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Balanus amphitrite

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Abstract

Natural microbial communities found on different substrata exposed to marine environment including the barnacle shell surfaces are reported to have varying influence on the settlement and metamorphosis of competent cypris larvae. Experiments were carried out to compare the influence of settlement-inducing compounds from the bacteria isolated from the shell surface of *Balanus amphitrite*, on its larval metamorphosis. The effect of multispecies bacterial film was also assessed. The production of different molecules by the bacteria was influenced by the nutrient media under which they were grown. It was observed that the promotory multispecies bacterial film turned to inhibition mode in the presence of the adult extract (AE) of the barnacle, indicating Bacteria-AE interactions to alter the synthesis of different compounds produced by bacteria. The studies also show that the water borne and the surface associated cues from the bacteria function differentially in mediating larval metamorphosis. Understanding the complexities involved in such interactions and identification of the factors governing them would be a step ahead.

Keywords: Metamorphosis; Cyprid; Bacterial film; Adult extract; Barnacle

Introduction

Barnacle cyprid larvae while exploring for potential habitats for settlement and metamorphosis come across a series of physical and chemical factors (Crisp, 1974). Previously, it was generally believed that larvae settle in a random fashion, however, the settlement and metamorphosis are now believed to take place in response to complex physical and biochemical cues, the most important being the specific environmental stimuli or chemical cues that are associated with the settling substratum (Kirchman *et al.*, 1982; Morse, 1984; Maki & Mitchell, 1985; Le Tourneux & Bourget, 1988; Pechenik, 1990; Szewzyk *et al.*, 1991; Pawlik, 1992; Rodriguez *et al.*, 1993; Qian *et al.*, 2000). Surface chemistry also seems to play an important role in the distribution of adults (Strathmann *et al.*, 1981; Roberts *et al.*, 1991; Holm *et al.*, 1997). Laboratory and field studies have demonstrated that barnacle cyprids prefer to metamorphose on or near conspecifics, a process termed as gregariousness and this feature has been related to arthropodin (settlement factor), a glycoprotein present in the adults (Knight-Jones & Crisp, 1953; Crisp & Meadows, 1963) which will be referred as adult extract (AE) in the present investigation. While exploring a substratum, *B. amphitrite* cyprids deposit footprints of temporary adhesive that stimulate the settlement of other cyprids, even in the absence of conspecific adults (Clare *et al.*, 1994).

Besides adult conspecifics, a substratum with a well-developed biofilm is often preferred by barnacle cyprids like most other invertebrate larvae (Crisp, 1984). The larvae may use specific chemical signatures from biofilms or characteristic microbial assemblages to indicate preferred ecological conditions (Unabia & Hadfield, 1999). Thus it was hypothesized that the availability of conflicting cues in such assemblages might help pilot the larvae to their settlement destination (Khandeparker *et al.*, 2003). Biofilms generally inhibited settlement of *B. amphitrite* cyprids (Olivier *et al.*, 2000), although some biofilms were inductive or had neutral effect (Wieczorek *et*

al., 1995). The inhibitory effect by biofilms has been mainly attributed to their bacterial components (Maki *et al.*, 1988; Holmstrom *et al.*, 1992; Avelin Mary *et al.*, 1993; Anil & Khandeparker, 1998; Khandeparker *et al.*, 2002a, 2003). Several studies have addressed the implications of bacterial films on larval settlement of *B. amphitrite* (Maki *et al.*, 1988; Avelin Mary *et al.*, 1993; Neal & Yule, 1994; Olivier *et al.*, 2000; Khandeparker *et al.*, 2002a, 2003, Lau *et al.*, 2003). The specific molecular components of bacterial extracellular polymers (EPS) have been reported to be involved in the cyprid settlement of *B. amphitrite* (Maki *et al.*, 1988). For a chemical cue to be effective against larvae it must be either expressed on the surface of the substratum or released into the surrounding water (waterborne cue) (Crisp & Meadows, 1963; Hadfield & Scheuer, 1985; Tamburri *et al.*, 1992). Our previous studies have reported the response of the cyprids towards gram negative bacteria (*Pseudomonas aeruginosa*) isolated from shell surface of *B. amphitrite* and the bacterial products to be regulated by both contact chemoreception and olfaction, depending on the properties of the settlement-inducing compounds (Khandeparker *et al.*, 2002a). These results are used in this paper for comparison.

Five different experiments were carried out in this investigation to compare the influence of settlement-inducing compounds from two bacterial isolates isolated from the shell surface of *B. amphitrite*, on the metamorphosis of its cyprids. Out of two, one was a gram-positive bacterium, *Bacillus pumilus* and the other was a gram-negative bacterium, *Citrobacter freundii*. An attempt was also made to assess the impact of these bacteria together along with *Pseudomonas aeruginosa*, also isolated from shell surface of *B. amphitrite*. All the settlement cues and the bacterial films were also assessed in presence of AE owing to the co-existence of biofilms with the barnacles.

Materials and methods

Preparation of the adult extract (AE)

Adult extract was prepared by following the method described by Larman *et al.* (1982). Adults of *B. amphitrite*, collected from the intertidal area of Dona Paula (15° 27.5' N, 73° 48' E), were brought to the laboratory and cleaned by brushing off the epibiotic growth on their shells using a nylon brush. The animals were then washed with deionised water (RO pure) and 100 g wet wt. of whole adults was crushed with a mortar and pestle using 100 ml of deionised water (RO pure). The supernatant of the crushed mixture was decanted, centrifuged at 12000 x g for 5 min and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged at 12000 x g for 5 min and the supernatant was then frozen at -20° C until further use. The protein content of the extract was estimated following the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as the standard. A protein concentration of 50 µg ml⁻¹ of AE was used for all assays.

Rearing of *B. amphitrite* larvae

B. amphitrite is euryhaline (Anil *et al.* 1995). The life cycle of *B. amphitrite* includes planktotrophic larval development consisting of six naupliar instars and a non-feeding cyprid instar. The first instar nauplii do not feed and molt into the second instar within 1-2 hours. Instars II to VI are phytoplanktonotrophic. The barnacles were brought to the laboratory and exposed to air for a short interval (2-3 hours) and then placed in filtered seawater, which triggered the release of larvae. These larvae were then mass reared in 2-liter glass beakers using filtered seawater (FSW) of 35‰ on a diet of *Chaetoceros calcitrans*, a unicellular diatom, at a cell concentration of 2 x 10⁵ cells ml⁻¹. The food organism was replenished every day while changing the water. After 5-

6 days the cyprids obtained were siphoned out and stored at 5° C prior to settlement assays. Two-day-old cyprids were used to carry out the assays. These methods have been described in detail previously by Rittschof *et al.* (1984).

Isolation of bacteria from shell surface of *B. amphitrite*

B. amphitrite brought to the laboratory were rinsed with deionised water (RO pure) to remove dirt. The animals were then scraped with a nylon brush using millipore filtered autoclaved seawater under sterile conditions. The sample was further diluted and spread plated on Zobell Marine Agar 2216 (HIMEDIA). The bacteria were grown at room temperature (30 ± 2 °C). The bacterial colonies thus isolated were maintained on Zobell Marine Agar 2216 slants. Streaking on Zobell Marine Agar 2216 checked the purity of the culture. The isolated bacteria were identified following Bergey's manual of systematic bacteriology (Krieg, 1984). Out of two, the gram-positive bacterium was *B. pumilus* and the gram-negative bacterium was *C. freundii*. The gram-positive bacterium was also screened for phylogenetic analysis in Germany by Dr. Hentschel. The complete 16S rDNA sequence reveals that the isolate is a *B. pumilus* strain (98.4% homology). PCR amplification, cloning, sequencing and phylogenetic analysis was carried out following the method by Hentschel *et al.* (2001).

