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Antifouling potentials of *Neopetrosia proxima* from Southern Guimaras, Philippines against the marine diatom *Navicula ramosissima*, barnacle *Balanus* sp., and green mussel *Perna viridis*

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Abstract. Methanolic crude extracts of marine sponge *Neopetrosia proxima* prepared in three different concentrations (1.0 µg mL^{-1}, 10.0 µg mL^{-1} and 100.0 µg mL^{-1}) were determined as potential antifouling agent against the settlement of selected fouling organisms *Navicula ramosissima*, *Balanus* sp., and *Perna viridis*. Results of one-way ANOVA (α = 0.05) showed significant differences at 24 hrs, 48 hrs, 72 hrs and 96 hrs between test concentrations and control on the settlement density of diatoms. Three test concentrations from sponge extracts also showed strong inhibition effect against the settlement of barnacles *Balanus* sp. and green mussel *Perna viridis*. As a preliminary investigation, results of the present study most probably attributed by the presence of active compounds in the marine sponge *Neopetrosia proxima* although this needs to be confirmed through isolation and purification in future studies. Thus, the present study demonstrated that marine sponge *N. proxima* can be a potential candidate as an antifouling agent.
Key Words: marine sponge, crude extracts, fouling organisms, growth, settlement, inhibition.

Introduction. Surface colonization and development of micro- and macrofoulers on submerged structures both on man-made and natural environment is a natural process called biofouling. It is considered one of the widespread global phenomenon that causes serious operational problems and huge economic losses worldwide (Ritschoff et al 2003). To prevent biofouling, man-made paints that contains TBT (tributyltin) and organotin are often been used. Although these substances are certainly effective, they are detrimental to non-target organisms (Qian et al 2009). A worldwide ban on the use of TBT was even implemented since 2008 because of its high persistence in the marine environment and its strong toxicity to non-target marine organisms. Therefore, it is important to search and develop non-toxic or less toxic antifouling agent (Ritschoff et al 2003).

Substances that prevent colonization on living surfaces are particularly relevant to antifouling technology and may encourage new solutions of finding safe antifoulants (Fusetani & Clare 2006). Marine macro-organisms such as sponges have been proven to be a good source of bioactive compounds, including antifouling compounds (Fusetani & Clare 2006; Hentschel et al 2006; Garson 1993). Their sessile and unarmored body propels the synthesis of secondary metabolites to defend themselves from predators and competitors, as also to maintain a clean body surface (Becerro et al 2003).

Accumulation of major fouling organisms causes serious problems in marine technology like roughness in ship's hulls that reduces efficiency, speed and maneuverability (Champ & Lowenstein 1992), cooling systems of power plants, and aquaculture materials (Holmes 1970; Houghton 1978). Diatoms are major component of

microbial slimes (Molino & Wetherbee 2008) and considerably provide foothold for macrofouling organisms for attachment (Anil et al 2006). They possibly offer differing cues to settling of higher sedentary organisms, which could lead in establishing macrofouling community. Thus, they are often selected as target organisms (Anil et al 2006). The prolific way of settlement of barnacle larvae and their global distribution has made them the most common fouling marine invertebrate. In addition, barnacles are easy to culture under laboratory conditions and their reproduction can be manipulated so that larvae are obtainable throughout the year for toxicity and settlement tests (Clare & Alred 2009). Likewise, mussels also cause serious problems affecting aquaculture nets, off-shore rigs and industrial coolant outflows (Nishida et al 2003). Mussels are highly suitable model organisms for adhesions because of their accessibility of its attachment apparatus (Waite & Qin 2001) and antifouling studies (Da Gama et al 2003).

Information on the potentials of marine sponge *Neopetrosia proxima* as antifoulant is scarce and scantily available. Therefore, this study preliminarily aims to determine the potential of *N. proxima* against the growth of *Navicula ramosissima* and settlement of *Balanus* sp., and *Perna viridis*.

