

## *Chapter 2*

# *Significance of sugars in exploration and metamorphosis*

## 2.1 Introduction

Most marine invertebrate larvae select certain environments by metamorphosing in response to cues associated with them (Pechenik 1990; Pawlik 1992). The identification of the exact nature of these cues remains an active field of study. 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 a few hours. Instars II to VI are phytoplanktotrophic. The cyprid, which is the settlement stage larva of the barnacle *B. amphitrite*, has been used to study the cues influencing settlement and metamorphosis (Rittschof et al. 1992; Crisp 1990; Maki et al. 1994; Holm 1990; Pechenik et al. 1993; Clare et al. 1992; Wieczorek et al. 1995; Yamamoto et al. 1995).

Many barnacle species show a gregarious response towards adult and juvenile conspecifics. Arthropodin or settlement factor, a glycoprotein present in the adults is thought to be responsible for this behavior (Knight-Jones 1953; Knight-Jones and Crisp 1953; Crisp and Meadows 1963). Clare et al. (1995) reported the involvement of cyclic AMP in the pheromonal modulation of barnacle settlement.

The barnacle cyprid is discriminating in its choice of settlement site and in order to exercise its power of discrimination it has to explore the surface. Cyprids leave behind 'footprints' of temporary adhesive (Walker and Yule 1984) while exploring some surfaces, which are believed to be secreted by

antennular glands that open out onto the antennular attachment disc (Nott and Foster 1969). This temporary adhesive serves to hold the cyprid onto the substratum while it searches for a suitable place to settle. The cyprid footprints have also been reported to induce settlement of other cyprids, even in the absence of conspecific adults (Walker and Yule 1984; Yule and Walker 1985; Clare et al. 1994).

Chemical cues such as bacterial exopolymers have also been shown to be involved, the composition of which can influence subsequent settlement by invertebrate larvae (Maki et al. 1988,2000). Three neutral sugars, D-mannose, D-glucose and D-galactose, form the most common constituents of bacterial exopolysaccharides from both marine and freshwater environments (Sutherland 1980). The interactions between bacterial exopolymers and cypris temporary adhesive are most likely to be effected via polar groups. The strength of these interactions will determine how well a cyprid adheres to a filmed surface, which in turn will provide a further cue in determining the settlement potential of that surface (Yule and Crisp 1983; Neal and Yule 1992).

Neal and Yule (1996) while studying the effects of dissolved sugars upon the temporary adhesion of cypris larvae of five barnacle species from four families reported D-glucose to show a common, concentration-dependent, inhibitory effect for the five species; maximum inhibition occurred at  $10^{-8}$ M glucose. D-mannose and D-galactose showed similar activity to D-glucose. Recently, *Lens culinaris* agglutinin (LCA)-binding sugar chains of the adult extract (AE)

have been implicated in the settlement of *B. amphitrite* (Matsumura et al. 1998a). LCA binds to glucose and mannose. The settlement-inducing activity is associated with 3 major subunits with estimated molecular masses of 76 (often present as a possible dimer), 88 and 98 kDa. Moreover, three LCA-binding subunits of settlement-inducing protein complex (SIPC) were isolated by SDS-PAGE and found that each individual subunit also induced larval settlement, suggesting an important role of specific sugar chain of SIPC in the settlement of *B. amphitrite* (Matsumura et al. 1998b). Immunostaining studies indicated that the SIPC was present in the footprints of the cyprids (Matsumura et al. 1998c).

Taking into consideration the above perspectives, experiments were carried out in order to assess how cypris larvae would explore and metamorphose when treated with LCA specific sugars (i.e. D-glucose and D-mannose). The influence of D-galactose was also assessed similarly. Evaluation of sugar-treated cyprids was carried out with AE-coated and non-coated multiwells containing filtered seawater (FSW). This was carried out in order to observe how a cyprid would behave when the polar groups associated with CTA are blocked by cues such as sugars and under such conditions how AE influences the search behavior and metamorphosis response. The settlement assays were conducted using single as well as multiple cyprids.

In this investigation, experiments were carried out to study the exploratory behavior and subsequent metamorphosis response of cyprids when subjected simultaneously to sugars and AE.

## 2.2 Materials and methods

### 2.2.1 Preparation of adult extract (AE)

The adult extract (AE), which stimulates the settlement of barnacle cyprids, was prepared by following the method of 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. These animals were then washed and ~100-g wet wt. of whole adults were crushed with a mortar and pestle using 100 ml of deionised water (RO pure). The supernatant of the crushed mixture was decanted, centrifuged (12,000 x g for 5 min) and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged (12,000 x g for 5 min) and then frozen at -20° C until further use. The protein content of the extract was estimated following the method described by Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard. A protein concentration of 50 µg ml<sup>-1</sup> of AE was used for all assays.

### 2.2.2 Rearing of *B. amphitrite* larvae

*B. amphitrite* nauplii were mass reared in 2-liter glass beakers, using filtered seawater of 35‰ salinity, on a diet of *Chaetoceros calcitrans*, a unicellular diatom, at a cell concentration of 2 x 10<sup>5</sup> cells ml<sup>-1</sup>. The feed 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. Rittschof et al. (1984) have described these methods in detail.

### 2.2.3 Treatment with sugars

The cyprids were immersed in seawater containing different concentrations ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-5}$  or  $10^{-3}$ M) of D-glucose, D-galactose or D-mannose for 5 min. In the case of single cyprid assays, cyprids were immersed individually into the sugar solutions and after removal were transferred each to a separate multiwell for the assessment of footprint deposition and metamorphosis. On the other hand, in case of multiple cyprid assays, approximately 25-30 cyprids were used and treated similarly for the assessment of metamorphosis. This method has been described by Neal and Yule (1996). All the sugar solutions were made up in millipore-filtered ( $0.22 \mu\text{m}$ ), UV- irradiated seawater.

### 2.2.4 Visualization of footprints

The sugar-treated, as well as non-treated cyprids, were siphoned out and introduced individually into six-well plates (Corning- 430343) coated with  $50 \mu\text{g ml}^{-1}$  AE and to non-coated wells, each containing 5 ml of millipore-filtered, autoclaved seawater at 35‰. The experiments were carried out with single cyprids in order to prevent larva-larva interactions. The experiments were repeated three times using three different batches of larvae with six replicates at each trial ( $n=6$ , with batch as an additional factor). The cyprids were allowed to explore the wells for 2 hours at  $20^{\circ} \text{C}$  (there was no settlement during this time), after which the wells were emptied and stained for footprints with a protein dye reagent (Bradford 1976) as described by Walker and Yule (1984).

### 2.2.5 Assay protocol for evaluation of metamorphosis

The schematic representation of the experimental set-up is shown in Figure 2.1.

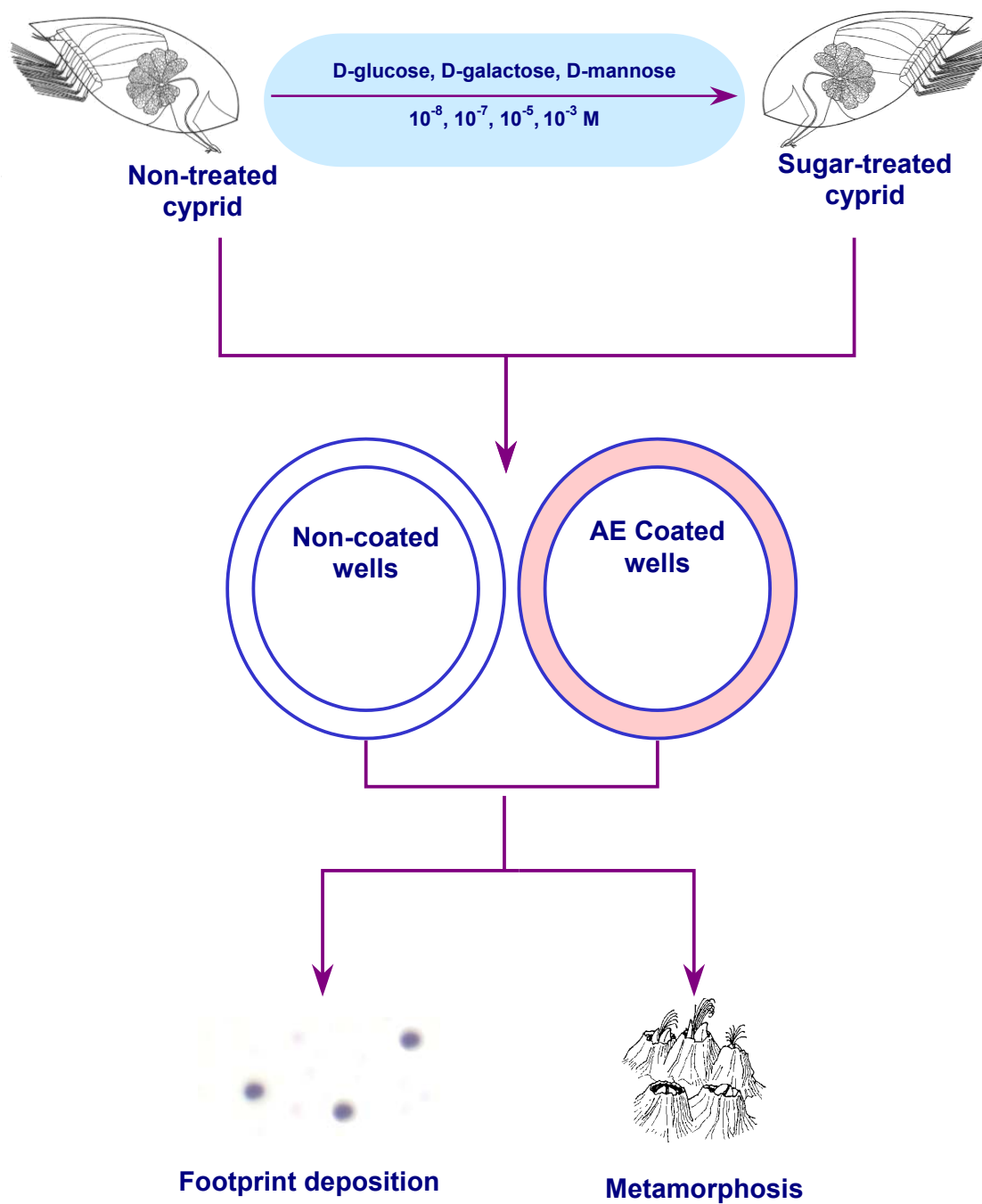


Fig. 2.1 Schematic representation of the experimental set-up

About 25-30 sugar-treated or non-treated cyprids were introduced into wells of 24-well plates coated with AE and to non-coated multiwells (Corning-430262) along with 2 ml of autoclaved, filtered seawater at 35‰ (Maki et al. 1990). The above treatments were repeated employing a single cyprid (single cyprid assay). The AE-coated surfaces were prepared by inoculating the multiwells with AE at a protein concentration of 50  $\mu\text{g ml}^{-1}$ . After 3 hours, the multiwells were washed three times with autoclaved filtered seawater after which the cyprids were introduced.

The assays were repeated for four times using four different batches of larvae with four replicates at each trial (n=4, with batch as an additional factor) and were maintained at  $26 \pm 1^\circ \text{C}$  on a 12 h Light: 12 h Dark photoperiod. They were monitored every 24 hours for a period of 4 days and metamorphosed cyprids were counted at the end of each day.

### 2.2.6 Statistical analysis

The influence of different concentrations of D-glucose, D-galactose and D-mannose and larval batch on footprint deposition by the cyprids was evaluated by three-way ANOVA (Sokal and Rohlf 1981). Two-way ANOVA was performed to evaluate the differences in metamorphosis with respect to cyprids treated with different concentrations of three sugars and the non-treated cyprids in presence or absence of AE. Three-way ANOVA was also carried out to evaluate the differences in metamorphosis with respect to sugar type, concentration and age of the cyprids exposed to AE-coated or non-coated surfaces. The data on metamorphosis (%) was arcsine transformed to ensure normality and homogeneity of variances before subjecting to statistical



analysis, whereas the raw data on footprint deposition was square-root transformed. The influence of sugar-treated and non-treated cyprids in the presence of AE on the deposition of footprints was evaluated by one-way ANOVA. A post-ANOVA Scheffe's test was done to test the difference between the treatments on footprint deposition (Sokal and Rohlf 1981). The methods such as preparation of adult extract and rearing of *B. amphitrite* larvae have been repeated in the subsequent chapters for the readers convenience.

## 2.3 Results

The footprints were densely stained and roughly oval in shape making them easily distinguishable from adsorbed glycoprotein of the adult extract; the footprints measured about 30-37 $\mu$ m across. The number of footprints deposited by sugar-treated cyprids in presence of AE in the assay wells at different concentrations of D-glucose, D-galactose and D-mannose is shown in (Fig. 2.2).

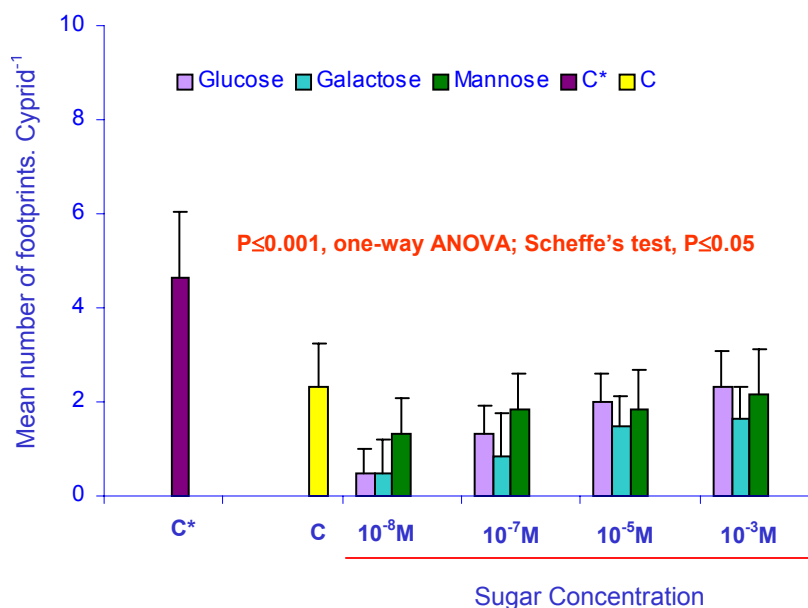


Fig. 2.2 Number of footprints deposited by sugar-treated cyprids exposed to AE-coated surface. C\*- non-treated cyprids exposed to AE-coated surface, C-non-treated cyprids exposed to non-coated surface

The number of footprints deposited by a cyprid increased with increasing concentration of sugars. In the absence of AE, footprints were not deposited by the cyprids treated with sugars. One-way ANOVA indicated that the number of footprints deposited by non-treated cyprids in the presence of AE were significantly different from the sugar-treated cyprids ( $p \leq 0.001$ , One-way ANOVA;  $p \leq 0.05$ , Scheffe's test). Three-way ANOVA performed between all three sugars, larval batch, and different sugar concentrations revealed a significant difference in the footprint deposition with respect to sugar type at different concentrations (Table 2.1).

Table 2.1 Three-way ANOVA. The influence of D-glucose, D-galactose or D-mannose (sugar type), larval batch and different concentrations of all the three sugars on the deposition of footprints by the sugar-treated cyprids exposed to AE-coated surfaces. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor	df	SS	MS	Fs
A (sugar type)	2	0.83	0.41	
B (larval batch)	2	0.08	0.04	
C (Conc.)	3	2.8	0.93	
A*B	4	0.04	0.01	1.75ns
A*C	6	0.63	0.10	18*****
B*C	6	0.033	0.005	0.94ns
A*B*C*	12	0.070	0.006	
Total	35	4.48		

(\*\*\*\*\* $p \leq 0.001$ , ns- not significant)

The metamorphosis response of the cyprids after 24 hours is shown in (Fig. 2.3a,c). The cyprids treated with D-mannose resulted in maximum metamorphosis at a concentration of  $10^{-8}$ M when exposed to wells devoid of AE and the metamorphosis rate was almost twice that observed with the non-treated cyprids (Fig. 2.3a).

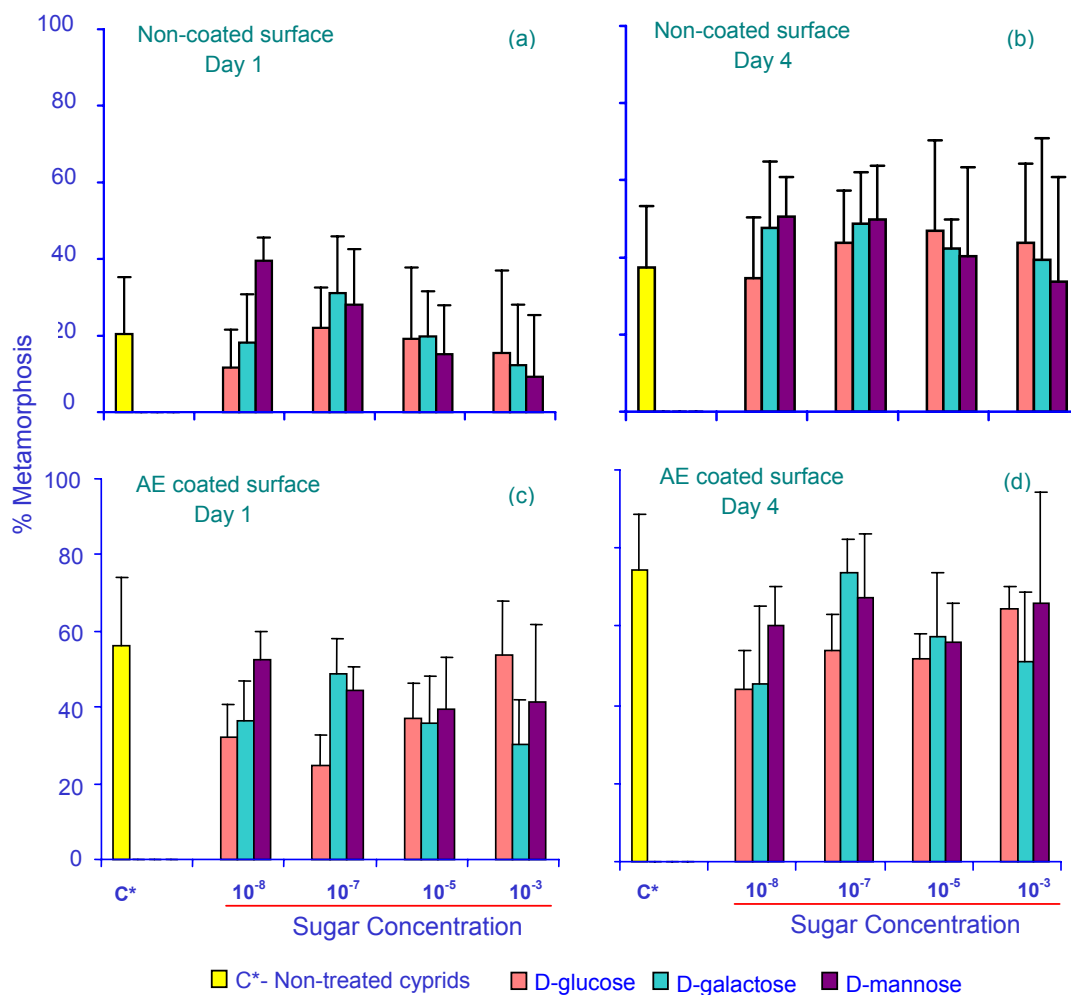


Fig. 2.3 Percentage metamorphosis of cyprids (Multiple cyprid assay). (a) and (b)- sugar-treated and non-treated cyprids (control) exposed to non-coated surface, (c) and (d)- sugar-treated and non-treated cyprids exposed to AE-coated surface. Vertical lines indicate the standard deviation from mean

Such a response was not given by cyprids treated with D-glucose or D-galactose. At 10<sup>-8</sup>M, D-glucose showed a reduced effect. Two-way ANOVA also indicated significant differences in the metamorphosis rates at 10<sup>-8</sup>M concentration with treatments (sugar-treated and non-treated) and with sugars in the presence and absence of AE ( $p \leq 0.025$  and  $p \leq 0.001$ ).

D-mannose treated cyprids metamorphosed in higher percentages in the presence of AE but less than the non-treated cyprids (Fig. 2.3c). The results on day 4, which reflect the response of ageing of cyprids, showed a substantial increase in the metamorphosis rates (Fig. 2.3b,d). Three-way ANOVA revealed that the metamorphosis differed significantly with respect to sugar type and cyprid age and with sugar type and concentration when exposed to AE-coated or non-coated surfaces (Table 2.2).

Table 2.2 Three-way ANOVA. The influence of D-glucose, D-galactose or D-mannose (sugar type), cyprid age and different concentrations of all the three sugars on the metamorphosis of cyprids exposed to AE-coated or non-coated surfaces in a multiple cyprid assay. (df. degree of freedom; SS. sum of the squares; MS. Mean of squares; Fs. Fischer constant).

Factor	AE-coated surfaces				Non-coated surfaces		
	df	SS	MS	Fs	SS	MS	Fs
A (Sugar type)	2	5.2	2.6		1.2	0.6	
B (Cyprid age)	1	2.2	2.2		4.2	4.2	
C (Conc.)	3	0.4	0.13		1	0.3	
A*B	2	0.9	0.5	4.85*	0.9	0.4	6.03**
A*C	6	2.8	0.5	4.89*	2.7	0.4	6.07**
B*C	3	0.8	0.3	2.95 ns	0.2	0.08	1.1 ns
A*B*C*	6	0.6	0.09		0.4	0.07	
Total	23	12.9			10.6		

(\*\*p≤0.05, \* p≤0.1 ns- not significant)

When all the treatments were assessed using single cyprids (Fig. 2.4), a trend similar to that observed with the assays employing multiple cyprids (Figs. 2.3 and 2.4) was obtained. The metamorphosis rates were higher in the presence of AE and D-mannose facilitated maximum metamorphosis at  $10^{-8}$ M when exposed to wells without AE.

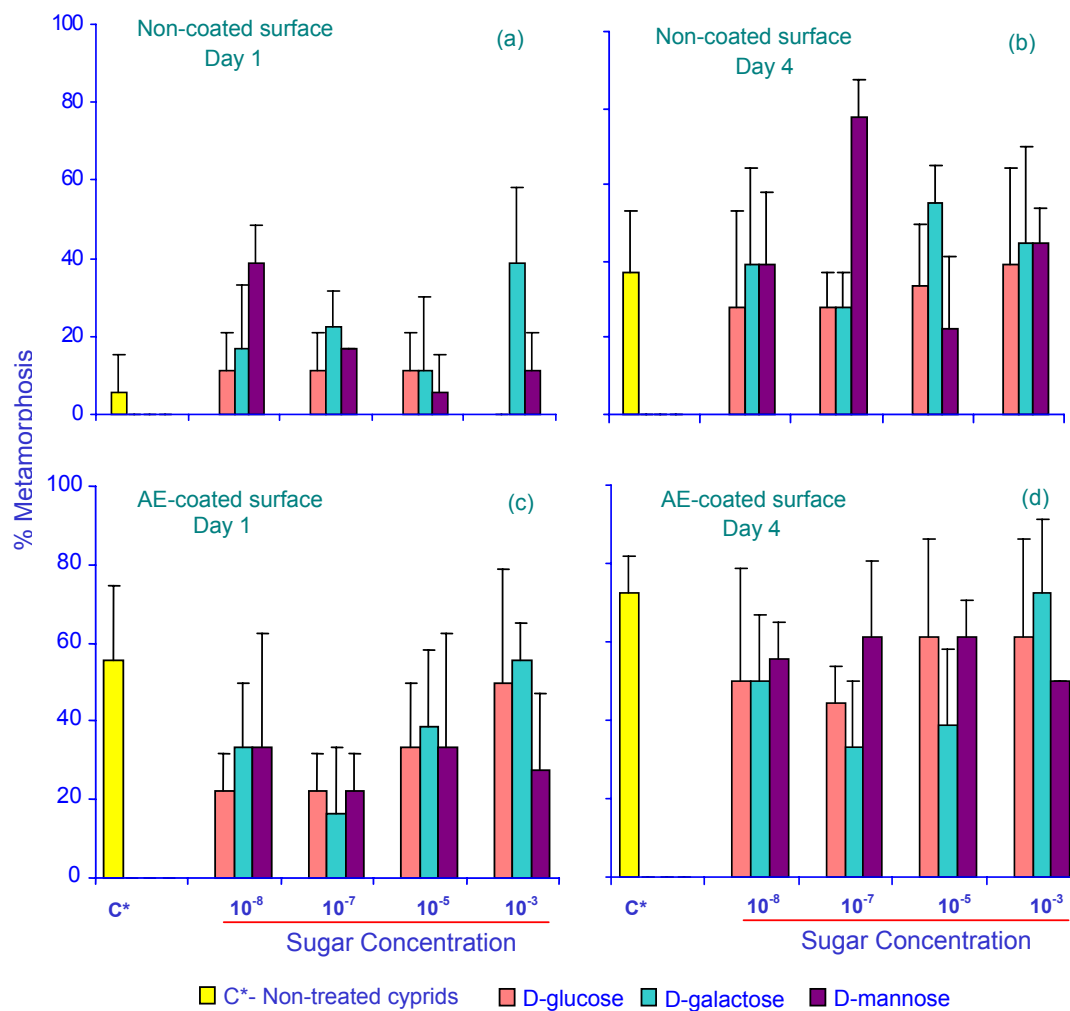


Fig. 2.4 Percentage metamorphosis of cyprids (single cyprid assay). (a) and (b)-sugar-treated and non-treated cyprids (control) exposed to non-coated surface, (c) and (d)- sugar-treated and non-treated cyprids exposed to AE-coated surface. Vertical lines indicate the standard deviation from mean

## 2.4 Discussion

It has been hypothesized that sugars in solutions adsorb electrostatically through  $-OH$  groups to polar groups associated with the CTA (Yule and Walker 1987). Higher sugar concentrations block more polar groups, thus nullifying their contribution to adhesion, resulting in lower adhesion thresholds

below those for cohesive failure (Neal and Yule 1996). Many of the extracellular signalling molecules act at very low concentrations (typically  $\leq 10^{-8}$  M) and the receptors that recognize them usually bind them with high affinity (affinity constant  $K_a \geq 10^8 \text{ l mol}^{-1}$ ) (Bruce et al. 1994).

The results of the present investigation showed that D-mannose triggered metamorphosis significantly at a concentration of  $10^{-8}$  M and the metamorphosis rate was almost twice that observed with the non-treated cyprids when assessed in the absence of AE. However, the other LCA specific sugar (D-glucose) showed a reduced effect, which suggests the involvement of D-mannose moieties of AE in the promotion of *B. amphitrite* settlement. Earlier investigations have reported stimulation, inhibition or no effect of bacterial films on the attachment of barnacle cyprids (Visscher 1928; Harris 1946; Crisp and Meadows 1962; Tighe-Ford et al. 1970). Previous studies on the effect of bacterial films on cypris larvae (Maki et al. 1988, 1990; Holmström et al. 1992; Avelin Mary et al. 1993; Neal and Yule 1994a,b) have generally found such films to reduce either settlement or adhesion. Neal and Yule (1996) consider that 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 and Walker 1984). Thus the results of the present investigation suggest that exopolysaccharides, rich in D-mannose, would be most effective in triggering metamorphosis. However, larvae are likely to respond to more than one sensory stimulus when searching for a settlement location, and some factors, such as naturally produced bacterial metabolites, may override the importance of others (Maki et al. 1989).