Experiment 1. Influence of bacteria and its products on cyprid metamorphosis

Effect of monospecies Bacterial film (BF)

The influence of bacterial films of *B. pumilus* and *C. freundii* on the metamorphosis of *B. amphitrite* cyprids was assessed at different salinities (15, 25, 35 and 45‰) and temperatures (20, 26 & 30 °C). The adult extract (AE) was used as positive control and autoclaved filtered

seawater (FSW) was used as negative control. Filming of the surfaces with bacteria was performed using polystyrene multiwells following the methods previously described by Maki *et al.* (1988). The dishes with the bacterial treatment were fixed with formaldehyde (final concentration 1 to 2%; v/v) to quantify the bacterial numbers. The quantification of attached bacteria was done by using acridine orange and epifluorescence microscopy (Daley & Hobbie, 1975). The adsorption technique resulted in bacterial densities of 10^6 to 10^7 attached bacteria cm^{-2} .

Culture supernatant and its fractions

The bacteria were grown in basal salt solution (BSS), pH 7.5, containing (g. l^{-1}): NaCl, 30.0; KCl, 0.75; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 7.0; NH_4Cl , 1.0; K_2HPO_4 , 0.7; KH_2PO_4 , 0.3; glucose, 10.0 and 1ml of trace metal solution (Bhosle, 1981). A 2% (v/v) inoculum of an 18h old culture grown in the same medium was used. The bacteria were cultivated at 30 ± 2 °C, under a cycle of 14-h light/10-h darkness. The culture was subjected to mild agitation by keeping it on a shaker. When the culture reached stationary phase, cells were centrifuged at $20,000 \times g$ for 15 min at 4° C. The supernatant was filtered through 0.22 μm millipore filter and the resultant filtrate was utilized as the culture supernatant (CS) in the assays. Subsequently, three sub-samples were concentrated in a stirred ultrafiltration cell (Amicon, Danvers, MA) to $1/10^{\text{th}}$ of their original volume using filters with a nominal molecular weight cut off of 3,000, 10,000 and 30,000 sequentially. The fractions between 3,000-10,000 mol. wt., 10,000-30,000 mol. wt., and 30,000 & above have been abbreviated as CS1, CS2 and CS3 respectively.

Culture supernatant and the fractions were characterized for total carbohydrate content following the method described by Dhople & Bhosle (1987) and protein content as described by

Lowry *et al.* (1951). D-glucose and BSA were used as the standards for carbohydrate and protein analysis respectively. The larval assays with culture supernatants and its fractions were rationalized at $50 \mu\text{g ml}^{-1}$ of carbohydrates and were carried out at different salinities (15, 25, 35 and 45‰).

Bacterial extract

The bacteria were extracted in butanol as described by Elyakov *et al.* (1996). This butanol-bacterial extract (BBE) was stored below 5°C for further use. The butanol extracted only low molecular weight polar metabolites. A concentration of $50 \mu\text{g ml}^{-1}$ of BBE was used to assess its effect on the cyprid metamorphosis at 15, 25, 35 & 45‰.

The bacterial film, culture supernatant, fractions and bacterial extract were also examined for their effect on cyprid metamorphosis in presence of AE ($50 \mu\text{g ml}^{-1}$).

Experiment 2

Effect of leachants (water-borne compounds) on cyprid metamorphosis.

Experiment 2 was carried out at 35‰. It differs from Experiment 1 as it was carried out with BBE, culture supernatant and fractions ($50 \mu\text{g ml}^{-1}$) without washing off the leachants. Hence the larvae were subjected to adsorbed (surface-bound) components as well as the leachants. The effect of AE ($50 \mu\text{g ml}^{-1}$) was also assessed similarly.

Experiment 3

Influence of culture supernatants extracted using different nutrient mediums on metamorphosis.

The bacteria were grown in marine broth (MB), basal salt solution (BSS) and organically rich modified Vishniac's medium (MV) as described by Perkins (1973) and by semi-solid culture (Abu *et al.*, 1991). The bacteria were cultivated at 30 ± 2 °C, the culture was subjected to mild agitation by keeping it on a shaker. The culture was centrifuged when it reached the stationary phase, at this phase maximum amount of exopolymer was produced. A 2% (v/v) inoculum of an 18h old culture grown in the same medium was used each time. The culture supernatants were harvested by centrifugation (20,000 x g for 30 min) and subsequently sterile filtered (0.22 µm, Millipore). They were then concentrated to 1/10th of their original volume in a stirred ultrafiltration cell (1,000 MW cut-off, Amicon). The culture supernatants were characterized by estimating total carbohydrates and proteins. The larval assays were carried out at a carbohydrate and protein concentrations of 25 and 50 µg ml⁻¹ respectively. These culture supernatants extracted from bacteria grown on different nutrient media i.e. CS(MB), CS(BSS), CS(MV) and CS(semi-solid) were examined on cyprid metamorphosis at 35‰. They were also examined in presence of AE (25/50 µg ml⁻¹).

Experiment 4. Influence of bacterial exopolysaccharides extracted using different nutrient mediums on metamorphosis

Extraction of bacterial exopolysaccharides (EPS)

For the extraction of exopolysaccharides, the culture supernatants extracted from bacteria grown under different nutritional conditions were treated with 5 volumes of absolute ethanol and left at 4° C overnight. The precipitate (EPS) was recovered by centrifugation at 30,000 x g for 15 min at 4° C, re-dissolved in distilled water and treated with DNase and RNase A (1-2 µg ml⁻¹ final concentration) for 3 hours at 37° C. The material was dialyzed (8,000 MW cut-off)

overnight at 4° C against distilled water and centrifuged at 30,000 x g for 20 min at 25° C to remove insoluble material. The supernatant was recovered and lyophilized. The exopolysaccharides extracted from bacteria grown on different nutrient media were evaluated for carbohydrate content and used at a concentration of 50 µg ml⁻¹ at 35‰. They were then subjected to the assays separately as well as in presence of AE (50 µg ml⁻¹).

Experiment 5

Effect of multispecies Bacterial film (BF)

The bacteria, *B. pumilus*, *C. freundii* were mixed along with *Pseudomonas aeruginosa* in a 1:1:1 ratio. They were grown in Marine Broth 2216 (Difco) to mid-exponential phase. The bacterial film was developed on the surfaces following the methods described by Maki *et al.* (1988). This resulted in bacterial densities of 10⁶ to 10⁷ attached bacteria cm⁻².

The influence of this multispecies bacterial film on the metamorphosis of *B. amphitrite* cyprids was assessed at 35‰ and in the presence of AE.

Assay protocol for Experiments 1, 2, 3, 4 and 5

The settlement assays were carried out using polystyrene multiwells (Corning-430343, 6 well multiwells). The multiwells were inoculated with bacterial film, culture supernatants, fractions, bacterial exopolysaccharides & bacterial extract. They were also assessed along with AE (3 replicates for each of the combination by taking three different batches of larvae, n=9). Controls were FSW (negative control) and AE (positive control). The inoculated multiwells were rinsed off after three hours by repeated rinsing with autoclaved filtered seawater under a laminar chamber except in Expt. 2 where the multiwells containing the dilutions were assessed along with

the leachants (without washing off). Subsequently ~ 35-40 cyprids were introduced with 5 ml of autoclaved filtered seawater (15/25/35/45‰ salinity) as required. The settlement assays were monitored for a period of four days with an intermittent observation everyday. Assay wells were maintained at $26 \pm 1^\circ \text{C}$ (12h light: 12h dark photoperiod).

Statistical analysis

Data in the form of percentage of larval settlement was arcsine transformed to ensure normality of means and homogeneity of variances before statistical analysis. The influence of bacterial film, culture supernatant, three fractions and the bacterial extract in presence and absence of AE at different salinities was evaluated on the larval metamorphosis at the end of day 1 and day 4 using Two-way ANOVA omitting the blanks (Sokal & Rohlf, 1981) followed by Tukey's multiple comparison test at the $\alpha=0.05$ significant level. These analysis were performed for each bacterial species and the differences between the treatments i.e. bacterial film and bacterial inducers/controls was evaluated. 2-way ANOVA was also carried out to evaluate the effect of different nutrient mediums on cyprid metamorphosis. This analysis was also performed for each species by taking nutrient mediums and concentration ($25 \mu\text{g ml}^{-1}$ and $50 \mu\text{g ml}^{-1}$ of protein/carbohydrate) as variables. The blank treatment was omitted from the analysis.