Material and Method

Collection and extraction preparation. Marine sponge *N. proxima* was collected on August 2011 through scuba diving between 5 to 10 meters depth on coastal waters of Southern Guimaras and were transported to chemistry laboratory, Department of Chemistry, University of the Philippines Visayas. Collected samples were washed with fresh water to remove epiphytes and sand particles. Samples were air-dried and cut into fine pieces using blender. Homogenized samples were soaked in 80% methanol and sonicated. Crude extracts were filtered using filter paper (Whatman no. 1) fitted in a buchner funnel and was subject to rotary vacuum evaporator. For efficient and gentle removal of solvents, sponge crude extracts were evaporated under reduced pressure at 40^{o}C, and were slowly added 20 to 40 mL n-Hexane. Methanolic crude extracts were collected and air-tight in labeled glass vials for bioassays.

Settlement assays. Antifouling potentials of methanolic crude extracts of *N. proxima* against fouling organisms were conducted in Marine Pollution and Ecotoxicological Laboratory, University of the Philippines Visayas. All glasswares and materials used in the experiments were sterilized.

Anti-settlement against diatom. Cultured diatoms were maintained at 24^{o}C and were fed on a daily diet of f2/Medium by Guillard & Ryther (1962). Anti-settlement assays against diatoms utilized 12 pieces of 400-mL beaker. Each beaker was placed 3 pieces of glass sides. Each sides of glass slides has 3 squares of equal measurements. 10,000 cells mL^{-1} were added into each beaker. Test concentrations (1.0 µg mL^{-1}, 10.0 µg mL^{-1}, 100.0 µg mL^{-1}) and control (filtered seawater) were prepared in triplicates. Provided with aeration, experimental beakers were covered with aluminum foil and exposed at 24-hour light period. Cells that attached to the glass slides were counted from 0, 24, 48, 72 and 96 hours under LB221 Binocular Digital Microscope.

Anti-settlement against barnacles. Rearing and culture of adult barnacles were followed using Hellio et al (2004) method. After 6 days when barnacle larvae were released, a 10-mL pipette was used to collect larvae from culturing tanks. 50 individuals of competitive 5th stage barnacle larvae were placed into each 400-mL beaker with 200-mL filtered seawater (Ritschoff et al 1992). A chosen cut smooth-surfaced bamboo were placed inside each beakers. Test concentrations tested were 1.0 µg mL^{-1}, 10.0 µg mL^{-1}, 100.0 µg mL^{-1}. Each test concentration and control (filtered seawater) were prepared in triplicates. Beakers were provided with mild aeration and covered with aluminum foil. 10-mL of *Chaetoceros calcitrans* were fed daily into each beaker. After 30 days, attached barnacles were counted using a high power microscope.

Anti-settlement against green mussel. Mussels were collected from local coastal waters through snorkeling during low tide. Sizes from 1 to 2 centimeters (cm) were used. Prior to the experiment, mussels were acclimatized before the assay was conducted. A total of 15 individuals of green mussel were placed into each Petri dish. Test concentrations (1.0 µg mL^{-1}, 10.0 µg mL^{-1}, 100.0 µg mL^{-1}) and control (filtered seawater) were prepared in 3 replicates. After 24 hours, test organisms were observed visually for attachment of byssus.

Data analysis. Settlement density of diatom *N. ramosissima* was determined. Meanwhile, attachment rate of barnacles and mussels were determined by which initial number of test organisms (50 = barnacles; 15 = mussels) minus final number of test organisms (N_{30}) = barnacles counted after 30 days; (N_{24}) = attached mussels counted after 24 hours, divided by the initial number test organisms (50 = barnacles; 15 = mussels) times 100.

Statistical computations were carried out using SPSS v16.0. Comparisons between test concentrations and control were done with one-way Analysis of Variance (ANOVA, $\alpha = 0.05$), followed by Post Hoc test Tukey's HSD and Dunnett's at 95% level of confidence (Conover & Iman 1981).

Results and Discussion

Settlement against diatom. Settlement density of diatom *N. ramosissima* showed significant differences between three control test concentrations at 24, 48, 72 and 96 hours (Table 1). At 24 and 48 hours, Tukey's test revealed no significant differences between test concentrations (Figure 1 and Figure 2, respectively). While at 72 and 96 hours, significant difference were observed between 1.0 µg mL^{-1} and 100.0 µg mL^{-1} (Figure 3 and Figure 4, respectively).