In the absence of AE, sugar-treated cyprids did not deposit footprints suggesting that the response of cyprids towards sugars was quick thus resulting in either metamorphosis promotion or reduction without further exploration. During search behavior the most obvious point of contact between the cyprid and the substratum is the attachment disc of the third antennular segment (Nott 1969; Nott and Foster 1969). However, settlement factor could also be detected in solution, which directs attention to the fourth antennular segment with its array of sensory setae (Gibson and Nott 1971; Clare and Nott 1994). Flicking of the fourth antennular segment with its associated setae is evident while a cyprid explores a substratum (*Balanus balanoides*, Gibson and Nott 1971; *B. amphitrite*, Clare and Nott 1994) and suggests an analogy to the flicking action of decapod antennules (Schmidt and Ache 1979). Secondly, recent evidence has been obtained in support of the role of cAMP in cyprid settlement (Clare et al. 1995). A laser ablation technique to evaluate the role of sensory setae of cyprid antennules was also advocated for identifying the sites of pheromone reception (Clare et al. 1994). Clare and Matsumura (2000) suggested that barnacle settlement induction involves receptor-ligand interactions and a signal transduction pathway(s) that translates into permanent attachment and metamorphosis. The detection of AE even after blockage of polar groups of CTA on the third antennular segment with its attachment disc, suggests the availability of alternate sites for pheromone reception. It is possible that the settlement proteins of AE are detected by the receptors on the fourth antennular segment via olfaction. The absence of AE rendered these sites non-functional, thus the cyprids responded to sugars in either the promotion or reduction of metamorphosis

without further search. This aspect needs attention and will be helpful in identifying the role of alternate pheromone reception sites.

Clare et al. (1994) recommended that whenever feasible, barnacle settlement assays should employ a single larva. However, a comparison of the results of single cyprid and multiple cyprid assays showed a similar trend, thus indicating that larva-larva interaction may not play an important role, when such pretreated larvae are subjected to assays.

Although D-mannose proved to be an effective cue in eliciting metamorphosis, all the cyprids did not show a similar response. The cyprids that metamorphosed successfully may be the ones that were physiologically fit. The non-feeding cypris larvae have to depend upon the energy reserves incorporated during planktotrophic naupliar development. The nutritional as well as environmental conditions seem to jointly determine the energy status of the larvae (Anil and Kurian 1996; Anil et al. 2001). Older larvae had increased rates of metamorphosis. Earlier research has indicated that larval age is known to affect settlement. In the laboratory, the older cyprids responded more readily to external cues than the recently formed ones due to the decrease in the response threshold with larval age (Rittschof et al. 1984). A possible explanation for this fact could be that young cyprids which are more discriminating during settlement than older cyprids become less discriminating with age (Rittschof et al. 1984; Crisp 1988) presumably due to the decline in their energy reserves and physiological quality.



## *Chapter 3*

# *Evaluation of different inducers from microorganisms that influence the metamorphosis*

### 3.1 Introduction

Cypris larvae test various areas before finally attaching to any substratum. A series of factors such as surface type, water flow, light, temperature, larval age, competitors and the chances of success in reproduction (Crisp 1974) are important in the choice of a settlement site. In addition, the most important essential factors or determinants are the specific chemical cues or triggers associated with the settling substratum (Kirchman et al. 1982a; Morse 1984 a,b; Maki and Mitchell 1985; Szewzyk et al. 1991; Qian et al. 2000). Competent larvae metamorphose only after encountering certain environmental cues associated with habitat appropriate for the juvenile (Pechenik 1990; Pawlik 1992). Surface chemistry is also very important to larval settlement and plays role in the distribution of adults (Strathmann et al. 1981; Roberts et al. 1991; Holm et al. 1997). Several studies have shown that many marine invertebrate larvae settle and metamorphose in response to extracellularly produced components and other environmental stimuli, hence the behavioral and morphogenetic responses may be triggered by different inducers (Rodriguez et al. 1993). The settlement and metamorphosis are shown to be controlled by larval sensory recognition, which transduce the external signals into the signals within the organism (Pawlik 1992).

*B. amphitrite* cyprids like *Balanus balanoides* (Walker and Yule 1984) have been shown to deposit footprints of temporary adhesive while exploring a substratum that stimulate the settlement of other cyprids, even in the absence of conspecific adults (Clare et al. 1994; Yule and Walker 1985).

Besides adult conspecifics, bacterial films coating the benthic substrates have been suggested as sources of water borne cues mediating settlement of oyster larvae (Bonar et al. 1986; Fitt et al. 1989; Tamburii et al. 1992). Barnacle cyprids like most other larvae prefer to settle on the substrata that possess a well-developed biofilm (Crisp 1984; Clare et al. 1992). The tenacity of temporary adhesion of cyprids to unfilmed substrata or bacterial films does not always correlate with their final fixation (Maki et al. 1994). The studies related to interactions between cypris larvae and bacterial films have generally found most bacterial species to inhibit attachment of *B. amphitrite* cyprids to polystyrene surfaces, although several bacterial species showed no effect (Maki et al. 1988,1990,1992; Avelin Mary et al. 1993; Neal and Yule 1994a,b). The influence of bacterial films and the culture supernatant containing extracellular materials on the settlement of *Balanus improvisus* Darwin cyprids have been shown to differ when examined in field from that observed in the laboratory (O'Connor and Richardson 1996). Recently thraustochytrid protists, which are found in marine microbial films have been shown to induce the settlement of *B. amphitrite* (Raghukumar et al. 2000).

The effect generated by bacterial strains, whether stimulatory or inhibitory for larval settlement, amplifies with the age of the film. (Maki et al. 1989; Holmström et al. 1992). The presence of a bacterial biofilm has been interpreted as a general signal that a surface is neither temporary nor toxic and larvae may use more specific chemical signatures from biofilms or characteristic microbial assemblages to indicate preferred ecological conditions at a site (Unabia and Hadfield 1999). The bacteria influence the settlement by changing the nature of the substratum either by altering the

surface wettability or by exposing different surface molecular domains for example, in the form of exopolymers (Anil et al. 1997). Bacteria can also produce surface-bound and soluble chemical cues that either stimulate or inhibit larval settlement (Kirchman et al. 1982a; Maki et al. 1990; Szewzyk et al. 1991; Maki et al. 1992).

A wide variety of bacterial supernatants also appeared to influence the search behavior of the oyster, *Crassostrea gigas* larvae via ammonia gas and other weak amine bases (Bonar et al. 1990). For a chemical cue to be effective against larvae it must be either present on the surface of the substratum or released into the surrounding water (waterborne cues), both of which have been documented in the literature (Crisp and Meadows 1962,1963; Morse et al. 1980; Jensen and Morse 1990; Hadfield and Scheuer 1985; Tamburri et al. 1992).

Adult conspecifics, the biofilms on their shell surfaces, or the interaction of both have been debated for their source of origin of various settlement inducing compounds which cause gregarious settlement in barnacles. Anil and Khandeparker (1998) and Anil et al. (1997) reported that in *B. amphitrite* cyprids the degree of inducement of metamorphosis varied with various combinations of exopolymers of different bacterial strains with or without AE.

Four different experiments (Fig. 3.1) were carried out to study the influence of settlement inducing compounds from *Pseudomonas aeruginosa*, *Bacillus pumilus* and *Citrobacter freundii*, bacteria isolated from the shell surface of *B. amphitrite*, on the cyprid metamorphosis of *B. amphitrite*. In Expt. 1 the influence of bacterial film was assessed at different environmental conditions

(salinity and temperature). The culture supernatant, its different molecular-weight fractions and bacterial extract were subjected to cyprid metamorphosis assays. The influence of bacterial film and its products were also assessed along with conspecific adult extract (AE). In Expt. 2 the effectiveness of leachants and surface-bound compounds was assessed. In Expt. 3 the effect of culture supernatants produced by the bacteria grown in different nutrient media was evaluated along with the AE. Whereas in Expt. 4 the effectiveness of bacterial exopolysaccharides extracted using different nutrient mediums was investigated.

Recent years have seen several publications on the prevalence of a group of osmoheterotrophic, fungoid protists, the thraustochytrids in the sea. The presence, and often dense populations of these single-celled microorganisms have been reported from numerous habitats, including living algae, marine detritus, phytoplankton aggregates, water column, invertebrates and numerous other habitats (Moss 1986; Raghukumar 1990,1996; Frank et al. 1994; Naganuma et al. 1998). The influence of thraustochytrid protist (MS2D) identified as a component of marine microbial films was also evaluated on the metamorphosis of *B. amphitrite* cyprids.

## **3.2 Materials and methods**

### **3.2.1 Preparation of the adult extract (AE)**

Adult extract was prepared by following the method described earlier 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 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 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<sup>-1</sup> of AE was used for all assays.

### **3.2.2 Rearing of *B. amphitrite* larvae**

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 phytoplanktotrophic. Nauplii were mass reared in 2-liter glass beakers using filtered seawater of 35‰ on a diet of *C. calcitrans*, a unicellular diatom, at a cell concentration of 2 x 10<sup>5</sup> cells ml<sup>-1</sup>. 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 by Rittschof et al. (1984).

### **3.2.3 Isolation of bacteria from shell surfaces 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. The

bacterial colonies thus isolated were maintained on Zobell Marine Agar 2216 slants. The purity of the culture was checked by streaking on Zobell Marine Agar 2216. The isolated bacteria were identified following Bergey's manual of systematic bacteriology (Krieg 1984) (Table 3.1a,b). Out of three bacteria, two were gram-negative and one was gram-positive. The gram-positive bacterium was also screened for phylogenetic analysis in Germany by Dr. Hentschel (Table 3.2). 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 of Hentschel et al. (2001).

Table 3.1a The results of the tests employed to identify gram-negative bacteria

Tests	Results	Tests	Results
Color	Cream	Color	Cream
Shape	Short rods	Shape	Short rods
Gram stain	-	Gram stain	-
Motility	+	Motility	+
Hugh Leifson's test	Aerobic oxidative	Hugh Leifson's test	Facultative fermentative
Growth at pH 3.6	-	Indole	-
Growth at 4° C	-	Methyl red	+
Growth at 41° C	+	Simmons citrate	+
Indole	-	H <sub>2</sub> S (KIA / TSI)	+
Methyl red	-	Urease	-
Simmons citrate	+	Phenylalanine deaminase	-
H <sub>2</sub> S (KIA / TSI)	-	Nitrate reduction	+
Urease	-	Oxidase	-
Phenylalanine deaminase	-	Catalase	+
Nitrate reduction	+	Denitrification	-
Oxidase	+	Gelatin liquefaction	-
Catalase	+	O-F glucose	Fermentative
Denitrification	+	Arginine dihydrolase	-
Gelatin liquefaction	+	Utilization of:	
Starch hydrolysis	-	Glucose	+
O-F glucose	Oxidative	D-Xylose	+
Arginine dihydrolase	+	Mannitol	+
Alkaline phosphatase heat resistance	+	D-Mannose	+
Litmus milk (peptonization)	+	L-Arabinose	+
Utilization of:		Lactose	-
Glucose	+	D-Fructose	+
D-Xylose	-	m-Inositol	-
D-Ribose	+	Sucrose	-
Mannitol	+	D-Galactose	-
Cellobiose	-	Acetamide	+
D-Mannose	-		
L-Arabinose	-		
Lactose	-		
Maltose	-		
D-Fructose	+		
m-Inositol	-		
Sucrose	-		
D-Galactose	-		
Acetamide	+		
Bacteria	<i>Pseudomonas aeruginosa</i>		<i>Citrobacter freundii</i>

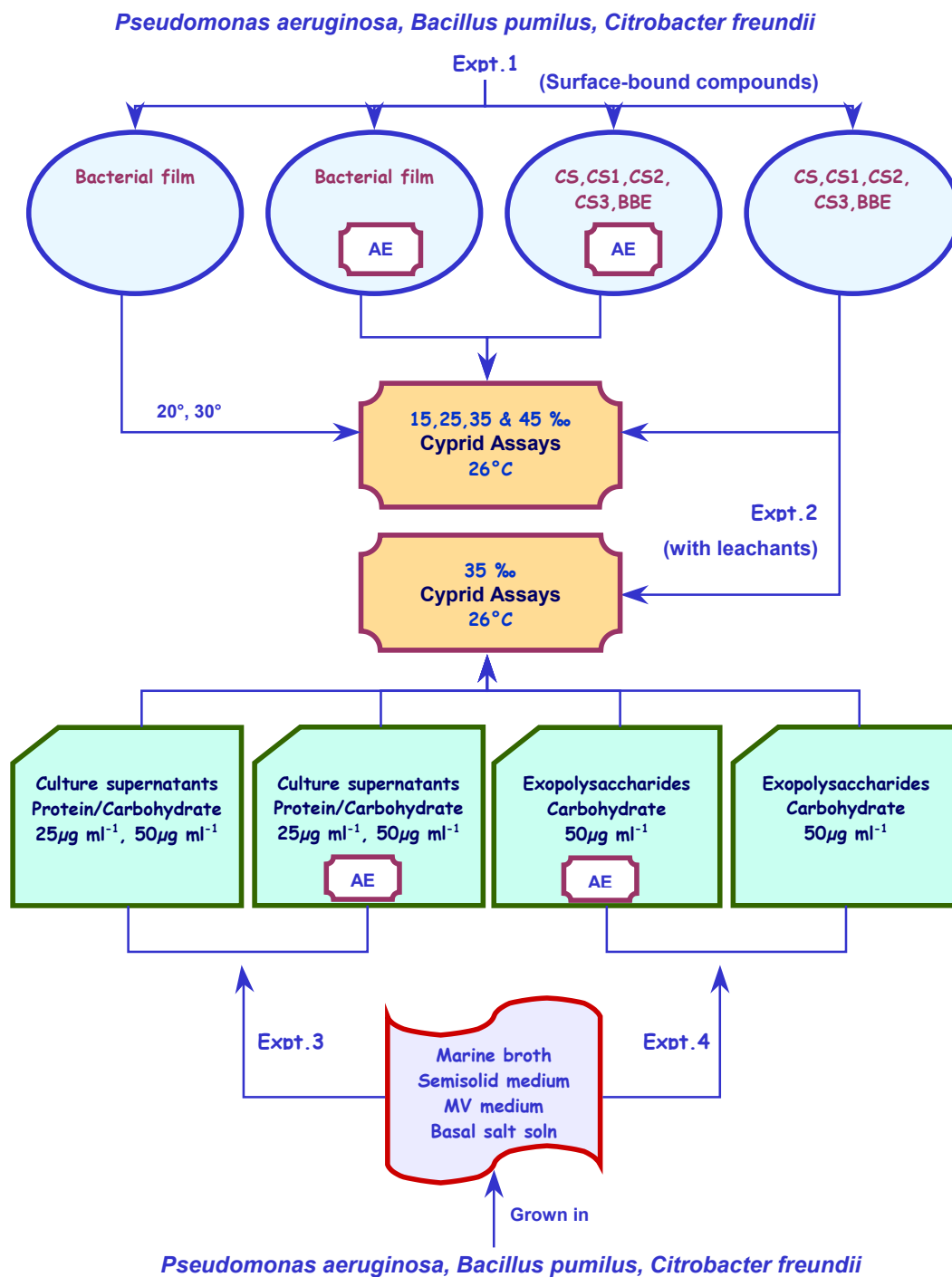
Table 3.1b The results of the tests employed to identify gram-positive bacteria

Tests	Results
Color	Cream
Shape	bacillus
Gram stain	+
Motility	+
O-F glucose	Oxidative
Growth at pH 5.7	+
Growth at 5° C	-
Growth at 40° C	+
Indole	-
Simmons citrate	+
Urease	-
Phenylalanine deaminase	-
Nitrate reduction	-
Oxidase	+
Catalase	+
Casein hydrolysis	+
Gelatin liquefaction	+
Starch hydrolysis	-
Voges-Proskauer test	+
Utilization of:	
Glucose	+
D-Xylose	+
D-Arabinose	+
Propionate	-
D-mannitol	+
Bacteria	<i>Bacillus pumilus</i>

Table 3.2 Phylogenetic identification of gram-positive bacteria.

Method	Bases sequenced	Nearest phylogenetic neighbor	% similarity	Phylogenetic Affiliation
cloned	1295	<i>Bacillus pumilus</i> strain KL-052	98.4%	<i>Bacillus pumilus</i> strain KL-052





CS1-3000-10000, CS2-10000-30000, CS3-<30000 mol. wt; MV- modified vishniac's medium  
All the experiments included adult extract (AE) as positive control and filtered seawater (FSW) as negative control

Fig. 3.1 Schematic representation of the experimental set-up

## Experiment 1. Influence of surface-bound compounds of bacteria and its products on metamorphosis

### 3.2.4 Bacterial film (BF)

The influence of bacterial film on the metamorphosis of *B. amphitrite* cyprids was assessed at different salinities (15, 25, 35 and 45‰) and temperatures (20, 26 and 30° C) and compared with AE (positive control) and FSW (negative control). Filming of the surfaces with bacteria was performed following the methods previously described by (Maki et al. 1988,1990). The dishes with the bacterial treatment were fixed with formaldehyde (final concentration 1 to 2%; v/v) and the quantification of attached bacteria was done by using acridine orange and epifluorescence microscopy. In this way the bacterial density was determined (Daley and Hobbie 1975). The adsorption technique resulted in densities of  $10^6$  to  $10^7$  attached bacteria  $\text{cm}^{-2}$ .

### 3.2.5 Culture supernatant and its fractions

The bacteria were grown in basal salt solution (BSS), pH 7.5, containing (g.  $\text{l}^{-1}$ ): NaCl, 30.0; KCl, 0.75;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 7.0;  $\text{NH}_4\text{Cl}$ , 1.0;  $\text{K}_2\text{HPO}_4$ , 0.7;  $\text{KH}_2\text{PO}_4$ , 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. After 48h, when the culture reached stationary phase, cells were centrifuged at 20,000 x g for 15 min at 4° C. The supernatant was filtered through 0.22  $\mu\text{m}$  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<sup>th</sup> of the 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 as well as the fractions were characterized for total carbohydrate content following the method described earlier by Dhople and Bhosle 1987 and protein content 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‰).

### **3.2.6 Bacterial extract**

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

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

### **Experiment 2. Effectiveness of leachants in presence of surface-bound compounds on metamorphosis**

Experiment 2 was carried out at 35‰. It differs from Experiment 1 as it was carried out with culture supernatant, fractions and bacterial extract ( $50 \mu\text{g ml}^{-1}$ ) 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), organically rich modified vishniac's medium (MV) as described by Perkins (1973) and by semi-solid culture (Abu et al. 1991). The culture supernatants were harvested by centrifugation ( $20,000 \times g$  for 30 min) and subsequently sterile filtered ( $0.22 \mu\text{m}$ , Millipore). They were then concentrated to  $1/10^{\text{th}}$  of the 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  $50 \mu\text{g ml}^{-1}$ , respectively. These culture supernatants extracted using different nutritional media i.e. CS(MB), CS(BSS), CS(MV) and CS(semi-solid) were examined at 35‰. They were also examined in presence of AE ( $25/50 \mu\text{g ml}^{-1}$ ).

#### **3.2.7 Semi-solid culture**

Since the growth conditions used in the semi-solid culture are different from that of others the details are provided. For this, bacterial cells grown in marine broth were washed with phosphate buffered saline - PBS (pH 7.3) and re-suspended in the same buffer. Five ml of the washed suspension were added to a petri plate of marine agar which had been overlaid with a sterile dialysis membrane (8,000 MW cut-off) presoaked in deionised water and rinsed with sterile PBS (pH 7.3). After 24h, polycarbonate nucleopore filters (25mm, 0.4

$\mu\text{m}$  pore size) were introduced into the thin liquid layer on the surface of the dialysis membrane. To extract and purify the exopolymer, the membranes were removed and surface growth was scraped into a minimum amount of PBS. Sodium chloride was added to a final concentration of 0.4 M. In order to preserve the osmotic stability glycerol was added to a final dilution of 1:24. The suspension was then agitated at 4° C overnight, and centrifuged at 30,000 x g for 35 min at 4° C. The recovered supernatant was dialysed (8,000 MW cut-off) overnight against distilled water. The non-dialysable material was filtered through Whatman #4 filter paper and lyophilized. The lyophilized material was re-dissolved in minimum amount of distilled water and filtered through Whatman #4 paper. The culture supernatant thus obtained was characterized by estimating total carbohydrates and proteins.

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

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

For the extraction of exopolysaccharides, the culture supernatants extracted 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, redissolved in distilled water and treated with DNase and RNase A (1-2  $\mu\text{g ml}^{-1}$ . 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 were evaluated for carbohydrate content and

rationalized at a concentration of  $50 \mu\text{g ml}^{-1}$  at 35‰. They were then subjected to the assays separately as well as in presence of AE ( $50 \mu\text{g ml}^{-1}$ ).

### 3.2.9 FTIR spectroscopy

The major structural groups of the culture supernatants of the bacteria cultivated in different nutrient media and the bacterial extract were detected using Fourier transformed infra-red spectroscopy (FTIR).

### 3.2.10 Assay protocol for Experiments 1, 2, 3 and 4

The settlement assays were carried out using (Corning-430343, 6 well multiwells). The multiwells were inoculated with bacterial film, culture supernatants, fractions, bacterial exopolysachharides & bacterial extract. They were also assessed along with AE (3 replicates for each of the combination by taking three different batches of larvae and repeated thrice,  $n=9$ ). 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 mutiwells 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). The influence of bacterial film, AE and FSW was also investigated at  $20^\circ$  and  $30 \pm 1^\circ \text{C}$ .

### 3.2.11 Assay protocol for thraustochytrid (#MS 2D)

Broth culture of thraustochytrid (#MS 2D) (5ml) was placed in the multiwells and the cells were allowed to grow on the surfaces for 24 hrs. The treated multiwells were washed off by repeated rinsing with autoclaved filtered sea water. Subsequently cyprids were introduced (approx. 50) in six replicates along with 5ml of autoclaved filtered sea water (35‰) to each well. Earlier such a method was used by Maki et al. (1988) for evaluating bacterial films. Controls were filtered sea water with no additions and adult extract (AE). The settlement assay was monitored for a period of four days at an interval of 24 hrs. The metamorphosed cyprids were counted at each of the observations.

### 3.2.12 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 bacteria, culture supernatants, fractions and the bacterial extract in presence and absence of AE on the metamorphosis of cyprids was evaluated using One-way ANOVA (Sokal and Rohlf 1981). A post ANOVA analysis was performed using Scheffe's test ( $\alpha=0.05$ ). Those that did not meet the normality assumption were analyzed using non-parametric statistical analysis such as Mann-Whitney U-Test ( $\alpha=0.025$ ). The analysis was performed by transforming the values to ranks and was used to compare the means between the two treatments. Three-way ANOVA was performed to evaluate the influence of temperature and salinities with respect to bacterial films, AE and FSW on the cyprid metamorphosis. The data in Experiment 1 was also subjected to cluster analysis in order to evaluate the influence of all

the bacterial inducers on larval metamorphosis. The dissimilarity levels were measured through squared euclidean distance and group average method (Pielou 1984). The procedure shows the result of the clustering as a tree diagram or dendrogram. Squared euclidean distance is used to measure the dissimilarity level. The intercluster distance is measured by group average method. The X-axis groupings are based on the clusters that are dissimilar beyond mid point of highest dissimilarity observed.

### **3A. *Pseudomonas aeruginosa***

#### **3A 3 Results**

##### **3A 3.1 Experiment 1**

When the bacterial film of *P. aeruginosa* was assessed at 26° C, and at different salinities, the cyprids metamorphosed in higher percentages when compared to control (FSW) at 25 and 35‰ ( $p \leq 0.025$ , Mann-Whitney), whereas at 15 and 45‰, metamorphosis was not facilitated (Fig. 3A.1). The metamorphosis rates of the cyprids exposed to bacterial film at different salinities were significantly different ( $p \leq 0.01$ , One-way ANOVA;  $p \leq 0.05$ , Scheffe's test). However such differences were not evident with the aging of the films and cyprids (Table 3A.1a). When assessed in presence of AE, metamorphosis was facilitated irrespective of salinity differences (Fig. 3A.1). The influence of bacteria in the presence of AE did not differ significantly with respect to salinity (Table 3A.1b).



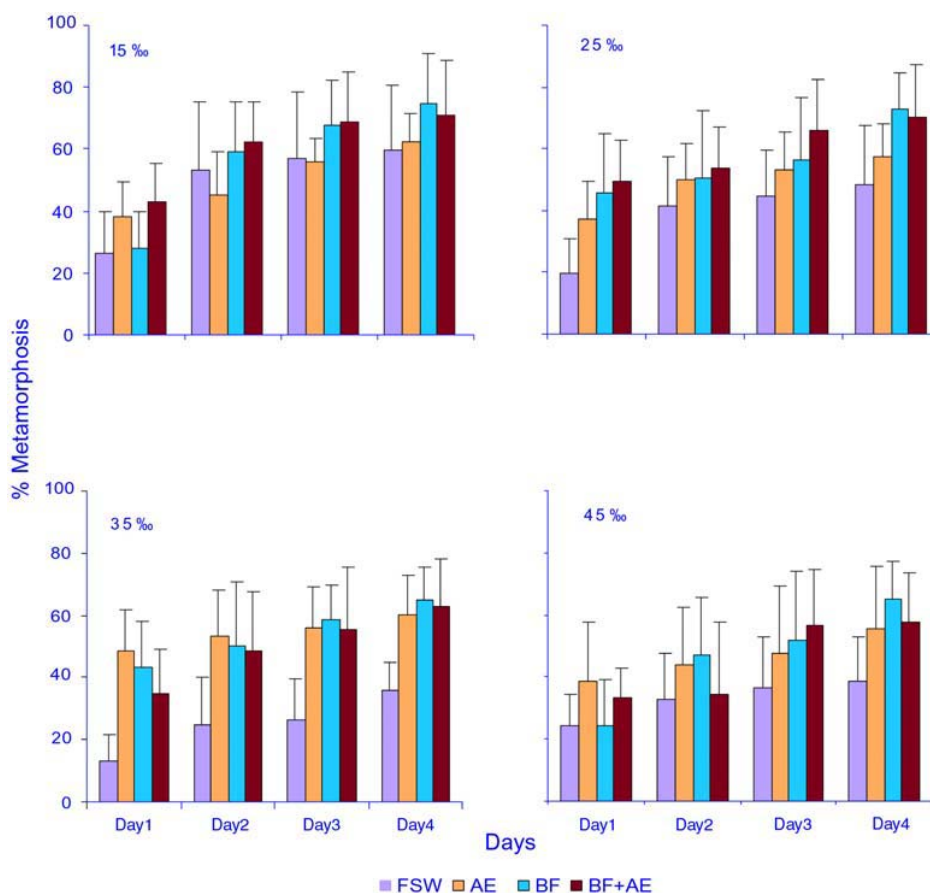


Fig. 3A.1 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*P. aeruginosa*) in presence of AE (adult extract) at different salinities. Vertical lines indicate the standard deviation from mean and are shown as positive

Bacterial film appeared to be the most influential in inducing the metamorphosis of cyprids when compared to AE at 20° and 30° C except at 45‰ (Fig. 3A.2). At these temperatures, the influence of bacterial film, AE and FSW was almost similar at 45‰ and comparatively less than that observed at 26° C. Three-way ANOVA indicated the differences between bacterial film, AE and FSW to be significant with respect to salinity and temperature at the end of day1, whereas on day 4 no significant differences were observed (Table 3A.2).