Results

The results obtained from the larval assays in comparison to positive and negative controls divided the settlement responses into three distinct categories, i.e. 1. 'Inductive' (I): percentage of metamorphosis is greater than or equal to positive control, 2. 'Weakly inductive' (WI): percentage of metamorphosis is greater than negative control but lesser than positive control, and

3. 'No effect' (NE): percentage of metamorphosis is equal to or less than negative control. This classification is done based on the one described by Harder *et al.* (2002).

Bacillus pumilus

Experiment 1

Films of *B. pumilus* when assessed at 26° C, showed no effect at 25, 35 and 45‰ (Fig. 1), whereas at 15‰, it was weakly inductive (Fig. 1; Table 1a). At 30° C, bacterial film was inductive at 15‰, whereas at 20° C, it induced metamorphosis at almost all salinities (Table 1b). In summary, *B. pumilus* film induced cyprid metamorphosis at lower salinity and temperature.

The influence of its culture supernatant and the fractions containing different molecular weight substances on larval metamorphosis was also different with respect to salinity (Fig. 2). Higher molecular weight fraction was inductive at lower salinity and vice versa (Table 1a). The bacterial extract was inhibitory at 35 and 45‰, whereas it was inductive at 15 and 25‰. Two-way ANOVA also showed significant variation in larval metamorphosis among the bacterial inducers and the salinities on day 1 and 4 (Table 2a). However, there was no significant difference among the bacterial inducers in presence of adult extract, but significant variation among salinities was evident at the end of day 1 (Table 2b). Tukey's multiple comparison tests (Table 3) revealed significant variation between the bacterial film and FSW at 15‰ and with respect to AE it was significantly different at 35 ‰ (Fig. 1). At 15‰, bacterial film was inductive, whereas, at 35‰, the adult extract was inductive. Also, bacterial film and the bacterial extract showed no significant difference at any salinity but with respect to the culture supernatant and the fractions it showed significant difference at particular salinity. The response of all the bacterial inducers at

different salinities changed resulting in significant differences in most of the cases showing non-significance at the end of day 4 (Table 3).

Experiment 2

The cyprids did not settle in presence of leachants of CS2 and CS3, while CS, CS1, BBE leachants were not effective in provoking metamorphosis when compared to negative control as well as positive control at the end of Day 1 (Fig. 3). However, a sudden hike in metamorphosis of cyprids by leachants of BBE was observed at the end of day 4.

Experiment 3

No culture supernatant was effective in inducing the metamorphosis of the cyprids. The influence of culture supernatants obtained using different nutrient mediums was non-significant on larval metamorphosis (Table 4a). However, in presence of AE significant differences were evident among the culture supernatants, but showed insignificant variation between the concentrations (Table 4b).

Experiment 4

The exopolysaccharides extracted using different nutritional media showed no effect on metamorphosis and their response was not comparable to that of AE.

Citrobacter freundii

Experiment 1

At 26° C, BF was weakly inductive at 15‰, whereas at other salinities it showed no effect on the metamorphosis (Table 1a). When the bacterial film was assessed in presence of AE a further decrease in the metamorphosis rates was evident at 25, 35 and 45‰, whereas at 15‰, the metamorphosis rates increased (Fig. 4). At 20 °C, the overall response of the bacterial film was inductive, whereas at 30°C, it induced metamorphosis at 15 and 45‰ at the end of day 1 (Table 1b).

Culture supernatant and fractions were inductive at all the salinities except at 35‰ where they were weakly inductive (Fig. 5; Table 1a). The bacterial extract induced metamorphosis at 15‰ and 45‰, whereas at 35‰ it was inhibitory. Except at 35‰, BBE induced metamorphosis in presence of AE. Two-way ANOVA showed significant variation in larval metamorphosis among the bacterial inducers and the salinities (Table 2c). When assessed in presence of adult extract also the variations among the inducers and salinities was significant at the end of day 1 and day 4 (Table 2d). Tukey's multiple comparison test revealed significant variation between bacterial film and CS3 at all the salinities. Whereas, it was significantly different from other culture supernatant fractions at some salinities (Table 3). The bacterial extract showed insignificant difference from the bacterial film at all the salinities at the end of day 1, however was significantly different on day 4 at 15 and 35‰ (Table 3). The adult extract showed higher metamorphosis and was significantly different from the bacterial film only at 35‰ (Fig. 4).

Experiment 2

The cyprids did not settle in presence of leachants of BBE and CS3 and the other inducers were inhibitory at the end of Day 1. (Fig. 6).

Experiment 3

C. freundii cultivated in BSS, MB and MV yielded culture supernatants with high carbohydrate content, whereas those extracted using semi-solid culture showed higher protein content. Two-way ANOVA showed significant variation between the culture supernatants extracted using different extraction protocols and the concentration of carbohydrate and protein at which they were used on day 1 & 4 (Table 4c). The larvae metamorphosed in higher percentages and were significantly different from other culture supernatants as indicated by Tukey's test when exposed to culture supernatant obtained by using BSS at a carbohydrate concentration of 25 & 50 $\mu\text{g ml}^{-1}$. When assessed in presence of AE a significant difference was observed between the concentrations at the end of day 1 (Table 4d).

Experiment 4

The exopolysaccharides extracted using different nutritional media did not induce metamorphosis and were not comparable to that of AE (data not shown).

Experiment 5

The multispecies bacterial film was facilitatory when compared to the monospecies bacterial films of *B. pumilus* and *C. freundii* (Fig. 7). The metamorphosis rates were almost similar as that shown by *P. aeruginosa* films. However, when the multispecies film was assessed in presence of AE, metamorphosis was not promoted.

Discussion

The influence of monospecies bacterial films on metamorphosis of *B. amphitrite* cyprids varied at different temperatures and salinities. In case of a phototrophic bacterium, *Rhodobacter sphaeroides*, the different stress factors such as adverse environmental conditions, caused modifications in its protein pattern (Nepple & Bachofen, 1997). Our previous study also indicated that variations in larval behavior towards bacterial film varied with the salinity and temperature and was attributed to alteration in metabolic activities of bacteria and expression of different cell surface organic molecules (Khandeparker *et al.*, 2002a). Dineen & Hines (1992) proposed a hypothesis that the cyprids of *Balanus improvisus*, respond to a change in conformation of settlement factor protein brought about by the experimental salinity. Salinity can change the protein conformation, thereby changing its three dimensional structure. The variation in response of cyprids towards the bacterial films at different salinities could be also possible due to the conformational changes brought about by salinity in different bacterial molecular domains.

The production of different molecules by the bacteria was influenced by the nutritional conditions under which they were grown and such differences governed larval metamorphosis. The metamorphosis inducement by the culture supernatant obtained from *P. aeruginosa* grown on semi-solid culture was greater than those obtained using any other nutrient media and was protein concentration dependent. The culture supernatant isolated from cells growing in a semi-solid environment represents a closer approximation to the natural environment existing between a bacterium and its substratum (Abu *et al.* 1991). According to Boyle and Reade (1983), such conditions may be similar to the effects of exposure to intertidal zones.

However, in case of *C. freundii*, cyprids metamorphosed in higher percentages when exposed to culture supernatant obtained by using BSS and this was carbohydrate concentration

dependent. Whereas, in case of culture supernatant of *B. pumilus* such an inducement was not evident. This indicates that the biochemical composition of the bacterial inducers change with the availability of different levels of nutrients and this provoked the larvae to settle or repel.

When *B. pumilus* and *C. freundii* were assessed along with *P. aeruginosa*, the metamorphosis was facilitated. The metamorphosis rates were almost similar to that showed by *P. aeruginosa* (Fig. 7). The multispecies film was assessed at 26° C and 35‰, at which *C. freundii* and *B. pumilus* films were inhibitory but *P. aeruginosa* was weakly inductive. Thus, it can be hypothesized that promotory bacteria send analogous (similar) signals to the other bacteria and modulate their physiological response rendering inhibitory bacteria into inductive ones. The cell-cell communication between their own species and perhaps others through N-acyl-L-homoserine lactones (AHL) has been demonstrated in natural biofilms (McLean *et al.*, 1997). Thus each bacterium seems to play an important role and the effect of promotory bacteria could overrule the others resulting in settlement and metamorphosis. The other possible reason could be related to the growth of viable bacteria in presence of each other. In the present investigation all three bacteria were mixed in 1:1:1 proportion, which may not be true in a natural community, also, it is quite possible that the growth of one bacteria is suppressed by the other while growing, such bacteria-bacteria interactions could result in production of some factors that either detoxify or promote different compounds produced by bacteria. Thomas & Robinson (1987) while evaluating the role of bacteria in the metal tolerance of the fouling diatom *Amphora coffeaeformis* suggested that bacteria or bacteria-algal interactions produce some soluble factor which either detoxifies copper and TBTF or increases the tolerance of *Amphora coffeaeformis* to these toxins. Understanding the complexities involved in such interactions and identification of the factors governing them would be a step ahead.

