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## **Significance of biofilm proteins in modulating cyprid metamorphosis of *Balanus amphitrite* (Cirripedia: Thoracica)**

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### **Abstract**

The role of biofilm proteins in modulating the cyprid metamorphosis is not well understood. In the present investigation this possibility was explored by disrupting biofilm proteins using commercially available protease. The influence of natural biofilm and artificial biofilms of *Aeromonas salmonicida salmonicida* and *Bacillus brevis* and their culture supernatants (CS) and exopolysaccharides (EPS) obtained under different nutritional conditions was evaluated.

Natural biofilm facilitated cyprid metamorphosis whereas monospecies biofilms did not. The treatment of biofilms with protease resulted in decline in metamorphosis, except in *B. brevis* where although metamorphosis was negated initially it increased with the protease concentration and observation days. Since the response of cyprids to EPS produced by *A. salmonicida salmonicida* was protein concentration dependent, protease treatment resulted in decrease in metamorphosis, whereas, in case of *B. brevis* biofilms, protease hydrolysis products could be important in providing the settlement cues. Thus, biofilm bacterial components play an important role in the cyprid metamorphosis of *Balanus amphitrite*.

**Keywords:** *Balanus amphitrite*; protease; biofilm; cyprid metamorphosis; EPS; protein

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## Problem

Biofouling is a prominent feature on artificial structures like ship hulls, underwater pipelines, bridges, offshore platforms etc. that results in their modification leading to deterioration of their performance. Barnacles are particularly pervasive foulers (Otani *et al.* 2007) causing a significant increase in drag (Schultz 2007) and the economic cost associated with marine fouling runs into billions of dollars annually (Yebra *et al.* 2004; Clare 1996; Callow & Callow 2002; Townsin 2003). This serious problem is being fought by developing different antifouling products (Ponasik *et al.* 1998; Evans *et al.* 2000; Kobayashi & Okamura 2002; Yebra *et al.* 2004; Schultz 2007). These products consist of mainly toxic metals or organometallic compounds that are prone to bioaccumulation and thus harmful to the marine environment. In this regard, role of enzymes in antifouling (AF) coatings have been studied over the past 20 years, as enzymes are rapidly degraded and are therefore expected to be environmentally friendly (Olsen *et al.* 2007). The antifouling potential of serine-proteases was evaluated on barnacle cyprids by Pettitt *et al.* 2004 and reported these enzymes to inhibit their settlement.

Biofilms play an important role in the settlement and metamorphosis of various marine invertebrate larvae, especially belonging to marine fouling organisms (Strathmann *et al.* 1981; Kirchman *et al.* 1982; Weiner *et al.* 1985; Maki *et al.* 1988,1990,1992; Szewzyk *et al.* 1991; O' Connor & Richardson 1998; Wieczorek & Todd 1998; Tsurumi & Fusetani 1998; Maki *et al.* 2000; Hadfield & Paul 2001; Harder *et al.* 2001; Dobretsov & Qian 2002; Lau *et al.* 2003; Khandeparker *et al.* 2003,2005,2006; Jouuchi *et al.* 2007). Settlement success of *B. amphitrite* cyprids is influenced by biofilms of varying species composition, physiological conditions and growth phase (Wieczorek & Todd 1998; Qian *et al.* 2003; Hung *et al.* 2008). Natural microbial communities on marine substrata, most likely containing a multiplicity of bacterial species, can stimulate, inhibit, or have no effect on settlement (permanent attachment) of barnacle cyprids (Strathmann *et al.* 1981; Qian *et al.* 2007). Surface chemistry is also important to larval settlement and plays an important role in distribution of adults (Holm *et al.* 1997). Furthermore, *in situ* growth of juvenile barnacles depends on both independent and interactive effect of cyprid energy reserves and habitat (Thiyagarajan *et al.* 2005). Surface wettability does not alter the positive effect of natural biofilms on larval attachment (Hung *et al.* 2008). However, the responses of biofilm changes with the age and bacterial density. Older films of *Deleya (Pseudomonas) marina* were found to be inhibitory to *B. amphitrite* cyprids (Maki *et al.* 1988). The relative proportion of inhibitory and stimulatory bacterial strains in biofilms differs with the environmental conditions (Dobretsov *et al.* 2006). Biofilms of *Pseudoalteromonas*

sp. caused significantly lower larval settlement of *Bugula neritina* and their metamorphosis decreased with increasing densities of *Pseudoalteromonas* sp. in the biofilm (Dahms *et al.* 2004).

Many microfouling as well as macrofouling species, such as bacteria, diatom, algal spores and invertebrate larvae employ protein or glycoprotein polymers while attaching to surfaces (Cooksey & Cooksey 1995; Kamino 2001). Thus, it is expected that specific proteases would hydrolyze these proteins/glycoprotein's thereby preventing their adhesion. The role of biofilm proteins in modulating cyprid metamorphosis is not well understood. This possibility was explored in this study by disrupting the proteins in biofilms using protease from *Bacillus licheniformis*. The effect of proteases on bacterial biofilms and its subsequent effect on the barnacle cyprid settlement was evaluated using *B. amphitrite* (= *Amphibalanus amphitrite*) (Pitombo 2004), an extensively studied barnacle in India for its ecology, understanding the mechanisms of cue detection and testing efficacy of antifouling compounds/paints (Anil & Kurian 1996; Anil & Khandeparkar 1998; Karande 1999; Khandeparker *et al.* 2002a,b,2003; Desai & Anil 2004,2005; Desai *et al.* 2006; Khandeparker *et al.* 2006; Desai 2008). The larval development in this organism consists of six naupliar stages followed by a non-feeding cyprid stage that explores surfaces to locate a suitable place for settlement. While exploring surfaces for attachment the cyprids use antennules that have highly developed chemo and mechano receptor systems (Lagersson & Høeg 2002). Barnacle cyprids prefer to metamorphose on or near conspecifics and this behavior is attributed to glycoprotein identified from adult conspecifics (Knight-Jones 1953; Knight-Jones & Crisp 1953; Crisp & Meadows 1963; Clare & Matsumura 2000), which is recently referred as Settlement Inducing Protein Complex (SIPC) (Head *et al.* 2003). Dreanno *et al.* (2006) reported the cloning and characterization of the full-length SIPC cDNA from *B. amphitrite*, and the results indicated that the SIPC mRNA is expressed widely in adults, juveniles and larvae. In the present study, SIPC is referred as Adult Extract (AE).

In view of the above two marine biofilm bacteria namely *Aeromonas salmonicida salmonicida*, *Bacillus brevis* were selected as *Aeromonas* and *Bacillus* sp. are specific producers of extracellular proteases (Priest, 1977; Shafee *et al.*, 2005) along with Natural biofilms (NBF). The following objectives were addressed (1) The influence of monospecies artificial bacterial films of *A. salmonicida salmonicida*, *B. brevis* and NBF (2-day-old) on the cyprid metamorphosis of *B. amphitrite* (2) Influence of extracellular polymeric substances produced by *A. salmonicida salmonicida* and *B. brevis* on the cyprid metamorphosis of *B. amphitrite* (3) Whether biofilms treated with protease provide different sets of signals to the settling cyprids of *B. amphitrite*?