Table 1

Settlement density of diatom *Navicula ramosissima* exposed between control and test concentrations of methanolic extracts of marine sponge *Neopetrosia proxima*

Time (hours)	*Concentration (µg mL^{-1})*			
	Control	1.0	10.0	100.0
	Average growth (cells/mm^2)			
24	0.0142^{a}	0.0032^{b}	0.0014^{b}	0.0005^{b}
48	0.0363^{a}	0.0120^{b}	0.0113^{b}	0.0023^{b}
72	0.0645^{a}	0.0344^{b}	0.0243^{b}	0.0055^{c}
96	0.1049^{a}	0.0661^{b}	0.0432^{bc}	0.0106^{c}
Mean	0.0550	0.0289	0.0200	0.0047

Data analysis for Dunnett's test also showed significant differences between control and three different test concentrations of methanolic extracts of *N. proxima* at 24, 48, 72, and 96 hours. The methanolic extracts from marine sponge *N. proxima* inhibited the growth of diatom *N. ramosissima* at three (3) test concentrations could be probably due to the active compound present in *N. proxima* that reduces the growth of the diatom. Because sponges are sessile and soft-bodied, they release chemical substances to act as antifoulants and prevent the overgrowth of neighboring organisms (Hartman 1981; Bakus & Green 1974). Qian et al (2007) found out that Hongkong sponges *Haliclona* sp. have antifouling activity against the growth of diatoms *Navicula* sp. It was also pointed out in the study conducted by Dobretsov et al (2004) that sponge metabolites reduced the recruitment of diatom *Navicula* sp. One possible reason for the inhibition of diatom growth in the present study is the toxicity of sponge compounds (Lee & Qian 2003; Dobretsov et al 2004).

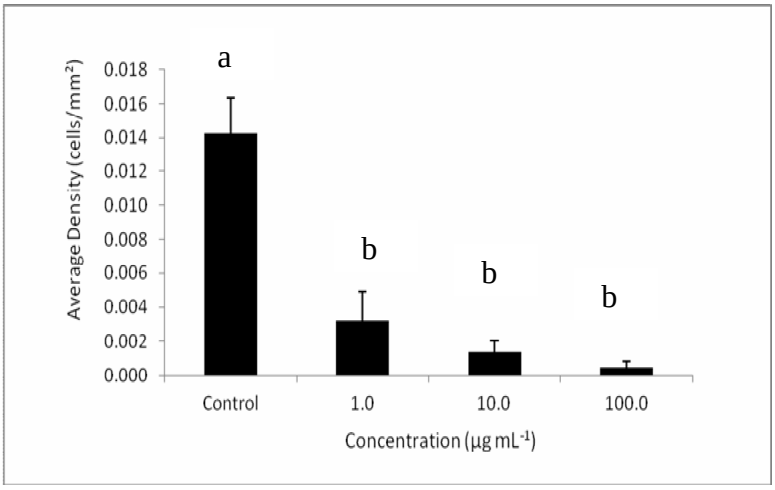

Figure 1. Growth of diatom *N. ramosissima* at 24 hours between control and test concentrations of methanolic extracts of marine sponge *N. proxima.*

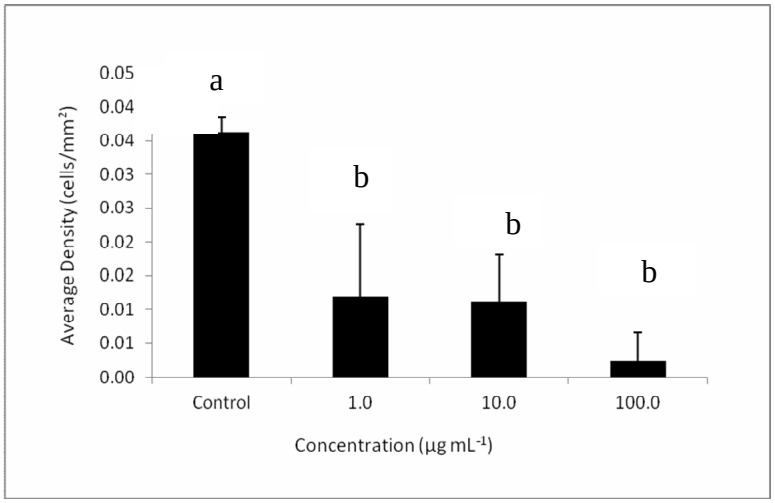

Figure 2. Growth of diatom *N. ramosissima* at 48 hours between control and test concentrations of methanolic extracts of marine sponge *N. proxima.*

