Title: Assessing Antifouling Activity by *Heteractis magnifica* against *Amphibalanus amphitrite* and *Escherichia coli*

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Abstract

Antifouling coatings are used to inhibit the growth of marine biofouling; however, current antifouling coatings are considered harmful to non-target species as they are not biodegradable. Copper and TBT are heavy metals in antifouling coatings that leachate and are destructive to non-target marine life. In order to prevent harm to non-target species, natural bioactive metabolites can be applied in antifouling coatings as opposed to metallic coatings. Sessile organisms like the sea anemone are an ideal candidate for natural antifoulants. Sea anemones are rich in secondary bioactive metabolites and produce natural chemical repellents to prevent the growth of fouling organisms on the surface of their body. This research project focused on the sea anemone *Heteractis magnifica* due to the potential of natural antifoulants present in the organism. A larval barnacle settlement inhibition assay and biofilm assay were performed to determine the ability of the sea anemone crude extract to inhibit biofouling. Through this research, a natural antifoulant can potentially be identified.

Introduction

A major problem for marine industries is biofouling, the accumulation of undesirable organisms on immersed artificial surfaces. Biofouling latches onto anthropogenic surfaces such as ships and aquaculture structures which hinders

efficiency. Marine industries look to counter biofouling because of increased fuel consumption of ships due to frictional resistance, reduced waterflow, and increased weight of infrastructures [6,14]. All submerged structures and vessels functioning in seawater are at risk of biofouling (Figure 1).

Figure 1 Marine biofouling. Coated steel grate after 9 months of exposure (left image) and uncoated steel grate after 7 months of exposure (right image) [20].

Microfouling and macrofouling organisms are the two main forms of marine biofouling. Microfouling is a physical, reversible adhesion while macrofouling is a chemical, irreversible adhesion. Microfouling organisms are microscopic organisms like fungal and bacterial biofilms while macrofouling consist of larvae or macroorganisms that attach and anchor themselves to submerged surfaces. The first step in the formation of biofouling is the occurrence of a physical reaction in which the organic material forms on the substrate surface (Figure 2). Following this adhesion, the bacteria or diatoms undergo reversible adsorption or irreversible adhesion. Biofilm rapidly forms on any immersed surface in seawater which leads to the eventual growth of macrofoulers. Macrofouling is dependent on the

formation of microfoulers; therefore, it would be ideal to prevent biofouling during the physical reactions rather than biochemical reactions [4].

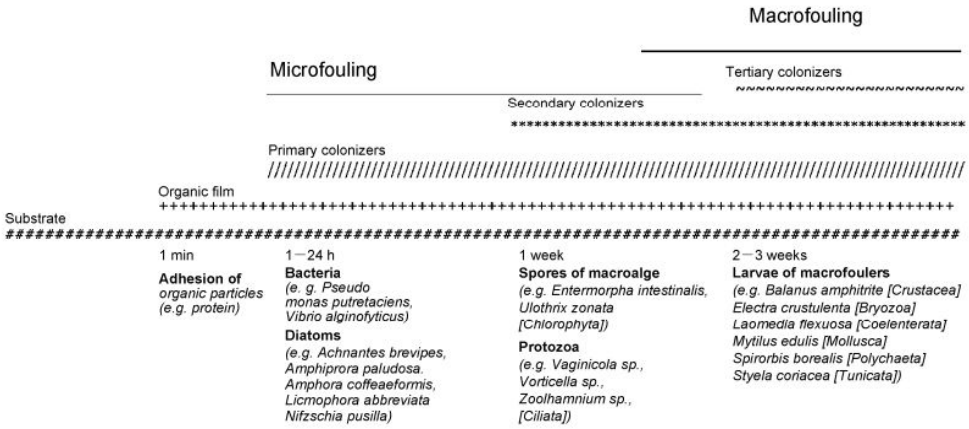

Figure 2 Temporal structure of settlement of biofouling organisms [1].