## 2. Material and methods

### Rearing of *B. amphitrite* larvae

Adult broods of *B. amphitrite* were collected from the intertidal area of Dona Paula Bay located at Zuari estuary, Goa, west coast of India (15° 27.5' N, 73° 48' E). Nauplii released by these broods were separated and mass reared in 2-liter glass beakers, using filtered sea water (0.22 µm) of 35‰, on a diet of *Chaetoceros calcitrans*, a unicellular diatom, at a cell concentration of  $1.5 \times 10^5$  cells ml<sup>-1</sup>. The feed organism was replenished every day while changing the seawater. The rearing beakers were maintained at 25 (±2) °C in an incubator on a 12 h Light: 12 h Dark photoperiod. After 6-7 days the cyprids obtained were siphoned out and stored at 5 °C prior to settlement assays. Two-day old cyprids were used in the assays, as this is a standard practice used (Anil *et al.* 2001; Clare *et al.* 1994).

### Preparation of adult extract (AE)

Adult extract was prepared by following the method described by Larman *et al.* (1982). Adult *B. amphitrite*, collected from the field were brought to the laboratory and cleaned using nylon brush to remove all the epiphytes. Equal weight/volume of deionized, RO pure water was used to crush the adults using a pestle and mortar. Subsequently, the mixture was centrifuged at 12 000 x g for 5 min. The supernatant was collected and boiled in a water bath for 10 min. The extract was centrifuged at 12,000 x g for 5 min. and supernatant was kept at -20°C until further use. The concentration of protein in the AE was determined following the method described by Lowry *et al.* (1951) using Bovine serum albumin (BSA) as the standard. A protein concentration of 50 µg ml<sup>-1</sup> of AE was prepared by diluting the AE in sterile filtered sea water and used for all assays and this was treated as positive control.

### Development of bacterial film

*A. salmonicida salmonicida* and *B. brevis* were grown in Marine Broth 2216 (MB) with an initial inoculum of 2% which was obtained by growing the bacterium in the same medium for 18h. Culture medium was maintained at 26 (±2) °C on a shaker. The surfaces of polystyrene multiwell plates (24-well; Nunclon R Delta) were coated with bacterial films following the method described by Maki *et al.* (1988,1990). The resultant bacterial density was  $10^6$  to  $10^7$  cells cm<sup>-2</sup>.

The dishes with the bacterial treatment were fixed with formaldehyde (final concentration 1%; v/v) and the quantification of attached bacteria was done by using acridine orange and epifluorescence microscopy.

### **Development of natural biofilm**

Polystyrene multiwells (24-well; Nunclon R Delta) were immersed at Dona Paula Bay from the existing jetty for a period of two days to obtain two-day old natural biofilm. These multiwells with natural biofilms were brought to the laboratory by immersing them in sterile filtered sea water (FSW) and were used immediately for the settlement assays.

### **Isolation and identification of bacteria**

The bacteria were isolated from the natural biofilms. For this, the biofilm was scraped in sterile filtered sea water (0.22 $\mu$ m), after which the sample was diluted and spread plated on Zobell Marine Agar 2216. The purity of the culture was checked by streaking on Zobell Marine Agar 2216. Bacteria were identified following 'Bergey's manual of systematic bacteriology' (Krieg 1984).

### **Extraction of culture supernatant (CS)**

*A. salmonicida salmonicida* and *B. brevis* were grown in MB and Basal Salt Solution (BSS) medium. The bacterium was grown in basal salt solution (BSS), pH 7.5, containing (g l<sup>-1</sup>) NaCl 30.0, KCl 0.75, MgSO<sub>4</sub>·7H<sub>2</sub>O 7.0, NH<sub>4</sub>Cl 1.0, K<sub>2</sub>HPO<sub>4</sub> 0.7, KH<sub>2</sub>PO<sub>4</sub> 0.3 and glucose 10.0, and 1 ml of trace metal solution (Bhosle 1981). A 2% (v/v) inoculum obtained by growing the bacterium in the same medium for 18h was used. Culture medium was kept at 26(±2) °C on a shaker. When the culture reached stationary phase, bacterial cells were centrifuged at 20 000 x g for 15 min. at 4°C. The culture supernatant was dialyzed overnight in 8000 MW cut off dialysis membrane at 4°C against distilled water. The dialyzed samples were then analyzed for protein concentration as described by Lowry et al. (1951) and carbohydrate concentration by phenol sulphuric acid method as described by Dhople & Bhosle (1987) and stored at 4°C until further use. The cyprid metamorphosis assays were carried out at protein and carbohydrate concentration of 50 and 25  $\mu$ g ml<sup>-1</sup> respectively.

### **Standardization of bacterial growth**

The growth of *A. salmonicida salmonicida* and *B. brevis* was determined in MB and BSS medium. The bacteria were inoculated in the above mediums. 2% v/v of bacteria pre grown in the same media for 18h was added to the medium. The medium was kept at 26 (±2) °C on a shaker. Optical Density (OD) of the cultures was observed at 540 nm at equal intervals to find the bacterial growth in

the culture medium. Total carbohydrate concentration was measured of the samples taken for O.D. In BSS medium, the samples were dialyzed using 8000 MW cut off dialysis membrane against distilled water at 4°C overnight before estimation of carbohydrate for removing the Dextrose added in the medium.

### **Extraction of bacterial exopolysaccharides (EPS)**

For the extraction of EPS, *A. salmonicida salmonicida* was grown in MB and BSS medium with an initial inoculum of 2% which was obtained by growing the bacterium in the same medium for 18h. Culture medium was kept at 26 ( $\pm 2$ ) °C on a shaker. When the culture reached stationary phase, the cells were centrifuged at 20 000 x g for 15 min at 4°C. The culture supernatant was collected and filtered through 0.22 $\mu$ m filter paper. To the extracted culture supernatant 5 volumes of absolute ethanol was added and left at 4°C overnight. Further the solution was centrifuged at 30,000 x g for 15 min. at 4°C. The precipitate (EPS) obtained was dissolved in autoclaved distilled water and dialyzed (8000 MW cut-off) overnight at 4°C against distilled water. The dialyzed samples were analyzed for protein (Lowry *et al.* 1951) and carbohydrate concentration (Dhople & Bhosle 1987) and preserved at 4°C until further use. The same protocol was followed for EPS extraction of *B. brevis*. The cyprid assays were carried out at protein and carbohydrate concentration of 50 and 25  $\mu$ g ml<sup>-1</sup>.

### **Assay protocol**

The bacteria (*A. salmonicida salmonicida* and *B. brevis*) treated multiwells and multiwells with two-day old natural biofilms were rinsed off repeatedly with autoclaved filtered sea water. Wells treated with adult extract at a protein concentration of 50 $\mu$ g ml<sup>-1</sup> were incubated for 3h and then rinsed off with autoclaved filtered sea water. This was taken as positive control. Wells with autoclaved filtered sea water with no treatment were taken as negative control. Subsequently, cyprids were introduced (approx. 10 to 15) along with 2 ml autoclaved filtered sea water of 35‰ to each well (Maki *et al.* 1988). The assays were repeated twice using 2 batches of larvae with 6 replicates at each trial (n=12). Assay wells were maintained at 26  $\pm$  2° C (12h Light: 12h Dark photoperiod). The settlement assays were monitored for the number of metamorphosed cyprids for a period of four days with an intermittent observation every 24 h.

