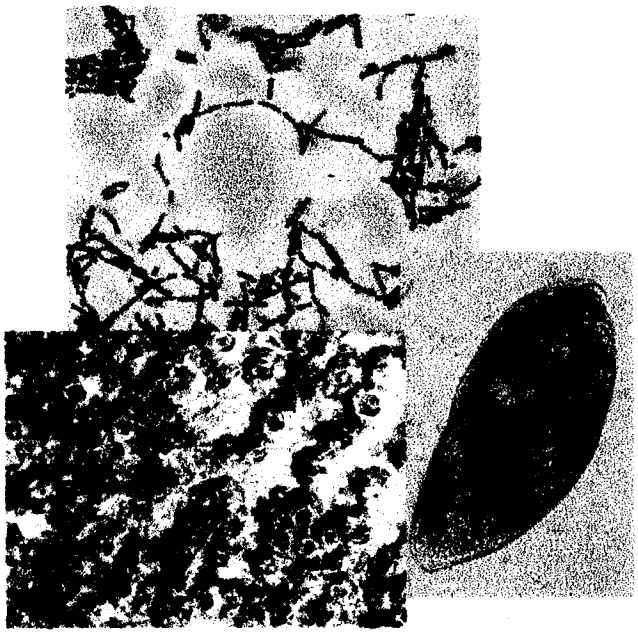
Thesis

Studies on some cues regulating metamorphosis of the larvae of *Balanus amphitrite* (Cirripedia:Thoracica)



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National Institute of Oceanography
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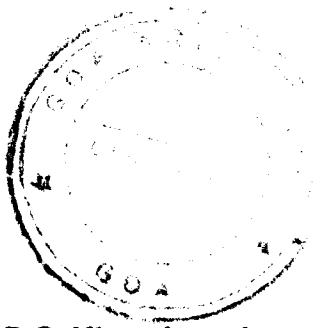
August 2002

Studies on some cues regulating metamorphosis of the larvae of *Balanus amphitrite* (Cirripedia:Thoracica)

Thesis Submitted to the
Goa University
for the degree of
Doctor of Philosophy in
Marine Science



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Statement

As required under the University ordinance 0.19.8 (vi), I state that the present thesis entitled "Studies on some cues regulating metamorphosis of the larvae of *Balanus amphitrite* (Cirripedia: Thoracica)" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

LIDITA D S KHANDEPARKER

Certificate

This is to certify that the thesis entitled "Studies on some cues regulating metamorphosis of the larvae of *Balanus amphitrite* (Cirripedia: Thoracica)", submitted by Ms. Lidita D S Khandeparker for the award of the degree of Doctor of Philosophy in Marine Science is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any Universities or Institutions.

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The corrections suggested by the experts are incorporated.

14/7/2003

S. Raghu Kumar
4-7-03.

Acknowledgements

I express my deep sense of gratitude to my guide Dr. S. Raghukumar, Scientist, National Institute of Oceanography, Goa, for his inspiring guidance, encouragement and support throughout the study.

I am equally grateful to Dr. A. C. Anil, Scientist and Head, MCMRD, National Institute of Oceanography, Goa, for his valuable guidance, suggestions and affectionate encouragements throughout the study period. I am also indebted to him for sharing his splendid experience and knowledge with me.

I am also thankful to Dr. C. U. Rivonkar, Lecturer, Goa University, for providing guidance and support during the study.

I am grateful to Dr. E. Desa, Director, National Institute of Oceanography for providing me the necessary laboratory facilities during the research work.

I am thankful to Council of Scientific and Industrial Research, India for the award of Senior Research Fellowship. I also thank Office of Naval Research, USA for support during the initial phases of the work.

Special thanks to Dr. A. B. Wagh for his encouragement.

I gratefully acknowledge the advise and support provided by Dr. N. B. Bhosle. I am also grateful to Dr. S. S. Sawant for providing generous help during my study.

I very much appreciate and gratefully acknowledge the efforts of Mr. K. Venkat during my research work as well as for the excellent job he has done during thesis compilation.

I am also indebted to Prof. Dr. U.M.X. Sangodkar, Dr. C. L. Rodrigues and Dr. G. N. Nayak of Goa University. I also acknowledge the help given by Dr. Lata Raghukumar, Dr. N Ramaiah and Dr. P. S. Parmeswaran.

I extend my sincere thanks to Dr. Ute Hentschel, Univ. of Würzburg, Germany for her help in bacterial identification.

I am thankful to Dr. N. L. Thakur, Ms. Smita Mitbavkar, Mr. Jagadish Patil, Mr. Y. Vishwakiran, Ms. Rakhee Khandeparker, Mr. Fraddry D'Souza, Dr. Prasanna Yennawar, Mr. P V Bhaskar, Ms. Brenda Fernandes, Ms. Ranee Veigas, Ms. Seema Araligidad, Ms. Priya D'Costa for their help during various stages of my research work.

I express my sincere thanks to Mr. Shyam Naik, Mr. N. S. Prabhu, Mr. A. P. Selvam and Mr. P.R. Kurle for their ever-helping attitude.

I acknowledge the help rendered by Ms. Anita Garg, Mr. Shripad Kunkolienkar, Ms. Vrushali Kolhe, Ms. Leena Prabhudesai, Ms. Sahana Hegde, Mr. Chetan Gaonkar for their cooperation during this study.

I am thankful to the staff of drawing section and workshop for all the help provided during the studies.

I am deeply indebted to my husband Dr. Dattesh V Desai for his constant encouragement and support without which this thesis would never have reached completion. I am greatly indebted to my parents, in-laws and all my family members.

(Lidita D S Khandeparker)

Chapter 1
General Introduction

General Introduction

On immersion of a surface in the marine environment the fundamental processes that contribute to the development of fouling communities are initiated. Fouling results in modification of the immersed structures leading to deterioration of its performance and is a complex mixture of physical, chemical and biological phenomenon. In general, the first stage is the adsorption of a conditioning film of organic and inorganic compounds to the surface. Conditioned surfaces are then colonized by various microorganisms such as bacteria, diatoms etc. (Marshall et al. 1971; Costerton et al. 1978) followed by attachment of algal spores and invertebrate larvae (Wahl 1989). These biofilms play an important role in mediating settlement and metamorphosis of invertebrate larvae.

Barnacles are dominant fouling organisms found all over the world and are the major target organisms in the development of antifouling technology. Among barnacles, *Balanus amphitrite* is an important model organism for these studies because of its rapid larval development, the ease of raising synchronous mass cultures and the predictable settlement in static conditions. *B. amphitrite* is euryhaline (Anil et al. 1995), breeds throughout the year (Karande 1967; Anil 1986) and is a dominant fouling organism in the fouling community in Indian waters (Karande 1967; Anil 1986; Venugopalan and Wagh 1990; Fernando 1990; Rao 1989; Santhakumaran 1989). It has been reported from both east and west coasts of India (Karande 1967,1974; Fernando 1978; Anil 1986; Pillai 1958).

The larval development of this organism includes six naupliar instars and a non-feeding pre-settling cyprid instar, specialized for exploration of a suitable surface for settlement and metamorphosis into the adult. Cypris larva is discriminating in its choice of settlement site (Knight-Jones 1953; Crisp and Meadows 1962, 1963). Laboratory and field studies have demonstrated that barnacle cyprids prefer to metamorphose on or near conspecifics. This gregarious feature has been related to settlement pheromone, a glycoprotein present in the adults, referred as arthropodin (Knight-Jones and Crisp 1953; Knight-Jones 1953; Crisp and Meadows 1963). Native barnacle pheromones are thought to be a heterogenous group of 3,000 to 5,000 Dalton peptides (Rittschof 1985). Recently, a settlement-inducing protein complex (SIPC) from the adult barnacle, *B. amphitrite* has been isolated which is composed of three major subunits with molecular weights of 76, 88 and 98 kDa (Matsumura et al. 1998b). The settlement-inducing activity of the adult extract (AE), which has SIPC, was suppressed by lentil lectin (LCA), suggesting that the carbohydrate moiety of the adult glycoprotein is important in this species (Matsumura et al. 1998a).

Two types of barnacle adhesion to a substratum have been observed, namely temporary and permanent adhesion (Maki et al. 1994). In the marine environment the cyprid employs the antennular disc, an adhesive organ, for temporary attachment to the substratum (Nott 1969; Nott and Foster 1969). Barnacle cement is used for permanent settlement and is an underwater adhesive insoluble protein complex. Temporary attachment by the antennules retains the larva on the substratum and enables exploration to

take place (Walker et al. 1987). However, if a substratum proves unsuitable, cyprid can detach and swim-off to locate other surfaces. The various factors influencing settlement site selection are outlined by Crisp (1974), among which the chemical factors are sensed by antennular disc apical sense organ and terminal setae of fourth antennular segment (Fig. 1).

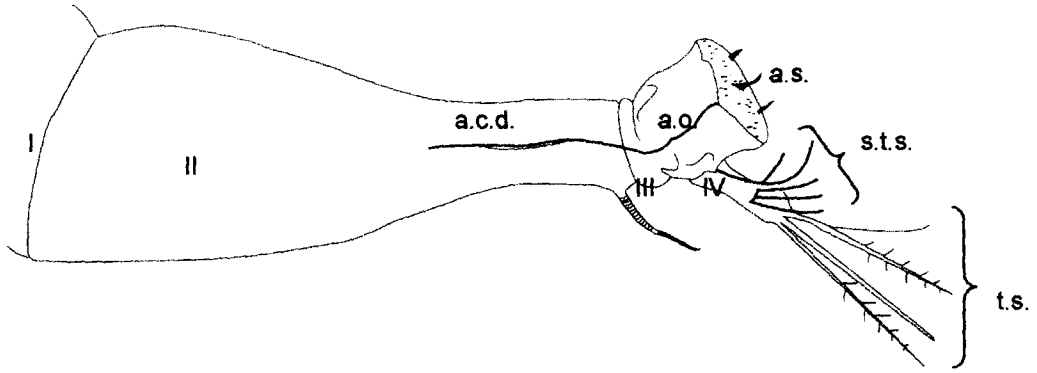


Fig. 1 Cypris antennule showing segments I-IV with attachment organ (a.o.) on third segment, a.c.d. – axial cement duct, a.s. – axial sensory seta, s.t.s – subterminal setae, t.s – terminal setae; Source: Walker G, Yule A.B. and Nott J.A. 1987, Barnacle biology: Crustacean Issues 5, A.A. Balkema, Rotterdam.

The barnacle cyprid is discriminating in its choice of settlement sites and in order to exercise its power of discrimination it has to explore the surface. The third antennular segment with its attachment disc is the most obvious point of contact between the cyprid and the substratum during the search (Nott 1969; Nott and Foster 1969). Darwin (1851,1854) observed that the attaching antennular segment consists of large, thin, circular sucking disc at the edge of which cement is secreted, and the antennular disc becomes attached to the substratum. The cyprid after settling and attachment to a surface molts its carapace and the body exoskeleton except for the embedded parts of the antennule after which it metamorphoses into an adult (Lindner 1984).

While exploring some surfaces, cyprids leave behind 'footprints' of temporary adhesive, which are believed to be secreted by the glands of the antennular disc. A relatively attractive substratum will acquire a correspondingly large number of footprints. The presence of footprints increase the attractiveness of a substratum and should result in gregarious settlement even in the absence of conspecific adults (Walker and Yule 1984; Yule and Walker 1985). However, the detection of settlement pheromone in the solution directs the attention to the fourth antennular segment that bears an impressive array of sensory setae (Gibson and Nott 1971; Clare and Nott 1994) as the putative site of pheromone reception. Flicking of the fourth antennular segment is evident while a cyprid explores a substratum (Clare et al. 1994). If a chemosensory mechanism of pheromone recognition is involved in the barnacle recruitment, there must be a signal transduction step in the pathway. Rittschof et al. (1986) suggested that settlement might be effected by an external protein/peptide receptor and a transduction pathway that involves the stimulation of adenylate cyclase. Yamamoto et al. (1995) demonstrated that a protein kinase C (PKC) signal transduction system plays an important role in larval metamorphosis of *B. amphitrite*. The evidence for the involvement of cyclic AMP (cAMP) in the settlement of this species is provided by Clare et al. (1995) and is in accord with mammalian olfaction for which cAMP is a signal transduction pathway component (Anholt 1991). Cyclic AMP acts as an intracellular signaling molecule in all prokaryotic and animal cells. It is synthesized from ATP by a plasma-membrane-bound enzyme adenylyl cyclase, and it is rapidly and continuously destroyed by one or more cyclic AMP phosphodiesterases, which hydrolyze cyclic AMP to adenosine 5'-

monophosphate (5'-AMP). Clare and Matsumura (2000) suggested that barnacle settlement induction involves receptor-ligand interactions and a signal transduction pathway(s) that translates into attachment and 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 many cases the recognition of the chemical cues and other environmental stimuli by the larvae is suggested to be mediated by the larval nervous system (Bonar et al. 1990; Morse 1990). It has been illustrated that 5HT, DOPA and dopamine induce the settlement of cyprid larvae (Kon-ya and Endo 1995) thus suggesting that neurotransmitters (biogenic amines) are regulators of barnacle settlement and/or metamorphosis. The surface wettability has also been showed to rearrange the bacterial components that are exposed to the larvae.

Marine invertebrate larvae are presented with a wide range of cues as they approach a substratum. These cues may be physical ones or biologically derived chemical cues associated with bacteria, microflora and microfauna. Microbial biofilms have generally been examined as a stimulus for the settlement of macrofouling organisms (Crisp 1974). The larvae may use specific chemical signatures from biofilms or characteristic microbial assemblages to indicate preferred ecological conditions at a site. Films composed of individual strains of bacteria can have varying effects on larval

attachment (Kirchman et al. 1982a; Weiner et al. 1985,1989; Maki et al. 1988,1989; Szewzyk et al. 1991; Holmstrom et al. 1992; Avelin Mary et al. 1993; O' Connor and Richardson 1996). A bacterium may also elicit different responses by different fouling organisms. The bacterium, *Deleya marina*, stimulated the settlement of spirorbid polychaete larvae but inhibited the settlement of both bryozoan (Maki et al. 1989) and barnacle larvae (Maki et al. 1992). Maki et al. (1990) showed that the same bacterium when adsorbed on different substrata it elicited different attachment responses by barnacle larvae. Neal and Yule (1994) demonstrated that the age of the biofilm, rather than the surface wettability determined the larval adhesion. 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 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).

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). The bacteria can change the nature of the substratum either by altering the surface wettabilty or by exposing different surface molecular domains for example, in the form of exopolymers (Anil et al. 1997; Khandeparker et al. 2002). Exopolymers and other excreted products produced by microorganisms have been shown to be involved in settlement of macrofoulers, metamorphosis induction, growth and

development of organisms (Maki et al. 1990, 1992; Holmström et al. 1992,1996; Holmström and Kjelleberg 1994; Mary et al. 1993; Keough and Raimondi 1996). 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).

Natural microbial communities on estuarine and marine substrata, presumably containing a variety of bacterial species, can stimulate, inhibit, or have no effect on settlement (permanent attachment) of larval barnacles (Strathmann et al. 1981; Maki et al. 1988,1990). The period over which a natural microbial film has developed can also influence the settlement response of cyprids (Keough and Raimondi 1995; Weiczorek et al. 1995).

The involvement of lectins in the settlement and metamorphosis of invertebrates has been hypothesized for many years. Lectins, a class of naturally occurring proteins or glycoproteins exists in almost all living organisms and can recognize and bind carbohydrates specifically and noncovalently. Lectins play a key role in cell adhesion and processes, which involve specific recognition between cells during development (Frazier and Glaser 1979; Barondes 1980).

The lectin model was hypothesized after a series of experiments (Kirchman and Mitchell 1981; Kirchman et al. 1982a,b, 1983,1984; Mitchell and Kirchman 1984). In the lectin model, lectins present on the invertebrate larvae recognize a specific carbohydrate molecule from the microbial biofilm inducing

settlement. Initial experiments utilized natural sugars to block the interaction between invertebrates and surface biofilms (Kirchman and Mitchell 1983). Lectin-mediated processes can be inhibited by low molecular-weight sugars, since the added sugar competes for the carbohydrate-binding site on the protein (Sharon and Lis 1972). The role of lectins in larval settlement has been investigated in several studies (Kirchman and Mitchell, 1981,1983,1984; Kirchman et al. 1982a,b; Maki and Mitchell 1985; Mitchell 1984; Mitchell and Kirchman 1984; Mitchell and Maki 1988). The settlement of a polychaete, *Janua brasiliensis* was also mediated by lectins on the larval surface that are proposed to recognize and bind to the bacterial polymer containing glucose (Kirchman et al. 1982a). However no such studies are reported in case of barnacles.

Cypris, the terminal larval instar, prolong their larval duration until a conducive substratum is available for settlement and subsequent metamorphosis. The influence of different cues is dependent on cypris larvae that have been raised through traditional rearing protocol and preconditioned at 5° C for a day or two prior to settlement assays. Holm (1990) also indicated that temporal variation in cyprid behavior may be a result of changes in larval culture conditions and/or matemal effects. Cyprids like other invertebrate larvae derive their energy from stored lipids. These competent larvae delay metamorphosis in the absence of stimuli. However, after postponement, larvae will settle in a less discriminatory manner (Rittschof et al. 1984) possibly because of depleting energy reserves (Lucas et al. 1979), which will jeopardize post metamorphic growth and/or survival (Pechenik and Cerulli 1991; Pechenik et

al. 1993). This signifies the importance of energy reserves and/ or nutritional stress of the larvae that is used.

Taking into consideration the above, following were addressed to study the cues that regulate metamorphosis of the larvae of *B. amphitrite*.

- **Significance of sugars in exploration and metamorphosis.**
- **Evaluation of different inducers from microorganisms that influence the metamorphosis.**
- **Lectins as probes to evaluate the role of signaling molecules from the bacteria.**
- **Influence of larval rearing conditions and ageing on the energetics and metamorphosis.**

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

antennulary 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 $\mu g\ ml^{-1}$ 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 2×10^5 cells ml^{-1}. 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 µm), 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 µg 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° 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.

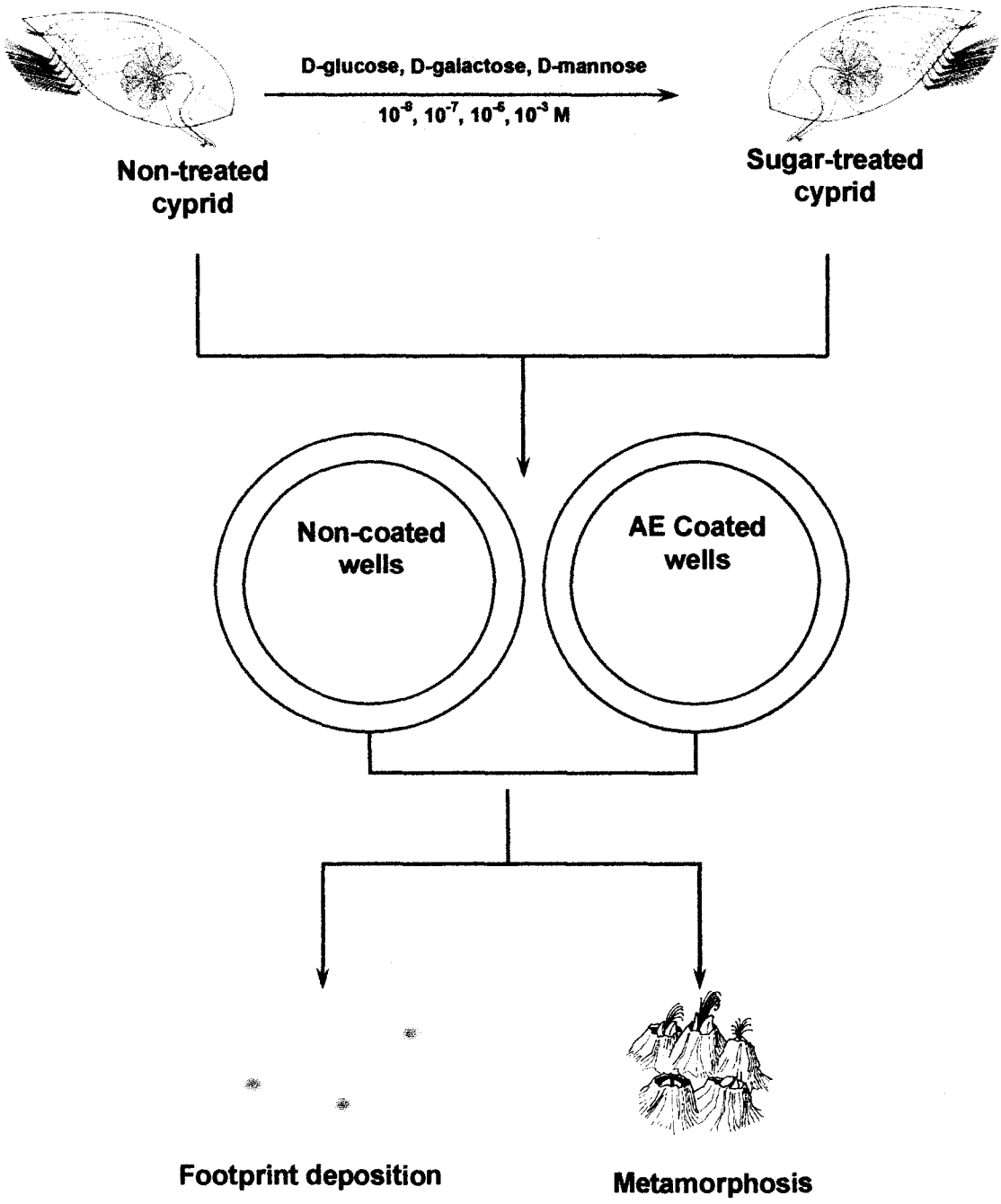


Fig. 2.1 Schematic representation of the experimental set-up

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 $\mu g\ ml^{-1}$. 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 ±1° 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 30-37µm 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).

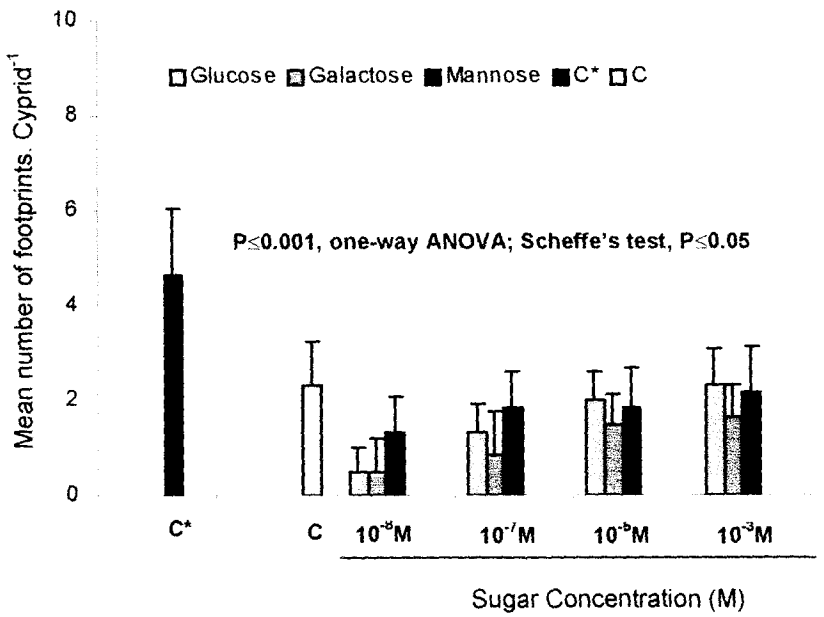


Fig. 2.2 Number of footprints deposited by sugar-treated cyprids exposed to AE-coated surface. C*- non-treated cyprids exposed to AE-coated surface, C-non-treated cyprids exposed to non-coated surface

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).

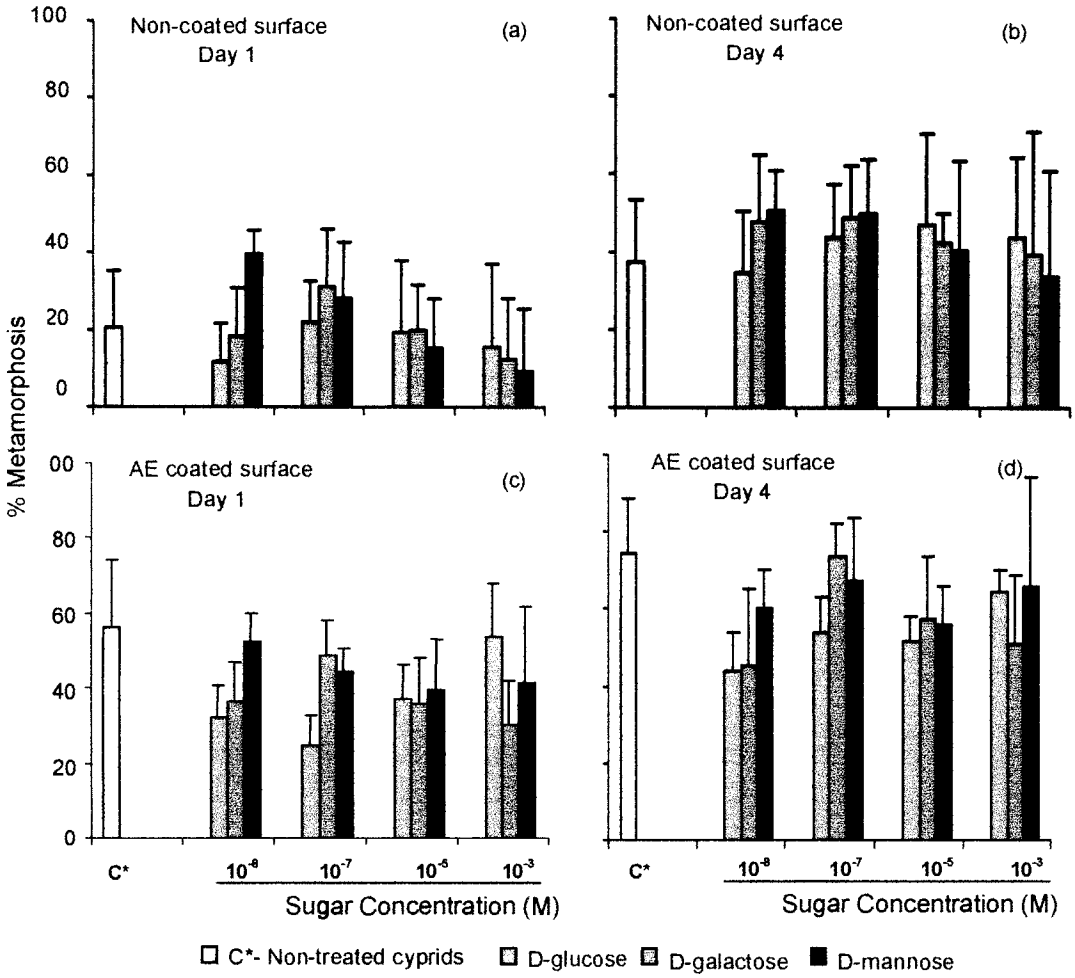


Fig. 2.3 Percentage metamorphosis of cyprids (Multiple cyprid assay). (a) and (b)- sugar-treated and non-treated cyprids (control) exposed to non-coated surface, (c) and (d)- sugar-treated and non-treated cyprids exposed to AE-coated surface. Vertical lines indicate the standard deviation from mean

Such a response was not given by cyprids treated with D-glucose or D-galactose. At 10^{-8}M, D-glucose showed an reduced effect. Two-way ANOVA also indicated significant differences in the metamorphosis rates at 10^{-8}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		

(**$p \leq 0.05$, * $p \leq 0.1$ 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.

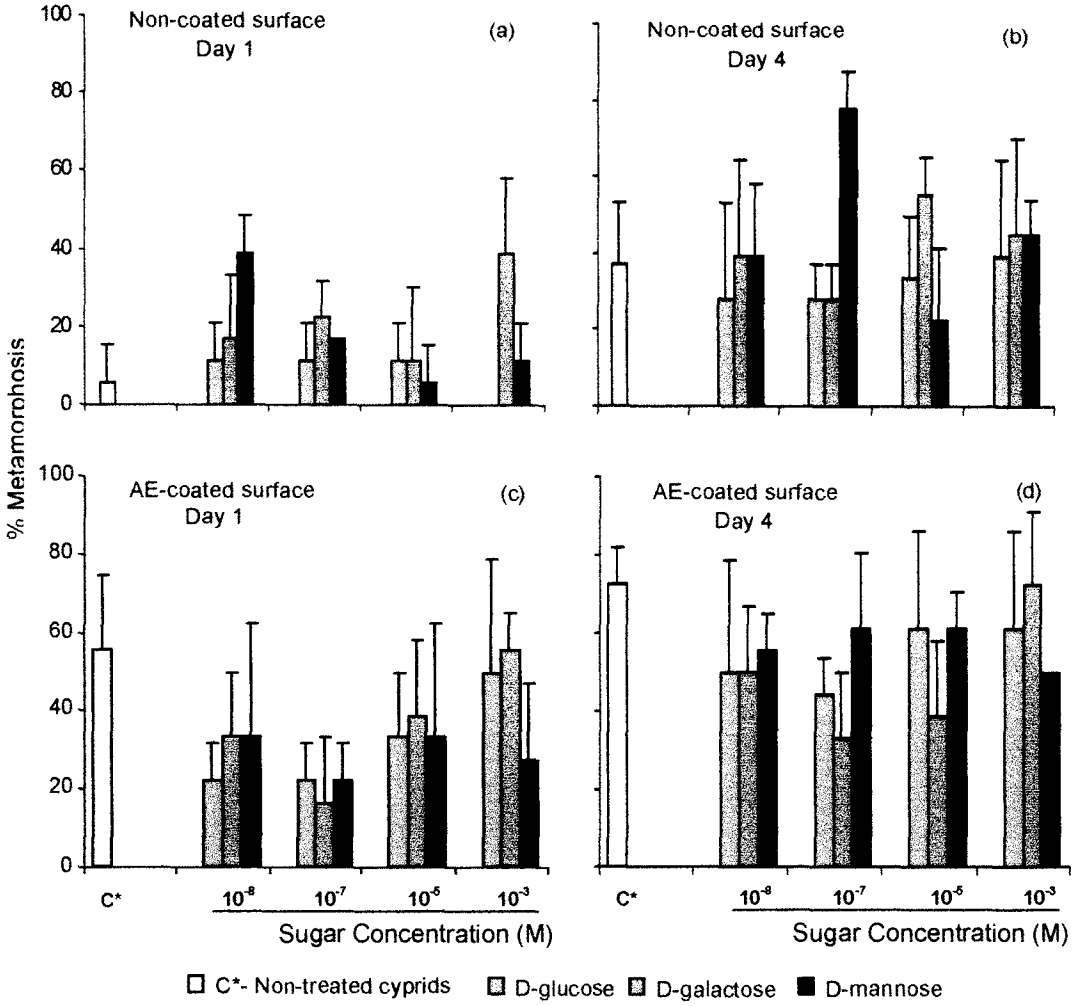


Fig. 2.4 Percentage metamorphosis of cyprids (single cyprid assay). (a) and (b)- sugar-treated and non-treated cyprids (control) exposed to non-coated surface, (c) and (d)- sugar-treated and non-treated cyprids exposed to AE-coated surface. Vertical lines indicate the standard deviation from mean

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 $K_a \geq 10^6$ 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 an 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 $\mu g\ ml^{-1}$ 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 2×10^5 cells ml^{-1}. The food organism was replenished every day while changing the water. After 5-6 days the cyprids obtained were siphoned out and stored at 5° C prior to settlement assays. Two-day-old cyprids were used to carry out the assays. These methods have been described in detail 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

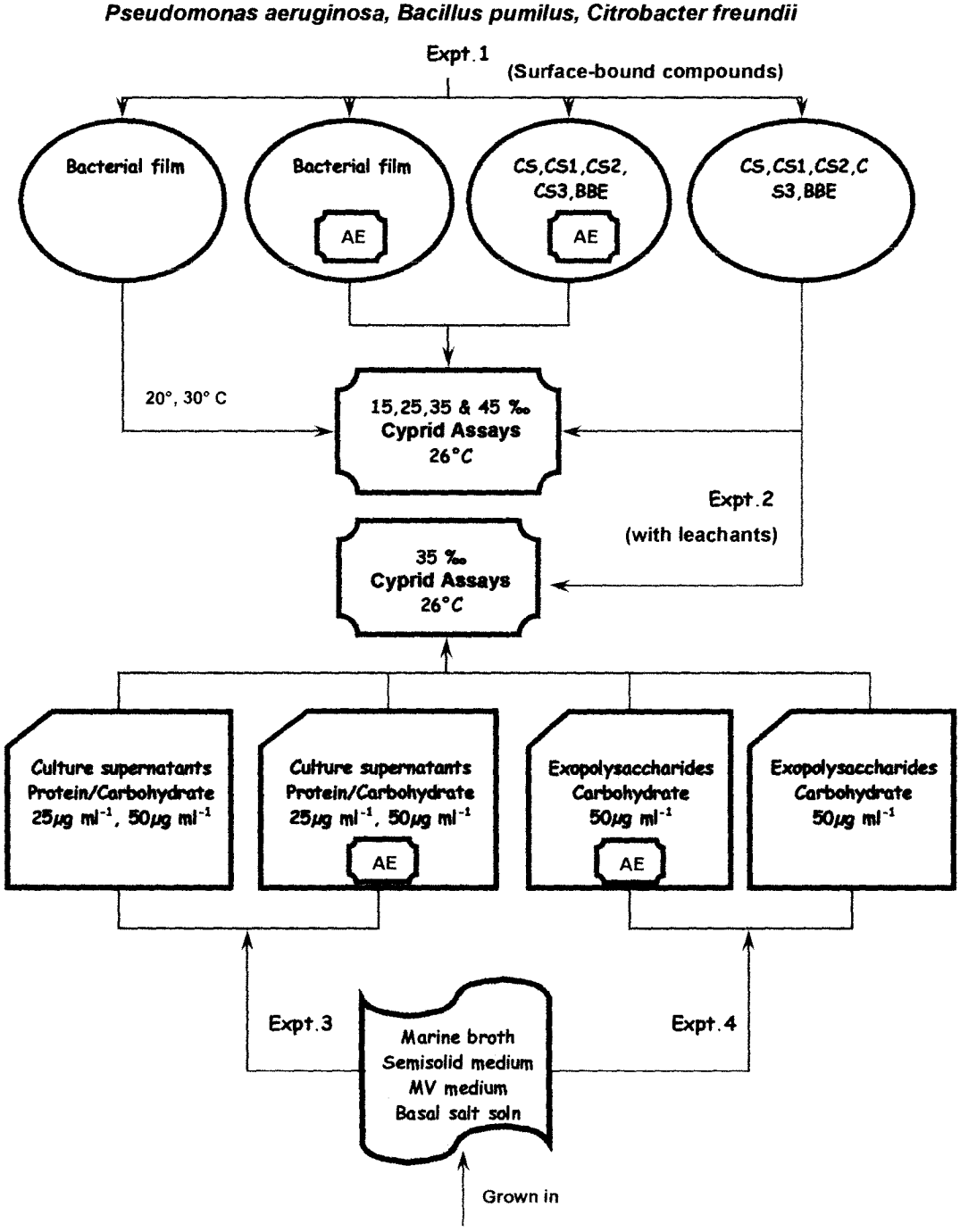
Tests	Results	Tests	Results
Color	Cream	Color	Cream
Shape	Short rods	Shape	Short rods
Gram stain	-	Gram stain	-
Motility	+	Motility	+
Hugh Leifson's test	Aerobic oxidative	Hugh Leifson's test	Facultative fermentative
Growth at pH 3.6	-	Indole	-
Growth at 4° C	-	Methyl red	+
Growth at 41° C	+	Simmons citrate	+
Indole	-	H_2S (KIA / TSI)	+
Methyl red	-	Urease	-
Simmons citrate	+	Phenylalanine deaminase	-
H_2S (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	+		
Mannitol	+		
Cellobiose	-		
D-Mannose	-		
L-Arabinose	-		
Lactose	-		
Maltose	-		
D-Fructose	+		
m-Inositol	-		
Sucrose	-		
D-Galactose	-		
Acetamide	+		
Bacteria	*Pseudomonas aeruginosa*		*Citrobacter freundii*

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	*Bacillus pumilus*

Table 3.2 Phylogenetic identification of gram-positive bacteria.

Method	Bases sequenced	Nearest phylogenetic neighbor	% similarity	Phylogenetic Affiliation
cloned	1295	*Bacillus pumilus* strain KL-052	98.4%	*Bacillus pumilus* 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

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 cm^{-2}.

3.2.5 Culture supernatant and its fractions

The bacteria were grown in basal salt solution (BSS), pH 7.5, containing (g. l^{-1}): NaCl, 30.0; KCl, 0.75; $MgSO_4.7H_2O$, 7.0; NH_4Cl, 1.0; K_2HPO_4, 0.7; KH_2PO_4, 0.3; glucose, 10.0 and 1ml of trace metal solution (Bhosle 1981). A 2% (v/v) inoculum of an 18h old culture grown in the same medium was used. 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 μm millipore filter and the resultant filtrate was utilized as the culture supernatant (CS) in the assays. Subsequently, three sub-samples were concentrated in a stirred ultrafiltration cell (Amicon, Danvers, MA) to $1/10^{th}$ 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 μg 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° C for further use. The butanol would extract only low molecular weight polar metabolites. A concentration of 50 μg 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 μg 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 μg 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 μg 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 x g for 30 min) and subsequently sterile filtered (0.22 μm, Millipore). They were then concentrated to $1/10^{th}$ 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 μg 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 μg 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

μm 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 μg ml^{-1}. final concentration) for 3 hours at 37° C.The material was dialyzed (8,000 MW cut-off) overnight at 4° C against distilled water and centrifuged at 30,000 x g for 20 min at 25° C to remove insoluble material. The supernatant was recovered and lyophilized. The exopolysaccharides were evaluated for carbohydrate content and

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

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 ± 1° C (12h light: 12h dark photoperiod). The influence of bacterial film, AE and FSW was also investigated at 20° and 30 ± 1° 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 (α=0.05). Those that did not meet the normality assumption were analyzed using non-parametric statistical analysis such as Mann-Whitney U-Test (α=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).

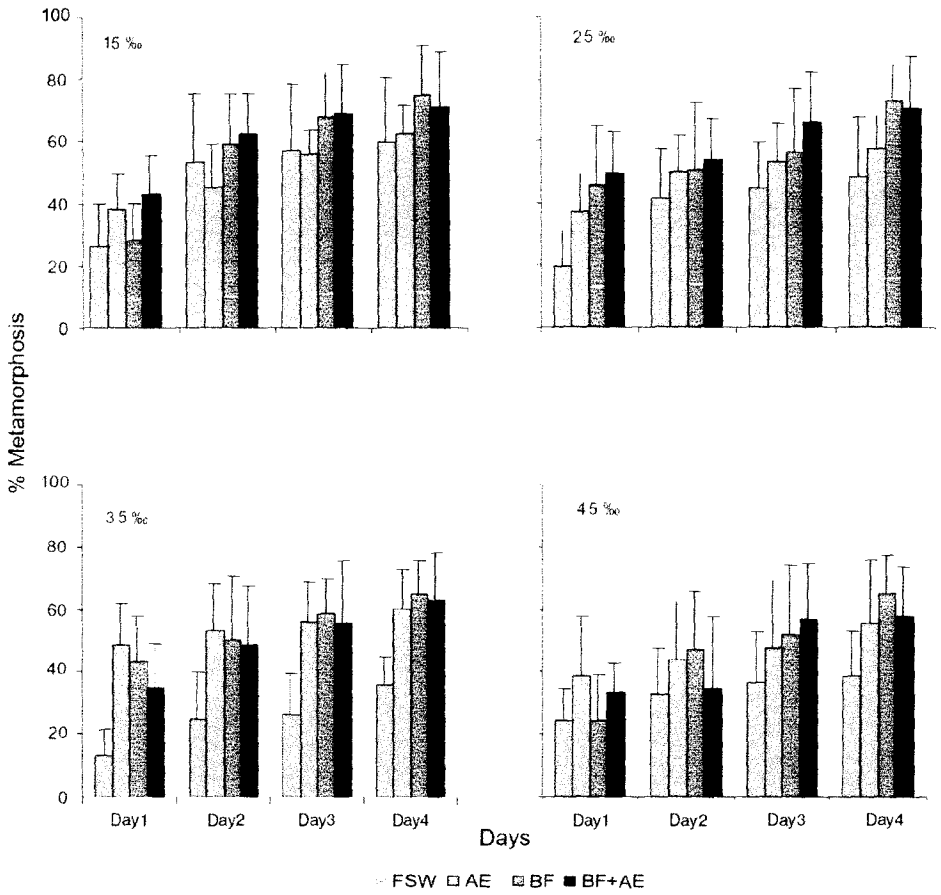


Fig. 3A.1 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*P. aeruginosa*) in presence of AE (adult extract) at different salinities. Vertical lines indicate the standard deviation from mean and are shown as positive

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
(a) BF	Salinity	3	1731	577	4.7***	502	167	0.8ns	**(b) BF+AE**	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		
(c) CS1	Salinity	3	64	21	0.4ns	179	59	0.5ns	**(d) CS1+AE**	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		
(e) CS2	Salinity	3	174	58	0.6ns	1643	547	1.7ns	**(f) CS2+AE**	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		
(g) CS3	Salinity	3	1399	466	7*****	629	209	1.9ns	**(h) CS3+AE**	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		
(i) CS	Salinity	3	843	281	2.9*	1512	504	2.2ns	**(j) CS+AE**	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		
(k) BBE	Salinity	3	1326	442	4.4**	570	190	1.6ns	**(l) BBE+AE**	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)

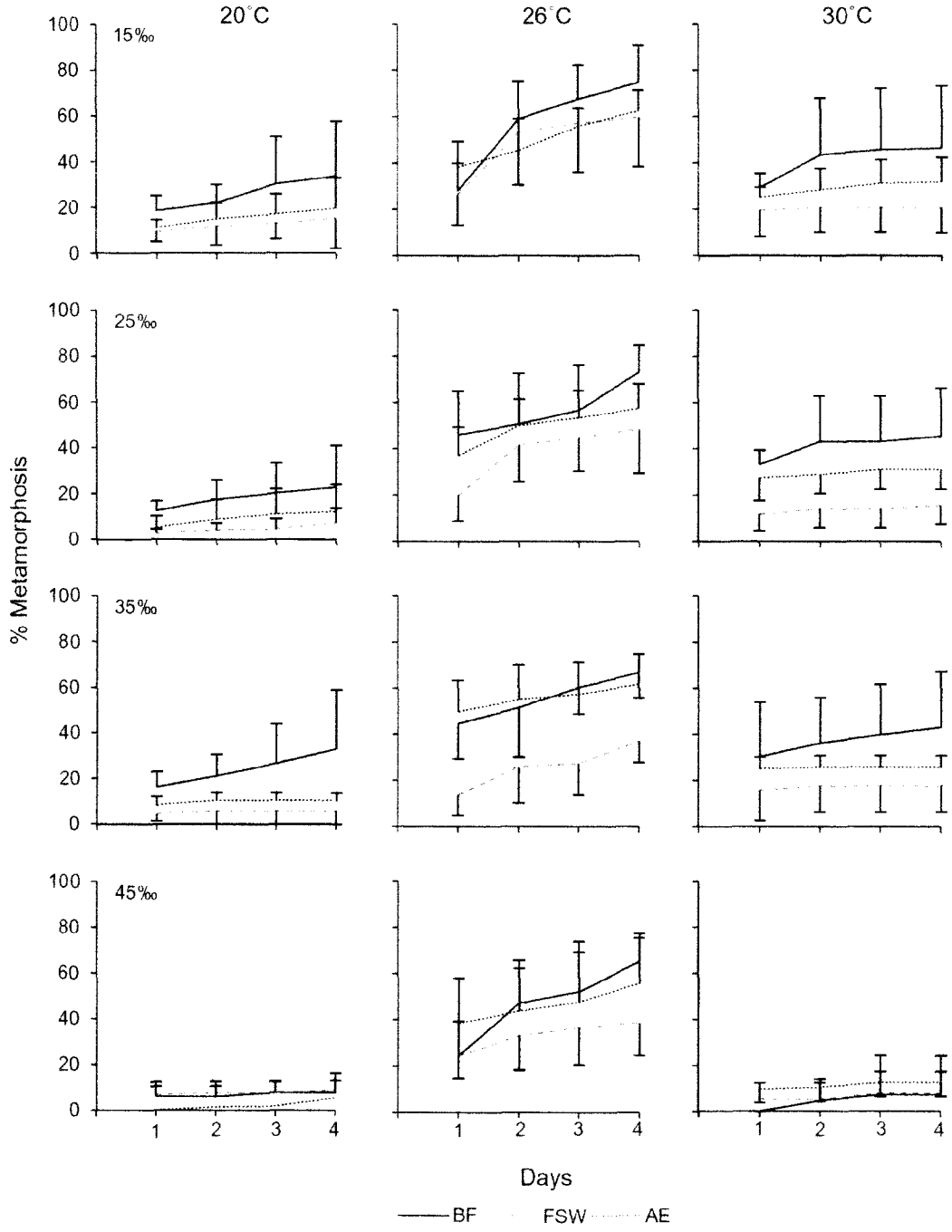


Fig. 3A.2 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (BF), adult extract (AE) and filtered sea water (FSW) at different salinities (15, 25, 35 and 45‰) and temperatures (20, 26 and 30° C)

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	Day 1				Day 4		
	df	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, *p≤0.05, 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).

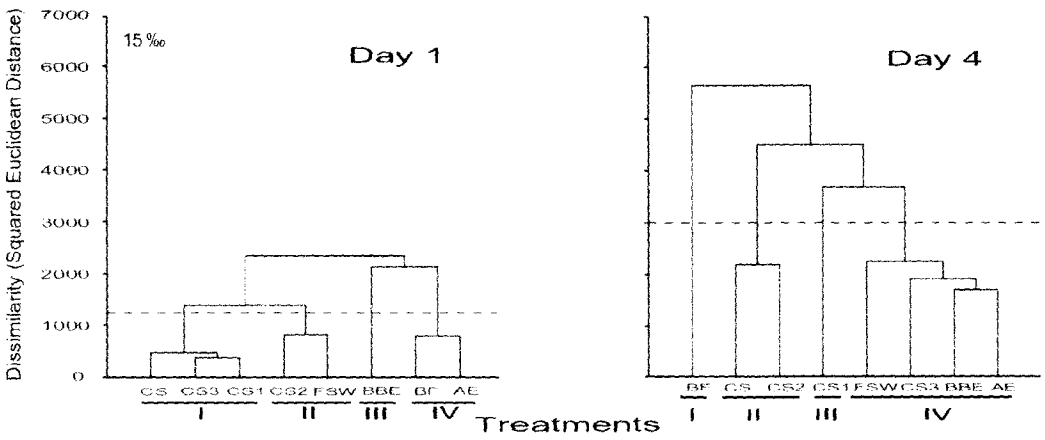


Fig. 3A.3 Dendrograms showing the dissimilarity between different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 15‰

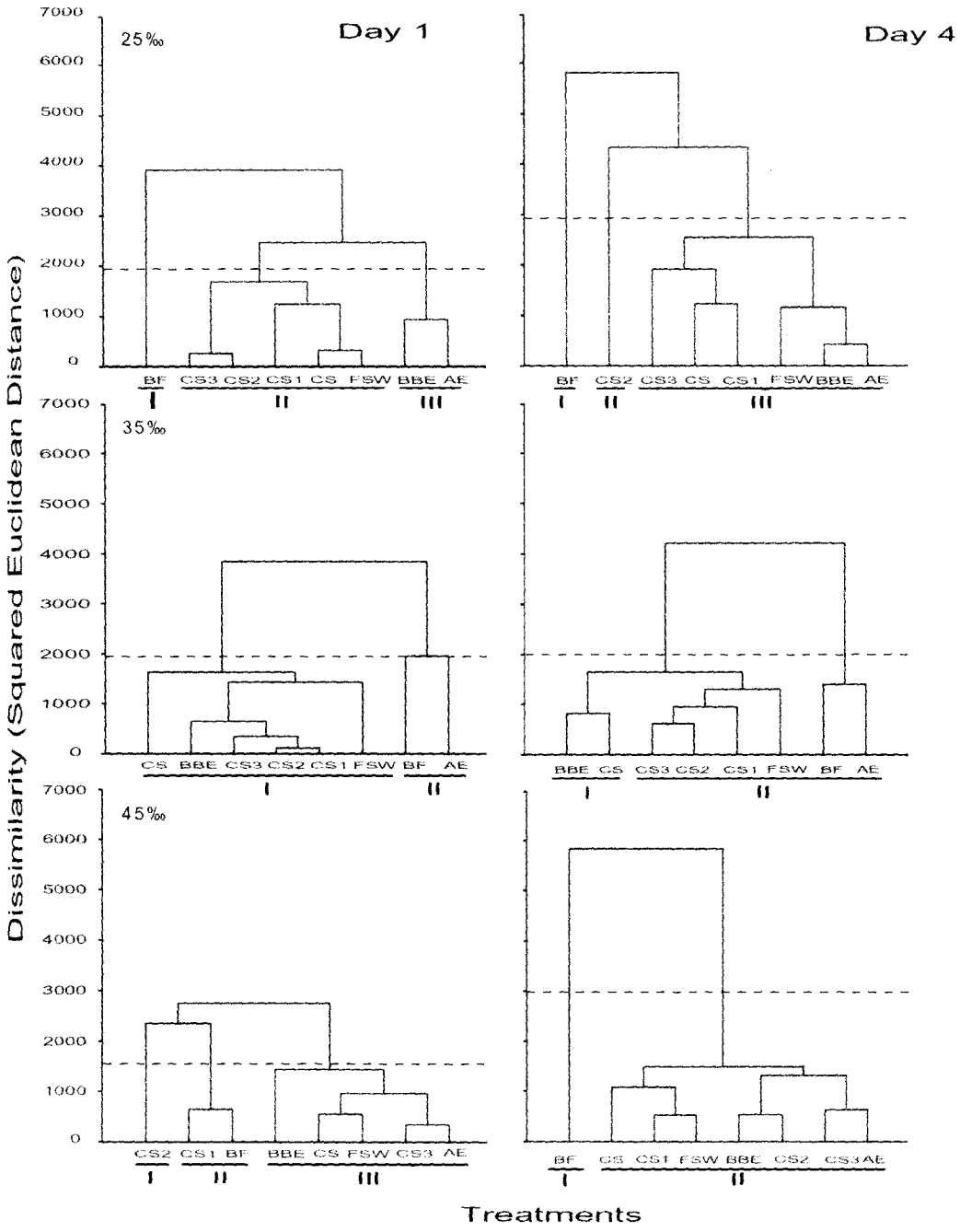


Fig. 3A.3 Dendrograms showing the dissimilarity between different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 25, 35 and 45‰. (The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed)

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 inducive 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).