To prevent the growth of these undesirable organisms, antifouling coatings are used. Antifouling agents are in chemical, biological, and physical methods to combat marine biofouling. Out of the respective methods, chemical methods are the most popular choice for industries because they are the most effective and inexpensive. Chemical methods involve the coating of self-polishing antifouling agents like tributyltin (TBT) and copper which inhibit growth and repel biofouling. However, these chemical coatings have adverse effects on the environment as they are not biodegradable. The two heavy metals mentioned above produce leachates which are defined as water that has percolated through a solid and leached out of its chemical constituents. The copper and TBT leachates produce ecotoxicological

effects on the environment. TBT based paints were banned on a worldwide scale in 2008 by the International Maritime Organization because the use of TBT causes shellfish deformities, the bioaccumulation of tin within a variety of organisms, and severe pollution as TBT absorbs on particles and aggregates in sediments [5,9]. However, many countries continue to use TBT-based coatings because they are the most effective antifouling coatings created in the marine industry.

Copper coatings also have the same ecotoxicological effect on a lower scale. In the marine environment, copper is one of the three most toxic heavy metals as it is detrimental to the abundance, growth, and reproduction of marine invertebrates [10]. Heavy-metal ions like copper induce oxidative stress by collecting in the gills of fish and interfering with breathing. It has also been found that the diversity of native and nonindigenous species is decreased in the presence of copper antifouling [3,15]. While copper is an essential nutrient for marine life, concentrations which exceed 20 micrograms per milliliter (μg/mL) are toxic to marine organisms. Fish and crustaceans are about 10 to 100 times more sensitive than mammals to the toxic copper alloys which leachate into the ocean [11]. Furthermore, metal bioavailability and toxicity to aquatic organisms can increase at lower salinity; this effect is due to less complexity of the metal ion with anions and a lack of competition of metal ions with cations for metal binding sites [16,17]. To

avoid these negative environmental impacts of copper antifouling, the use of environmentally benign antifoulants is an important strategy to be researched.

The importance of using natural products for antifouling has been recognized because of their ability to inhibit biofouling organisms while remaining non-toxic to non-target organisms and the environment. One of the most ecologically relevant antifouling strategies involves the application of the chemical defenses of sessile marine organisms, as these organisms have the ability to naturally repel biofouling on the surface of their invertebrate bodies [12]. Sessile organisms are rich in secondary bioactive metabolites. These metabolites would be important to use in antifouling coatings because of their natural biological effect on marine life to repel the settlement of fouling.

A potential source of natural antifouling agents is the sea anemone. Sea anemones contain secondary bioactive metabolites and nematocysts which are natural toxins that show antimicrobial activity [2]. The use of these metabolites and nematocysts would be ideal for antifouling since they are natural chemical repellents of marine organisms and can even be applied to antimicrobial drugs. There have been many biologically active compounds separated from marine invertebrates for the use of antifouling. The respective repellents have been hypothesized to produce the bioactive compounds necessary to prevent parasitic or

microbial colonization of their surface [8,19].

The sea anemone is a member of the phylum Cnidaria and the class Anthozoa. Organisms of this phylum and class protect themselves against the lethal or debilitating consequences of microbial and parasitic invasion through discriminatory tissue reactions [20]. Through these reactions, they produce natural repellents that can be potentially applied to antifouling coatings. Due to easy availability of *Heteractis magnifica* and previous research that indicates that it is rich in potential antifouling agents, this organism was chosen to be screened in the preceding research.

To analyze the antifouling activity of compounds, larval barnacle bioassays can be conducted since barnacles are major target organisms for antifouling activity [7]. Larval barnacle at the cyprid stage are tested for antifouling assays because it is the life stage in which barnacles function to find a surface to cement themselves on. The cyprid larvae secrete a glycoproteinous substance to settle on surfaces and cease to eat at this stage since they must settle. The inhibition of settlement for larval barnacle culture shows the ability of the crude extract to prevent barnacles from attaching to the surface of the multi-well plate that they are tested in.

Further, biofilm assays are conducted to determine antifouling activity

because the disruption in the formation of biofilm can be measured through absorbance. The susceptibility of biofilms to antifouling activity cannot be determined through standard microdilution testing since the above tests are based on the response of planktonic (suspended) organisms rather than biofilm (surface-associated) organisms [18].

Purpose

The purpose of this study is to analyze the antifouling activity of the sea anemone *Heteractis magnifica* through a larval barnacle inhibition assay and biofilm assay. If the crude extract of *Heteractis magnifica* inhibits the growth of the respective organisms, it can be concluded that antifouling agents are present. Therefore, the purpose would also be to isolate and identify the active antifouling components which are presumed to be present in the *H. magnifica* crude extract.