The monospecies bacterial biofilms of *A. salmonicida salmonicida* and *B. brevis* and natural biofilms were treated with Concanavalin - A (1mg ml<sup>-1</sup>) and protease of (0.5ng ml<sup>-1</sup>, 2ng ml<sup>-1</sup>, 5ng ml<sup>-1</sup>) and incubated for 30 min. Protease enzyme from *Bacillus licheniformis* (Subtilisin Carlsberg) was

obtained from Sigma. The protease was dissolved in filtered sea water. The wells after incubation were rinsed off with autoclaved filtered sea water and subsequently cyprids (approx. 10 to 15) were introduced in the multiwells along with 2 ml of autoclaved filtered sea water of 35‰. The assays were repeated three times using different batches of larvae with four replicates at each trial (n=12). We also conducted larval assay using BSA as control. BSA was inoculated at a protein concentration of  $50\mu\text{g ml}^{-1}$ , incubated for 3h and then rinsed off with autoclaved filtered sea water. Subsequently, multiwells coated with BSA were incubated with different concentration of protease and incubated for 30 min. The total protein was quantified in the supernatant before and after the protease treatment after which the multiwells were rinsed off with autoclaved filtered sea water. Subsequently, cyprids (approx. 10 to 15) were introduced in the multiwells (n=12) along with 2 ml of autoclaved filtered sea water of 35‰. The untreated biofilms with FSW alone were treated as control.

Culture supernatant (CS) and EPS extracted from *A. salmonicida salmonicida* and *B. brevis* by growing in BSS and MB medium were assessed at different concentrations of protein and carbohydrate (25 and  $50\mu\text{g ml}^{-1}$ ). These were also inoculated in multiwells, incubated for 3h and subjected to larval assays.

### **Statistical analysis**

The data on settlement (%) was arcsine transformed to ensure normality and homogeneity of variances before subjecting to statistical analysis. The influence of different biofilms (natural as well as monospecies biofilms) when exposed to protease on cyprid metamorphosis was evaluated using One-way analysis of variance (ANOVA) (Sokal & Rohlf 1981). The AE and FSW were taken as positive and negative controls respectively for the analysis. The influence of CS and EPS extracted from *A. salmonicida salmonicida* and *B. brevis* on the cyprid metamorphosis was also evaluated. A post-ANOVA Tukey's test was done to test the difference between the treatments.

## **Results**

### **Effect of natural biofilm (NBF) on cyprid metamorphosis**

Cyprids exposed to NBF and AE showed almost similar metamorphosis response on day 1 (Fig. 1). When the biofilm was assessed in presence of AE, a significant increase in metamorphosis was observed ( $p \leq 0.05$ , Tukey's test; Fig. 1). However, when treated with protease significant inhibition in metamorphosis was observed at the lowest concentration i.e.  $0.5\text{ ng ml}^{-1}$  ( $p \leq 0.05$ , Tukey's test).

An increase in the concentration of protease did not inhibit further the cyprid metamorphosis and the response was comparable with NBF. The settlement assays were conducted for a period of four days. The metamorphosis rates increased on day 2 and thereafter remained almost similar until day 4. The results of day 1 and day 4 are presented.

#### **Effect of *A. salmonicida salmonicida* biofilm on cyprid metamorphosis**

Cyprids exposed to *A. salmonicida salmonicida* biofilms did not facilitate metamorphosis as observed at the end of day 1, the response was almost similar to FSW (Fig. 2). When these films were assessed in presence of AE, metamorphosis was facilitated and was comparable with that of AE. The treatment of these biofilms with protease resulted in decline in cyprid metamorphosis with an increase in protease concentration.

A significant increase in metamorphosis was observed from day 1 to 4 in case of *A. salmonicida salmonicida* biofilms. Although the treatment of biofilm with protease at different concentrations resulted in increased metamorphosis on day 4, it was lower when compared to the untreated biofilm ( $p \leq 0.05$ , Tukey's test; Fig. 2).

#### **Effect of *B. brevis* biofilm on cyprid metamorphosis**

Cyprids exposed to *B. brevis* biofilms (Fig. 3) did not metamorphose as observed at the end of day 1. When these biofilms were assessed with AE also did not induce cyprids to metamorphose. *B. brevis* films both in presence/absence of AE had no effect on cyprid metamorphosis even when aged. However, when treated with protease although metamorphosis was negated initially an increase was evident with the protease concentration and observation days. A significant facilitation of metamorphosis was evident at higher concentration ( $2 \text{ ng ml}^{-1}$ ) on day 2, while there was significant decrease in cyprid metamorphosis from day 1 to 4 when the biofilm was exposed to protease at  $0.5 \text{ ng ml}^{-1}$  ( $p \leq 0.05$ , Tukey's test; Fig. 3).

The influence of BSA was more when compared to natural or artificial bacterial films and AE on day 1. When BSA was treated with different concentration of protease although showed increased metamorphosis the trend was similar as that observed in case of natural and artificial films (Fig. 4). However, when BSA was incubated with different concentration of protease and the total protein quantified in the supernatant before washing off the multiwells, it was observed that with the increasing concentration of protease, the amount of protein in the supernatant decreased suggesting the degradation of protein.



## **Influence of culture supernatant (CS) and exopolysachharides (EPS) on cyprid metamorphosis**

### *Aeromonas salmonicida salmonicida*

The influence of CS and EPS obtained by growing the bacteria using different nutrient mediums (BSS and MB) at carbohydrate concentration of 25 and 50  $\mu\text{g ml}^{-1}$  on cyprid metamorphosis is shown in Figure 5. Among all the treatments, EPS obtained by growing the bacteria in BSS at carbohydrate concentration of 50  $\mu\text{g ml}^{-1}$  showed significant inhibition in metamorphosis with respect to AE and FSW (Fig. 5) on day 1. The influence of CS and EPS produced under different nutrient conditions by *A. salmonicida salmonicida* was significantly lower than the AE except for EPS obtained by growing the bacteria in MB, at a protein concentration of 50  $\mu\text{g ml}^{-1}$  that showed significant facilitation in metamorphosis (Fig. 6).

### *Bacillus brevis*

The influence of CS and EPS obtained by growing the bacteria in different nutrient mediums (BSS and MB) at carbohydrate concentration of 25 and 50  $\mu\text{g ml}^{-1}$  on cyprid metamorphosis is depicted in Figure 6. EPS obtained by growing the bacteria in BSS at a carbohydrate concentration of 50  $\mu\text{g ml}^{-1}$  resulted in significant inhibition of metamorphosis with respect to AE and FSW (Fig.7). However, EPS and CS obtained by growing the bacteria in MB at a protein concentration of 25  $\mu\text{g ml}^{-1}$  facilitated metamorphosis higher than FSW but significantly lower than the AE on day 1 (Fig. 8).

## **Discussion**

The influence of natural biofilm on cyprid metamorphosis was similar as that of adult extract (AE). When the natural biofilm was assessed along with the adult extract the cyprid metamorphosis increased significantly. Previous study by Khandeparker *et al.* (2006) suggested that bacteria-adult extract interaction can alter the synthesis of compounds produced by bacteria. Also, waterborne and the surface-associated cues from the same bacteria function differentially in mediating larval metamorphosis. In the present study, the binding of adult extract to the natural biofilm was patchy as observed under the microscope after staining with the Bradford Reagent (Plate 1). Studies by Khandeparker *et al.* (2003) have indicated the possible role of patchiness in the marine environment in piloting the barnacle cyprids to their settlement destination. The availability of a strong promoter like AE in patches probably facilitated the metamorphosis. When BSA was incubated with different concentration of protease and the total protein quantified in the supernatant before washing off the multiwells it was observed that with the increasing concentration of protease, the amount of protein

in the supernatant decreased suggesting the degradation of protein and although the metamorphosis rates were higher there was no trend when BSA was treated with different concentration of protease, suggesting that the type of protein is important in the cyprid metamorphosis.