The presence of AE did not alter the response of *B. pumilus* film at different salinities. However, a significant decrease in metamorphosis by the mixed film was observed in presence of AE. Our previous study has shown that when the viable films of *P. aeruginosa* were examined in presence of AE, the metamorphosis was facilitated irrespective of the salinity differences (Khandeparker *et al.*, 2002a). Lau *et al.* (2003) showed that conspecific settlement factor (AE) reverts the inhibitive effect of natural biofilms developed in the intertidal region but the inhibitive effect of nonviable monospecies bacterial films prevailed over AE. A decrease in the metamorphosis of cyprids by multispecies film in presence of AE could be possibly due to complex interactions between different bacterial domains and the proteins of the AE. These interactions could alter the synthesis of some compounds, by expressing the previously unexpressed genes. Characterization of the bacterial molecules obtained by growing different bacteria in presence of AE would give a better understanding of the mechanism by which AE reverted the effect produced by the mixed bacterial film.

The present study emphasizes the importance of bacteria and AE interactions suggesting adult settlement proteins to play a vital role in bacterial modulations of metamorphosis behavior. In one of our previous study the bacteria-lectin interaction was suggested to play an important role in piloting the cyprids to their destination (Khandeparker *et al.*, 2003). The role of lectins in bacteria-AE interactions needs further attention.

In an earlier investigation, the effects of bacteria and diatoms mixed at different ratios on the larval settlement of *Hydroides elegans* has been investigated (Harder *et al.*, 2002) and most of the treatments showed higher percentages of settlement on a mixed diatom-bacteria biofilm than on a monospecies biofilm. Recently, Patil & Anil (2005) reported that differential responses of *B. amphitrite* cyprids to different diatom species and their EPS to indicate that each diatom

species provides a different set of physico-chemical signals to settling larvae. Understanding the possible interactions among the biofilm components in providing the cues to the settling larvae would shed more light on this aspect.

In this investigation the culture supernatant containing the extracellular materials was fractionated into different molecular weight categories, which were characterized in terms of protein and carbohydrates. The higher molecular weight fraction of *B. pumilus* was effective at lower salinity, whereas, in case of *C. freundii*, culture supernatant and fractions were inductive at all the salinities except at 35‰, where they were weakly inductive. The response of cyprids to bacterial supernatants can thus be attributed to the activity of specific molecular weight compounds. Our previous study hypothesized that the effect of bacterial culture supernatants on cyprid metamorphosis with respect to salinity could be the result of positional effect due to isomerism (Khandeparker *et al.*, 2002a).

The cyprids did not settle in presence of waterborne compounds from high molecular weight exopolymers of *B. pumilus* and *C. freundii*. The response of the cyprids to the leachants of BBE of *B. pumilus* and *C. freundii* was also negative showing no settlement. However, the leachants of BBE extracted from *P. aeruginosa* showed a two-fold increase in the metamorphosis rates. The bacteria were extracted in n-butanol, which would extract only low molecular weight polar metabolites. In oysters, low-molecular weight peptides with arginine at the C-terminal were identified as natural water-soluble cue inducing settlement (Zimmer-Faust & Tamburri, 1994). It was proposed that the deterrents appear to be primary non-polar, low soluble secondary metabolites effective at low concentrations (Steinberg *et al.*, 2001, 2002). The inhibitory compounds excreted from bacteria were also shown to be water-soluble proteins and carbohydrates (Dobretsov & Qian, 2004). The EPS obtained from *P. aeruginosa* grown in BSS

exhibited similar metamorphosis effects as that of bacteria and AE, and hence was shown to be a responsible surface-bound component of the bacterial film, which supplied positive cues for the settling cyprids (Khandeparker *et al.*, 2002a). However, in the present study the EPS produced by *B. pumilus* and *C. freundii* did not induce metamorphosis of the cyprids. The structure of bacterial exopolymers is capable of either determining the effectiveness of the cypris temporary adhesive or affecting the cyprid's 'willingness to detach' (Yule & Walker, 1984).

The presence of bacteria with the important surface-associated cues and the water borne ones from the same surface could have ecological significance. Microscale patchiness of bacteria is evidenced in marine communities. In our earlier investigation the bacterial films were probed with lectins and it was found that the lectins can transform the signals by the bacteria. Thus it was hypothesized that presence of lectins could provide *B. amphitrite* with an ability in altering the signals or cues (Khandeparker *et al.*, 2003). It was also shown that the response of cyprids was sugar dependent in the absence of adult extract (AE), however, the sugar-treated cyprids when exposed to surface coated with AE continued exploring or searching the surface (Khandeparker *et al.*, 2002b). Thus the cues associated with the substratum could alter the effects by the water-borne cues. Hence, it can be hypothesized that the surface associated cues mediate the settlement and metamorphosis whereas the water borne ones could be more significant in locating the destination for example the presence of biofilm by the exploring larvae.

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Table 1a. Influence of different settlement-inducing compounds from *B. pumilus*, *C. freundii* & *P. aeruginosa* on cyprid metamorphosis at different salinities. BF: Bacterial Film; CS: culture supernatant; CS1: culture supernatant fraction between 3,000-10,000 mol. wt.; CS2: culture supernatant fraction between 10,000-30,000 mol. wt.; CS3: culture supernatant fraction from 30,000 & above; BBE: butanol-bacterial extract. I: Inductive; WI: Weakly Inductive; NE: No Effect.

26 °C	<i>B. Pumilus</i>						<i>C. freundii</i>						<i>P. aeruginosa</i>					
	BF	CS	CS1	CS2	CS3	BBE	BF	CS	CS1	CS2	CS3	BBE	BF	CS	CS1	CS2	CS3	BBE
15‰	WI	I	NE	I	I	I	WI	I	I	I	I	I	NE	NE	NE	WI	NE	NE
25‰	NE	I	I	I	WI	WI	NE	I	I	I	I	WI	I	WI	WI	WI	NE	I
35‰	NE	WI	WI	WI	WI	NE	NE	WI	WI	WI	WI	NE	WI	WI	WI	WI	WI	NE
45‰	NE	WI	I	NE	NE	NE	NE	I	I	I	I	I	NE	I	NE	WI	I	I

Table 1b. Influence of different bacterial films on the metamorphosis of *B. amphitrite* cyprids at different temperatures and salinities

20 °C	<i>B. Pumilus</i>	<i>C. freundii</i>	<i>P. aeruginosa</i>
15 ‰	I	I	I
25 ‰	I	I	I
35 ‰	WI	WI	I
45 ‰	I	I	I
30 °C			
15 ‰	I	I	I
25 ‰	WI	NE	I
35 ‰	NE	NE	I
45 ‰	NE	I	NE

Table 2. Two-way ANOVA. Influence of different bacterial inducers (BF, CS, CS1, CS2, CS3, BBE) at different salinities (15, 25, 35 and 45‰) on the metamorphosis of *B. amphitrite* cyprids on day 1 and 4. BF: Bacterial Film; CS: culture supernatant; CS1: culture supernatant fraction between 3,000-10,000 mol. wt.; CS2: culture supernatant fraction between 10,000-30,000 mol. wt.; CS3: culture supernatant fraction from 30,000 & above; BBE: butanol-bacterial extract; ♣: The bacterial inducers were assessed in presence of AE. (a) *B. pumilus*; (b) *B. pumilus* in presence of AE; (c) *C. freundii*; (d) *C. freundii* in presence of AE. (df: degree of freedom; SS: sum of the squares; MS: mean of squares; Fs: Fischer constant).