Hypothesis

The sea anemone *H. magnifica* will produce bioactive agents which inhibit biofouling organisms like the barnacle *Amphibalanus amphitrite* and *Escherichia coli* and can be isolated and identified. The acetone crude extract will be the most efficient extract in inhibiting larval barnacle growth and biofilm formation based on past research of this crude extract.

Null Hypothesis

Heteractis magnifica crude extract will show no activity against larval barnacle settlement and biofilm formation when compared to the control.

Variables

Variables for the larval barnacle assay include the control of the aged sea water, the negative control of the DMSO treatment, and the positive control of the CuSO4. The experimental group consists of the acetone, ethanol, and methanol crude extract treatments of 25 μg/mL. The dependent variable are the rates of settlement, death, and swimming larvae. The independent variable is the type of extract. The variables for the biofilm assay include the control of LB and the negative control of PBS. The standard is LB and *E. coli* culture. The experimental group is the differing concentrations of the acetone crude extract. The dependent variable is absorbance. The independent variable is the concentration of the crude extract.

Safety

Sea anemones should be handled with care because they release neurotoxins that may come in contact with the skin and cause swelling and redness. *E. coli* can cause infections if not properly handled; eye protection, gloves, and a lab coat are necessary whenever handling *E. coli*. Acetone, ethanol, and methanol should be

kept away from flame because they are highly flammable. The autoclave must be loaded properly to avoid explosions. To avoid burns, it is important to take caution of hot surfaces when loading and unloading the autoclave. DMSO can cause mild skin irritations and is combustible and flammable. While handling DMSO, protective clothing should be worn.

Materials

- 24-well & 96-well polystyrene multiwell plates (located in E8)
- Autoclave Benchmark Bioclave (located in E8)
- *Amphibalanus amphitrite;* barnacle larvae (generously provided by Daniel Rittschof and Beatriz Orihuela of Duke University)
- Acetone Flinn Scientific (located in E8)
- Air pump (located in E8)
- Aquarium (located in E8)
- Biospec Tissue Tearor Model 985370 (located in E8)
- Copper sulfate (located in E8)
- Cover slips (located in E8)
- 0.4% crystal violet Sigma Aldrich (located in E8)
- Dissecting Microscope
- DMSO (located in E8)
- *E. coli* (located in E8)
- Ethanol Flinn Scientific (located in E8)
- 15 mL and 45 mL falcon tubes (located in E8)
- 250 mL Erlenmeyer flasks (located in E8)
- 500 mL beakers (located in E8)
- 150 mL beaker (located in E8)
- 100 mL beaker (located in E8)
- Deionized water (located in E8)
- Fisher Scientific™ Multiskan™ FC Microplate Reader (located in E8)
- *Heteractis magnifica* (Ritteri anemone; obtained from Blue Zoo Aquatics)

- Incubator (located in E8)
- Difco ™ Luria-Bertani Broth and Agar, Miller (located in E8)
- Methanol Flinn Scientific (located in E8)
- Pipettor Biopette (located in E8)
- Multichannel pipettor Biopette (located in E8)
- Pipette tips (located in E8)
- Scalpel (located in E8)
- Tissue homogenizing system glass tube (located in E8)
- Millipore Filter paper 0.4 µm (located in E8)
- 1 mL microcentrifuge tubes (located in E8)
- Analytical balance (located in E8)
- Laboratory refrigerator (located in E8)
- Centrifuge (located in E8)
- Aged seawater (generously provided by Daniel Rittschof and Beatriz Orihuela of Duke University)
- Buffer tray (located in E8)

Methods

Methods were conducted in E8 at TOHS.