When *A. salmonicida salmonicida* was assessed in presence of AE, it promoted metamorphosis but the same was not observed with *B. brevis* films indicating that AE always do not supersede the inhibitory cues. Since the biofilms were viable, the production of extra cellular inhibitors by the bacteria possibly degraded the glycoprotein (AE) and this needs consideration in future when the viable biofilms are assessed for larval metamorphosis. Recently, it has been shown that biofilms inducing attachment of bryozoan, *Bugula neretina* did not produce proteolytic enzymes while the majority of settlement inhibiting biofilms did express proteases (Dobretsov *et al.* 2007).

Exopolymers and other excreted products produced by microorganisms have been shown to be involved in settlement, metamorphosis, growth and development of macro fouling community (Maki *et al.* 1990,1992; Holmström *et al.* 1992; Mary *et al.* 1993; Holmström & Kjelleberg 1994; Holmström *et al.* 1996; Keough & Raimondi 1996; Khandeparker *et al.* 2002,2003,2006). In the present investigation the EPS obtained by growing *A. salmonicida salmonicida* in Marine Broth at a protein concentration of 50  $\mu\text{g ml}^{-1}$  showed significant facilitation in metamorphosis. In case of EPS and CS from *B. brevis* no such inducement was observed. However, EPS obtained by growing the bacteria in BSS at a carbohydrate concentration of 50  $\mu\text{g ml}^{-1}$  resulted in significant inhibition of metamorphosis with respect to AE and FSW. When these monospecies biofilms were treated with protease from *B. licheniformis* it was found that metamorphosis decreased with the increasing concentration of protease in *A. salmonicida salmonicida*, whereas in *B. brevis* although metamorphosis was negated initially, metamorphosis increased with the concentration of protease and observation days. Since the response of cyprids to EPS produced by *A. salmonicida salmonicida* films was protein concentration dependent, the protease treatment resulted in decrease in metamorphosis. In case of *B. brevis* possibly the protease hydrolysis products provided positive cues to cyprids. It is also possible that at higher concentration, protease digested most of the proteins from cells and EPS, thus resulting in no real biofilm left on the surface. This signifies the importance of proteins in the biofilms.

The natural biofilm also showed reduction in metamorphosis after protease treatment. When treated with protease significant inhibition in metamorphosis was observed at 0.5  $\text{ng ml}^{-1}$ . An increase in the concentration of protease did not further inhibit the cyprid metamorphosis and the response was comparable with NBF. Currently, most of the antifouling paints are based on copper (Finnie 2006) supplemented with booster biocides (Liu *et al.* 1997; Readman *et al.* 1993; Yebra *et al.* 2004; Kostantinou & Albanis 2004; Turley *et al.* 2005), which are toxic. Alternatively, bacteria

produce diverse chemical compounds forming an important source of antifouling compounds (Dobretsov et al., 2006a, b). Previously it has been reported by Zentz *et al.* (2002) that crude proteases from *Pseudoalteromonas* sp. significantly decreased larval attachment at concentrations of 0.03 to 1 mIU ml<sup>-1</sup>, which is six folds lower than effective concentration of Tributyltin (TBT). Effects of different commercial enzymes on the adhesion of a marine biofilm forming *Pseudoalteromonas* sp. indicated that hydrolase's could either prevent adhesion, or remove adhered bacterial cells effectively, or conversely increase bacterial adhesion depending on enzymatic concentrations and type of enzymes tested (Leroy *et al.* 2008). In the present study, initial response of cyprids could be attributed to surface bound cues, as most of the exopolymers in biofilms are glycoproteins. Thus, biofilm components are important in larval metamorphosis. The treatment of biofilms with protease is hypothesized to disrupt the effect caused by proteins directly thereby changing the surface characteristics or indirectly by the release of the hydrolysis products and this has implications in the control of biofouling and the present study is a beginning towards the same.

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## Legends to figures

Fig.1 Influence of Natural biofilm (NBF), Natural Biofilm treated with Adult Extract (NBF + AE), protease at different concentrations, (NBF+ 0.5 ng ml<sup>-1</sup> , NBF + 2 ng ml<sup>-1</sup> , NBF + 5 ng ml<sup>-1</sup>) on cyprid metamorphosis of *B. amphitrite*. Controls are Adult extract (AE), Filtered Sea Water (FSW). The values are presented as mean numbers ± S.E. of metamorphosed cyprids using different batches of larvae.

Fig. 2 Influence of *A. salmonicida salmonicida* biofilm (BF), biofilm treated with Adult Extract (BF + AE), protease at different concentrations, (BF+ 0.5 ng ml<sup>-1</sup> , BF + 2 ng ml<sup>-1</sup> , BF + 5 ng ml<sup>-1</sup>) on cyprid metamorphosis of *B. amphitrite*. Controls are Adult extract (AE), Filtered Sea Water (FSW). The values are presented as mean numbers ± S.E. of metamorphosed cyprids using different batches of larvae.

Fig.3 Influence of *Bacillus brevis* biofilm (BF), biofilm treated with adult extract (BF + AE), protease at different concentrations, (BF+ 0.5 ng ml<sup>-1</sup> ,BF + 2ng ml<sup>-1</sup> ,BF + 5 ng ml<sup>-1</sup>) on cyprid metamorphosis of *B. amphitrite*. Controls are Adult extract (AE), Filtered Sea Water (FSW). The values are presented as mean numbers ± S.E. of metamorphosed cyprids using different batches of larvae.

Fig. 4 Influence of Bovine Serum Albumin (BSA), BSA treated with protease at different concentrations, (BSA+ 0.5 ng ml<sup>-1</sup> ,BSA + 2ng ml<sup>-1</sup> ,BSA + 5 ng ml<sup>-1</sup>) on cyprid metamorphosis of *B. amphitrite*. The values are presented as mean numbers ± S.E. of metamorphosed cyprids using different batches of larvae.

Fig. 5 Influence of CS and EPS produced by *A. salmonicida salmonicida* when grown in Marine Broth and Basal salt solution at a carbohydrate concentration of 25 and 50 µg ml<sup>-1</sup> respectively on the cyprid metamorphosis of *B. amphitrite*. AE – Adult extract, FSW – Filtered Sea water. The values are presented as mean numbers ± S.E. of metamorphosed cyprids using different batches of larvae.

\* significantly different data with respect to Adult Extract according to One-way ANOVA (p ≤ 0.001) followed by Tukey's test (α= 0.05).

● significantly different data with respect to Filtered Sea Water according to One-way ANOVA (p ≤0.001) followed by Tukey's test (α = 0.05).

Fig. 6 Influence of CS and EPS produced by *A. salmonicida salmonicida* when grown in Marine Broth at a protein concentration of 25 and 50 µg ml<sup>-1</sup> respectively on the cyprid metamorphosis of *B. amphitrite*. AE – Adult extract, FSW – Filtered Sea water. The values are presented as mean numbers ± S.E. of metamorphosed cyprids using different batches of larvae.