Table 3A.1 One-way ANOVA. The influence of *P. aeruginosa*, culture supernatant, fractions and butanol-bacterial extract at different salinities on the metamorphosis of cyprids of *B. amphitrite*. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor	Day 1				Day 4					Day 1			Day 4		
	df	SS	MS	Fs	SS	MS	Fs	SS		MS	Fs	SS	MS	Fs	
<b>(a) BF</b>	Salinity	3	1731	577	4.7***	502	167	0.8ns	<b>(b) BF+AE</b>	509	170	2.2ns	372	124	0.4ns
	Within sub. Gr.err.	32	3879	121		6421	200			2420	76		9547	298	
	Total	35	5610			6923				2929			9919		
<b>(c) CS1</b>	Salinity	3	64	21	0.4ns	179	59	0.5ns	<b>(d) CS1+AE</b>	313	104	0.7ns	310	103	0.5ns
	Within sub. Gr.err.	32	1387	43		3405	106			4735	148		6907	216	
	Total	35	1451			3584				5048			7217		
<b>(e) CS2</b>	Salinity	3	174	58	0.6ns	1643	547	1.7ns	<b>(f) CS2+AE</b>	163	54	0.2ns	1533	511	1.4ns
	Within sub. Gr.err.	32	2848	89		9771	305			7162	224		11894	372	
	Total	35	3022			11414				7325			13427		
<b>(g) CS3</b>	Salinity	3	1399	466	7*****	629	209	1.9ns	<b>(h) CS3+AE</b>	1649	550	3.3*	2810	937	4.3**
	Within sub. Gr.err.	32	2121	66		3561	111			5303	166		6919	216	
	Total	35	3520			4190				6952			9729		
<b>(i) CS</b>	Salinity	3	843	281	2.9*	1512	504	2.2ns	<b>(j) CS+AE</b>	480	160	0.6ns	2477	826	2.8ns
	Within sub. Gr.err.	32	3063	95		7235	226			9137	285		9299	291	
	Total	35	3906			8747				9617			11776		
<b>(k) BBE</b>	Salinity	3	1326	442	4.4**	570	190	1.6ns	<b>(l) BBE+AE</b>	160	53	0.4ns	2052	684	3.1*
	Within sub. Gr.err.	32	3223	101		3760	117			3884	121		7016	219	
	Total	35	4549			4330				4044			9068		

(\*\*\*\*\*p≤0.001,\*\*\*\*p≤0.005, \*\*\*p≤0.01,\*\* p≤0.025, \*p≤0.05, ns- not significant)

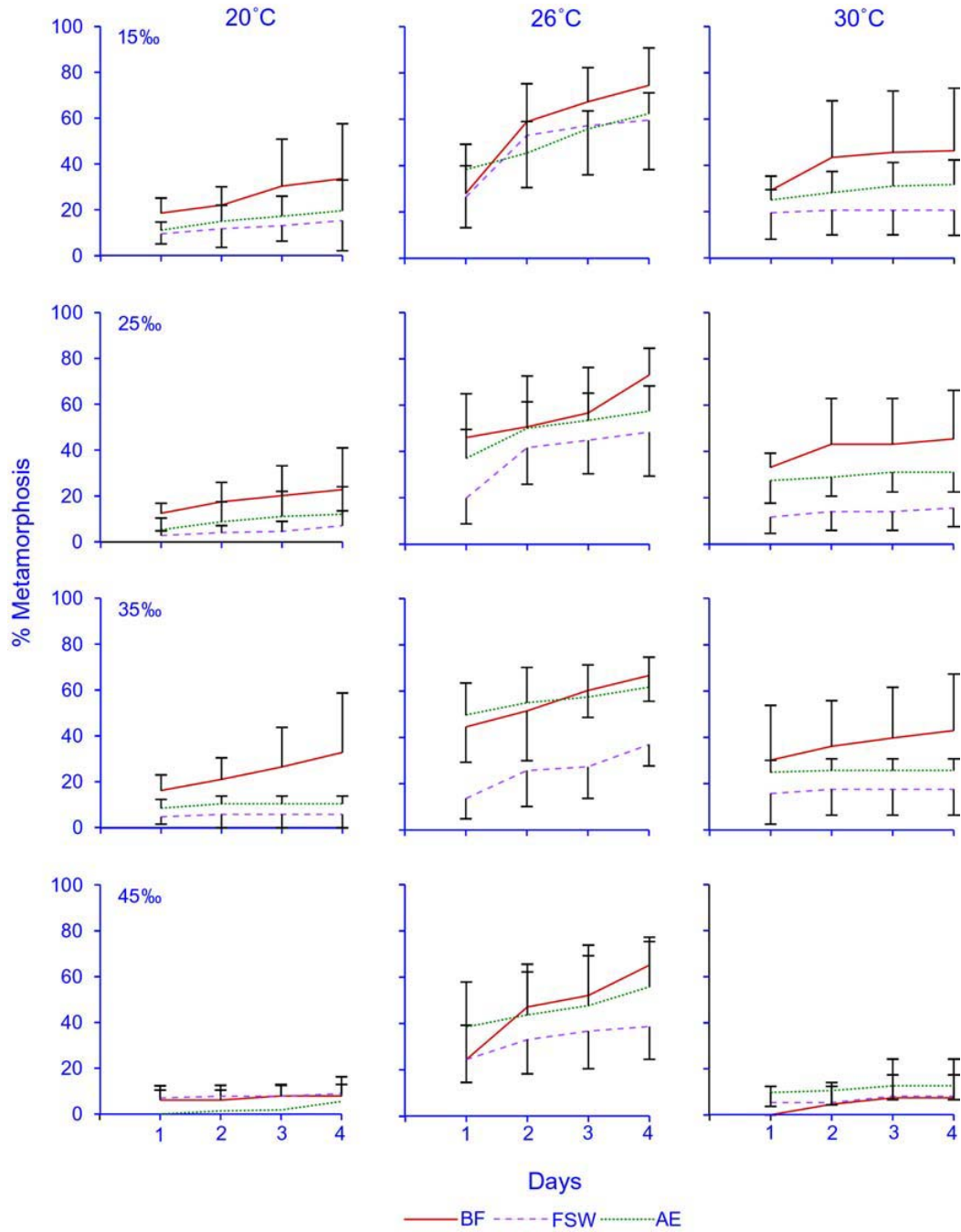


Fig. 3A.2 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (BF), adult extract (AE) and filtered sea water (FSW) at different salinities (15, 25, 35 and 45‰) and temperatures (20, 26 and 30° C)

Table 3A.2 Three-way ANOVA. The influence of temperatures (20, 26 & 30° C) and salinities (15, 25, 35 & 45‰) with respect to treatments (bacterial film, AE and FSW) on the metamorphosis of *B. amphitrite* cyprids on day 1 and day 4. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor	df	Day 1			Day 4		
		SS	MS	Fs	SS	MS	Fs
A (temperature)	2	756	378		4231	2115	
B (salinity)	3	208	104		689	344	
C (treatments)	2	259	86		517	172	
A*C	4	189	47	6***	86	21	2ns
A*B	6	105	17	2.2ns	146	24	2.2ns
C*B	6	199	33	4.3**	163	27	2.5ns
A*B*C*	12	94	8		130	11	
Total	35	1810			5962		

(\*\*\*\*p<0.001, \*\*\*p<0.005, \*\*p<0.01, \*p<0.025, ns- not significant)

Cluster analysis indicated that the response of the cyprids to some of the bacterial cues, which showed similarity in larval induction of metamorphosis at one salinity, differed at the other (Fig. 3A.3). At 15‰, bacterial film and AE were similar in inducing larval metamorphosis owing to which they formed one cluster, whereas, at 25‰ bacterial film was most dissimilar from rest of the cues. At 35‰, bacterial film and AE were highly dissimilar from rest of the bacterial cues whereas at 45‰ bacterial film and CS1 showed a similar response (Fig. 3A.3; Day 1). The response differed as the cyprids as well as the cues aged (Fig. 3A.3; Day 4).

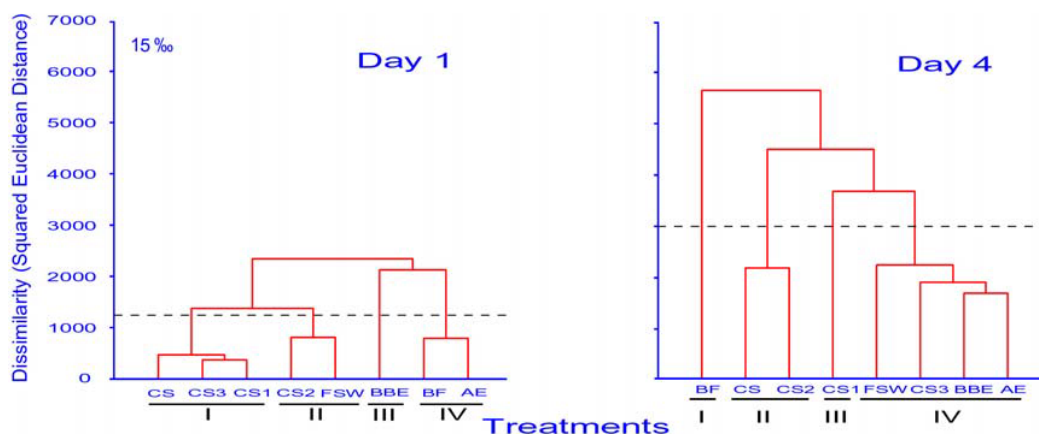


Fig. 3A.3 Dendrograms showing the dissimilarity between different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 15‰

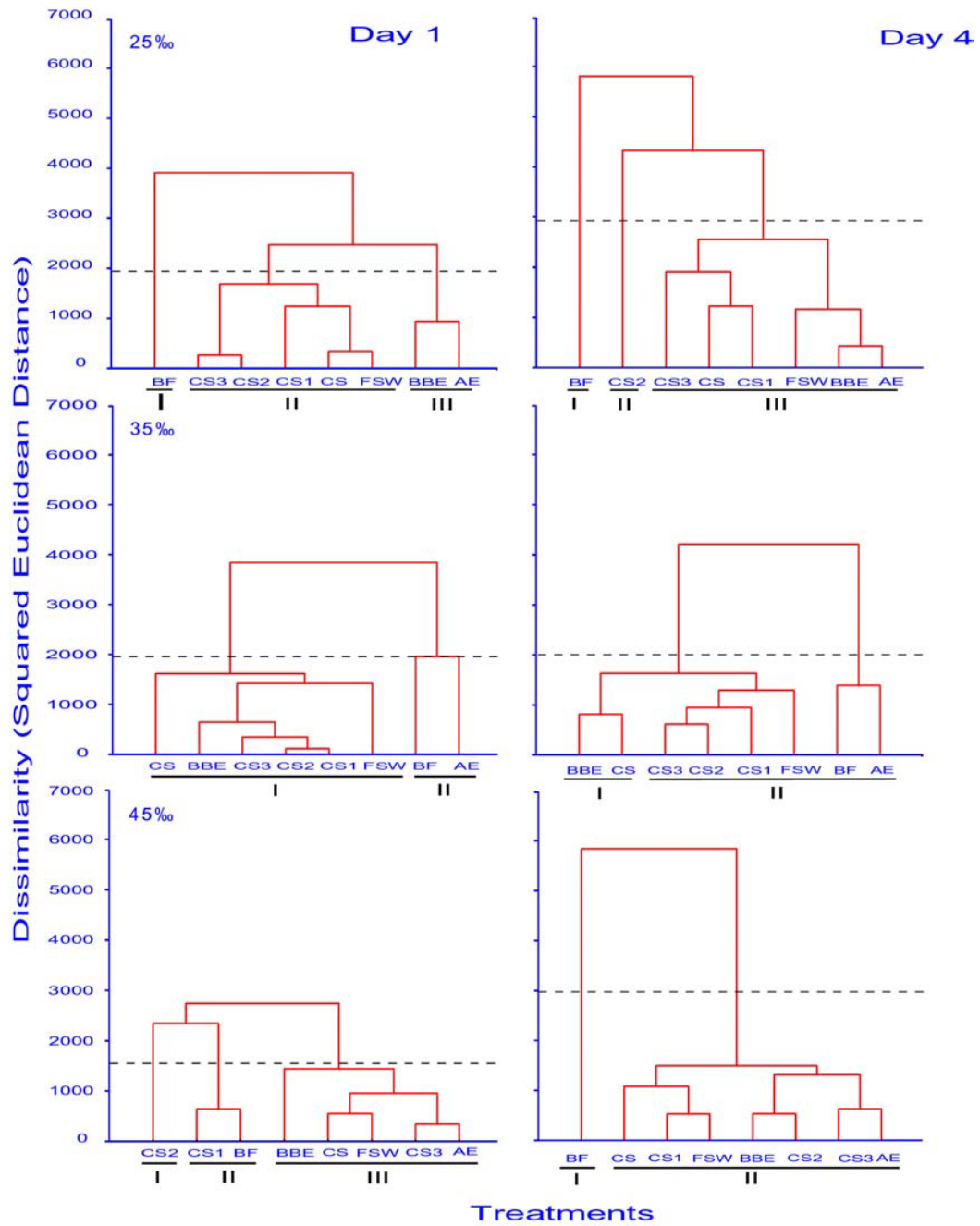


Fig. 3A.3 Dendrograms showing the dissimilarity between different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 25, 35 and 45%. (The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed)

The response of cyprids towards bacterial culture supernatant varied with the fractions containing different molecular weight substances. A significant difference in metamorphosis inducement with respect to salinity was seen with only CS3 ( $p \leq 0.001$ , One-way ANOVA;  $p \leq 0.05$ , Scheffe's test) on day 1, whereas on day 4 these differences were not significant (Table 3A.1g). The percentage of larvae metamorphosing in response to CS2 at 15 and 25‰ and CS at 35‰ were higher when compared to other fractions at the end of day 1 (Fig. 3A.4). However, neither CS2 nor CS was as inductive as AE at these salinities. At 45‰, the fraction CS3 and CS were as effective as AE in provoking metamorphosis of cyprids. In summary, higher molecular weight fraction of the culture supernatant was inductive at higher salinity. When they were assessed in presence of AE the metamorphosis rates increased. However significant differences with respect to salinities were observed with only CS3 (Table 3A.1h).

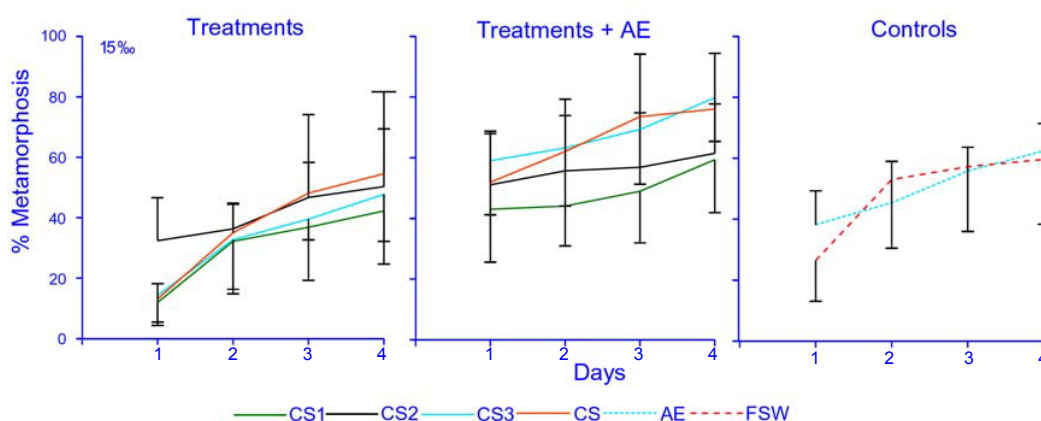


Fig. 3A.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution in presence and absence of adult extract (AE) at 15‰. Vertical lines indicate the standard deviation from the mean and are indicated as either positive or negative

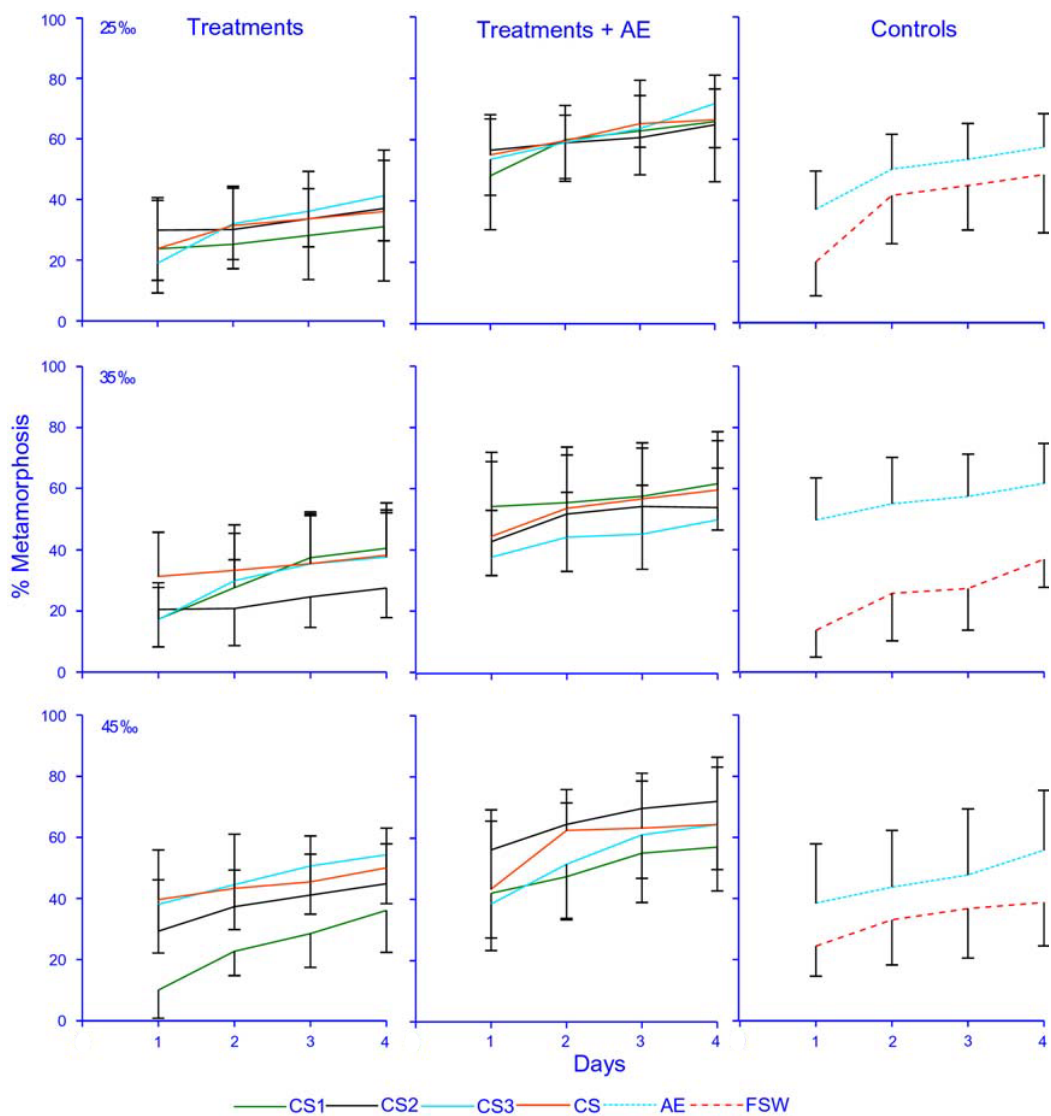


Fig. 3A.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution in presence and absence of adult extract (AE) at 25, 35 and 45‰. Vertical lines indicate the standard deviation from the mean and are indicated as either positive or negative

Bacterial extract showed ketonic compounds as indicated by FTIR and their influence varied with the salinity. The inhibitory effect of the extract at 15‰ and 35‰ was nullified in presence of AE (Fig. 3A.5).

A marginal increase in the metamorphosis rates was observed with the aging of the cyprids and the settlement cues.

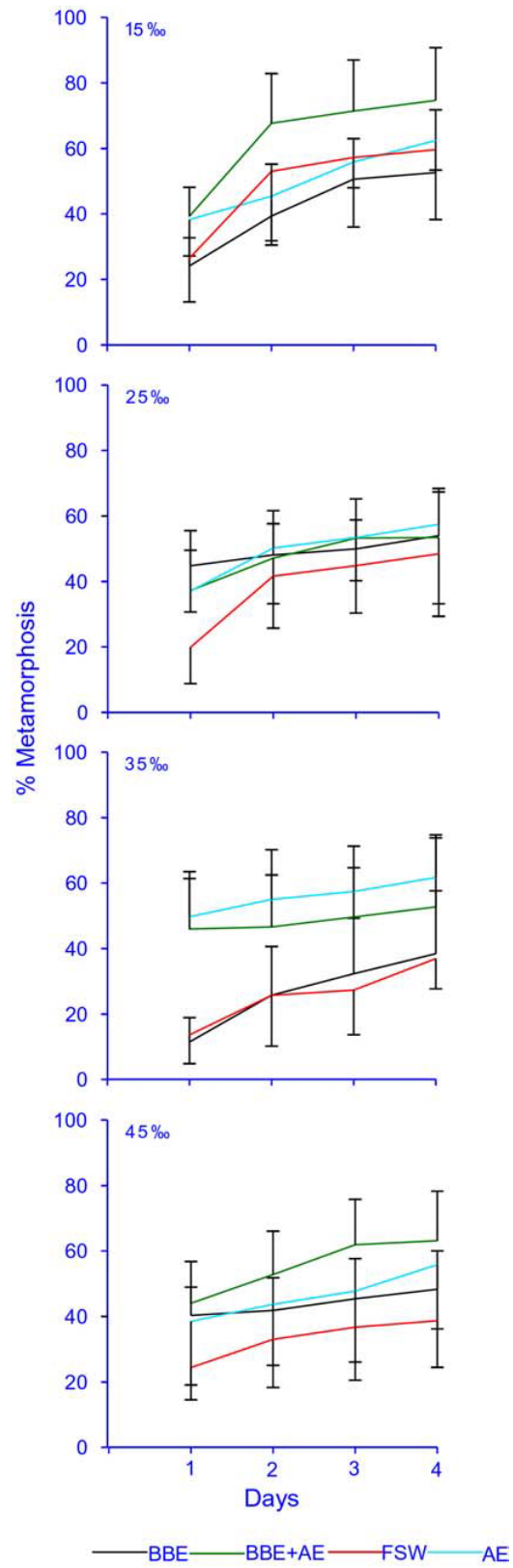


Fig. 3A.5 Percentage metamorphosis of *B. amphitrite* cyprids exposed to Butanol-bacterial extract at different salinities. Vertical lines indicate the standard deviation from the mean and are indicated as either positive or negative



### 3A 3.2 Experiment 2

When the surface-bound components of bacterial extract was assessed along with the leachants, a two-fold increase in the metamorphosis rates was observed, where surface-bound components alone were inhibitory ( $p \leq 0.025$ , Mann-Whitney). The leachants of fraction CS1 resulted in decrease in the metamorphosis rates to as low as 4.5% when assessed along with its adsorbed components (Fig. 3A.6).

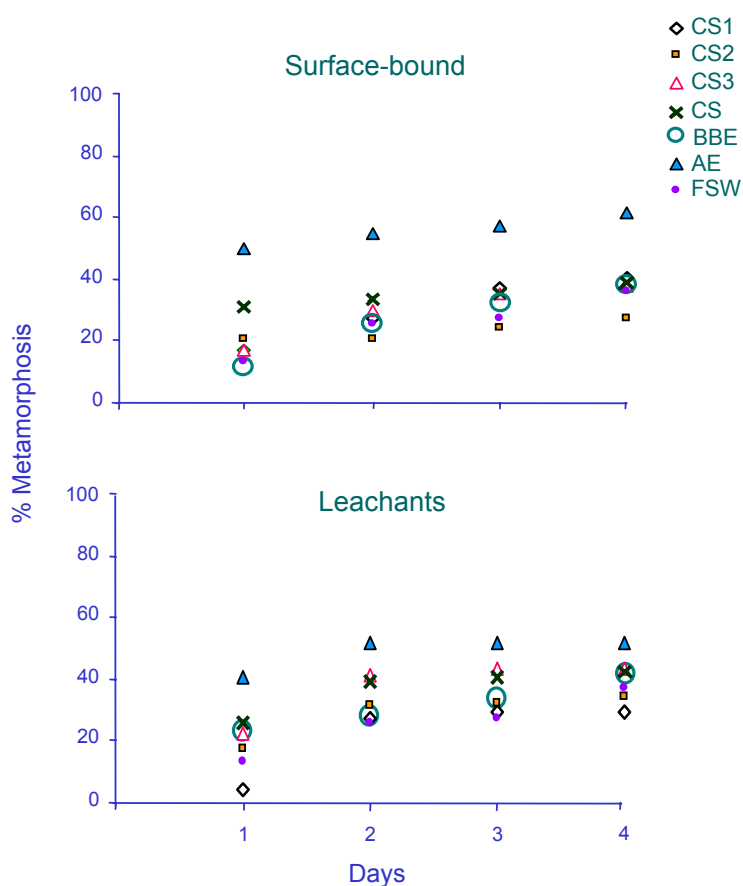


Fig. 3A.6 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial inducers (surface-bound) used in experiment 1 in presence of leachants at 35‰

### 3A 3.3 Experiment 3

*P. aeruginosa* cultivated in BSS, MB and MV resulted in culture supernatants with higher carbohydrate content, whereas when extracted using semi-solid culture showed higher protein content.

The larvae metamorphosed in higher percentages when exposed to culture supernatant obtained by semi-solid culture when compared to that extracted using other nutritional media at a protein concentration of  $25 \mu\text{g ml}^{-1}$ . The differences among these supernatants were highly significant ( $p \leq 0.001$ , One-way ANOVA;  $p \leq 0.05$ , Scheffe's test). An increase in protein concentration to  $50 \mu\text{g ml}^{-1}$  resulted in non-significant differences (Table 3A.3a). At this concentration culture supernatant produced by semi-solid culture showed an inhibition. At the end of day 4 there was no significant difference in the response of the culture supernatants to cyprid metamorphosis at a carbohydrate concentration of  $25 \mu\text{g ml}^{-1}$ . Except for the culture supernatants produced by semi-solid culture no other culture supernatant was comparable with AE or bacterial film (Fig. 3A.7b,e). Mann-Whitney U-Test showed no significant difference between bacterial films, AE and the culture supernatant extracted by semi-solid culture.

When these culture supernatants were assessed in presence of AE, significant variations in metamorphosis was observed at a carbohydrate and protein concentration of  $50 \mu\text{g ml}^{-1}$  and  $25 \mu\text{g ml}^{-1}$ , respectively, at the end of day 1 (Table 3A.3b). Whereas day 4 observations indicated non-significant differences among culture supernatants only at a protein concentration of  $50 \mu\text{g ml}^{-1}$ .