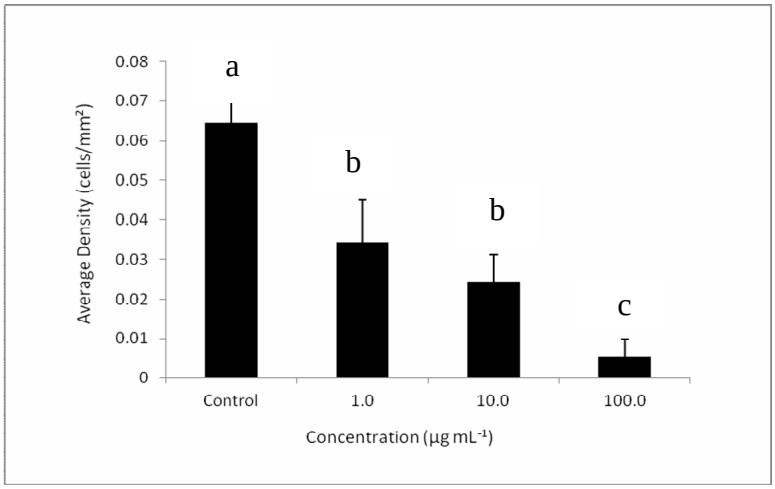

Figure 3. Growth of diatom *N. ramosissima* at 72 hours between control and test concentrations of methanolic extracts of sponge *N. proxima*.

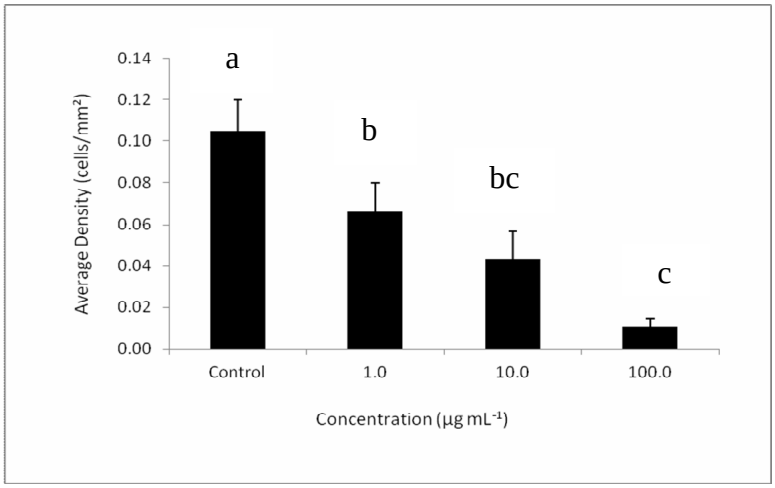

Figure 4. Growth of diatom *N. ramosissima* at 96 hours between control and test concentrations of methanolic extracts of sponge *N. proxima*.

Settlement against barnacle and mussel. Mean density of barnacles *Balanus* sp. showed that one-way ANOVA showed significant differences between test concentrations (Figure 5). Moreover, Post Hoc Tukey's test also revealed significant differences between each extract concentrations 100.0 µg mL^{-1}, 10.0 µg mL^{-1}, and 1.0 µg mL^{-1}.