\* significantly different data with respect to Adult Extract according to One-way ANOVA (p ≤0.001) followed by Tukey's test (α = 0.05).

● significantly different data with respect to Filtered Sea Water according to One-way ANOVA (p ≤0.001) followed by Tukey's test (α = 0.05).

Fig. 7 Influence of CS and EPS produced by *B. brevis* when grown in Marine Broth and Basal Salt Solution at a carbohydrate concentration of 25 and 50 µg ml<sup>-1</sup> respectively on the cyprid metamorphosis of *B. amphitrite*. AE – Adult extract, FSW – Filtered Sea water. The values are presented as mean numbers ± S.E. of metamorphosed cyprids using different batches of larvae.

\* significantly different data with respect to Adult Extract according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

● significantly different data with respect to Filtered Sea Water according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

Fig. 8 Influence of CS and EPS produced by *B. brevis* when grown in Marine Broth at a protein concentration of 25 and 50  $\mu\text{g ml}^{-1}$  respectively on the cyprid metamorphosis of *B. amphitrite*. AE – Adult extract, FSW – Filtered Sea water. The values are presented as mean numbers  $\pm$  S.E. of metamorphosed cyprids using different batches of larvae.

\* significantly different data with respect to Adult Extract according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

● significantly different data with respect to Filtered Sea Water according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