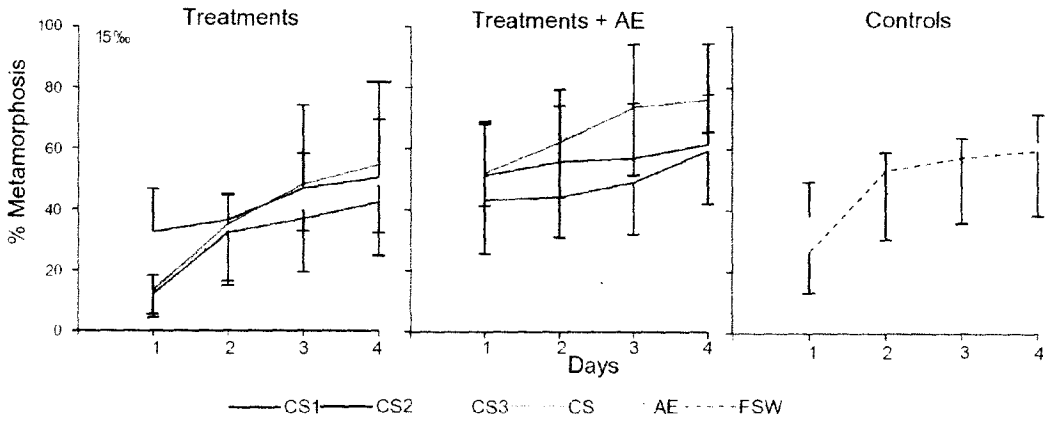


Fig. 3A.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution in presence and absence of adult extract (AE) at 15‰. Vertical lines indicate the standard deviation from the mean and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

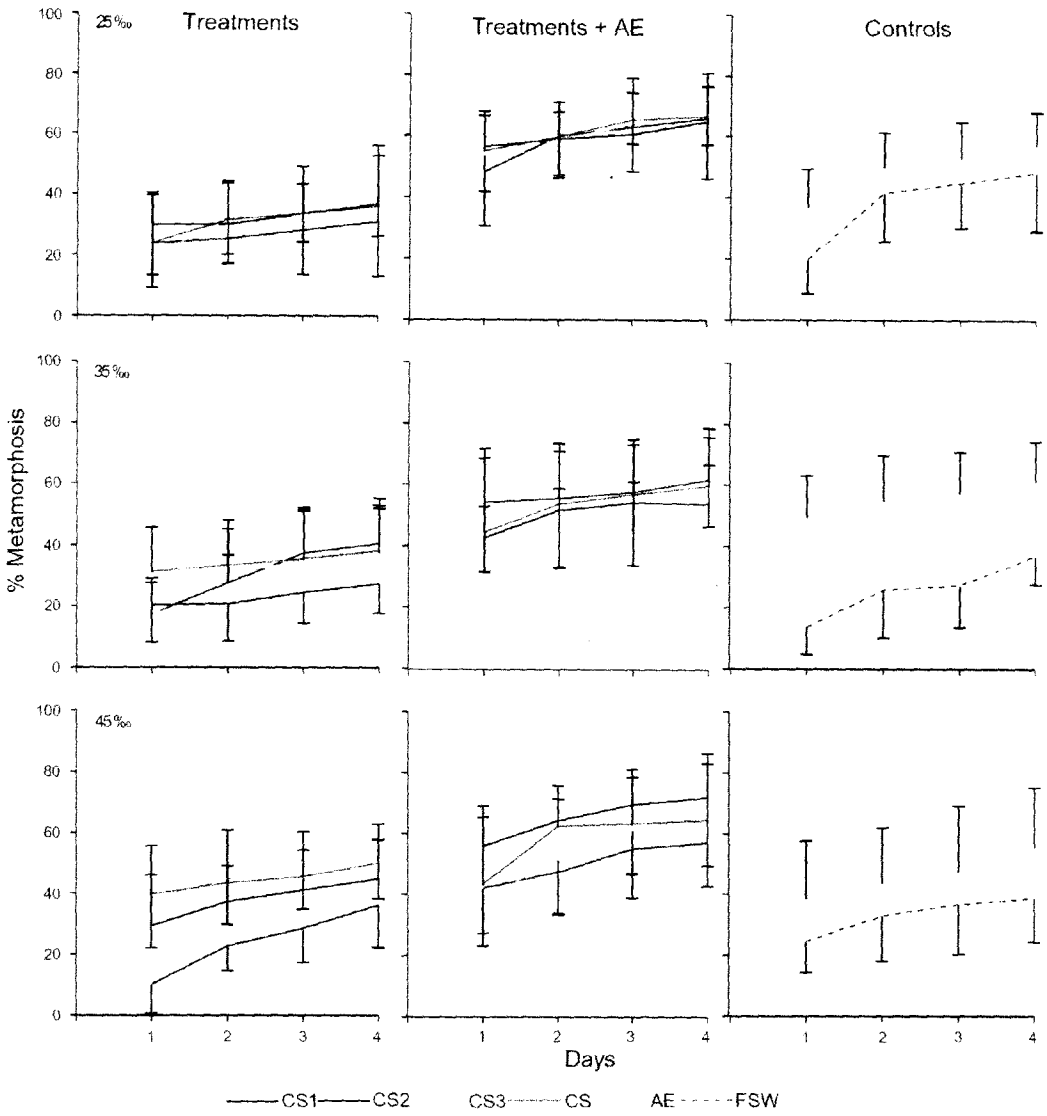


Fig. 3A.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution in presence and absence of adult extract (AE) at 25, 35 and 45‰. Vertical lines indicate the standard deviation from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

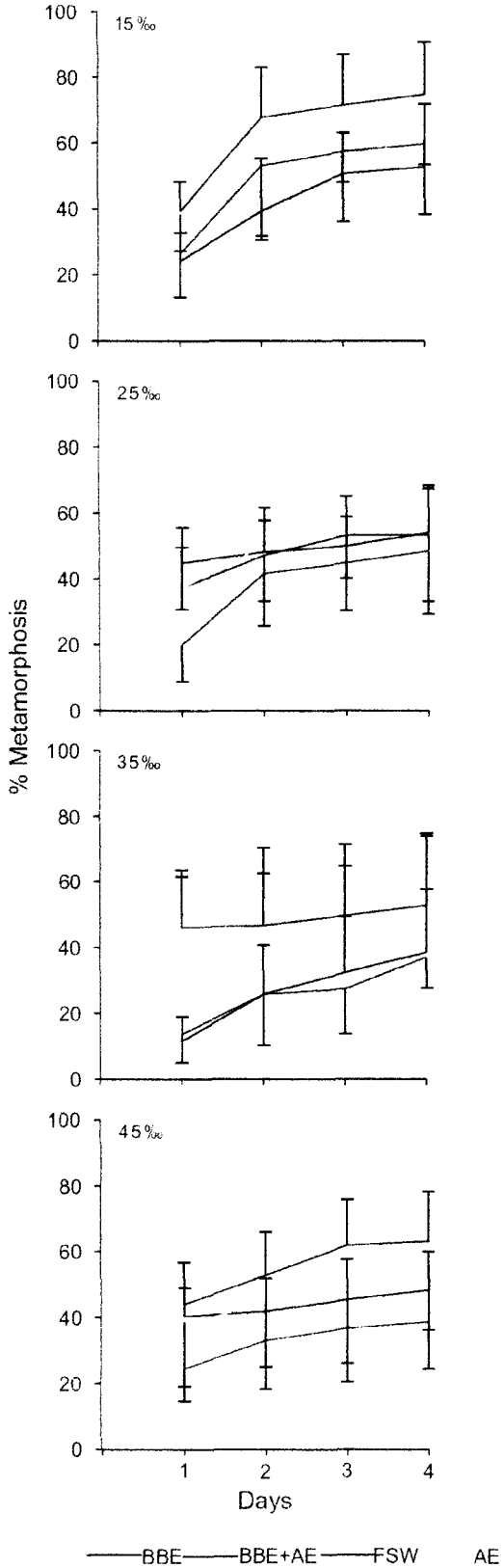


Fig. 3A.5 Percentage metamorphosis of *B. amphitrite* cyprids exposed to Butanol-bacterial extract at different salinities. Vertical lines indicate the standard deviation from the mean and are indicated as either positive or negative

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).

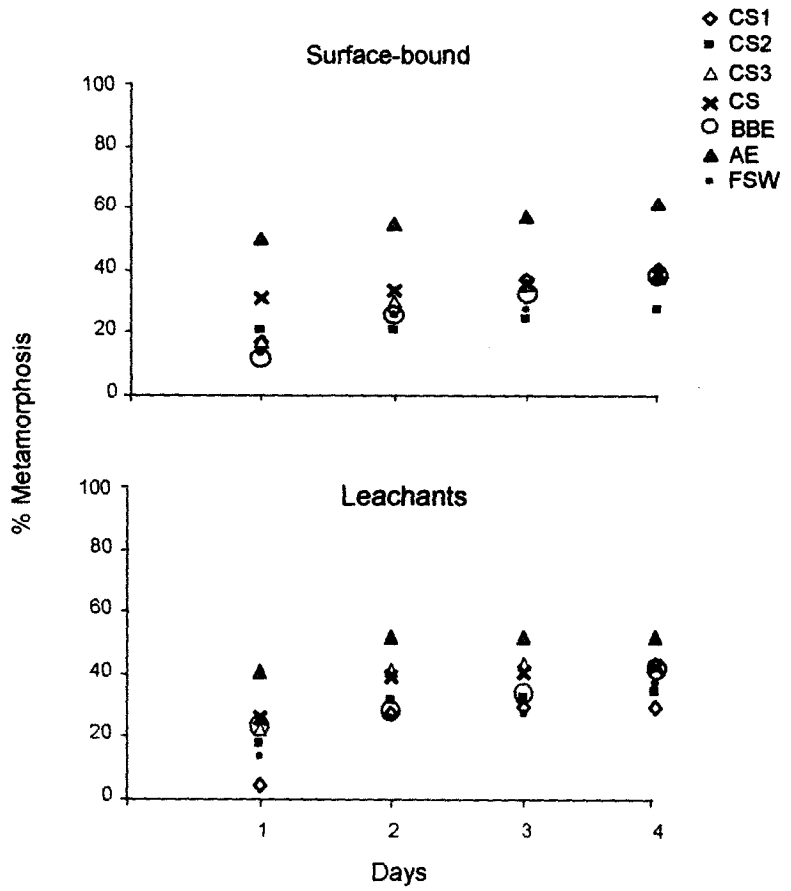


Fig. 3A.6 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial inducers (surface-bound) used in experiment 1 in presence of leachants at 35‰

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 μg 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 μg 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 μg 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 μg ml^{-1} and 25 μg 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 μg 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	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
Day 1													
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		
Day 4													
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	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
Day 1													
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		
Day4													
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)

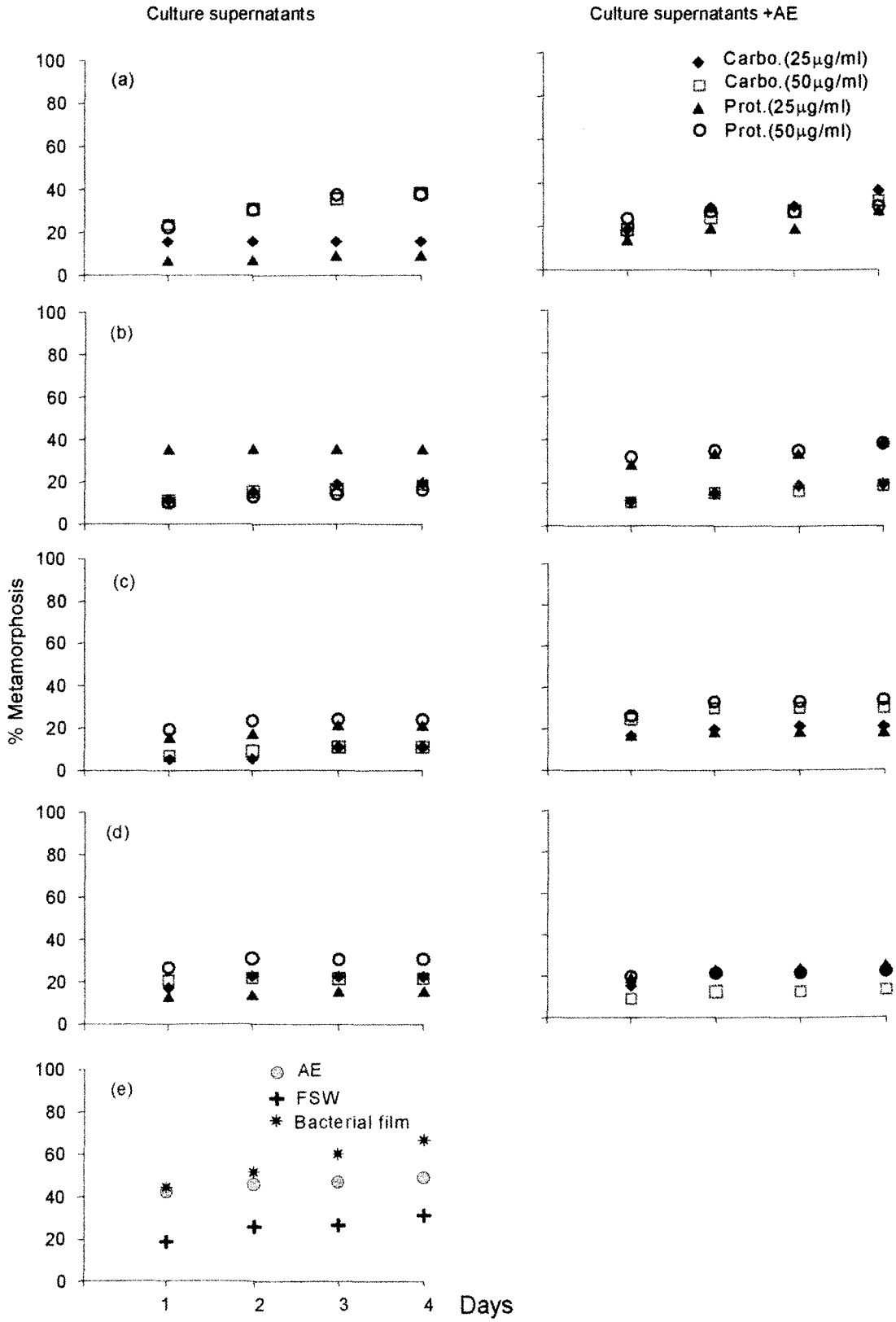


Fig. 3A. 7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutrient media at 35‰ ir presence and absence of adult extract. (a) CS(BSS) (b) CS(semi-solid) (c) CS(MV) (d CS(MB) (e) Controls

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).

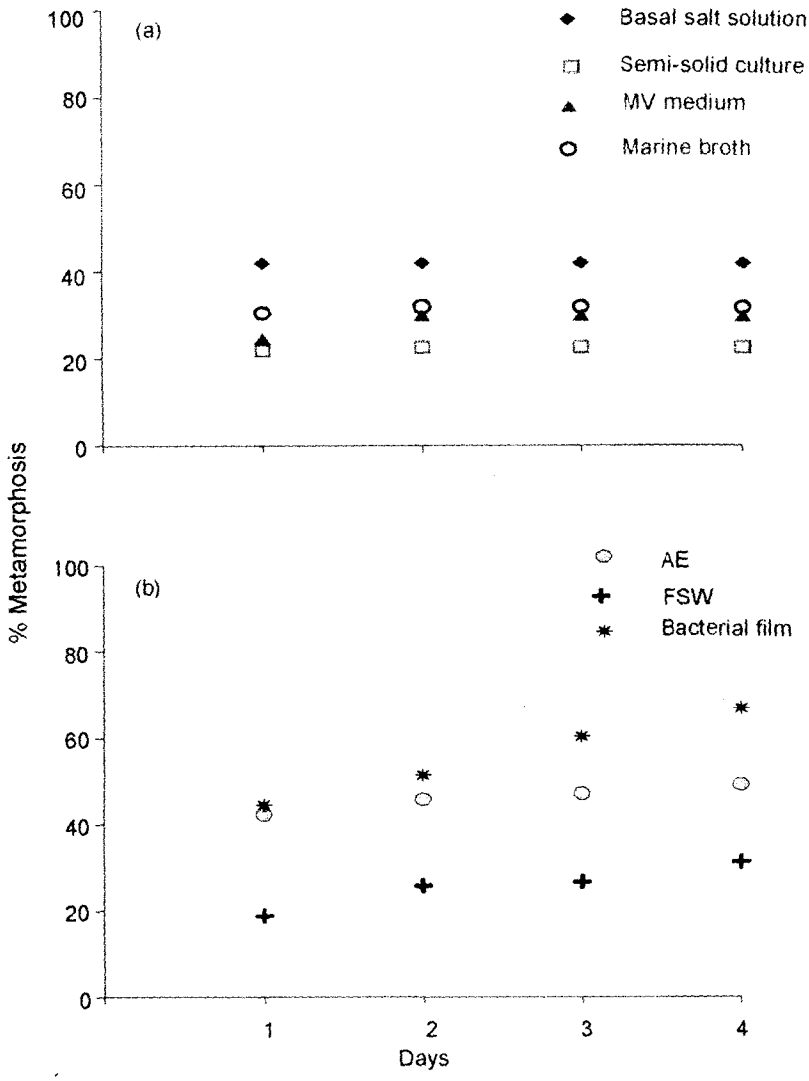


Fig. 3A. 8 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial exopolysaccharides obtained after growing the bacteria under different nutritional conditions at 35‰

3A 3.5 FTIR spectroscopy

Fourier transformed infrared (FTIR) spectrum of CS of BSS media revealed characteristic strong peaks around 3550-3200 cm^{-1}, 1640 cm^{-1} and 1076-1122 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).

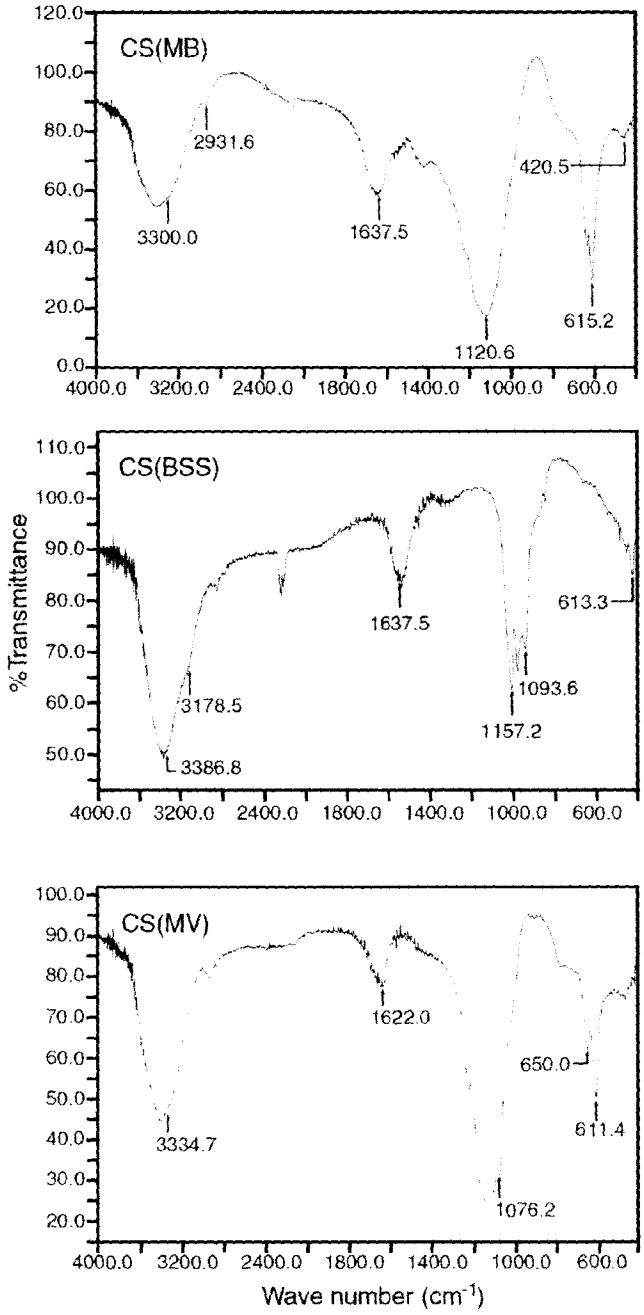


Fig. 3A.9 FTIR spectras of culture supernatants of MB, BSS and MV media

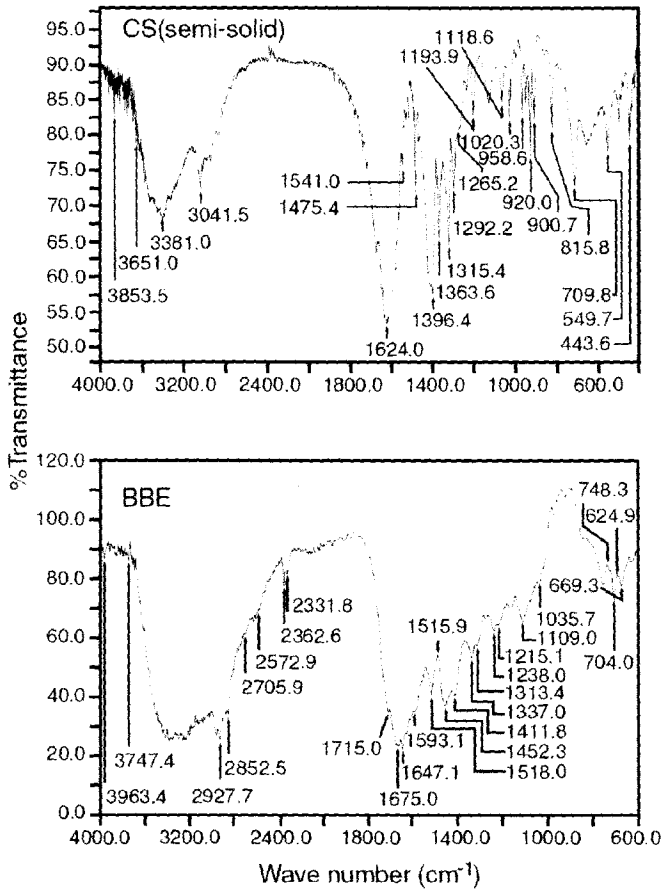


Fig. 3A.9 FTIR spectras of culture supernatant of semi-solid media and bacterial extract

Culture supernatant of semi-solid culture and the bacterial extract revealed strong peaks at 1624 and 1647 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 cm^{-1} & 1363.6 cm^{-1}, the spectra also indicated the presence of unsaturation evident from C-H bending vibrations located around 1000 and 900 cm^{-1}. Bacterial extract showed peaks at 1715 & 1675 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).

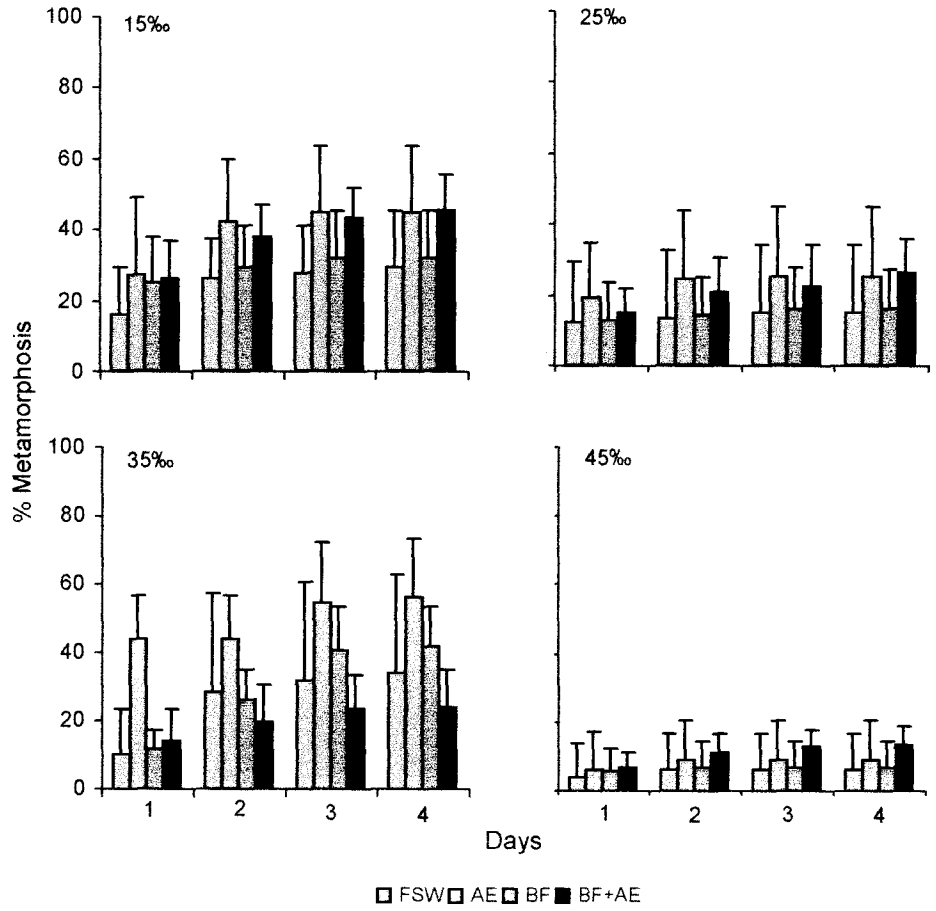


Fig. 3B.1 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*B. pumilus*) in presence of adult extract (AE) at different salinities (15, 25, 35 and 45‰). Vertical lines indicate the standard deviation from mean and are shown as positive.

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).

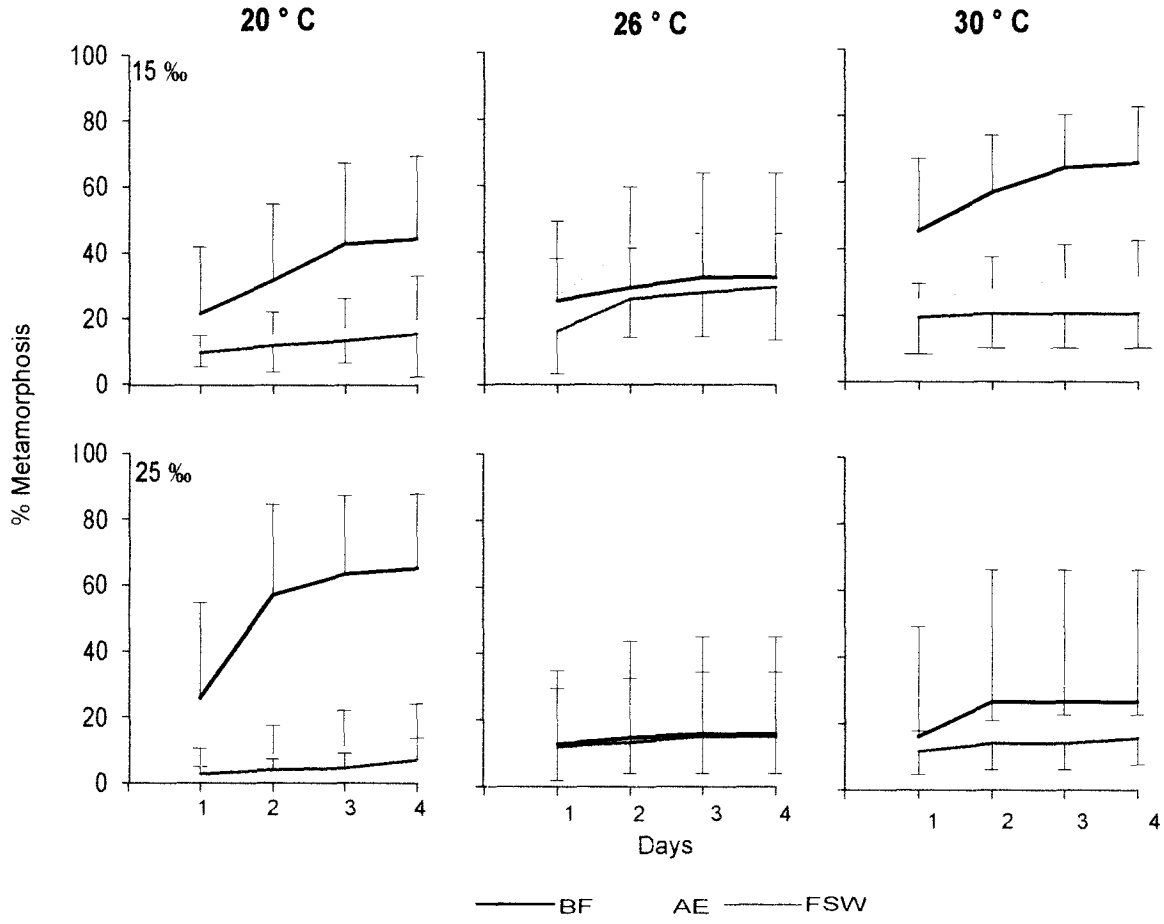


Fig. 3B.2 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*B. pumilus*), adult extract (AE) and filtered sea water (FSW) at different salinities (15 and 25‰) and temperatures (20, 26 and 30° C). Vertical lines indicate the standard deviation from mean and are indicated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

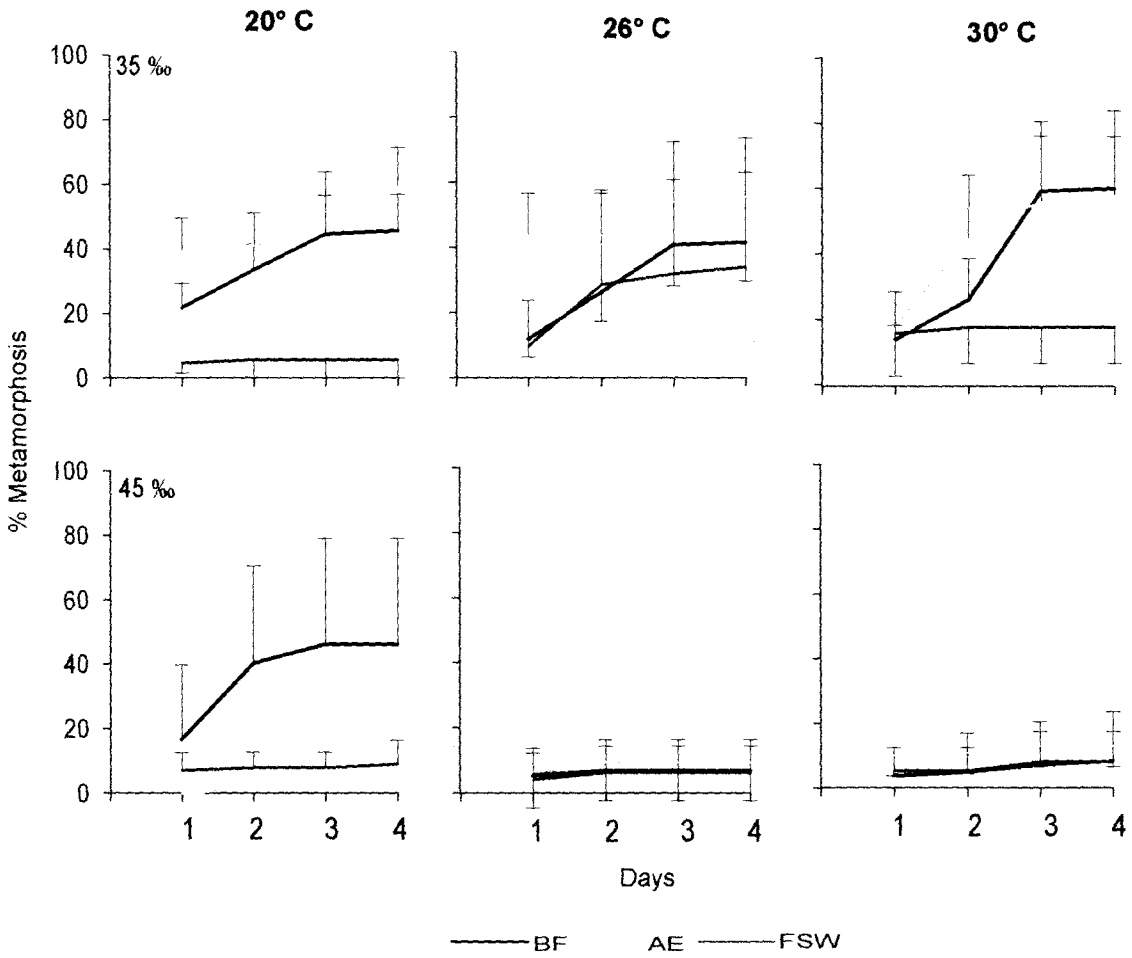


Fig. 3B.2 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*B. pumilus*), adult extract (AE) and filtered sea water (FSW) at different salinities (35 and 45‰) and temperatures (20, 26 and 30° C). Vertical lines indicate the standard deviation from mean and are indicated 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				Day 1			Day 4		
	df	SS	MS	Fs	SS	MS	Fs		SS	MS	Fs	SS	MS	Fs
(a) BF Salinity	3	868	289	9.5*****	3174	1058	22*****	**(b) BF+AE**	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		
(c) CS1 Salinity	3	748	249	4***	475	158	0.7ns	**(d) CS1+AE**	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		
(e) CS2 Salinity	3	2281	760	6****	4371	1457	4***	**(f) CS2+AE**	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		
(g) CS3 Salinity	3	2761	920	14*****	4742	1580	20*****	**(h) CS3+AE**	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		
(i) CS Salinity	3	3857	1286	14*****	6545	2182	12*****	**(j) CS+AE**	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		
(k) BBE Salinity	3	1889	630	7*****	3664	1221	10*****	**(l) BBE+AE**	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).

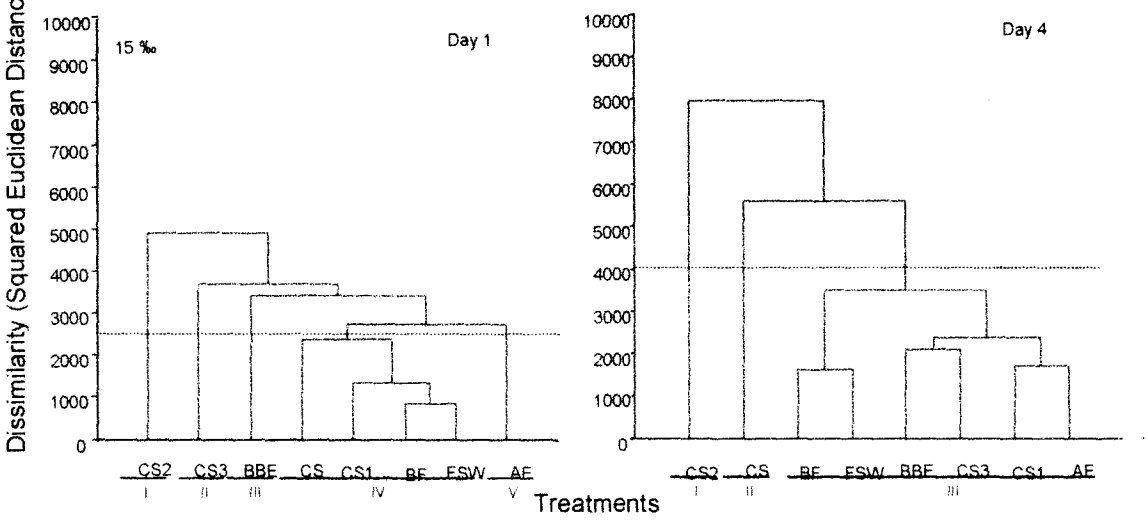


Fig. 3B.3 Dendrograms showing the dissimilarity among different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 15‰. The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed

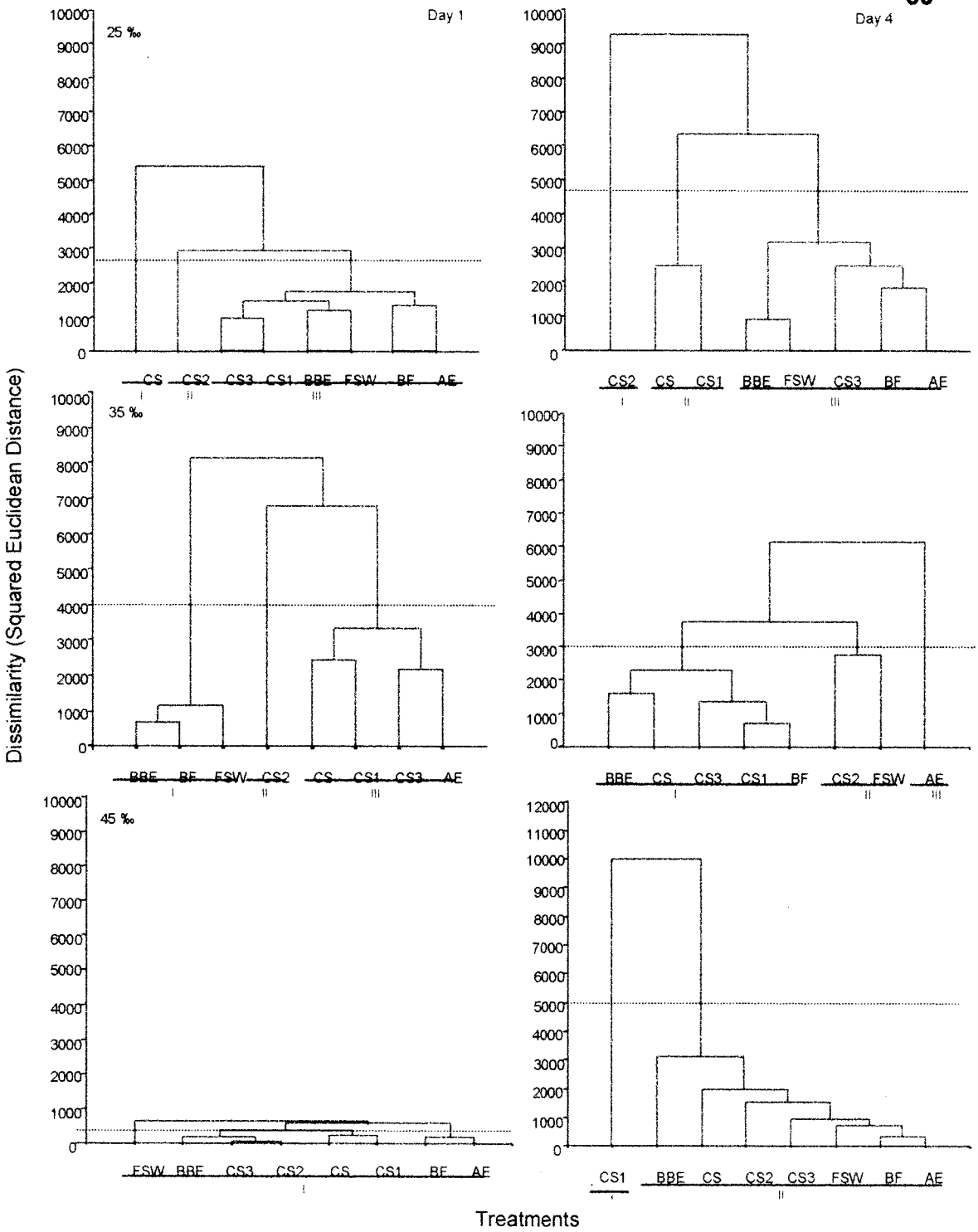


Fig. 3B.3 Dendrograms showing the dissimilarity among different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 25, 35 and 45‰. The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed

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

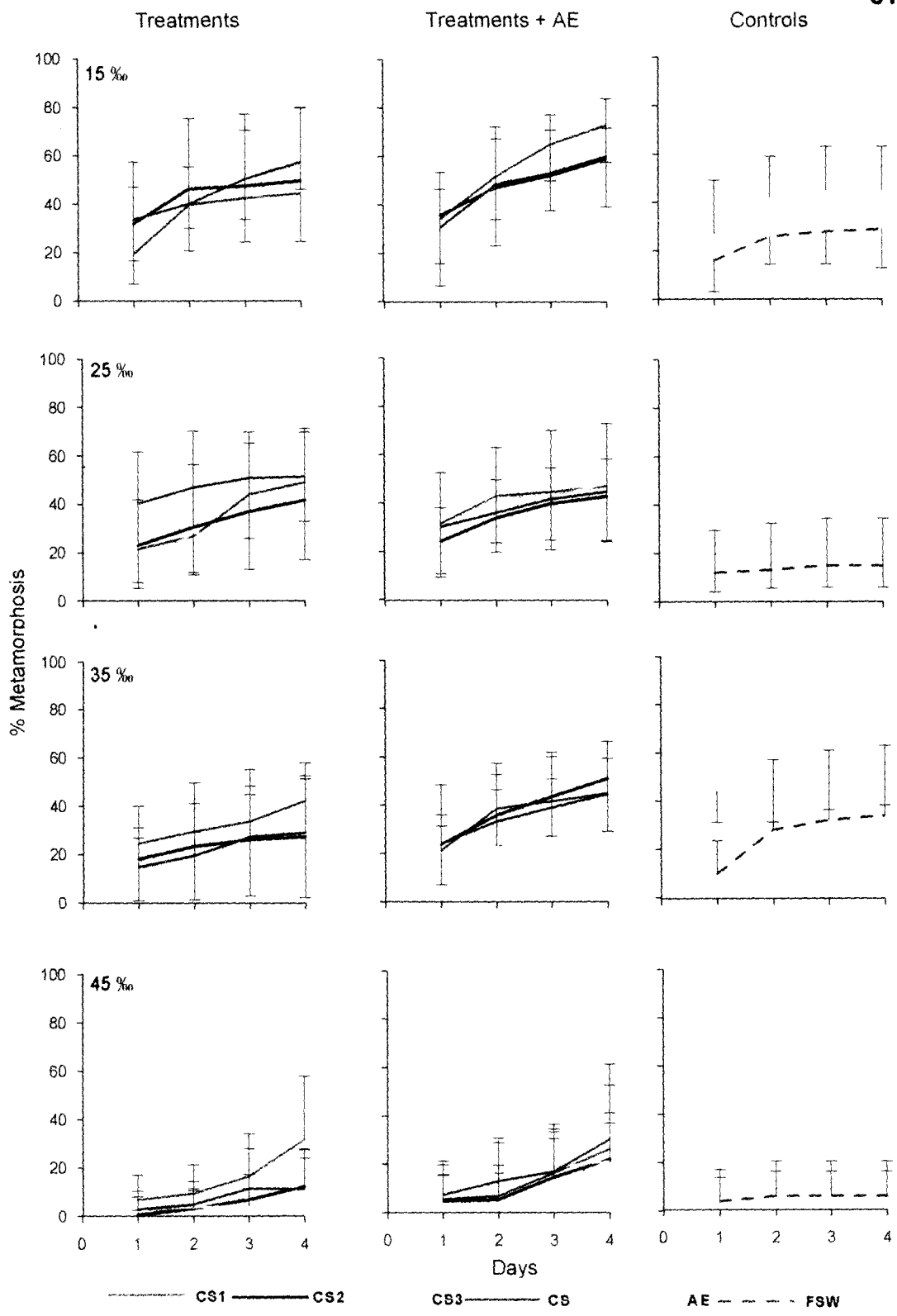


Fig. 3B.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution in presence and absence of adult extract (AE). Vertical lines indicate the standard deviation from mean and are indicated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

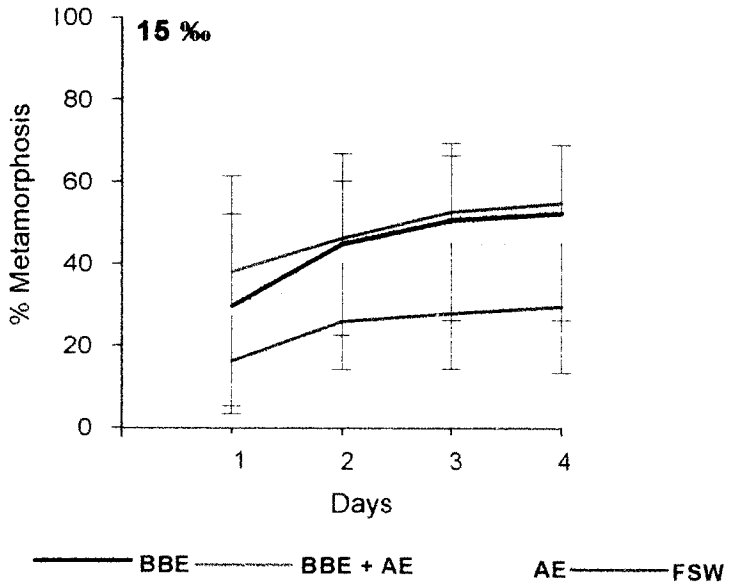


Fig. 3B.5 Percentage metamorphosis of *B. amphitrite* cyprids exposed to Butanol- bacterial extract at 15‰. Vertical lines indicate the standard deviation from mean and are indicated as either positive or negative

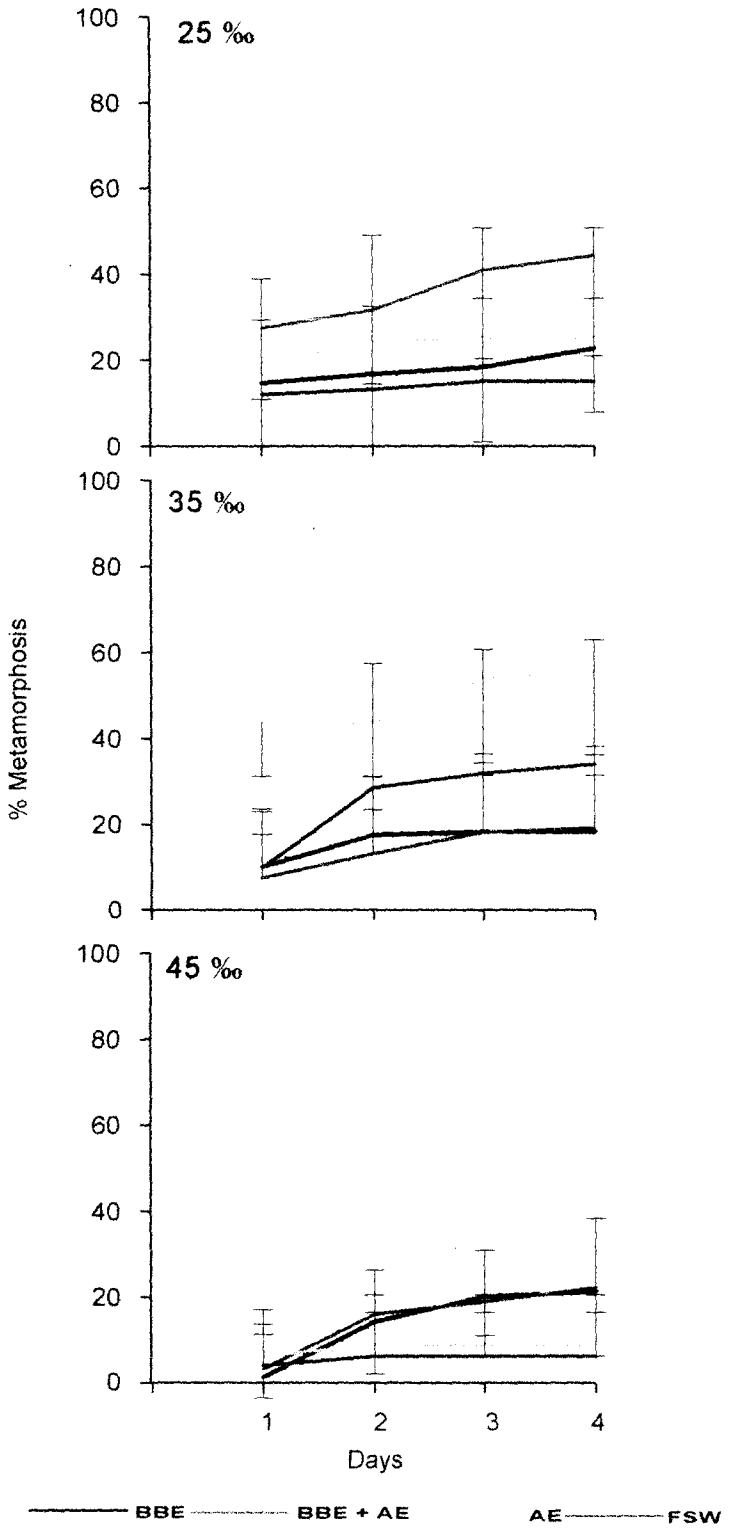


Fig. 3B.5 Percentage metamorphosis of *B. amphitrite* cyprids exposed to Butanol-bacterial extract at 25, 35 and 45‰. Vertical lines indicate the standard deviation from mean and are indicated as either positive or negative

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.

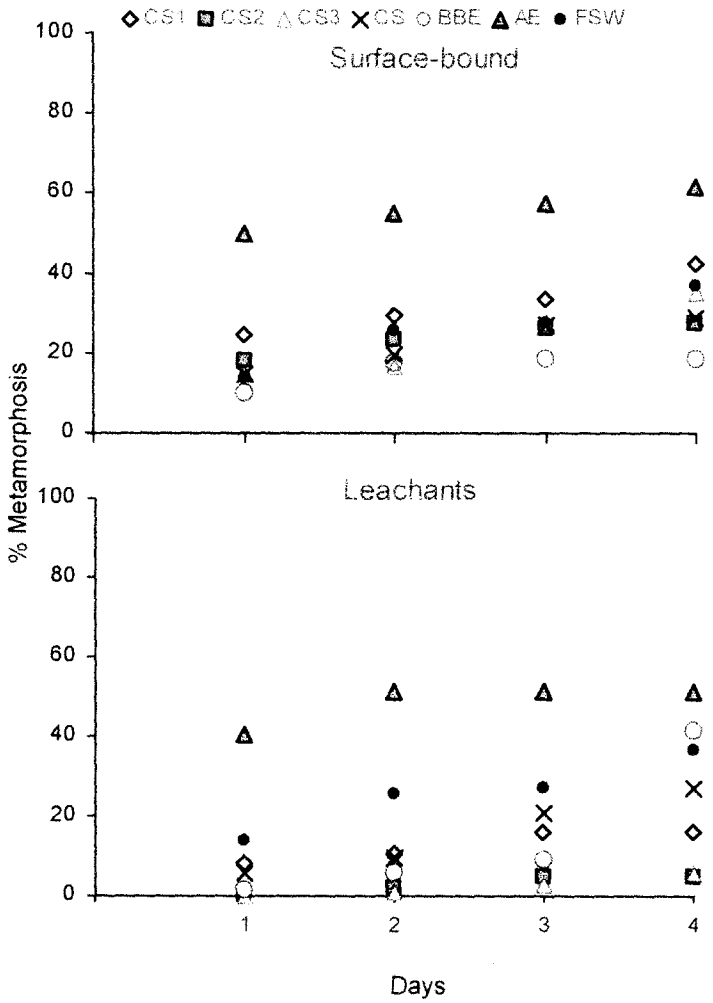


Fig. 3B.6 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial inducers (surface-bound) used in experiment 1 in presence of leachants at 35‰

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 μg ml^{-1} on day 1 and 4 (Table 3B.3b).