	df	SS	Day 1		Day 4		Fs
			MS	Fs	SS	MS	
(a) <i>B. pumilus</i>							
Bacterial inducers	5	1101	220	2.7*	3847	769	4.3****
Salinities	3	8991	2997	36.7****	13866	4622	26****
Combined	15	2981	198	2.4****	8271	551	3.1****
Within sub. Gr. Err.	264	21567	81		46830	177	
Total	287	34641			72816		
(b) <i>B. pumilus</i> ♣							
Bacterial inducers	5	1060	212	1.6 ns	5387	1077	4***
Salinities	3	10028	3342	25.7****	20561	6853	26****
Combined	15	1304	87	0.67 ns	8692	579	2.1**
Within sub. Gr. Err.	264	34242	129		70548	267	
Total	287	46636			105191		
(c) <i>C. freundii</i>							
Bacterial inducers	5	11811	2362	13.3****	12796	2559	8.7****
Salinities	3	13612	4537	25.5****	20949	6983	24****
Combined	15	3619	241	1.3 ns	8455	563	1.9*
Within sub. Gr. Err.	264	46884	177		77249	292	
Total	287	75928			119451		
(d) <i>C. freundii</i> ♣							
Bacterial inducers	5	14584	2916	20****	14332	2866	13****
Salinities	3	14628	4876	34****	26631	8877	40****
Combined	15	5628	375	2.6****	7306	487	2.1****
Within sub. Gr. Err.	264	38092	144		58689	222	
Total	287	72934			106959		

(**** $p \leq 0.001$, *** $p \leq 0.005$, ** $p \leq 0.01$, * $p \leq 0.025$, ns: non-significant)

Table 4. Influence of different culture supernatants obtained by growing the bacteria under different nutrient mediums (BSS, ThM, MB and semi-solid culture) on the metamorphosis of *B. amphitrite* cyprids at two different concentrations of carbohydrates and proteins ($25 \mu\text{g ml}^{-1}$ and $50 \mu\text{g ml}^{-1}$) at the end of day 1 and 4. ♣: The culture supernatants were assessed in presence of AE. (a) *B. pumilus*; (b) *B. pumilus* in presence of AE; (c) *C. freundii*; (d) *C. freundii* in presence of AE. (df: degree of freedom; SS: sum of the squares; MS: mean of squares; Fs: Fischer constant).

	df	SS	Day 1 MS	Fs	SS	Day 4 MS	Fs
(a) <i>B. pumilus</i>							
Culture supernatants	3	406	135	1.5 ns	454	151	0.82 ns
Concentration	3	318	106	1.18 ns	816	272	1.48 ns
Combined	9	531	59	0.65 ns	2202	245	1.33 ns
Within sub. Gr. Err.	176	15776	90		32157	183	
Total	191	17033			35630		
(b) <i>B. pumilus</i> ♣							
Culture supernatants	3	1508	502	6.1***	2135	711	2.5 ns
Concentration	3	182	60	0.74 ns	1775	591	2.1 ns
Combined	9	1168	130	1.6 ns	3604	400	1.4 ns
Within sub. Gr. Err.	176	14298	81		49401	280	
Total	191	17156			56916		
(c) <i>C. freundii</i>							
Culture supernatants	3	1422	474	16.9***	3467	1156	7.07***
Concentration	3	794	264	9.4***	1463	488	2.98*
Combined	9	1917	213	7.6***	5170	574	3.51***
Within sub. Gr. Err.	176	4948	28		28739	163	
Total	191	9084			38841		
(d) <i>C. freundii</i> ♣							
Culture supernatants	3	785	261	2.9*	2459	819	2.7*
Concentration	3	1961	653	7.2***	1377	459	1.53 ns
Combined	9	2506	278	3.09**	3614	401	1.34 ns
Within sub. Gr. Err.	176	15839	89.9		52545	298	
Total	191	21094			59997		

(*** $p \leq 0.001$, ** $p \leq 0.005$, * $p \leq 0.05$, ns: non-significant).

Legends to Figures

Fig. 1. Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*B. pumilus*) in presence of adult extract at different salinities (15, 25, 35 and 45‰). Vertical lines indicate the standard deviation from mean and are shown as positive. FSW - Filtered Sea Water ; AE - Adult Extract; BF - Bacterial Film. * - Significantly different data with respect to FSW according to Two-way ANOVA ($p \leq 0.001$) followed by Tukey's test ($\alpha = 0.05$); • - significantly different data with respect to AE according to Two-way ANOVA ($p \leq 0.001$) followed by Tukey's test ($\alpha = 0.05$).

Fig. 2. Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions obtained from *B. pumilus* grown in basal salt solution in presence and absence of adult extract (AE). Vertical lines indicate the standard deviation from mean and are indicated as positive. CS1 - Culture Supernatant (3,000-10,000 mol. wt.); CS2 - Culture Supernatant (10,000-30,000 mol. wt.); CS3 - Culture Supernatant (30,000 & above mol. wt.); CS - Culture Supernatant; AE - Adult Extract; FSW - Filtered Sea Water; I- Inductive.

Fig. 3. Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial inducers (surface-bound) from *B. pumilus* used in experiment 1 in presence of leachants at 35‰. CS1 - Culture Supernatant (3,000-10,000 mol. wt.); CS2 - Culture Supernatant (10,000-30,000 mol. wt.); CS3 - Culture Supernatant (30,000 & above mol. wt.); CS - Culture Supernatant; BBE - Butanol-Bacterial Extract; AE - Adult Extract; FSW - Filtered Sea Water.

Fig. 4. Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*C. freundii*) in presence of adult extract (AE) at different salinities (15, 25, 35 and 45‰). Vertical lines indicate the standard deviation from mean and are shown as positive. FSW - Filtered Sea Water;

AE - Adult Extract; BF - Bacterial Film, • - significantly different data with respect to AE according to Two-way ANOVA ($p \leq 0.001$) followed by Tukey's test ($\alpha = 0.05$).

Fig. 5. Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions obtained from *C. freundii* grown in basal salt solution in presence and absence of adult extract (AE). Vertical lines indicate the standard deviation from mean and are indicated as positive. CS1 - Culture Supernatant (3,000-10,000 mol. wt.); CS2 - Culture Supernatant (10,000-30,000 mol. wt.); CS3 - Culture Supernatant (30,000 & above mol. wt.); CS - Culture Supernatant; AE - Adult Extract; FSW - Filtered Sea Water; I- Inductive.

Fig. 6. Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial inducers (surface-bound) from *C. freundii* used in experiment 1 in presence of leachants at 35%. CS1 - Culture Supernatant (3,000-10,000 mol. wt.); CS2 - Culture Supernatant (10,000-30,000 mol. wt.); CS3 - Culture Supernatant (30,000 & above mol. wt.); CS - Culture Supernatant; BBE - Butanol-Bacterial Extract; AE - Adult Extract; FSW - Filtered Sea Water.

Fig. 7. The influence of multispecies bacterial film (*P. aeruginosa*, *B. pumilus* and *C. freundii*) on the cyprid metamorphosis of *B. amphitrite*. AE - Adult Extract; FSW - Filtered Sea Water.