Table 3A.3a. One-way ANOVA. The influence of culture supernatants (CS) extracted by four different extraction protocols at two different concentrations of carbohydrates and proteins on the metamorphosis of *B. amphitrite* cyprids.

Factor	df	25µg/ml (carbohydrates)			50µg/ml (carbohydrates)			25µg/ml (proteins)			50µg/ml (proteins)		
		SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
<b>Day 1</b>													
CS	3	258	86	4**	588	196	3.6**	1455	485	9.9*****	448	149	2.7ns
Within sub. Gr.err.	32	698	21.8		1711	53.5		1562	48.8		1751	54.7	
Total	35	956			2299			3017			2199		
<b>Day 4</b>													
CS	3	227	75.5	1.7ns	1297	432	7.07*****	1204	401	7.5*****	831	277	4.53***
Within sub. Gr.err.	32	1437	45		1955	61.1		1701	53.2		1955	61.1	
Total	35	1664			3252			2905			2786		

(\*\*\*\*\*p<0.001, \*\*\*\*p<0.005, \*\*\*p<0.01, \*\* p<0.025, \*p<0.05, ns- not significant)

Table 3A.3b. One-way ANOVA. The influence of culture supernatants (CS) extracted by four different extraction protocols at two different concentrations of carbohydrates and proteins in presence of AE on the cyprid metamorphosis of *B. amphitrite*. (df.degree of freedom; SS. sum of the squares; MS. mean of squares; Fs.Fischer constant).

Factor	df	25µg/ml (carbohydrates)			50µg/ml (carbohydrates)			25µg/ml (proteins)			50µg/ml (proteins)		
		SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
<b>Day 1</b>													
CS	3	82	27.2	0.5ns	449	150	4.1**	392	130	4.7****	271	90	1.4ns
Within sub. Gr.err.	32	1665	52		1164	36.4		873	27.3		2030	63.4	
Total	35	1747			1613			1265			2301		
<b>Day4</b>													
CS	3	1028	342	4.6***	1182	394	3.8**	695	232	6.7*****	491	164	1.7ns
Within sub. Gr.err.	32	2390	75		3277	102		1109	35		2955	92.4	
Total	35	3418			4459			1804			3446		

(\*\*\*\*\*p<0.001, \*\*\*\*p<0.005, \*\*\*p<0.01, \*\* p<0.025, \*p<0.05, ns- not significant)

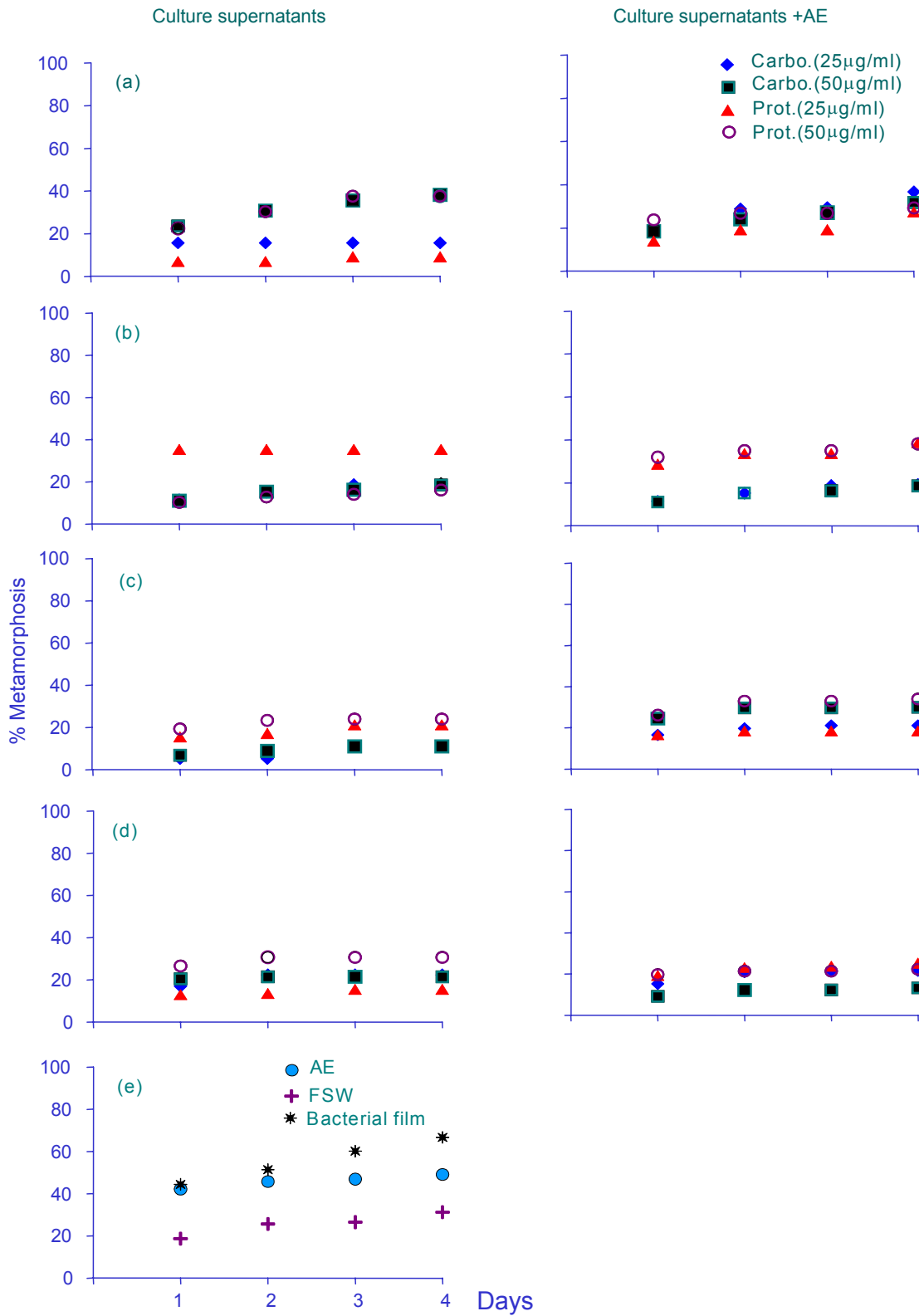


Fig. 3A. 7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutrient media at 35‰ in presence and absence of adult extract. (a) CS(BSS) (b) CS(semi-solid) (c) CS(MV) (d) CS(MB) (e) Controls

### 3A 3.4 Experiment 4

The exopolysaccharides (EPS) obtained from the bacteria grown in semi-solid culture showed high protein content whereas those obtained from BSS, MB and MV mediums were mainly carbohydrates. The EPS obtained from BSS exhibited similar metamorphosis as that shown by bacteria and AE at 35‰ (Fig. 3A.8a,b).

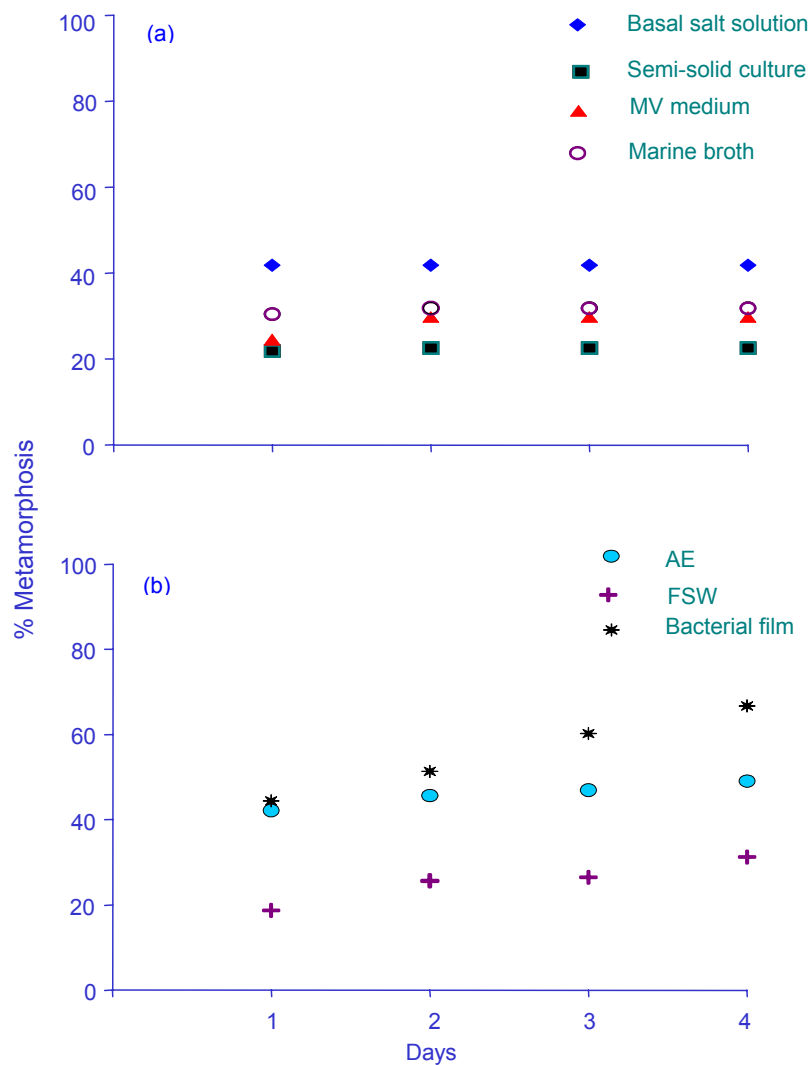


Fig. 3A. 8 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial exopolysaccharides obtained after growing the bacteria under different nutritional conditions at 35‰

### 3A 3.5 FTIR spectroscopy

Fourier transformed infrared (FTIR) spectrum of CS of BSS media revealed characteristic strong peaks around  $3550\text{--}3200\text{ cm}^{-1}$ ,  $1640\text{ cm}^{-1}$  and  $1076\text{--}1122\text{ cm}^{-1}$  for O-H stretching, C=O stretching and C-O stretching. The structural identity of CS from FTIR results was mainly of carbohydrates. Similar results were obtained with the culture supernatants of MB and MV media (Fig. 3A.9).

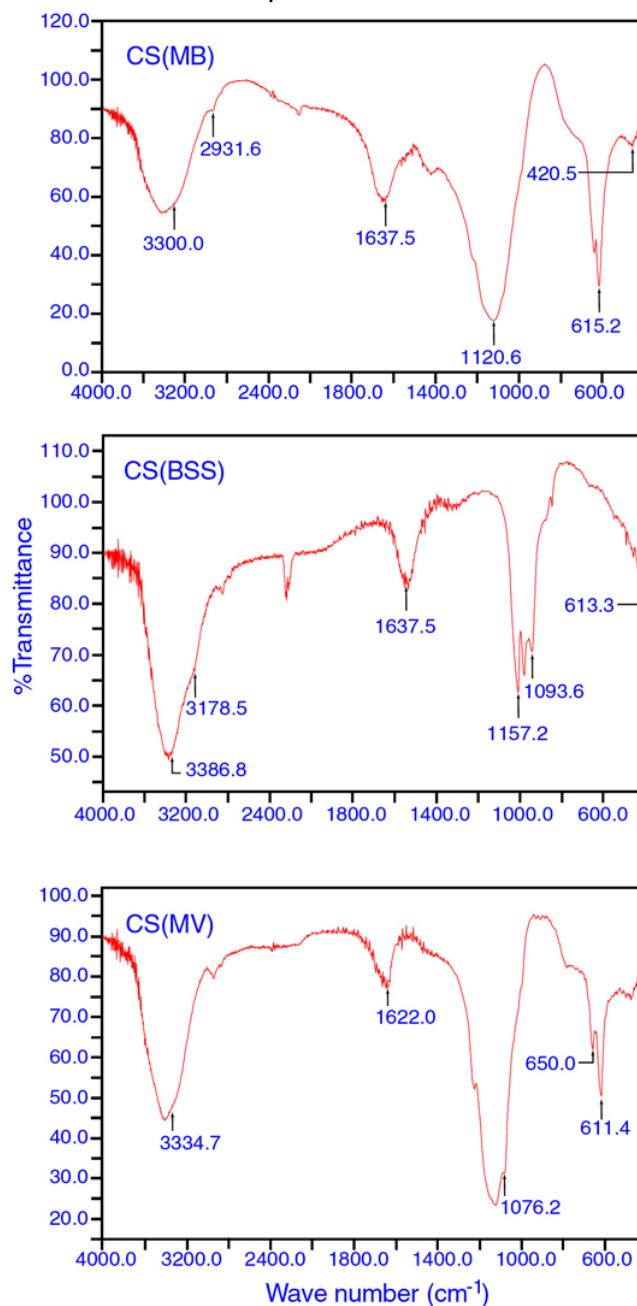


Fig. 3A.9 FTIR spectra of culture supernatants of MB, BSS and MV media

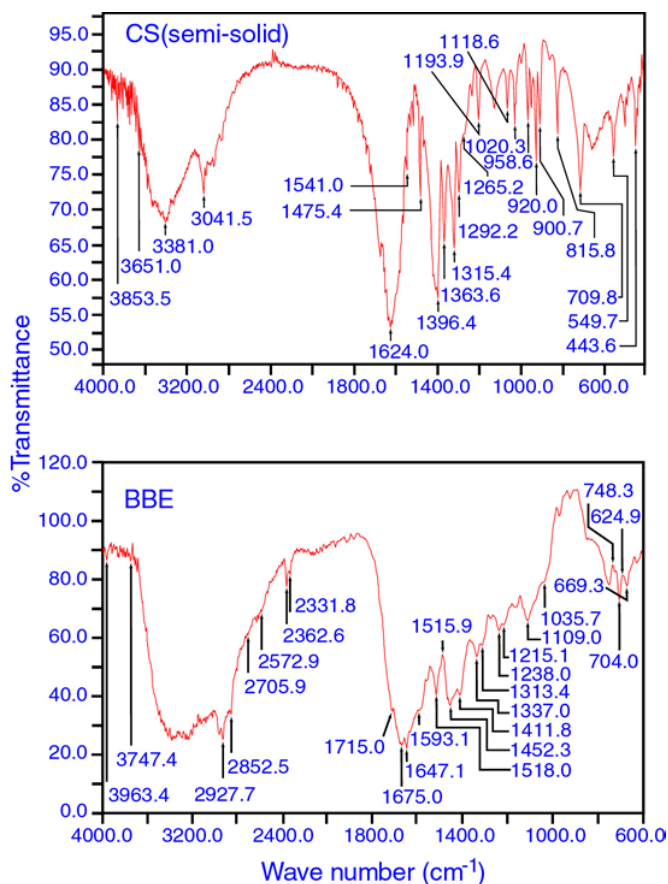


Fig. 3A.9 FTIR spectras of culture supernatant of semi-solid media and bacterial extract

Culture supernatant of semi-solid culture and the bacterial extract revealed strong peaks at 1624 and 1647  $\text{cm}^{-1}$  for N-H bend and were proteinaceous. In case of semi-solid culture supernatant presence of terpenoids and steroids were evidenced from IR absorption at 1475  $\text{cm}^{-1}$  & 1363.6  $\text{cm}^{-1}$ , the spectra also indicated the presence of unsaturation evident from C-H bending vibrations located around 1000 and 900  $\text{cm}^{-1}$ . Bacterial extract showed peaks at 1715 & 1675  $\text{cm}^{-1}$  characteristic for ketones.

### 3B. *Bacillus pumilus*

#### 3B 3.1 Experiment 1

The bacterial film (BF) of *B. pumilus* when assessed at 26° C, facilitated metamorphosis similar to that of AE at 15‰. The metamorphosis inducement by AE and BF was almost similar and comparatively lower at 45‰. At 35‰, BF did not promote metamorphosis (Fig. 3B.1).

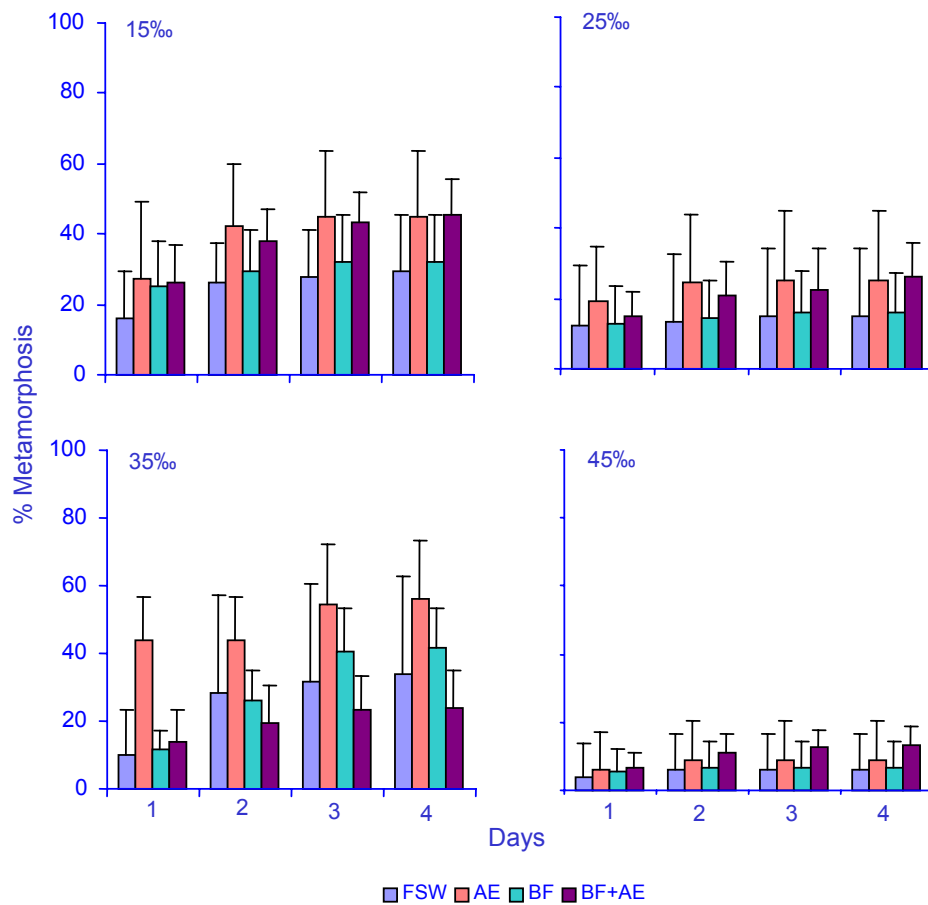


Fig. 3B.1 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*B. pumilus*) in presence of adult extract (AE) at different salinities (15, 25, 35 and 45‰). Vertical lines indicate the standard deviation from mean and are shown as positive.



The differences in metamorphosis rates with respect to salinity was highly significant (Table 3B.1a,b) in presence and absence of AE on day 1 & 4 ( $p \leq 0.001$ , one-way ANOVA;  $p \leq 0.05$ , Scheffe's test).

At 30° C, bacterial film was the most influential at 15‰, whereas at 20° C, it induced metamorphosis at 15, 25 & 45‰. However, the metamorphosis rates were not higher then that observed at 30° C (Fig. 3B.2).

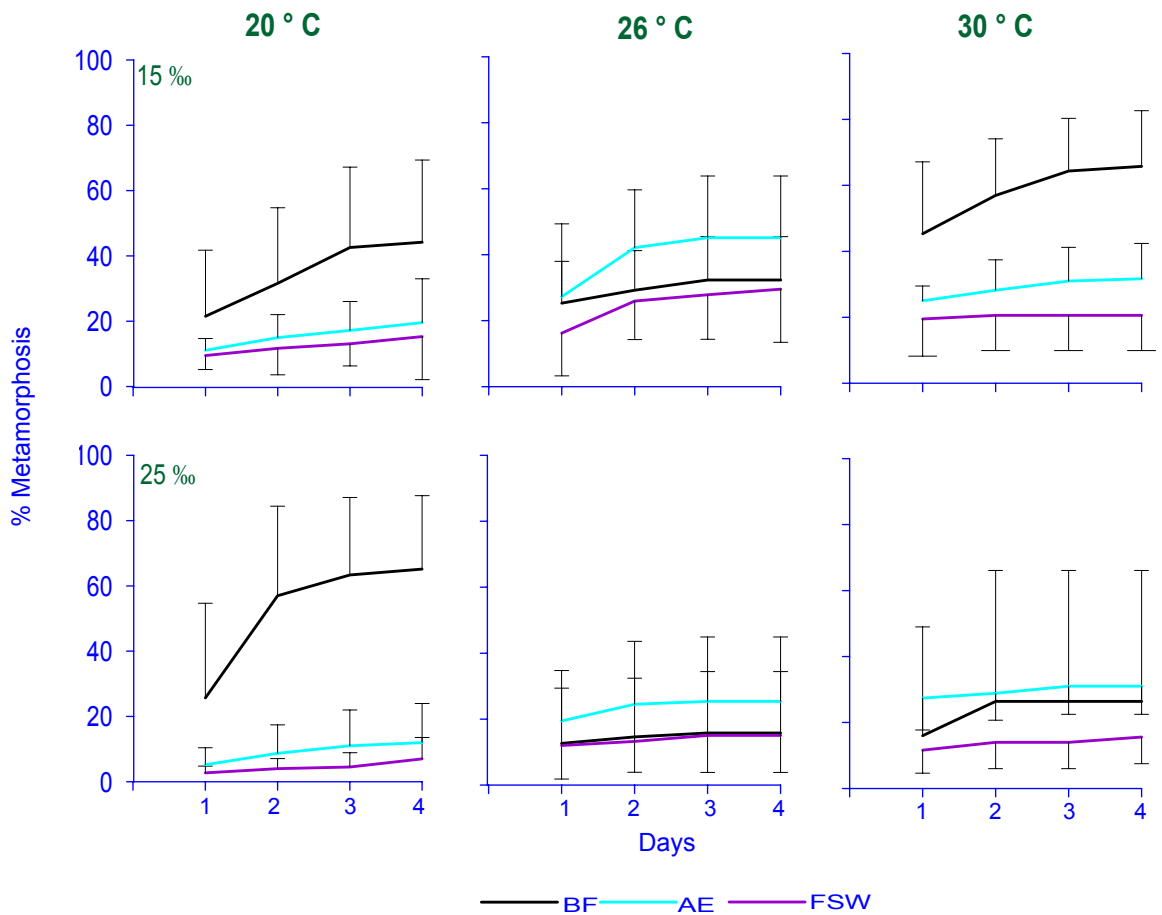


Fig. 3B.2 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*B. pumilus*), adult extract (AE) and filtered sea water (FSW) at different salinities (15 and 25‰) and temperatures (20, 26 and 30° C). Vertical lines indicate the standard deviation from mean and are indicated as either positive or negative.

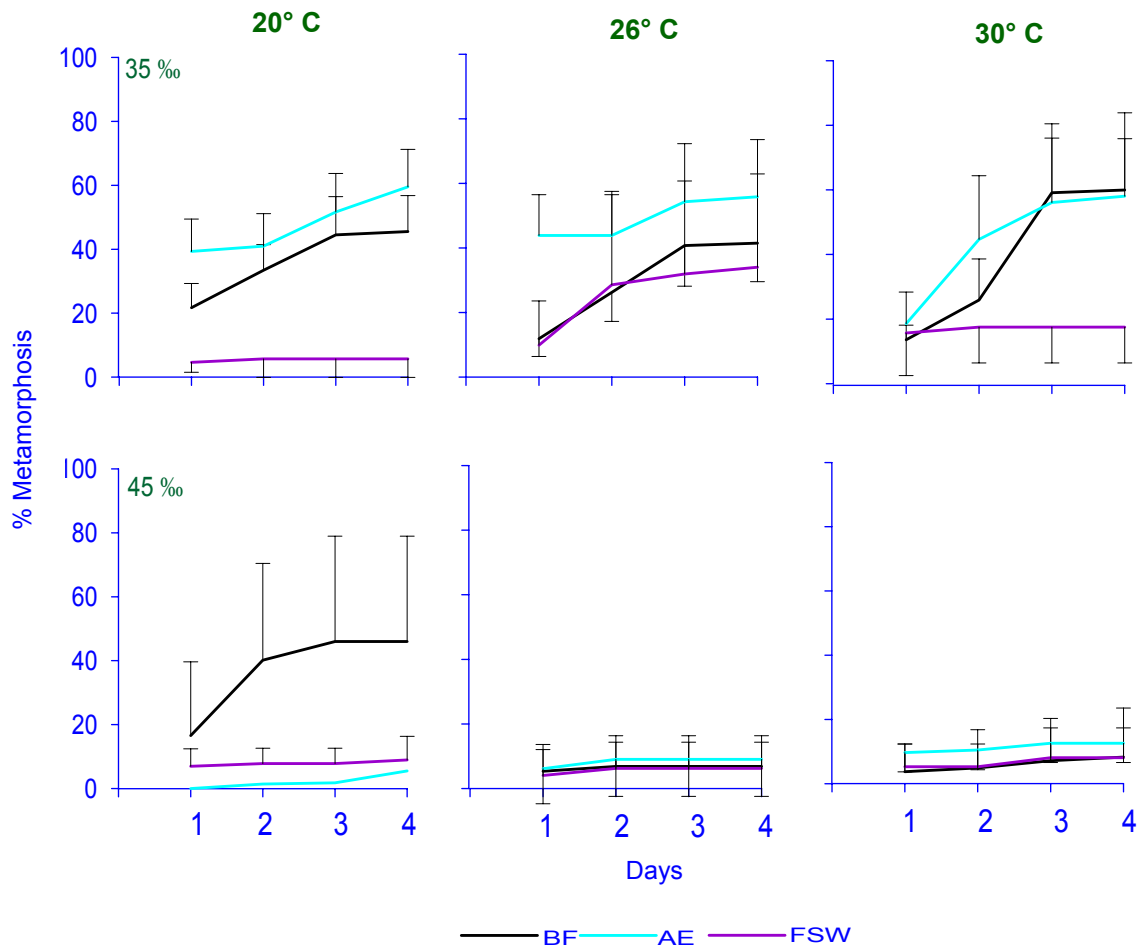


Fig. 3B.2 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*B. pumilus*), adult extract (AE) and filtered sea water (FSW) at different salinities (35 and 45‰) and temperatures (20, 26 and 30°C). Vertical lines indicate the standard deviation from mean and are indicated as either positive or negative

Table 3B.1 One-way ANOVA. The influence of *B. pumilus*, culture supernatant, fractions and butanol-bacterial extract at different salinities on the metamorphosis of cyprids of *B. amphitrite*. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor	Day 1							Day 4							
	df	SS	MS	Fs	SS	MS	Fs	df	SS	MS	Fs	SS	MS	Fs	
<b>(a) BF</b>	Salinity	3	868	289	9.5*****	3174	1058	22*****	<b>(b) BF+AE</b>	820	273	12*****	2439	813	25*****
	Within sub. Gr.err.	44	1345	30		2107	48			978	22		1424	32	
	Total	47	2213			5281				1798			3863		
<b>(c) CS1</b>	Salinity	3	748	249	4***	475	158	0.7ns	<b>(d) CS1+AE</b>	2252	751	7*****	8670	2890	15*****
	Within sub. Gr.err.	44	2592	59		10341	235			4564	104		8273	188	
	Total	47	3340			10816				6816			16943		
<b>(e) CS2</b>	Salinity	3	2281	760	6****	4371	1457	4****	<b>(f) CS2+AE</b>	2090	697	8*****	3997	1332	7*****
	Within sub. Gr.err.	44	5610	127		14278	324			3582	81		8204	186	
	Total	47	7891			18649				5672			12201		
<b>(g) CS3</b>	Salinity	3	2761	920	14*****	4742	1580	20*****	<b>(h) CS3+AE</b>	1572	524	1.6ns	5126	1709	5****
	Within sub. Gr.err.	44	2903	66		3462	79			14476	329		14338	326	
	Total	47	5664			8204				16048			19464		
<b>(i) CS</b>	Salinity	3	3857	1286	14*****	6545	2182	12*****	<b>(j) CS+AE</b>	1526	508	5****	1434	478	1ns
	Within sub. Gr.err.	44	4035	92		7730	176			4446	101		14767	336	
	Total	47	7892			14276				5972			16201		
<b>(k) BBE</b>	Salinity	3	1889	630	7*****	3664	1221	10*****	<b>(l) BBE+AE</b>	3716	1239	11*****	4320	1440	
	Within sub. Gr.err.	44	4112	93		5080	115			4730	107		6910	157	9*****
	Total	47	6001			8744				8446			11230		

(\*\*\*\*\*p≤0.001,\*\*\*\*p≤0.005,\*\*\*p≤0.01,\*\*p≤0.025,\*p≤0.05, ns- not significant)

Three-way ANOVA indicated the differences between bacterial film, AE and FSW to be significant with respect to temperature at the end of day 1 & 4 (Table 3B.2).