Preparation of crude extract

On arrival the entire body of *H. magnifica* was washed in DI water before usage to allow time for undigested food and adherent debris to be removed. 300 g of the sea anemone was cut into small pieces with a scalpel. 100 g of the separate sea anemone pieces were placed in a tissue homogenizing system glass tube and extracted with distilled water (1:2, w/v). The Biospec Tissue Tearor (handheld homogenizer) was used for homogenization. The homogenized sea anemone pieces

were then centrifuged at 13 675 rpm for 20 min at room temperature. The supernatant of the distilled water was removed. The homogenate of the sea anemone pieces was then immersed in a beaker of 150 mL of methanol. This process was applied with separate 100 g pieces of anemone for ethanol and acetone extraction, respectively. The homogenized sea anemone pieces were extracted with the respective solvents and the crude extract was centrifuged at 13 675 rpm for 20 min at room temperature. The supernatants were removed by pipette and the solutions were evaporated to dryness by blowing a stream of gas over the solutions to remove the volatile solvents. This method of evaporation was conducted in a fume hood for 48 hrs and the resultants were stored at 4ºC for further use. The preparation of the crude extract was conducted in a modified manner of the research of *Antimicrobial properties of sea anemone Anthopleura nigrescens from Pacific coast of Costa Rica* [4].

Larval barnacle culture

Amphibalanus amphitrite nauplii were obtained from the field and reared in mass culture to cyprids (the settlement stage) on *Skeletonema costatum*. Cyprids were collected from the culture by a sieve cascade after 4 days, cleaned of debris and held at 4 - 6°C [regards to Daniel Rittschof and Beatriz Orihuela]. The cyprids *Amphibalanus amphitrite* were then maintained in the laboratory in 35 ppt

seawater at 4°C and used at 3 and 4 days old.

The experiments were conducted in sterile 24-well polystyrene multiwell plates. Before testing the crude extract, a pre-test of larval tolerance was performed to decide the maximum percentage of the organic solvent allowed to be used to dissolve the extract. The crude extract was dissolved in DMSO and then added to aged seawater to obtain a concentration of 100 μg/ml with DMSO concentration less than 1%. Ten competent larvae were added by micropipette to each of the 6 replicates of 2 mL of the test solution. CuSO4 solution was tested at the concentration of 25 μg/mL. The respective substances were added by pipette so that each well had 2 mL of solution. The plates were incubated for a 24 h period under similar conditions as when the larvae were reared. After the incubation period, the larval settlement, swimming, and death were calculated. Cyprids were considered dead if they did not move, their appendages were extended, and they did not respond when touched lightly with a metal probe. After counting the dead and swimming larvae, the multiwell plates were rinsed with DI water to remove unattached larvae. Permanently attached larvae were then counted to determine the settlement rate.

After determining the maximum amount of organic solvent allowed, the experiment was conducted with the acetone, ethanol, and methanol crude extracts

and new batches of larvae. There were 6 replicates of the acetone, ethanol, and methanol crude extracts with the concentrations of 25 µg/mL which is the concentration screened for in antifouling assays [13].

Antifouling activity of Heteractis magnifica against E. coli biofilm

Using a multipipettor, the wells in a 96-well microtiter plate were filled with varying volumes of crude extract consisting of 10 µL, 20 µL, 30 µL, 50 µL, 100 µL, and 200 µL. Each group had 3 replicate wells. The groups all had the same volume by adding LB until the individual well reached 300 µL. Another microtiter plate was prepared with fresh LB and 1 µL of the growth culture and was incubated at 37°C for 24 hours. One µL of the *E. coli* culture from the respective plate was then pipetted into each well of the second plate and was incubated at 37°C for 24 hours. The medium from the wells were removed and the wells were washed three times with 200 µl per well of distilled water using a multichannel pipette and allowed to dry for 15 minutes. The wells were stained with 200 µL of 0.4% crystal violet at room temperature for 20 min. The unbound stain was disposed of and the wells were then washed gently three times with 200 µl of distilled water without contact of the well's wall. The wells were air-dried for 15 minutes and the crystal violet in each well was solubilized by adding 200 µl of dimethyl sulfoxide (DMSO). DMSO was disposed of by multichannel pipette. A

microplate reader read the results at ~~The microtiter plate was read at~~ 590 nm ~~with a microplate reader~~ to analyze the plate spectrophotometrically. These methods were conducted according to a modified version of the method employed in the studies *Antifouling Properties of Zinc Nitrate in Seawater* and *Effect of Cinnamon Oil on Quorum Sensing-Controlled Virulence Factors and Biofilm Formation in Pseudomonas aeruginosa* [21,12].

Biofilm formation is considered microfouling. Therefore, if biofilm formation is impaired in the presence of the crude extract, it can be inferred that the crude extract inhibited *E. coli* growth which is comparable to biofouling.