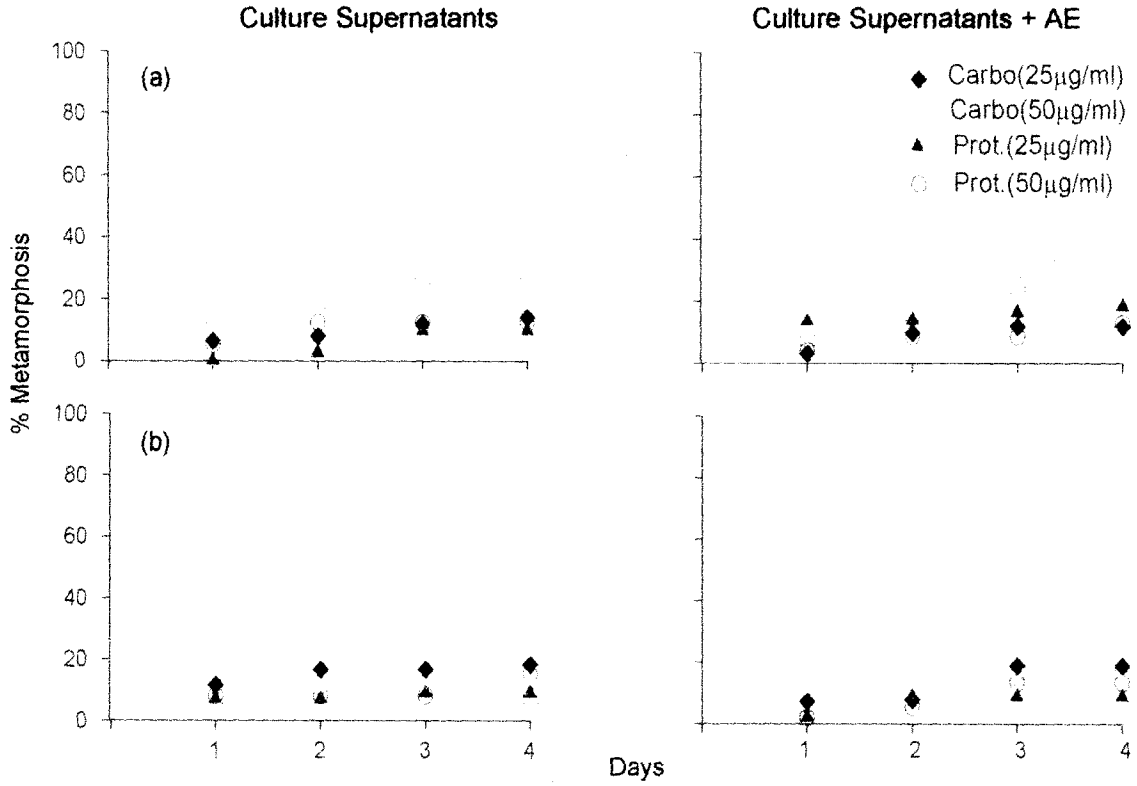


Fig. 3B.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutrient media at 35‰. (a) CS(BSS) (b) CS(semi-solid)

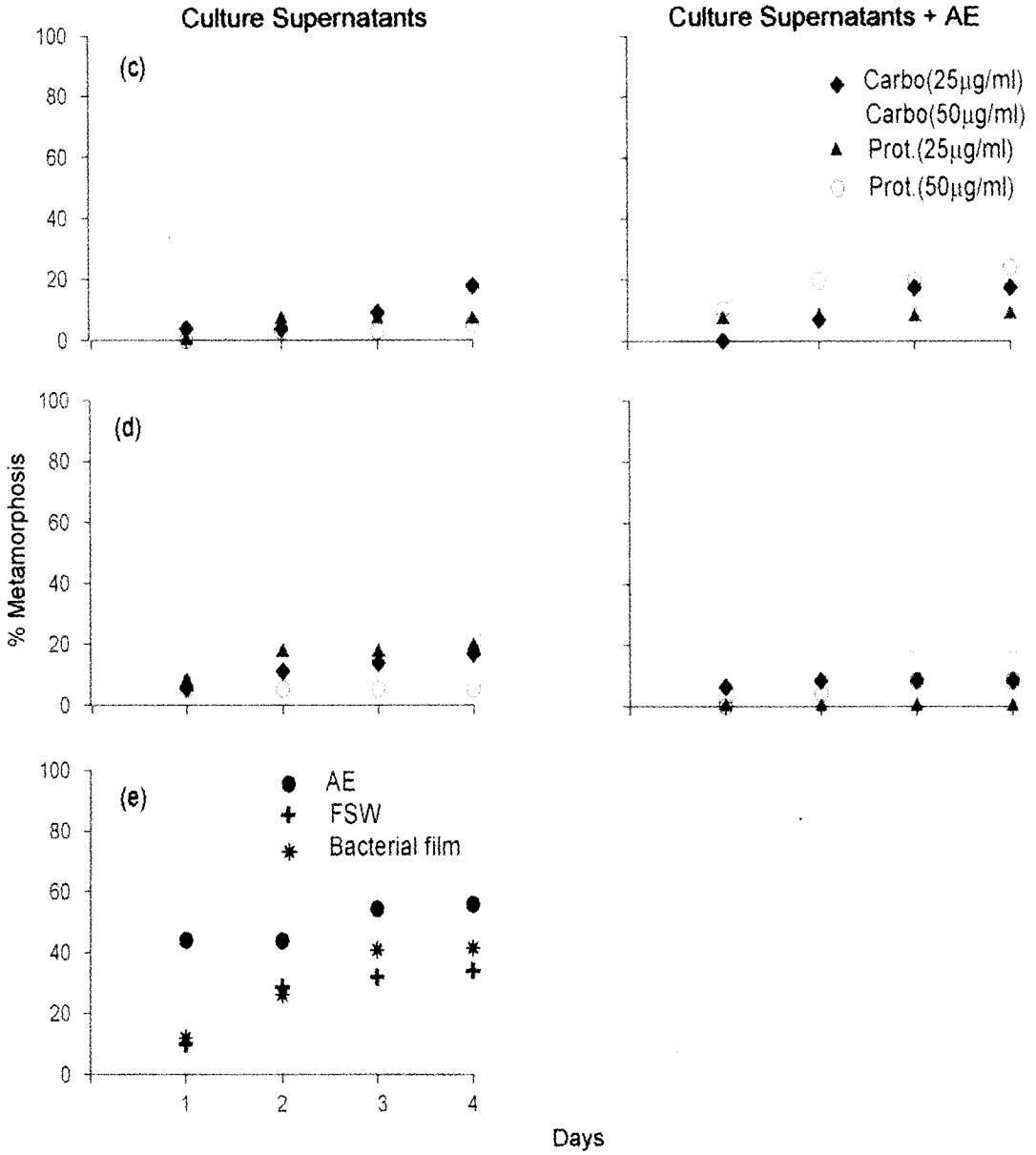


Fig. 3B.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutrient media at 35‰. (c) CS(MV) (d) CS(MB) (e) Controls

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 µg ml^{-1} on day 1 and 4 (Table 3B.3b).

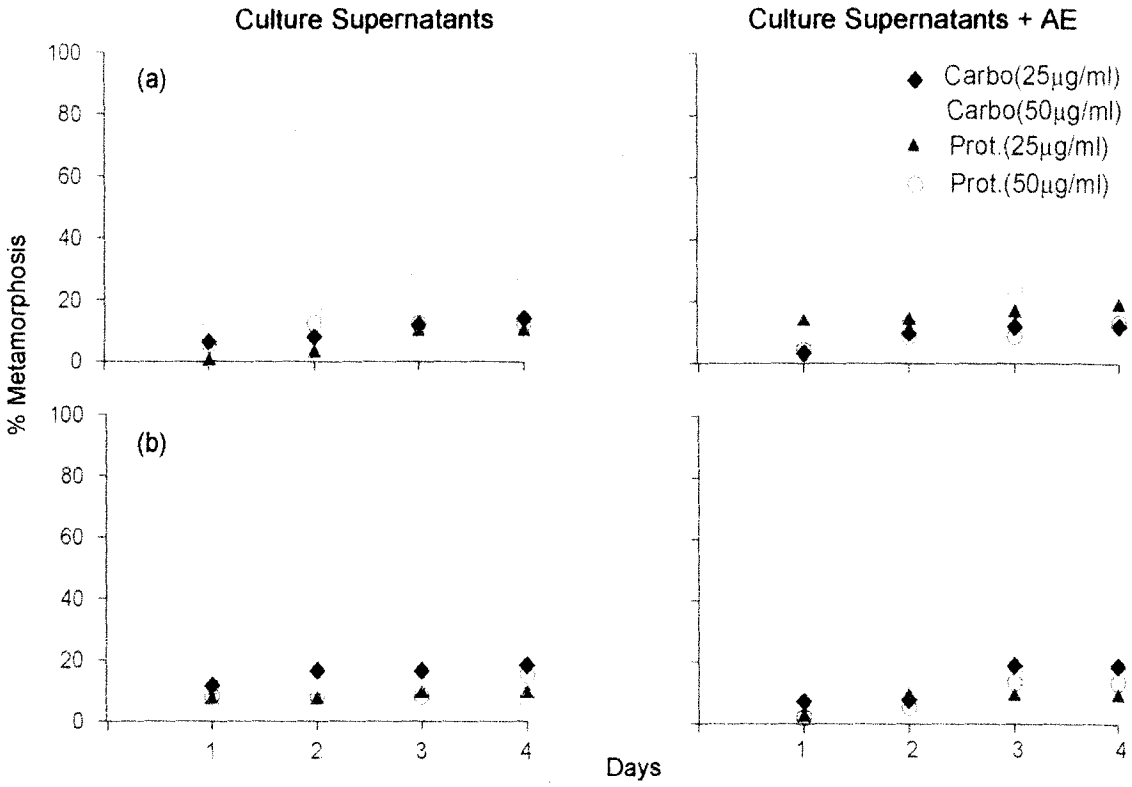


Fig. 3B.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutrient media at 35‰. (a) CS(BSS) (b) CS(semi-solid)

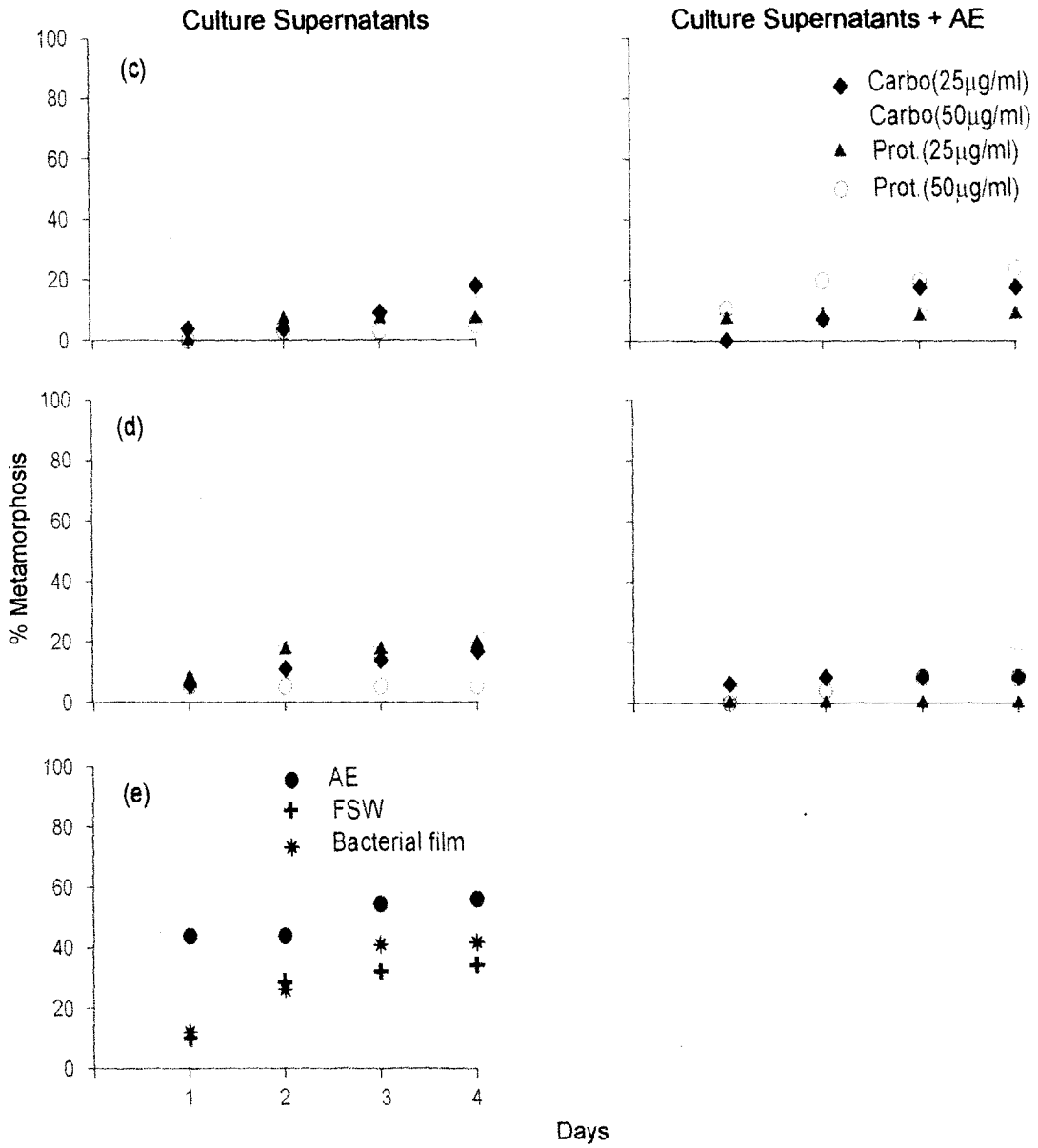


Fig. 3B.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutrient media at 35‰. (c) CS(MV) (d) CS(MB) (e) Controls

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
Day1													
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		
Day4													
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
Day1													
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		
Day4													
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		

(*****$p \leq 0.001$,****$p \leq 0.005$, ***$p \leq 0.01$,** $p \leq 0.025$, *$p \leq 0.05$, 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).

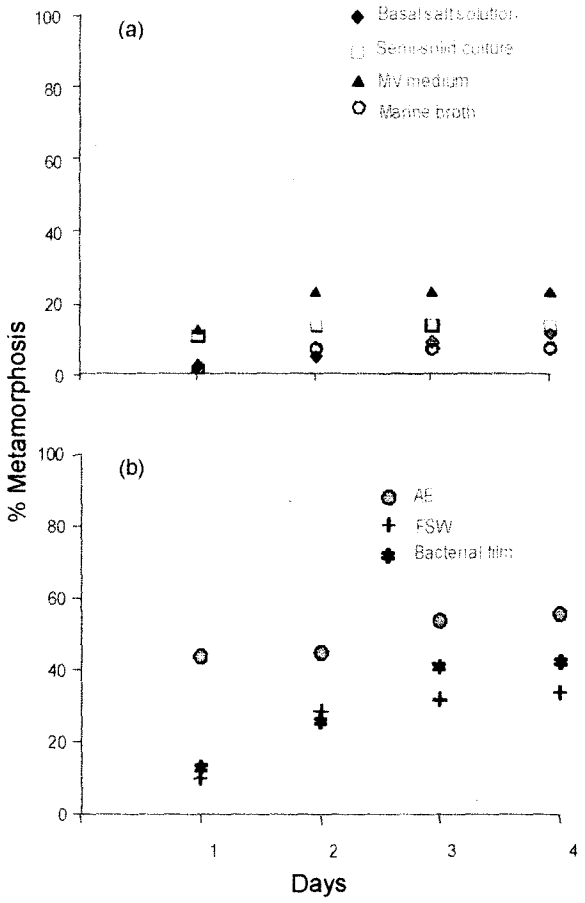


Fig. 3B.8 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial exopolysaccharides obtained after growing the bacteria under different nutritional conditions at 35‰

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 cm^{-1}, 1640 cm^{-1} and 1090 -1117 cm^{-1} for O-H stretching, C=O stretching and C-O stretching and hence were mainly carbohydrates.

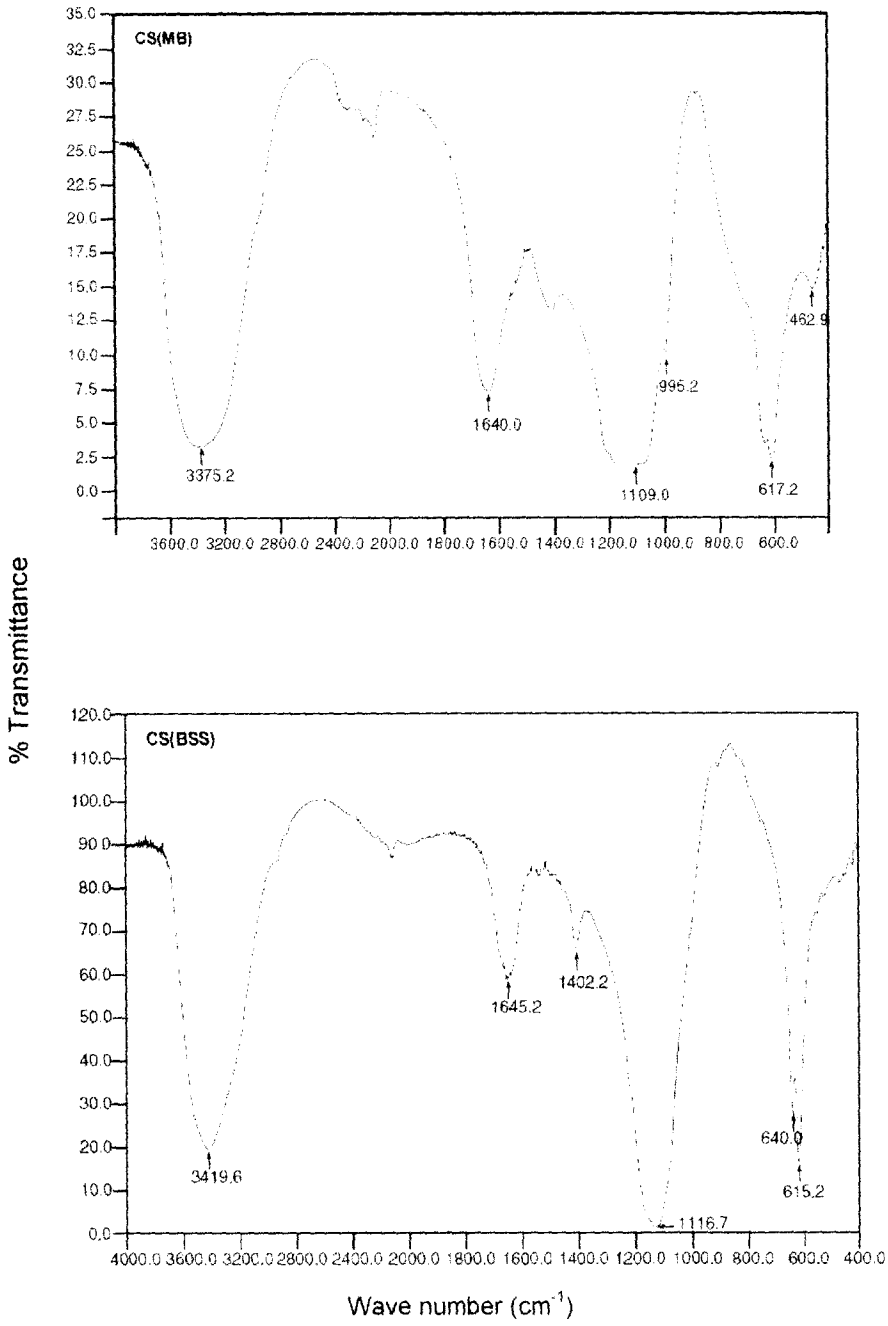


Fig. 3B.9 FTIR spectras of culture supernatants of bacteria grown in MB and basal salt solution (BSS)

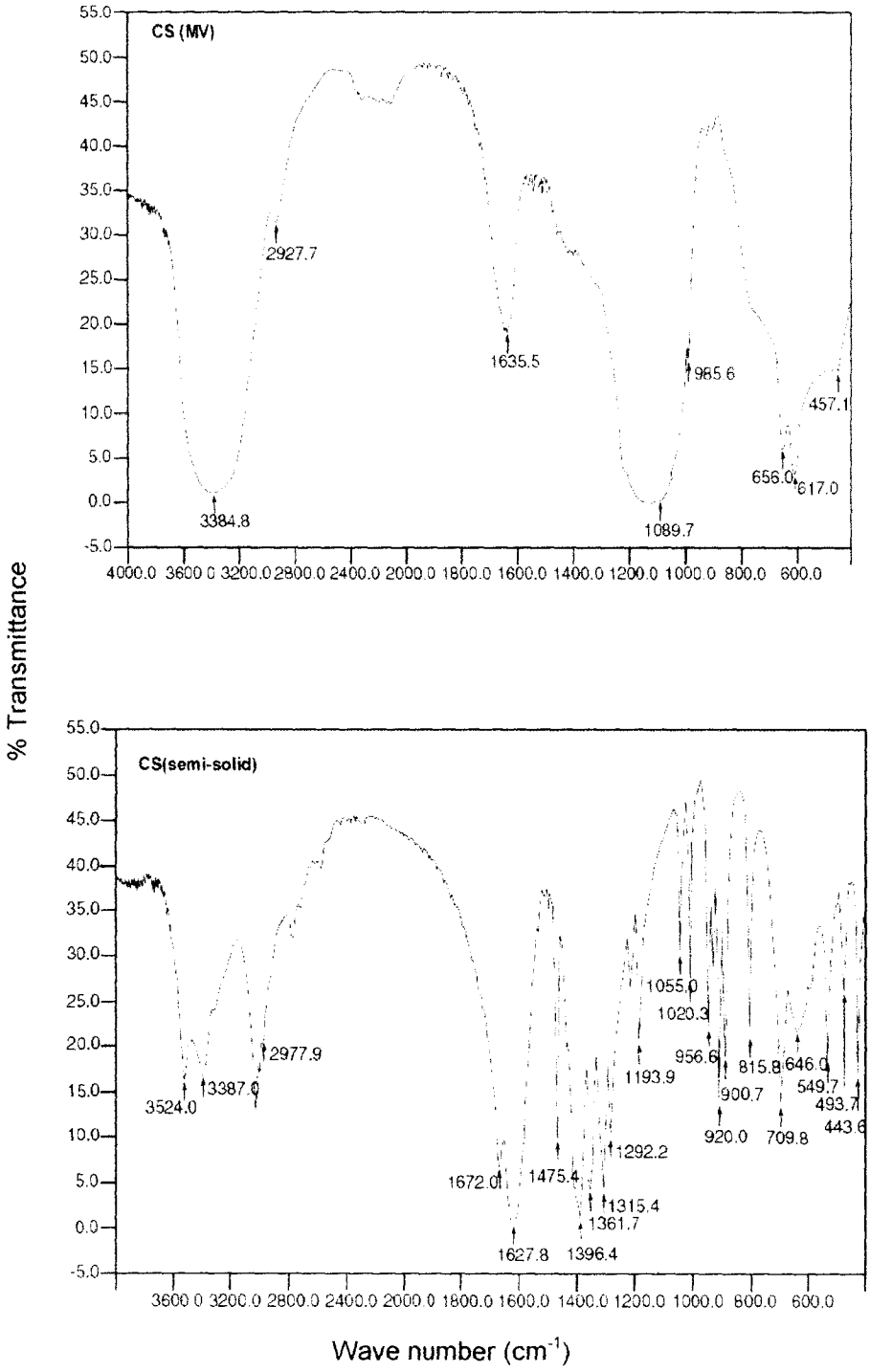


Fig. 3B.9 FTIR spectras of culture supernatants of bacteria grown in MV and semi-solid medium

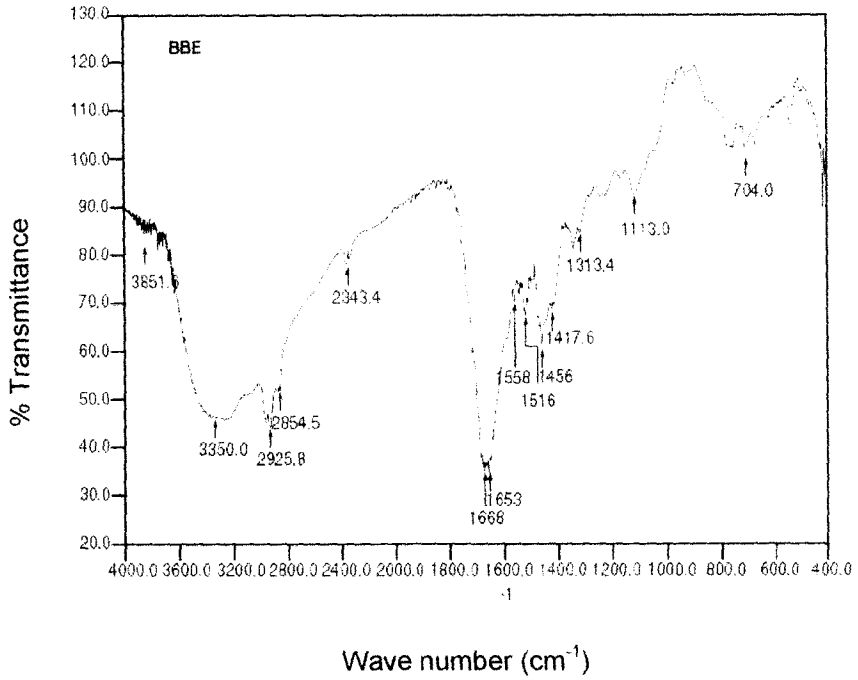


Fig 3B.9 FTIR spectra of Butanol-bacterial extract

Culture supernatant of semi-solid culture and bacterial extract revealed strong peaks at 1628 and 1653 cm^{-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 and 1362 cm^{-1}. The spectra also indicated the presence of unsaturation evident from C-H bending vibrations located around 1000 and 900 cm^{-1}. Bacterial extract showed peak at 1668 cm^{-1} 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).

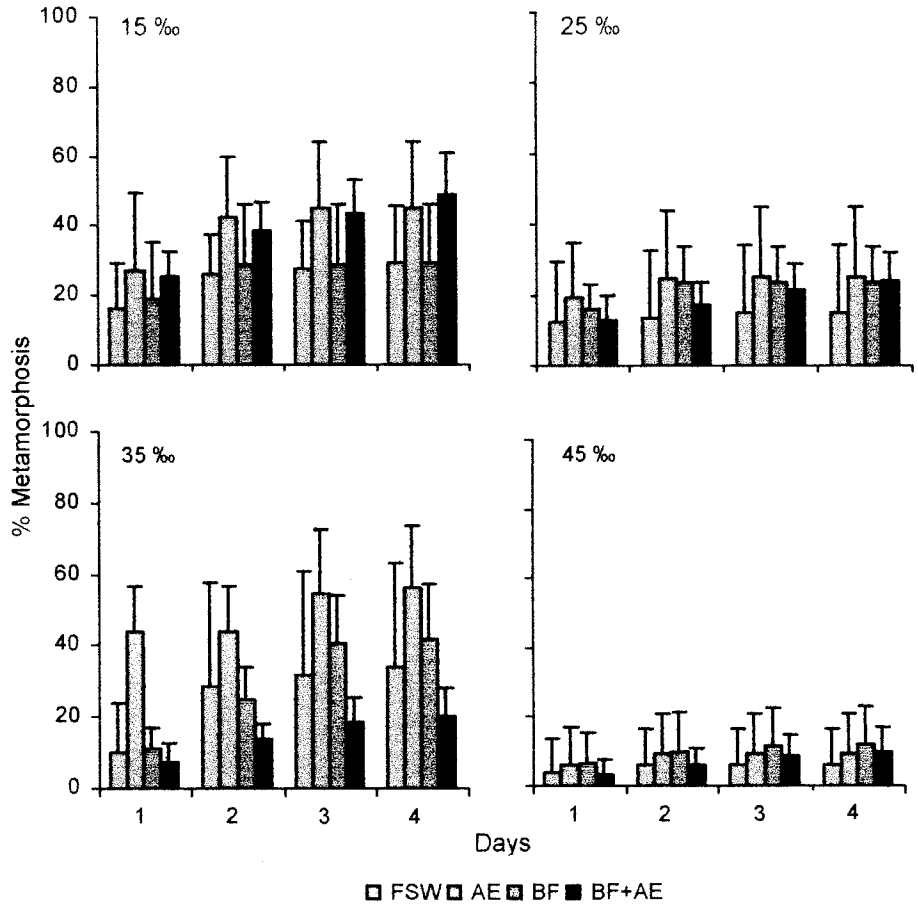


Fig. 3C.1 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*C. freundii*) in presence of adult extract (AE) at different salinities. Vertical lines indicate the standard deviation from mean and are shown as positive

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
(a) BF	Salinity	3	378	126	3**	2046	682	9*****	**(b) BF+AE**	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		
(c) CS1	Salinity	3	5365	1788	4.5***	10985	3661	7*****	**(d) CS1+AE**	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		
(e) CS2	Salinity	3	5673	1891	20*****	6191	2064	10*****	**(f) CS2+AE**	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		
(g) CS3	Salinity	3	4161	1387	6.3*****	2815	938	2.8*	**(h) CS3+AE**	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		
(i) CS	Salinity	3	1096	365	2ns	2549	850	2.5ns	**(j) CS+AE**	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		
(k) BBE	Salinity	3	1807	602	7*****	8435	2811	20*****	**(l) BBE+AE**	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.

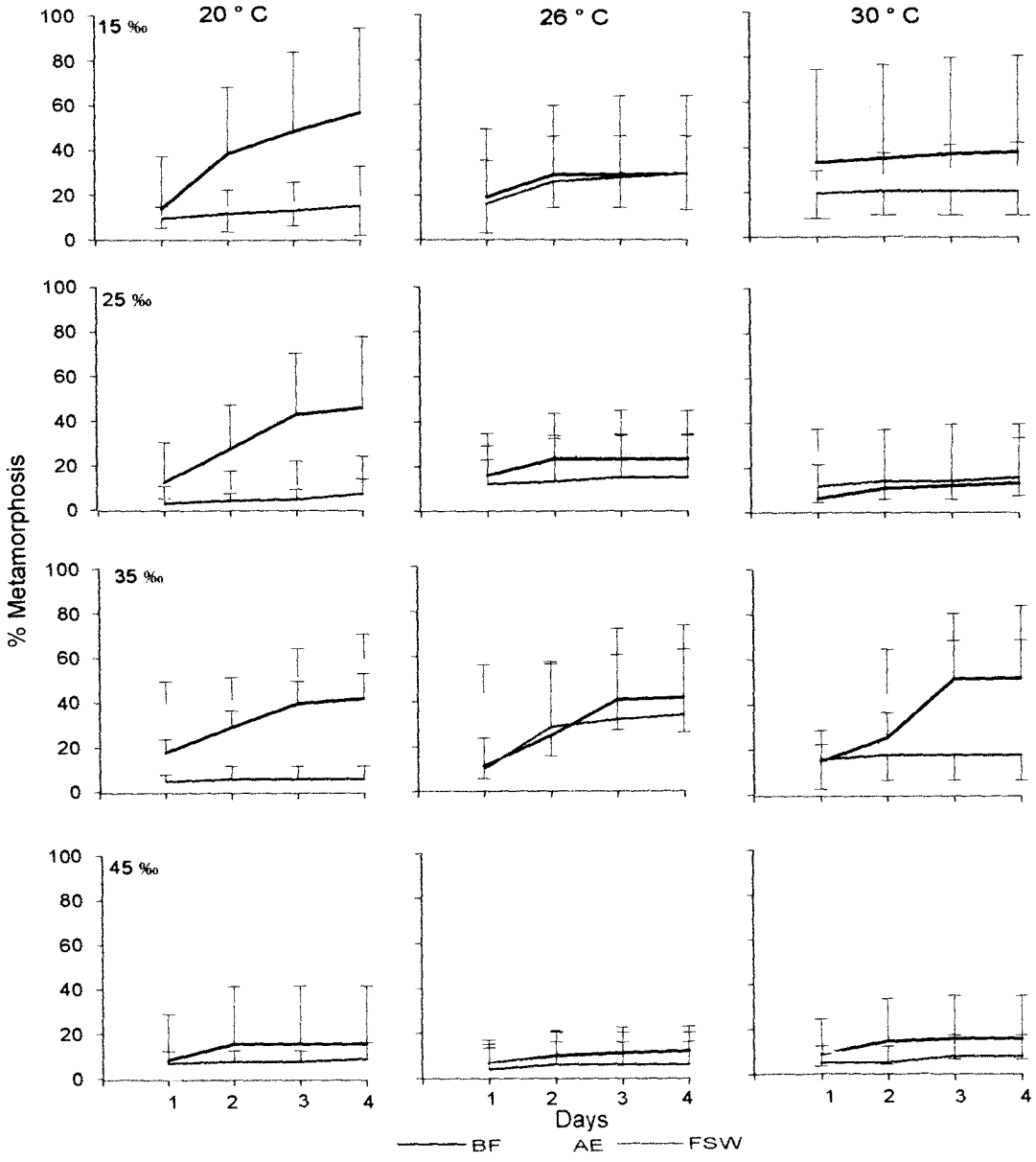


Fig. 3C.2 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial film (*C. freundii*), adult extract (AE) and filtered sea water (FSW) at different salinities and temperatures. Vertical lines indicate the standard deviation from mean and are shown as either positive or negative

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).

		Day 1			Day 4		
	df	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).

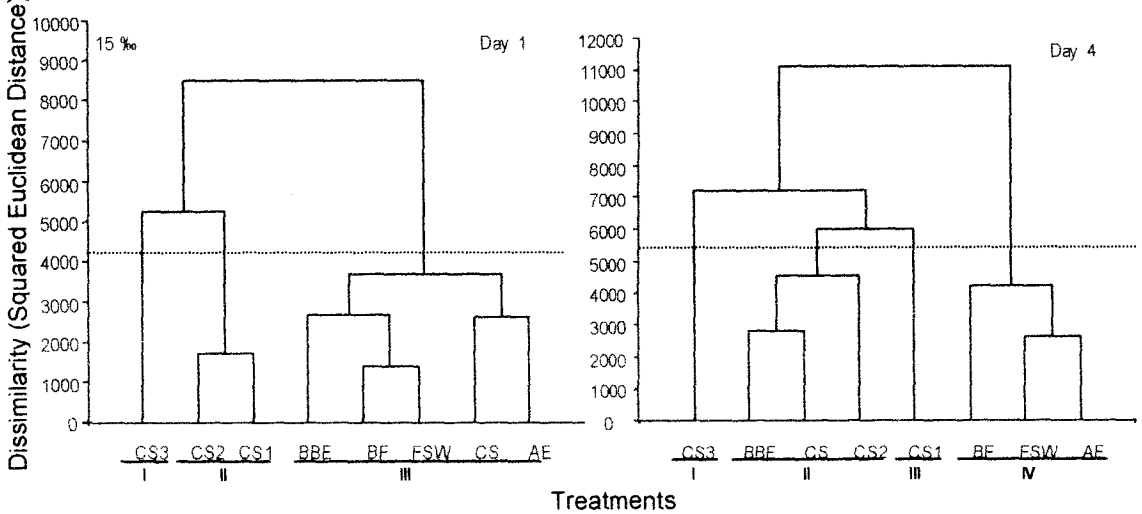


Fig. 3C.3 Dendrograms showing the dissimilarity among different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 15‰. The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed

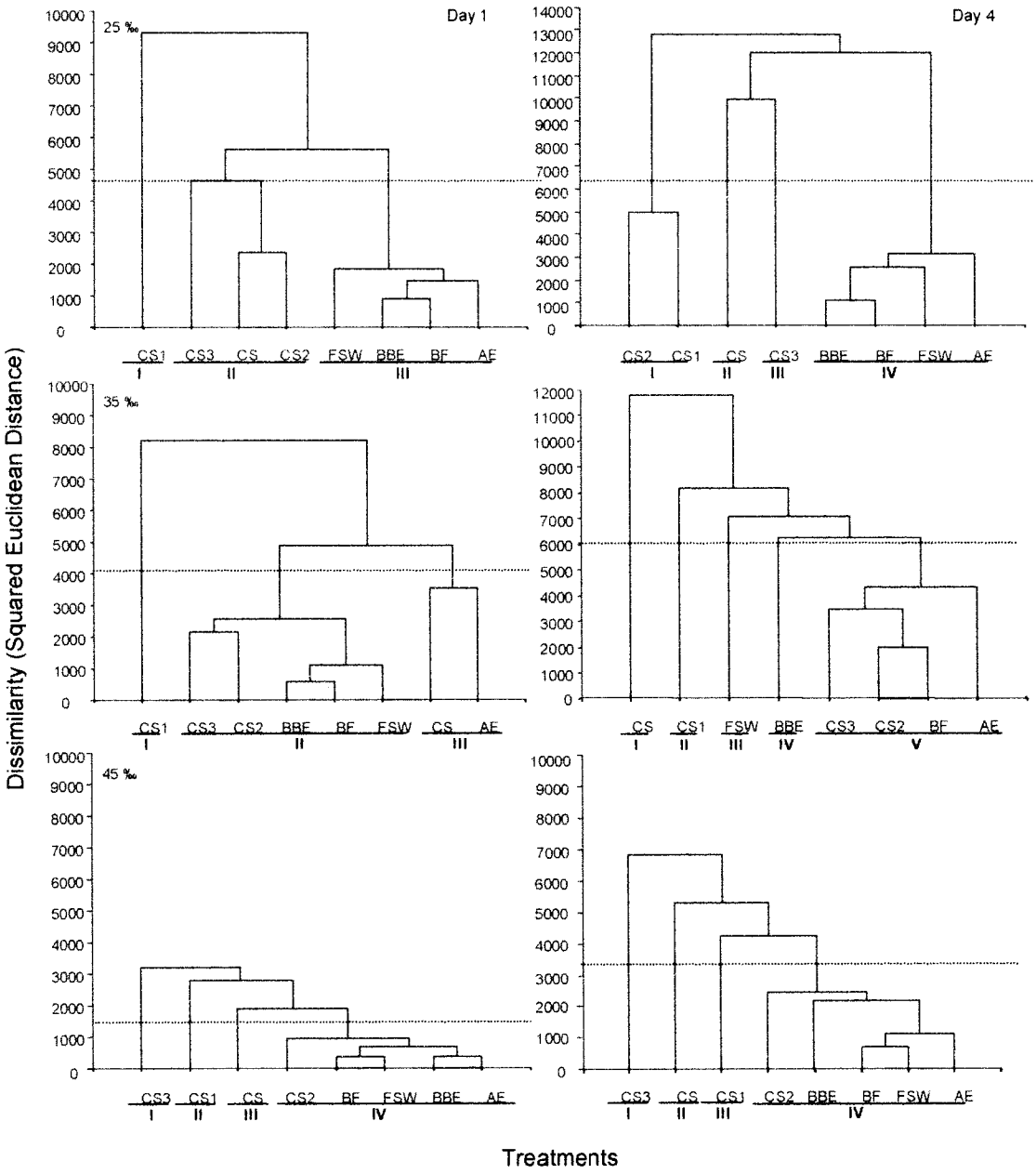


Fig. 3C.3 Dendrograms showing the dissimilarity among different bacterial inducers (treatments) towards metamorphosis of *B. amphitrite* cyprids on day 1 and day 4 at 25, 35 and 45‰. The X-axis groupings are based on the clusters that are dissimilar beyond mid-point of highest dissimilarity observed

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.

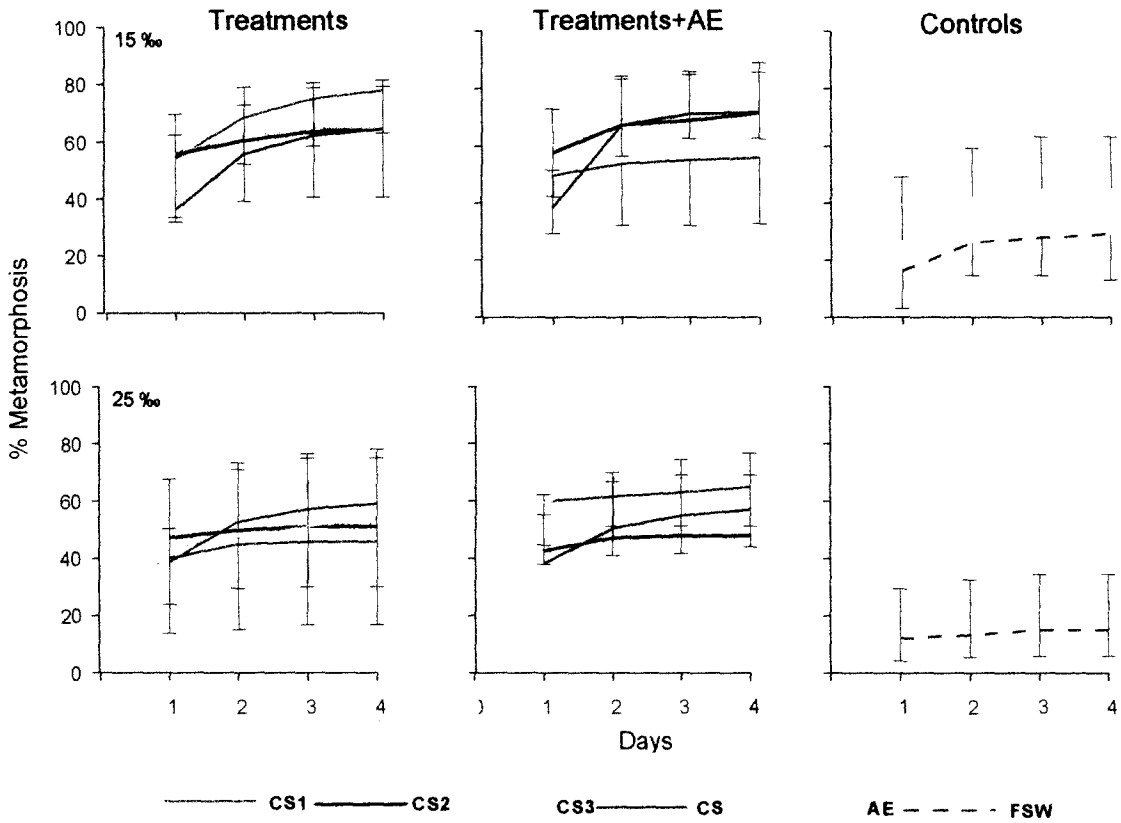


Fig. 3C.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution (BSS) in presence and absence of adult extract (AE) at 15 and 25‰. Vertical lines indicate the standard deviation from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

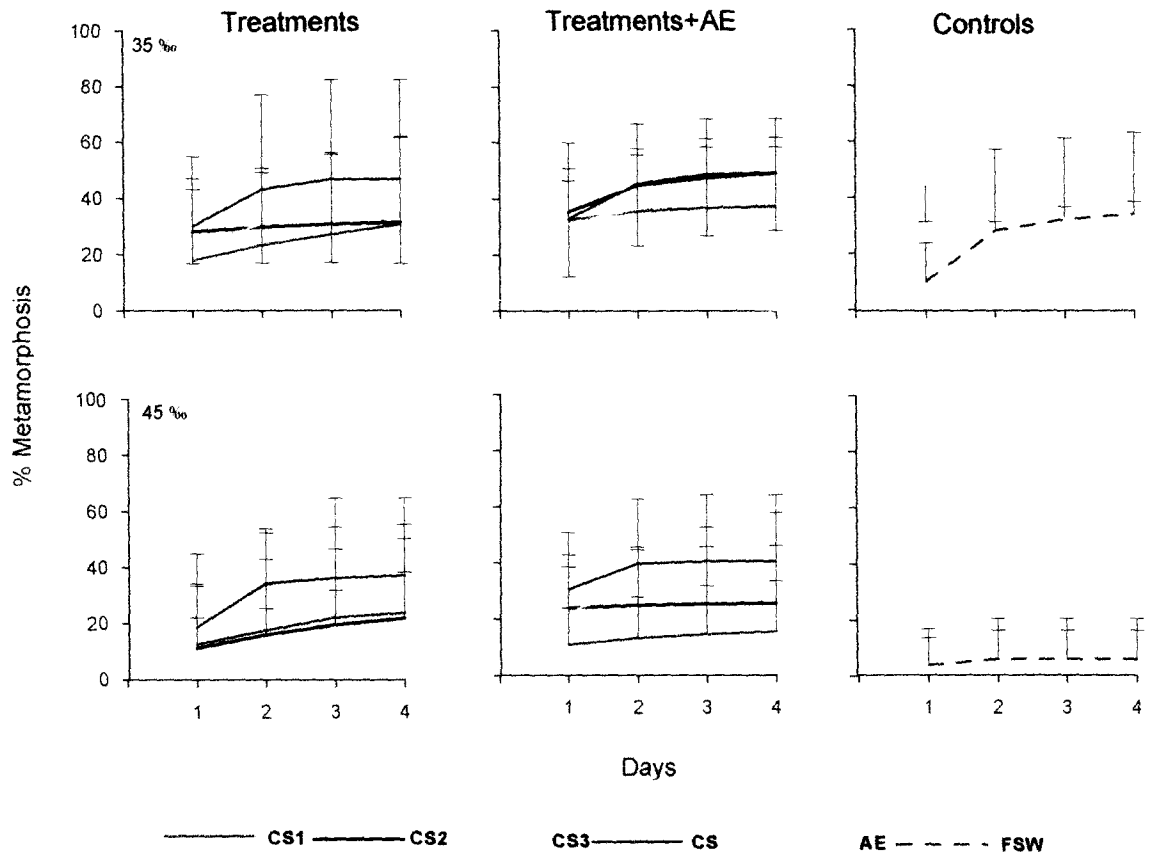


Fig. 3C.4 Percentage metamorphosis of *B. amphitrite* cyprids in response to culture supernatant and its fractions (treatments) obtained from the bacteria grown in basal salt solution (BSS) in presence and absence of adult extract (AE) at 35 and 45‰. Vertical lines indicate the standard deviation from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

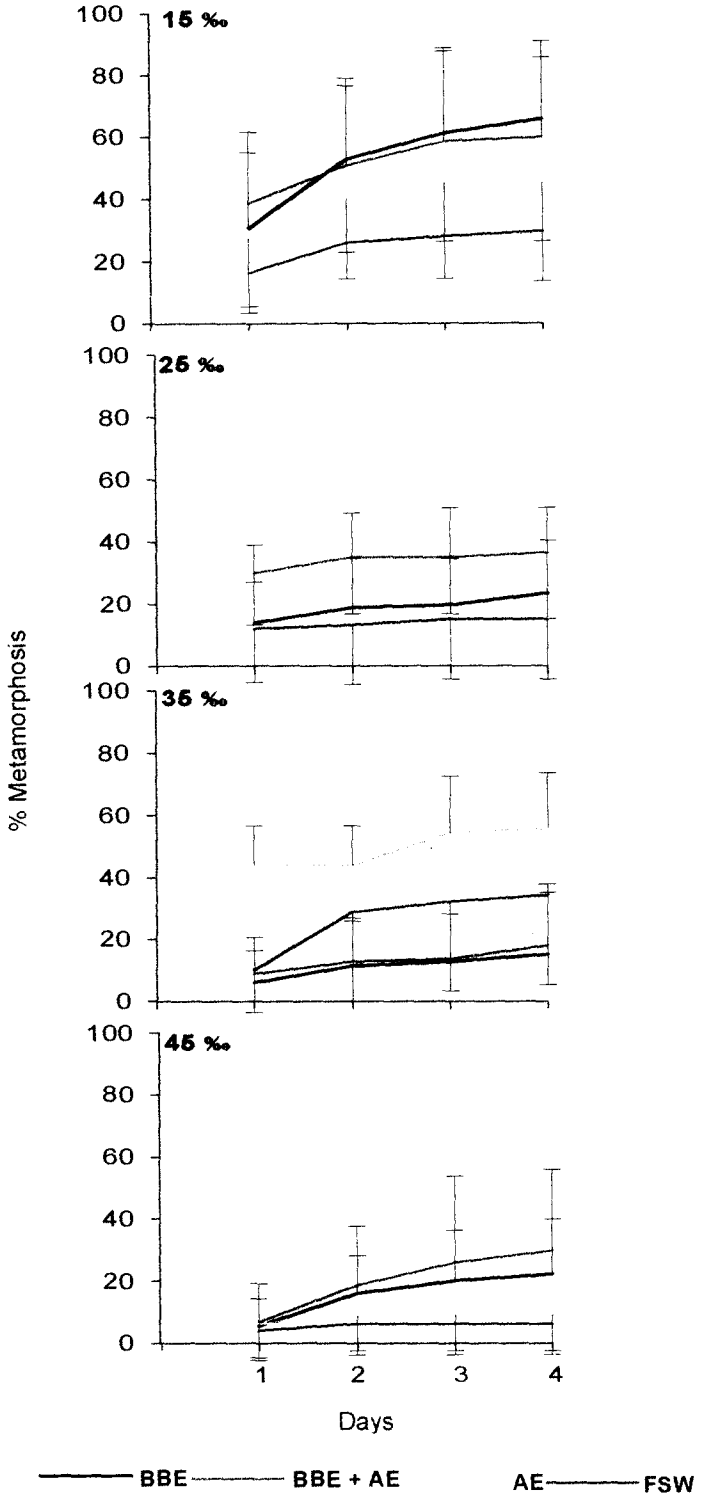


Fig. 3C.5 Percentage metamorphosis of *B. amphitrite* cyprids exposed to Butanol-bacterial extract at different salinities. Vertical lines indicate the standard deviation from mean and are shown as either positive or negative

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.

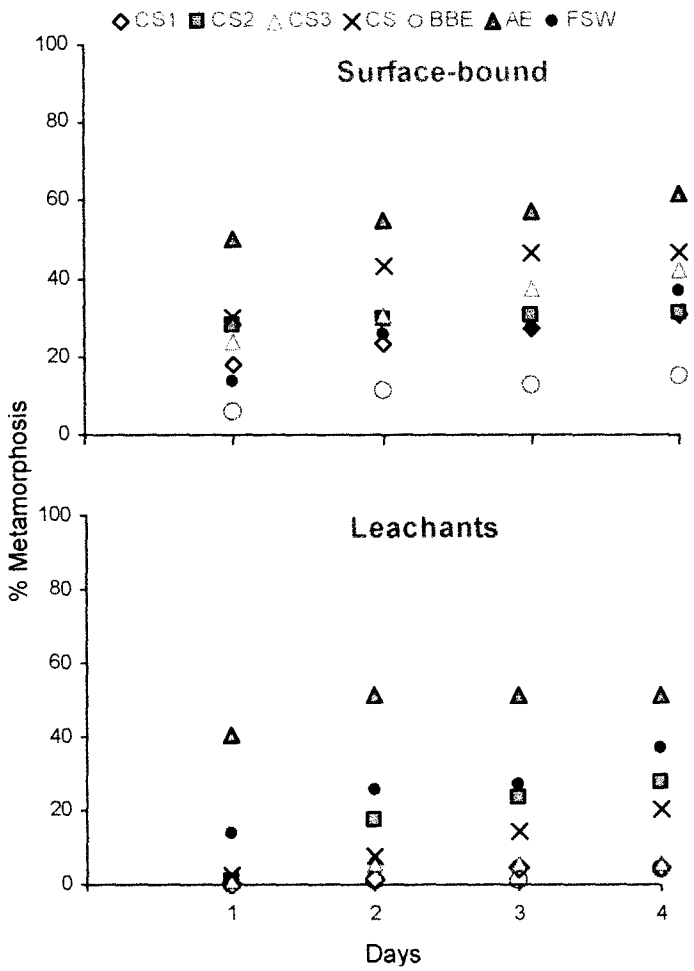


Fig. 3C.6 Percentage metamorphosis of *B. amphitrite* cyprids in response to bacterial inducers (surface-bound) used in experiment 1 in presence of leachants at 35‰

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 50µg 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 50µg ml^{-1}.