Table 3B.2 Three-way ANOVA. The influence of temperature (20, 26 & 30° C) and salinities (15, 25, 35 & 45‰) with respect to treatments (bacterial film, AE and FSW) on the metamorphosis of *B. amphitrite* cyprids on day 1 and day 4. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor	Day 1				Day 4		
	df	SS	MS	Fs	SS	MS	Fs
A (temperature)	2	102	52		21	11	
B (salinity)	3	408	136		943	314	
C (treatments)	2	122	61		1304	652	
A*B	6	130	22	1.5ns	687	115	3.1*
A*C	4	189	47	3.2*	1105	276	7.4****
B*C	6	162	28	1.8ns	81	13	0.4ns
A*B*C	12	177	15		446	37	
Total	35	1290			4588		

(\*\*\*\*p<0.001, \*\*\*\*p<0.005, \*\*\*p<0.01, \*\*p<0.025, \*p<0.05, ns- not significant)

Cluster analysis indicated the response of cyprids to differ with the salinity, age of the cyprids as well as the cues. At 25‰, CS was the most dissimilar whereas at 45‰, there was no much difference in response of the different cues towards cyprid metamorphosis (Fig. 3B.3; Day 1). The response differed with the age of the cyprids and the cues (Fig. 3B.3; Day 4).

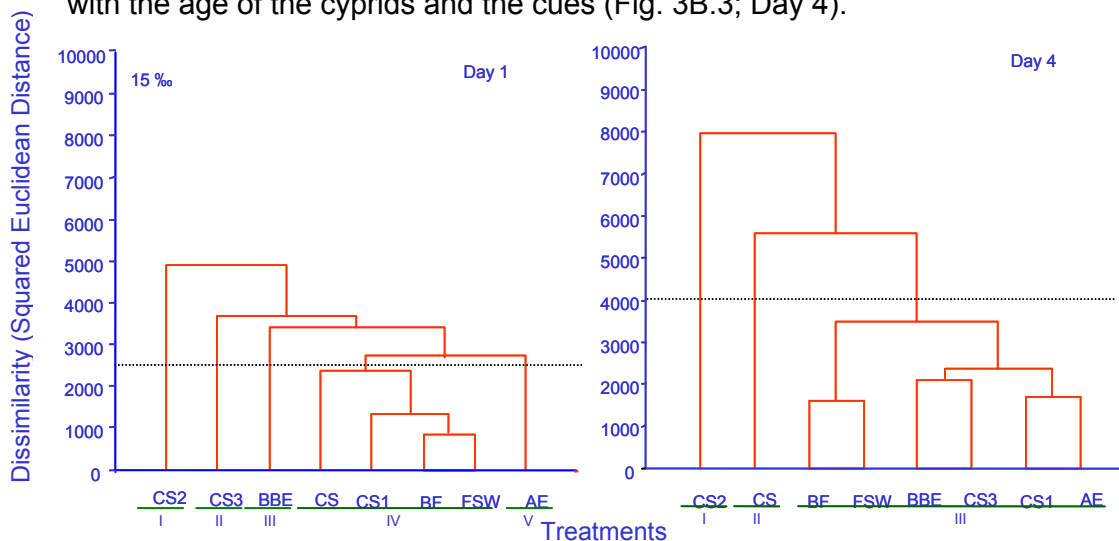


Fig. 3B.3 Dendrograms showing the dissimilarity among different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 15‰. The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed

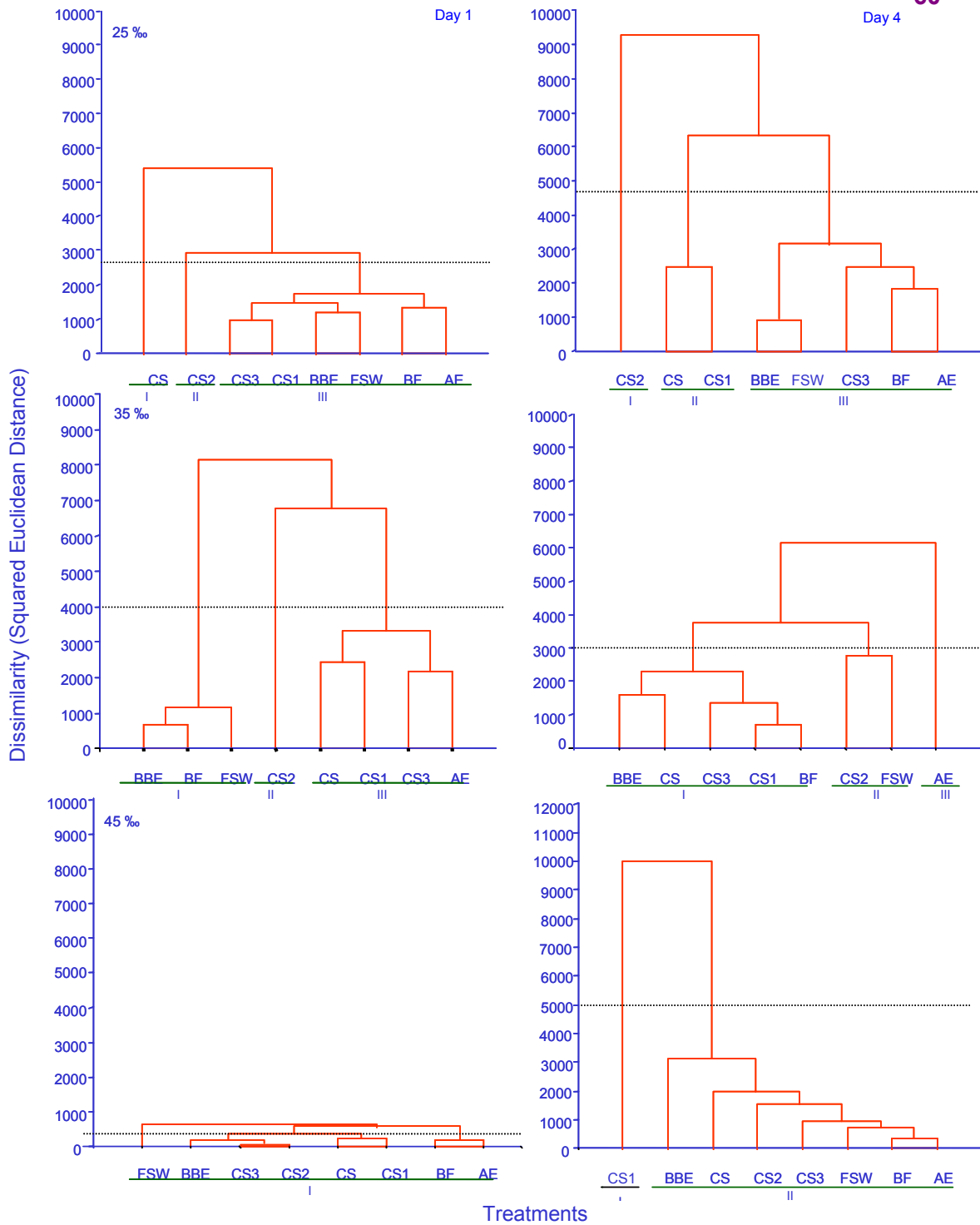


Fig. 3B.3 Dendrograms showing the dissimilarity among different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 25, 35 and 45%. The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed

The effect of the culture supernatant fractions containing different molecular weight substances was different on metamorphosis (Fig. 3B.4).

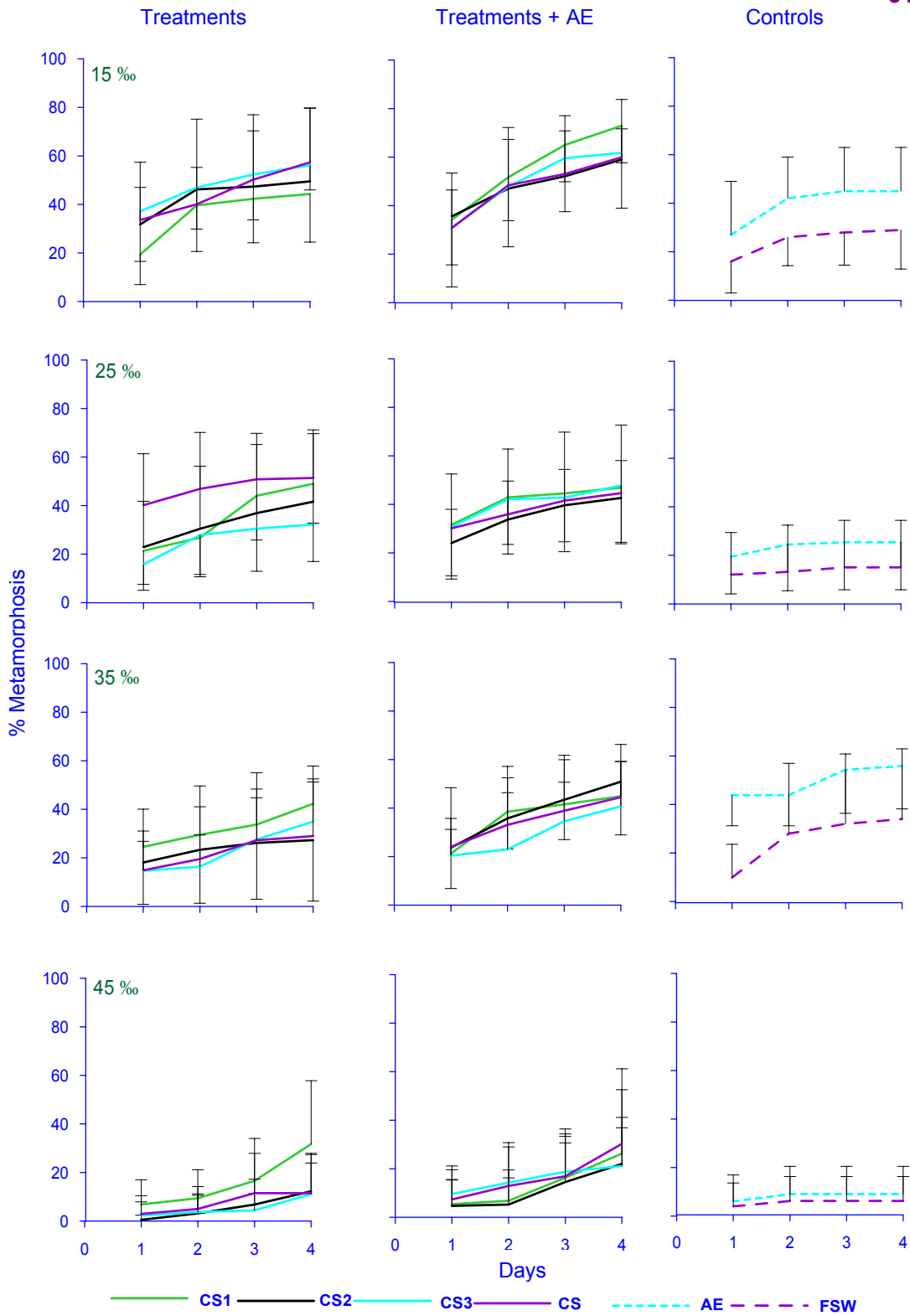


Fig. 3B.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution in presence and absence of adult extract (AE). Vertical lines indicate the standard deviation from mean and are indicated as either positive or negative

A significant difference in the metamorphosis inducement with respect to salinity was evident (Table 3B.1c,e,g & i) at the end of day 1. Culture supernatant (CS) facilitated higher metamorphosis than that observed with AE at 25‰, whereas CS3 was effective at 15‰. Higher molecular weight fraction was effective at lower salinity.

When CS and its fractions were assessed in presence of AE, there was no increase in the metamorphosis rates. Except for CS3, the differences in metamorphosis rates were significant at different salinities.

The influence of bacterial extract varied with salinity (Fig. 3B.5). The influence of bacterial extract in presence and absence of AE was significantly different with respect to salinity on day 1 & 4 (Table 3B.1 k,l). BBE was inhibitory at 35‰, whereas at 15 and 25‰, BBE facilitated metamorphosis more than that of AE when assessed in presence of AE.

A marginal increase in metamorphosis rates was evident with the aging of the settlement cues and cyprids.

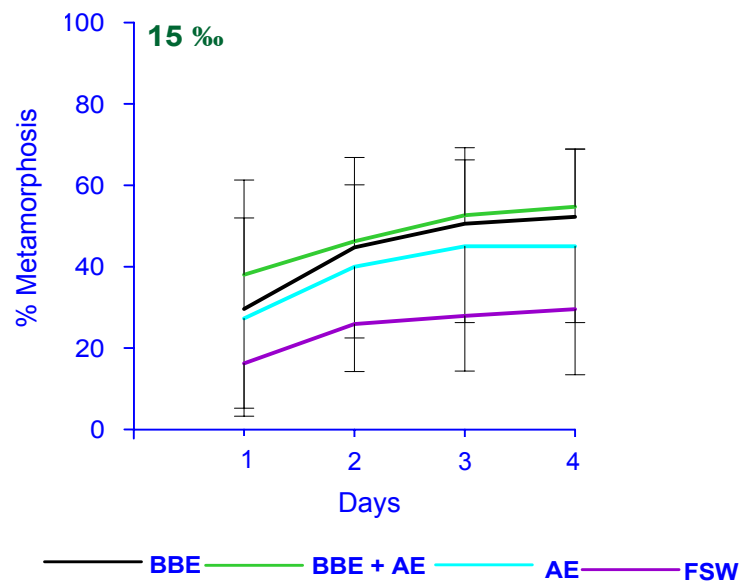


Fig. 3B.5 Percentage metamorphosis of *B. amphitrite* cyprids exposed to Butanol- bacterial extract at 15‰. Vertical lines indicate the standard deviation from mean and are indicated as either positive or negative

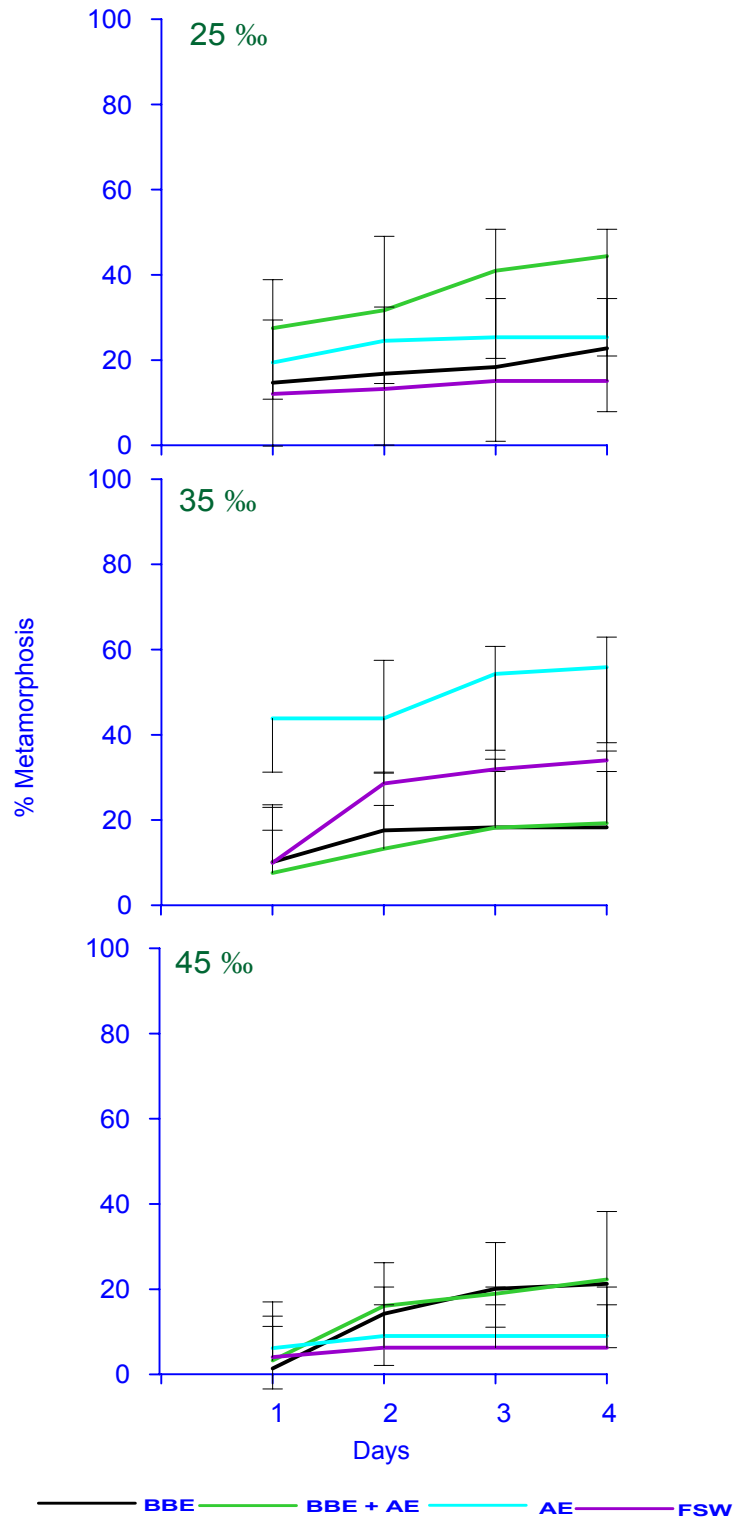


Fig. 3B.5 Percentage metamorphosis of *B. amphitrite* cyprids exposed to Butanol-bacterial extract at 25, 35 and 45%. Vertical lines indicate the standard deviation from mean and are indicated as either positive or negative



### 3B 3.2 Experiment 2

Metamorphosis rates were lower when the surface-bound components of the bacterial extract were assessed along with the leachants (Fig. 3B.6). The cyprids did not settle in presence of leachants of BBE and CS3 at the end of Day 1.

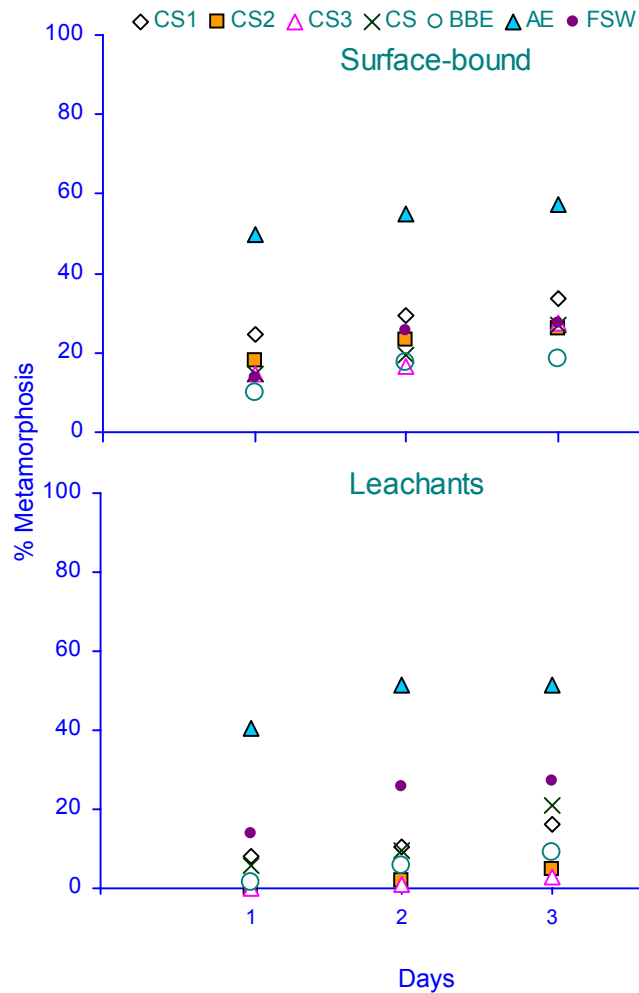


Fig. 3B.6 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial inducers (surface-bound) used in experiment 1 in presence of leachants at 35%

### 3B 3.3 Experiment 3

No culture supernatant was effective in inducing the metamorphosis of the cyprids (Fig. 3B.7). The differences in the responses of culture supernatants obtained using different nutritional conditions were not significant (Table 3B.3a). However, in presence of AE significant differences were evident at the carbohydrate concentration of 25 & 50  $\mu\text{g ml}^{-1}$  on day 1 and 4 (Table 3B.3b).

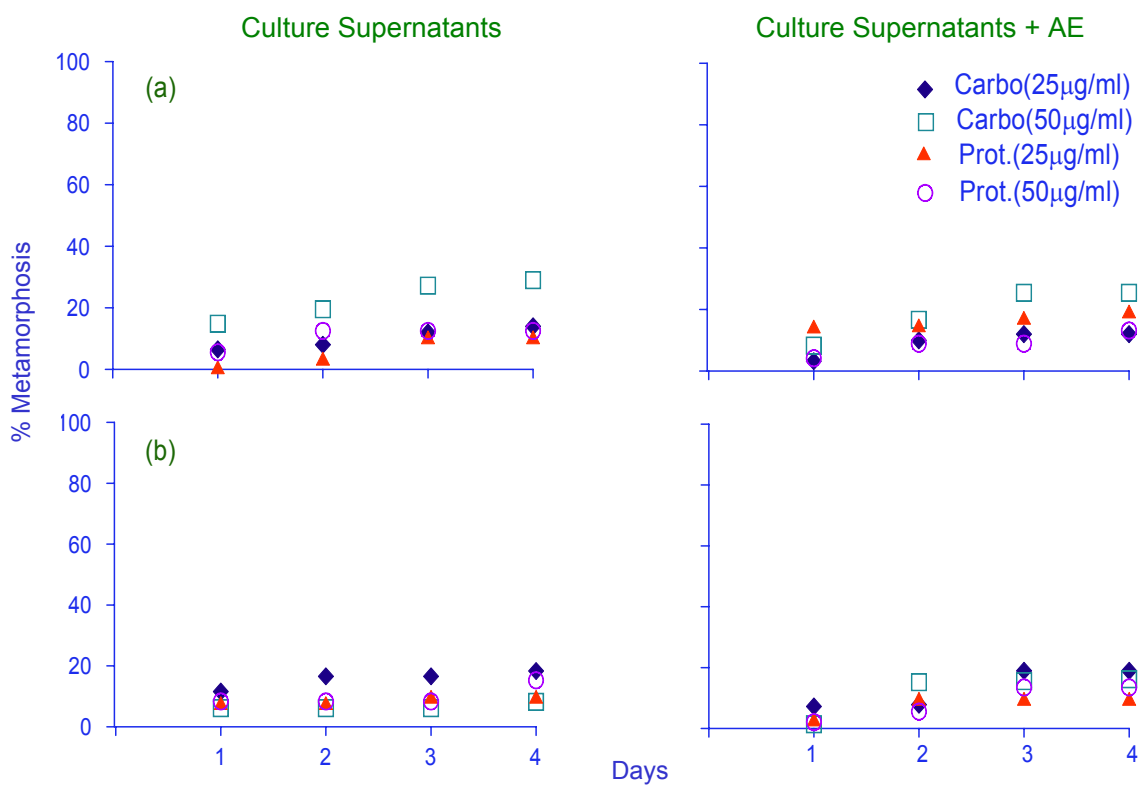


Fig. 3B.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutrient media at 35‰. (a) CS(BSS) (b) CS(semi-solid)

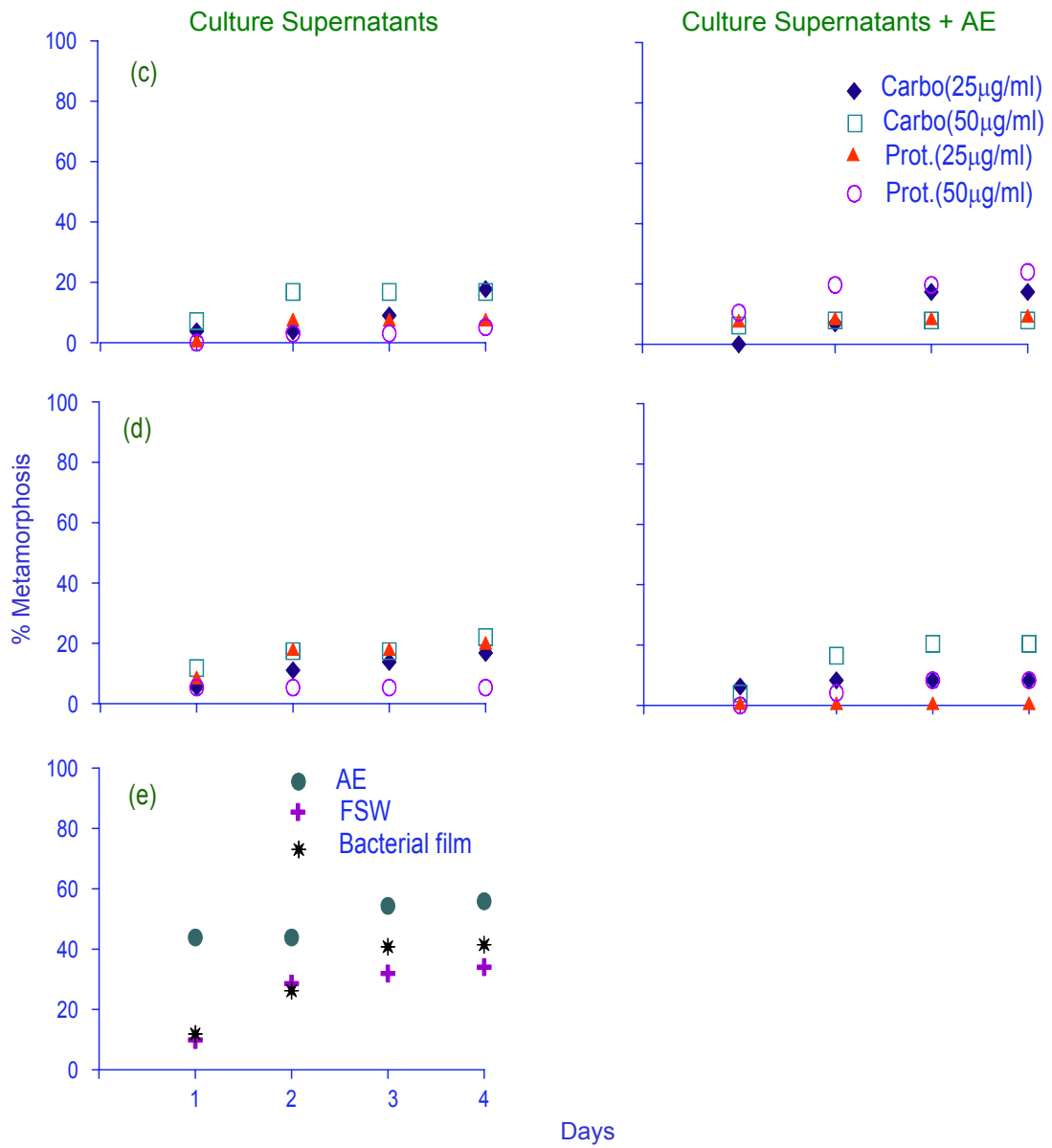


Fig. 3B.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutrient media at 35%. (c) CS(MV) (d) CS(MB) (e) Controls

Table 3B.3a One-way ANOVA. The influence of culture supernatants (CS) extracted by four different extraction protocols at two different concentrations of carbohydrates and proteins on the metamorphosis of *B. amphitrite* cyprids.

