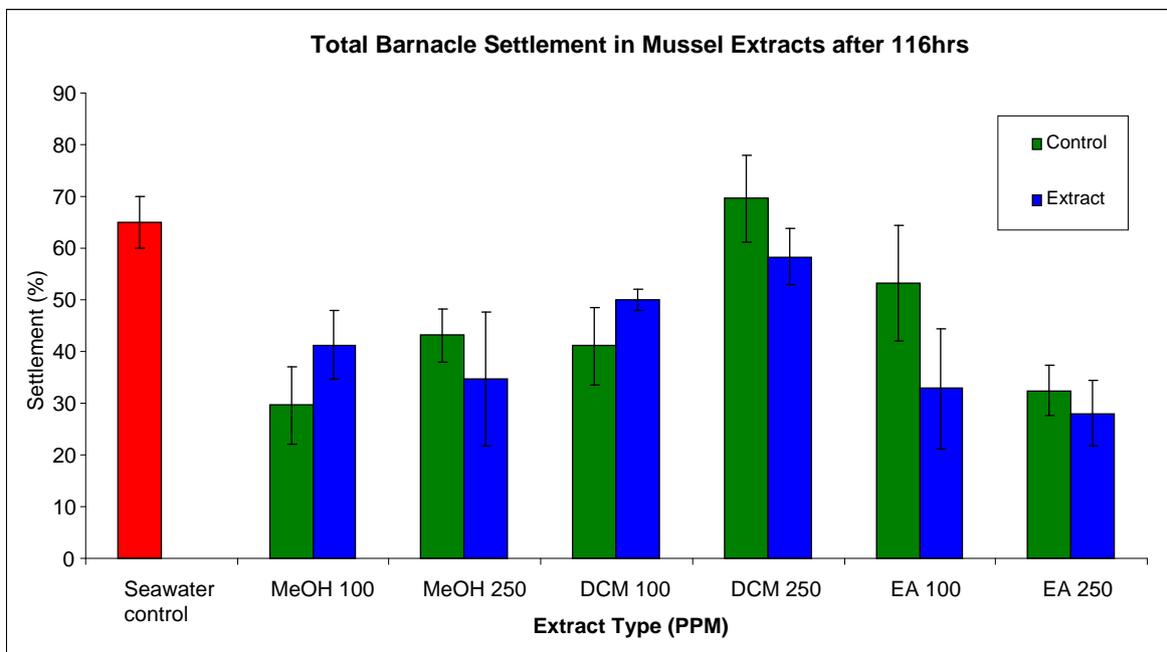


Figure 18 Graph of barnacle percentage settlement after 116 hours for different extract fractions

Single factor ANOVA showed no significant difference at 48 hours or 116 hours between the paired extracts and their respective controls or the same extract at different concentrations (for example DCM 100 ppm compared with DCM at 250 ppm) ($p > 0.05$). A significant difference was shown at 116 hours between the DCM 250 ppm extract and the EA 250 ppm extract ($p = 0.03$); however all other comparisons of the different extracts at the same concentrations showed no significant difference. At 250 ppm the DCM extract showed significantly greater percentage settlement rates than the EA 250 ppm extract. Seawater controls (indicated in red) confirm the viability of the larvae for settlement.

3.4 NMR

Analysis of the generated ^1H NMR spectra (representative spectra of high activity fractions are shown in Figure 19), indicates the major component of the periostracum extract is likely a fatty compound, as highlighted by observed resonances (indicated by the blue box) at δ 1.60, δ 1.20 and δ 0.85. Chemical shifts recorded in the spectra were highly characteristic for the presence of such a compound. The large resonance in the ^1H NMR spectrum is indicative of residual CHCl_3 within the deuterated NMR solvent CDCl_3 . Shifts associated with hydroxy methines are highlighted by the group of diagnostic resonances occurring around δ 4.00 contained in the red box. Resonances associated with aromatic groups were also recorded and are indicated by the two small black boxes containing shifts occurring around δ 7.00. There are many possibilities as to the structure and identity of the compound responsible for the observed activity including sphingosines, acylated glycerides, straight chain or hydroxylated fatty compounds. Further isolation and structure elucidation using other NMR experiments is required to elucidate the structure of the compound(s) responsible for the activity.