When assessed in presence of AE a significant difference was observed at a carbohydrate concentration of 50 µg 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).

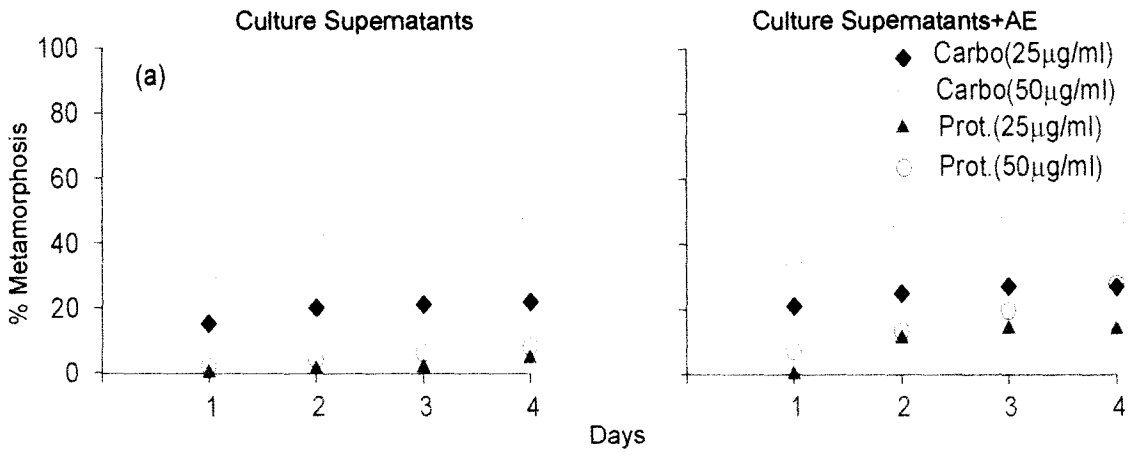


Fig. 3C.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatant obtained by growing the bacteria under basal salt solution (BSS) at 35‰

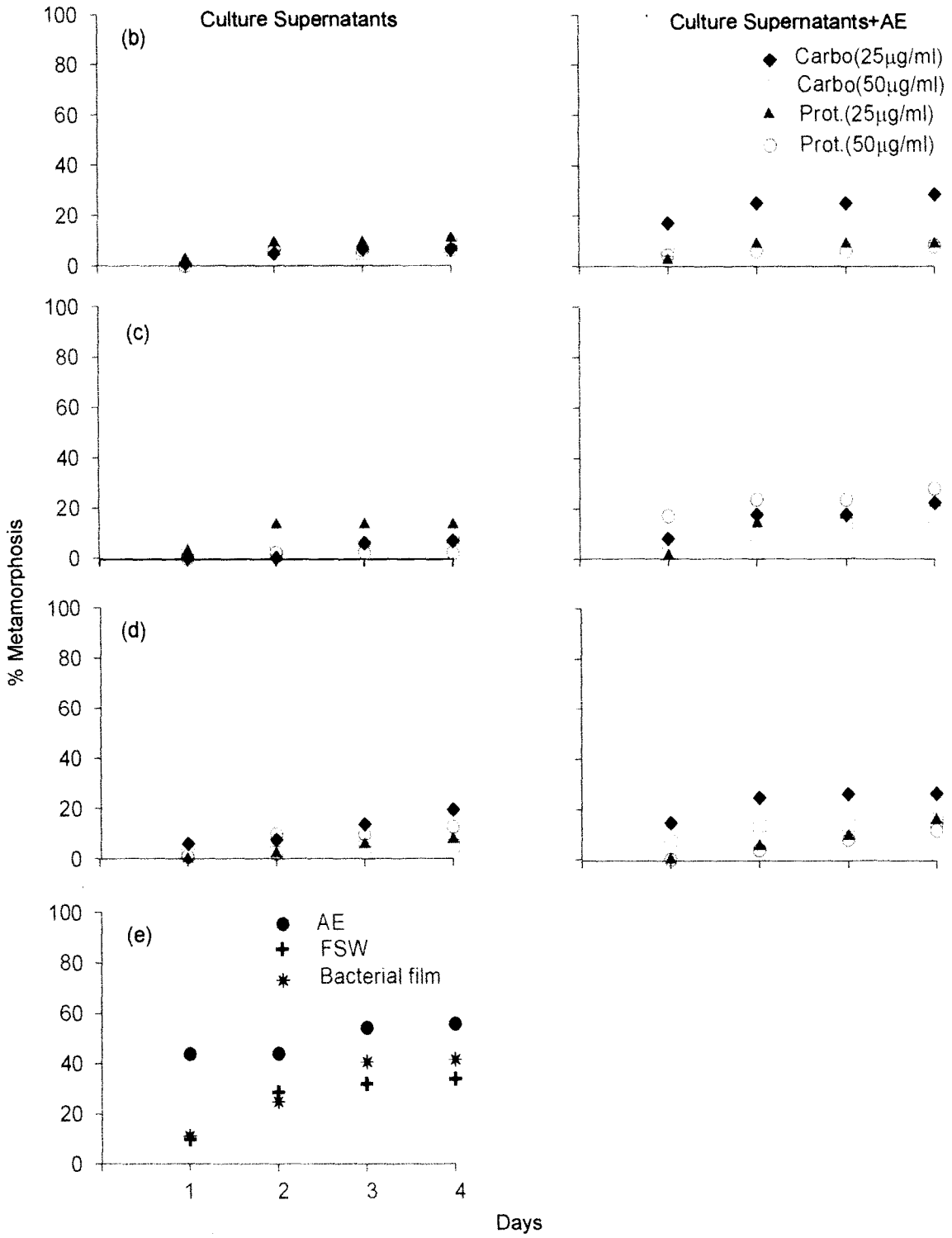


Fig. 3C.7 Percentage metamorphosis of *B. amphitrite* cyprids exposed to culture supernatants obtained by growing the bacteria under different nutritional media at 35‰. (b) CS(semi-solid) (c) CS(MV) (d) CS(MB) (e) Controls

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
Day1													
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		
Day4													
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
Day1													
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		
Day4													
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		

(*****$p \leq 0.001$,****$p \leq 0.005$, ***$p \leq 0.01$,** $p \leq 0.025$, *$p \leq 0.05$, 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).

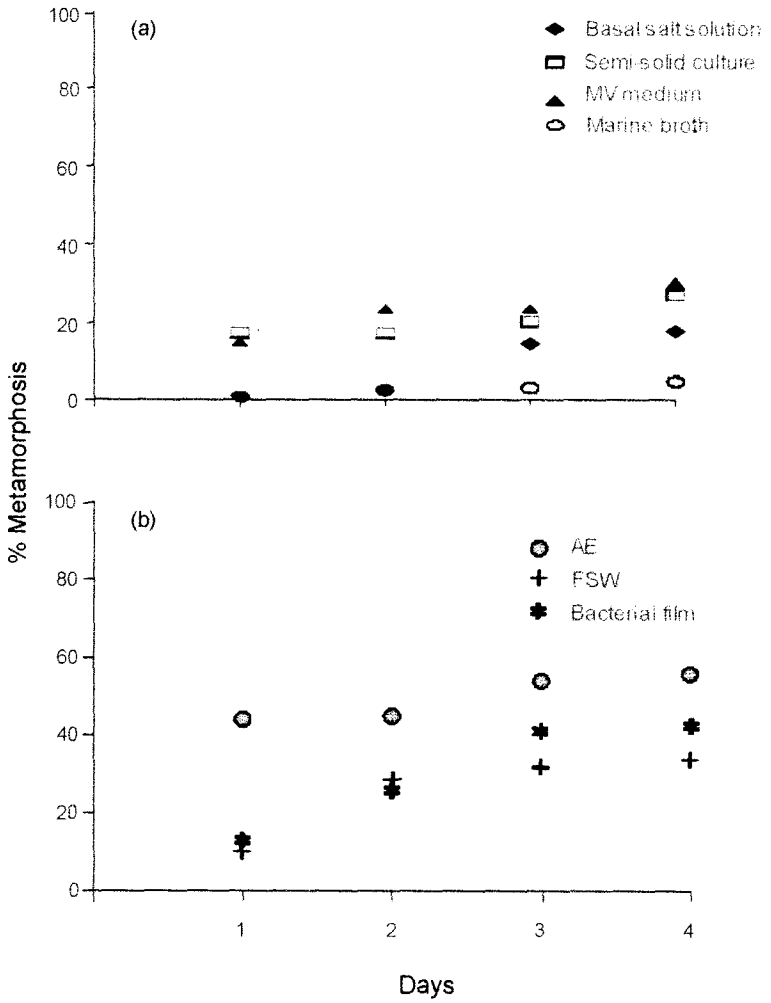


Fig. 3C.8 Percentage metamorphosis of *B. amphitrite* cyprids exposed to bacterial exopolysaccharides obtained after growing the bacteria under different nutritional conditions at 35‰

3C 3.5 FTIR spectroscopy

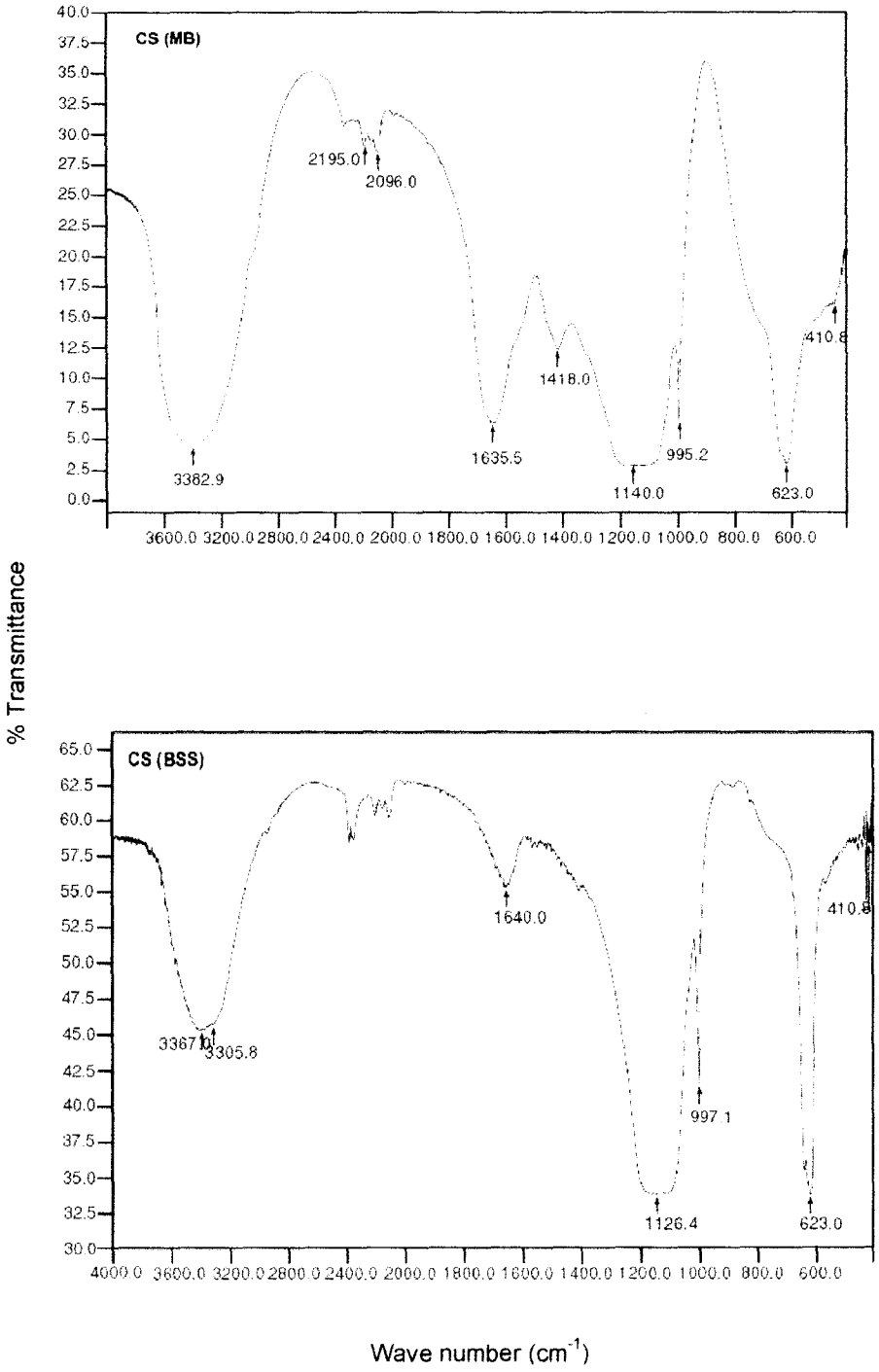


Fig. 3C.9 FTIR spectras of culture supernatants of bacteria grown in MB and basal salt solution (BSS)

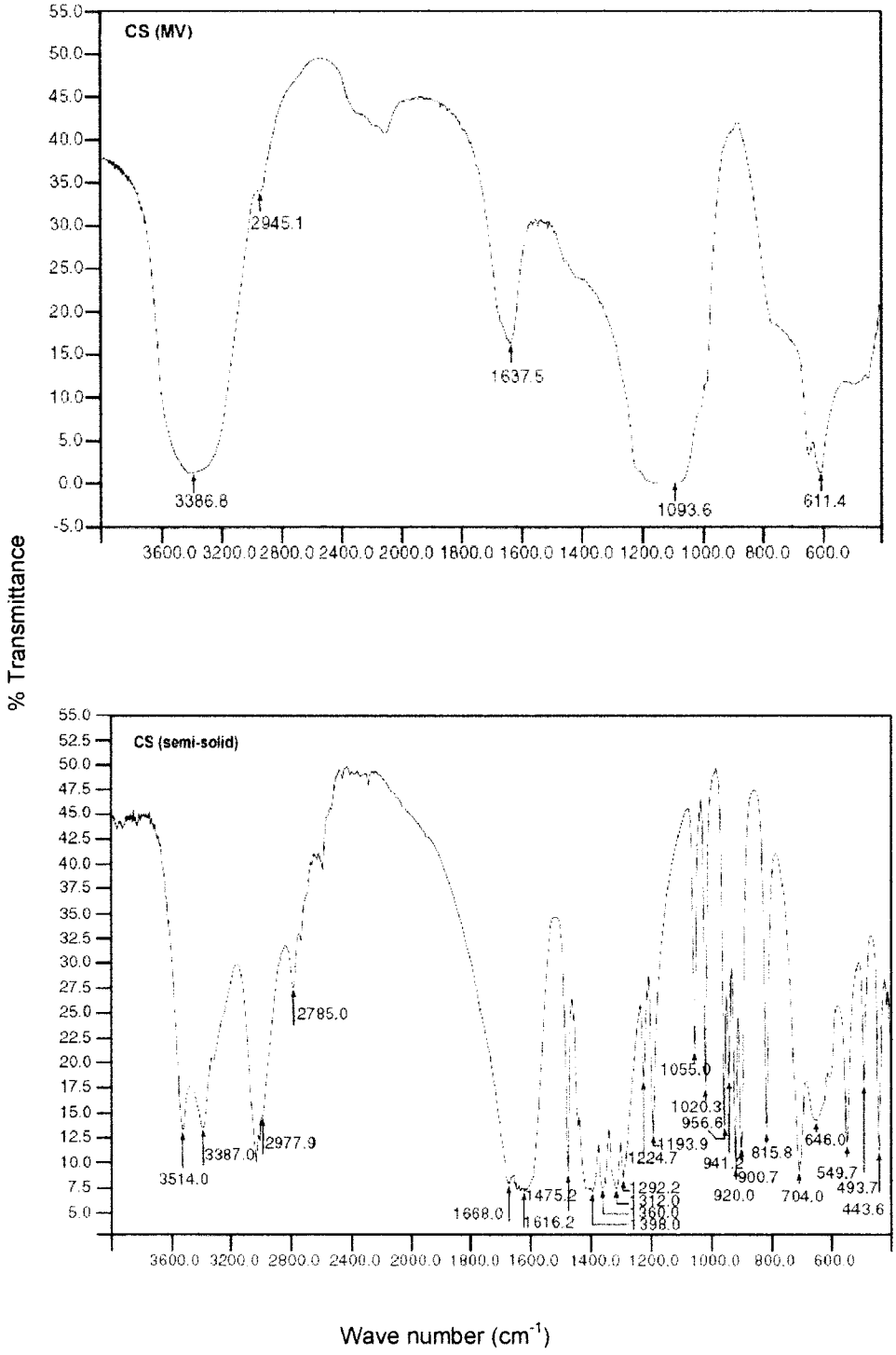


Fig. 3C.9 FTIR spectras of culture supernatants of bacteria grown in MV and semi-solid medium

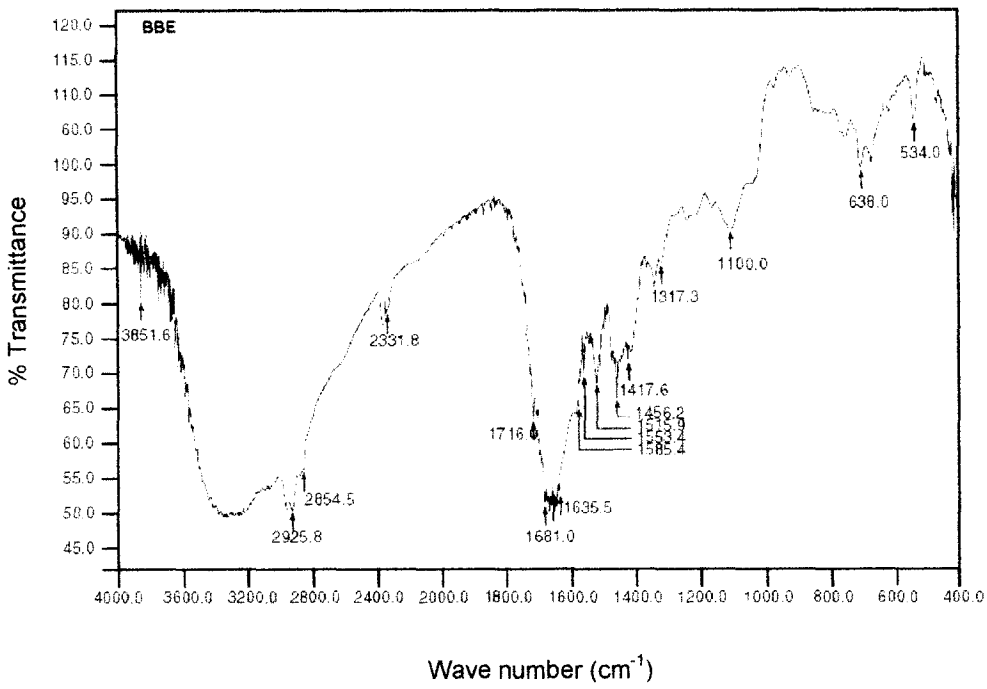


Fig. 3C.9 FTIR spectra of Butanol-bacterial extract

FTIR spectrum of culture supernatants of bacteria grown in MB, BSS and MV revealed characteristic strong peaks around 3550-3200 cm^{-1}, 1640 cm^{-1} and 1094-1126 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 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 cm^{-1} and 1360 cm^{-1}. The spectra also indicated presence of unsaturation evident from C-H bending vibrations located around 1000 and 900 cm^{-1}. Bacterial extract showed peaks at 1716 and 1681 cm^{-1} characteristic for ketones.

3D. Thraustochytrid (#MS 2D)

In general percentage metamorphosis increased from day one to day four. The presence of thraustochytrid (#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 thraustochytrid exhibited highest percentage metamorphosis facilitation followed by adult extract and control (FSW).

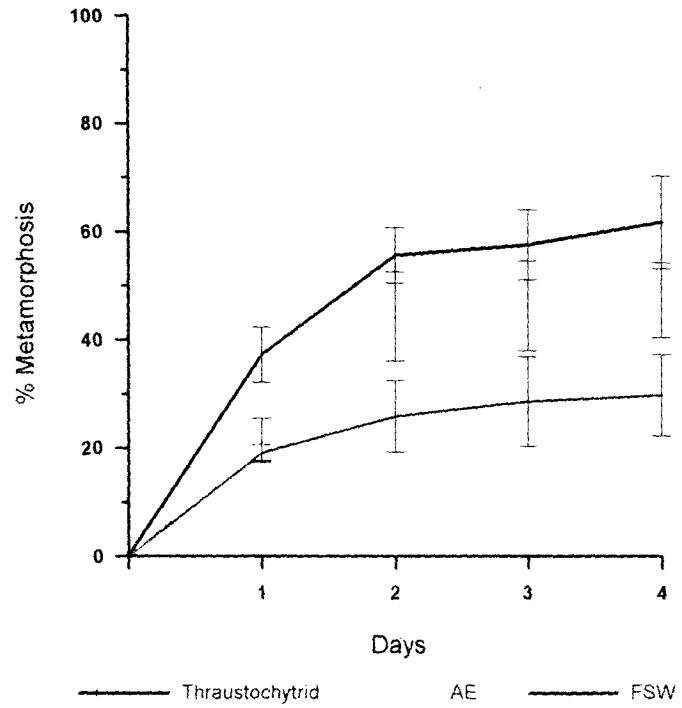


Fig. 3D.1 Percentage metamorphosis of *B. amphitrite* cyprids in presence of thraustochytrid, adult extract (AE) and filtered sea water (FSW)

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 salilities. 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; Kuttenreich 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 μg ml^{-1} ($p \leq 0.001$). An increase in protein concentration to 50 μg 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 μg 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.

Chapter 4

Lectins as probes to evaluate the role of signaling molecules from the bacteria

4.1 Introduction

Bacterial biofilms have been indicated to be important in the settlement processes of planktonic larvae of most marine invertebrate groups (Crisp 1984; Mitchell 1984). The larvae are attracted towards specific chemicals in the microbial films that serve as cues for the settling larvae. Examples include spirorbid polychaetes (Knight-Jones 1951; Meadows and Williams 1963; Kirchman et al. 1982a), bryozoans (Mihm et al. 1981; Brancato and Woollacott 1982), ascidians (Szewzyk et al. 1991; Wieczorek and Todd 1997), echinoderms (Cameron and Hinegardner 1974; Johnson et al. 1991), phoronids (Herrmann 1975), sponges (Woollacott and Hadfield 1996), cnidarians (Müller et al. 1976; Hofmann et al. 1978; Neumann 1979), mollusks (Scheltema 1961; Weiner et al. 1985) and oysters (Weiner et al. 1985).

Barnacle cyprids, like most other larvae, also prefer to settle on substrata that possess a well-developed biofilm (Crisp 1984; Clare et al. 1992). A number of investigators have assessed the effects of pure cultures of bacteria isolated from biofilms on the larvae of choice in the laboratory (Maki et al. 1988,1992,1994; Holmström et al. 1992,1996; Avelin Mary et al. 1993; O'Connor and Richardson 1996,1998; Anil and Khandeparker 1998; Khandeparker et al. 2002). Earlier investigations have reported stimulation, inhibition or no effect of bacterial films on the attachment of barnacle cyprids (Crisp and Meadows 1962; Harris 1946; Visscher 1928; Tighe-Ford et al. 1970; Neal and Yule 1994a,b; Maki 1999). Olivier et al. (2000) also showed that the degree of microbial pre-colonization was the main parameter affecting the settlement of *B. amphitrite*. Recently a thraustochytrid protist, detected in

marine microbial films has been shown to induce the settlement of *B. amphitrite* (Raghukumar et al. 2000).

After attaching to a surface, bacteria not only reproduce but also exude mucous materials to form a layer of biofilm (Costerton et al. 1978,1995), known as exopolymers. These are composed of polysaccharides with variable amount of proteins. The extracellular polymeric substances (EPS) either contribute to the biofilm matrix (Cooksey 1992; Costerton et al. 1994) or are released by the microorganisms to the surrounding medium as planktonic or free EPS (Sutherland 1985; Beech 1990; Beech et al. 2000; Khandeparker and Bhosle 2001). The larval responses thus depend on the surface, which is changed by the extracellular molecules (Baier 1984) and the nature of the extracellular products. Maki et al. (1988) has reported the involvement of exopolymers produced by the bacteria in the attachment response of the larvae of *B. amphitrite*. Bacteria can also produce surface-bound and soluble chemical cues that either stimulate or inhibit larval settlement (Kirchman et al. 1982a; Maki et al. 1990,1992; Szewzyk et al. 1991). Bacterial extracellular products are also reported to be involved in the attachment of larvae of a spirorbid polychaete (Kirchman et al. 1982b) and an oyster (Weiner et al.1985).

Lectins, a class of naturally occurring proteins or glycoproteins exist in almost all living organisms and can recognize and bind carbohydrates specifically and noncovalently. The role of lectins in larval settlement has been investigated in several studies (Kirchman and Mitchell 1981,1983,1984;

Kirchman et al. 1982a,b; Maki and Mitchell 1985; Mitchell 1984; Mitchell and Kirchman 1984; Mitchell and Maki 1988). The settlement of a polychaete, *Janua brasiliensis* was also mediated by lectins on the larval surface that are proposed to recognize and bind to the bacterial polymer containing glucose (Kirchmann et al. 1982a). In case of *Crassostrea*, an oyster, neither the lectins nor monoclonal antibodies blocked the ability of *Alteromonas colwelliana* films to cue set the larvae (Weiner et al. 1989). No such studies are reported in case of barnacles to the best of our knowledge, although *Lens culinaris* agglutinin (LCA)-binding sugar chains of the settlement-inducing protein complex (SIPC) which induces gregarious settlement in this species have been suggested to be involved in the settlement of *B. amphitrite* (Matsumura et al. 1998). The present investigation was undertaken in order to identify the specific carbohydrate molecules from the bacterial film that are responsible for metamorphosis facilitation.

The bacteria, *P. aeruginosa*, *B. pumilus*, *C. freundii* isolated from the biofilms present on the shell surfaces of *B. amphitrite* were used in the present investigation. The primary strategy was to block the determinants of the bacterial films using lectins that would bind to specific carbohydrate moieties present in the extracellular polysaccharides on the bacterial surfaces. A comparison of the responses of the larvae to lectin-treated bacterial films and untreated bacterial films would unravel the possible mechanisms for the induction of larvae of *B. amphitrite* by bacterial films and the specific carbohydrate moiety of the polysaccharide involved in signaling the larvae.

4.2 Materials and methods

4.2.1 Preparation of adult extract (AE)

AE was prepared by following the method described earlier by Larman et al. (1982). Adult specimens of *B. amphitrite* were collected from the intertidal area of Dona Paula (15° 27.5'N, 73° 48'E). After being brought to the laboratory they were cleaned by brushing off the epibiotic growth on their shells using a nylon brush. The animals were then washed with deionised water. Approximately 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 (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 of Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard. A protein concentration of 50µg ml^{-1} was used for all assays.

4.2.2 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 10^5 cells ml^{-1}. The feed

organism was replenished every day while changing 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 (Rittschof et al. 1984).

4.2.3 Isolation of bacteria from the shell surfaces of *B. amphitrite*

B. amphitrite brought to the laboratory were scraped with a nylon brush using Millipore filtered 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).

4.2.4 Bacterial film

The influence of single-species films of *P. aeruginosa*, *B. pumilus* and *C. freundii* films was assessed at 35‰. Filming of the surfaces with bacteria was performed following the methods 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 density of attached bacteria was determined using acridine orange and epifluorescence microscopy (Daley and Hobbie 1975). The adsorption technique resulted in densities of 10^6 to 10^7 attached bacteria per cm^2.

4.2.5 Extraction of planktonic EPS

All three bacteria grown in marine broth (MB) were harvested by centrifugation (20,000 x g for 30 min) and subsequently sterile filtered (0.22µm, Millipore). The culture supernatant thus obtained was 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 µg ml^{-1} final concentration) for three 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.

4.2.6 EPS characterization

The EPS was characterized for monosaccharide composition. For this, the EPS was hydrolyzed with 2N HCl for 2h at 100° C in ampoules flushed with nitrogen before sealing. After hydrolysis, the solution was evaporated to dryness under reduced pressure at 40° C. The neutral sugars were converted to alditol acetates and were analyzed by capillary gas chromatography (GC) as described earlier (Bhosle et al. 1995; Khandeparker and Bhosle 2001) (Chrompack, Middleburg, The Netherlands, Model CP 9002) equipped with a fused silica capillary column coated with CP sil-88 (25m, id=0.32mm, df=0.12; Chrompack, Middleburg, The Netherlands). A flame ionization detector was used to separate the alditol acetate mixture. A known amount (0.4µl) of the sample was applied using an on-column injector when the oven temperature

was 70° C. The oven temperature was rapidly raised to 150° C and further programmed at 3° C min^{-1} to 230° C and maintained at this temperature for ca 10 min. Quantification of the components was done by peak area integration of the GC results using the data handling system installed in the instrument. The response factors were calculated using standard sugar alditol acetates and myoinositol as an internal standard and were used for the quantification of the results.

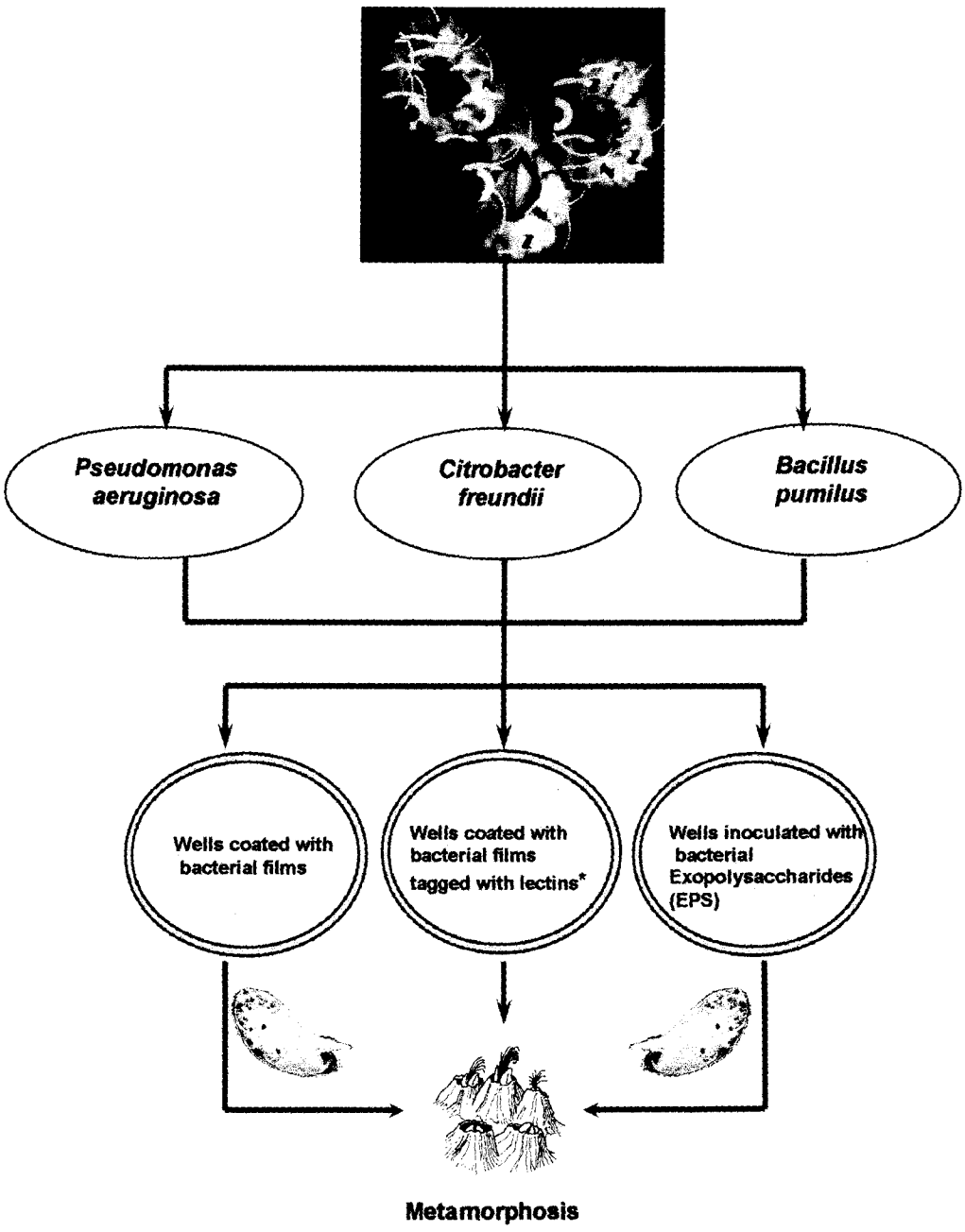
4.2.7 Treatment of bacterial films with lectins

The lectins, *Lens culinaris* agglutinin (LCA), Concanavalin A (ConA), *Limulus polyphemus* (Limulin), Wheat germ agglutinin (WGA) and Glycine max were obtained from Sigma chemicals each conjugated with FITC (fluorescein isothiocyanate).

The bacterial films of *P. aeruginosa*, *B. pumilus* and *C. freundii* were treated with 250µl of the lectin (stock solution of 1 or 5 mg/ml in distilled deionised water) as described by Hood and Schmidt (1996). After incubation for 30 min the multiwells were rinsed with autoclaved filtered seawater thrice to which ~25-30 cyprids were introduced with 5 ml of autoclaved filtered seawater of 35‰.

4.2.8 Assay protocol

The schematic representation of the experimental set-up is given in Figure 4.1.



**Lens culinaris* agglutinin (LCA); Concanavalin A (Con A); *Limulus polyphemus* (Limulin); Wheat germ agglutinin (WGA); Glycine max (Gly max)

Fig. 4.1 The schematic representation of the experimental set-up

The multi-wells were inoculated with the bacteria and planktonic EPS (50µg ml^{-1}). The multi wells inoculated with the bacterial films were rinsed off after three hours by repeated rinsing with autoclaved filtered seawater under a laminar chamber. Subsequently ~25-30 cyprids were introduced with 5 ml of autoclaved filtered seawater of 35‰. All the experiments were repeated four times using four different batches of larvae with six replicates at each trial (n=6, with batch as an additional factor). Controls were filtered seawater (FSW, negative control) and adult extract (AE, positive control). The settlement assays were carried out using Corning-430343, 6 well multiwells and were monitored for a period of four days with an intermittent observation everyday. Assay wells were maintained at 26±1° C (12h light: 12h dark photoperiod). The films probed with lectins were examined using Olympus (CX40) microscope equipped with a V-LH50HG fluorescence attachment.

4.2.9 Statistical analysis

Data in the form of percentage of larval settlement was arcsine transformed to ensure normality of means and homogeneity of variances before subjecting to statistical analysis. The influence of lectin-treated bacterial films on the cyprid metamorphosis was evaluated using one-way analysis of variance (ANOVA) (Sokal and Rohlf 1981). A post ANOVA analysis was performed using Scheffe's test (α=0.05). Those that did not meet the normality assumption were analyzed using non-parametric statistical analysis such as Mann-Whitney U-Test (α=0.025). The analysis was performed by transforming the values to ranks and was used to compare the means between the two treatments.

4.3 Results

Among the three bacterial species isolated from shell surfaces of *B. amphitrite*, *P. aeruginosa* positively influenced metamorphosis of cyprids. The cyprids exposed to *P. aeruginosa* facilitated (44%) metamorphosis similar as that of AE (44%) at the end of day 1 (Fig. 4.2).

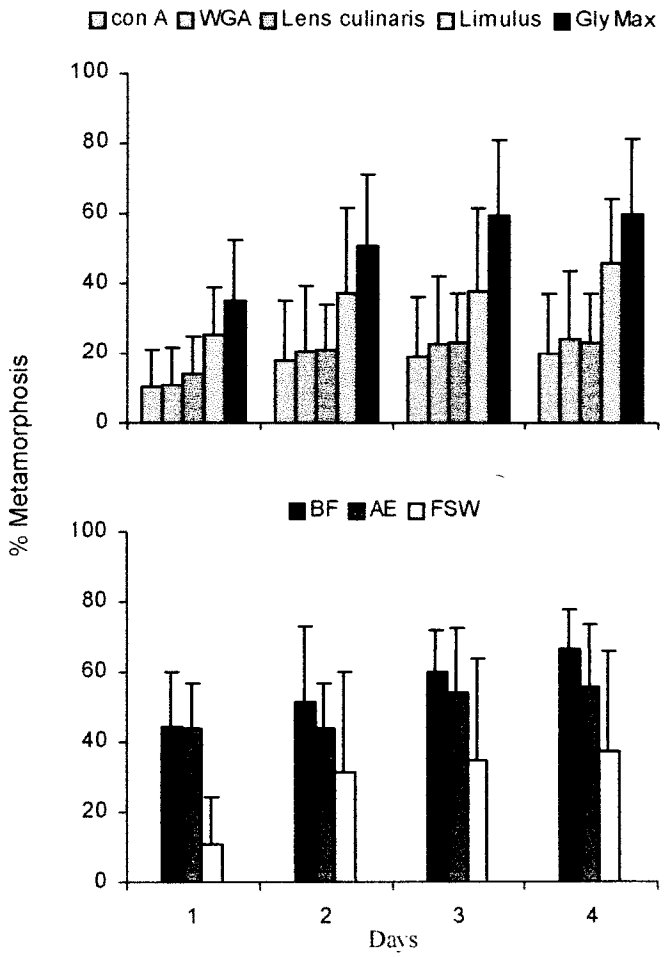


Fig. 4.2 Percentage metamorphosis of cyprids exposed to lectin-treated and non-treated films of *P. aeruginosa*. Vertical lines indicates the standard deviation from mean. AE- adult extract, FSW- filtered seawater, BF- bacterial film

C. freundii and *B. pumilus* films did not facilitate metamorphosis of cyprids (Figs. 4.3 and 4.4), the response was almost similar as that observed with plain filtered seawater (-ve control).

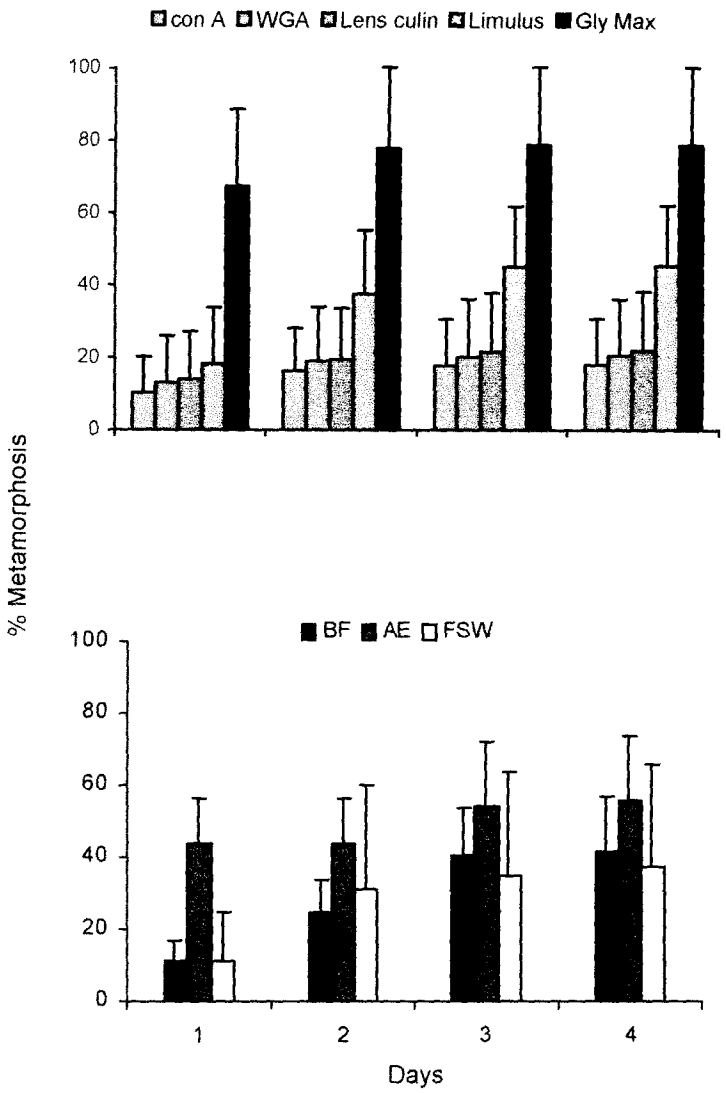


Fig. 4.3 Percentage metamorphosis of cyprids exposed to lectin-treated and non-treated films of *C. freundii*. Vertical lines indicates the standard deviation from mean. AE- adult extract, FSW- filtered seawater, BF- bacterial film

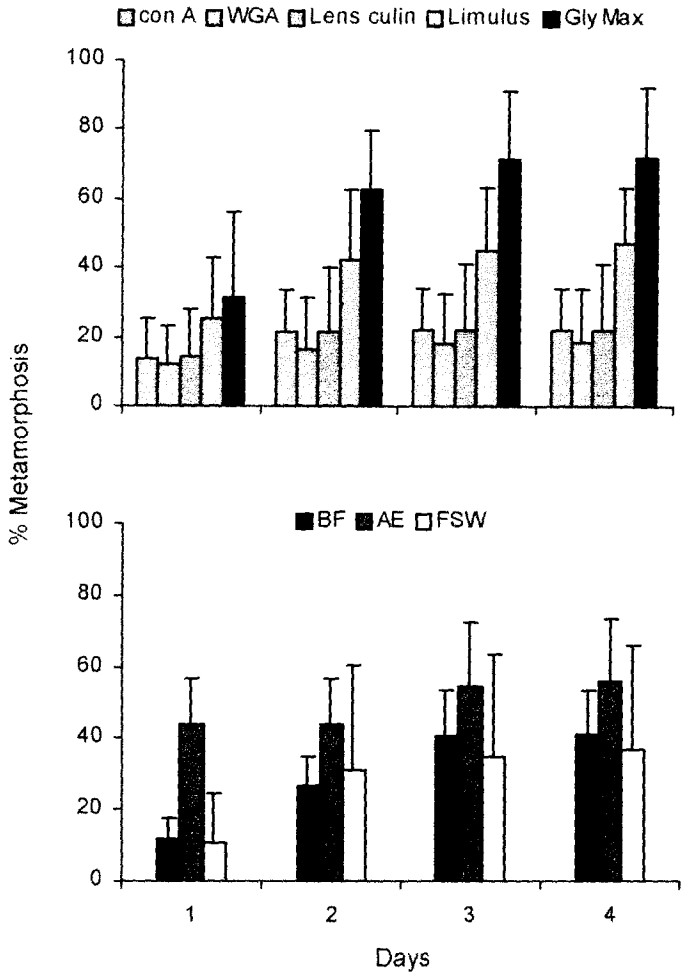


Fig. 4.4 Percentage metamorphosis of cyprids exposed to lectin-treated and non-treated films of *B. pumilus*. Vertical lines indicate the standard deviation from mean. AE- adult extract, FSW- filtered seawater, BF- bacterial film

The influence of planktonic or free exopolysaccharide (EPS) obtained from *P. aeruginosa* was comparatively more than the EPS produced by the other two bacteria on the metamorphosis of cyprids. However, the metamorphosis rates were lower than the *P. aeruginosa* films (Figs. 4.2 and 4.5). The EPS produced by *B. pumilus* and *C. freundii* were strongly inhibitory (Fig. 4.5).

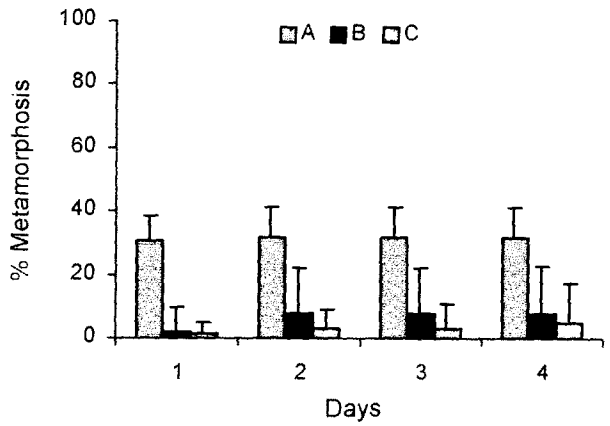


Fig. 4.5 Percentage metamorphosis of cyprids exposed to planktonic exopolysaccharides obtained from *P. aeruginosa* (A), *B. pumilus* (B) and *C. freundii* (C). Vertical lines indicate the standard deviation from mean

Capillary gas chromatography analysis result for the relative monosaccharide composition (%) of the planktonic EPS produced by all the three bacteria is presented in Table 4.1.

Table 4.1 Relative monosachharide composition (%) of the EPS produced by *P. aeruginosa, B. pumilus* and *C. freundii.*

Microorganism	Mannose (%)	Glucose (%)	Ribose (%)
Pseudomonas aeruginosa	86.28	13.71	-
Citrobacter freundii	80.17	14.95	4.86
Bacillus pumilus	86.17	13.10	0.72

All the three bacteria showed the presence of mannose and glucose, the former being the major constituent. *B. pumilus* and *C. freundii* showed the presence of ribose, which was absent in *P. aeruginosa*. The percentage of ribose in case of *B. pumilus* was less (0.72%) than that observed in *C. freundii* (4.86%).

The EPS intimately associated with the bacterial cell surface were probed through application of different lectins. The sugar specificity of different lectins used in the present experiment is shown in Table 4.2. The cyprids exposed to *P. aeruginosa* films treated with lectins, Con A, WGA, LCA and Limulin showed a decline in metamorphosis. The treatment of Glycine max to the films of *P. aeruginosa* showed almost similar metamorphosis response as that observed with untreated bacterial films ($p \leq 0.025$, Mann-Whitney).

Table 4.2 Sugar specificity of lectins

Abbreviation lectin	Common name	Sugar specificity
Con A	Concanavalin A	D-mannose
		D-glucose
		Fructofuranose
LCA	*Lens culinaris* agglutinin	D-mannose
		D-glucose
		N-acetyl-D-glucosamine
WGA	Wheat germ agglutinin	N-acetyl-D-glucosamine
Limulus polyphemus	Limulin	N-acetylneuraminic acid
		Glucuronic acid
		Phosphorycholine analogs
Gly max	Glycine max	N-acetyl-D-galactosamine

A significant difference in the metamorphosis rates was observed (Table 4.3) when the bacterial films were treated with different lectins ($p \leq 0.001$, one-way ANOVA; $p \leq 0.05$, Scheffe's test).

The films of *C. freundii* did not facilitate metamorphosis of cyprids, but resulted in metamorphosis promotion higher than that observed with AE when treated with Glycine max (67%) at the end of day 1. The difference in the

metamorphosis rates between the untreated bacterial film and Glycine max treated film was highly significant ($p \leq 0.025$, Mann-Whitney).

Table 4.3 One-way ANOVA. Influence of lectin-treated and non-treated bacterial films (Treatment) of *P. aeruginosa*, *B. pumilus* and *C. freundii* on the metamorphosis of *B. amphitrite* cyprids. (df.degree of freedom; SS. sum of the squares; MS. mean of squares; Fs.Fischer constant).

	Pseudomonas aeruginosa				*Bacillus pumilus*			*Citrobacter freundii*		
	df	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
Day 1										
Treatment	5	4788	958	9*****	3580	716	9*****	15486	3097	26*****
Within sub.Gr.err.	138	14564	105		10707	78		16332	118	
Total	143	19352			14287			31818		
Day 4										
Treatment	5	18662	3732	26*****	23377	4675	36*****	36634	7327	47*****
Within sub.Gr.err.	138	19927	144		17705	128		21557	156	
Total	143	38589			41082			58191		

(*****$p \leq 0.001$)

The films of *B. pumilus* treated with Glycine max and Limulin positively influenced metamorphosis while untreated films did not. The inducement by Glycine max-treated *B. pumilus* films was not comparable with *C. freundii* (32 and 67%) and was less than the AE (44%). However, the films treated with Glycine max showed a considerable increase in metamorphosis on day 2 (63%).

A marginal increase in the metamorphosis rates was observed as the cyprids as well as the bacterial films aged. However the pattern almost remained same except in case of *B. pumilus* where a considerable hike in metamorphosis was seen in the Glycine max-treated films.

4.4 Discussion

Epifluorescence microscopy coupled with the lectin specificity has been suggested as a new way to probe rapidly and precisely spatial relationships of complex distributions of molecules on marine surface when films are present (Michael and Smith 1995). Among the bacteria isolated from the shell surface of *B. amphitrite*, *P. aeruginosa* facilitated metamorphosis. However, when probed with Concanavalin A, WGA and LCA, which binds specifically to glucose and its derivatives, mannose and fructofuranose, negated the promotory effect. Hence, these carbohydrate moieties associated with the cell surface of *P. aeruginosa* are suggested to be involved in the larval induction of *B. amphitrite*. A decrease in the metamorphosis was also evident in case of *P. aeruginosa* films treated with Limulin suggesting the presence of N-acetylneuraminic acid, glucuronic acid or phosphocholine analogues in the EPS that could be involved in attracting the cyprids. However when probed with Glycine max there was not much difference in the metamorphosis response when compared to an untreated bacterial film indicating either absence or non-involvement of galactose derivatives associated with its EPS.

The films of *B. pumilus* and *C. freundii* did not promote metamorphosis. Whereas the Glycine max-treated bacterial films of *B. pumilus* and *C. freundii* showed a considerable increase in the metamorphosis, the metamorphosis rates being higher than that observed with the AE. Hence the probability of N-acetyl-galactosamine being associated with the capsular EPS of these bacteria cannot be ruled out. The blocking of the galactose derivatives on the films of *B. pumilus* and *C. freundii* enhanced the settlement, which were otherwise inhibitory to the cyprids of *B. amphitrite*. Three galactose-binding

lectins have been described in the haemolymph of the acorn barnacle *M. rosa* (Marques and Baracco 2000). The lectin present in the haemolymph of *B. rostrata* has been also reported to recognize the same monosaccharide (Toda et al. 1998). The fact that primary urine production is the result of ultrafiltration of the haemolymph (White and Walker 1981) it is possible that the lectins could find their way to the surface via the excretory canal. Hence, the presence of lectins in these animals other than enhancing the phagocytosis of microorganisms could also possibly play an important role in altering the signals or cues and may be ecologically important. The immobilization of marine bacteria in hydrogels has been developed as a new concept for the prevention of macrofouling (Gatenholm et al. 1995). The inhibitory components of the bacteria for e.g. in this study galactose residues can thus be protected by immobilizing them in the hydrogels and could be of great help in antifouling technology which needs further validation.