Factor		25µg/ml (carbohydrates)			50µg/ml (carbohydrates)			25µg/ml (proteins)			50µg/ml (proteins)			
		df	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
<b>Day1</b>														
	CS	3	338	112	1.7ns	206	69	1ns	244	81	1.5ns	341	114	0.6ns
	Within sub. Gr.err.	44	2910	66		2803	64		2411	55		8411	191	
	Total	47	3248			3009			2655			8752		
<b>Day4</b>														
	CS	3	455	151	1.3ns	1015	339	3.2**	738	246	0.9ns	616	205	0.8ns
	Within sub. Gr.err.	44	4979	113		4598	104		12580	286		10775	245	
	Total	47	5434			5613			13318			11391		

Table 3B.3b One-way ANOVA. The influence of culture supernatants (CS) extracted by four different extraction protocols at two different concentrations of carbohydrates and proteins in presence of AE on the cyprid metamorphosis of *B. amphitrite*. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor		25µg/ml (carbohydrates)			50µg/ml (carbohydrates)			25µg/ml (proteins)			50µg/ml (proteins)			
		df	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
<b>Day1</b>														
	CS	3	1174	391	10*****	1130	377	7.5*****	820	273	1.4ns	266	89	2.0ns
	Within sub. Gr.err.	44	1765	40		2208	50		8714	198		1909	43	
	Total	47	2939			3338			9534			2175		
<b>Day4</b>														
	CS	3	2753	918	4.2***	3025	1008	2.7*	1132	377	1.9ns	1000	333	1.0ns
	Within sub. Gr.err.	44	9440	214		16467	374		8733	198		13352	303	
	Total	47	12193			19492			9865			14352		

(\*\*\*\*\*p≤0.001, \*\*\*\*p≤0.005, \*\*\*p≤0.01, \*\* p≤0.025, \*p≤0.05, ns- not significant)

### 3B 3.4 Experiment 4

The exopolysaccharides extracted using different nutritional media did not induce metamorphosis and their response was not comparable to that of AE (Fig. 3B.8a,b).

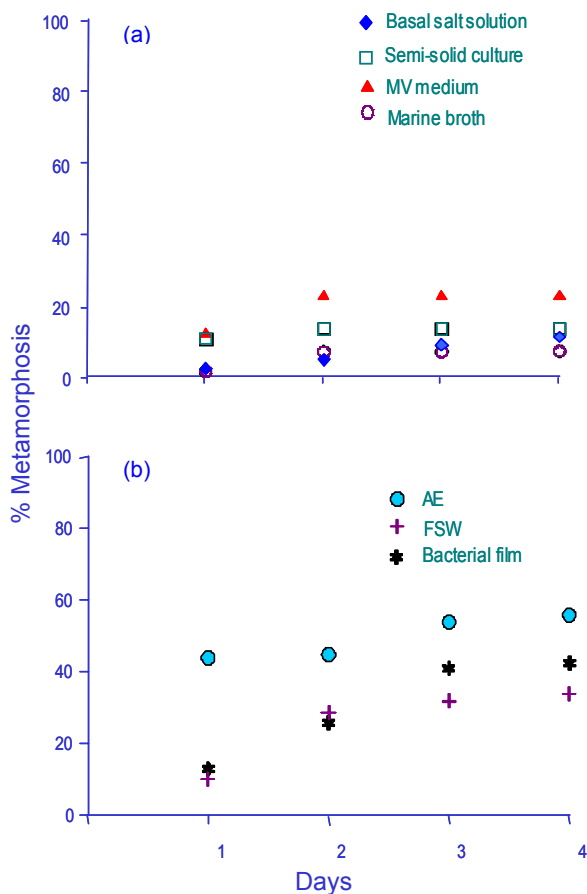


Fig. 3B.8 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial exopolysaccharides obtained after growing the bacteria under different nutritional conditions at 35‰

### 3B 3.5 FTIR spectroscopy

Fourier transformed infrared spectrum of culture supernatants grown in MB, BSS and MV revealed characteristic strong peaks around  $3550-3200\text{ cm}^{-1}$ ,  $1640\text{ cm}^{-1}$  and  $1090-1117\text{ cm}^{-1}$  for O-H stretching, C=O stretching and C-O stretching and hence were mainly carbohydrates.

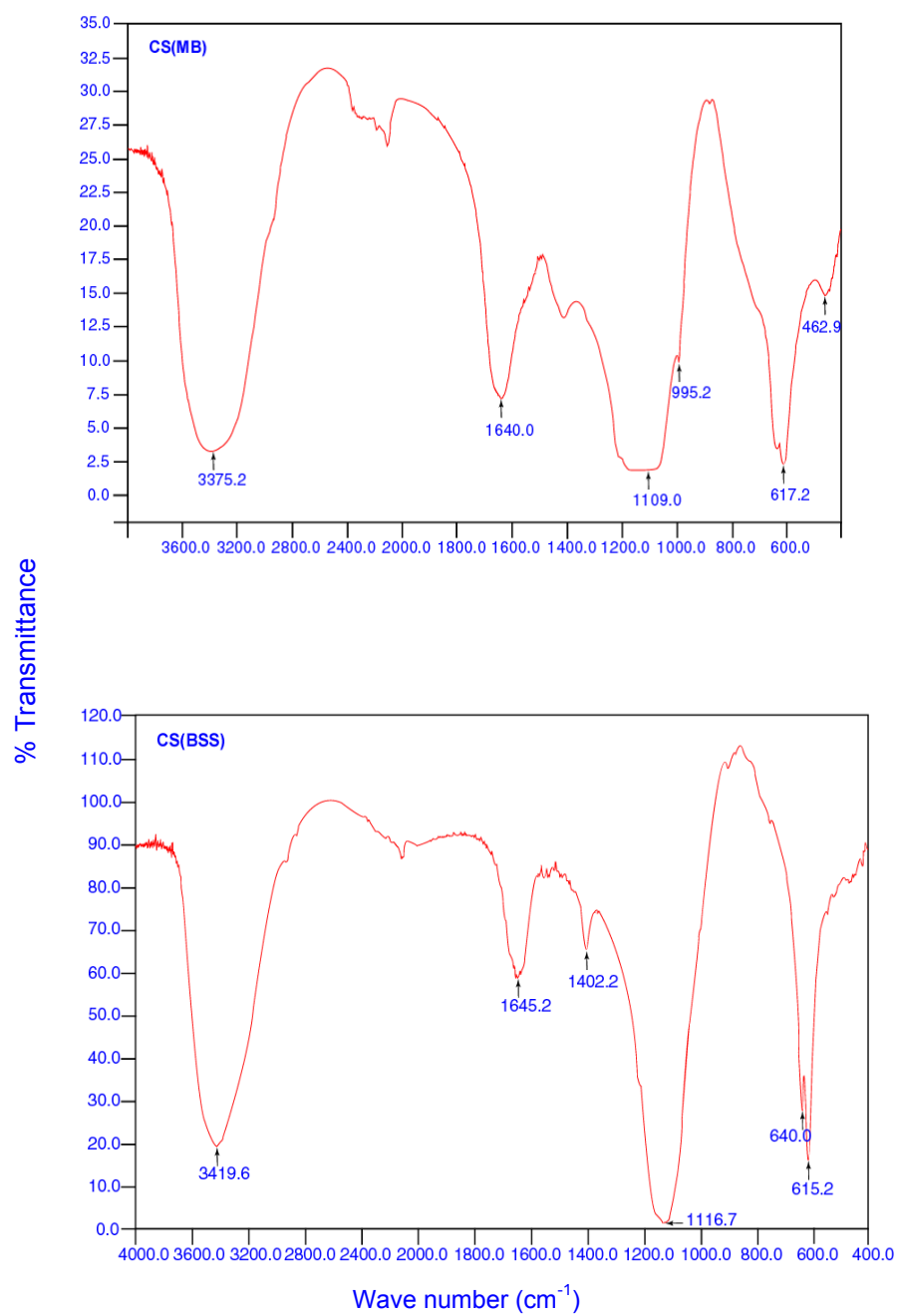


Fig. 3B.9 FTIR spectras of culture supernatants of bacteria grown in MB and basal salt solution (BSS)

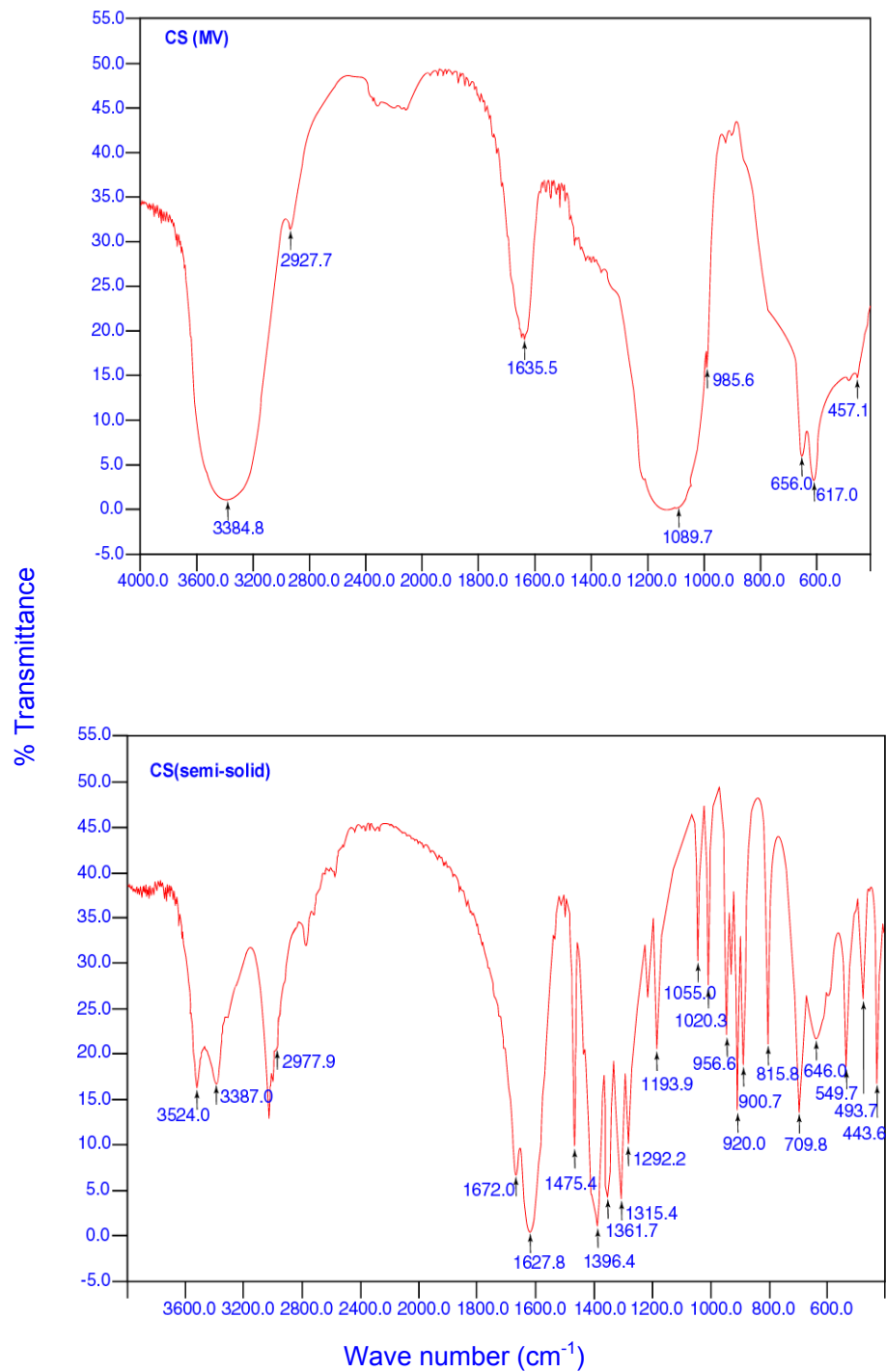


Fig. 3B.9 FTIR spectra of culture supernatants of bacteria grown in MV and semi-solid medium

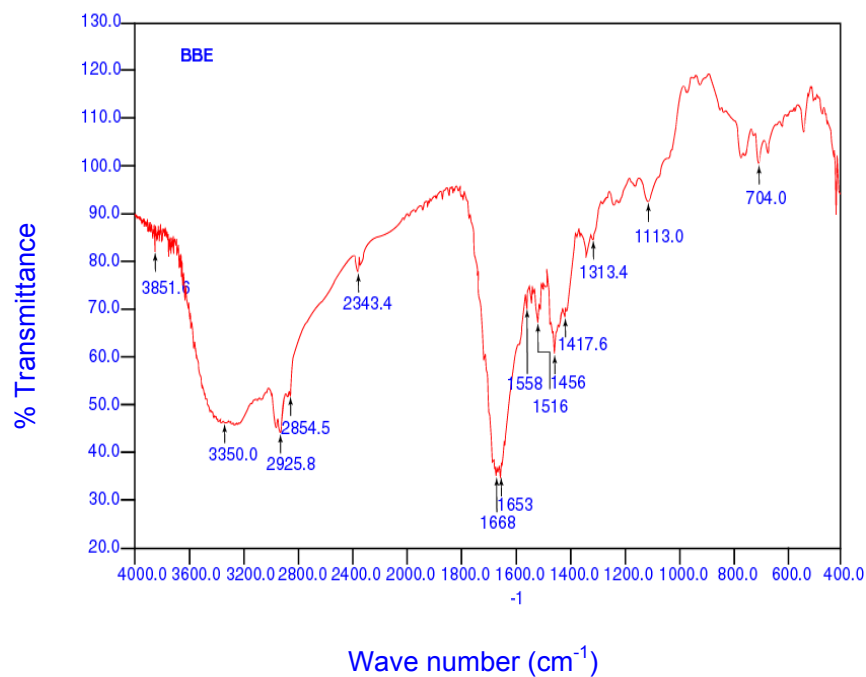


Fig 3B.9 FTIR spectra of Butanol-bacterial extract

Culture supernatant of semi-solid culture and bacterial extract revealed strong peaks at 1628 and 1653 cm<sup>-1</sup> for N-H bend and were proteinaceous. In case of semi-solid culture supernatant presence of terpenoides and steroids were evidenced from IR absorption at 1475 and 1362 cm<sup>-1</sup>. The spectra also indicated the presence of unsaturation evident from C-H bending vibrations located around 1000 and 900 cm<sup>-1</sup>. Bacterial extract showed peak at 1668 cm<sup>-1</sup> characteristic for ketones.



### 3C. *Citrobacter freundii*

#### 3C 3.1 Experiment 1

The bacterial film of *C. freundii* did not facilitate metamorphosis at 35‰ (Fig. 3C.1). A significant difference in the metamorphosis rates with respect to salinity was evident on day 1 ( $p \leq 0.025$ , one-way ANOVA;  $p \leq 0.05$ , Scheffe's test).

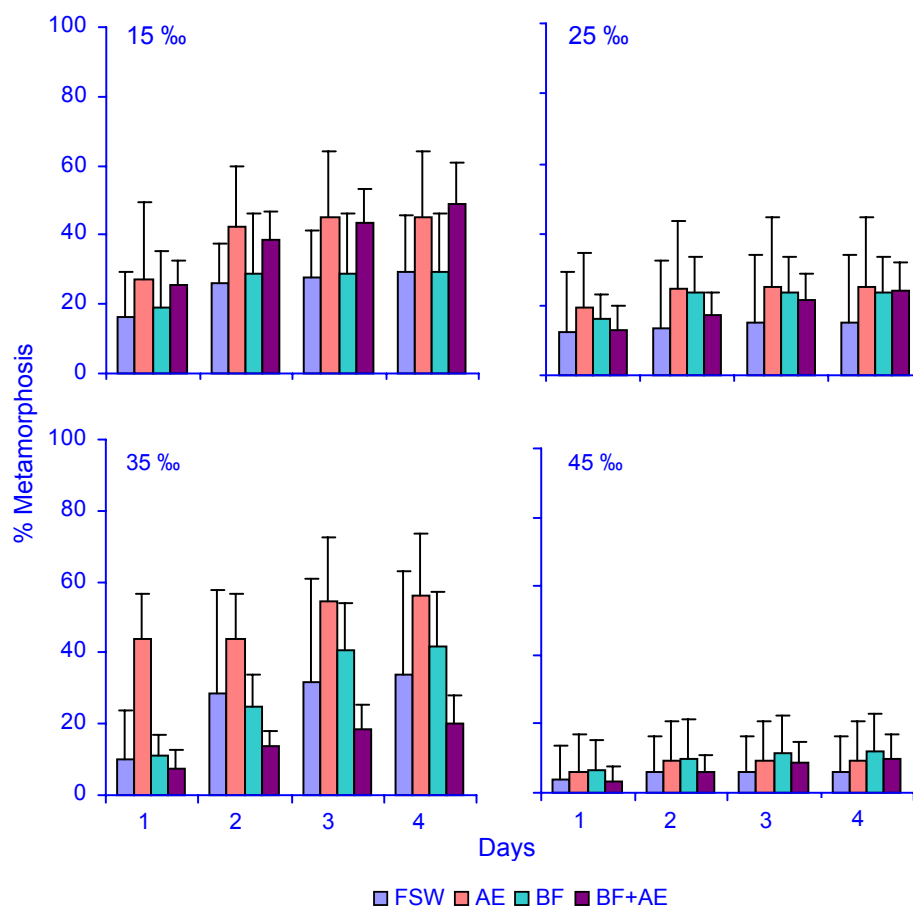


Fig. 3C.1 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*C. freundii*) in presence of adult extract (AE) at different salinities. Vertical lines indicate the standard deviation from mean and are shown as positive

The differences were highly significant with the aging of the film and the cyprids (Table 3C.1a) and in presence of AE (Table 3C.1b).

Table 3C.1. One-way ANOVA. The influence of *C. freundii*, culture supernatant, fractions and butanol-bacterial extract at different salinities on the metamorphosis of cyprids of *B. amphitrite*. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor		Day 1			Day 4				Day 1			Day 4			
		df	SS	MS	Fs	SS	MS		Fs	SS	MS	Fs	SS	MS	Fs
<b>(a) BF</b>	Salinity	3	378	126	3**	2046	682	9*****	<b>(b) BF+AE</b>	1148	383	34*****	3754	1251	40*****
	Within sub. Gr.err.	44	1695	38		3377	77			500	11		1383	31	
	Total	47	2073			5423				1648			5137		
<b>(c) CS1</b>	Salinity	3	5365	1788	4.5***	10985	3661	7*****	<b>(d) CS1+AE</b>	7513	2504	13*****	8164	2721	10*****
	Within sub. Gr.err.	44	17463	397		21863	497			8687	197		11706	266	
	Total	47	22828			32848				16200			19870		
<b>(e) CS2</b>	Salinity	3	5673	1891	20*****	6191	2064	10*****	<b>(f) CS2+AE</b>	2982	994	6*****	6771	2257	11*****
	Within sub. Gr.err.	44	4240	96		8650	196			6834	155		9225	210	
	Total	47	9913			14841				9816			15996		
<b>(g) CS3</b>	Salinity	3	4161	1387	6.3*****	2815	938	2.8*	<b>(h) CS3+AE</b>	6224	2075	13*****	7625	2542	12*****
	Within sub. Gr.err.	44	9672	220		14781	336			6967	158		9453	215	
	Total	47	13833			17596				13191			17078		
<b>(i) CS</b>	Salinity	3	1096	365	2ns	2549	850	2.5ns	<b>(j) CS+AE</b>	171	57	0.4ns	3150	1050	6****
	Within sub. Gr.err.	44	7334	167		14745	335			6948	158		7817	178	
	Total	47	8430			17294				7119			10967		
<b>(k) BBE</b>	Salinity	3	1807	602	7*****	8435	2811	20*****	<b>(l) BBE+AE</b>	3252	1084	11*****	5813	1937	6*****
	Within sub. Gr.err.	44	3727	85		6258	142			4411	100		13295	302	
	Total	47	5534			14693				7663			19108		

(\*\*\*\*\*p≤0.001, \*\*\*\*p≤0.005, \*\*\*p≤0.01, \*\* p≤0.025, \*p≤0.05, ns- not significant)

When the bacterial film was assessed in presence of AE a decrease in the metamorphosis rates was evident at 25, 35 and 45‰, whereas at 15‰, the metamorphosis rates were similar as that shown by AE.

At 20° C, the bacterial film facilitated metamorphosis higher than that of AE at 15 & 25‰. Whereas, at 30° C, the metamorphosis was facilitated at 15‰ at the end of day 1 (Fig. 3C.2). At 26° C, BF did not facilitate metamorphosis.

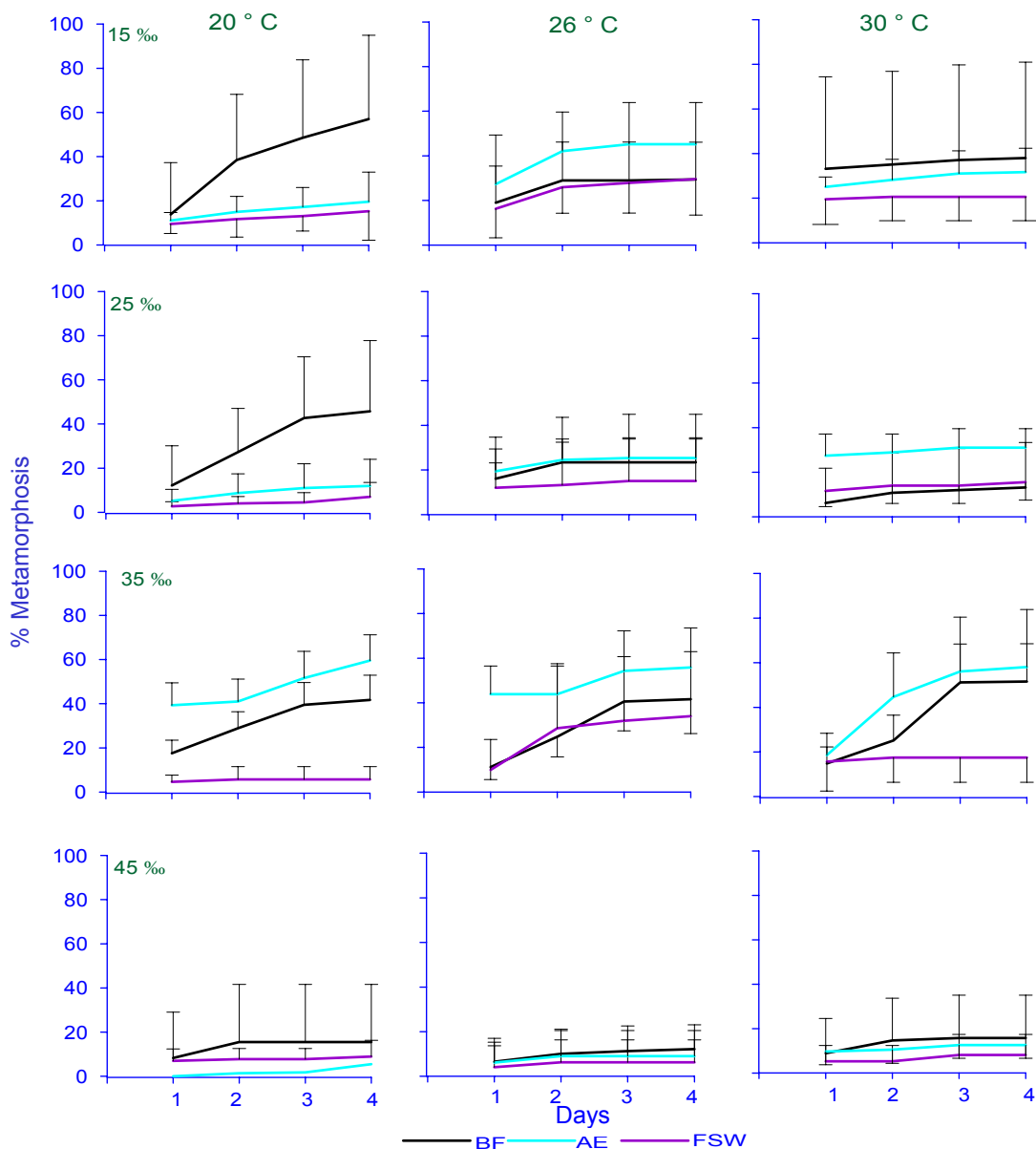


Fig. 3C.2 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*C. freundii*), adult extract (AE) and filtered sea water (FSW) at different salinities and temperatures. Vertical lines indicate the standard deviation from mean and are shown as either positive or negative

However, three-way ANOVA indicated the differences between bacterial film, AE and FSW to be non-significant with respect to salinity and temperature at the end of day 1 and 4 (Table 3C.2).

Table 3C.2 Three-way ANOVA. The influence of temperature (20, 26 & 30° C) and salinities (15, 25, 35 & 45‰) with respect to treatments (bacterial film, AE and FSW ) on the metamorphosis of *B. amphitrite* cyprids on day 1 and day 4. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

	df	Day 1			Day 4		
		SS	MS	Fs	SS	MS	Fs
A (temperature)	2	155	78		53	26	
B (salinity)	3	348	116		700	233	
C (treatments)	2	98	49		959	480	
A*B	6	102	17	0.98ns	334	56	1.1ns
A*C	4	101	25	1.4ns	303	76	1.6ns
B*C	6	115	19	1.1ns	238	40	0.8ns
A*B*C	12	207	17		583	48	
Total	35	1126			3170		

(ns- not significant)

Cluster analysis indicated the response of cyprids to differ with the salinity, age of the cyprids as well as the cues. At 15‰, CS2 and CS1 showed a similar response, whereas at 25 & 35 ‰ CS1 was the most dissimilar fraction. (Fig. 3C.3; Day 1). The response differed with the age of the cyprids and the cues (Fig. 3C.3; Day 4).