Results

Table 1. Settled cyprids in presence of controls

Treatment	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Aged Sea Water	70%	81%	83%	78%	70%	67%
CuSO4	0%	0%	0%	9%	9%	0%
DMSO	78%	81%	81%	70%	77%	70%

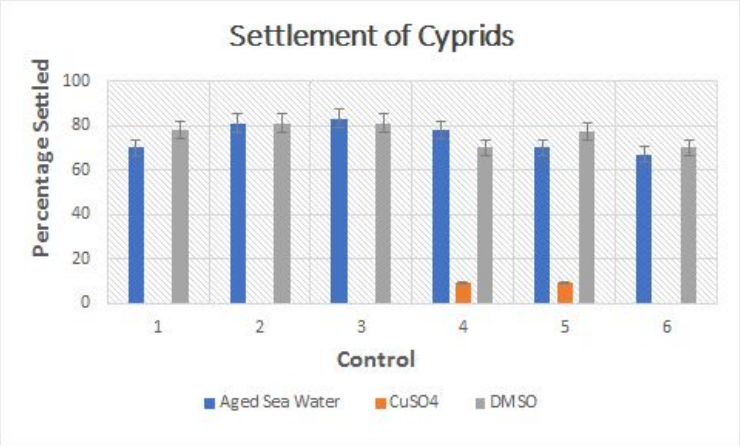

Figure 1. Represents the amount of cyprids after 24 h exposure to controls at 28 C.

Table 2. Swimming cyprids in presence of controls

Treatment	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Aged Sea Water	30%	8%	7%	22%	30%	33%
CuSO4	20%	22%	30%	37%	19%	40%
DMSO	14%	10%	19%	30%	23%	30%

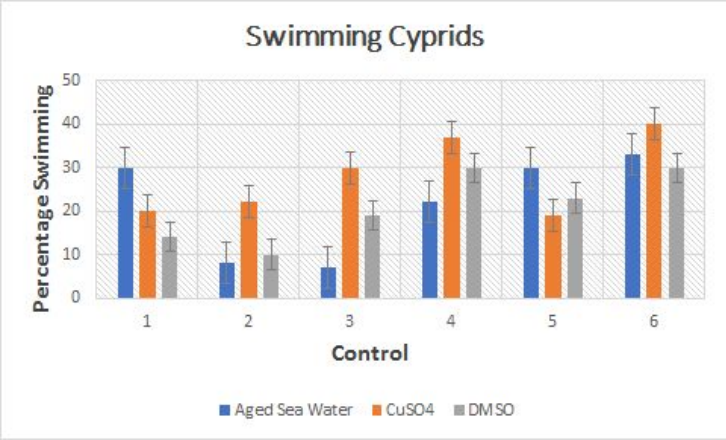

Figure 2. Represents the amount of swimming cyprids after 24 h exposure to controls at 28 C.

Table 3. Dead cyprids in presence of controls

Treatment	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Aged Sea Water	0%	11%	0%	0%	0%	8%
CuSO4	80%	78%	70%	54%	72%	60%
DMSO	8%	9%	0%	0%	0%	0%

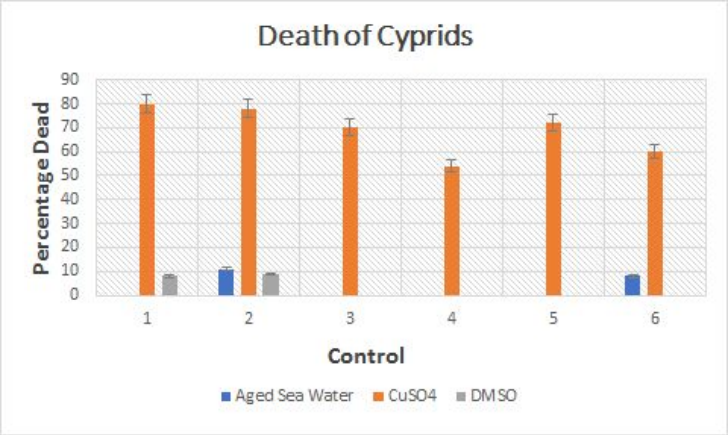

Figure 3. Represents the amount dead cyprids after 24 h exposure to controls at 28 C.