When the planktonic EPS of *P. aeruginosa* was assessed against the cyprids, although metamorphosis was facilitated, it was not as effective as the EPS associated with the bacterial cell surface. An earlier investigation has reported that the response of the cyprids of *B. amphitrite* to bacteria and its products is regulated by both contact chemoreception and/or olfaction, depending on the properties of the settlement inducing compounds (Khandeparker et al. 2002). The planktonic EPS of *B. pumilus* and *C. freundii* were strongly inhibitory. Capillary gas chromatographic analysis of the planktonic EPS revealed the presence of glucose and mannose which occurred as the most abundant carbohydrates in all the three bacteria, whereas ribose was additionally present in *B. pumilus* and *C. freundii*. The presence of ribose in the EPS may

be a responsible component for strong inhibitory effect of EPS produced by *B. pumilus* and *C. freundii.*

Aged cyprids showed higher rates of metamorphosis. A possible explanation could be that young cyprids are more discriminating than old cyprids but become less discriminating with age during settlement (Rittschof et al. 1984; Crisp 1988) presumably due to decline in their energy reserves and thus the physiological quality (Anil et al. 2001).

As all three bacteria were isolated from the shell surface of *B. amphitrite,* their involvement in the gregarious behavior cannot be ruled out. The presence of both facilitatory and inhibitory bacteria may have some adaptive implications wherein the adult barnacle avoids the epibiosis on some of its parts e.g. tergum and scutum / apical region from where the barnacle cirri open out for feeding, respiration etc.

In the present investigation the bacteria were assessed separately, whereas, in nature they occur in conjunction and the contradictory signals from each bacterium would give different set of signals to the settling larvae. Spatial and chemical heterogeneities of surfaces in marine environment have been illustrated by lectin probes and its role in possible microscale cues for biofouling is suggested (Michael and Smith 1995). The understanding of the synchronization of all the contradictory signaling molecules in presence of each other needs further validation. It has been reported earlier 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 (Anil et al. 1997; Anil and Khandeparker 1998). Some bacteria in

biofilms are capable of genetic exchange (Fry and Day 1990). The cell-cell communication between their own species and perhaps others through N-acyl-L-homoserine lactones (AHL) cannot be ruled out as AHL activity has been demonstrated in natural biofilms (McLean et al. 1997). The ability of larval receptors to distinguish between the EPS that are intimately associated with the cell surface and those are released in the planktonic form would be a key for production of probes for such saccharides and the genes that produce them can then be explored.

Chapter 5

Influence of larval rearing conditions and ageing on the energetics and metamorphosis

5.1 Introduction

It has been suggested that larval experiences other than predation may have an impact on recruitment. Though metamorphosis was successful, competitive ability was impaired due to reduced growth rate (Jarrett and Pechenik 1997). Pechenik et al. (1993) observed that to delay metamorphosis may be a double-edged sword for *B. amphitrite,* increasing an individual's chance of locating a site appropriate for metamorphosis, but simultaneously reducing the ability to compete for space during the first few weeks of juvenile life. The cypris larvae are non-feeding and have to depend upon the energy reserves incorporated during the earlier planktotrophic naupliar development.

Nucleic acids play a major role in growth and development. It has been shown that the RNA:DNA ratio is an indicator of nutritional condition (Clemmesen 1996) and has been routinely used for estimating growth rates or nutritional condition of larval fish in the field. Only a few studies have applied this technique to crustaceans and their larvae (Dagg and Littlepage 1972; Sulkin 1975; Anger and Hirche 1990; Wagner et al. 1998). In this study, the influence of rearing temperature and food concentration on ensuing changes in the content of RNA and DNA content of cyprids is examined. Ageing cyprids at 5 °C was adopted as it has been shown that the major cyprid protein content remained constant at this temperature (Satuito et al. 1996). The impact of these factors on the metamorphosis of the cypris larvae is presented.

5.2 Materials and methods

5.2.1 Rearing of *B. amphitrite* larvae

Larval development consists of six naupliar instars and a non-feeding cypris instar. The first nauplius stage is non-feeding and is of short duration. Nauplii obtained from *B. amphitrite* were mass reared in 2 L. glass beakers (1 larva ml^{-1}) on a daily diet of *C. calcitrans*, a unicellular diatom, at a concentration of 1 $x10^5$ and 2 $x10^5$ cells ml^{-1}. The cultures were maintained at 20 and 30° C (±1° C) in an incubator (12h light:12h dark photoperiod). The larval density and food concentrations were maintained by monitoring mortality of the larvae every day. The rearing experiments for each of the conditions were carried out three times.

5.2.2 Cyprid metamorphosis assay

The cyprids obtained were filtered through a tier of plankton netting (300, 230 and 160µm). Cyprids (~10) were sampled for their length and breadth measurements as and when they appeared. The cyprids obtained on day 5 at 30 °C (2 $x10^5$ cells ml^{-1}), day 6 at 30° C (1 $x10^5$ cells ml^{-1}) and day 10 at 20° C (2 $x10^5$ and 1 $x10^5$ cells ml^{-1}) were used in metamorphosis assays.

The adult extract (AE) which is known to promote metamorphosis in *B. amphitrite* cyprids was used as a positive control and was prepared following the method described by Larman et al. (1982). In addition, cyprid metamorphosis was also monitored in the absence of the adult extract (control). The protein content of the extract was estimated using the method described by Lowry et al. (1951) using BSA (Bovine Serum Albumin) as the standard. A concentration of 50 µg ml^{-1} was used in the assays. The assays in

each condition were run in triplicates. About 20-25 cyprids were introduced into polystyrene multiwells (Nunclon delta 1-52795) with 5ml of filtered, autoclaved seawater (35‰). Assays were carried out at 25±1° C.

5.2.3 Estimation of nucleic acids in *B. amphitrite* larvae

The technique described by Clemmesen (1988,1993), which is based on fluorimetric determination of RNA and DNA content of an individual fish larva, was adopted for estimation of nucleic acids in the larvae of *B. amphitrite*. All the chemicals used were analytical grade. Ethidium bromide was obtained from BDH laboratories and Ribonuclease A was obtained from Merck. Yeast ribonucleic acid (RNA) and Calf thymus deoxyribonucleic acid (DNA) were procured from Hi media. Sodium dodecyl sulphate was procured from Sigma chemicals (USA). Standard regression curves of DNA and RNA were plotted. Larval homogenate (10 in no) was prepared, and then spiked with known amounts of standard DNA and RNA to evaluate the precision of the method, which was found to be ±5 %.

5.2.4 Statistical analysis

Influence of rearing temperature and food concentration on the size (total length and total breadth) of cyprids, DNA and RNA content and RNA:DNA ratio were evaluated by two-way ANOVA (Sokal and Rohlf 1981).

5.3 Results

The cyprids formed at 30° C had total length ranging from 451 to 480 µm and total breadth from 218 to 227 µm and were larger than those formed at 20° C, which had total length ranging from 421 to 448 µm and total breadth from 211 to 230 µm.

Two-way ANOVA showed that the food concentration and temperature influenced cypris length and breadth significantly. The food concentration and temperature interaction showed an insignificant effect indicating the influence to be equal at both the temperature and food concentration (Table 5.1).

Table 5.1 Two-way ANOVA of the influence of food concentration and temperature on cyprid length, cyprid bredth, DNA, RNA and RNA/DNA of cyprids.

Factor		Cyprid length			Cyprid breadth		
	df	SS	MS	Fs	SS	MS	Fs
Food Conc.	1	5198	5198.4	26.5*****	1172	1172	5.19*
Temperature	1	15920	15920	81.3*****	2030	2030	9.0****
Food conc.x Temp	1	636	636.8	3.25 ns	159	159	0.70 ns
Within sub. Gr.err.	36	7043	195.6		8122	225	
Total	39	28797			11483		

Factor		DNA			RNA			RNA/DNA		
	df	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
Food Conc.	1	0.03	0.03	0.3 ns	0.79	0.79	3.09 ns	0.01	0.01	0.07ns
Temperature	1	0.34	0.34	3.05 ns	0.96	0.96	3.78 ns	0.14	0.14	0.75ns
Food conc.x Temp	1	0.007	0.007	0.06 ns	2.17	2.17	8.5***	0.36	0.36	1.99ns
Within sub. Gr.err.	28	3.16	0.11		7.17	0.25		5.19	0.18	
Total	31	3.55			11.1			5.71		

(*****$p \leq 0.001$, ****$p \leq 0.005$, ***$p \leq 0.01$, *$p \leq 0.05$, ns- not significant)

The average DNA content of the cyprids from the different rearing conditions (Figs. 5.1a,e and 5.2a,e; 0 day values) averaged 1.9 to 2.1 µg larva^{-1}. The RNA content averaged 3.2 µg larva^{-1} (2x10^5 cells ml^{-1}) and 2.4 µg larva^{-1} (1x10^5 cells ml^{-1}) at 30° C (Figs. 5.1b and 5.2b), whereas at 20° C the average RNA content was 2.3 µg larva^{-1} at 2x10^5 cells ml^{-1} and 1.7 µg larva^{-1} at 1x10^5 cells ml^{-1} (Figs. 5.1f and 5.2f).

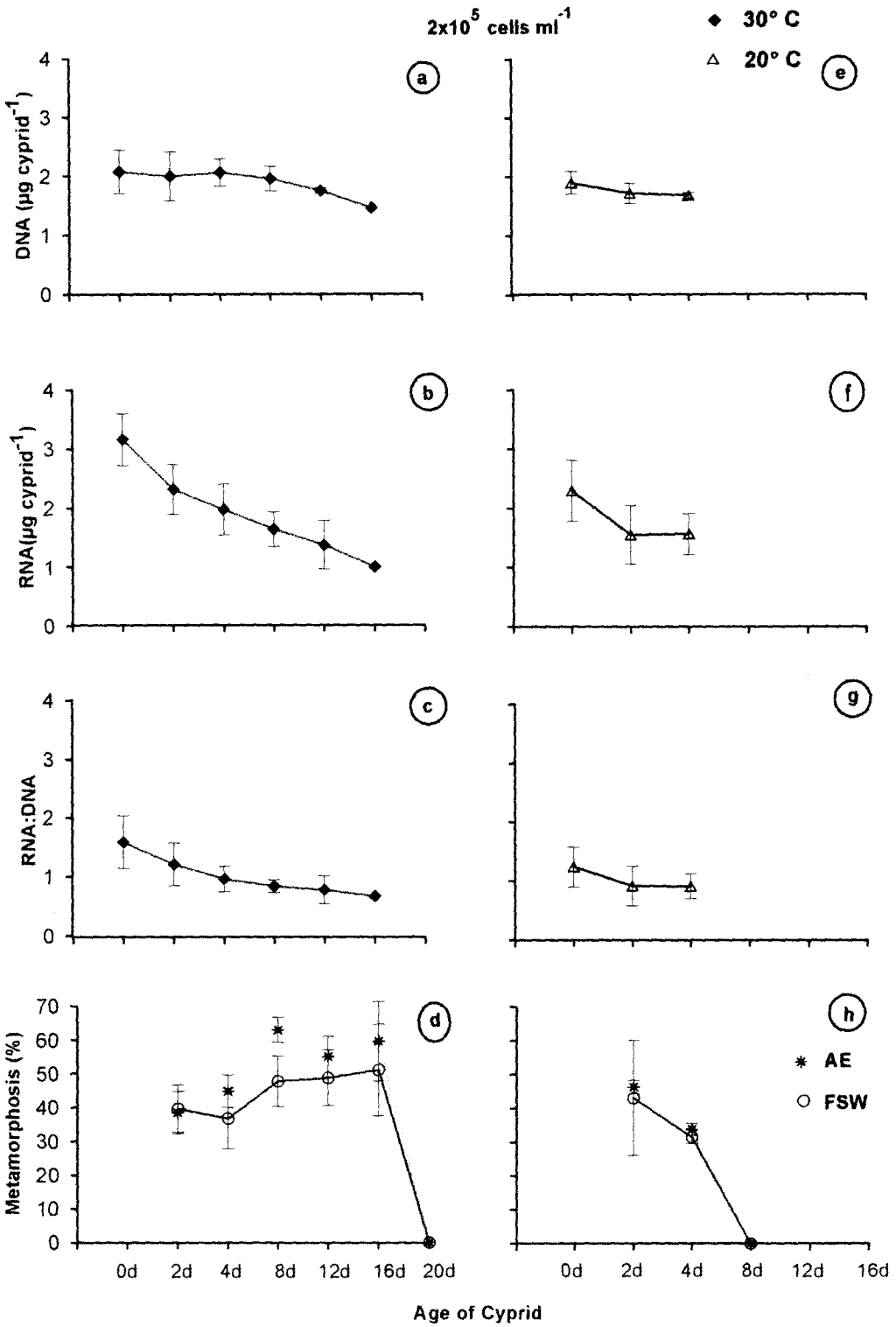


Fig. 5.1 Influence of ageing of cyprids at 5° C on DNA, RNA and RNA/DNA ratio and percentage metamorphosis. (a), (b), (c) and (d) represent larvae raised at 30° C and (e), (f), (g) and (h) represent larvae raised at 20° C at 2 x 10^5 cells ml^{-1}

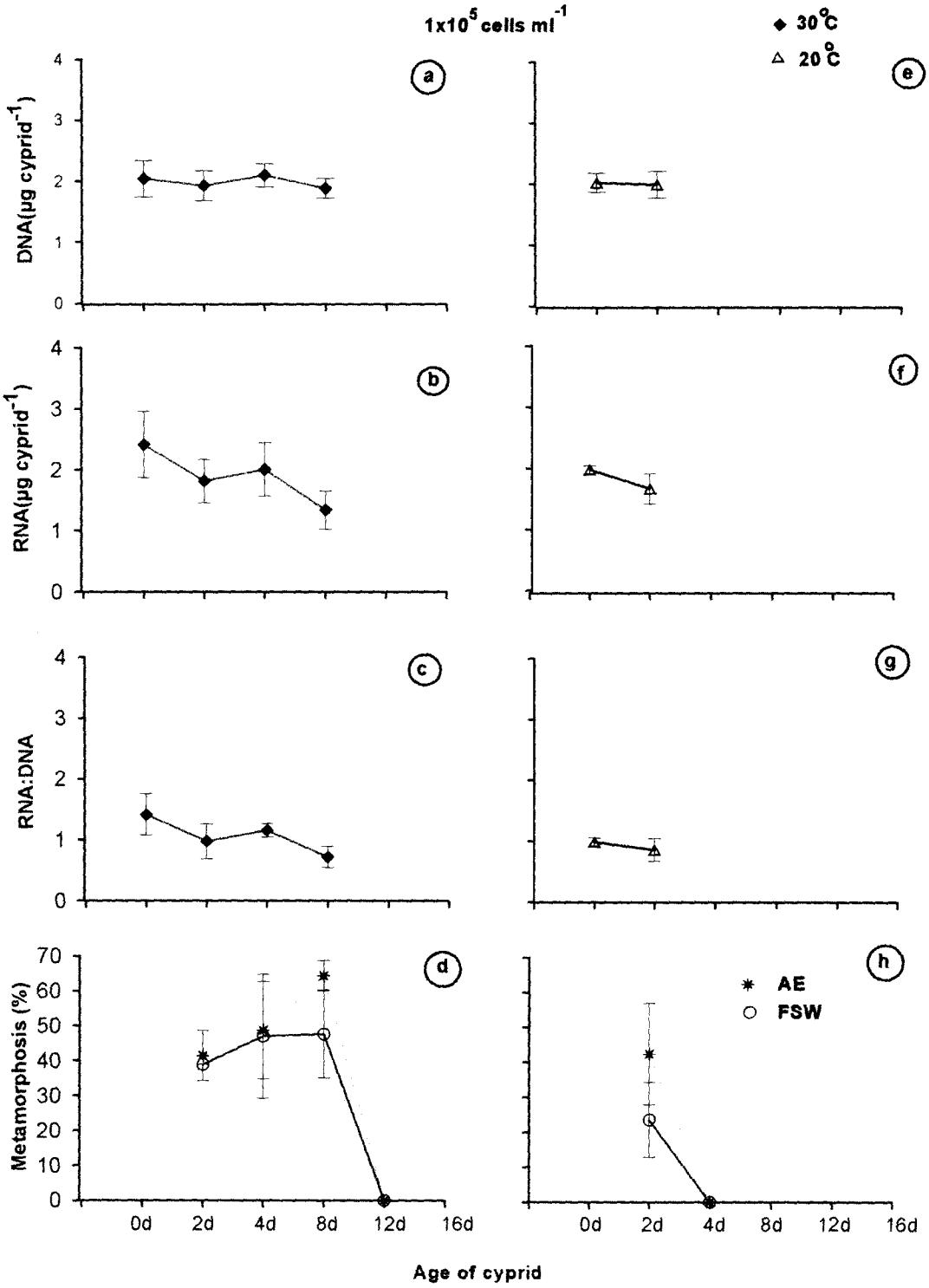


Fig. 5.2 Influence of ageing of cyprids at 5° C on DNA, RNA and RNA/DNA ratio and percentage metamorphosis. (a), (b), (c) and (d) represent larvae raised at 30° C and (e), (f), (g) and (h) represent larvae raised at 20° C at 1 x 10^5 cells ml^{-1}

The cyprids on aging at 5° C showed that RNA (Figs. 5.1b,f and 5.2b,f) and the RNA:DNA ratio progressively decreased (Figs. 5.1c,g and 5.2c,g). The cyprid attachment assay (Figs. 5.1d and 5.2d) results, evaluating the influence of aging showed that the larvae reared at 30° C, $2x10^5$ cells ml^{-1} and $1x10^5$ cells ml^{-1}, were viable for 16 and 8 days respectively. The metamorphosis rate peaked with 8-16 day aged cyprids and was positively influenced by AE when compared to the control (FSW) (Figs. 5.1d and 5.2d).

The cyprids raised at 20° C could only metamorphose successfully for 4 days ($2x10^5$ cells ml^{-1}) and 2 days ($1x10^5$ cells ml^{-1}). The metamorphosis rate decreased with aging and was similar in both AE and control conditions (Figs. 5.1h and 5.2h).

5.4 Discussion

Spawning activity in some marine invertebrates coincides with phytoplankton blooms (Barnes and Barnes 1958; Starr et al. 1990). This coupling is explained as one of the survival measures offered to the planktotrophic offspring. Barnes and Barnes (1958), while examining the recruitment of *Balanus (=Semibalanus) balanoides* over a period of ten years in the Firth of Clyde, attributed recruitment failure to abnormal increase in larval predation, unusual physical parameters, presence of deleterious chemicals as well as defective sampling. Olson and Olson (1989), however, felt that recruitment largely depended on the abundance of larval food. Cyprids like other invertebrate larvae derive their energy from stored lipids. The extent of this energy reserve in cyprids depends on the condition under which the nauplii grow (Lucas et al. 1979). In the case of cyprids, both food concentration and

temperature influenced length and breadth. This fact coupled with the information that larvae take longer duration to complete development at the lower temperature indicate that assimilation of energy for growth is significantly influenced by these two variables.

Cypris, the terminal larval instar, prolong their larval duration until a conducive substratum is available for settlement and subsequent metamorphosis. Freeman and Costlow (1983) showed that moulting is distinctly biphasic in cyprids of *B. amphitrite*. In free-swimming cyprids moulting is arrested at an early stage and is only resumed after settlement. Pechenik et al. (1998) state that growth rate of newly-metamorphosed barnacles slows down significantly if *B. amphitrite* cyprids were prevented from metamorphosing for as few as 3 days. It was observed by Connell (1961) that reduced growth rates would compromise the ability of juvenile barnacles to compete for space and seriously reduce their likelihood of successfully recruiting to the adult population. In the light of these observations, Jarrett and Pechenik (1997) and Pechenik et al. (1998) opined that larval experience influences juvenile performance. The evidence gathered from the present experiments indicate that the naupliar experience clearly determines the capability of the cyprids to metamorphose as well. The RNA content of larvae raised at 20° C, which had longer total naupliar duration, was considerably less compared to those raised at 30° C. This difference in RNA content was reflected in the capability of the cyprids to survive ageing at 5° C. The larvae raised at 20° C could only successfully metamorphose until 2 to 4 days, whereas, those raised at 30° C could do so for 8 to 16 days. Clare et al. (1994) observed that the cyprid settlement of *B. amphitrite* is uncharacteristically low when reared with *D.*

tertiolecta and that, although the cyprids look normal, they may be deficient in some respect. It was also observed earlier that, by day 3, the cyprids lose their power to discriminate the substrata and are less useful for assaying metamorphosing inducers (Rittschof et al. 1984; Crisp 1988; Clare et al. 1994). Maki et al. (1988) showed from their experimental results on inhibition of attachment of *B. amphitrite* to bacterial films, 4 day old cyprids showed an increase in larval attachment as compared to 2 day old cyprids. It was observed with cyprids of *Balanus (=Semibalanus) balanoides* after 4-5 weeks at 10° C a final threshold level was reached wherein they possessed insufficient energy to accomplish metamorphosis into a feeding juvenile barnacle (Lucas et al.1979). Høeg and Ritchie (1987) observed with *Lernaeodiscus porcellanae* (Cirripedia: Rhizocephala) larvae, which have lecithotrophic nauplii and small sized cyprids, the energy threshold, where metamorphosis was no longer possible, was seemingly reached in less than 15 days and attributed this to the rearing temperature. The results of the present investigation show cypris metamorphosis rate and the influence of ageing are governed by naupliar rearing conditions and will be of critical importance to recruitment and early post-settlement mortality.

Chapter 6

Summary

Summary

Recruitment of the larvae of fouling organisms and their metamorphosis is the most important step in the fouling process. Cypris larvae does a substratum search before undergoing metamorphosis. The third antennular segment with its attachment disc is the most obvious point of contact between the cyprid and the substratum during the search. Flicking of the fourth antennular segment is also evident while a cyprid explores a substratum (Clare et al. 1994). While exploring some surfaces, cyprids leave behind 'footprints' of temporary adhesive, which are believed to be secreted by the glands of the antennular disc. The exploratory behavior and subsequent metamorphosis response of cypris when subjected simultaneously to sugars and adult extract (AE) is reported through this study. Evaluation of sugar-treated cyprids was carried out with AE coated and non-coated multiwells containing filtered seawater (FSW). This was done in order to observe how a cyprid would behave when the polar groups associated with CTA are blocked by cues like sugars and under such conditions how AE influences cyprid's search behavior and metamorphosis response.

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 promotion or inhibition of metamorphosis without further search.

This aspect needs attention and will be helpful in identifying the role of alternate pheromone reception sites.

Gregarious settlement in barnacles has been related to the settlement inducing compounds from adult conspecifics, microbes and their interaction. Efforts have been made to elucidate these settlement cues from these sources. Bacteria, *P. aeruginosa*, *B. pumilus* and *C. freundii* isolated from the shell surface of *B. amphitrite* and a thraustochytrid protist identified as a component of marine microbial films were used as candidate organisms. The facilitation of metamorphosis by *P. aeruginosa, B. pumilus or C. freundii* depended on the salinity or temperature. The variations in larval behavior in response to salinity and temperature can be attributed to alteration in metabolic activities 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 leachants of *P. aeruginosa* culture supernatant fraction CS1 proved to be most inhibitory, whereas 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; Kuttenreich et al. 1988). Culture supernatant fractions obtained from the bacteria by growing them

in BSS consisted mainly of carbohydrates. The differences in metamorphosis induction by them with respect to salinity may be the result of positional effect due to isomerism.

The culture supernatant of *P. aeruginosa* obtained by 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 and 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. freun*dii, cyprids metamorphosed in higher percentages when exposed to culture supernatant obtained by using BSS and was carbohydrate concentration dependent. In the case of culture supernatants of *B. pumilus,* such an inducement was not evident.

In the present investigation the bacteria were assessed separately, whereas, in nature they occur in conjunction. The contradictory signals from each bacterium would give different set of signals to the settling larvae. Spatial and chemical heterogeneities of surfaces in marine environment have been illustrated by lectin probes and its role in possible microscale cues for biofouling is suggested (Michael and Smith 1995). The understanding of the synchronization of all the

contradictory signaling molecules in presence of each other needs further validation.

It has been reported earlier 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 (Anil et al. 1997; Anil and Khandeparker 1998). Some bacteria in biofilms are capable of genetic exchange (Fry and Day 1990). The cell-cell communication between their own species and perhaps others through N-acyl-L-homoserine lactones (AHL) cannot be ruled out as AHL activity has been demonstrated in natural biofilms (McLean et al. 1997). The ability of larval receptors to distinguish between the EPS that are intimately associated with the cell surface and those that are released in surrounding medium would be a key for production of probes for such saccharides and the genes that produce them can then be explored. The response of the cyprids to bacteria and its products thus seems to be regulated by both contact chemoreception and/or olfaction, which depend on the properties of the settlement inducing compounds.

The involvement of lectins in the settlement and metamorphosis of invertebrates has been hypothesized for many years. Lectins belong to a class of naturally occurring proteins or glycoproteins and can recognize and bind carbohydrates specifically and noncovalently. In the present investigation the bacterial films were probed with lectins in order to identify the specific carbohydrate molecules responsible in signaling the cyprids. *P. aeruginosa* when tagged with lectins

specific to glucose and its derivatives, mannose and fructofuranose negated the promotory effect. Tagging of galactose derivatives translated the inhibitory effect of *B. pumilus* and *C. freundii* into a promotory one. This shows that the lectins can transform the signals in either direction. Galactose-binding lectins have been identified in the haemolymph of barnacles, which could find their way through the excretory system to the surface. It is possible that the presence of lectins could provide this organism with an ability in altering the signals or cues. Microscale patchiness of bacteria is also evidenced in marine communities. In view of this, and the conflicting cues available, such patchiness can help piloting the larvae to the destination by the basibionts. Understanding these controlling mechanisms and interfering with the pathways that are involved in lectin synthesis would be a step ahead in antifouling technology.

Thraustochytrid protists that have been detected in microbial films recently facilitated metamorphosis of cyprids to an even greater extent than adult extract (AE). 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, will advance our understanding of the complexities of invertebrate larval recruitment.

Cypris prolong their larval duration until a conductive substratum is available for settlement and subsequent metamorphosis. Cyprids, like other invertebrate larvae derive their energy from stored lipids. In the absence of stimuli, these

competent larvae delay metamorphosis. In the present study the aged cyprids showed higher rates of metamorphosis. A possible explanation could be that young cyprids are more discriminating than the old ones but become less discriminating with age during settlement (Rittschof et al. 1984; Crisp 1988) presumably due to decline in their energy reserves and thus the physiological quality (Anil et al. 2001). The evidence gathered from the present experiments indicate that the naupliar experience clearly determines the capability of the cyprids to metamorphose as well. The RNA content of larvae raised at 20° C, which had longer total naupliar duration, was considerably less compared to those raised at 30° C. This difference in RNA content was reflected in the capability of the cyprids to survive ageing at 5° C. The larvae raised at 20° C could only successfully metamorphose until 2 to 4 days, whereas, those raised at 30° C could do so for 8 to 16 days. Clare et al. (1994) observed that the cyprid settlement of *B. amphitrite* is uncharacteristically low when reared with *D. tertiolecta* and that, although the cyprids look normal, they may be deficient in some respect. It was also observed earlier that, by day 3, the cyprids lose their power to discriminate the substrata and are less useful for assaying metamorphosing inducers (Rittschof et al. 1984; Crisp 1988; Clare et al. 1994). Maki et al. (1988) showed from their experimental results on inhibition of attachment of *B. amphitrite* to bacterial films, 4 day old cyprids showed an increase in larval attachment as compared to 2 day old ones. It was observed with cyprids of *Balanus (=Semibalanus) balanoides* after 4-5 weeks at 10° C a final threshold level was reached wherein they possessed insufficient energy to

accomplish metamorphosis into a feeding juvenile barnacle (Lucas et al.1979). Høeg and Ritchie (1987) observed with *Lemaeodiscus porcellanae* (Cirripedia: Rhizocephala) larvae, which have lecithotrophic nauplii and small sized cyprids, the energy threshold, where metamorphosis was no longer possible, was seemingly reached in less than 15 days and attributed this to the rearing temperature.

The results of the present investigation show that the variations in the larval response to different cues and the influence of aging of cyprids on larval metamorphosis can thus be related to the larval quality and / or nutritional stress experienced by the larvae which is governed by naupliar rearing conditions. This will be of critical importance to recruitment and early post-settlement mortality.

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Journal of Experimental Marine Biology and Ecology
289 (2003) 1–13

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

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Barnacle larval destination: piloting possibilities by bacteria and lectin interaction

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Received 27 October 2002; received in revised form 6 December 2002; accepted 14 January 2003

Abstract

Modulation of metamorphosis in barnacles in response to cues of biological origin is established. The bacteria associated with the barnacles also have a role in such modulations. We isolated the bacteria, *Pseudomonas aeruginosa*, *Bacillus pumilus* and *Citrobacter freundii* from the shell surface of *Balanus amphitrite* and assayed against its cypris larvae. The former species was promotory while the latter two inhibited cyprid metamorphosis. *P. aeruginosa* however, when tagged with lectins specific to glucose and its derivatives, mannose and fructofuranose negated the promotory effect. Whereas, tagging of galactose derivatives translated the inhibitory effect of *B. pumilus* and *C. freundii* into a promotory one showing that lectins can alter the signals in either direction. Galactose-binding lectins have been identified in the haemolymph of barnacles, which could find their way through the excretory system to the surface. The presence of such lectins could probably provide this organism with an ability to alter the signals or cues. Microscale patchiness of bacteria is also evident on surfaces in the sea. The availability of conflicting cues in patches may help pilot the larvae to their settlement destination. Understanding these controlling mechanisms and interfering with the pathways that are involved in lectin synthesis would be a step forward in antifouling technology.

Keywords: Cypid; Metamorphosis; *Balanus amphitrite*; Lectins; *Pseudomonas aeruginosa*; *Bacillus pumilus*; *Citrobacter freundii*

1. Introduction

Bacterial biofilms are implicated in the settlement process of planktonic larvae of most marine invertebrate groups (Crisp, 1984; Mitchell, 1984). The larvae are attracted

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0022-0981/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0022-0981(03)00024-8

towards specific chemicals in the microbial films that serve as cues for the settling larvae.

Balanus amphitrite Darwin is a dominant fouling organism cosmopolitan in tropical and warm temperate seas. The larval development of this organism includes six planktotrophic naupliar instars and a non-feeding, settling cyprid instar. The cypris larvae explore various areas before attaching to a substratum. Barnacle cyprids, like most other larvae, also prefer to settle on substrata that possess a well-developed biofilm (Crisp, 1984; Clare et al., 1992). A number of investigators have assessed the effects of pure cultures of bacteria isolated from biofilms on the larvae of choice in the laboratory (Maki et al., 1988, 1992, 1994; Holmström et al., 1992, 1996; Avelin Mary et al., 1993; O'Connor and Richardson, 1996, 1998; Anil and Khandeparker, 1998; Khandeparker et al., 2002). 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; Neal and Yule, 1994a,b; Maki, 1999). Recently a thraustochytrid protist, detected in marine microbial films has been shown to induce the settlement of *B. amphitrite* (Raghukumar et al., 2000).

After attaching to a surface, bacteria not only reproduce but also exude mucous materials known as exopolymers to form a biofilm (Costerton et al., 1978, 1995). These exopolymers are composed of polysaccharides with variable amount of proteins. These extracellular polymeric substances (EPS) either contribute to the biofilm matrix (Cooksey, 1992; Costerton et al., 1994) or are released by the microorganisms to the surrounding medium as free EPS (Sutherland, 1985; Beech, 1990; Beech et al., 2000; Khandeparker and Bhosle, 2001). The larval responses thus depend on the surface, which is changed by the extracellular molecules (Baier, 1984). Maki et al. (1988) has reported the involvement of exopolymers produced by bacteria in the attachment response of the larvae of *B. amphitrite*. Bacterial extracellular products are also reported to be involved in the attachment of larvae of a spirorbid polychaete (Kirchman et al., 1982b) and an oyster (Weiner et al., 1985).

Lectins, a class of naturally occurring proteins or glycoproteins exist in almost all living organisms and can recognize and bind carbohydrates specifically and non-covalently. The role of lectins in larval settlement has been investigated in several studies (Kirchman and Mitchell, 1981, 1983, 1984; Kirchman et al., 1982a,b; Maki and Mitchell, 1985; Mitchell, 1984; Mitchell and Kirchman, 1984; Mitchell and Maki, 1988). The settlement of a polychaete, *Janua brasiliensis* was also mediated by lectins on the larval surface that are proposed to recognize and bind to the bacterial exopolymer containing glucose (Kirchman et al., 1982a). In the case of *Crassostrea*, an oyster, neither the lectins nor monoclonal antibodies blocked the ability of *Alteromonas colwelliana* films to cue set the larvae (Weiner et al., 1989). No such studies are reported for barnacles to the best of our knowledge. However, recently *Lens culinaris* agglutinin (LCA)-binding sugar chains of the settlement-inducing protein complex (SIPC) have been implicated in the settlement of *B. amphitrite* (Matsumura et al., 1998). The present investigation was undertaken in order to identify the specific carbohydrate molecules from the bacterial film that are responsible for metamorphosis facilitation.

The bacteria, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Citrobacter freundii* isolated from the biofilms present on the shell surfaces of *B. amphitrite* were used in the present investigation. The primary strategy was to block the determinants of the bacterial films using lectins that would bind to specific carbohydrate moieties present in the extracellular polysaccharides. A comparison of the responses of cypris larvae to lectin-treated bacterial films and untreated bacterial films should unravel the possible mechanism(s) for the induction of larvae of *B. amphitrite* by bacterial films by identifying the specific carbohydrate moiety of the polysaccharide involved in signalling the larvae.

2. Materials and methods

2.1. Preparation of settlement-inducing protein complex (SIPC)

SIPC was prepared by following the method by Larman et al. (1982). Adult specimens of *B. amphitrite* were collected from the intertidal area of Dona Paula (15°27.5′N, 73°48′E). The epibiotic growth on the shells was cleaned using a nylon brush. The animals were then washed with deionised water. Approximately 100-g wet weight 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 (12,000 × *g* for 5 min), and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged (12,000 × *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^{-1} was used for all assays.

2.2. Rearing of B. amphitrite larvae

The life-cycle of *B. amphitrite* includes a larval phase consisting of six planktotrophic naupliar instars and a non-feeding cypris instar. The first instar nauplii do not feed and molt into the second instar within 1–2 h. Instars II through VI are phytoplanktotrophic. Nauplii were mass reared in 2-l glass beakers using filtered seawater (35‰ salinity) with diet of *Chaetoceros calcitrans*, a diatom, at a concentration of 2×10^5 cells ml^{-1}. The feed organism was replenished every day when 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 elsewhere (see Rittschof et al., 1984).

2.3. Isolation of bacteria from the shell surfaces of B. amphitrite

B. amphitrite brought to the laboratory were scraped with a nylon brush using Millipore-filtered 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 Bergy's manual of systematic bacteriology (Krieg, 1984).

2.4. Bacterial film

The influence of single-species films of *P. aeruginosa*, *B. pumilus* and *C. freundii* films was assessed at 35 ‰. Filming of the surfaces with bacteria was performed following the methods described by Maki et al. (1988, 1990). The dishes with the bacterial treatment were fixed with formaldehyde (final concentration 1–2%, v/v) and the density of attached bacteria was determined using acridine orange and epifluorescence microscopy (Daley and Hobbie, 1975). The adsorption technique resulted in densities of 10^6–10^7 attached bacteria/cm^2.

2.5. Extraction of free EPS

All three bacteria grown in marine broth (MB) were harvested by centrifugation (20,000 × *g* for 30 min) and subsequently sterile filtered (0.22 μm, Millipore). The culture supernatant thus obtained was treated with five volumes of absolute ethanol and left at 4 °C overnight. The precipitate (EPS) was recovered by centrifugation (30,000 × *g* for 15 min) at 4 °C, redissolved in distilled water and treated with DNAse and RNAse A (1–2 μg ml^{-1} final concentration) for 3 h at 37 °C. The material was dialyzed (8000 MW cut-off) overnight at 4 °C against distilled water and centrifuged (30,000 × *g* for 20 min) at 25 °C to remove insoluble material. The supernatant was recovered and lyophilized.

2.6. EPS characterization

The EPS was characterized for monosaccharide composition. For this, the EPS was hydrolyzed with 2 N HCl for 2 h at 100 °C in ampoules flushed with nitrogen before sealing. After hydrolysis, the solution was evaporated to dryness under reduced pressure at 40 °C. The neutral sugars were converted to alditol acetates and were analyzed by capillary gas chromatography (GC) as described previously (Bhosle et al., 1995; Khandeparker and Bhosle, 2001) (Chrompack, Middleburg, The Netherlands, Model CP 9002) equipped with a fused silica capillary column coated with CP sil-88 (25 m, id = 0.32 mm, *df* = 0.12; Chrompack). A flame ionization detector was used to separate the alditol acetate mixture. A known amount (0.4 μl) of the sample was applied using an on-column injector when the oven temperature was 70 °C. The oven temperature was rapidly raised to 150 °C and further programmed at 3 °C min^{-1} to 230 °C and maintained at this temperature for ca. 10 min. Quantification of the components was done by peak area integration of the GC results using the data handling system installed in the instrument. The response factors were calculated using standard sugar alditol acetates and myoinositol as an internal standard and were used for the quantification of the results.

2.7. Treatment of bacterial films with lectins

The lectins, *L. culinaris* agglutinin (LCA), Concanavalin A (ConA), *Limulus polyphemus* (Limulin), Wheat germ agglutinin (WGA) and Glycine max were obtained from Sigma each conjugated with FITC (fluorescein isothiocyanate).

The bacterial films of *P. aeruginosa*, *B. pumilus* and *C. freundii* were treated with 250 μl of the lectin (stock solution of 1 or 5 mg/ml in distilled, deionised water) as described by Hood and Schmidt (1996). After incubation for 30 min, the multiwells were rinsed with autoclaved filtered seawater thrice to which ~ 25–30 cyprids were introduced with 5 ml of autoclaved filtered seawater of 35‰ salinity.

2.8. Assay protocol

The multiwells were inoculated with the bacteria and free EPS (50 μg ml^{-1}). The multiwells inoculated with the bacterial films were rinsed off after 3 h by repeated rinsing with autoclaved filtered seawater under a laminar chamber. Subsequently ~ 25–30 cyprids were introduced with 5 ml of autoclaved filtered seawater of 35‰ salinity. All the experiments were repeated four times using four different batches of larvae with six replicates at each trial ($n=6$, with batch as an additional factor). Controls were filtered seawater (FSW, negative control) and settlement-inducing protein complex (SIPC, positive control). The settlement assays were carried out using Corning-430343, six well multiwells and were monitored for a period of 4 days with intermittent observations everyday. Assay wells were maintained at 26 ± 1 °C (12 h light:12 h dark cycle). The films probed with lectins were examined using an Olympus (CX40) microscope equipped with a V-LH50HG fluorescence attachment.

2.9. Statistical analysis

Data in the form of percentage of larval settlement were arcsine transformed to ensure normality of means and homogeneity of variances before subjecting to statistical analysis. The influence of lectin-treated bacterial films on the cyprid metamorphosis was evaluated using one-way analysis of variance (ANOVA) (Sokal and Rohlf, 1981). A post-ANOVA analysis was performed using Scheffe's test ($\alpha=0.05$). Data 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.

3. Results

Among the three bacterial species isolated from shell surfaces of *B. amphitrite*, *P. aeruginosa* positively influenced metamorphosis of cyprids. The cyprids exposed to *P. aeruginosa* facilitated (44%) metamorphosis similar as that of SIPC (44%) at the end of day 1 (Fig. 1). *C. freundii* and *B. pumilus* films did not facilitate metamorphosis of cyprids (Fig. 2), the response was almost similar as that observed with plain filtered seawater (-ve

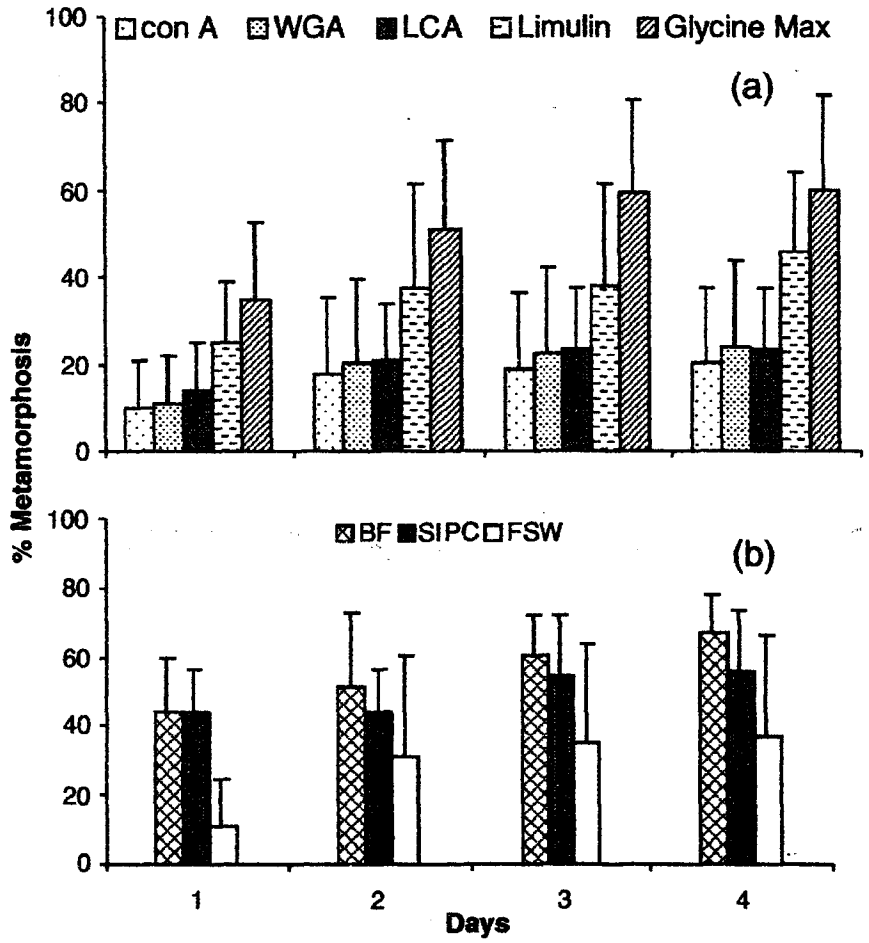
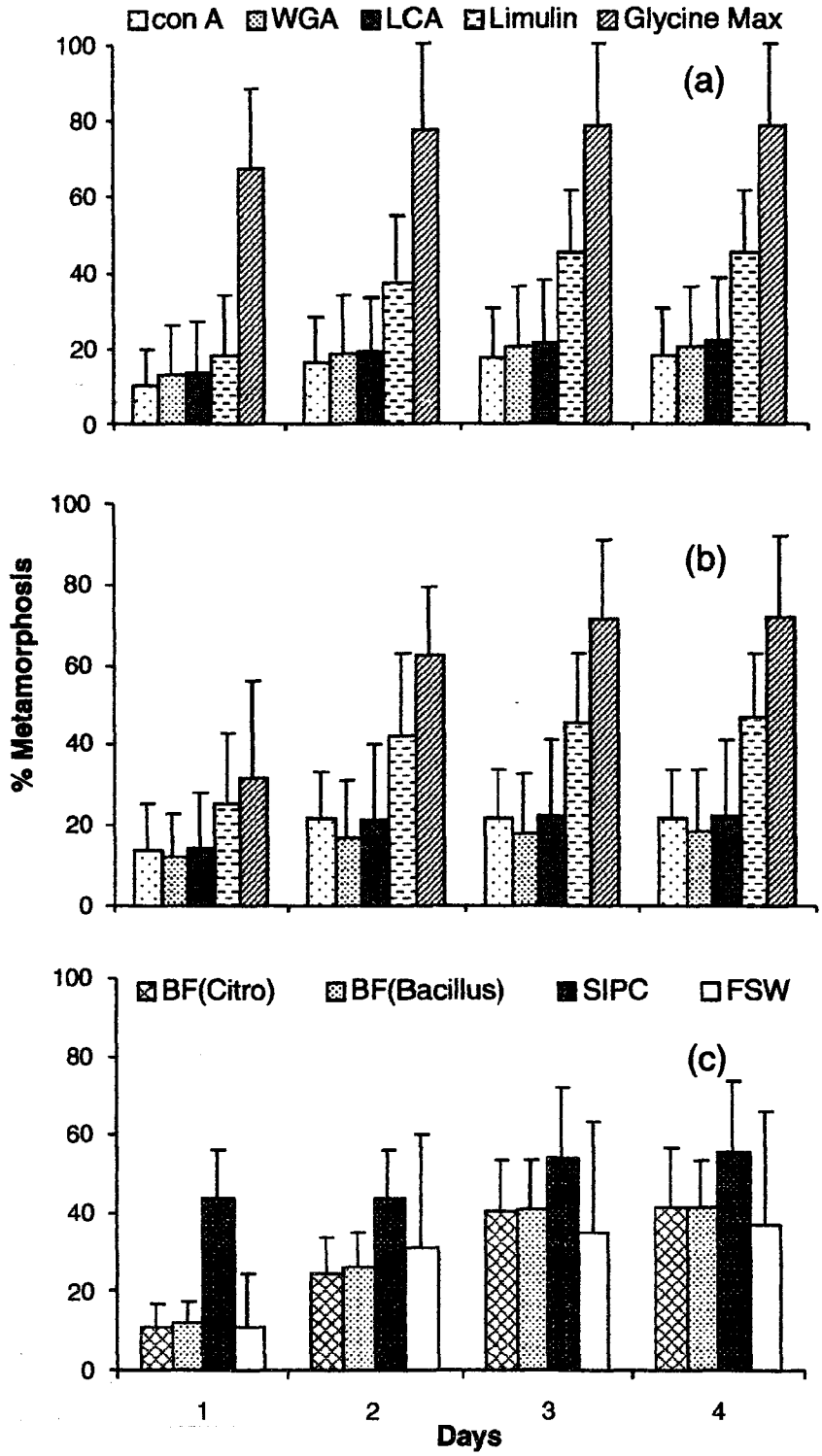


Fig. 1. Percentage metamorphosis of cyprids exposed to (a) lectin-treated and (b) non-treated films of *P. aeruginosa*. Vertical lines indicate the standard deviation from mean (BF—bacterial film). SIPC—settlement-inducing protein complex; FSW—filtered seawater (Lectin abbreviations: Con A—Concanavalin A, WGA—wheat germ agglutin, LCA—*L. culinaris* agglutinin).

control). The influence of free exopolysaccharide (EPS) obtained from *P. aeruginosa* was comparatively more than the EPS produced by the other two bacteria on the metamorphosis of cyprids. However, the metamorphosis rates were lower than the *P. aeruginosa* films (Figs. 1 and 3). The EPS produced by *B. pumilus* and *C. freundii* were strongly inhibitory (Fig. 3).

Capillary gas chromatography analysis showed the presence of mannose and glucose in the free EPS of all the three bacteria, mannose being the major constituent (Table 1). *B. pumilus* and *C. freundii* also showed the presence of ribose. The percentage of ribose in case of *B. pumilus* was less (0.72%) than that observed in *C. freundii* (4.86%) (Table 1).

Fig. 2. Percentage metamorphosis of cyprids exposed to lectin-treated and non-treated films of (a) *C. freundii*, (b) *B. pumilus* and (c) non-treated films of *C. freundii* and *B. pumilus*. Vertical lines indicate the standard deviation from mean [BF (Citro)—bacterial film (*C. freundii*); BF (Bacillus)—bacterial film (*B. pumilus*). SIPC—settlement-inducing protein complex; FSW—filtered seawater (Lectin abbreviations: Con A—Concanavalin A, WGA—wheat germ agglutin, LCA—*L. culinaris* agglutinin).


con A
WGA
LCA
Limulin
Glycine Max
(a)
(b)
(c)
% Metamorphosis
BF(Citro)
BF(Bacillus)
SIPC
FSW
Days
0
20
40
60
80
100
1
2
3
4

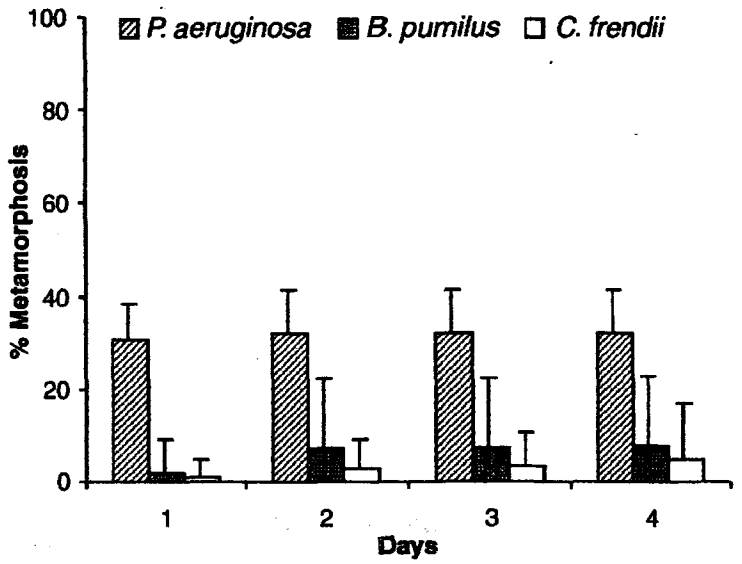


Fig. 3. Percentage metamorphosis of cyprids exposed to free exopolysacchrides obtained from *P. aeruginosa*, *B. pumilus* and *C. freundii*. Vertical lines indicate the standard deviation from mean.

The EPS intimately associated with the bacterial cell surface was probed through application of different lectins. The sugar specificity of different lectins used in the present experiment is given in Table 2. The cyprids exposed to *P. aeruginosa* films treated with lectins, Con A, WGA, LCA and Limulin showed a decline in metamorphosis. The treatment of Glycine max to the films of *P. aeruginosa* showed an almost similar metamorphosis response as that observed with untreated bacterial films ($p \leq 0.025$, Mann–Whitney). A significant difference in the metamorphosis rates was observed when the bacterial films were treated with different lectins ($p \leq 0.001$, one-way ANOVA; $p \leq 0.05$, Scheffe's test).

The films of *C. freundii* did not facilitate metamorphosis of cyprids, but resulted in metamorphosis promotion higher than that observed with SIPC when treated with Glycine max (67%) at the end of day 1. The difference in the metamorphosis rates between the untreated bacterial film and Glycine max treated film was highly significant ($p \leq 0.025$, Mann–Whitney).

The films of *B. pumilus* treated with Glycine max and Limulin positively influenced metamorphosis while untreated films did not. The inducement by Glycine max-treated *B. pumilus* films was not comparable with *C. freundii* (32% and 67%) and was less than the SIPC (44%). However, the films treated with Glycine max showed a considerable increase in metamorphosis on day 2 (63%).