## Legends to Plate

Plate 1 Biofilm exposed to the Adult Extract tagged with Bradford Reagent

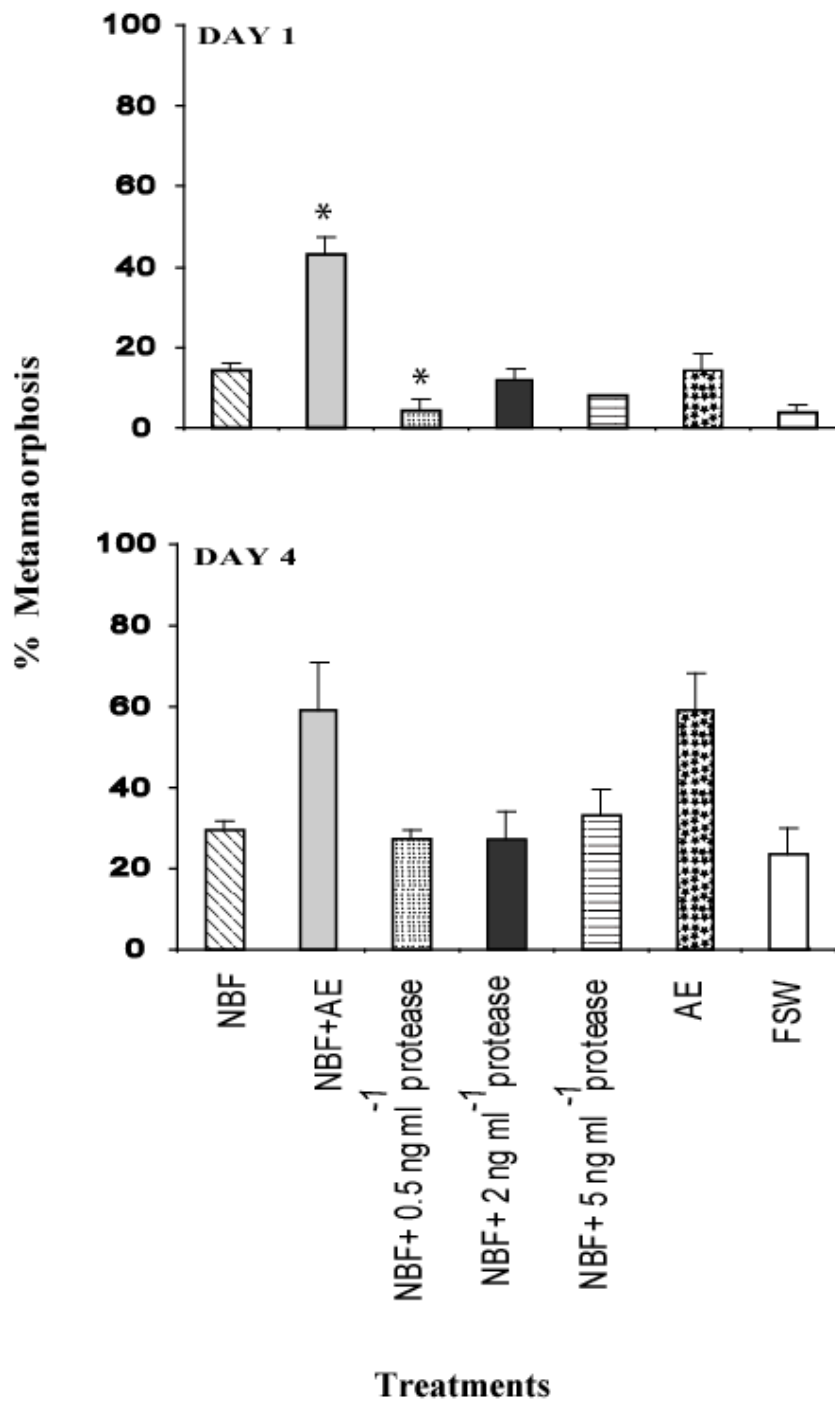


Figure 1

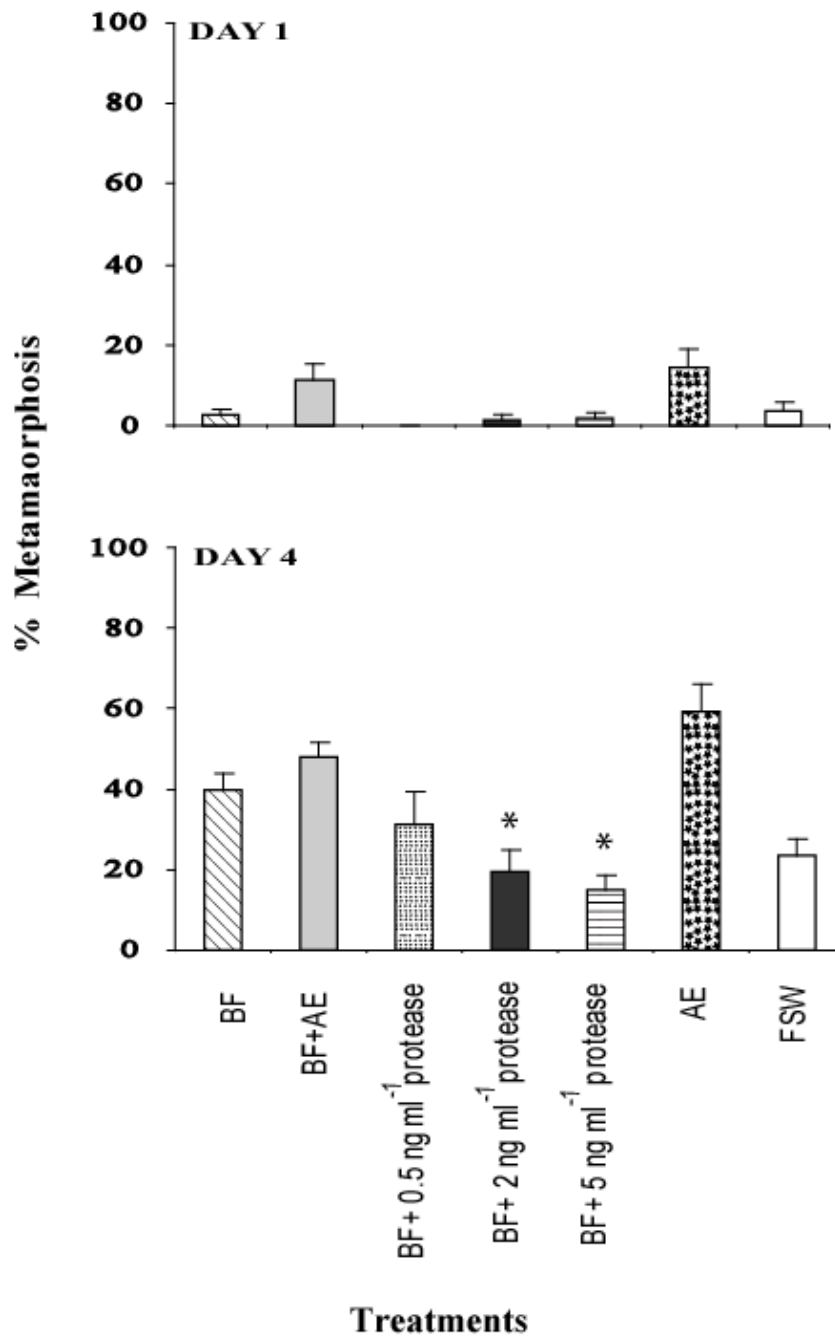


Figure 2

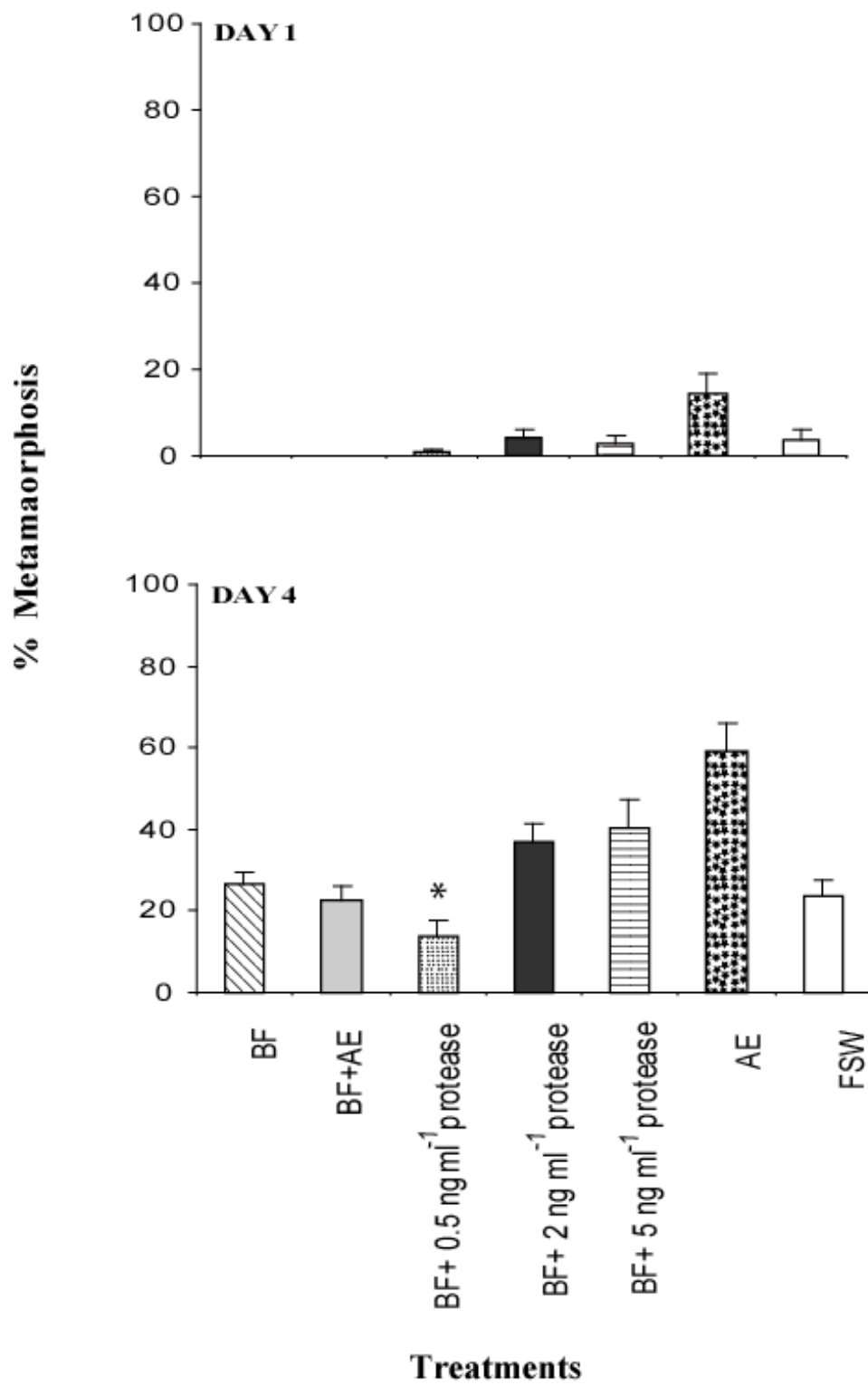


Figure 3

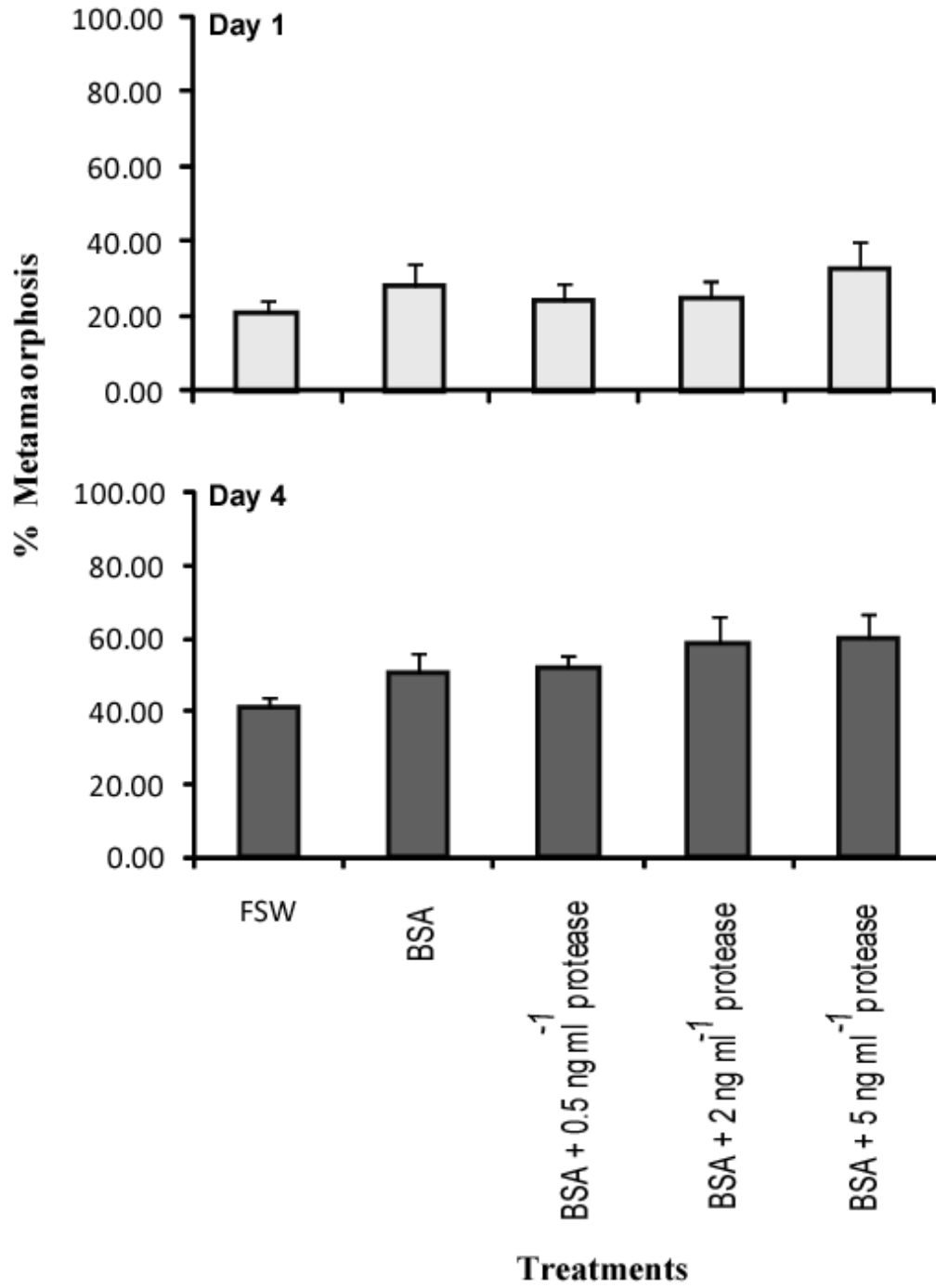
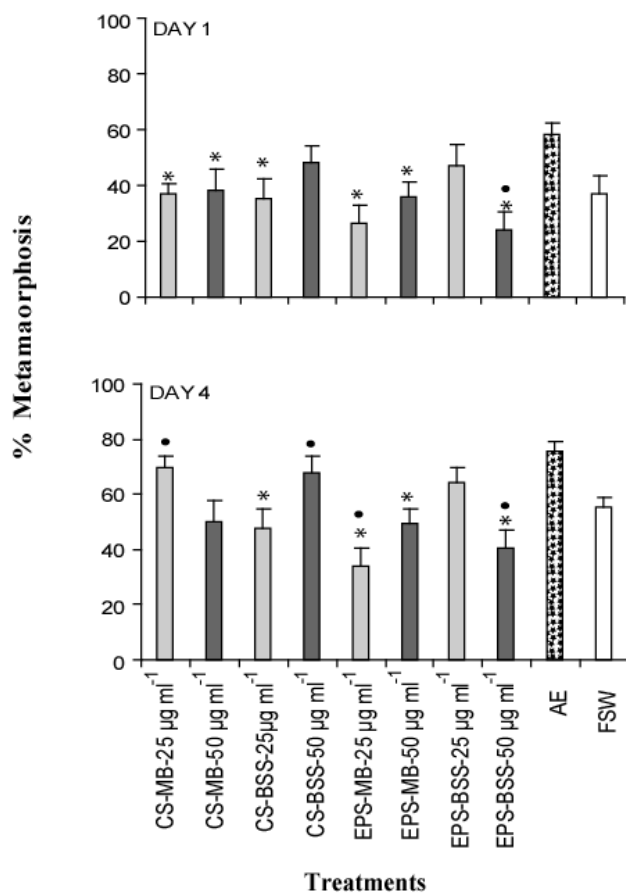