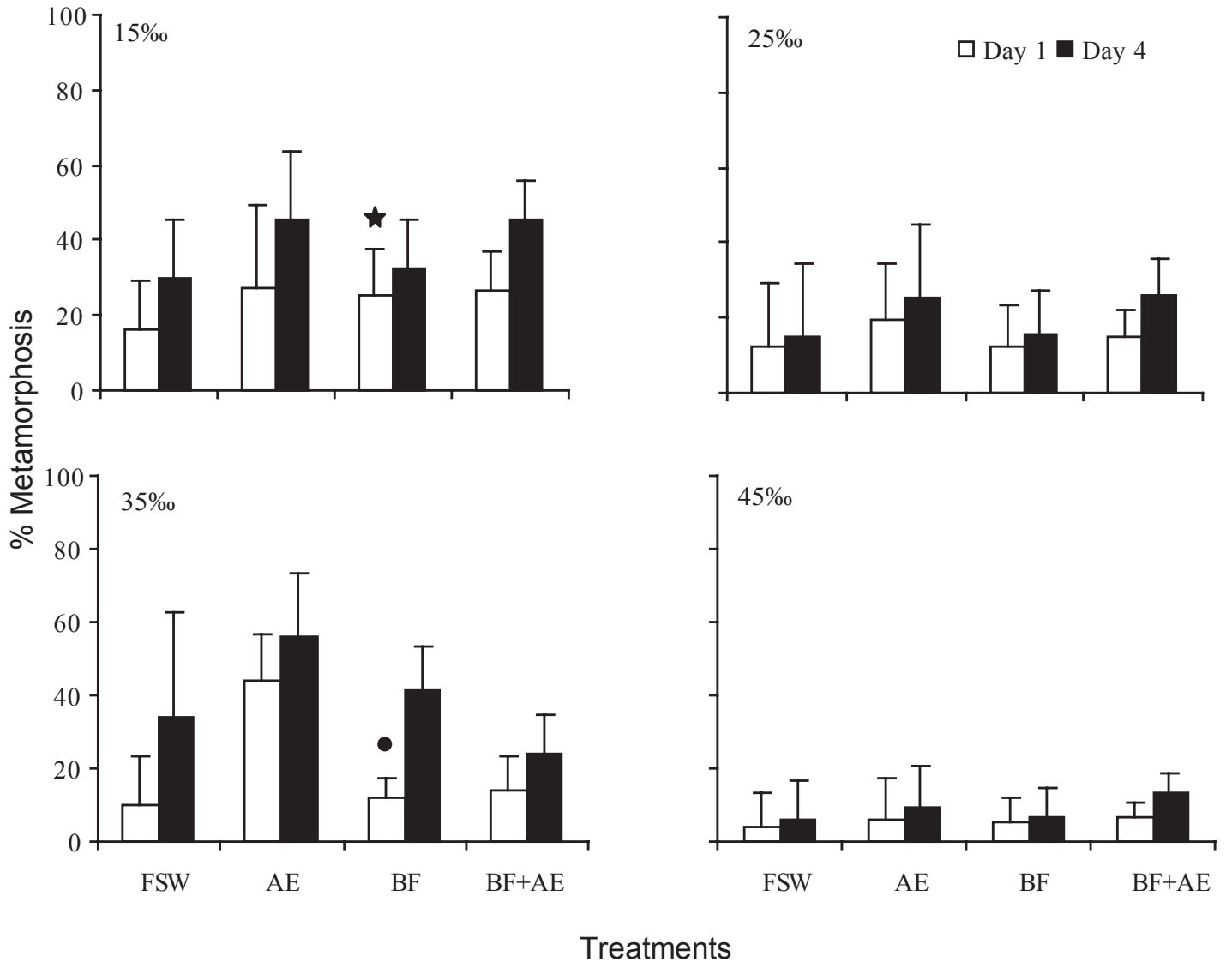


Fig. 1

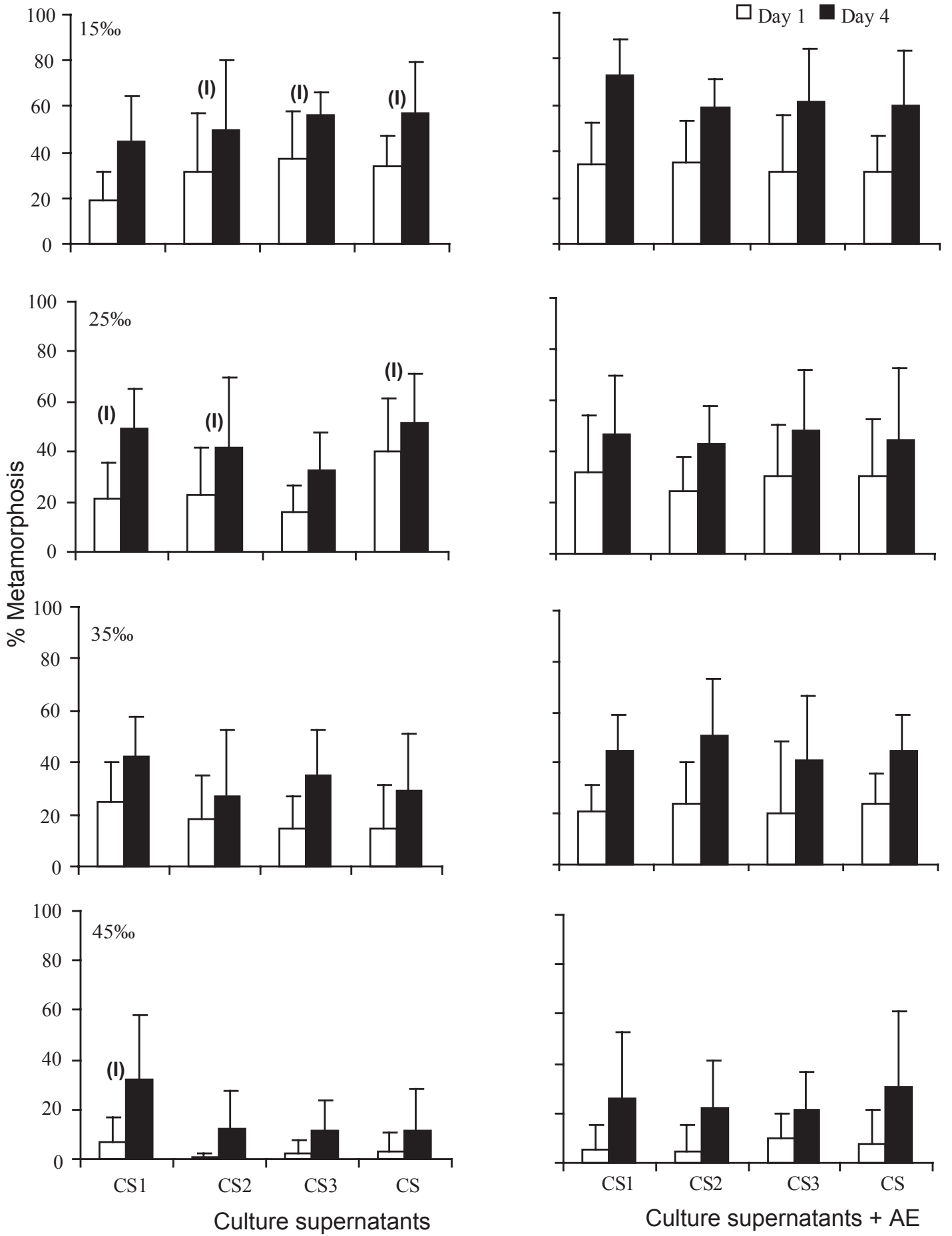


Fig. 2