Table 4. Settled cyprids in presence of crude extract treatments

Treatment	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Acetone Extract	18%	10%	0%	0%	8%	0%
Ethanol Extract	8%	22%	0%	10%	18%	0%
Methanol Extract	9%	25%	10%	23%	8%	15%

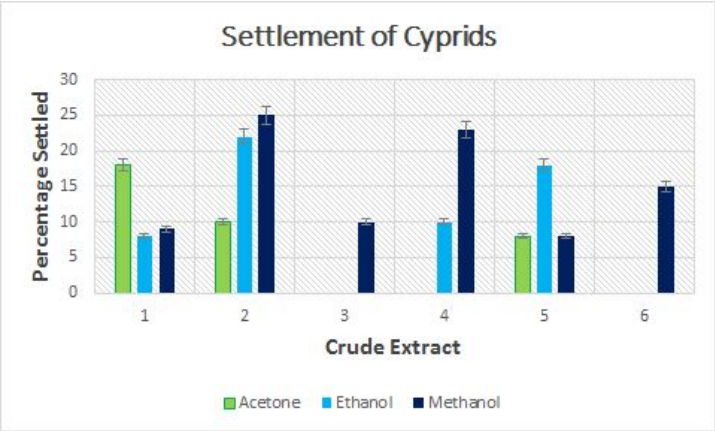

Figure 4. Represents the amount settled cyprids after 24 h exposure to crude extract at 28 C.

Table 5. Swimming cyprids in presence of crude extract treatments

Treatment	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Acetone Extract	10%	30%	22%	30%	17%	40%
Ethanol Extract	23%	23%	13%	40%	46%	43%
Methanol Extract	36%	25%	20%	15%	34%	47%

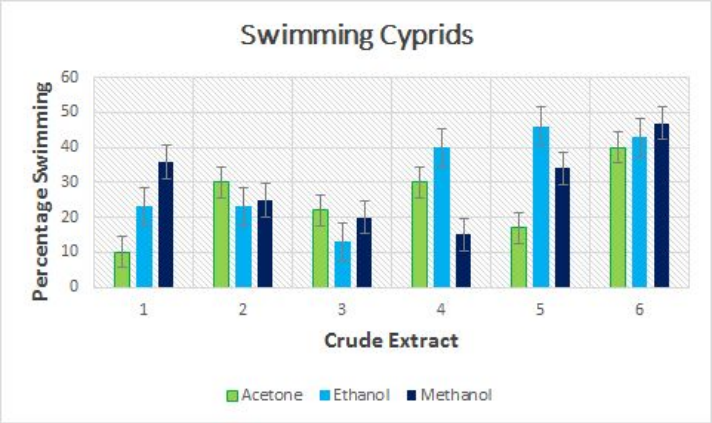

Figure 5. Represents the amount swimming cyprids after 24 h exposure to crude extract at 28 C.

Table 6. Dead cyprids in presence of crude extract treatments.

Treatment	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Acetone Extract	72%	60%	78%	70%	75%	60%
Ethanol Extract	69%	55%	87%	50%	36%	57%
Methanol Extract	55%	50%	70%	62%	58%	38%

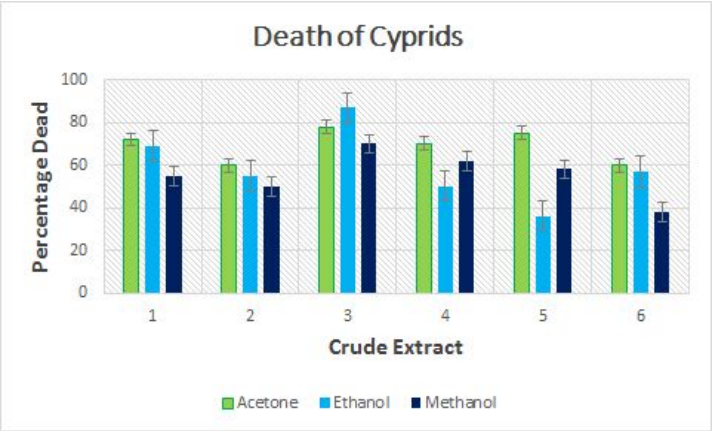

Figure 6. Represents the amount dead cyprids after 24 h exposure to crude extract at 28 C.