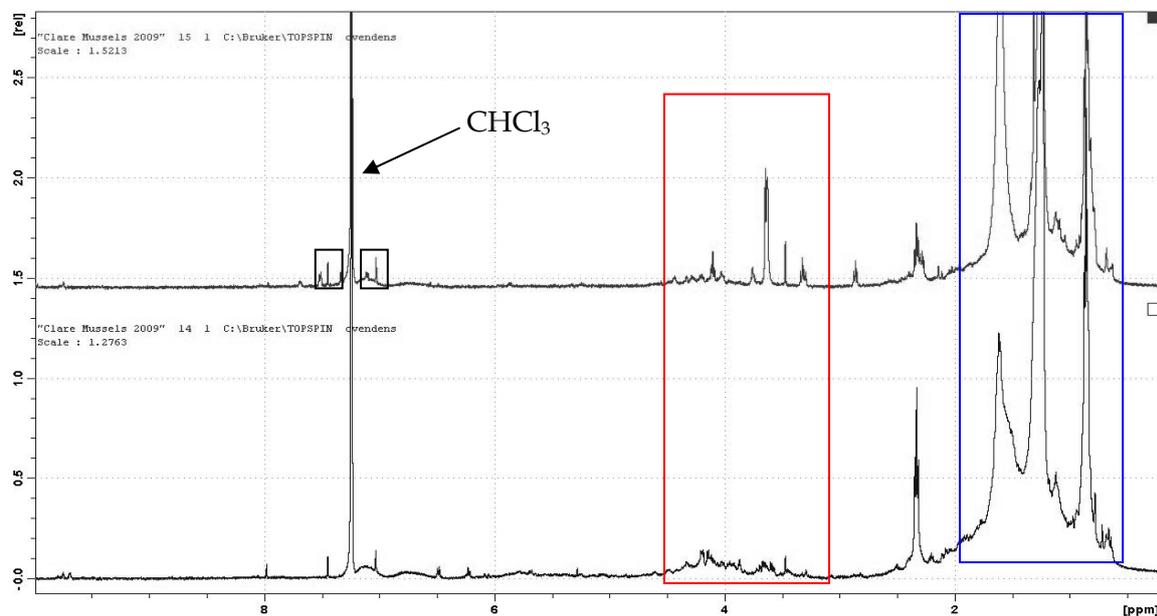


Figure 19 Stack plot of two high activity ^1H NMR spectra. Top spectrum: DCM extract fractionated with 100% DCM; bottom spectrum: DCM extract fractionated with 80:20 DCM:EA

4. Discussion

This research program has shown antifouling activity of extracts of marine mussel periostracum against a variety of common fouling organisms. The results of this research project support previous published findings regarding biocidal activity of mussel periostracum extracts. Research into novel, natural biocides has shown some antifouling activity in extracts of the periostracum obtained from *Mytilus sp.* (Bers *et al.*, 2006a). This research program has supported Bers finding and has extended and refined the work, due to the isolation of the periostracum from the mussel shell and testing of only those extracts recovered from the periostracum itself. Further, the research has narrowed down where the antifouling activity occurs, and has given some indication as to the compound(s) responsible for the observed activity.

Diatom assays show that biocidal activity is greater in extracts produced from DCM and EA rather than MeOH. This suggests that the compound responsible for the activity is relatively non-polar and hence able to be extracted by increasingly less polar solvents. Similarly, bryozoan settlement and metamorphosis was adversely affected by extract produced from DCM and EA. No significant difference was observed between extracts and control in the barnacle cyprids attachment assay.

Initial NMR analysis supports the suggestion of a non-polar compound being responsible for the observed activity, with results highly characteristic of a non-polar fatty compound such as a triglyceride or steroid. NMR analyses of the extracts have shown the presence of fatty compounds, hydroxylated groups and aromatic compounds.

Statistical analysis of results was unable to be performed for this study due to a number of factors. The high levels of auto-fluorescence demonstrated by the periostracum extract prevented pixel counting of the diatom assay images, and hence no accurate quantitative analysis of the biocidal effect of the extracts could be made. The small data set from the bryozoan assay meant that any statistical interpretation would not be robust. However, it was shown that the tested extracts had a deterrent effect on the metamorphosis and settlement of bryozoan larvae. The barnacle cyprid attachment assay showed variable test results and did not demonstrate any significant difference between the performance of the controls and the performance of the extracts in preventing settlement. Previous research into the antifouling characteristics of marine mussel shells has shown that the microtopography of the shell may be a physical deterrent to barnacle cyprid settlement, and hence, chemical activity toward barnacle cyprids may not be as pronounced (Scardino & de Nys, 2004).

If future investigations into the antifouling potential of the marine mussel periostracum are to be successful, greater amounts of raw materials (i.e. mussels) and improved periostracum removal and extraction techniques are needed. Larger bioassays to verify the initial 'screening' assays performed during this research project will be required. Field trials may be of assistance to validate these and future laboratory test results. Identification and structural investigation of the chemical responsible for the antifouling activity would also benefit from greater extract amounts, as High Performance Liquid Chromatography (HPLC) could be employed to fraction and purify the extract, enabling more accurate analysis by NMR, Gas

Chromatography Mass Spectrometry (GCMS), Liquid Chromatography Mass Spectrometry (LCMS) or Fourier Transform Mass Spectrometry (FTMS).

Even if the particular compound responsible for the apparent activity is able to be isolated and definitively identified, challenges may still exist. The synthesis of a stable compound is often problematic, as is the successful integration into a marine coating matrix, whilst still retaining antifouling efficacy. As a result, any use of natural antifouling compounds derived from and inspired by marine mussel natural defence systems, will take considerable development time before application into any new product. Similarly to the strategies employed by marine organisms, any natural products may form only one component of a multifaceted antifouling defence system.

Due to the complexity of natural products research, and the time restrictions of this research program, a definitive identification of the active compound has proved elusive. This indicates that further long range research into chemical antifouling defence systems of marine mussels is warranted and should include bioassays on a wider range of common fouling organisms and expanded field trials. Further elucidation of the chemical structure using a variety of analytical techniques and comparison against databanks may provide information as to the chemical identity of the compound responsible for the antifouling activity witnessed.

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19. ABSTRACT This report details the research program investigating the potential antifouling activity of extracts from the periostracum of the marine mussel <i>Mytilus sp.</i> . Previous research has suggested that marine mussels utilise multi-faceted defence mechanisms to ensure their fitness does not suffer due to heavy fouling colonisation of their shells. The shell coating known as the periostracum is indicated as a possible physical and chemical antifouling defence component. In this study, the periostracum was separated from the shell and extracts of the periostracum obtained by serial extraction with three solvents of increasing polarity. The extracts were tested against common marine fouling organisms: diatoms, bryozoans and barnacle larvae. Considerable activity against marine diatoms and bryozoans was shown by dichloromethane and ethyl acetate extracts. Initial NMR fingerprinting of the extracts suggests the presence of fatty compounds (potentially triglycerides), hydroxylated groups and aromatics. Further purification by HPLC and NMR analysis would be required to elucidate the structure of the compound responsible for the noted antifouling activity. This would require a significant increase in the amount of mussel periostracum to be acquired.						