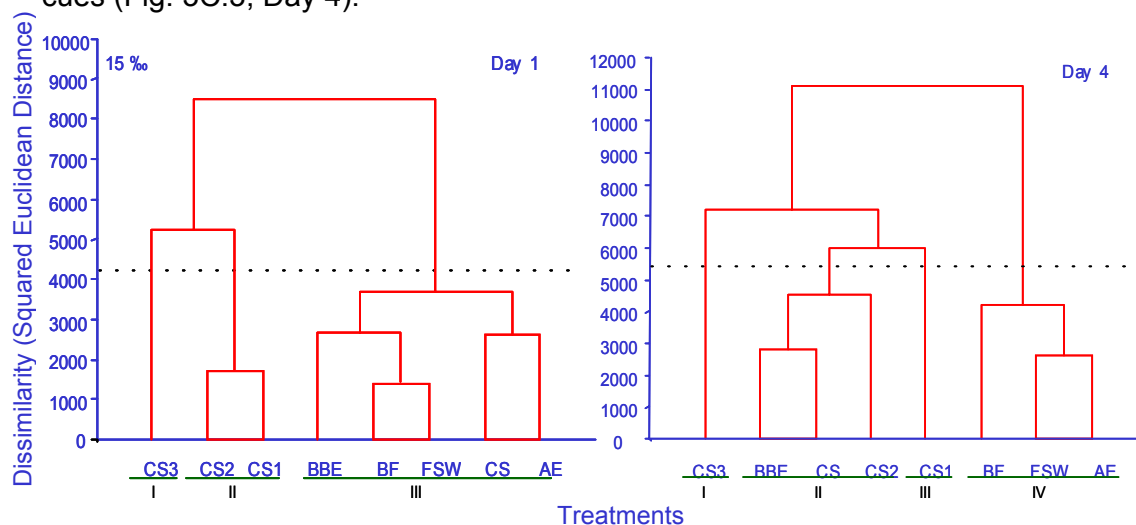


Fig. 3C.3 Dendrograms showing the dissimilarity among different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 15‰. The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed

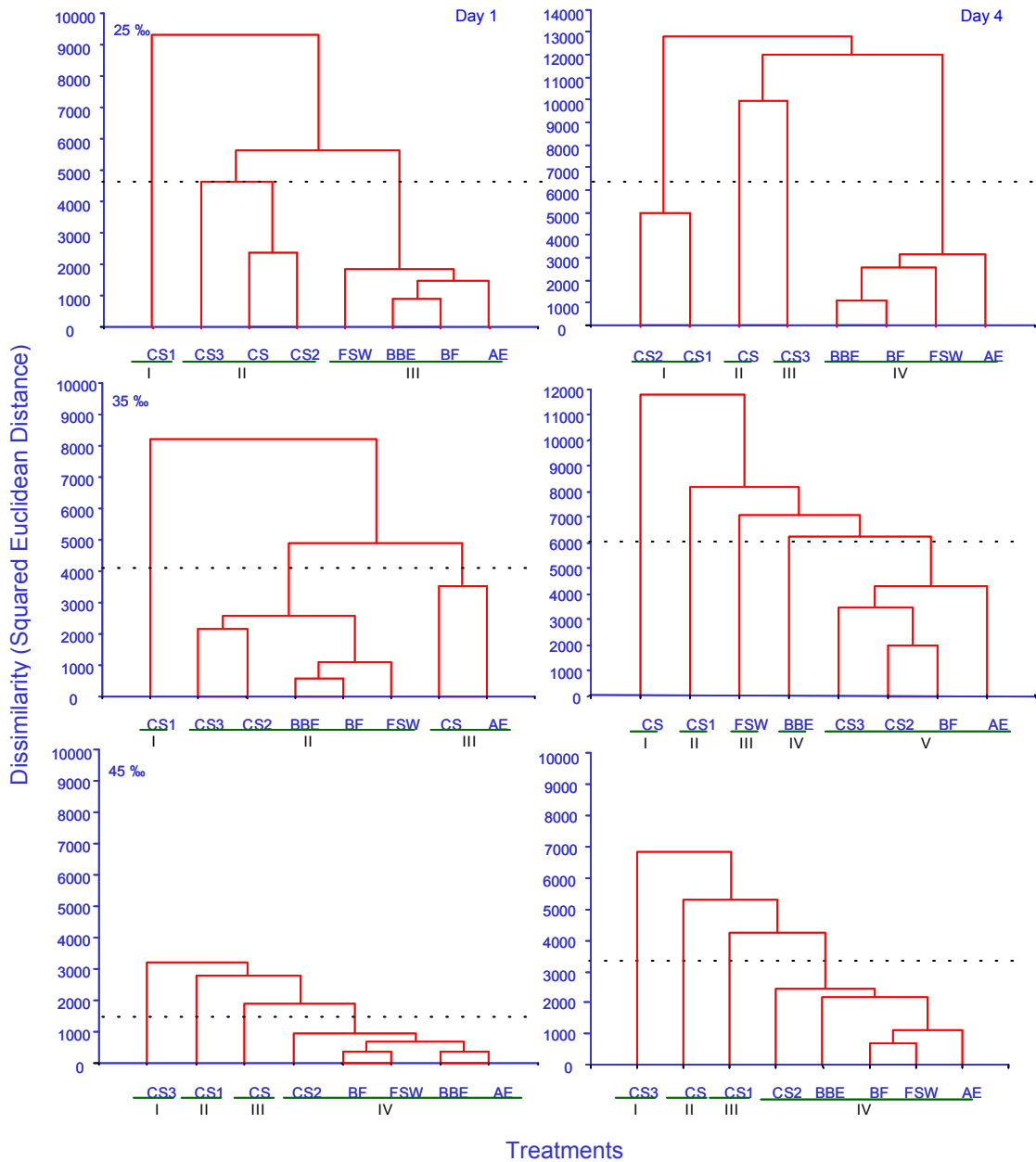


Fig. 3C.3 Dendrograms showing the dissimilarity among different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 25, 35 and 45%. The X-axis groupings are based on the clusters that are dissimilar beyond midpoint of highest dissimilarity observed

Metamorphosis rates of culture supernatant and fractions were comparatively higher than that of AE at 15 & 25‰ (Fig. 3C.4). A significant difference in metamorphosis inducement with respect to salinity was evident with CS1, CS2, CS3 on day 1 and 4 (Table 3C.1 c,e,g), whereas, no significant difference was observed with the culture supernatant (Table 3C.1i). When they were assessed in presence of AE, a significant difference in the metamorphosis was observed with respect to salinities ( $p \leq 0.001$ , one-way ANOVA;  $p \leq 0.05$ , Scheffe's test) on day 1, except for CS. Lower molecular weight fraction was effective at lower salinity and vice-versa.

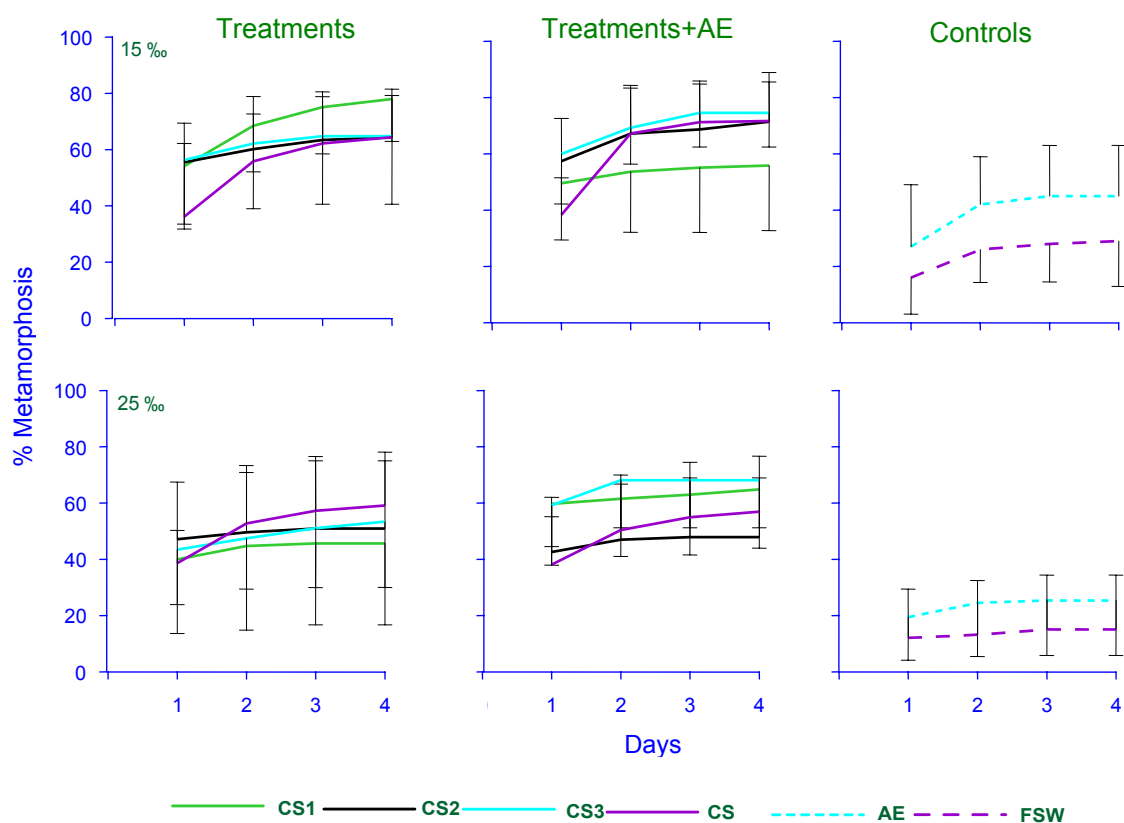


Fig. 3C.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution (BSS) in presence and absence of adult extract (AE) at 15 and 25‰. Vertical lines indicate the standard deviation from mean and are shown as either positive or negative

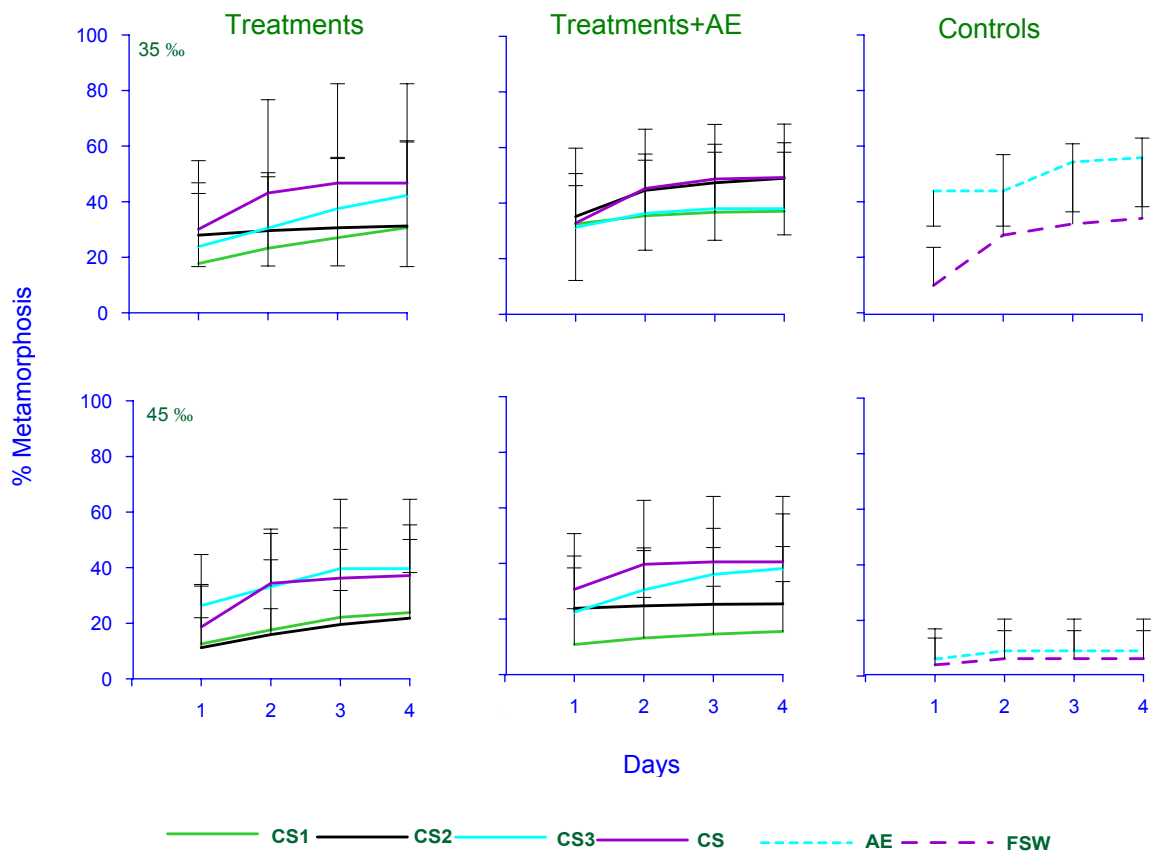


Fig. 3C.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution (BSS) in presence and absence of adult extract (AE) at 35 and 45‰. Vertical lines indicate the standard deviation from mean and are shown as either positive or negative

The bacterial extract facilitated metamorphosis at 15‰, whereas, it was inhibitory at all other salinities. The inhibitory effect was nullified in presence of AE at 25‰ (Fig. 3C.5). The influence of bacterial extract in presence and absence of AE was significantly different with respect to salinity on day 1 and 4 (Table 3C.1 k,l).

A marginal increase in the metamorphosis rates was evident with the aging of the settlement cues and cyprids.

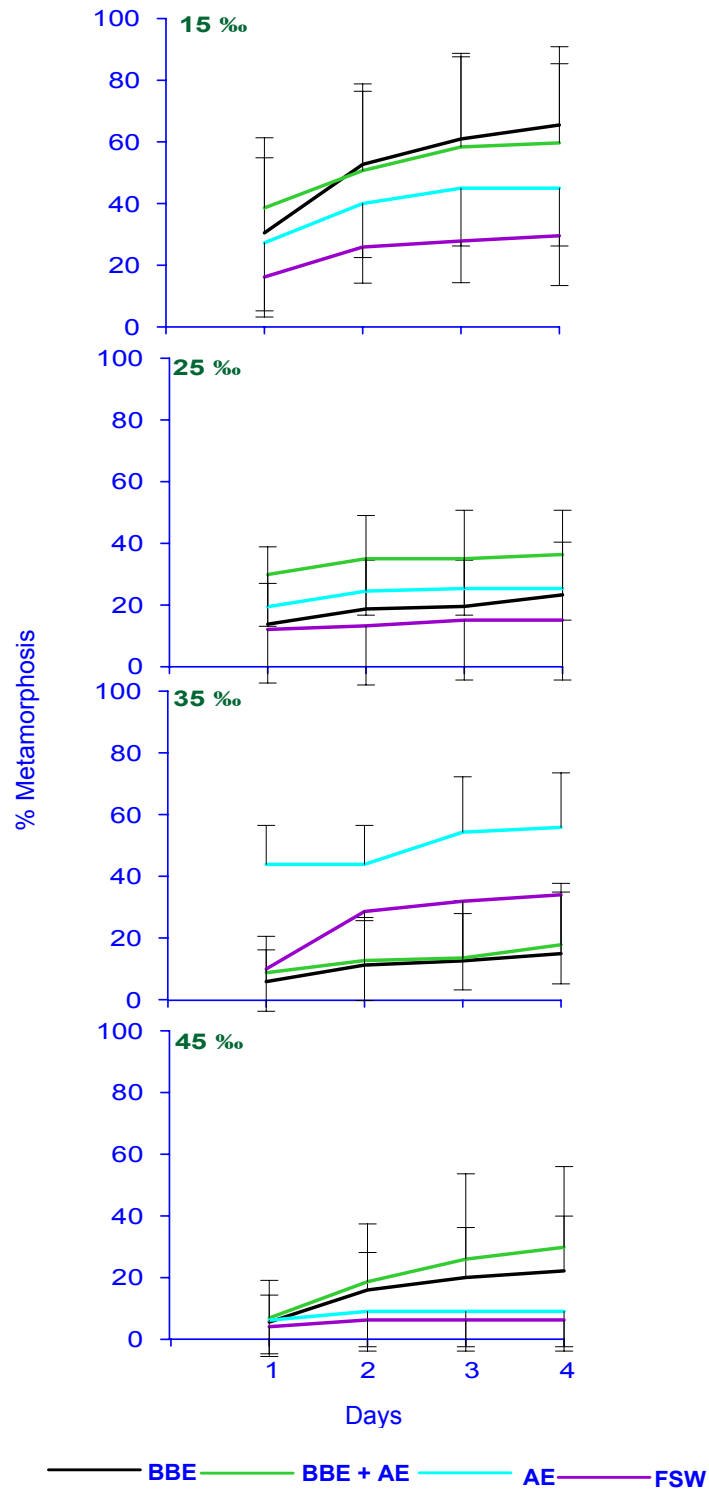


Fig. 3C.5 Percentage metamorphosis of *B. amphitrite* cyprids exposed to Butanol-bacterial extract at different salinities. Vertical lines indicate the standard deviation from mean and are shown as either positive or negative



### 3C 3.2 Experiment 2

When the surface-bound components of the bacterial extract were assessed along with the leachants, a decrease in the metamorphosis rates was evident (Fig. 3C.6). The cyprid did not settle in presence of leachants of BBE and CS3 at the end of Day 1.

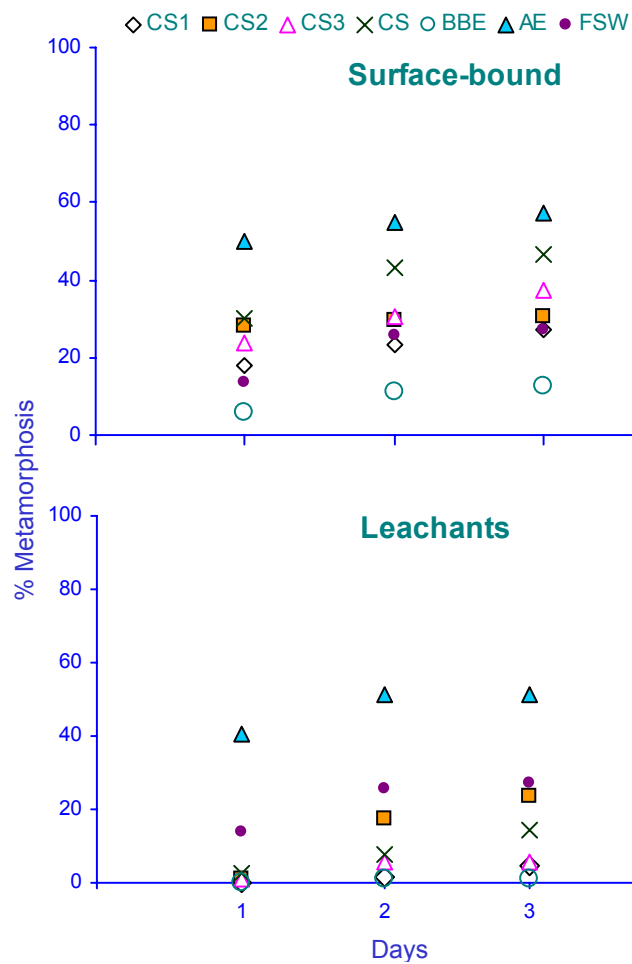


Fig. 3C.6 Percentage metamorphosis of *B. amphitrite* cyprids in response to bacterial inducers (surface-bound) used in experiment 1 in presence of leachants at 35%

### 3C 3.3 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.

The differences among the culture supernatants extracted using different extraction protocols were significant at carbohydrate concentrations of 25 and 50  $\mu\text{g ml}^{-1}$  ( $p \leq 0.001$ , one-way;  $p \leq 0.05$ , Scheffe's test) on Day 1 and 4 (Fig. 3C.7, Table 3C.3a). The larvae metamorphosed in higher percentages when exposed to culture supernatant obtained by using BSS at a carbohydrate concentration of 50  $\mu\text{g ml}^{-1}$ .

When assessed in presence of AE a significant difference was observed at a carbohydrate concentration of 50  $\mu\text{g ml}^{-1}$  on day 1 and 4 ( $p \leq 0.001$ , one-way;  $p \leq 0.05$ , Scheffe's test; Fig. 3C.7, Table 3C.3b).

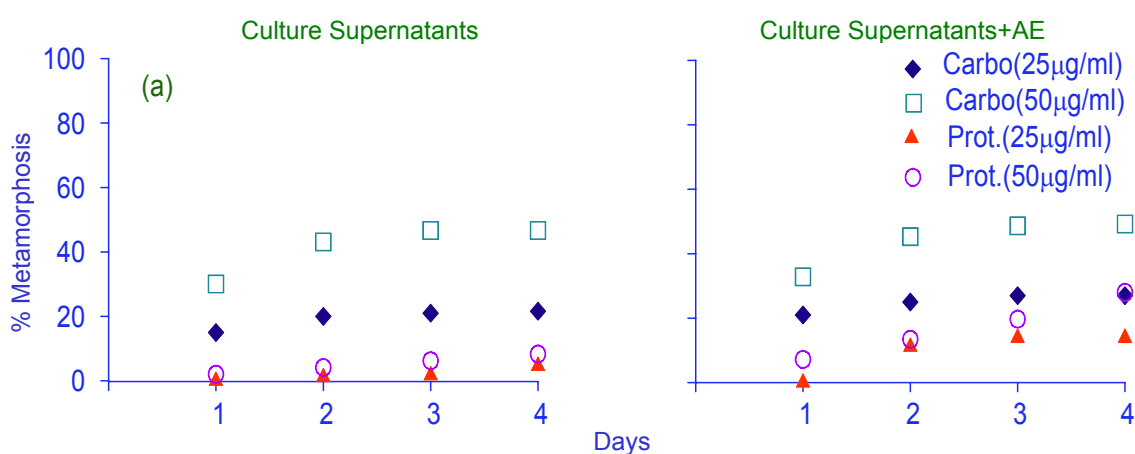


Fig. 3C.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatant obtained by growing the bacteria under basal salt solution (BSS) at 35‰

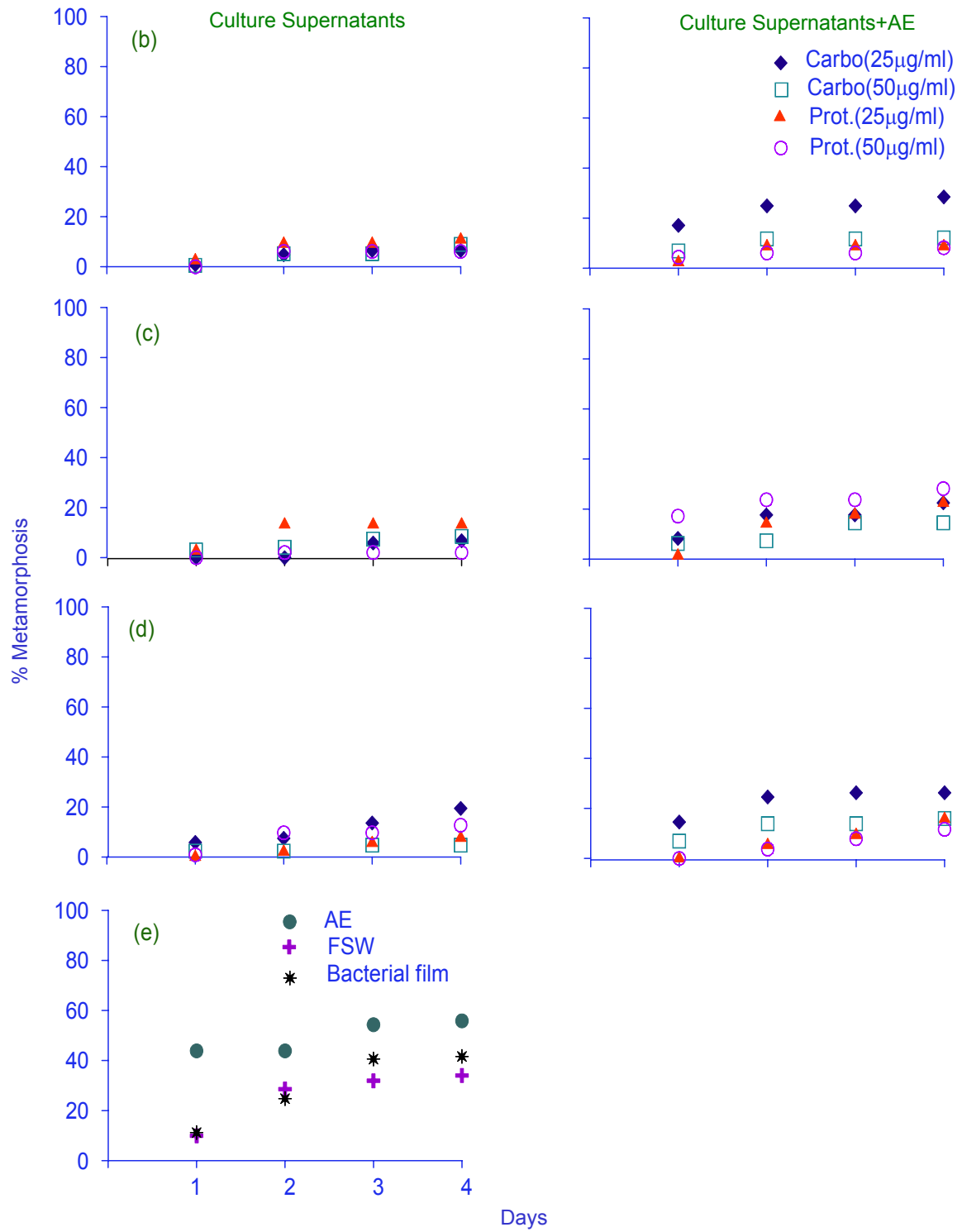


Fig. 3C.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutritional media at 35‰. (b) CS(semi-solid) (c) CS(MV) (d) CS(MB) (e) Controls

Table 3C.3a One-way ANOVA. The influence of culture supernatants (CS) extracted by four different extraction protocols at two different concentrations of carbohydrates and proteins on the metamorphosis of *B. amphitrite* cyprids. (df. degree of freedom; SS. sum of the squares; MS. mean of squares; Fs. Fischer constant).

Factor	25µg/ml (carbohydrates)				50µg/ml (carbohydrates)			25µg/ml (proteins)			50µg/ml (proteins)		
	df	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
<b>Day1</b>													
CS	3	2695	898	12*****	2665	888	12*****	28	9.5	0.7ns	12	4	0.75ns
Within sub. Gr.err.	44	3303	75		3239	74		626	14		229	5.2	
Total	47	5998			5904			654			241		
<b>Day4</b>													
CS	3	6358	2119	9*****	6922	2307	9.6*****	204	68	0.5ns	348	116	0.5ns
Within sub.Gr.err.	44	10207	232		10610	241		5805	132		9725	221	
Total	47	16565			17532			6009			10073		

Table 3C.3b One-way ANOVA. The influence of culture supernatants (CS) extracted by four different extraction protocols at two different concentrations of carbohydrates and proteins in presence of AE on the cyprid metamorphosis of *B. amphitrite*.