Table 7. *E. coli* Biofilm Absorbance

Treatment	Replicate 1	Replicate 2	Replicate 3
Control (LB)	0.29320 nm	0.26080 nm	0.24010 nm
Standard (LB & *E. coli*)	0.85630 nm	0.98790 nm	1.27750 nm
10 µL Extract	0.44330 nm	0.58560 nm	0.23660 nm
20 µL Extract	0.18990 nm	0.12190 nm	0.13320 nm
30 µL Extract	0.28750 nm	0.22910 nm	0.15080 nm
50 µL Extract	0.40720 nm	0.36330 nm	0.50430 nm
100 µL Extract	0.18960 nm	0.23480 nm	0.10090 nm
200 µL Extract	0.27540 nm	0.31090 nm	0.25630 nm

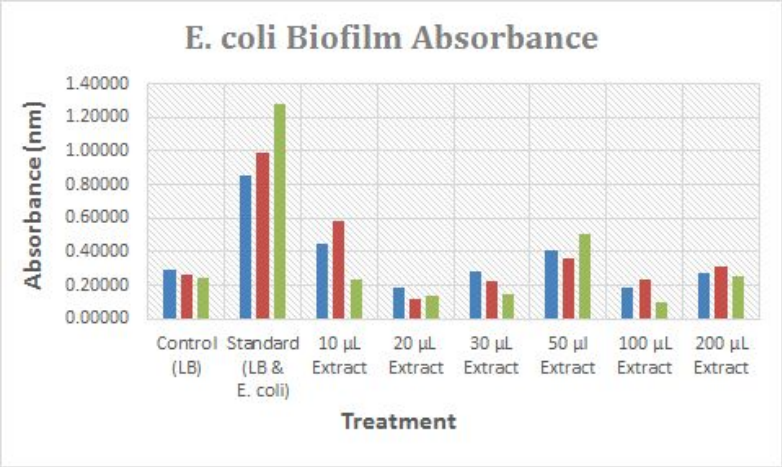

Figure 7. Represents the absorbance of the crystallized biofilm after 24 h exposure to control, standard, and acetone crude extract at 37 C.

Discussion

This experiment was conducted with the purpose of calculating the antifouling activity of *Heteractis magnifica* and comparing its crude extracts to the common antifoulant of CuSO4. The data and results collected indicate that there are potential antifoulants in *Heteractis magnifica* that can be applied to an antifouling coating. The acetone crude extract of *Heteractis magnifica* outperformed the positive control of CuSO4 in the aspects of the larval barnacle settlement rate and death rate.

To transfer the crude extracts of *Heteractis magnifica* to the wells, the crude extracts were dissolved in DMSO. There is no significant statistical change between the control of aged seawater and the negative control of DMSO. Since the

two respective controls yielded similar results, the crude extract was dissolved in DMSO without affecting the larval barnacle culture. The percentage of settlement, death, and swimming larvae was then calculated for the acetone crude extract, ethanol crude extract, and methanol crude extract. The acetone crude extract had the average settlement rate of 6.1% which was the lowest settlement rate out the crude extracts. The methanol crude extract had the average settlement rate of 15% and the ethanol crude extract had the average settlement rate of 9.7%. The results of the settlement rate for the crude extracts were all significantly lower than the control of the aged seawater in which the settlement rate was 74.83%. Further, the positive control of CuSO4 had the average settlement rate of 9%. The acetone crude extract yielded higher levels of settling-inhibitory activity against the barnacle larvae compared to CuSO4, the positive control. This inhibition of settlement for larval barnacles shows the ability of the crude extract to prevent the cyprid stage from attaching to submerged surfaces.

The highest average larval barnacle death rate was 69.1% for the acetone extract. The control of aged sea water had the average death rate of 3.1%. The death rate of the acetone crude extract is comparable to that of the CuSO4 which was 72.0%, indicating that there are potent antifouling agents present in the acetone crude extract. The swimming percentage for the acetone crude extract was

24.8% which was the lowest out of the crude extracts. Therefore, the null hypothesis of this research was rejected for the larval barnacle settlement assay.