Table 1
Monosaccharide composition (%) of the free EPS produced by *P. aeruginosa*, *C. freundii* and *B. pumilus*

Microorganism	Mannose (%)	Glucose (%)	Ribose (%)
Pseudomonas aeruginosa	86.28	13.71	–
Citrobacter freundii	80.17	14.95	4.86
Bacillus pumilus	86.17	13.10	0.72

Table 2
Sugar specificity of lectins

Lectin abbreviation	Common name	Sugar specificity
Con A	Concanavalin A	D-mannose D-glucose Fructofuranose
LCA	*Lens culinaris* agglutinin	D-mannose D-glucose *N*-acetyl-D-glucosamine
WGA	Wheat germ agglutinin	*N*-acetyl-D-glucosamine
Limulus polyphemus	Limulin	*N*-acetylneuraminic acid Glucuronic acid Phosphorycholine analogs
Gly max	Glycine max	*N*-acetyl-D-galactosamine

A marginal increase in the metamorphosis rates was observed as the cyprids as well as the bacterial films aged. However, in case of *B. pumilus*, a considerable hike in metamorphosis was evident in the Glycine max-treated films.

4. Discussion

Epifluorescence microscopy coupled with the lectin specificity has been suggested as a new way to probe rapidly and precisely spatial relationships of complex distributions of molecules on marine surfaces when biofilms are present (Michael and Smith, 1995). Among the bacteria isolated from the shell surface of *B. amphitrite*, *P. aeruginosa* facilitated cyprid metamorphosis. However, when probed with Concanavalin A, WGA and LCA, which binds specifically to glucose and its derivatives, mannose and fructofuranose, negated the promotory effect. Hence, these carbohydrate moieties associated with the cell surface of *P. aeruginosa* are suggested to be involved in the larval induction of *B. amphitrite*. A decrease in the metamorphosis was also evident in case of *P. aeruginosa* films treated with Limulin suggesting the presence of *N*-acetylneuraminic acid, glucuronic acid or phosphocholine analogues in the EPS that could be involved in attracting the cyprids. However, when probed with Glycine max there was not much difference in the metamorphosis response when compared to an untreated bacterial film, indicating either absence or non-involvement of galactose derivatives associated with its EPS.

The films of *B. pumilus* and *C. freundii* did not promote cyprid metamorphosis. Whereas, the Glycine max-treated bacterial films of *B. pumilus* and *C. freundii* showed a considerable increase in the metamorphosis, (metamorphosis rates being higher than that observed with the SIPC). This indicates that *N*-acetyl-galactosamine could probably be associated with the capsular EPS of these bacteria, the blocking of which by Glycine max transformed the inhibitory effect of *B. pumilus* and *C. freundii* into a promotory one. Three galactose-binding lectins have been described in the haemolymph of the acorn barnacle *M. rosa* (Marques and Barracco, 2000). The lectin present in the haemolymph of *Balanus rostrata* has been also reported to recognize the same monosaccharide (Toda et al., 1998). The fact that primary urine production is the result of ultrafiltration of the haemolymph

(White and Walker, 1981) it is possible that the lectins could find their way to the surface via excretory system. Hence, the presence of lectins in these animals other than enhancing the phagocytosis of microorganisms could also possibly play an important role in altering the signals or cues and may be ecologically important. The immobilization of marine bacteria in hydrogels has been developed as a new concept for the prevention of macrofouling (Gatenholm et al., 1995). Some of the components of the bacteria that are strongly inhibitory for the larvae (e.g. in this study, galactose residues) can thus be immobilized in the hydrogels and could be of great help in antifouling technology.

When the free EPS of *P. aeruginosa* was assayed against the cyprids, although metamorphosis was facilitated, it was not as effective as the EPS associated with the bacterial cell surface. An earlier investigation has reported that the response of the cyprids of *B. amphitrite* to bacteria and its products is regulated by both contact chemoreception and/or olfaction, depending on the properties of the settlement inducing compounds (Khandeparker et al., 2002). The free EPS of *B. pumilus* and *C. freundii* were strongly inhibitory. Capillary gas chromatographic analysis of the free EPS revealed the presence of glucose and mannose in all the three bacteria, whereas ribose was additionally present in *B. pumilus* and *C. freundii*. The presence of ribose in the EPS may be the component responsible for strong inhibitory effect of EPS produced by *B. pumilus* and *C. freundii*.

Aged cyprids showed higher rates of metamorphosis. A possible explanation could be that young cyprids are more discriminating than old cyprids, but become less discriminating with age during settlement (Rittschof et al., 1984; Crisp, 1988) presumably due to the decline in their energy reserves and perhaps physiological quality (Anil et al., 2001).

In the present investigation, the bacteria were assessed separately, whereas, in nature they occur in conjunction and the contradictory signals from each bacterium would give a different set of signals to the settling larvae. Lectin probes have illustrated spatial and chemical heterogeneities of surfaces in the marine environment and its role in possible microscale cues for biofouling is suggested (Michael and Smith, 1995). The understanding of the synchronization of contradictory signalling molecules responsible for metamorphosis needs further validation. It has been reported earlier that in *B. amphitrite* cyprids the degree of inducement of metamorphosis varied with various combinations of exopolymers of different bacterial strains with or without SIPC (Anil et al., 1997; Anil and Khandeparker, 1998). Some bacteria in biofilms are capable of genetic exchange (Fry and Day, 1990). The cell–cell communication between their own species and perhaps others through *N*-acyl-L-homoserine lactones (AHL) cannot be ruled out as AHL activity has been demonstrated in natural biofilms (McLean et al., 1997). The ability of larval receptors to distinguish between the EPS that are intimately associated with the cell surface and those released in the free form would be a key for production of probes for such saccharides and the genes that produce them can then be explored.

Acknowledgements

We are grateful to Dr. E. Desa, Director of the National Institute of Oceanography, for his support and encouragement. We are thankful to Dr. N B Bhosle for his advice during the course of this work. We thank Dr. P.S. Parameswaran, Dr. S.S. Sawant, Mr. K.

Venkat, and other colleagues of MCMRD for their help. We also thank Ms. Rakhee and Mr. Fraddry for the help provided during GC analysis due to which we could complete the process in minimum time. L. Khandeparker acknowledges CSIR, India for the award of a Senior Research Fellowship. We thank the anonymous reviewers for their effort in improving the manuscript. This work was supported by ONR Grant No. N 0014-940423 and is a NIO contribution [3813]. [SS]

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Vol. 28: 37–54, 2002 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published May 16

Factors regulating the production of different inducers in *Pseudomonas aeruginosa* with reference to larval metamorphosis in *Balanus amphitrite*

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ABSTRACT: Gregarious settlement in barnacles has been related to the settlement-inducing compounds from adult conspecifics, bacteria in the biofilms, and their interaction. Elucidation of larval settlement cues from these sources is limited. The effectiveness of larval settlement cues under different environmental conditions (salinity, temperature) needs evaluation. *Pseudomonas aeruginosa*, a bacterium isolated from the shell surface of *Balanus amphitrite* Darwin, was used as a candidate. The influence of bacterial film, culture supernatant and its molecular-weight fractions, and bacterial extract was investigated along with the conspecific adult extract (AE). The influence of culture supernatants and exopolysaccharides obtained from the bacterium cultivated in different nutrient media, effectiveness of leachants and adsorbed (surface-bound) compounds on the metamorphosis of cyprids of *B. amphitrite* was also assessed. The influence of *P. aeruginosa* on cyprid metamorphosis varied with salinity and temperature. The differences were not significant as the film and the cyprids aged. When the bacterial film was examined in the presence of an active substance (agonist) such as AE, metamorphosis was facilitated, suggesting the role of competitive antagonism in cue perception. The higher molecular-weight fraction of the bacterial-culture supernatant was inductive at higher salinity. Conversely, the lower molecular-weight fraction of the culture supernatant showed maximum inhibition when the adsorbed (surface-bound) compounds were assessed along with the leachants. Bacterial extract showed the presence of ketonic compounds, and its influence differed with salinity. The inhibitory effect of the extract was nullified in the presence of AE. When the extract was examined in the presence of leachants, a 2-fold increase in the metamorphosis rates was evident where only surface-bound components were inhibitory. Fourier transformed infrared spectroscopy analysis revealed that bacteria grown in different nutrient media yielded culture supernatants with different chemical composition, thus altering their ability to induce metamorphosis of cyprids. Maximum inducement was provoked by the culture supernatant obtained from semi-solid culture, and this positive effect was protein concentration dependent. The exopolysaccharides obtained from bacteria grown in basal salt solution facilitated metamorphosis similar to that of the bacterial film and AE. The response of the cyprids to bacteria and its products seems to be regulated by both contact chemoreception and olfaction, depending on the properties of the settlement-inducing compounds. 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.

KEY WORDS: *Pseudomonas aeruginosa* · *Balanus amphitrite* · Cyprid · Metamorphosis · Exopolysaccharides · Culture supernatant

INTRODUCTION

Balanus amphitrite Darwin is a dominant fouling organism found all over the world. The larval development of this organism includes 6 naupliar instars and a non-feeding presettling cyprid instar. *B. amphitrite* breeds throughout the year in Indian waters (Karande 1967, Anil 1986) and is euryhaline (Anil et al. 1995).

Cypris larvae test various areas before finally attaching to any substratum. A series of factors such as

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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 factors or determinants for settlement are the specific chemical cues or triggers associated with the substratum (Kirchman et al. 1982a, Morse 1984a,b, Maki & 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 for larval settlement and plays a role in the distribution of adults (Strathmann et al. 1981). 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). Settlement and metamorphosis are shown to be controlled by larval sensory recognition, which transduces the external signals into signals within the organism (Pawlik 1992).

Field and laboratory studies have shown that barnacle cyprids prefer to metamorphose on or near conspecifics. The responsible settlement pheromone or positive cue has been recognized as arthropodin or settlement factor (SF+), a glycoprotein present in the adults (Knight-Jones 1953, Knight-Jones & Crisp 1953, Crisp & Meadows 1963). *Balanus amphitrite* cyprids like *B. balanoides* (Walker & Yule 1984) have been shown to deposit footprints of temporary adhesive while exploring a substratum that stimulates the settlement of other cyprids, even in the absence of conspecific adults (Yule & Walker 1985, Clare et al. 1994).

Besides adult conspecifics, bacterial films (BF) coating the benthic substrates have been suggested as sources of waterborne cues mediating settlement of oyster larvae (Bonar et al. 1986, Fitt et al. 1989, Tamburri 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 BF does not always correlate with their final fixation (Maki et al. 1994). The studies related to interactions between cypris larvae and BF have generally found most bacterial species to inhibit attachment of *Balanus amphitrite* cyprids to polystyrene surfaces, although several bacterial species showed no effect (Maki et al. 1988, 1990, 1992, Avelin Mary et al. 1993, Neal & Yule 1994a,b). The influence of BF and the culture supernatant (CS) containing extracellular materials on the settlement of *B. improvisus* Darwin cyprids has been shown to differ when examined in the field from that observed in the laboratory (O'Connor & 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, is amplified 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 & Hadfield 1999). The bacteria influence 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, 1992) Szewzyk et al. 1991.

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 & Meadows 1962, 1963, Morse et al. 1980, Hadfield & Scheuer 1985, Jensen & Morse 1990, 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 that cause gregarious settlement in barnacles. Anil & Khandeparker (1998) and Anil et al. (1997) reported that in *Balanus amphitrite* cyprids the degree of inducement of metamorphosis varied with various combinations of exopolymers of different bacterial strains with or without adult extract (AE).

We carried out 4 different experiments (Fig. 1) to study the influence of settlement-inducing compounds from *Pseudomonas aeruginosa*, a bacterium isolated from the shell surface of *Balanus amphitrite*, on the cyprid metamorphosis of *B. amphitrite*. In Expt 1 the influence of BF was assessed under different environmental conditions (salinity and temperature). The CS, its molecular-weight (MW) fractions and bacterial extract were subjected to cyprid metamorphosis assays. The influence of BF and its products was also assessed along with conspecific AE. In Expt 2 the effectiveness of leachants and surface-bound compounds was assessed. In Expt 3 the effect of CS produced by the bacteria

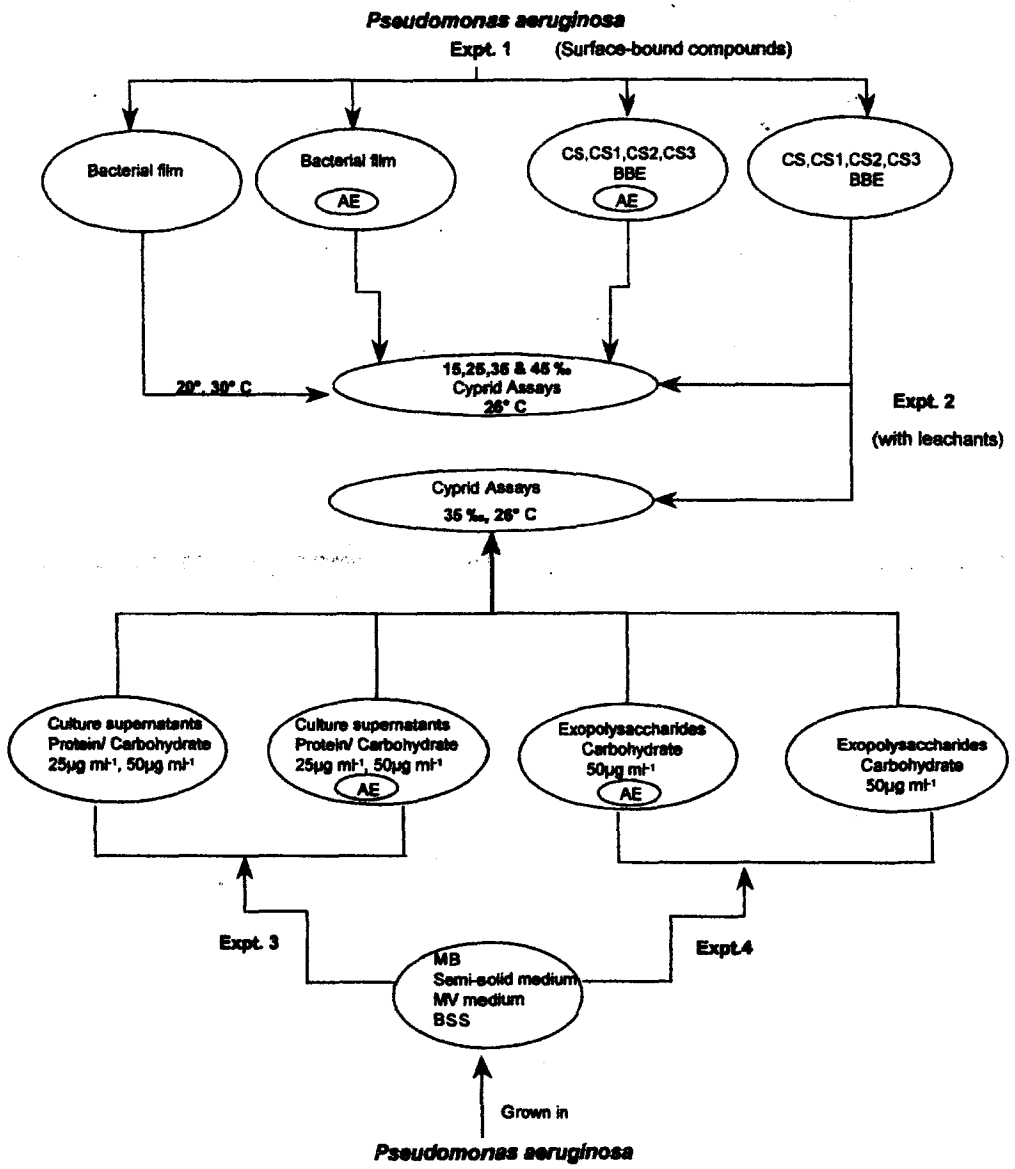

Fig. 1. Schematic representation of the experimental set-up. All the experiments included adult extract (AE) as the positive control and filtered seawater (FSW) as the negative control. BF: bacterial film; BBE: butanol-bacterial extract; CS: culture supernatant; CS1: MW < 10 000; CS2: MW = 10 000 to 30 000; CS3: MW > 30 000; MV: modified Vishniac's medium; MB: marine broth; BSS: basal salt solution

grown in different nutrient media was evaluated along with the AE. In Expt 4 the effectiveness of bacterial exopolysaccharides (EPS) extracted using different nutrient media was investigated.

MATERIALS AND METHODS

Preparation of the AE. AE was prepared by following the method described earlier by Larman et al. (1982). Adults of *Balanus 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 deionized water (reverse osmosis [RO] pure). The supernatant of the crushed mixture was decanted, centrifuged at 12 000 × *g* for 5 min and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged at 12 000 × *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^{-1} of AE was used for all assays.

Rearing of *Balanus amphitrite* larvae. The life cycle of *B. amphitrite* includes planktotrophic larval development consisting of 6 naupliar instars and a non-feeding cyprid instar. The first instar nauplii do not feed, and they molt into the second instar within a few hours. Instars II to VI are phytoplanktotrophic. Nauplii were mass reared in 2 l glass beakers using filtered seawater (FSW) of 35‰ salinity on a diet of *Chaetoceros calcitrans*, a unicellular diatom, at a cell concentration of 2×10^5 cells ml^{-1}. The food organism was replenished every day while changing the water. After 5 to 6 d the cyprids obtained were siphoned out and stored at 5°C prior to settlement assays. Cyprids (2 d old) were used for the assays. These methods have been described in detail by Rittschof et al. (1984).

Isolation of bacteria from shell surfaces of *Balanus amphitrite*. *B. amphitrite* were brought to the laboratory and rinsed with deionized 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. Bacteria were identified following 'Bergy's manual of systematic bacteriology' (Krieg 1984). *Pseudomonas aeruginosa* was chosen for the present investigation. The results of the morphological and biochemical tests employed to identify the bacterium are given in Table 1.

Expt 1. *BF:* The influence of BF on the metamorphosis of cyprids of *Balanus amphitrite* 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). The surfaces were covered with bacterial film 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 attached bacteria were quantified by using acridine orange and epifluorescence microscopy. In this way the bacterial density was determined (Daley & Hobbie 1975). The adsorption technique resulted in densities of 10^6 to 10^7 attached bacteria cm^{-2}.

CS and its fractions: The bacterium was grown in basal salt solution (BSS), pH 7.5, containing (g l^{-1}) NaCl 30.0, KCl 0.75, $MgSO_4 \cdot 7H_2O$ 7.0, NH_4Cl 1.0, K_2HPO_4 0.7, KH_2PO_4 0.3 and glucose 10.0, and 1 ml of trace metal solution (Bhosle 1981). A 2% (v/v) inoculum of an 18 h old culture grown in the same medium was used. After 48 h, when the culture reached stationary phase, cells were centrifuged at $20\,000 \times g$ for 15 min at 4°C. The supernatant was filtered through a 0.22 µm Millipore filter and the resultant filtrate was used as the CS in the assays. Subsequently, 3 subsamples were concentrated in a stirred ultrafiltration cell (Amicon) to one-tenth of the original volume using filters with a nominal MW cut-off of 3000, 10000 and 30000 sequentially. The fractions between 3000–10000 MW, 10000–30000 MW, and MW > 30000 have been abbreviated to CS1, CS2 and CS3, respectively.

CS, as well as the fractions, was characterized for total carbohydrate content following the method described earlier by Dhople & 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 CS and its fractions were rationalized at

Table 1. *Pseudomonas aeruginosa*. Results of the tests employed to identify the bacterium. KIA/TSI: Kligler's Iron Agar/Triple Sugar Iron Agar; O-F glucose: oxidation-fermentation text using glucose; +:positive; –:negative

Test	Results
Color	Cream
Shape	Short rods
Gram stain	–
Motility	+
Hugh Leifson's test	Aerobic oxidative
Growth at pH 3.6	–
Growth at 4°C	–
Growth at 41°C	+
Indole	–
Methyl red	–
Simmons citrate	+
H_2S (KIA/TSI)	–
Urease	–
Phenylalanine deaminase	–
Nitrate reduction	+
Oxidase	+
Catalase	+
Denitrification	+
Gelatin liquefaction	+
Starch hydrolysis	–
O-F glucose	Oxidative
Arginine dihydrolase	+
Alkaline phosphatase heat resistance	+
Litmus milk (peptonization)	+
Utilization of:	
Glucose	+
D-xylose	–
D-ribose	+
Mannitol	+
Cellobiose	–
D-mannose	–
L-arabinose	–
Lactose	–
Maltose	–
D-fructose	+
m-inositol	–
Sucrose	–
D-galactose	–
Acetamide	+

50 µg ml^{-1} of carbohydrates and were carried out at different salinities (15, 25, 35 and 45‰).

Bacterial extract: The bacterium was extracted in butanol following the method described by Elyakov et al. (1996). This butanol-bacterial extract (BBE) was stored below 5°C for further use. The butanol extracted only low MW polar metabolites. A concentration of 50 µg ml^{-1} of BBE was used to examine its effect on the cyprid metamorphosis at 15, 25, 35 and 45‰.

The BF, CS, fractions and bacterial extract were also examined in the presence of AE (50 µg ml^{-1}).

Expt 2. This experiment was carried out at 35‰. It differs from Expt 1 as it was carried out with CS, fractions and bacterial extract (50 µg 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 µg ml^{-1}) was similarly assessed.

Expt 3. The bacteria were grown in marine broth (MB), BSS, organically rich modified Vishniac's medium (MV) as described by Perkins (1973) and semi-solid culture (Abu et al. 1991). The CS were harvested by centrifugation (20 000 × *g* for 30 min) and subsequently sterile filtered (0.22 µm, Millipore). They were then concentrated to one-tenth of the original volume in a stirred ultrafiltration cell (1000 MW cut-off, Amicon). The CS were characterized by estimating total carbohydrates and proteins. The larval assays were carried out at carbohydrate and protein concentrations of 25 and 50 µg ml^{-1}, respectively. These CS, 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 the presence of AE (25/50 µg ml^{-1}).

Semi-solid culture: Since the growth conditions used in the semi-solid culture are different from those of other cultures, the details are provided. For this, *Pseudomonas aeruginosa* cells grown in MB were washed with phosphate-buffered saline (pH 7.3) and resuspended in the same buffer. Five milliliters of the washed suspension was added to a petri plate of marine agar that had been overlaid with a sterile dialysis membrane (8000 MW cut-off) presoaked in deionized water and rinsed with sterile PBS (pH 7.3). After 24 h, polycarbonate Nuclepore filters (25 mm, 0.4 µm 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 × *g* for 35 min at 4°C. The recovered supernatant was dialysed (8000 MW cut-off) overnight against distilled water. The non-dialysable material was filtered through Whatman #4 filter paper and lyophilized. The lyophilized material was redissolved in a minimum amount of distilled water and filtered through Whatman #4 paper. The CS thus obtained was characterized by estimating total carbohydrates and proteins.

Expt 4. *Extraction of bacterial EPS:* For the extraction of EPS, the CS 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 × *g* for 15 min at 4°C, redissolved in distilled water, and treated with DNAse and RNAse A (1 to 2 µg ml^{-1} final concentration) for 3 h at 37°C. The material was dialyzed (8000 MW cut-off) overnight at 4°C against distilled water and centrifuged at 30 000 × *g* for 20 min at 25°C to remove insoluble material. The supernatant was recovered and lyophilized. The EPS were evaluated for carbohydrate content and rationalized at a concentration of 50 µg ml^{-1} at 35‰. They were then subjected to the assays separately, as well as in the presence of AE (50 µg ml^{-1}).

Fourier transformed infrared spectroscopy (FTIR). The major structural groups of the CS of the bacteria cultivated in different nutrient media, MW fractions and the bacterial extract were detected using FTIR.

Assay protocol. The settlement assays were carried out using Corning-430343 6-well multiwells. The multiwells were inoculated with BF, CS, fractions, bacterial EPS and bacterial extract. They were also assessed along with AE (3 replicates for each of the combination by taking 3 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 3 h by repeated rinsing with autoclaved FSW 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 to 40 cyprids were introduced with 5 ml of autoclaved FSW (15, 25, 35 and 45‰ salinity) as required. The settlement assays were monitored for a period of 4 d with an intermittent observation every day. Assay wells were maintained at 26 ± 1°C (12:12 h light:dark cycle). The influence of BF, AE and FSW was also investigated at 20 and 30 ± 1°C.

Statistical analysis. Data in the form of percentage of larval settlement were arcsine transformed to ensure normality of means and homogeneity of variances before statistical analysis. The influence of *Pseudomonas aeruginosa*, CS, fractions and the bacterial extract in the presence and absence of AE on the metamorphosis of cyprids was evaluated using 1-way ANOVA (Sokal & Rohlf 1981). A post ANOVA was performed using Scheffé's test ($\alpha = 0.05$). Those factors that did not meet the normality assumption were

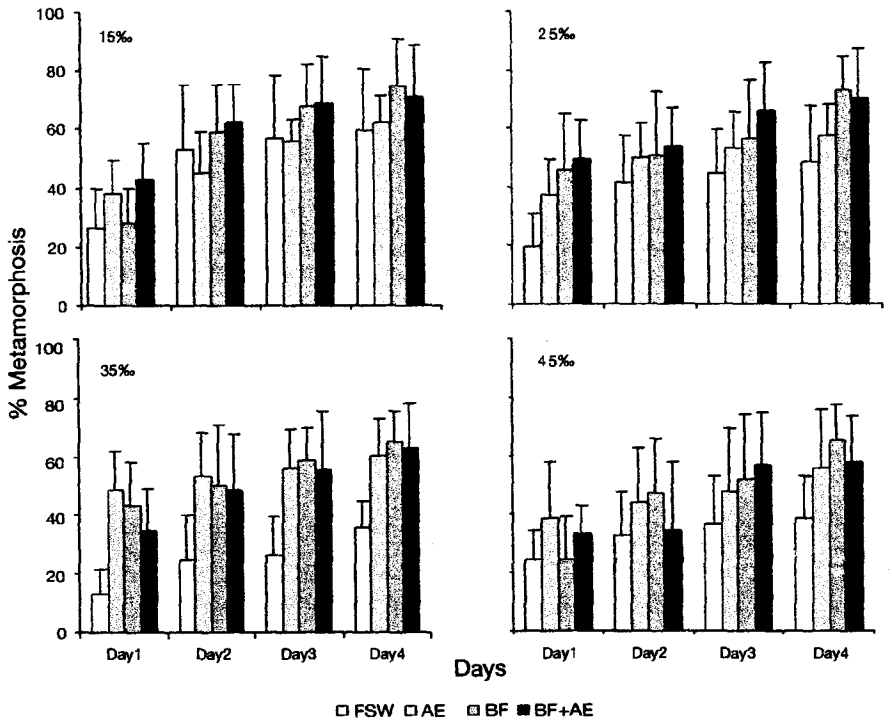

Fig. 2. *Balanus amphitrite*. Expt 1. Percentage metamorphosis of cyprids exposed to bacterial film (*Pseudomonas aeruginosa*) in the presence of AE at different salinities. Vertical lines indicate mean + SD. Abbreviations as in Fig. 1

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 2 treatments. Three-way ANOVA was performed to evaluate the influence of temperature and salinities with respect to BF, AE and FSW on the cyprid metamorphosis. The data in Expt 1 were 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 the 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 inter-cluster distance is measured by the group average method. The *x*-axis groupings are based on the clusters that are dissimilar beyond the mid-point of the highest dissimilarity observed.

RESULTS

Expt 1

When the BF of *Pseudomonas aeruginosa* was assessed at 26°C and at different salinities, the cyprids metamorphosed at higher percentages than the control (FSW) at 25 and 35‰ ($p \leq 0.025$, Mann-Whitney), whereas at 15 and 45‰, metamorphosis was not facilitated (Fig. 2). The metamorphosis rates of the cyprids exposed to BF at different salinities were significantly different ($p \leq 0.01$, 1-way ANOVA; $p \leq 0.05$, Scheffé's test). However, such differences were not evident with the aging of the films and cyprids (Table 2a). When assessed in the presence of AE, metamorphosis was facilitated irrespective of salinity differences (Fig. 2). The influence of bacteria in the presence of AE did not differ significantly with respect to salinity (Table 2b).

BF 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. 3). At these temperatures, the influence of BF, AE and FSW was almost similar at 45‰ and comparatively less than that observed at 26°C. Three-way ANOVA indicated the differences between BF, AE and FSW to be significant with respect to salinity and temperature at the end of Day 1, whereas on Day 4 no significant differences were observed (Table 3).

Cluster analysis indicated that the response of the cyprids to some of the bacterial cues that showed similarity in larval induction of metamorphosis at one salinity differed at the other (Fig. 4). At 15‰, BF and AE were similar in inducing larval metamorphosis, owing to which they formed 1 cluster, whereas at 25‰ BF was

Table 2. One-way ANOVA. Influence of *Pseudomonas aeruginosa*, culture supernatant (CS), CS fractions and butanol-bacterial extract (BBE) at different salinities on the metamorphosis of *Balanus amphitrite* cyprids. AE: adult extract; BF: bacterial film; CS1: MW < 10000; CS2: MW = 10000 to 30000; CS3: MW > 30000; Fs: Fischer constant; MS: mean of squares; ns: not significant; SS: sum of squares; err.: error. $^{*}p \leq 0.05$; $^{**}p \leq 0.025$; $^{***}p \leq 0.01$; $^{*****}p \leq 0.001$

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

most dissimilar to the rest of the cues. At 35‰, BF and AE were highly dissimilar to rest of the bacterial cues, whereas at 45‰ BF and CS1 showed a similar response (Fig. 4, Day 1). The response differed as the cyprids and the cues aged (Fig. 4, Day 4).

The response of cyprids toward bacterial CS varied with the fractions containing different MW substances. A significant difference in metamorphosis inducement with respect to salinity was seen only with CS3 ($p \leq 0.001$, 1-way ANOVA; $p \leq 0.05$, Scheffé's test) on Day 1, whereas on Day 4 these differences were not significant (Table 2g). The percentage of larvae metamorphosing in response to CS2 at 15 and 25‰ and CS at 35‰ was higher than other fractions at the end of Day 1 (Fig. 5). 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, the higher MW fraction of the CS was inductive at higher salinity. When the fractions were assessed in the presence of AE the metamorphosis rates increased. However, significant differences with respect to salinities were observed only with CS3 (Table 2h).

Bacterial extract showed ketonic compounds as indicated by FTIR, and their influence varied with salinity. The inhibitory effect of the extract at 15 and 35‰ was nullified in the presence of AE (Fig. 6).

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

Table 3. *Balanus amphitrite*. Three-way ANOVA. Influence of temperature (20, 26 and 30°C) and salinities (15, 25, 35 and 45‰) with respect to treatments BF, AE and FSW) on the metamorphosis of *B. amphitrite* cyprids on Day 1 and Day 4. $^{**}p \leq 0.025$; $^{***}p \leq 0.01$. Abbreviations as in Table 2

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	2 (ns)
A × B	6	105	17	2.2 (ns)	146	24	2.2 (ns)
C × B	6	199	33	4.3**	163	27	2.5 (ns)
A × B × C	12	94	8		130	11	
Total	35	1810			5962		

Expt 2

When the surface-bound components of bacterial extract were assessed along with the leachants, a 2-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 a decrease in the metamorphosis rates to as low as 4.5% when assessed along with its adsorbed components (Fig. 7).

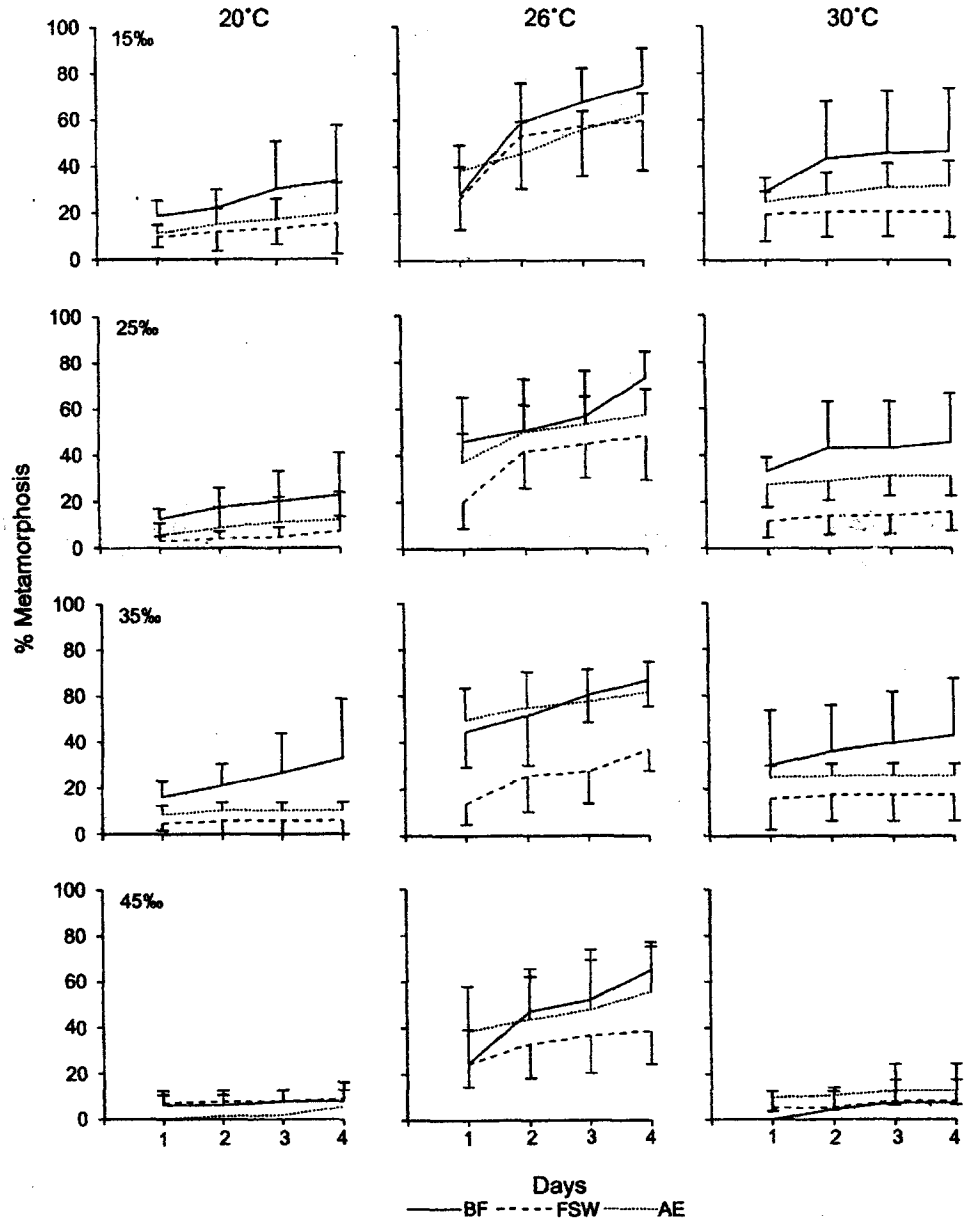

Fig. 3. *Balanus amphitrite*. Expt 1. Percentage metamorphosis of cyprids exposed to BF, FSW and AE at different salinities and temperatures. Vertical lines indicate mean ± SD. Abbreviations as in Fig. 1

Expt 3

Pseudomonas aeruginosa cultivated in BSS, MB and MV resulted in CS with higher carbohydrate content, whereas those extracted using semi-solid culture showed higher protein content.

The larvae metamorphosed at higher percentages when exposed to CS obtained by semi-solid culture than those extracted using other nutritional media at a protein concentration of 25 µg ml^{-1}. The differences between these supernatants were highly significant ($p \leq 0.001$, 1-way ANOVA; $p \leq 0.05$, Scheffé's test). An increase in protein concentration to 50 µg ml^{-1} resulted in non-significant differences (Table 4a). At this concentration CS produced by semi-solid culture showed an inhibition. At the end of Day 4, there was no significant difference in the response of the CS to cyprid metamorphosis at a carbohydrate concentration of 25 µg ml^{-1}. Except for the CS produced by semi-solid culture, no other CS was comparable with AE or BF

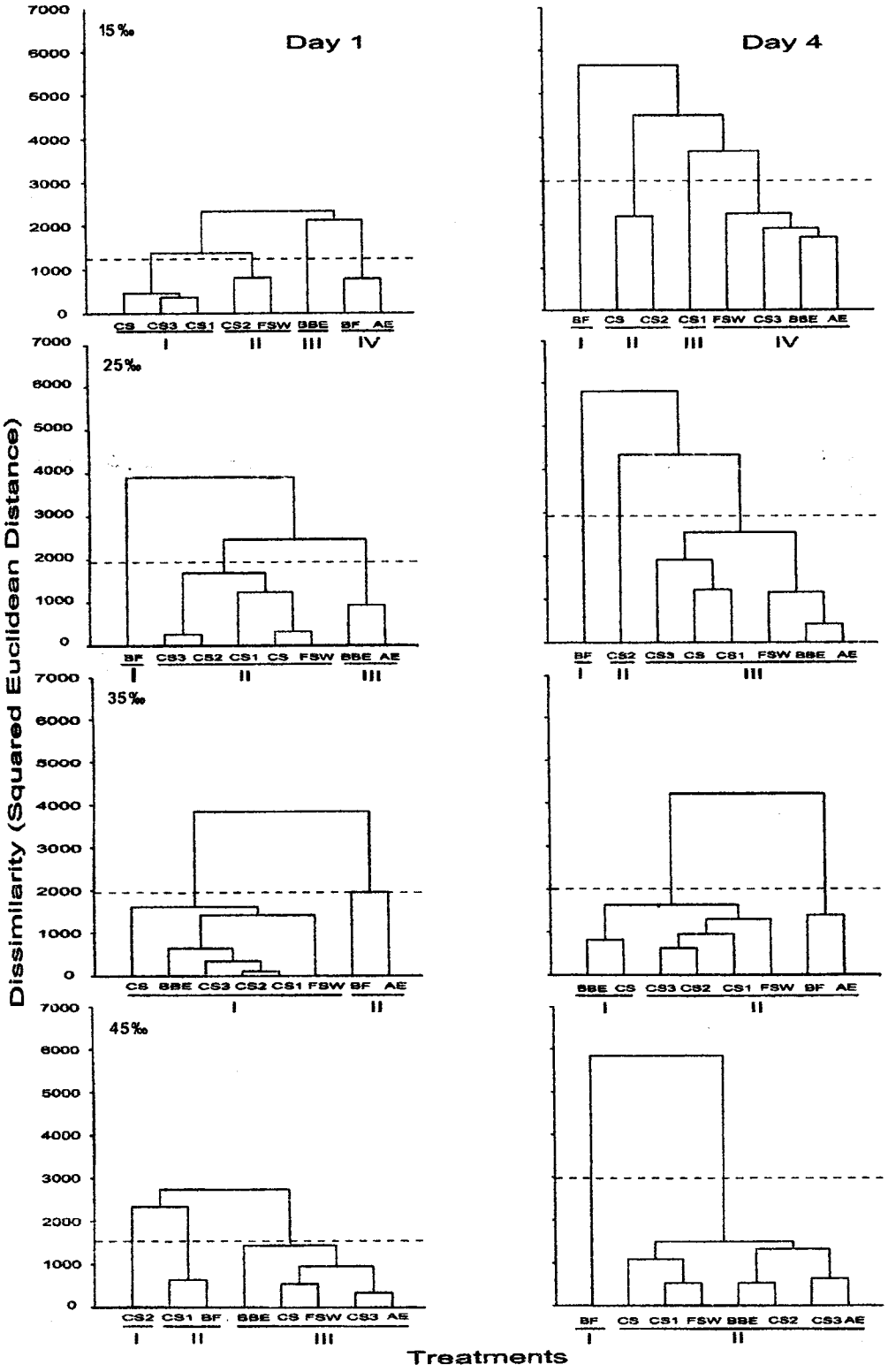

Fig. 4. *Balanus amphitrite*. Expt 1. Dendrograms showing the dissimilarity between different bacterial inducers (treatments) toward metamorphosis of cyprids on Days 1 and 4. Abbreviations as in Fig. 1. (The *x*-axis groupings are based on the clusters that are dissimilar beyond the mid-point of the highest dissimilarity observed)

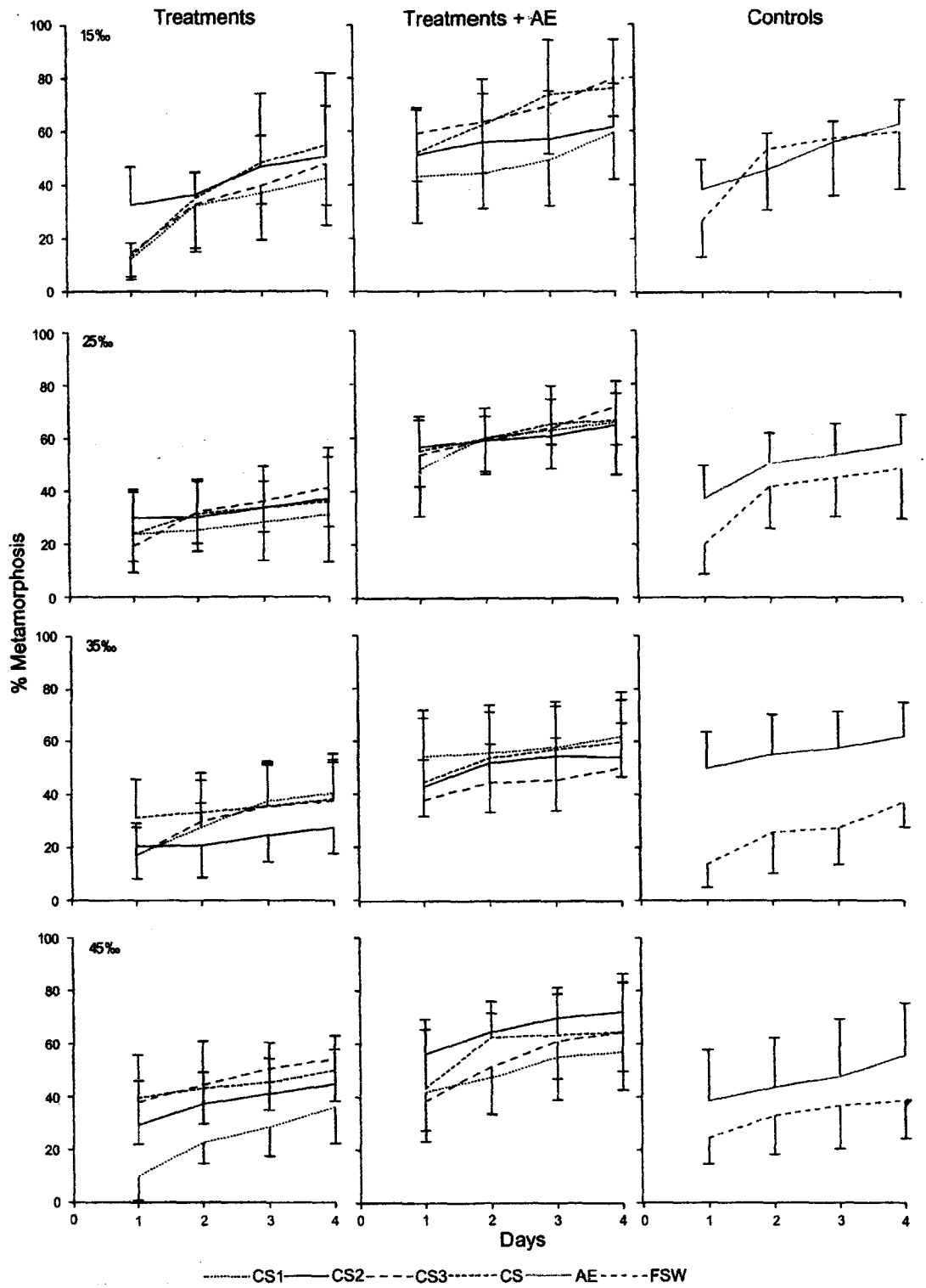

Fig. 5. *Balanus amphitrite*. Expt 1. Percentage metamorphosis of cyprids in response to CS and its fractions (treatments) obtained from the bacteria grown in basal salt solution (BSS) in the presence and absence of AE at different salinities. Vertical lines indicate mean ± SD. Abbreviations as in Fig. 1

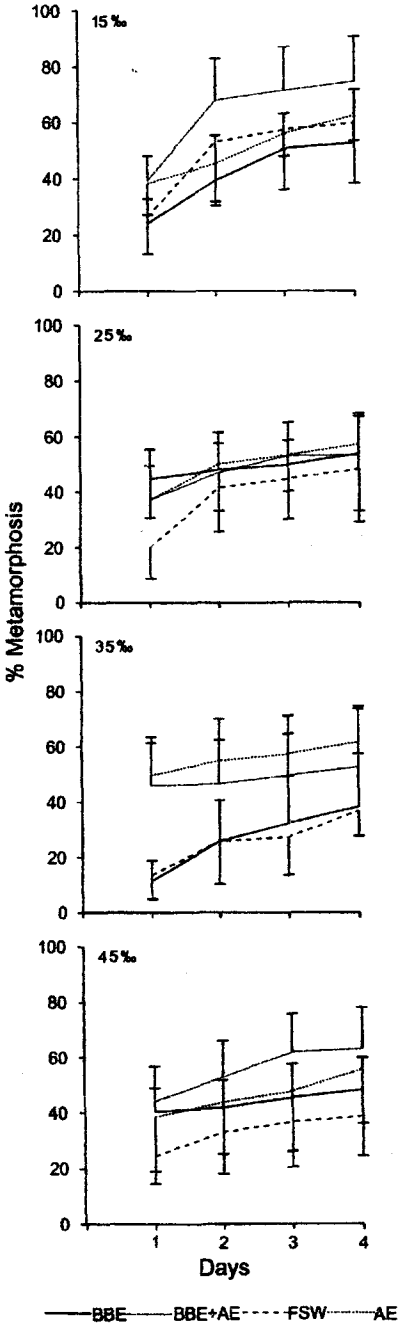

Fig. 6. *Balanus amphitrite*. Expt 1. Percentage metamorphosis of cyprids exposed to BBE at different salinities. Vertical lines indicate mean ± SD. Abbreviations as in Fig. 1

(Fig. 8b,e). Mann-Whitney *U*-test showed no significant difference between BF, AE and the CS extracted by semi-solid culture.

When these CS were assessed in the presence of AE, significant variations in metamorphosis were observed at a carbohydrate and protein concentration of 50 and

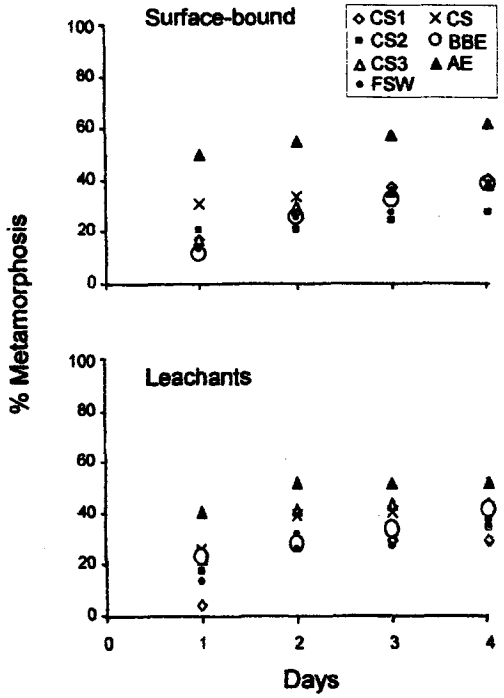

Fig. 7. *Balanus amphitrite*. Expt 2. Percentage metamorphosis of cyprids and bacterial inducers (surface-bound) used in Expt 1 in the presence of leachants at 35‰. Abbreviations as in Fig. 1

25 µg ml^{-1}, respectively, at the end of Day 1 (Table 4b). However, Day 4 observations indicated non-significant differences between CS only at a protein concentration of 50 µg ml^{-1}.

Expt 4

The EPS obtained from the bacteria grown in semi-solid culture showed high protein content, whereas those obtained from BSS, MB and MV media were mainly carbohydrates. The EPS obtained from BSS exhibited similar metamorphosis to that shown by bacteria and AE at 35‰ (Fig. 9).

FTIR

The FTIR spectra of CS1, CS2, CS3 and CS revealed characteristic strong peaks around 3550 to 3200, 1640 and 1076 to 1122 cm^{-1} for O-H stretching, C = O stretching and C-O stretching. The structural identity of CS1, CS2, CS3 and CS from FTIR results was mainly of carbohydrates. Similar results were obtained with the CS of MB and MV media (Fig. 10).

CS of semi-solid culture and the bacterial extract revealed strong peaks at 1624 and 1647 cm^{-1} for N-H bend

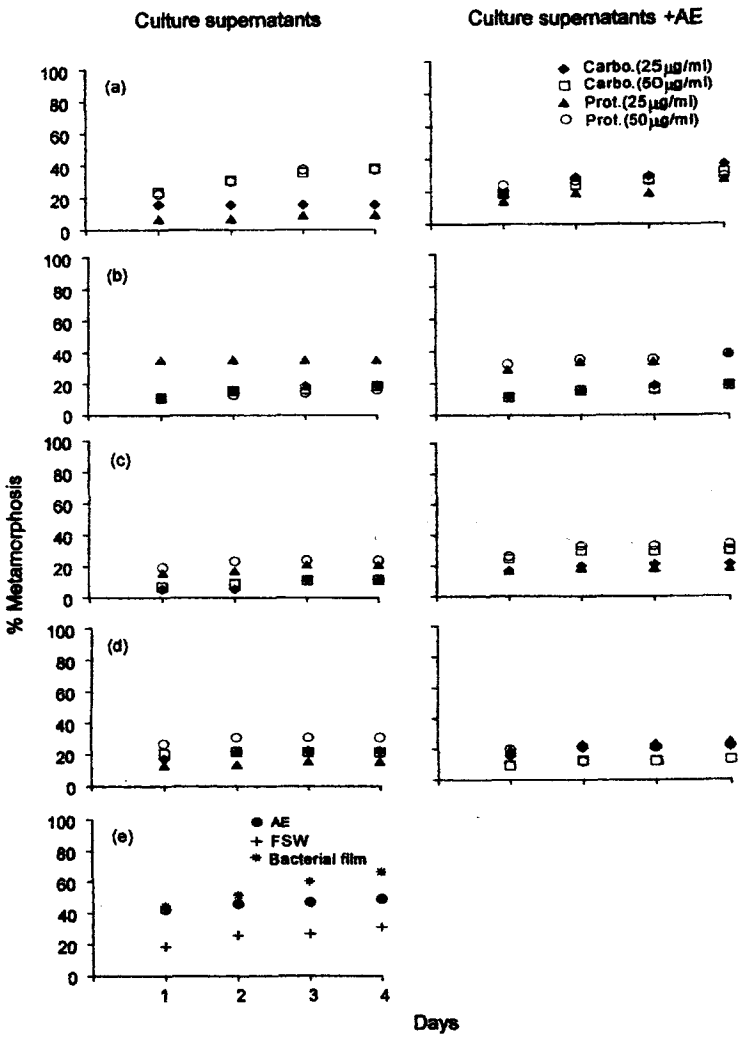

Fig. 8. *Balanus amphitrite*. Expt 3. Percentage metamorphosis of cyprids exposed to CS obtained by growing the bacteria under different nutritional media at 35‰ in the presence and absence of AE. (a) CS(BSS); (b) CS(semi-solid); (c) CS(MV) (d); CS(MB); (e) controls. Carbo: carbohydrate; Prot: protein; other abbreviations as in Fig.1

and were proteinaceous. In the case of semi-solid CS, the presence of terpenoids and steroids was evidenced from infrared absorption at 1475 and 1363.6 cm^{-1}; the spectra also indicated the presence of unsaturation evident from C-H bending vibrations located around 1000 and 900 cm^{-1}. Bacterial extract showed peaks at 1715 and 1675 cm^{-1}, characteristic of ketones.