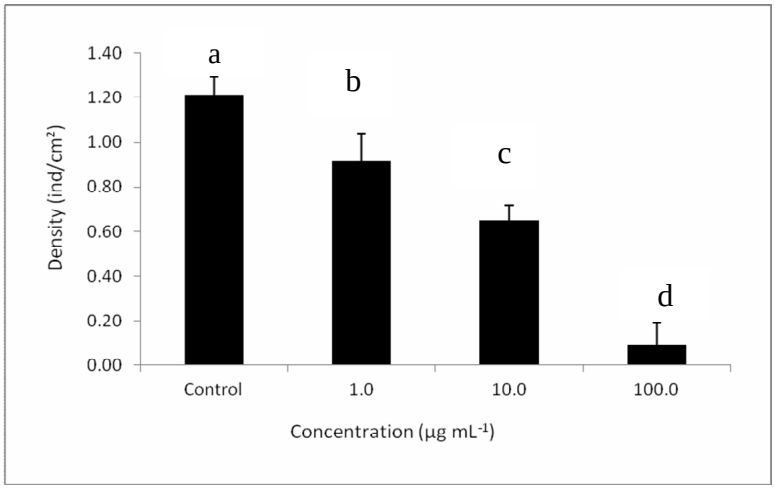

Figure 5. Mean density of barnacle *Balanus* sp. between control and test concentrations of methanolic extracts of sponge *N. proxima.*

Settlement activity of three (3) different test concentrations of methanolic extracts of *N. proxima* revealed highest attachment at 1.0 µg mL^{-1} (55.3% ± 7.6) and lowest at 100.0 µg mL^{-1} (5.3% ± 6.1). Statistical analysis of one-way ANOVA showed significant difference among test concentrations (Figure 6). Tukey's and Dunnet's test also revealed significant differences between test concentrations. In the settlement against the barnacle *Balanus* sp., methanolic extracts of *N. proxima* inhibited the settlement of *Balanus* sp. The significant differences observed indicate that methanol extracts of *N. proxima* showed considerable antifouling activity exhibited against *Balanus* sp. This significant effect is possibly due to the chemical substances present in the sponge *N. proxima.* This result is parallel with the findings of Limna Mol et al (2009) who reported significant inhibitory effect of marine sponge *Haliclona exigua* against the larval settlement of *Balanus amphitrite*.

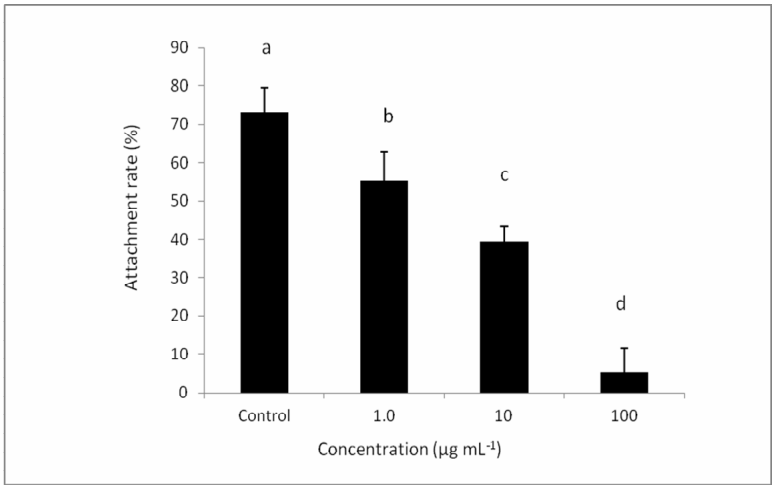

Figure 6. Mean attachment rate of barnacle *Balanus* sp. between control and test concentrations of methanolic extracts of sponge *N. proxima*.

In the settlement against the green mussel, among test concentrations, 100.0 µg mL^{-1} showed highest inhibitive effect. One-way ANOVA revealed significant differences among mean density of test concentrations. In addition, Tukey's revealed significant difference between 1.0 µg mL^{-1} and 100.0 µg mL^{-1} (Figure 7). Significant differences were also observed between control and 100.0 µg mL^{-1}, 10.0 µg mL^{-1} (Dunnett's).