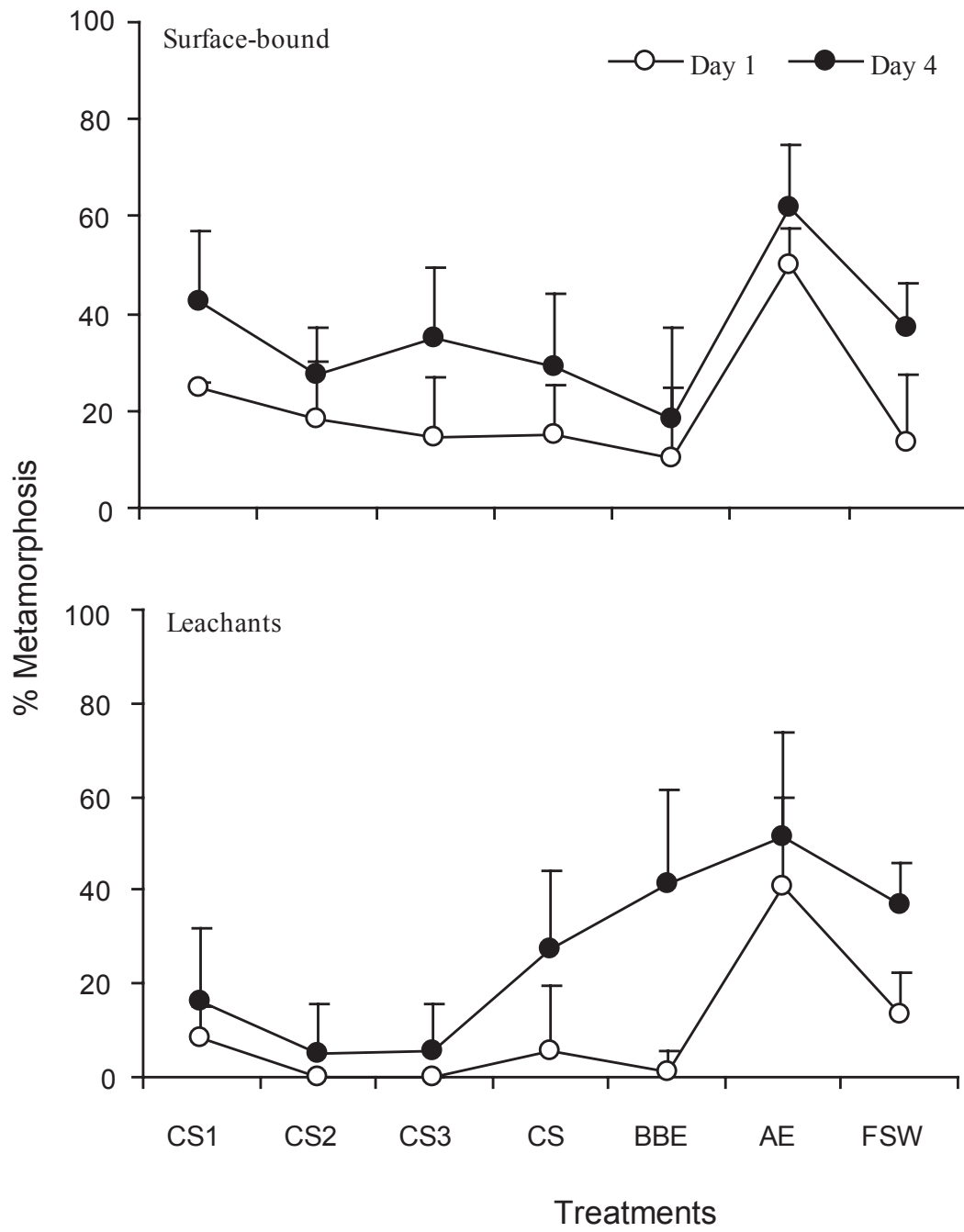


Fig. 3

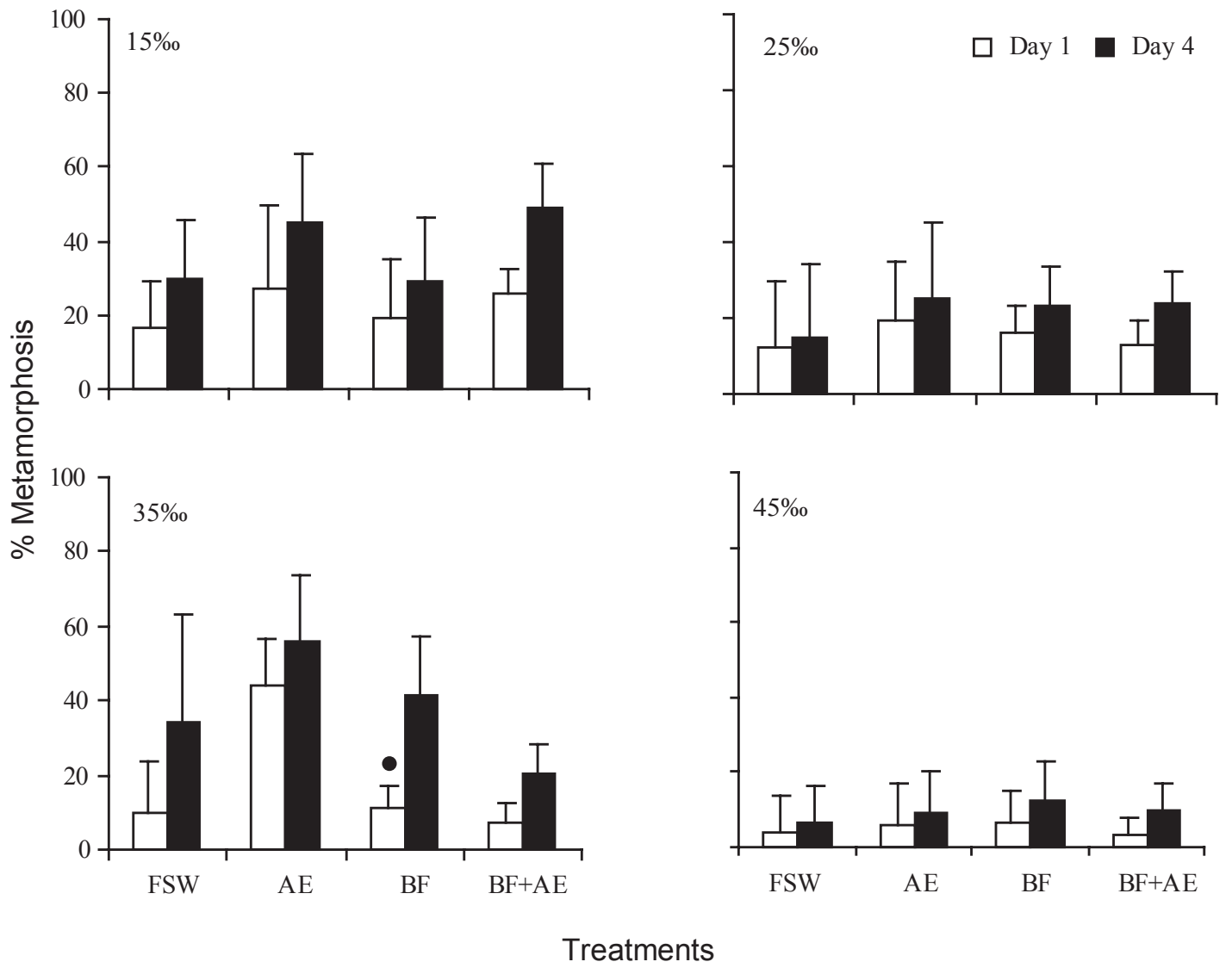


Fig. 4

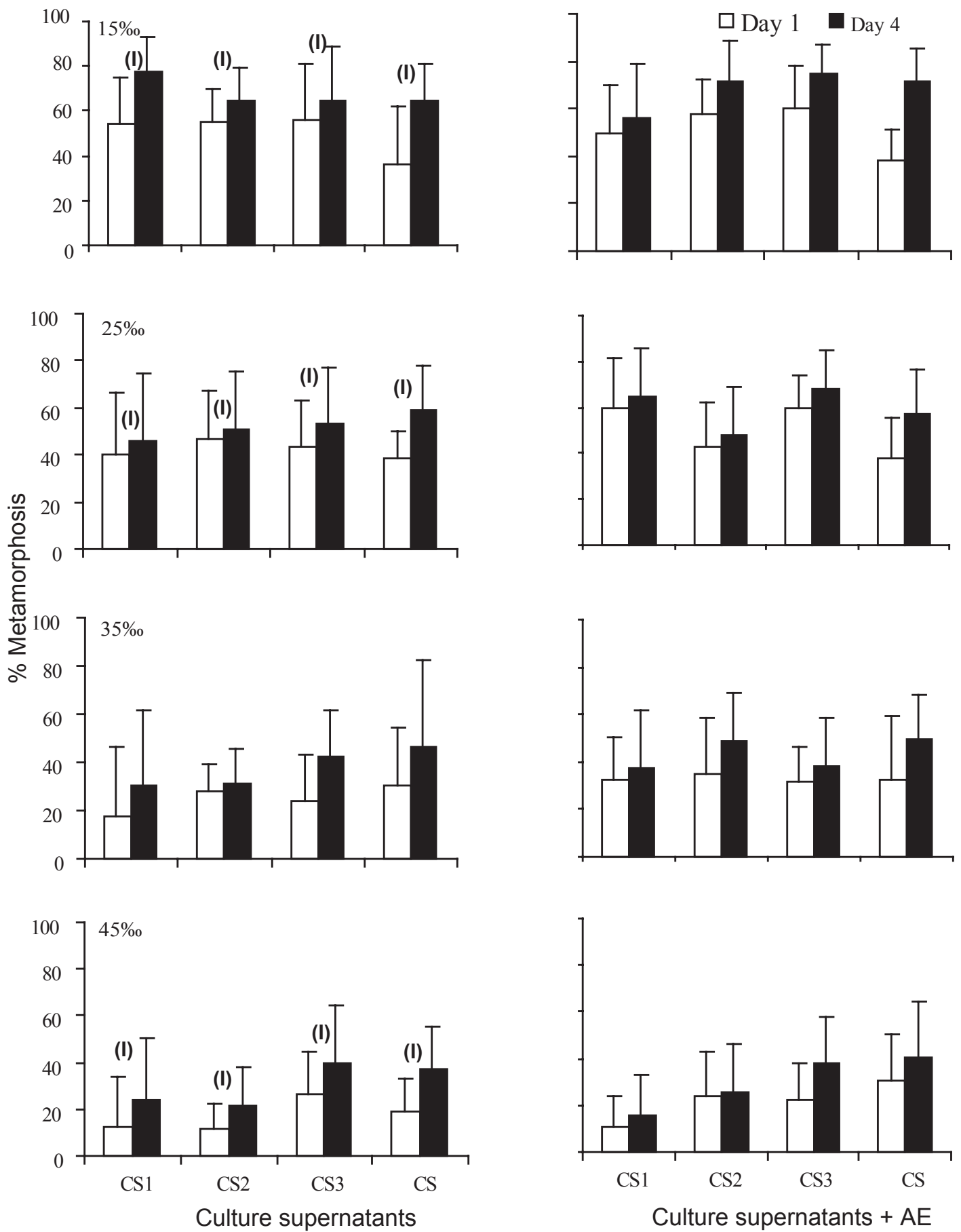