DISCUSSION

The facilitation of metamorphosis by *Pseudomonas aeruginosa* depended on the 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 determines how well a cyprid adheres to a filmed surface (Yule & Crisp 1983, Neal & Yule 1992). Also, changes in environmental conditions can turn on the previously unexpressed genes (Dagostino et al. 1990, Davies et al. 1993, Davies & 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 and expression of different cell surface organic molecules. These surface-associated specific biochemicals not only may function in the role of stimulating or inhibiting larvae but also 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 used 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 & Mitchell 1985, Schmahl 1985, Szewzyk et al. 1991). It has been reported that a metabolically active BF is needed to maintain the putative cue at a concentration that surpasses the threshold for induction of larval settlement (Lau & Qian 2001). Maki et al. (1994) reported that *Deleya marina* films on polystyrene dishes showed a negative effect on attachment of *Balanus 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 & Richardson 1998).

When the BF was examined in the presence of AE, metamorphosis was facilitated irrespective of the salinity differences. The BF 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 the 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 or internal neuronal processes as early transducers and mediators of recruitment process is suggested by the fact that γ-aminobutyric acid (GABA) and GABA

Table 4. *Balanus amphitrite*. (a) One-way ANOVA: Influence of CS extracted by 4 different extraction protocols at 2 different concentrations of carbohydrates and proteins on the metamorphosis of *B. amphitrite* cyprids. (b) One-way ANOVA: influence of CS extracted by 4 different extraction protocols at 2 different concentrations of carbohydrates and proteins in the presence of AE on the cyprid metamorphosis of *B. amphitrite*. *$p \leq 0.05$; **$p \leq 0.025$; ***$p \leq 0.01$; ****$p \leq 0.005$; *****$p \leq 0.001$. Abbreviations as in Table 2

Factor	df	25 µg ml^{-1} (carbohydrates)			50 µg ml^{-1} (carbohydrates)			25 µg ml^{-1} (proteins)			50 µg ml^{-1} (proteins)		
		SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
(a)													
Day 1													
CS	3	258	86	4**	588	196	3.6**	1455	485	9.9*****	448	149	2.7 (ns)
Within sub-group err.	32	698	21.8		1711	53.5		1562	48.8		1751	54.7	
Total	35	956			2299			3017			2199		
Day 4													
CS	3	227	75.5	1.7 (ns)	1297	432	7.07*****	1204	401	7.5*****	831	277	4.53***
Within sub-group err.	32	1437	45		1955	61.1		1701	53.2		1955	61.1	
Total	35	1664			3252			2905			2786		
(b)													
Day 1													
CS	3	82	27.2	0.5 (ns)	449	150	4.1**	392	130	4.7***	271	90	1.4 (ns)
Within sub-group err.	32	1665	52		1164	36.4		873	27.3		2030	63.4	
Total	35	1747			1613			1265			2301		
Day 4													
CS	3	1028	342	4.6***	1182	394	3.8**	695	232	6.7****	491	164	1.7 (ns)
Within sub-group err.	32	2390	75		3277	102		1109	35		2955	92.4	
Total	35	3418			4459			1804			3446		

analogs are potent inducers of settlement, attachment and metamorphosis (Morse 1984a,b, 1985, 1990, Pawlik 1990, Morse 1991a,b). Recently, Yamamoto et al. (1995) reported that a protein-kinase C (PKC) signal transduction system plays an important role in the metamorphosis of *Balanus amphitrite* cyprids. Clare et al. (1995) provided 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. The need to characterize and distinguish the receptors, which act via such signaling systems on particular settlement cues, is apparent.

Previous studies have reported increases in invertebrate larval recruitment with aged and more heavily filmed surfaces (Wieczorek et al. 1995, Keough & Raimondi 1995, 1996). In contrast, Maki et al. (1990) demonstrated that a 3 d old natural biofilm, but not 1 d old biofilm, inhibited barnacle larvae. Avelin Mary et al. (1993) found that all *Vibrio* sp. films and most other isolates from biofilms associated with *Balanus amphitrite* were inhibitory and no film stimulated barnacle settlement. In another study (Wieczorek et al. 1995) the 'older' films (comprising larger proportions of the shape category '*Vibrios*') clearly facilitated settlement. *Pseudomonas fluorescence* and *Alteromonas macleodii* films yielded weak and inconsistent effects on cyprid attachment of *B. amphitrite*. Despite variability between assays, bacterial effects on larval attach-

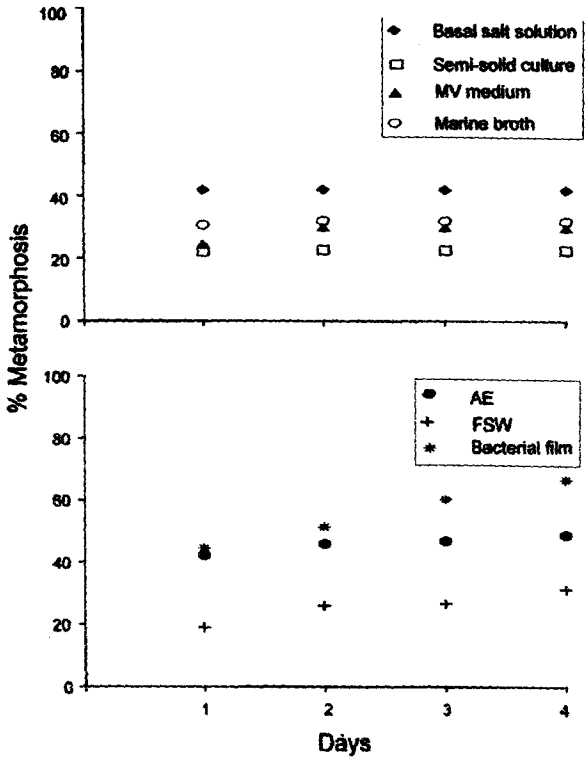

Fig. 9. *Balanus amphitrite*. Expt 4. Percentage metamorphosis of cyprids exposed to bacterial exopolysaccharides obtained after growing the bacteria under different nutritional conditions at 35‰. Abbreviations as in Fig. 1

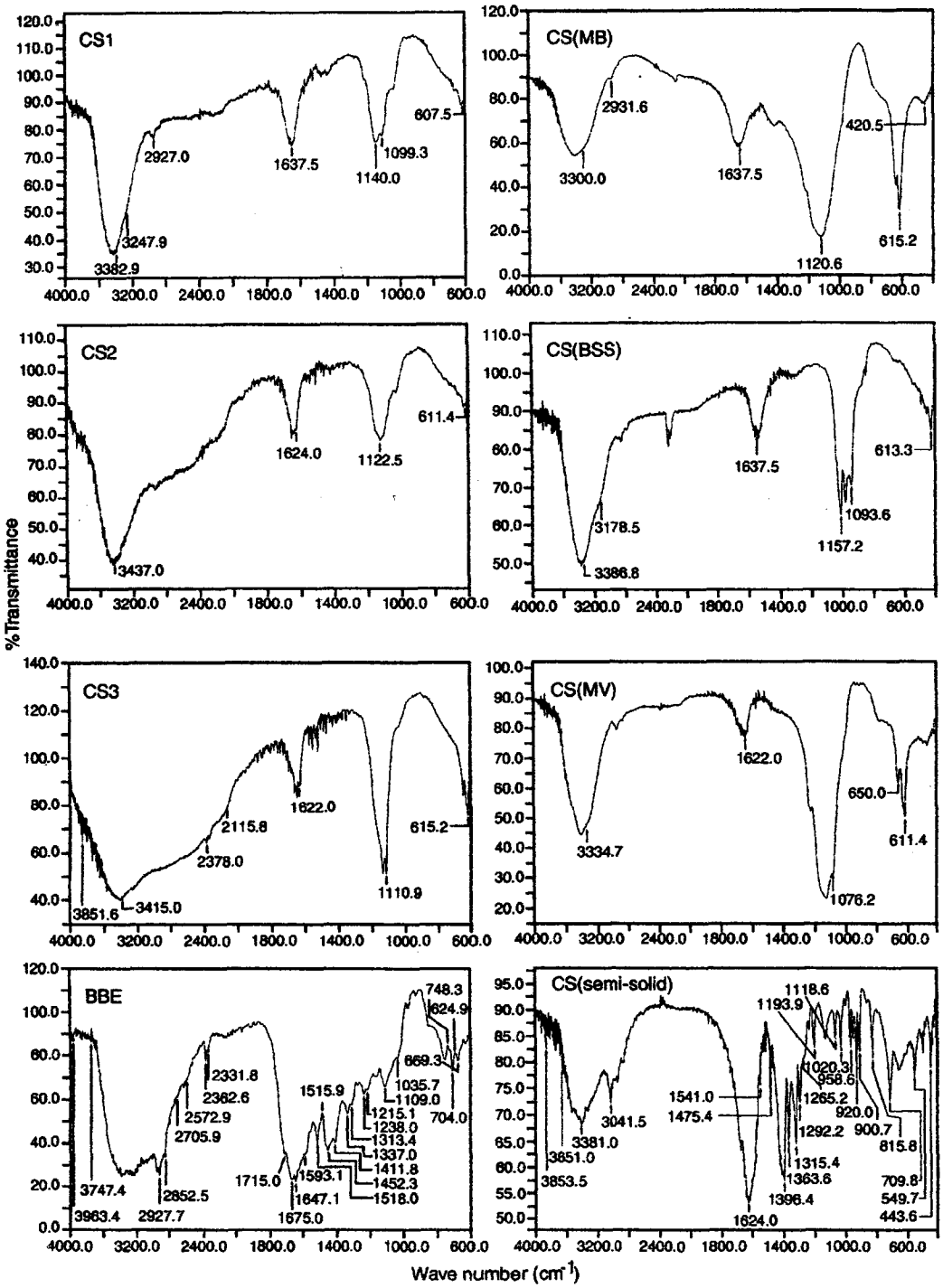

Fig. 10. Fourier transformed infrared spectroscopy (FTIR) spectra of culture supernatants, fractions and the bacterial extract. AE was the positive control and FSW the negative control. Abbreviations as in Fig. 1

ment within a particular assay generally were consistent for the duration of the assay (up to a week) (O'Connor & Richardson 1998). In the present study, although the facilitation of metamorphosis by *P. aeruginosa* initially depended upon the salinity and temperature, 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. There was no significant difference in the bacterial multiplication with respect to salinity and temperature on Day 4. Although the BF promoted metamorphosis, the inability of all the cyprids to show a similar response can be attributed to their physiological conditions. The nutritional and environmental conditions seem to jointly determine the energy status of the larvae (Anil & Kurian 1996, Anil et al. 2001). The cyprids that metamorphosed in response to the BF may be the ones that 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 a 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 influences 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, few have tested 2 or more factors on settlement (Pawlik 1992, Anil et al. 1997). The CS from stationary-phase cultures of *Deleya marina* consistently retarded cyprid attachment compared to attachment observed on control polystyrene petri dishes, but not glass vials (O'Connor & Richardson 1998). In this investigation the CS containing the extracellular materials was fractionated into different MW categories, which were characterized in terms of protein and carbohydrates. The structural identity of CS1, CS2, CS3 and CS from FTIR results was mainly that of carbohydrates. The influence of CS and its fractions on the metamorphosis of cyprids also varied with the salinity. At lower salinity CS3 and CS1 did not induce metamorphosis, while CS2 promoted metamorphosis; however, the response of the CS was negative. The promotive 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 response of cyprids to bacterial supernatants can thus be attributed to the activity of specific MW compounds.

The leachants of the CS and its fractions were associated with a decrease in the metamorphosis rates, whereas the low MW fraction CS1 proved to be the most inhibitory. The interaction of sugars with water is highly specific and depends strongly on the stereoisomerism of the hydroxyl group (Maggio et al. 1985, Kuttenreich 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 the bacteria grown in BSS exhibited similar metamorphosis effects to that of bacteria and AE, and hence may be a responsible surface-bound component of the BF, which supplied positive cues for the settling cyprids. The settlement of *Janua* sp. larvae was lectin mediated and involved bacterial EPS, where lectins on the larval surface are proposed to recognize and bind to the bacterial polymer containing glucose (Kirchman et al. 1982a,b). The extent of attachment of *Ciona intestinalis* larvae was also increased by the EPS produced by the bacteria (Szewzyk et al. 1991).

The bacterial extract was proteinaceous, showing the presence of ketonic compounds, and its influence varied with salinity. The inhibitory effect of the extract was nullified in the presence of AE. The leachants of the bacterial extract showed a 2-fold increase in the metamorphosis rates where only surface-bound components were inhibitory. The bacterium was extracted in n-butanol, which extracted only low MW 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 and needs further validation. The increased detection of the bacterial extract in solution also suggests the role of the fourth antennular segment with its impressive array of sensory setae (Gibson & Nott 1971, Clare & Nott 1994) involving the olfactory receptors. In oysters, low MW peptides with arginine at the C-terminal were identified as a natural water-soluble cue inducing settlement (Zimmer-Faust & Tamburri 1994). Rittschof (1985) partially purified water-soluble peptides (3000 to 5000 Da) released by adult conspecifics, which initiated attachment by larvae of *Balanus amphitrite*. The possible 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 CS containing the extracellular materials. Although the MB consisted mainly of peptic digest of animal tissue and the yeast extract, the CS was mainly carbohydrate in nature.

The CS obtained by 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 $\mu g\ ml^{-1}$ ($p \leq 0.001$). An increase in protein concentration to 50 $\mu g\ ml^{-1}$ resulted in inhibition of metamorphosis. The CS isolated from cells growing in a semi-solid environment also more closely approximates the

natural environment existing between a bacterium and its substratum (Abu et al. 1991). According to Boyle & Reade (1983), such conditions may be similar to the effects of exposure to intertidal zones. The response of the cyprids to bacteria and its products seems to be regulated by both contact chemoreception and olfaction, which depend on the properties of the settlement-inducing compounds.

Acknowledgements. We are grateful to Dr. E. Desa, Director of the National Institute of Oceanography, for his support and encouragement. We are thankful to Dr. N. B. Bhosle for his advice during the course of this work. We thank Dr. P. S. Parameswaran, Dr. S. S. Sawant, Mr. K. Venkat and other colleagues of MCMRD for their help. L.K. acknowledges CSIR, India, for the award of a Senior Research Fellowship. This work was supported by ONR Grant No. N 0014-940423 and is an NIO contribution (No. 3748)

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Editorial responsibility: John Dolan, Villefranche-sur-Mer, France

Submitted: November 8, 2001; Accepted: March 12, 2002
Proofs received from author(s): April 23, 2002

ELSEVIER

Journal of Experimental Marine Biology and Ecology
281 (2002) 77–88

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

Exploration and metamorphosis in *Balanus amphitrite* Darwin (Cirripedia; Thoracica) cyprids: significance of sugars and adult extract

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Received 3 May 2002; received in revised form 14 August 2002; accepted 10 September 2002

Abstract

Earlier observations have shown that sugars in solution adsorb electrostatically through –OH groups to polar groups associated with the cypris larva temporary adhesive (CTA). *Lens culinaris* agglutinin (LCA)-binding sugar chains of the adult extract (AE) have been suggested to be involved in the settlement of *Balanus amphitrite*. In the present study experiments were carried out 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 as well as nontreated cyprids was carried out with AE-coated and non-coated multiwells containing filtered sea water (FSW). D-mannose-treated cyprids resulted in higher metamorphosis than the nontreated cyprids at 10^{-8} M, whereas cyprids treated with the other LCA-specific sugar (D-glucose) showed an inhibitory effect in the absence of AE. This result implies the involvement of D-mannose moieties of AE in the settlement promotion of *B. amphitrite*. The barnacle cyprid, while exploring some surfaces, leaves behind 'footprints' of temporary adhesive. In the absence of AE, sugar-treated cyprids did not deposit footprints. Concurrently, the sugar-treated cyprids deposited footprints when exposed to multiwells coated with AE. A comparison of observations between 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. The third antennular segment with its attachment disc is the most obvious point of contact between the cyprid and the substratum during exploration. The detection of AE, even after blockage of polar groups of CTA on the third antennular segment, suggests availability of alternate sites for pheromone reception.

Keywords: *Balanus amphitrite*; Cyprid; Metamorphosis; Footprints; Sugars

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0022-0981/02/$ - see front matter
PII: S0022-0981(02)00414-8

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 *Balanus amphitrite* includes planktotrophic larval development consisting of six naupliar instars and a non-feeding cypris 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 (Clare et al., 1992; Crisp, 1990; Holm, 1990; Maki et al., 1994; Pechenik et al., 1993; Rittschof 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. While exploring some surfaces, cyprids leave behind 'footprints' of temporary adhesive (Walker and Yule, 1984), which are believed to be secreted by antennulary 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 three 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, we present the first study on the exploratory behavior and subsequent metamorphosis response of cyprids when subjected simultaneously to sugars and AE.

2. Materials and methods

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 weight of whole adults were crushed with a mortar and pestle using 100 ml of deionised water. The supernatant of the crushed mixture was decanted, centrifuged (12,000 × *g* for 5 min) and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged (12,000 × *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^{-1} of AE was used for all assays.

2.2. Rearing of B. amphitrite larvae

B. amphitrite nauplii were mass reared in 2-l glass beakers, using filtered seawater of 35‰ salinity, on a diet of *Chaetoceros calcitrans*, at a concentration of 2×10^5 cells ml^{-1}. 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.

2.3. 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 the 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 μm), UV-irradiated seawater.

2.4. Visualization of footprints

The sugar-treated as well as nontreated cyprids were siphoned out and introduced individually into six-well plates (Corning-430343) coated with 50 μg ml^{-1} AE and to non-coated wells, each containing 5 ml of millipore-filtered, autoclaved seawater at 35 ‰ salinity. 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 h at 20 °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).

2.5. Assay protocol for evaluation of metamorphosis

About 25–30 sugar-treated or nontreated 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 ‰ salinity (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 μg ml^{-1}. After 3 h, the multiwells were washed three times with autoclaved filtered seawater; after which the cyprids were introduced.

The assays were repeated 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 ± 1 °C on a 12 h light: 12 h dark cycle. They were monitored every 24 h for a period of 4 days and metamorphosed cyprids were counted at the end of each day. Fresh wells were used each time while repeating the assays.

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 nontreated cyprids in presence or absence of AE. A

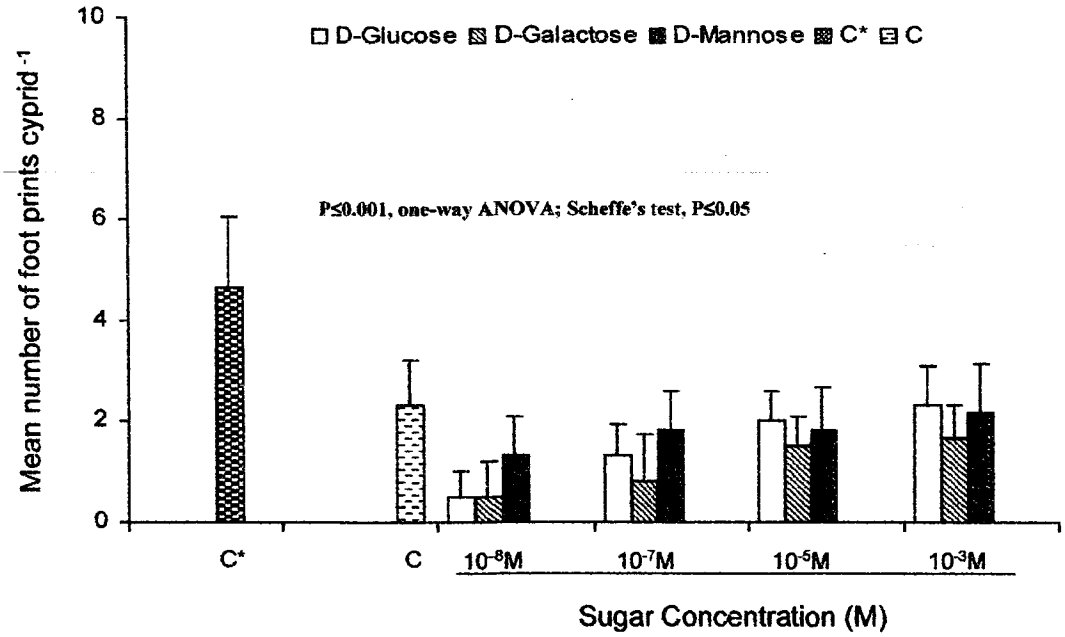

Fig. 1. Mean number of footprints deposited by sugar-treated cyprids exposed to AE-coated surface. (C*—nontreated cyprids exposed to AE-coated surface; C—nontreated cyprids exposed to non-coated surface). Please note: Footprints were not deposited by sugar-treated cyprids when exposed to non-coated surface.

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 (%) were arcsine transformed to ensure normality and homogeneity of variances before subjecting to statistical analysis, whereas the raw data on footprint deposition were square-root transformed. The

Table 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

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

df, degree of freedom; SS, sum of the squares; MS, mean of squares; Fs, Fischer constant.
ns, not significant.
***** $P \leq 0.001$.

influence of sugar-treated and nontreated 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).

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

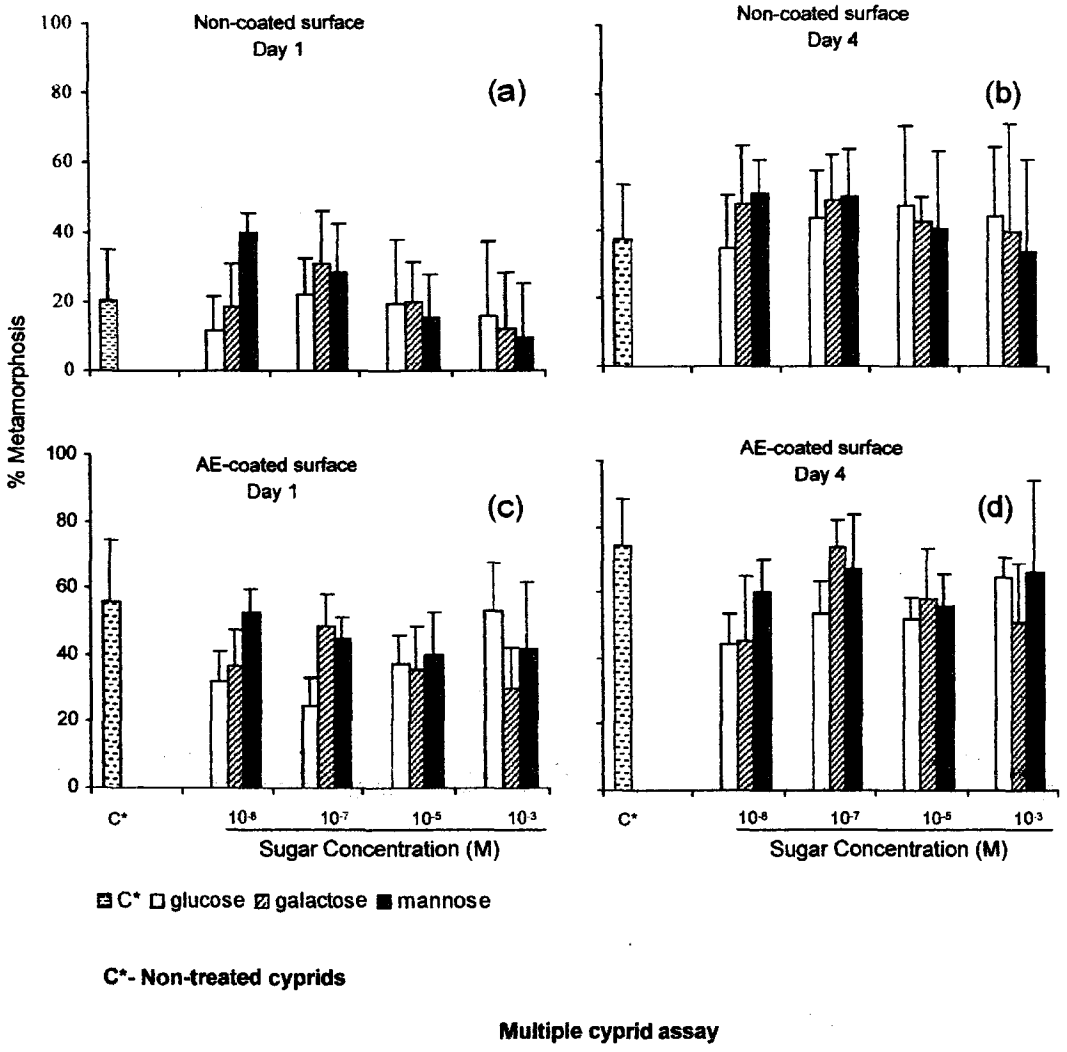

Fig. 2. Percentage metamorphosis of cyprids (multiple cyprid assay). (a and b) Sugar-treated and nontreated cyprids (control) exposed to non-coated surface. (c and d) Sugar-treated and nontreated cyprids exposed to AE-coated surface. Vertical lines indicate the standard deviation from mean.

about 30–37 μm across. The numbers of footprints deposited by sugar-treated cyprids in the presence of AE in the assay wells at different concentrations of D-glucose, D-galactose and D-mannose are shown in Fig. 1. 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 nontreated 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 1).

The metamorphosis response of the cyprids after 24 h is shown in Fig. 2a and 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 nontreated cyprids (Fig. 2a). Such a response was not given by cyprids treated with D-glucose or D-galactose. At 10^{-8} M, D-glucose showed an reduced effect. Two-way ANOVA also indicated significant differences in the metamorphosis rates at 10^{-8} M concentration with treatments (sugar-treated and nontreated) 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 nontreated cyprids (Fig. 2c). The results on day 4, which reflect the response of ageing of cyprids, showed a substantial increase in the metamorphosis rates (Fig. 2b and 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).

When all the treatments were assessed using single cyprids (Fig. 3), a trend similar to that observed with the assays employing multiple cyprids (Figs. 2 and 3) was

Table 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 (concentration)	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		

* $p \leq 0.1$, ** $p \leq 0.05$, ns = not significant.

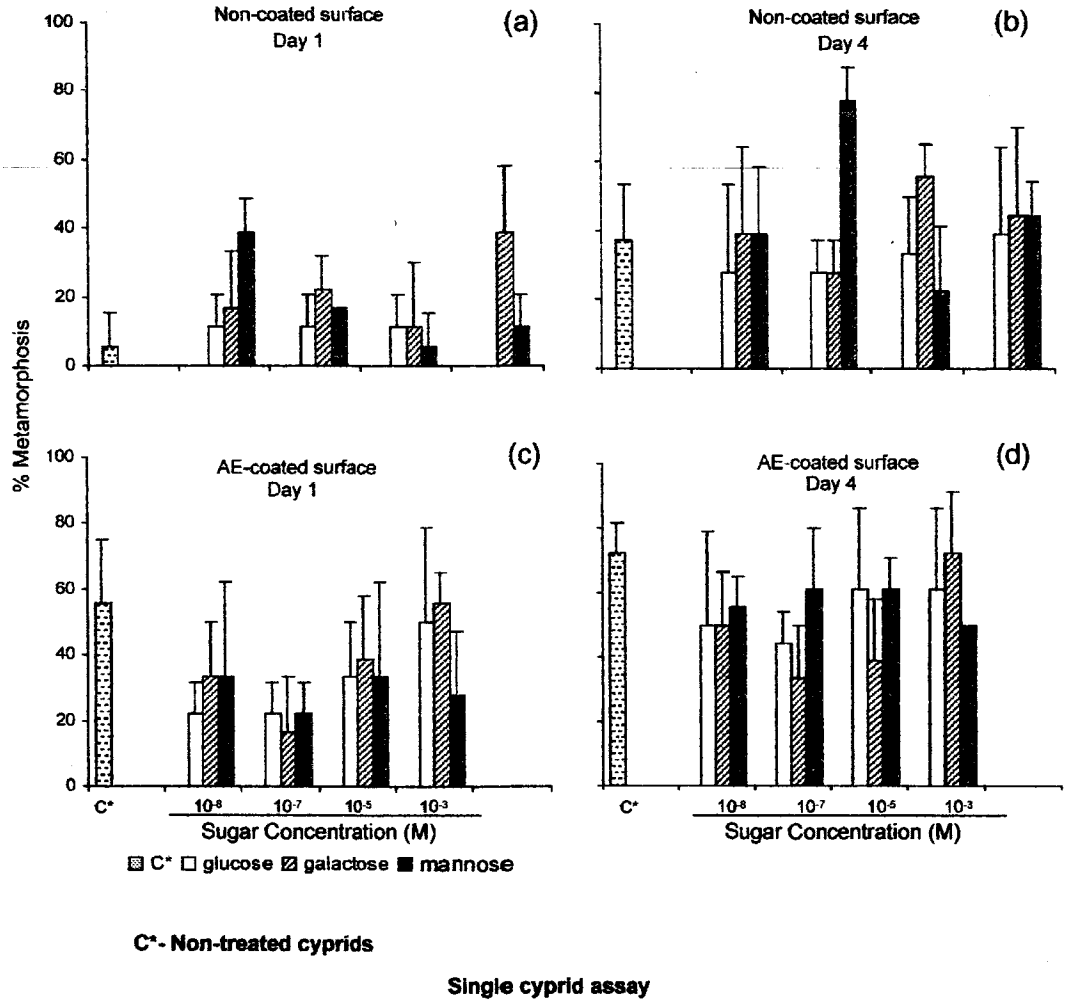

Fig. 3. Percentage metamorphosis of cyprids (single cyprid assay). (a and b) Sugar-treated and nontreated cyprids (control) exposed to non-coated surface. (c and d) Sugar-treated and nontreated cyprids exposed to AE-coated surface. Vertical lines indicate the standard deviation from mean. C*—nontreated cyprids.

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.

4. Discussion

It has been hypothesized that sugars in solution adsorb electrostatically through –OH groups to polar groups associated with the cypris temporary adhesive (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 to them with high affinity (affinity constant $K_a \geq 10^8$ 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 nontreated cyprids when assessed in the absence of AE. However, the other LCA-specific sugar (D-glucose) showed an 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 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 nonfunctional, 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.

Acknowledgements

We are grateful to Dr. E. Desa, Director National Institute of Oceanography, for his support and encouragement. We are thankful to Dr. N.B. Bhosle, Head MCMRD, for his advice during the course of this work. We thank Dr. S.S. Sawant and Mr. K. Venkat and other colleagues of MCMRD for their help. One of the authors (L. Khandeparker) acknowledges CSIR, India, for the award of a Senior Research Fellowship. This work was supported by ONR Grant no. N 0014-940423 and is an NIO contribution (No. 3789). [SS]

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Journal of Experimental Marine Biology and Ecology
263 (2001) 125–141

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

Larval development and metamorphosis in *Balanus amphitrite* Darwin (Cirripedia; Thoracica): significance of food concentration, temperature and nucleic acids

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Received 11 December 2000; received in revised form 21 March 2001; accepted 28 April 2001

Abstract

The influence of food concentration (*Chaetoceros calcitrans* at 1×10^5 and 2×10^5 cells ml^{-1}) and temperature (20°C and 30°C) on the nucleic acid content of the nauplii and the cyprids of *Balanus amphitrite*, an acorn barnacle, was evaluated. Food concentration and temperature jointly determined the naupliar instar duration. The total naupliar development period lasted 9–11 days at 20°C and was longer when compared to 5–6 days observed at 30°C. Increase in rearing temperature and food concentration positively influenced the size of the larvae. Food concentration influenced the DNA of III and VI naupliar instars and temperature influenced the DNA of IV and V instar nauplii. The RNA content of IV–VI naupliar instars was also influenced by the rearing temperature. Naupliar experience determined cyprid metamorphosis capability. The RNA content of larvae, that had longer total naupliar duration at 20°C, was considerably less than those raised at 30°C. This difference in RNA content was reflected in the capability of the cyprids to survive at 5°C. The resultant cyprids at 20°C could only successfully metamorphose over 2–4 days, whereas, those at 30°C could do so over 8–16 days. Such differences in the capability to metamorphose will be of critical importance to recruitment and early post-settlement mortality.

Keywords: Barnacle; *Balanus amphitrite*; Cirripedia; Invertebrate larva

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0022-0981/01/$ - see front matter
PII: S0022-0981(01)00280-5

1. Introduction

Barnacles have drawn the attention of many investigations in marine ecology owing to their easy accessibility on the rocky intertidal regions and also because some species are dominant in marine fouling (Barnes, 1957; Connell, 1961a, 1985; Strathmann et al., 1981; Sutherland, 1984, 1990; Crisp, 1988; Holm, 1990; Raimondi, 1990, 1991; Bertness et al., 1991). *Balanus amphitrite* (Darwin), an acorn barnacle, occurs in warm and temperate seas throughout the world (Karande, 1999). It can be easily maintained in the laboratory, and has a naupliar developmental period of about 5 days when raised under optimal rearing conditions. This species has been widely used in studies related to larval development, metamorphosis, influence of different chemical cues and antifouling assays (Rittschof et al., 1984; Maki et al., 1988, 1990; Clare et al., 1994; Anil et al., 1995; Anil and Khandeparker, 1998).

It has been suggested that larval experiences other than predation may have an impact on recruitment. Though metamorphosis was successful, competitive ability was impaired due to reduced growth rate (Jarrett and Pechenik, 1997). Pechenik et al. (1993) observed that to delay metamorphosis may be a double-edged sword for *B. amphitrite*, increasing an individual's chance of locating a site appropriate for metamorphosis, but simultaneously reducing the ability to compete for space during the first few weeks of juvenile life. The cypris larvae are non-feeding and have to depend upon the energy reserves incorporated during the earlier planktotrophic naupliar development.

Nucleic acids play a major role in growth and development. It has been shown that the RNA/DNA ratio is an indicator of nutritional condition (Clemmesen, 1996), and has been routinely used for estimating growth rates or nutritional condition of larval fish in the field. Only a few studies have applied this technique to crustaceans and their larvae (Dagg and Littlepage, 1972; Sulkin et al., 1975; Anger and Hirche, 1990; Wagner et al., 1998). In this study, we examine the influence of rearing temperature and food concentration on larval development, and ensuing changes in the content of RNA and DNA. We also examine the impact of these factors on the metamorphosis of the non-feeding cypris larvae.

2. Materials and methods

2.1. Rearing of *B. amphitrite* larvae

Larval development consists of six naupliar instars and a non-feeding cypris instar. The first nauplius stage is non-feeding and is of short duration. Nauplii obtained from *B. amphitrite* were mass reared in 2-l glass beakers (1 larva ml^{-1}) on a daily diet of *Chaetoceros calcitrans*, a unicellular diatom, at a concentration of 1×10^5 and 2×10^5 cells ml^{-1}. The cultures were maintained at 20°C and 30°C (± 1°C) in an incubator (12 h:12 h light/dark cycle). The larval density and food concentrations were maintained by monitoring mortality of the larvae every day. The rearing experiments for each of the conditions were carried out three times.

From each rearing experiment, larvae (~ 10) were sub-sampled every day for making observations on the different larval instars. The average instar number of this observation is presented as the instar index, and utilized in determining progression in larval development at a particular rearing condition. Simultaneously, measurement of size in terms of total length and total breadth of these larvae was carried out using a microscope equipped with an ocular micrometer.

2.2. Cyprid metamorphosis assay

The cyprids obtained were filtered through a tier of plankton netting (300, 230 and 160 μm). Nauplii were retained by the 300-μm screening. Cyprids trapped on the 230- and 160-μm screens were stored in a refrigerator at 5°C for ageing, and were used in the settlement assays as described by Rittschof et al. (1984). Ageing cyprids at 5°C was adopted as it has been shown that the major cyprid protein content remained constant at this temperature (Satuito et al., 1996). Cyprids (~ 10) were sampled for their length and breadth measurements as and when they appeared.

The cyprids obtained on day 5 at 30°C (2×10^5 cells ml^{-1}), day 6 at 30°C (1×10^5 cells ml^{-1}) and day 10 at 20°C (2×10^5 and 1×10^5 cells ml^{-1}) were used in attachment assays.

The adult extract (SF +), which is known to promote metamorphosis in *B. amphitrite* cyprids, was used as a positive control in the assay. The adult extract was prepared following the method described by Larman et al. (1982). In addition, cyprid metamorphosis was also monitored in the absence of the adult extract (control). The protein content of the extract was estimated using the method described by Lowry et al. (1951) using BSA (Bovine Serum Albumin) as the standard. A concentration of 50 μg ml^{-1} was used in the assays. The assays in each condition were run in triplicates. About 20–25 cyprids were introduced into polystyrene multiwells (Nunclon delta 1-52795) with 5 ml of filtered, autoclaved seawater (35‰ salinity). Assays were carried out at 25 ± 1°C.

2.3. Estimation of nucleic acids in B. amphitrite larvae

The technique described by Clemmesen (1988, 1993), which is based on fluorimetric determination of RNA and DNA content of an individual fish larva, was adopted for estimation of nucleic acids in the larvae of *B. amphitrite*. All the chemicals used were analytical grade. Ethidium bromide was obtained from BDH laboratories and Ribonuclease A was obtained from Merck. Yeast ribonucleic acid (RNA) and Calf thymus deoxyribonucleic acid (DNA) were procured from Hi-media. Sodium dodecyl sulphate was procured from Sigma (USA). Standard regression curves of DNA and RNA were plotted in order to calculate the final concentration in larvae. Larval homogenate (10 in number) was prepared, and then spiked with known amounts of standard DNA and RNA to evaluate the precision of the method, which was found to be ±5%.

2.4. Statistical analysis

Influence of rearing temperature and food concentration on the size (total length and total breadth) of nauplii and cyprids, DNA and RNA content and RNA/DNA ratio were

evaluated by linear regression analysis and Two-way ANOVA (Sokal and Rohlf, 1981). For the analysis of naupliar instars vs. DNA and RNA content, an average of the replicates ($n = 5$) from each of the three sets was used.

3. Results

Larval mortality was minimal at 30°C using both food concentration (5% and 10% at 2×10^5 and 1×10^5 cells ml^{-1}, respectively). Whereas, at 20°C the mortality differed between the two food concentrations. At 2×10^5 cells ml^{-1}, the mortality was 21% and

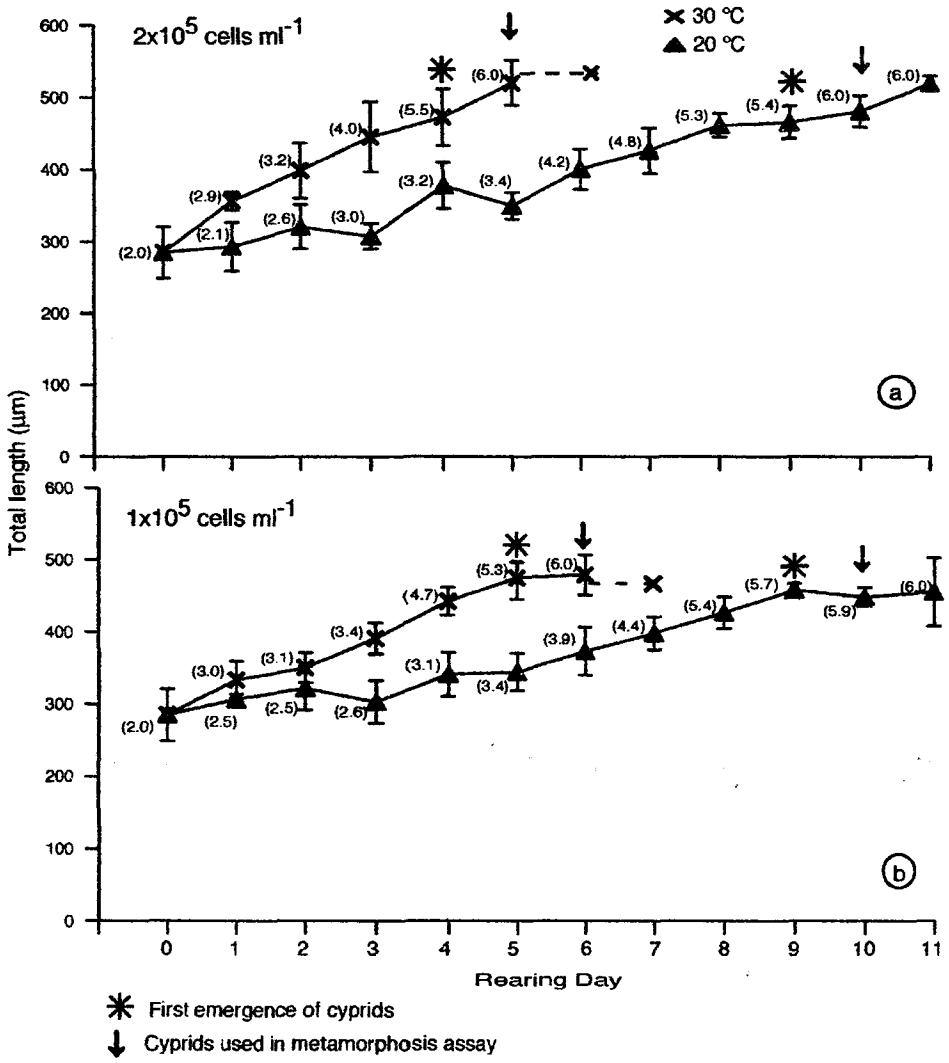

Fig 1. Variations in the length of the nauplii reared at different temperatures (30°C and 20°C) and food concentrations. (a) 2×10^5 cells ml^{-1}. (b) 1×10^5 cells ml^{-1}. Numbers in parentheses indicate the naupliar instar index and the bars represent the standard deviation.

at 1×10^5 cells ml^{-1} it was 46%. Naupliar development was completed in 6 (2×10^5 cells ml^{-1}) or 7 days (1×10^5 cells ml^{-1}) and cyprids were obtained as early as 4 days at the 30°C rearing temperature, whereas, at 20°C it was up to 12 days. The first cyprid at 20°C appeared on day 9. Average instar index on different rearing days is shown in Fig. 1a and b.

Temperature influenced the larval length of all the instars significantly, whereas, the food concentration influenced the larval length of instars V and VI (Table 1a–d). Variation in the breadth of the larvae was not significantly influenced by food concentration and temperature. Exceptions were the observed influence of food concentration on instar III and food concentration and temperature on the IV instar.

Variations in total length and breadth of the nauplii raised under different rearing conditions are shown in Fig. 2a and b. Larger larvae developed at 30°C, 2×10^5 cells ml^{-1} (larval length: $Y = 43.7x + 253.9$ μm, $r = 0.84$, $p \leq 0.001$; larval breadth: $Y = 36.07x + 179.3$ μm, $r = 0.82$, $p \leq 0.001$) and smallest ones at 20°C, 1×10^5 cells ml^{-1} (larval length: $Y = 39.8x + 222.5$ μm, $r = 0.86$, $p \leq 0.001$; larval breadth: $Y = 40.2x + 133.3$ μm, $r = 0.8$, $p \leq 0.001$). Larvae raised at 30°C, 1×10^5 cells ml^{-1} (larval length: $Y = 50.5x + 191.6$ μm, $r = 0.84$, $p \leq 0.001$; larval breadth: $Y = 36.5x + 154.6$ μm, $r = 0.81$, $p \leq 0.001$) and 20°C, 2×10^5 cells ml^{-1} were more similar in size (larval length: $Y = 41.8x + 226.2$ μm, $r = 0.82$, $p \leq 0.001$; larval breadth: $Y = 30.8x + 183.5$ μm, $r = 0.66$, $p \leq 0.001$).

3.1. Nucleic acid content

The newly hatched nauplii were either as the I or II instars. First instar duration is short and lasts for ~2 h. Estimation of nucleic acid was initially carried out with II instar larvae. The DNA and RNA content in newly hatched larvae (instar II) averaged 1.3 ± 0.3 and 1.9 ± 0.4 μg $larva^{-1}$, respectively, and the mean RNA/DNA ratio (II instar) was found to be 1.5 ± 0.6 (Fig. 3c and f).

The DNA and RNA content of the 5-day old larvae at 30°C (2×10^5 cells ml^{-1}) with an instar index VI averaged 2.5 ± 0.4 and 2.9 ± 0.3 μg $larva^{-1}$, respectively (Fig. 3a and b). The larvae reared at 30°C (1×10^5 cells ml^{-1}) reached instar index VI on day 6 and had an average DNA content of 2.1 ± 0.2 μg $larva^{-1}$ and RNA content of 2.8 ± 0.5 μg $larva^{-1}$ (Fig. 3d and e).

In the case of larvae reared at 20°C, instar index VI was observed on day 10 (2×10^5 cells ml^{-1}) and on day 11 at 1×10^5 cells ml^{-1} food concentration. The corresponding DNA and RNA contents varied from 2.2 ± 0.2, 2.4 ± 0.1 at 2×10^5 cells ml^{-1} (Fig. 3a and b) and 2.1 ± 0.3, 2.1 ± 0.3 μg $larva^{-1}$ at 1×10^5 cells ml^{-1}, respectively (Fig. 3d and e).

The regression of DNA and RNA content with reference to larval instars at different rearing conditions revealed that the slope is greater at 30°C, 2×10^5 cells ml^{-1} (DNA: $Y = 0.15x + 1.46$ μg, $r = 0.54$, $p \leq 0.02$; RNA: $Y = 0.27x + 1.11$ μg, $r = 0.81$, $p \leq 0.001$) when compared to those larvae reared at 30°C, 1×10^5 cells ml^{-1} (DNA: $Y = 0.08x + 1.58$ μg, $r = 0.69$, $p \leq 0.001$; RNA: $Y = 0.15x + 1.84$ μg, $r = 0.51$, $p \leq 0.02$). The case is similar when a comparison is made between the two food

Table 1
Two-way ANOVA of the influence of food concentration and temperature on total length, total breadth, DNA, RNA and RNA/DNA

Factor	*df*	Total length			Total breadth			DNA			RNA			RNA/DNA		
		SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
(a) III instar nauplii																
Food concentration	1	1178	1178	1.35 ns	7801	7801	6.1**	0.36	0.36	4.7*	0.02	0.02	0.055 ns	0.17	0.17	1.466 ns
Temperature	1	7163	7163	8.24***	1173	1173	0.9 ns	0.05	0.05	0.7 ns	0.423	0.42	1.15 ns	0.15	0.15	1.307 ns
Food concentration × temperature	1	14,603	14,603	16.8*****	81	81	0.06 ns	0.006	0.006	0.08 ns	0.003	0.003	0.008 ns	0.0005	0.0005	0.004 ns
Within sub. Gr.err.	36	31,276	868		46,019	1278		2.77	0.77		13.23	0.367		4.27	0.11	
Total	39	54,220			55,074			3.207			13.68			4.60		
(b) IV instar nauplii																
Food concentration	1	2924	2924	2.39 ns	7188	7188	4.9*	0.04	0.04	0.64 ns	0.39	0.39	2.59 ns	0.04	0.04	0.33 ns
Temperature	1	5731	5731	4.7***	7188	7188	4.9*	0.51	0.51	7.07***	0.56	0.56	3.72*	0.25	0.25	1.97 ns
Food concentration × temperature	1	3756	3755	3.0 ns	28	28	0.01 ns	0.08	0.08	1.14 ns	0.07	0.07	0.48 ns	0.47	0.47	3.73***
Within sub. Gr.err.	36	43,874	1218		51,796	1438		2.61	0.07		5.44	0.15		4.62	0.12	
Total	39	56,285			66,200			3.26			6.47			5.39		

(c) V instar nauplii																
Food concentration	1	6875	6875	8.75***	1433	1432	1.64 ns	0.11	0.11	1.60 ns	0.04	0.04	0.08 ns	0.02	0.02	0.38 ns
Temperature	1	15,024	15,024	19.14*****	549	549	0.63 ns	0.94	0.94	12.81*****	5.73	5.73	11.14****	1.24	1.24	17.38*****
Food concentration × temperature	1	−9.31× 10E−10	−9.31× 10E−10	−1.19× 10E−12	393	393	0.45 ns	0.50	0.50	6.90**	1.12	1.12	2.19 ns	0.003	0.003	0.05 ns
Within sub. Gr.err.	36	28,253			31,281	869		2.65	0.07		18.53	0.51		2.57	0.07	
Total	39	50,251			33,656			4.22			25.44			3.85		
(d) VI instar nauplii																
Food concentration	1	10,712	10,712	12.06****	637	637	0.65 ns	0.63	0.63	10.73****	0.25	0.25	1.72 ns	0.005	0.005	0.10 ns
Temperature	1	27,825	27,825	31.33*****	208	208	0.21 ns	0.08	0.08	1.53 ns	2.46	2.46	16.41*****	1.04	1.04	18.87*****
Food concentration × temperature	1	140	140	0.158 ns	117	117	0.12 ns	0.03	0.03	0.58 ns	0.19	0.19	1.31 ns	0.12	0.12	2.29 ns
Within sub. Gr.err.	36	31,963	888		34,855	968		2.11	0.05		5.39	0.14		1.99	0.05	
Total	39	70,640			35,817			2.86			8.31			3.17		

ns — Not significant; *df*—degree of freedom; SS—sum of the squares; MS—mean of squares; Fs—Fischer constant.
* $p \leq 0.05$, ** $p \leq 0.025$, *** $p \leq 0.01$, **** $p \leq 0.005$, ***** $p \leq 0.001$.