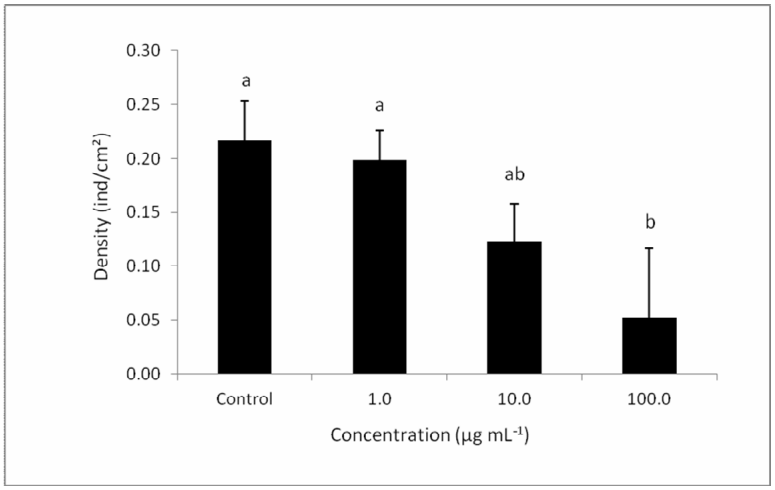

Figure 7. Mean density of green mussel *P. viridis* between control and test concentrations of methanolic extracts of sponge *N. proxima*.

Results of attachment rate of *P. viridis* exhibited highest rate at 1.0 µg mL^{-1} (75.6% ± 10.2) and lowest at 100 µg mL^{-1} (20.0% ± 24.0). Data analysis showed significant difference among mean attachment rate in different test concentrations (Figure 8). Furthermore, Tukey's HSD test showed significant difference between 100.0 µg mL^{-1} and 1.0 µg mL^{-1}. Post Hoc test revealed that attachment rate between control and 100.0 µg mL^{-1}, 10.0 µg mL^{-1} were significantly different (Dunnett's). Although their byssus thread failed to attach, they were determined alive because the mantle is still visible. Mussels that failed to produce byssus thread were due to the role of active extracts as defense substances. It might be possibly concluded that of *N. proxima* has a symbiotic effect on green mussel *P. viridis*. This is an important point since the goal of the study is to search for eco-friendly antifoulant. Furthermore, the present study revealed positive inhibition of settlement of *P. viridis* was due to the sizes of mussels used, where 1 to 2 centimeters (cm) was decided to utilize in the experiment. Devi et al (1998) pointed out that mature mussels gave more reliable results as compare to young ones. Young mussels were sensitive and retracted their foot even under minor stimulus. Since this study is

considered as preliminary investigation to future antifouling researches, young mussels were used first. However, this need to be confirmed and must be conducted in field studies. Similar results were obtained in the study conducted by Prabhu et al (2014), where *Desmosponge sp*. did not only showed antibacterial activity against *Salmonella* sp*., Staphylococcus* sp*., Bacillus* sp. but also exhibited significant activity against the green mussel *P. viridis*.

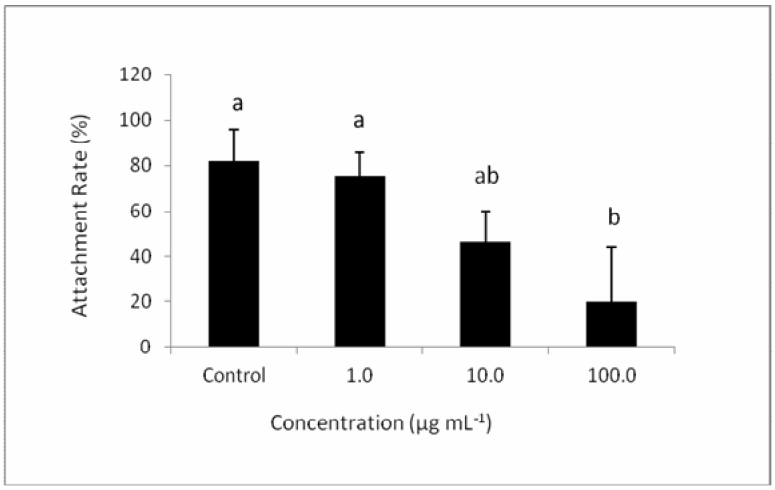

Figure 8. Comparison of attachment rate of *P. viridis* between control and test concentrations of methanolic extracts of sponge *N. proxima*.

Conclusions. As a summary, marine sponge such as *N. proxima* have received the authors' attention because it plausibly represents a source of unique and diverse metabolites and could have potential biological activities. The present study suggests that marine sponge *N. proxima* can be a potential antifoulant. This possible antifouling product can provide new insights that could be efficient bioactive compounds. Specifically, positive inhibitions were observed against selected target organisms. Although this speculations needs to be confirmed in the future research by doing isolation and purification studies.

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