Factor	25µg/ml (carbohydrates)				50µg/ml (carbohydrates)			25µg/ml (proteins)			50µg/ml (proteins)		
	df	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
<b>Day1</b>													
CS	3	1520	507	2.5ns	2375	791	7*****	16	5.4	0.7ns	676	225	2.4ns
Within sub. Gr.err.	44	8759	199		5172	117		336	7.6		4046	92	
Total	47	10279			7547			352			4722		
<b>Day4</b>													
CS	3	1613	538	1.1ns	4349	1449	8*****	406	135	0.5ns	1983	661	2.0ns
Within sub.Gr.err.	44	20358	463		7934	180		11414	259		14344	326	
Total	47	21971			12283			11820			16327		

(\*\*\*\*\*p≤0.001, \*\*\*\*p≤0.005, \*\*\*p≤0.01, \*\* p≤0.025, \*p≤0.05, ns- not significant)

### 3C 3.4 Experiment 4

The exopolysaccharides extracted using different nutritional media did not induce metamorphosis and were not comparable to that of AE (Fig. 3C.8a,b).

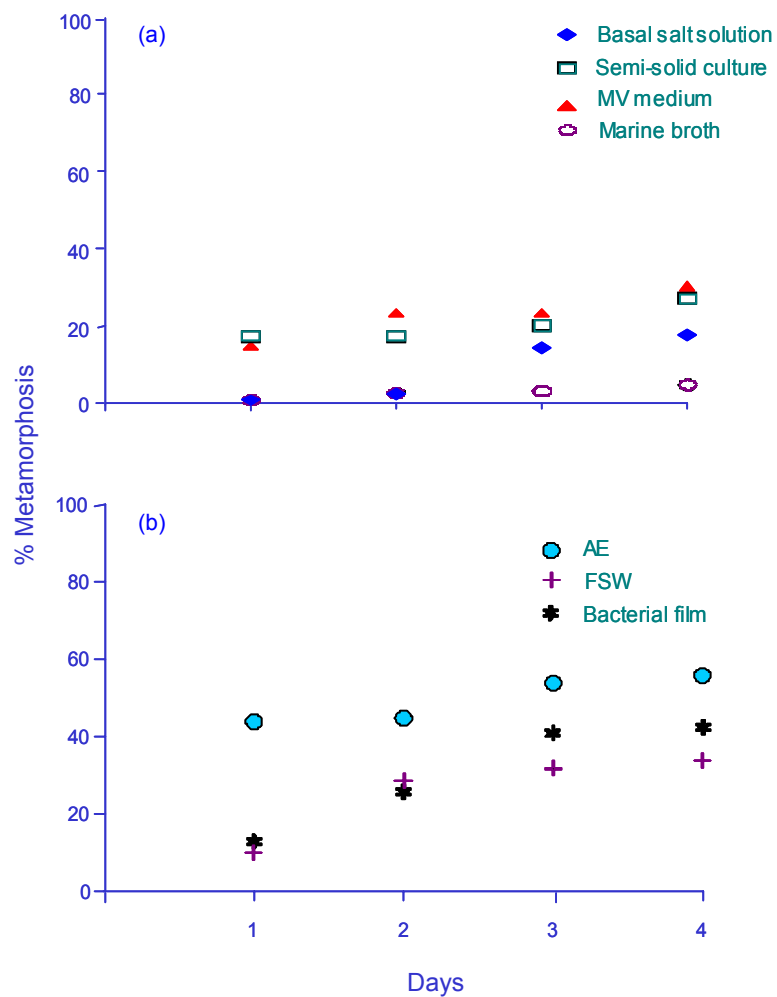


Fig. 3C.8 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial exopolysaccharides obtained after growing the bacteria under different nutritional conditions at 35‰

## 3C 3.5 FTIR spectroscopy

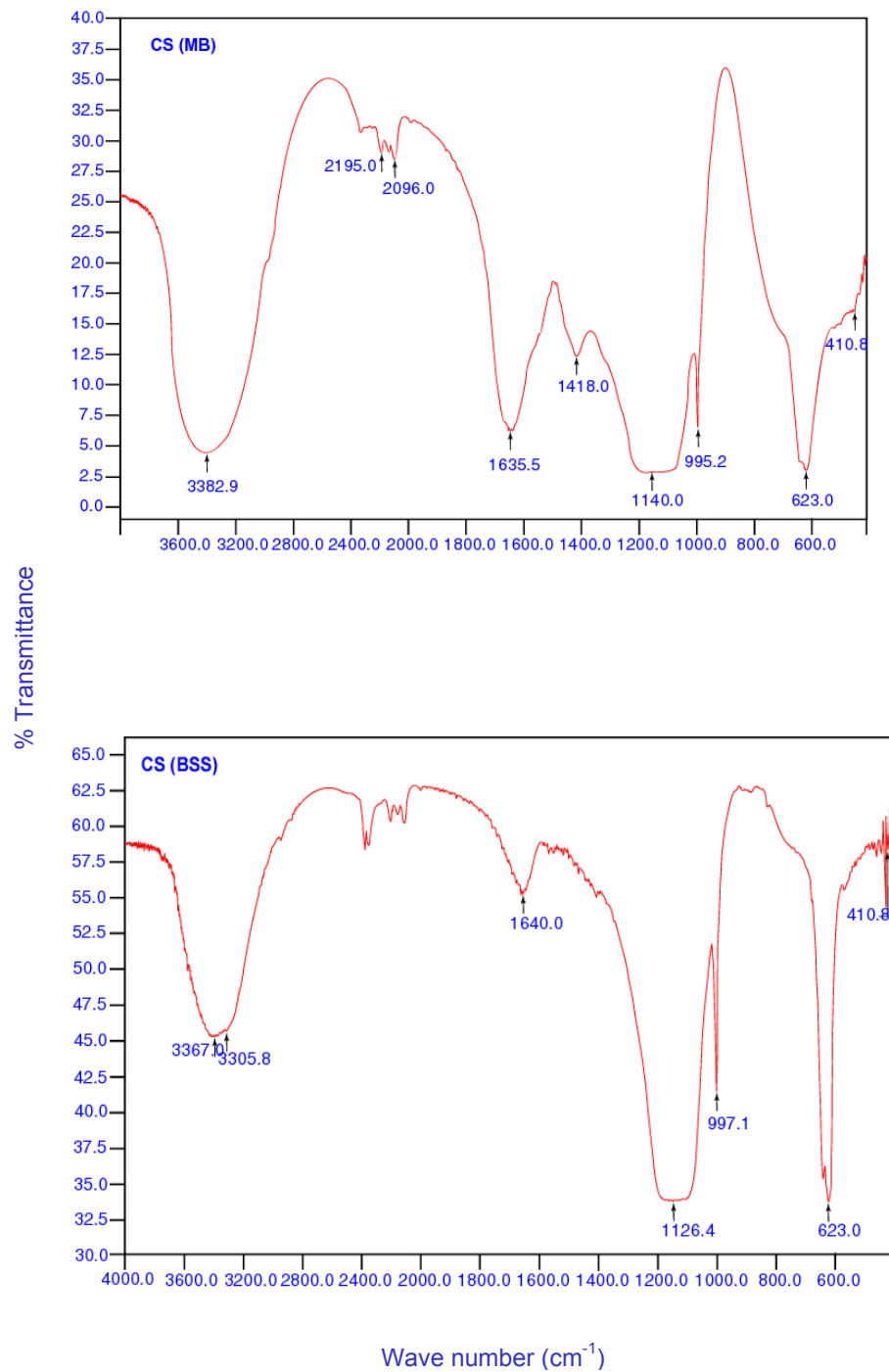


Fig. 3C.9 FTIR spectras of culture supernatants of bacteria grown in MB and basal salt solution (BSS)

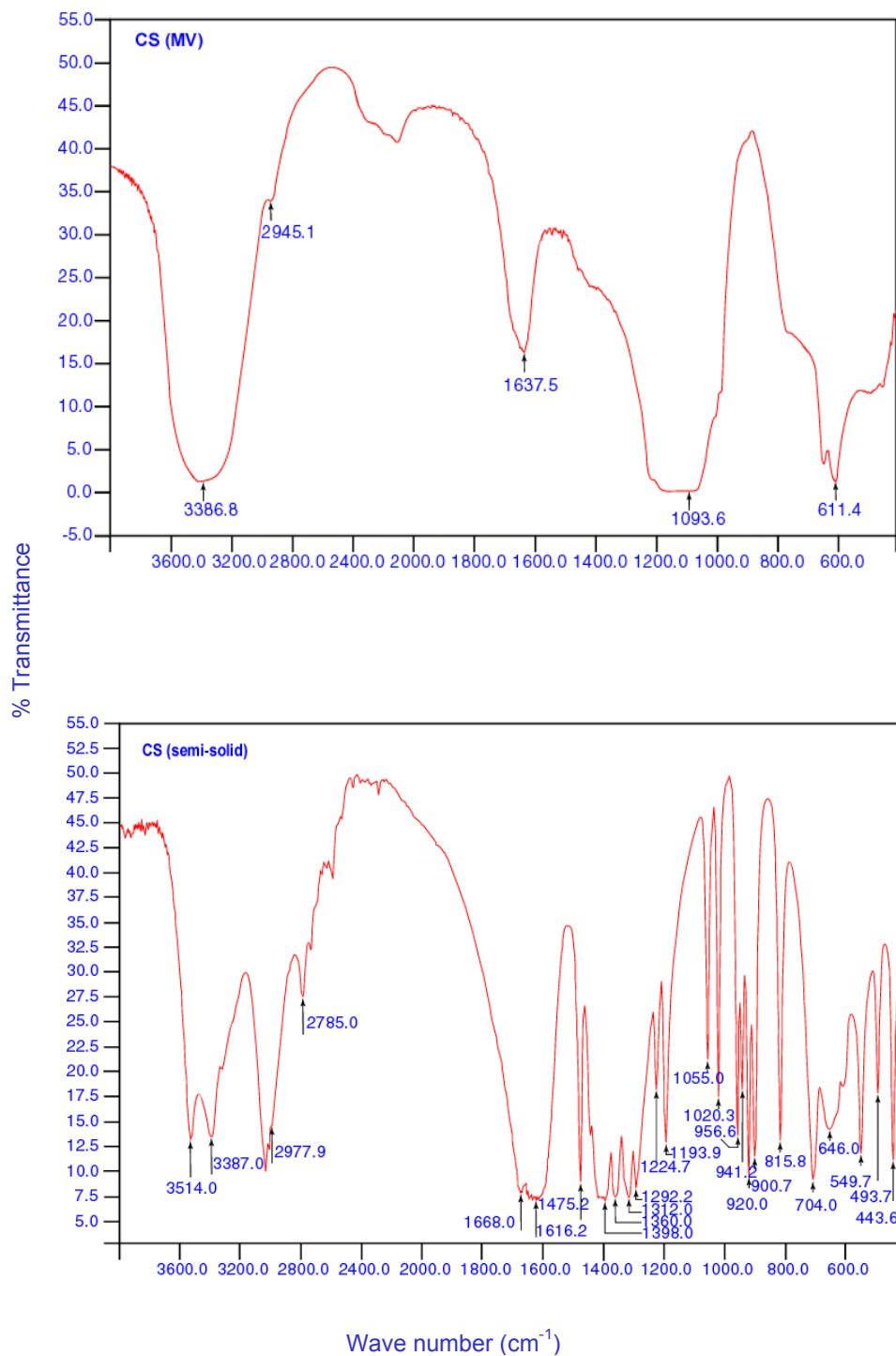


Fig. 3C.9 FTIR spectras of culture supernatants of bacteria grown in MV and semi-solid medium

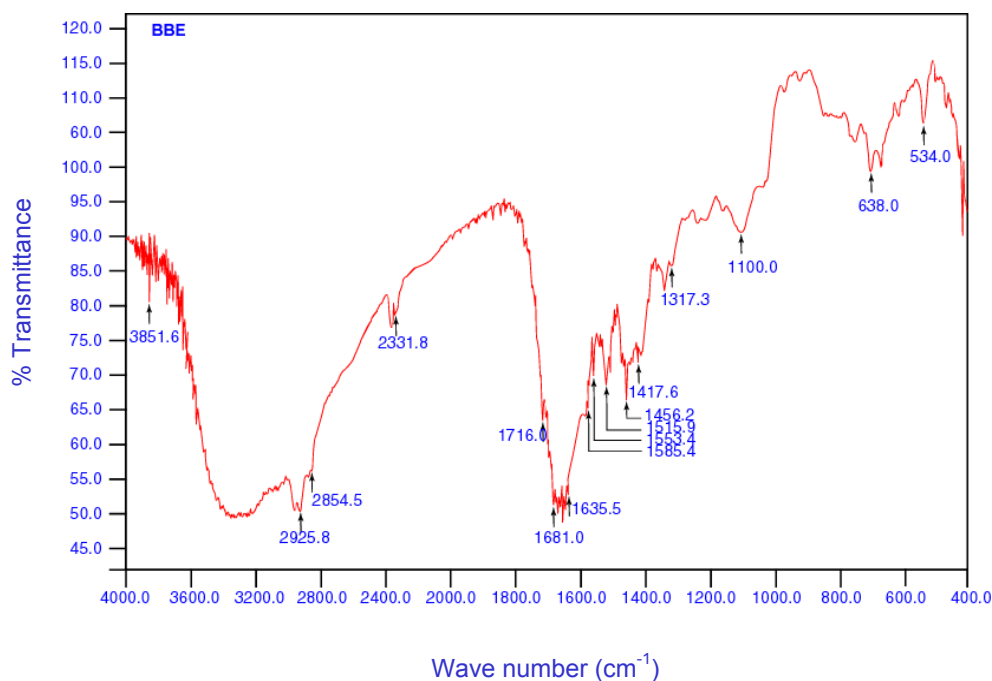


Fig. 3C.9 FTIR spectra of Butanol-bacterial extract

FTIR spectrum of culture supernatants of bacteria grown in MB, BSS and MV revealed characteristic strong peaks around  $3550\text{--}3200\text{ cm}^{-1}$ ,  $1640\text{ cm}^{-1}$  and  $1094\text{--}1126\text{ cm}^{-1}$  for O-H stretching, C=O stretching and C-O stretching and hence were mainly carbohydrates. Culture supernatant of semi-solid culture and bacterial extract revealed strong peaks at  $1616$  and  $1635\text{ cm}^{-1}$  for N-H bend and were proteinaceous. In case of semi-solid culture supernatant presence of terpenoides and steroids were evidenced from IR absorption at  $1475\text{ cm}^{-1}$  and  $1360\text{ cm}^{-1}$ . The spectra also indicated presence of unsaturation evident from C-H bending vibrations located around  $1000$  and  $900\text{ cm}^{-1}$ . Bacterial extract showed peaks at  $1716$  and  $1681\text{ cm}^{-1}$  characteristic for ketones.



### 3D. Thraustochyrid (#MS 2D)

In general percentage metamorphosis increased from day one to day four. The presence of thraustochyrid (#MS 2D) substantially increased the metamorphosis rate of *B. amphitrite* cyprids to an extent of 62(±8%) as compared to adult extract (AE) and the control (FSW) which was 47(±7%) and 30(±7%) respectively at the end of day four (Fig. 3D.1). The trend obtained was same for all the four days wherein thraustochyrid exhibited highest percentage metamorphosis facilitation followed by adult extract and control (FSW).

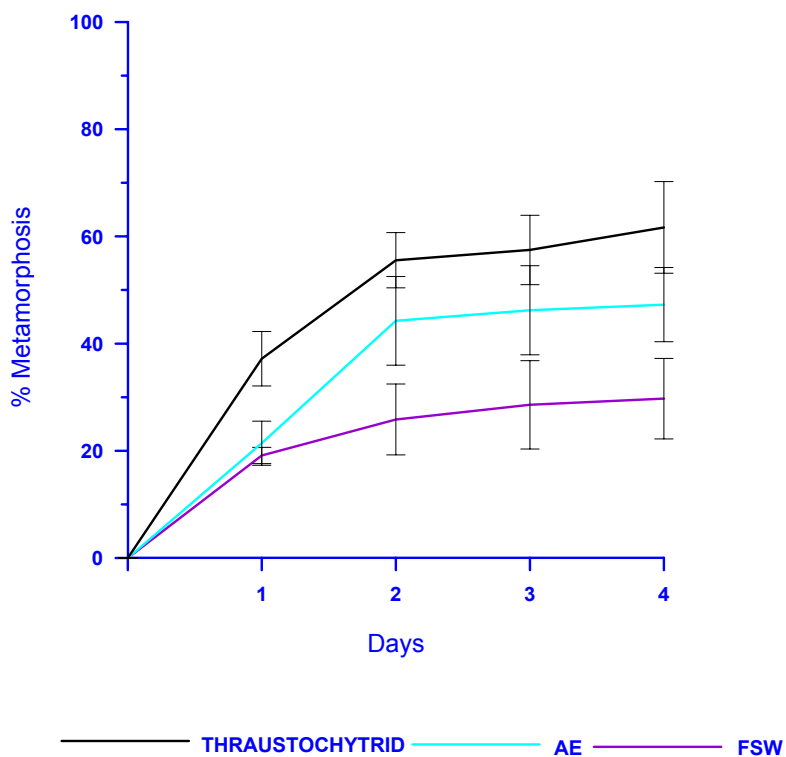


Fig. 3D.1 Percentage metamorphosis of *B. amphitrite* cyprids in presence of thraustochyrid, adult extract (AE) and filtered sea water (FSW)

### 3.4 Discussion

The facilitation of metamorphosis by *P. aeruginosa*, *B. pumilus* or *C. freundii* depended on salinity and temperature. Maki et al. (1990) suggested that a bacterium might produce different compounds at different salinities that result in differential binding with the cyprid's temporary adhesive. The strength of these interactions will determine how well a cyprid adheres to a filmed surface (Yule and Crisp 1983; Neal and Yule 1992). Also, changes in environmental conditions can turn on previously unexpressed genes (Dagostino et al. 1990; Davies et al. 1993; Davies and Geesey 1995) and stimulate changes in bacterial morphology (Dalton et al. 1994). The variations in larval behavior in response to salinity and temperature thus can be attributed to alteration in metabolic activities of bacteria and expression of different cell surface organic molecules. These surface associated specific biochemicals may not only function in the role of stimulating or inhibiting larvae but may change the surface chemistry in a more general fashion. By doing so they either mask important signals or block the receptors responsible for eliciting the larval responses (Maki 1999).

The major bacterially derived chemicals, which are utilized as cues for settlement by many invertebrate larvae, include waterborne products (Neumann 1979; Fitt et al. 1990) and substances associated with the bacterial cell surface (Müller 1973; Kirchman et al. 1982a,b; Maki and Mitchell 1985; Schmahl 1985; Szewzyk et al. 1991). It has been reported that a metabolically active bacterial film is needed to maintain the putative cue at a concentration that surpasses the threshold for induction of larval settlement (S C K Lau and

P-Y Qian 2001). Maki et al. (1994) reported *Deleya marina* films on polystyrene dishes to show negative effect on attachment of *B. amphitrite* whereas on tissue-culture polystyrene or borosilicate glass it did not. Hence the composition of the substratum can influence the effect a bacterial species has on the attachment of cyprids (O'Connor and Richardson 1998).

When *P. aeruginosa* film was examined in presence of AE the metamorphosis was facilitated irrespective of the salinity differences. The presence of AE did not alter the response of *B. pumilus* film at different salinities. Whereas, *C. freundii* film showed a decrease in the metamorphosis rates at 25, 35 and 45‰, whereas at 15‰, the metamorphosis rates were almost similar as that shown by AE when assessed in presence of AE. The bacterial film may contain substances (antagonists) for which a particular receptor site does have an affinity, but whose binding to that site causes a smaller or no effect. In presence of a particular concentration of an active substance (agonist), such as AE, a positive effect may be obtained by competitive antagonism (Musch 1996). The involvement of chemosensory and/or internal neuronal processes as early transducers and mediators of recruitment process is suggested by the fact that GABA and GABA analogs are potent inducers of settlement, attachment and metamorphosis (Morse 1984a,b,1985,1990; Morse A 1991a,b; Pawlik 1990). Recently Yamamoto et al. (1995) reported that a protein kinase C (PKC) signal transduction system to play an important role in metamorphosis of *B. amphitrite* cyprids. Clare et al. (1995) provided an evidence for the involvement of cyclic AMP (cAMP) in the settlement of this species. Yamamoto et al. (1996) have also reported that (5-hydroxytryptamine, 5-HT) is involved in the larval settlement of barnacles.

Previous studies have reported increases in invertebrate larval recruitment with aged and more heavily filmed surfaces (Wieczorek et al. 1995; Keough and Raimondi 1995,1996). In contrast Maki et al. (1990) demonstrated that a 3-day old natural biofilm, but not 1-day old biofilm, inhibited barnacle larvae. Avelin Mary et al. (1993) found that all *Vibrio* films and most other isolates from biofilms associated with *B. amphitrite* were inhibitory and no film stimulated barnacle settlement. In an other study (Wieczorek et al. 1995) the 'older' films (comprising larger proportions of the shape category 'Vibrios') clearly facilitated settlement. *P. fluorescence* and *Alteromonas macleodii* films, yielded weak and inconsistent effects on cyprid attachment of *B. amphitrite*. Despite variability among assays, bacterial effects on larval attachment within a particular assay generally were consistent for the duration of the assay (up to a week) (O'Connor and Richardson 1998). In the present study although the facilitation of metamorphosis by *P. aeruginosa* depended upon the salinity and temperature initially, the differences were not significant as the films and the cyprids aged. The aged film of *P. aeruginosa* promoted metamorphosis of cyprids at all salinities and temperatures. The films of *B. pumilus* and *C. freundii* showed a significant difference in the metamorphosis rate as the films and the cyprids aged. The aged films promoted metamorphosis at 35‰ at which they were inhibitory initially.

There was no significant difference in the multiplication of all three bacteria with respect to salinity and temperature on day 4. In case of bacterial films that promoted metamorphosis, the inability of all the cyprids to show a similar response can be attributed to their physiological conditions. The nutritional as

well as environmental conditions seem to jointly determine the energy status of the larvae (Anil and Kurian 1996; Anil et al. 2001). The cyprids that metamorphosed in response to the bacterial film may be the ones, which were physiologically fit. Earlier studies have shown that larval age is known to affect settlement where the older cyprids responded more readily to external cues (Rittschof et al. 1984; Crisp 1988; Satuito et al. 1997), presumably due to decline in their energy reserves and thus physiological quality.

Maki et al. (1988) reported that exopolymers produced by the bacteria were involved in the attachment response of the larvae, the composition of which influence subsequent fouling by the invertebrate larvae presumably by providing chemical cues for settlement. Although several studies have been carried out on the metamorphosis induction of the cyprid larvae only a few have tested two or more factors on settlement (Pawlik 1992; Anil et al. 1997). The culture supernatant from stationary-phase cultures of *D. marina* consistently retarded cyprid attachment compared to attachment observed on control polystyrene petri dishes, but not glass vials (O'Connor and Richardson 1998). 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 influence of *P. aeruginosa* culture supernatant and its fractions on the metamorphosis of cyprids varied with salinity. At lower salinity, the fractions CS3 and CS1 did not induce metamorphosis, while CS2 promoted metamorphosis. However, the response of the culture supernatant was negative. The promotory effect of CS2 thus seems to be masked rendering CS ineffective in provoking larval settlement. At 45‰, CS3 and CS induced maximum metamorphosis

comparable to that of AE. The higher molecular weight fraction of *B. pumilus* was effective at lower salinity, whereas, in case of *C. freundii*, lower molecular weight fraction was effective at lower salinity and vice-versa. The response of cyprids to bacterial supernatants can thus be attributed to the activity of specific molecular weight compounds.

The leachants of *P. aeruginosa* culture supernatant and its fractions were associated with a decrease in the metamorphosis rates, whereas the low molecular weight fraction CS1 proved to be the most inhibitory. The cyprids did not settle in presence of leachants of the fraction, CS3 of *B. pumilus* and *C. freundii* at the end of day 1. The interaction of sugars with water is highly specific and depends strongly on the stereoisomerism of the hydroxyl group (Maggio et al. 1985; Kutteneich et al. 1988). The bacterial supernatants obtained from the bacteria grown in BSS were mainly carbohydrates. The differences in metamorphosis induction by them with respect to salinity may be the result of positional effect due to isomerism.

The EPS obtained from *P. aeruginosa* grown in BSS exhibited similar metamorphosis effects as that of bacteria and AE, and hence may be a responsible surface-bound component of the bacterial film, which supplied positive cues for the settling cyprids. However, the EPS produced by *B. pumilus* and *C. freundii* did not induce metamorphosis of the cyprids.

The bacterial extract of all the three bacteria was proteinaceous showing the presence of ketonic compounds and its influence varied with the salinity. The leachants of the bacterial extract of *P. aeruginosa* showed a two-fold increase in the metamorphosis rates where only surface-bound components were

inhibitory. The inhibitory effect of the extract was nullified in the presence of AE. The response of the cyprids to the leachants of BBE of *B. pumilus* and *C. freundii* was negative showing no settlement, however, the surface-bound components were also inhibitory at the end of day 1 at 35‰. The bacterium was extracted in n-butanol, which would extract only low molecular weight polar metabolites. The reason that surface-bound components of the bacterial extract did not provoke metamorphosis could be its highly polar nature resulting in diffusion into the surrounding water in the multiwell. The variation in response of the surface-bound compounds of the extract at other salinities can also be related to the same reason but needs further validation. The increased detection of the bacterial extract in solution also suggests the role of fourth antennular segment with its impressive array of sensory setae (Gibson and Nott 1971; Clare and Nott 1994) involving the olfactory receptors. In oysters, low-molecular weight peptides with arginine at the C-terminal were identified as natural water-soluble cue inducing settlement (Zimmer-Faust and Tamburri 1994). Rittschof (1985) partially purified water-soluble peptides (3,000-5,000 Da) released by adult conspecifics, which initiated attachment by larvae of *B. amphitrite*. The possibility of involvement of ketonic compounds in altering the response of the bacterial extract cannot be ruled out.

The nutrient status of the bacteria was found to influence the quality and chemical composition of the culture supernatant containing the extracellular materials. Although the marine broth mainly consisted of peptic digest of animal tissue and the yeast extract, the culture supernatant was mainly carbohydrate in nature.

The culture supernatant obtained by growing *P. aeruginosa* in semi-solid culture was proteinaceous and showed the presence of terpenoids and steroids. The degree of inducement by this was greater than the supernatant obtained from bacteria using any other nutrient media at a protein concentration of  $25 \mu\text{g ml}^{-1}$  ( $p \leq 0.001$ ). An increase in protein concentration to  $50 \mu\text{g ml}^{-1}$  resulted in metamorphosis inhibition. The positive effect of culture supernatant obtained by growing *P. aeruginosa* under semi-solid culture was protein concentration dependent. The culture supernatant isolated from cells growing in a semi-solid environment also 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 was carbohydrate concentration dependent as it induced metamorphosis at a carbohydrate concentration of  $50 \mu\text{g ml}^{-1}$ . Whereas in case of culture supernatants of *B. pumilus* such an inducement was not evident. The response of the cyprids to bacteria and its products seems to be regulated by both contact chemoreception and/or olfaction, which depend on the properties of the settlement inducing compounds.

The thraustochytrids that were detected for the first time in microbial films in the present investigation, facilitated metamorphosis of barnacle cyprids to an even greater extent than adult extract (AE) of barnacles. It is likely that in nature, thraustochytrids on marine surfaces play an important role in the settlement of larvae belonging to macrofouling invertebrates. The need to



characterize and distinguish the receptors, which act via different signaling systems on a particular settlement cue, may be a step ahead to resolve the complexities of invertebrate larval recruitment.