Fig. 5

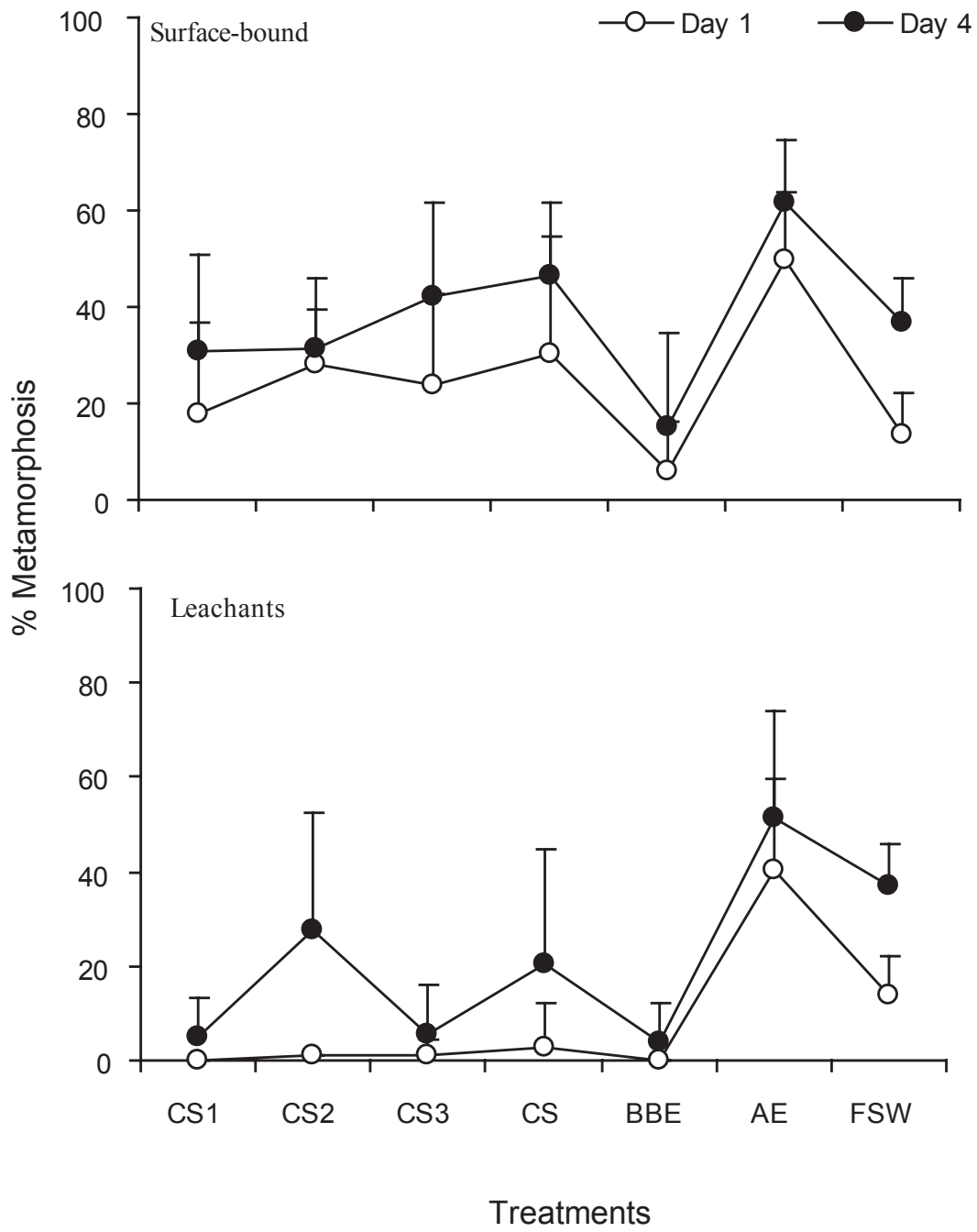


Fig. 6

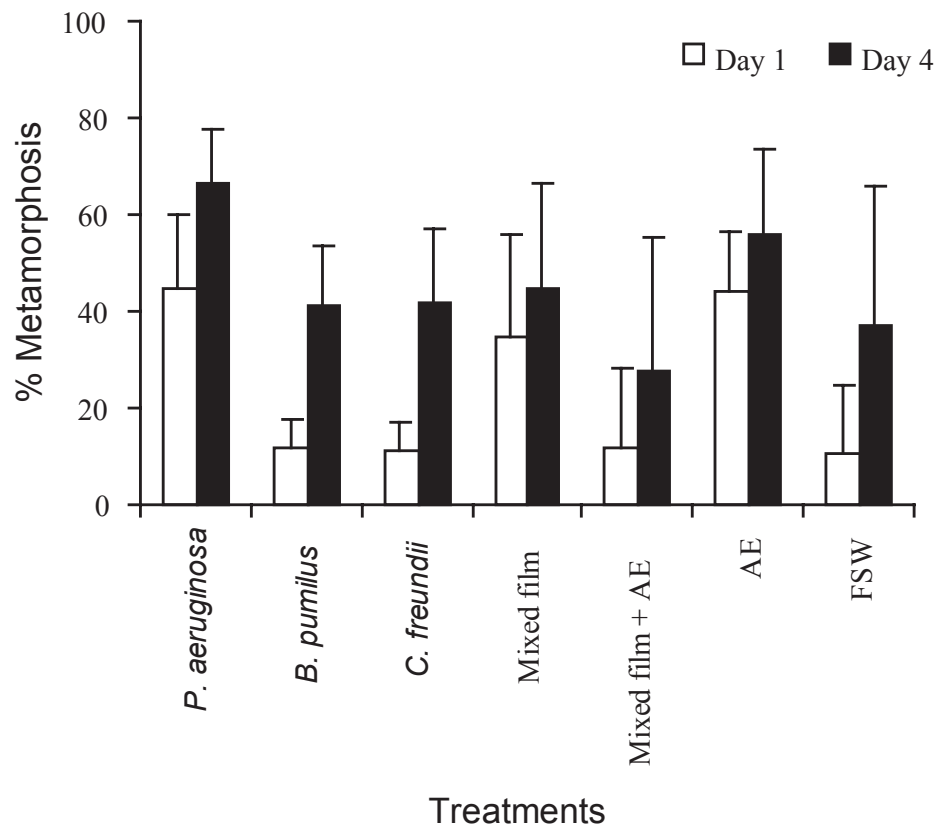


Fig. 7