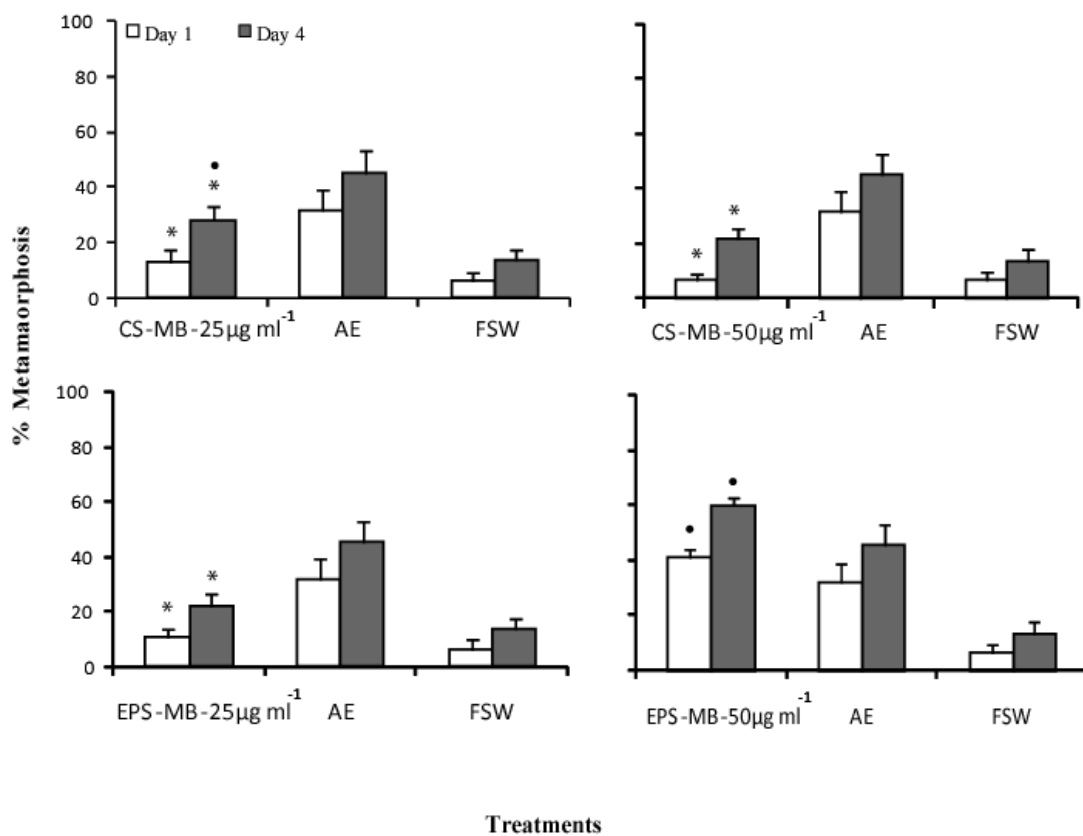


Figure 4



- \* Significantly different data with respect to Adult Extract according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).
- Significantly different data with respect to Filtered Sea Water according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