While conventional antifouling paints contain copper ions and compounds such as CuSO4, their high levels of toxicity to marine life pose a drawback. However, the acetone crude extract of *Heteractis magnifica* is considered safer because of its higher levels of settlement-inhibitory activity and lower death rate. Since the antifoulants in *Heteractis magnifica* are naturally produced by marine invertebrates, the bioactive fraction decomposes at a faster rate than copper which has a lower impact on the surrounding environment. However, natural antifouling coatings also have disadvantages since the coatings would need to be applied more often to submerged surfaces since the performance of the natural antifoulant would decrease with consistent exposure to seawater conditions.

Because the acetone crude extract was the most effective extract in the larval barnacle settlement inhibition assay, the respective extract was analyzed for the biofilm assay. The absorbance was significantly lower in the presence of crude extract compared to the standard of *E. coli*. The most effective concentration of acetone crude extract was the 20 μL aliquot. The absorbance decreased by an average of 0.63 nm against all crude extracts. This disruption in the formation of biofilm indicates antifouling activity in the acetone crude extract. However, the

anti-biofilm results showed a dose-independent effect. This could be a result of remaining crude extract from the higher concentrations of crude extract that was dissolved in PBS.

Sources of Error

For the centrifugation of the crude extract, it is directed by Henry Borbon *et. al* to centrifuge the extract at 4 C [4]. However, the lab used lacked a temperature changing system for centrifuging and the methods were altered to centrifuge the extract at room temperature. Immediately after centrifugation, the resultants were then stored in the laboratory refrigerator to bring the temperature to 4 C. This change could have caused the bioactive agents in the solution to degrade. However, results show antifouling activity which indicates that the agents were still active in the crude extract.

Further, due to the lack of a Buchi Rotavapor in the lab, the crude extract was not evaporated within 20 min at reduced pressure as directed by Al-Hazimi *et. al* [2]. The crude extract was instead evaporated within 48 h in the fume hood which could have lowered the activity of the bioactive fraction in the crude extract. Further, the time allotted to evaporate the volatile solvents could have allowed for bacteria to settle in the crude extract. Also, not all of the volatile solvents of acetone, ethanol, and methanol may have been effectively evaporated within that

time frame which would alter the results of the experiment.

Conclusion

The outcome obtained has the potential to provide an alternate antifouling agent to coatings containing copper. The inhibitory activity of the *H. magnifica* extract against larval barnacle culture represents an opportunity to discover anti-macrofouling agents from the extract. Anti-microfouling agents are also present in the extract due to the disruption in the formation of *E. coli* biofilm. The results indicate that *H. magnifica* crude extract is active against the two main groups of biofouling which are microfouling and macrofouling, and the active fraction of the crude extract can be further purified and identified.

Further Work

Preparation of sea anemone crude extract for HPLC analysis

The acetone crude extract showed the most activity against barnacle larvae and was used for HPLC analysis. An aliquot of the extract (0.5 mg) was dissolved in 0.2 mL HPLC-grade methanol, and filtered through Whatman® Nº1. Filter paper 0.22 µm syringe tip. For HPLC analysis, a 10 µL aliquot of the extract solution was injected onto an Agilent 1100 C18 reversed-phase HPLC column (5 µm, 250 mm × 4.6 mm i.d.) and analyzed with a Hewlett Packard Series 1100 HPLC system by a gradient elution (A: CH3CN with 0.1% trifluoroacetic acid (TFA); B: H2O with

0.1% TFA: 5–70% A over 25 min, 70–100% A from 25 to 30 min, and 100% A from 30 to 35 min; flow rate 0.7 mL/min; DAD detector wavelength 200-600 nm).

Disc diffusion assay

The disc diffusion method was used to determine if the pure sample identified from *H. magnifica* is an active antifouling agent. 25 mL of Zobell agar media was prepared. *E. coli* and collected biofilm were added to the mixture. A disc was autoclaved for sterilization. The agar mixture was poured into the 6 mm sterile disc and stored until solid. The crude extract was placed in the center of a sterile petri dish. The zone of inhibition was measured after 24 hr incubation at 37°. This disc diffusion method was repeated 3 times for each pure sample from *Heteractis magnifica*. To determine if the pure samples from *Heteractis magnifica* performed similarly to antifouling paints, ten different samples of antifouling paints were used in the above disc diffusion method.

Safety Data Sheets

SDS Acetone
SDS DMSO
SDS *E. coli*
SDS Ethanol
SDS Methanol

Acknowledgements

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