Figure 5

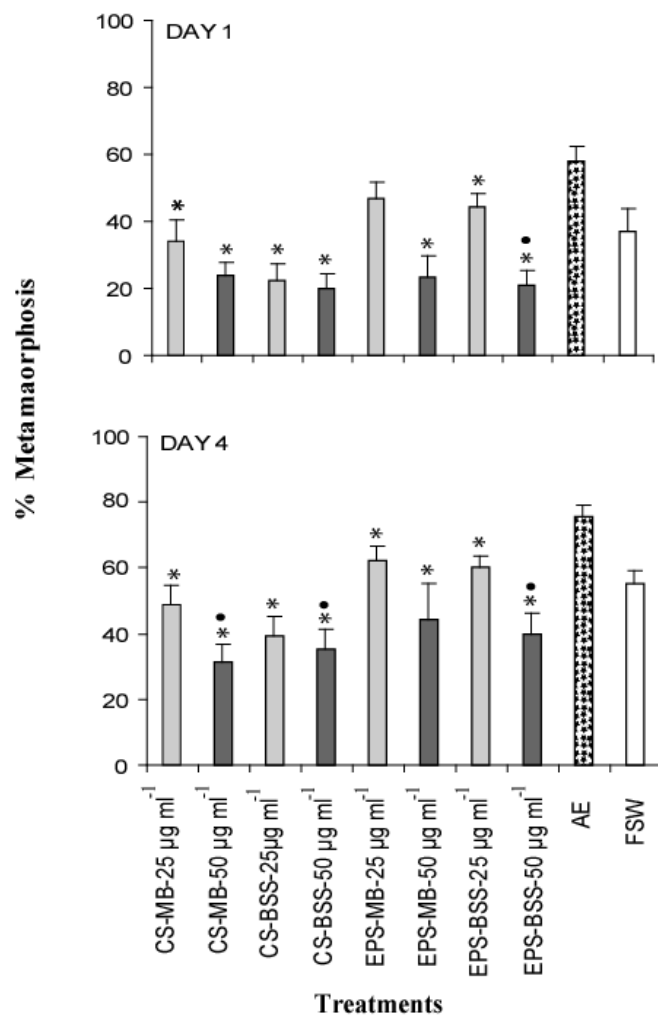


\* Significantly different data with respect to Adult Extract according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

• Significantly different data with respect to Filtered Sea Water according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

Figure 6

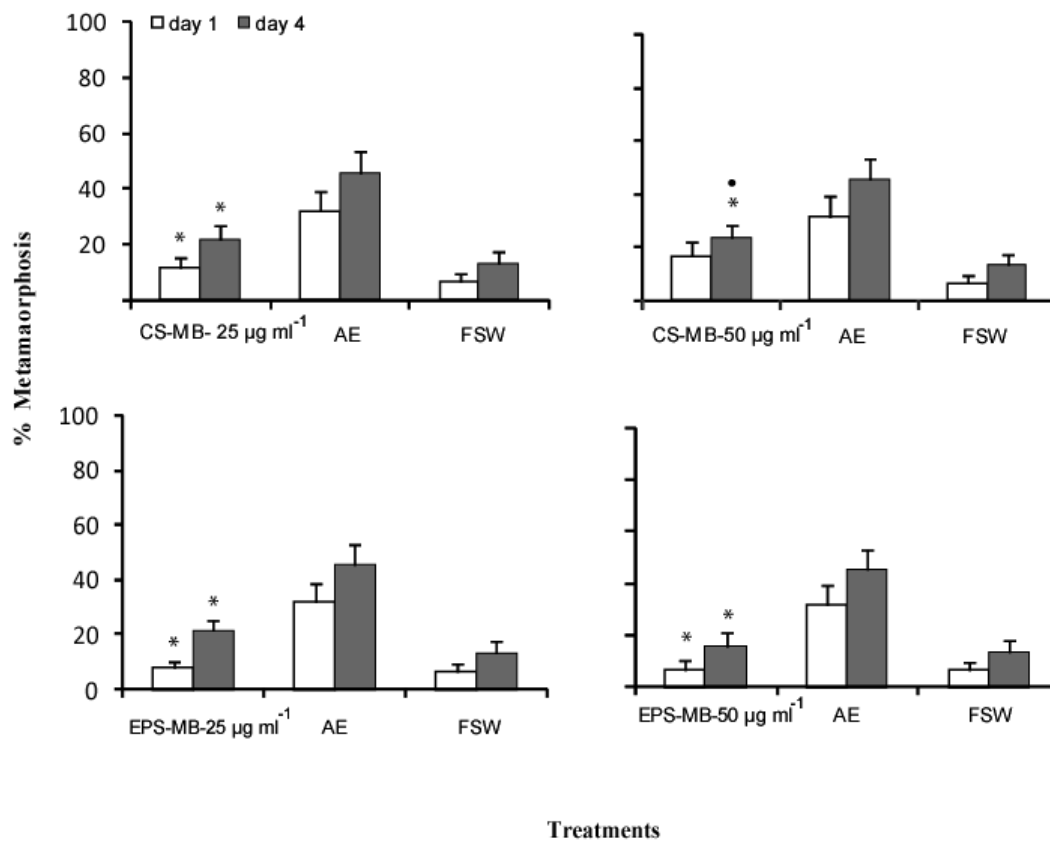




\* Significantly different data with respect to Adult Extract according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

• Significantly different data with respect to Filtered Sea Water according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

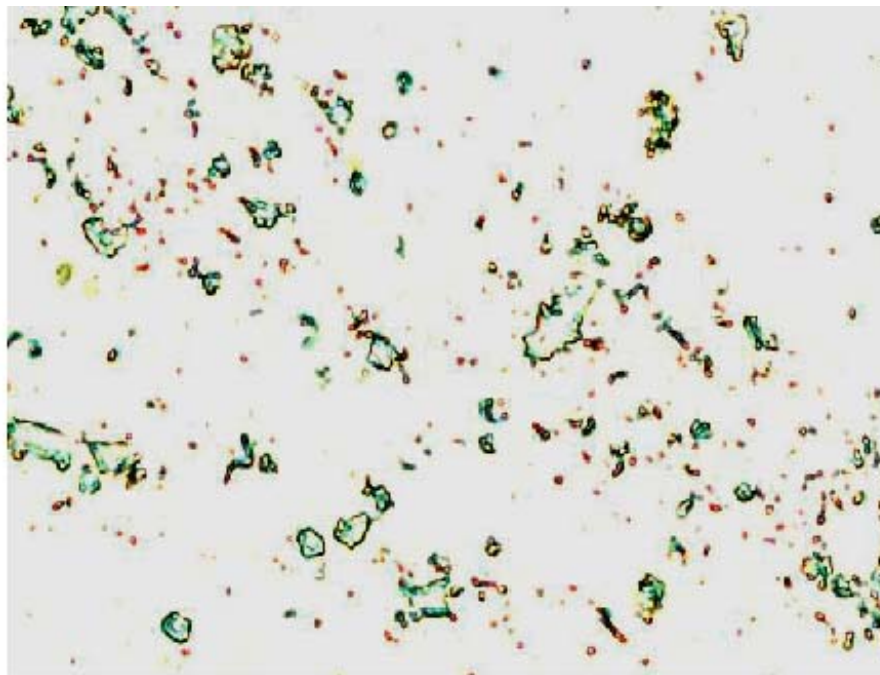
Figure 7



\* Significantly different data with respect to Adult Extract according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

• Significantly different data with respect to Filtered Sea Water according to One-way ANOVA ( $p \leq 0.001$ ) followed by Tukey's test ( $\alpha = 0.05$ ).

Figure 8



Natural Biofilm exposed to the Adult Extract tagged with Bradford Reagent

Plate 1