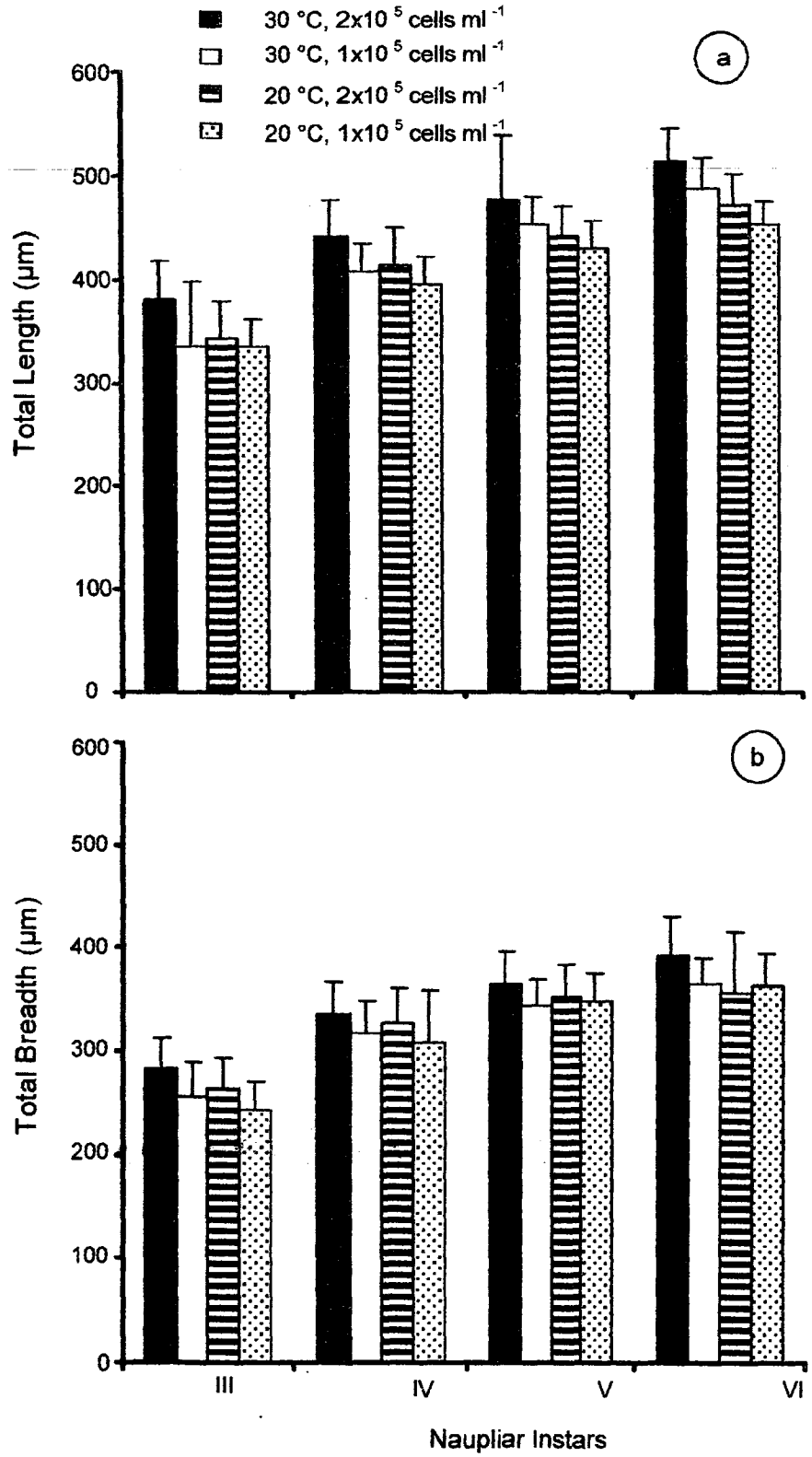

Fig 2. Variations in the size of the naupliar instars reared under different food and temperature conditions. (a) Total length. (b) Total breadth.

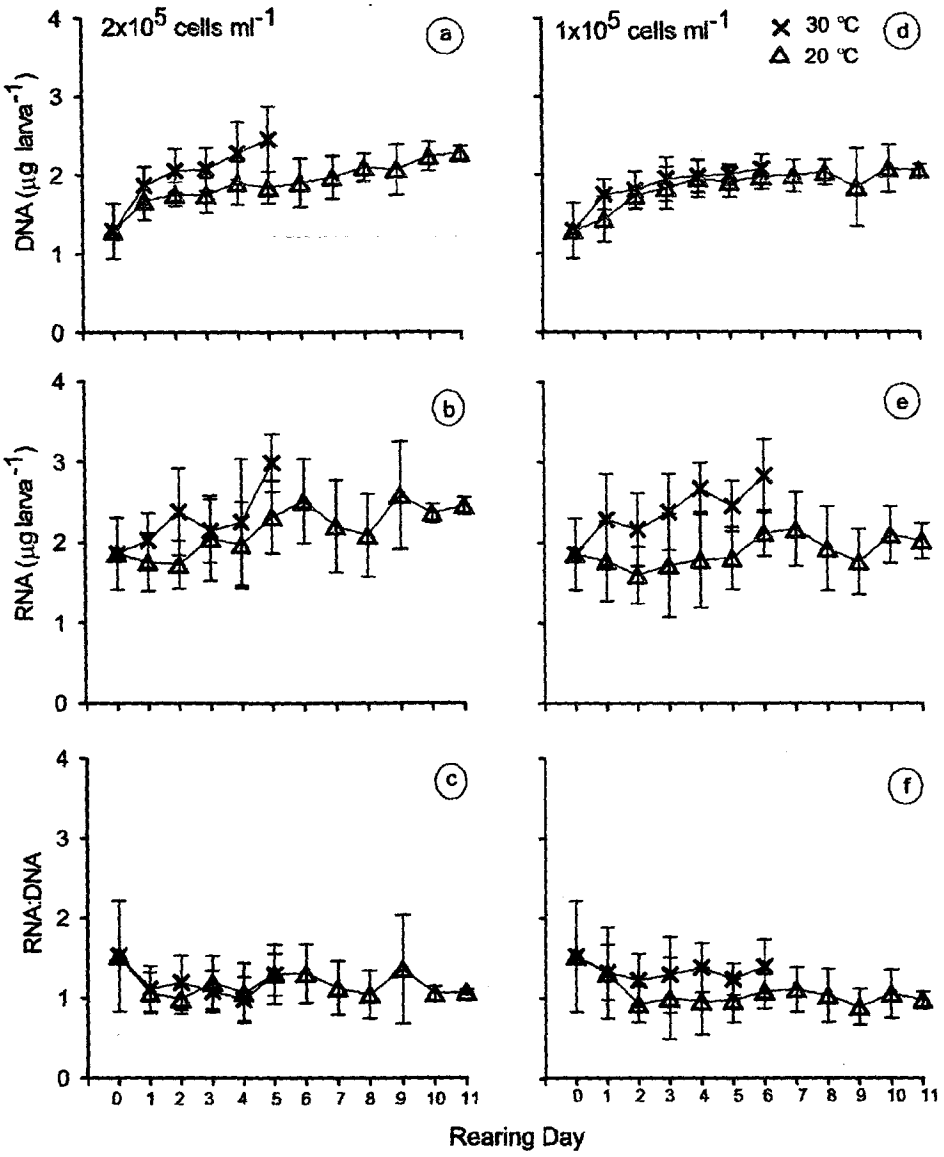

Fig 3. Variations in the DNA, RNA content and RNA/DNA ratio with reference to rearing days at 30°C and 20°C temperatures. (a), (b) and (c) refer to 2×10^5 cells ml^{-1} and (d), (e) and (f) refer to 1×10^5 cells ml^{-1} food concentration. Bars represent standard deviation.

concentrations at 20°C (at 2×10^5 cells ml^{-1} DNA: $Y=0.13x+1.34$ µg, $r=0.79$, $p\leq0.001$; RNA: $Y=0.18x+1.44$ µg, $r=0.59$, $p\leq0.001$; and at 1×10^5 cells ml^{-1} DNA: $Y=0.05x+1.76$ µg, $r=0.57$, $p\leq0.01$; RNA: $Y=0.02x+1.85$ µg, $r=0.07$, not significant).

A two-way ANOVA indicated food concentration influenced the DNA content of III and VI instar significantly, and the variable temperature influenced the DNA content of IV and V instars. The RNA content of IV–VI instars was significantly influenced by variable temperature, whereas, food concentration influence was not significant for any of the instars (Table 1a–d).

Table 2
Two-way ANOVA of the influence of food concentration and temperature on cyprid length, cyprid breadth, DNA, RNA, RNA/DNA of cyprids

Factor	*df*	Cyprid length			Cyprid breadth			DNA			RNA			RNA/DNA		
		SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs	SS	MS	Fs
Food concentration	1	5198	5198.4	26.5 *****	1172	1172	5.19 *	1, 0.03	0.03	0.3 ns	0.79	0.79	3.09 ns	0.01	0.01	0.07 ns
Temperature	1	15,920	15,920.1	81.3 *****	2030	2030	9.0 ****	1 0.34	0.34	3.05 ns	0.96	0.96	3.78 ns	0.14	0.14	0.75 ns
Food concentration × temperature	1	636	636.8	3.25 ns	159	159	0.70 ns	1 0.007	0.007	0.06 ns	2.17	2.17	8.5 ***	0.36	0.36	1.99 ns
Within sub. Gr.err.	36	7043	195.6		8122	225		28 3.16	0.11		7.17	0.25		5.19	0.18	
Total	39	28,797			11,483			31 3.55			11.12			5.71		

ns — Not significant; *df*—degree of freedom; S—sum of the squares; MS—mean of squares; Fs—Fischer constant.
* $p \leq 0.05$, *** $p \leq 0.01$, **** $p \leq 0.005$, ***** $p \leq 0.001$.

4. Cyprids

The cyprids formed at 30°C had total length ranging from 451 to 480 μm and total breadth from 218 to 227 μm and were larger than those formed at 20°C, which had total

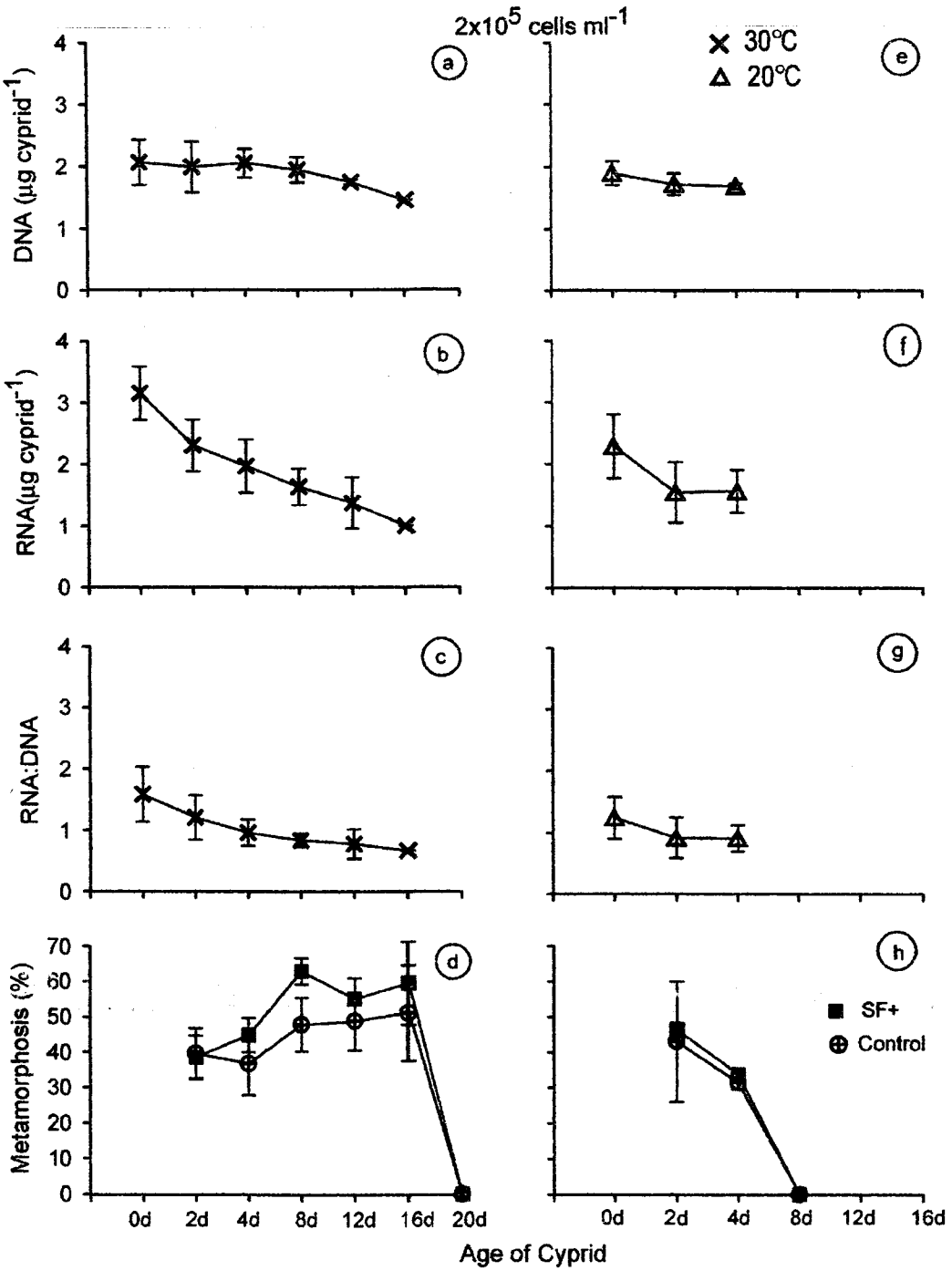

Fig 4. Influence of ageing of cyprids at 5°C on DNA, RNA and RNA/DNA ratio and percentage metamorphosis. (a), (b), (c) and (d) represent larvae raised at 30°C and (e), (f), (g) and (h) represent larvae raised at 20°C at 2×10^5 cells ml^{-1}.

length ranging from 421 to 448 μm and total breadth from 211 to 230 μm. A regression analysis of the day of cyprid emergence in each of the rearing conditions did not influence cypris size at both the temperatures. Two-way ANOVA showed that the food concentration and temperature influenced cypris length and breadth significantly. The food concentration and temperature interaction showed an insignificant effect indicating the influence to be equal at both the temperature and food concentration (Table 2).

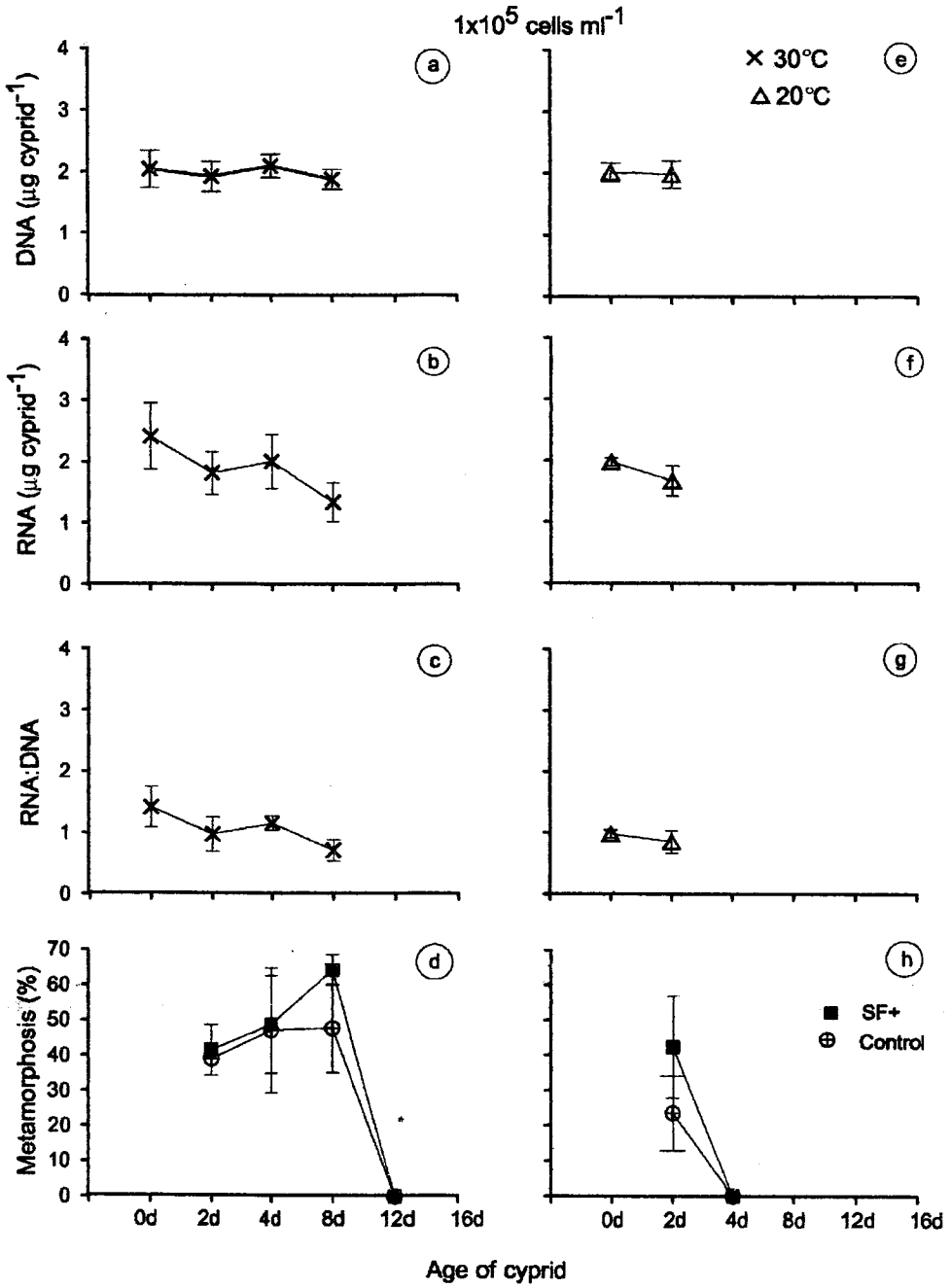

Fig 5. Influence of ageing of cyprids at 5°C on DNA, RNA and RNA/DNA ratio and percentage metamorphosis. (a), (b), (c) and (d) represent larvae raised at 30°C and (e), (f), (g) and (h) represent larvae raised at 20°C at 1×10^5 cells ml^{-1}.

The average DNA content of the cyprids from the different rearing conditions (Figs. 4a,e and 5a,e; 0 day values) averaged 1.9–2.1 μg larva^{-1}. The RNA content averaged 3.2 μg larva^{-1} (2×10^5 cells ml^{-1}) and 2.4 μg larva^{-1} (1×10^5 cells ml^{-1}) at 30°C (Figs. 4b and 5b), whereas, at 20°C the average RNA content was 2.3 μg larva^{-1} at 2×10^5 cells ml^{-1} and 1.7 μg larva^{-1} at 1×10^5 cells ml^{-1} (Figs. 4f and 5f).

The cyprids on ageing at 5°C showed that RNA (Figs. 4b,f and 5b,f) and the RNA/DNA ratio progressively decreased (Figs. 4c,g and 5c,g). The cyprid attachment assay (Figs. 4d and 5d) results, evaluating the influence of ageing showed that the larvae reared at 30°C, 2×10^5 and 1×10^5 cells ml^{-1}, were viable for 16 and 8 days, respectively. The metamorphosis rate peaked with 8–16 day aged cyprids and was positively influenced by SF + when compared to the control (Figs. 4d and 5d).

The cyprids raised at 20°C could only metamorphose successfully for 4 days (2×10^5 cells ml^{-1}) and 2 days (1×10^5 cells ml^{-1}). The metamorphosis rate decreased with ageing and was similar in both SF + and control conditions (Figs. 4h and 5h).

5. Discussion

Spawning activity in some marine invertebrates coincides with phytoplankton blooms (Barnes and Barnes, 1958; Starr et al., 1990). This coupling is explained as one of the survival measures offered to the planktotrophic offspring. Barnes and Barnes (1958), while examining the recruitment of *B.* (= *Semibalanus*) *balanoides* over a period of 10 years in the Firth of Clyde, attributed recruitment failure to abnormal increase in larval predation, unusual physical parameters, presence of deleterious chemicals as well as defective sampling. Olson and Olson (1989), however, felt that recruitment largely depended on the abundance of larval food. Cyprids like other invertebrate larvae derive their energy from stored lipids. The extent of this energy reserve in cyprids depends on the condition under which the nauplii grow (Lucas et al., 1979). In an earlier effort to evaluate the effect of food concentration, temperature and salinity on the larval development of *B. amphitrite,* it was observed that food availability and temperature jointly influence the energy allocation (Anil and Kurian, 1996). The results from this investigation also reflected these observations. Naupliar development duration was prolonged at 20°C (9–11 days) when compared to that at 30°C (5–6 days). West and Costlow (1987) observed that the effect of food concentration on naupliar size was slight, whereas, the size of the cyprid varied directly with food concentration. In this investigation, it was observed that temperature influenced the larval length of all the instars, whereas, food concentration influenced only the more advanced instars (V and VI). In the case of cyprids, both food concentration and temperature influenced length and breadth. This fact coupled with the information that larvae take longer duration to complete development at the lower temperature indicate that assimilation of energy for growth is significantly influenced by these two variables.

Yule (1984) evaluating the effect of temperature on the swimming activity of barnacle nauplii observed an increase in stroke rate with increase in temperature from 20°C to 30°C in *B. amphitrite* larvae. Temperature, through its effect on physiological processes, also influences motion, because water viscosity is physically coupled to

temperature. Thus, changes in temperature can influence the activity of microscopic organisms through both physiological and physical means (Podolsky and Emlet, 1993). Change in viscosity, therefore, impacts suspension feeding independently of physiology and has implications for many small scale biological processes (Podolsky, 1994). Rearing of *B. eburneus* nauplii at different temperatures and food concentrations showed that increased algal cell concentration at lower temperature compensated lower feeding efficiency (Scheltema and Williams, 1982). The larvae raised at 30°C, 1×10^5 cells ml^{-1} and 20°C, 2×10^5 cells ml^{-1} in the present experiments were similar in size and nucleic acid content. Using the regression constants from the equations, it is evident that VI instar larvae raised at 30°C, 1×10^5 cells ml^{-1} have an RNA content of 2.7 μg $larva^{-1}$, whereas, those raised at 20°C, 2×10^5 cells ml^{-1} have an RNA content of 2.5 μg $larva^{-1}$.

Jian-Wen and Pei-Yaun (1997) found that the larvae of *B. amphitrite* from a single brood responded in the same way as those from multiple broods. They also observed that food concentration had a clear impact on larval survival, development time and growth, whether they were obtained from single or multiple broods. The larvae used in the present experiments were collected from multiple broods. The DNA and RNA content in the newly hatched nauplii showed considerable variation (Fig. 3; 0 day). However, with development the variations in RNA/DNA ratio narrowed down in each of the rearing conditions (Fig. 3). This further indicates that even though initially different, under a given rearing condition, this variability will have insignificant impact on the quality of cyprids.

Cypris, the terminal larval instar, prolong their larval duration until a conducive substratum is available for settlement and subsequent metamorphosis. Freeman and Costlow (1983) showed that moulting is distinctly biphasic in cyprids of *B. amphitrite*. In free-swimming cyprids moulting is arrested at an early stage and is only resumed after settlement. Pechenik et al. (1998) state that growth rate of newly-metamorphosed barnacles slows down significantly if *B. amphitrite* cyprids were prevented from metamorphosing for as few as 3 days. It was observed by Connell (1961b) that reduced growth rates would compromise the ability of juvenile barnacles to compete for space and seriously reduce their likelihood of successfully recruiting to the adult population. In the light of these observations, Jarrett and Pechenik (1997) and Pechenik et al. (1998) opined that larval experience influences juvenile performance. The evidence gathered from the present experiments indicate that the naupliar experience clearly determines the capability of the cyprids to metamorphose as well. The RNA content of larvae raised at 20°C, which had longer total naupliar duration, was considerably less compared to those raised at 30°C. This difference in RNA content was reflected in the capability of the cyprids to survive ageing at 5°C. The larvae raised at 20°C could only, successfully, metamorphose until 2–4 days, whereas, those raised at 30°C could do so for 8–16 days. Clare et al. (1994) observed that the cyprid settlement of *B. amphitrite* is uncharacteristically low when reared with *D. tertiolecta* and that, although the cyprids look normal, they may be deficient in some respect. It was also observed earlier that, by day 3, the cyprids lose their power to discriminate the substrata and are less useful for assaying metamorphosing inducers (Rittschof et al., 1984; Crisp, 1988; Clare et al., 1994). Maki et al. (1988) showed from their experimental results on inhibition of attachment of *B.*

amphitrite to bacterial films, 4-day old cyprids showed an increase in larval attachment as compared to 2-day old cyprids. It was observed with cyprids of *B.* (= *Semibalanus) balanoides* after 4–5 weeks at 10°C a final threshold level was reached wherein they possessed insufficient energy to accomplish metamorphosis into a feeding juvenile barnacle (Lucas et al.,1979). Høeg and Ritchie (1987) observed with *Lernaeodiscus porcellanae* (Cirripedia: Rhizocephala) larvae, which have lecithotrophic nauplii and small sized cyprids, the energy threshold, where metamorphosis was no longer possible, was seemingly reached in less than 15 days and attributed this to the rearing temperature. The results of the present investigation show cypris metamorphosis rate and the influence of ageing are governed by naupliar rearing conditions and will be of critical importance to recruitment and early post-settlement mortality.

Acknowledgements

We are grateful to Dr. E. Desa, Director, National Institute of Oceanography, for his support and encouragement. We are thankful to Dr. N.B. Bhosle, Head, MCMRD, for his advice during the course of this work. We thank Dr. S.S. Sawant and Mr. K. Venkat and other colleagues of MCMRD for their help. The authors (D.D and L.K) greatly acknowledge CSIR, India for the award of Senior Research Fellowships. This work was supported by ONR Grant no.: N 0014-940423 and is NIO contribution (#3680). *[SS]*

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Marine Biology (2000) 136: 603-609

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Thraustochytrid protists as a component of marine microbial films

Received: 26 July 1999 / Accepted: 21 January 2000

Abstract Although ubiquitous in the marine environment, the presence and importance of thraustochytrid fungoid protists in primary film formation of freshly immersed surfaces in the sea have not been investigated. We isolated thraustochytrids from surfaces of glass, aluminium, mild steel and fibre glass panels immersed in sea water for 1 to 4 d, but not from those of copper and cupro-nickel. Microscopic examination of the primary film on glass surfaces revealed the presence of thraustochytrids. Thraustochytrids grew to varying population densities on surfaces of glass, aluminium and fibre glass in the laboratory. Scanning electron microscopy of the growth of *Ulkenia profunda* Gaertner, isolated from the primary film, on surfaces of glass and aluminium suggested that cells of the thraustochytrid attached directly to the surfaces, without producing copious extracellular polysaccharides. The presence of ectoplasmic net elements was not a prerequisite for attachment of cells to surfaces. Cell surface hydrophobicity of the thraustochytrid, as estimated by the MATH assay, might play an important role in adhesion. The presence of thraustochytrid cells on a polystyrene surface markedly induced settlement of barnacle larvae (*Balanus amphitrite*), as compared to barnacle extract and a control.

Introduction

Recent years have seen several publications on the prevalence in the sea of a group of osmohetrotrophic, fungoid protists, the thraustochytrids. The presence, and often dense populations, of these single-celled microorganisms has 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; Raghukumar et al. 1995; Naganuma et al. 1998). The mode of reproduction in these protists is by means of motile zoospores, which settle on suitable substrata that offer organic nutrients. Vegetative cells developing from encysted zoospores derive their nutrition by producing extracellular hydrolytic enzymes (Bahnweg 1970a, b; Coleman and Vestal 1987). We surmised that thraustochytrid zoospores would settle rapidly on freshly submerged substrata in the sea. Such materials have received a great deal of attention focused on the primary film that forms on them (Characklis and Eschers 1988; Bhosle et al. 1994). Observations include the development of an organic layer within a few hours and settlement of bacteria in less than a day and their further growth (Sonak and Bhosle 1995). This microbial fouling encourages macrofouling that leads to economic problems in shipping, submarine pipelines and marine constructions (Bhosle 1993). Marine invertebrate larvae are presented with a wide range of physical or biologically derived chemical cues as they approach a substratum. Biological cues are generally associated with bacteria, microflora and microfauna. In the case of barnacles, "settlement factor proteins" secreted by adults also induce settlement (Anil and Khandeparker 1998). Bacteria have been studied in great detail with relation to the process of microbial film formation and are known to play an important role in this process. In this paper, we present for the first time information on the rapid settlement of thraustochytrids on marine surfaces and their presence in a few substrata immersed in the sea.

Communicated by O. Kinne, Oldendorf/Luhe

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Materials and methods

Isolation of thraustochytrids

Fibre glass, aluminium, mild steel, copper and cupro-nickel, in the form of panels of 15 × 10 cm, and glass, as microscope slides,

were immersed in sea water at 3 m depth at Dona Paula Bay, Goa, on the west coast of India (15°25'N; 73°95'E). Two sets of the panels and the glass slides were removed at 24 h intervals over a period of 4 d. The materials were placed in an ice bucket filled with sterile sea water and immediately returned to the laboratory. Thraustochytrids were isolated essentially according to the method described by Gaertner (1968) and Sathe-Pathak et al. (1993). After draining the excess water from the surfaces of the panels and the slides, they were scraped using a porcelain scraper, and the scraped material placed in 5 ml of sterile sea water in sterile 5 cm Petri dishes. Pine pollen, sterilised at 90 to 100 °C for 24 to 48 h was added to this. After 2 to 5 d, the pine pollen was microscopically examined for growth of thraustochytrids on the particles. Some of the glass slides were stained overnight with the standard mycological stain lactophenol-cotton blue (Dring 1971) and examined under a microscope to detect growth of thraustochytrids.

Some of the thraustochytrids growing in these initial isolations were purified in axenic cultures by subculturing them in sea water and pine pollen, containing 1 mg streptomycin and 40 units penicillin ml^{-1}. Axenic cultures thus obtained were verified for absence of bacteria by streaking the culture on a nutrient agar medium, where colonies of thraustochytrids were produced. Such colonies were subsequently maintained in sea water/pine pollen. An isolate from mild steel panels, MS2D, was identified by examining various stages of its development under a microscope and used for scanning electron microscope studies, as outlined below.

Growth of thraustochytrids on various materials

Growth of two isolates of thraustochytrids, ML13 and COR1, on various materials was studied in the laboratory. Small squares of approximately 1 cm^2 of glass, fibre glass and mild steel were cut, sterilised by autoclaving and placed in 5 ml of sterile sea water in 5 cm Petri dishes. These were inoculated with a loopful of the cultures of thraustochytrids growing in sea water/pine pollen, and a small amount of sterile pine pollen was added. After 3 to 5 d growth, the materials were fixed in 3% formalin till further observations. Growth on triplicates of each material was examined by staining with 0.01% aqueous solution of the optical brightener Calcofluor (Sigma) and examining under an Olympus BX2 epifluorescence microscope, with a blue-violet excitation filter, using a 20x objective. Twenty microscope fields per sample were examined, and the number of cells per microscope field area was counted to give the density of growth.

Scanning electron microscopy

Small squares of glass and aluminium were inoculated with an isolate of the thraustochytrid (MS2D), as above. The culture used for this purpose was not axenic and still contained bacteria from the initial isolation. After growth for 3 d, the pieces were fixed in 3% formalin, sequentially dehydrated in 30, 50, 70, 95 and 100% acetone, critical-point dried, coated with gold-palladium in a sputter-coater and examined under a JEOL JSM-5800 LV scanning electron microscope.

Microbial adhesion to hydrocarbons (MATH) assay

The MATH assay was used to measure the hydrophobicity of the cells of the thraustochytrid isolate MS2D, isolated from mild steel panels. The assay is based on the fact that when hexadecane is vigorously mixed with cultures and the phases allowed to separate, cells with hydrophobic cell walls will separate into the organic phase (Rosenberg et al. 1980). The assay was carried out by adding 5 ml of seawater containing cells of the thraustochytrid, 0.5 ml of hexadecane to glass tubes and vortexing the tubes. The initial turbidities were immediately measured at 550 nm using a spectrophotometer. The tubes were then left for various time intervals, up to 75 min, for separation of the hexadecane phase. The turbiditiy of the aqueous phase was once again measured spectrophotometrically. Fraction of adhesion is expressed as:

(Initial turbidity – Final turbidity)/Initial turbidity.

Barnacle cyprids

The larval phase of *Balanus amphitrite*, one of the dominant macrofouling organisms, consists of six naupliar stages, followed by a final, non-feeding cyprid stage which explores for a suitable surface for settlement and subsequent metamorphosis. The larvae were reared in the laboratory through their naupliar instars. The day of metamorphosis from the sixth stage nauplius to a cyprid was considered Day 0. Cyprids were aged by storing them in the dark at 5 °C. Two-day-old cyprids were used in the assay, following a well-established method (Rittschof et al. 1984, 1992).

Assay protocol

The settlement assay was carried out using polystyrene multiwells (Nunclon R. Delta 1-52795). Five millilitres of a broth culture of the thraustochytrid MS2D were placed in the multiwells, and the cells were allowed to grow on the surfaces for 24 h. Another set of multiwells was inoculated with the settlement factor "arthropodin", a heat-stable preparation of glycoproteins extracted from *Balanus amphitrite*. Arthropodin was obtained by crushing whole adult barnacles in distilled water, removing solids by centrifugation and boiling the resulting supernatant. The extract, termed SF+ (settlement factor +), was applied to the multiwells at a concentration of 50 µg protein ml^{-1} (Rittschof et al. 1984; Dineen and Hines 1992).

The treated multiwells were washed off by repeated rinsing with autoclaved, filtered sea water. Subsequently, about 50 cyprids were introduced in six replicates along with 5 ml of autoclaved, filtered sea water of 35 ppt salinity, to each well. This method is similar to that used by Maki et al. (1988) for evaluating bacterial films. The control consisted of filtered sea water with no additions. The settlement assay was monitored for a period of 4 d at an interval of 24 h. The metamorphosed cyprids were counted at each of the observations.

Results

Thraustochytrids were isolated just after 24 h immersion of aluminium, glass and mild steel panels and continued to be isolated for 4 d (Table 1). In the case of aluminium panels, both replicates yielded thraustochytrids after 1 d immersion. Thraustochytrids were found on fibre glass panels after only 2 d of immersion. None were found on copper and cupro-nickel panels. Under laboratory conditions, glass, aluminium and fibre glass panels supported settlement and growth of the thraustochytrid isolate ML13. Fibre glass supported the least growth (Fig. 1). The isolate COR1 grew less densely on glass and aluminium panels and completely failed to grow on fibre glass.

Globose cells of 3 to 8 µm, resembling thraustochytrid cells, were detected when glass slides immersed for 48 h were stained with lactophenol-cotton blue and examined microscopically. In one instance, two elliptical

Table 1 Isolation of thraustochytrids from different substrata immersed at Dona Paula Bay, Goa, India (*A*, *B* are replicates; +, thraustochytrid isolated; –, thraustochytrid not isolated)

Substrate	Days			
	1	2	3	4
Aluminium				
A	+	+	+	+
B	+	+	+	+
Copper				
A	–	–	–	–
B	–	–	–	–
Cupric nickel				
A	–	–	–	–
B	–	–	–	–
Glass				
A	–	+	+	+
B	+	+	+	+
Fibre glass				
A	–	+	+	+
B	–	+	+	+
Mild steel				
A	–	+: *Ulkenia profunda*	+	+
B	+	+	+: *Labyrinthuloides minuta*	+

cells, 2.7 to 3.0 µm, connected by an "ectoplasmic net element"-like structure, resembling *Labyrinthuloides minuta* (Watson and Raper) Perkins, were observed. This species was also isolated in culture from mild steel panels immersed for 1 d.

The thraustochytrid MS2D was isolated from mild steel panels in axenic cultures and was identified as *Ulkenia profunda* Gaertner (Gaertner 1977), based on its developmental stages (Fig. 2a to e). The protist developed vegetative cells which were 4.0 to 7.0 µm and produced ectoplasmic net elements in sea water/pine pollen cultures (Fig. 2a to d). The contents of the cell escaped from the cell wall as an amoeboid mass, with the ectoplasmic net element still attached to it (Fig. 2c). This cytoplasmic mass divided by cleaving into several small cells, each of which escaped as a biflagellate zoospore (Fig. 2d). One to two cells, attached to the ectoplasmic net element, remained behind as proliferation bodies (Fig. 2e).

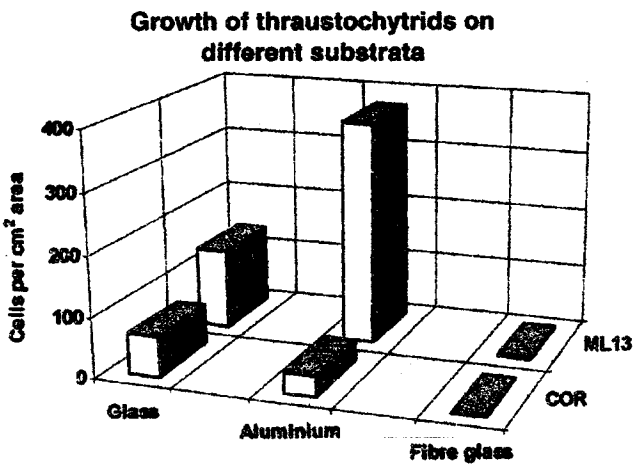

Fig. 1 Growth of two isolates (ML13, COR1) of thraustochytrids on three different substrata, laboratory experiments

The isolate MS2D was grown on aluminium and glass panels and examined under a scanning electron microscope. This experiment was carried out prior to the isolation of the thraustochytrid in axenic cultures, and, therefore, in the presence of bacteria. Growth and behaviour of the thraustochytrid were similar on both surfaces. Biflagellate zoospores attached to the surface (Fig. 3a). No signs of an adhesive material were observed in this attachment process. Mature vegetative cells often lacked ectoplasmic net elements and seemed to attach directly to the surface. Cells were much smaller (2.0 to 2.5 µm) on these surfaces, than those attached to pine pollen. Adjacent cells were frequently cemented to each other (Fig. 3b). Ectoplasmic net elements, in the form of branched filaments from the cells, were observed in some (Fig. 3c, d), but not all cells. A structure suggesting an adhesion cap was observed in one instance (Fig. 3d).

The MATH assay revealed that starting from the beginning of the observations at 15 min till the end at 75 min, > 50% of the cells of the thraustochytrid MS2D adhered to the hexadecane phase. The fraction of adherence ranged from 0.49 to 0.53.

The presence of the thraustochytrid MS2D substantially increased the percent metamorphosis of *Balanus amphitrite* cyprids to 61.67% (±8.55), as compared to the settlement factor arthropodin (SF+) and the control (Fig. 4). Metamorphosis in the latter two treatments was 47.27% (±6.94) and 29.73% (±7.51), respectively, at the end of Day 4. In general, percentage metamorphosis increased from the first to the fourth day. The trend obtained was the same for all 4 d, whereby the thrau-

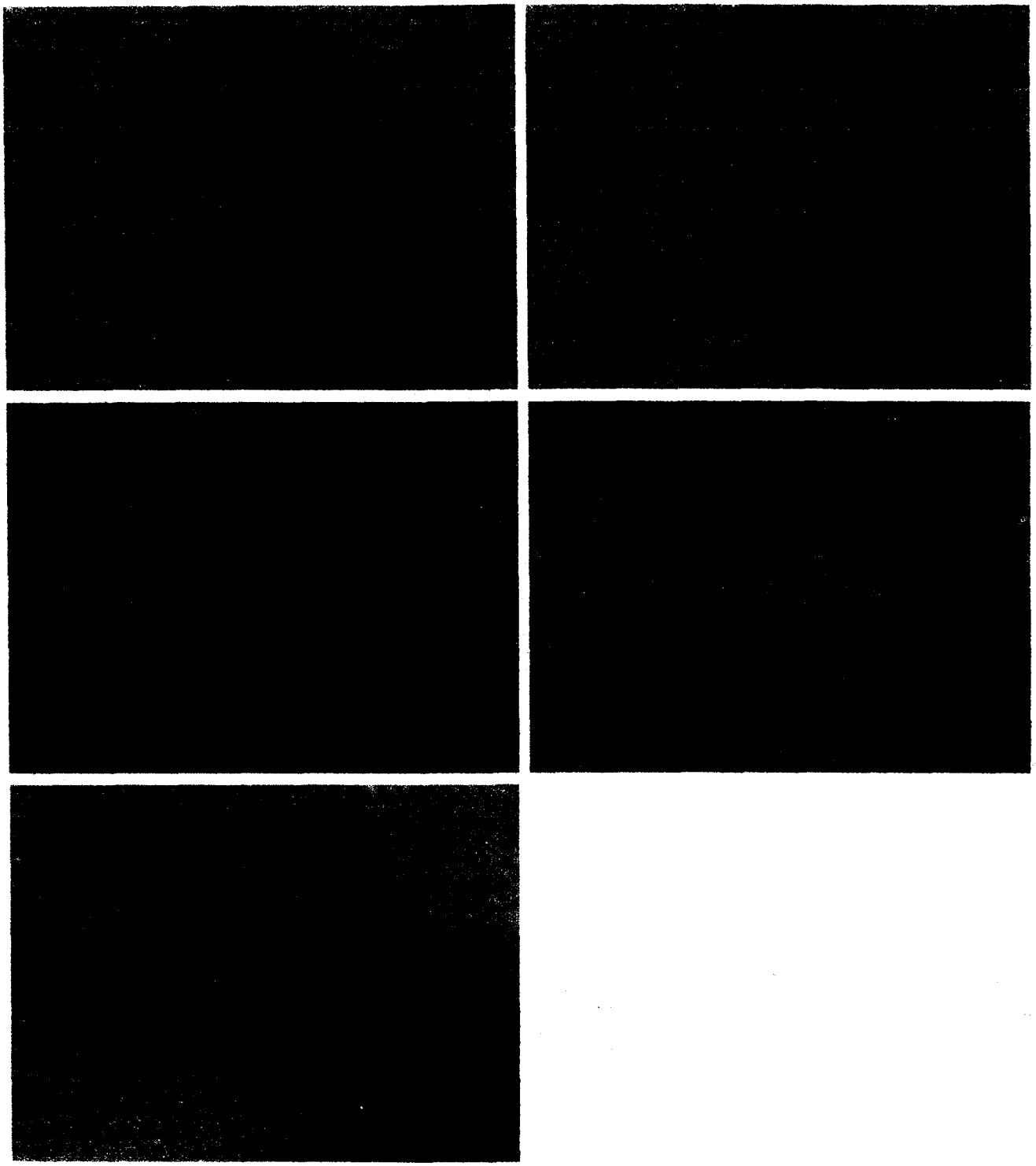

Fig. 2a–e *Ulkenia profunda.* Developmental stages on pine pollen (arrow in **a**) of isolate MS2D from mild steel panels immersed in Dona Paula Bay. Arrows in **c** indicate ectoplasmic net elements. Bar represents ten microns

stochytrid exhibited the highest percentage metamorphosis facilitation, followed by SF+ and then the control.

Discussion

The zoosporic mode of life of thraustochytrids can be expected to serve the ecological purpose of rapidly exploring substrate suitability for settlement and subsequent growth. In the marine environment, zoospores of

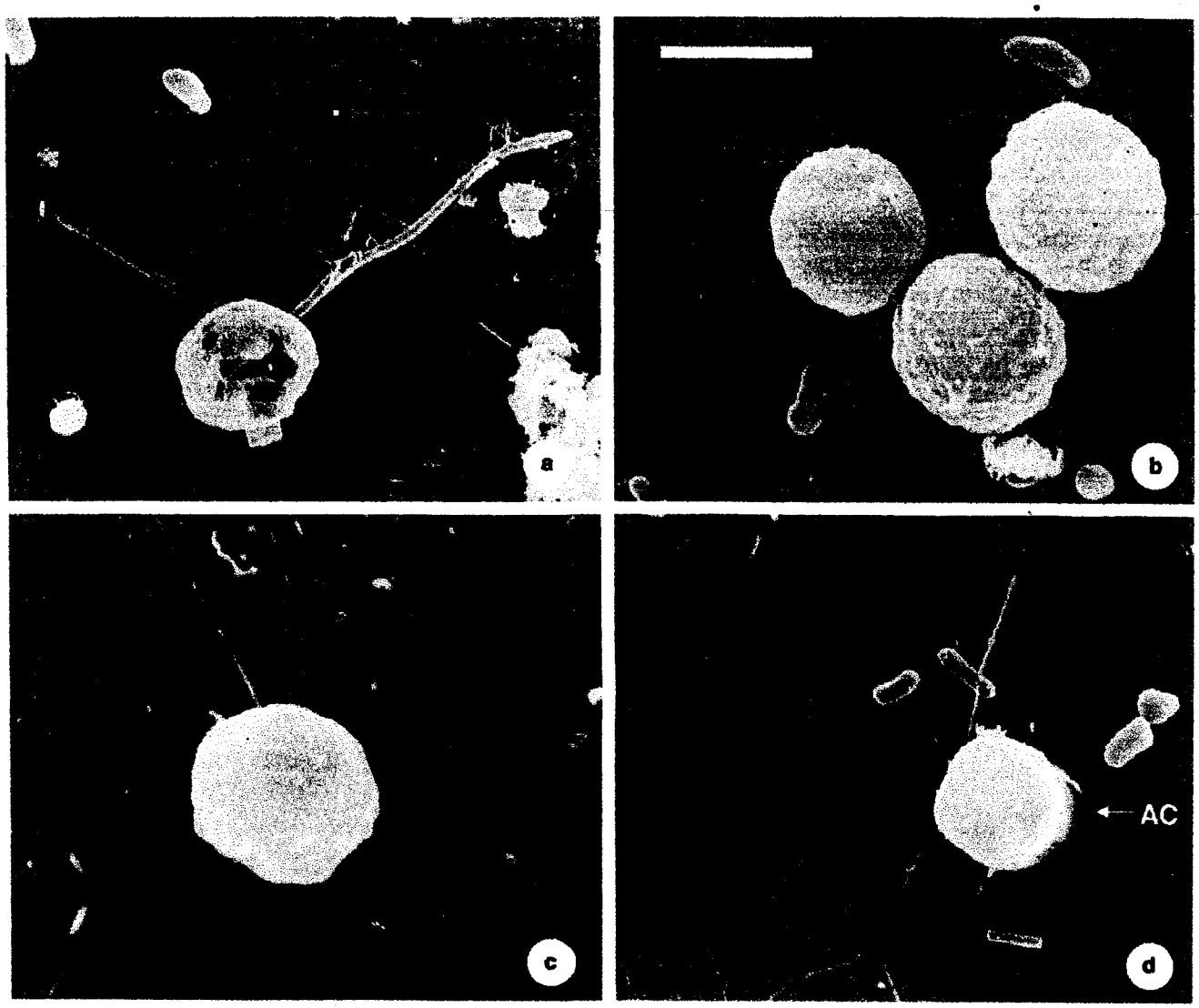

Fig. 3a–d *Ulkenia profunda*. Scanning electron micrographs of isolate MS2D grown on aluminium panels. Arrows in **c** and **d** indicate ectoplasmic net elements. Bars represent two microns (*AC* adhesion cap structure)

aquatic fungi such as *Halophytophtora* species may settle on organic detritus within a few hours (Newell et al. 1987). Such information is not available for thraustochytrids, the most abundant fungi-like organisms in the sea. Raghukumar et al. (1995) isolated thraustochytrids from mangrove leaf detritus that had been in water for 4 d. Our present study indicates that these protists can settle on inorganic particulate material in the sea within 24 h (Table 1). Microscopic examinations confirmed the presence of thraustochytrids on glass surfaces.

The next important step in future studies would be to estimate the cell densities of thraustochytrids on such surfaces. Baiting methods that are commonly used, like any other culture method, would not give an accurate picture of the actual population. A direct detection method using epifluorescence microscopy is available for

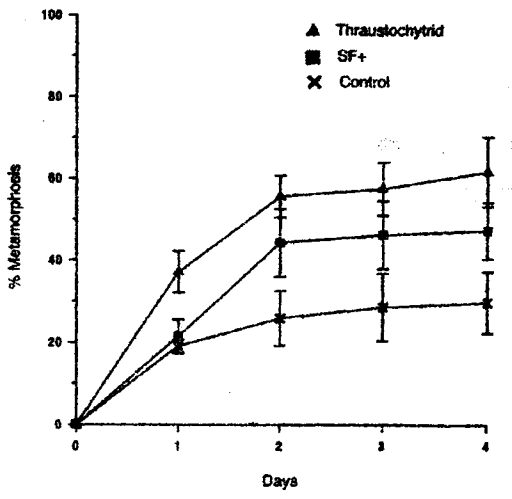

Fig. 4 *Balanus amphitrite*. Percent metamorphosis of cyprids in the presence of thraustochytrids and barnacle extracts (*SF+* settlement factor +, see "Materials and methods – Assay protocol")

thraustochytrids (Raghukumar and Schaumann 1993). However, this technique relies on the presence of a detectable cell wall, a criterion that may not be present in young and developing cells. Therefore, this technique may not be applicable to freshly immersed substrata.

Settlement of bacteria on various surfaces is influenced by the nature of both the material and the settling organism. Important among these parameters are the wettability or hydrophobicity and electrostatic properties of both the cell wall of the settling organism and the substratum as well as the toxicity of the material (Zutic and Tomaic 1988; Hussmark and Rönner 1992; Bhosle 1993). Copper and cupro-nickel, because of their biocidal properties, can deter the settlement and recruitment of bacteria and macrofouling organisms, especially in the initial periods of exposure. The absence of thraustochytrids on these panels, therefore, is understandable.

Hydrophobic interactions, including the hydrophobicity of the cell wall and that of the substratum, play an important role in adhesion (Stenström 1988; Rönner et al. 1990). Hydrophobic surfaces are believed to promote bacterial film formation better than hydrophilic surfaces (Characklis and Escher 1988; Pedersen 1990). Bhosle (1993) observed higher bacterial densities on the more hydrophobic surface of fibre glass, compared to aluminium. Our observations on thraustochytrids are contrary to these (Fig. 1); both isolates grew poorly on fibre glass, while attaining higher densities on glass and aluminium.

Hydrophobicity of the surface of the settling organism is also an important factor that encourages their settlement on various substrata (Rosenberg and Kjelleberg 1986; Sonak and Bhosle 1995). A positive relation is known to exist between cell wall hydrophobicity and settlement. Cells of isolate MS2D in the present study showed 50% separation in the hexadecane phase, a measure of cell wall hydrophobicity commonly used (Rosenberg and Kjelleberg 1986). This percentage could have been much higher but for the fact that thraustochytrid cells often clump to form large aggregates, which sink to the aqueous phase in the MATH test. Even our conservative estimate of the hydrophobicity values of thraustochytrids are higher than those reported by Sonak and Bhosle (1995) for a majority of bacteria during a prolonged period of immersion of materials in the sea. These authors reported >40% attachment of bacterial cells to hexadecane only in a maximum of 22.22% of their isolates from aluminium surfaces.

The initial settlement of thraustochytrid cells to surfaces deserves attention. Zoospores of phytopathogenic fungi are attracted to substrates chemotactically, and attachment is often mediated through lectins (Kiran 1996). Thraustochytrid zoospores may be attracted by the presence of the film of organic material, the glycocalyx, which develops on immersed marine surfaces within a few hours (Characklis and Eschers 1988). Alternatively, thigmotropism may play a role. In the case of bacteria, after an initial period of weak adhesion, extensive amounts of extracellular polysaccharides are produced that anchor them even more firmly to the substratum (Marshall 1985; Read and Costerton 1987; Vandevivere and Kirchman 1993). No elaborate formation of attaching polysaccharides was noted in the case of thraustochytrid cells in this study, although a localised adhesion cap may play a role (Fig. 3d). We suggest that cell walls of thraustochytrids are highly adhesive and play a direct role in their attachment to surfaces. Adhesion of thraustochytrid cells to each other to form clumps, as seen in Fig. 3b, is a regular feature in most species. Cell walls of these protists are known to be composed of sulphated polysaccharides, predominantly made of galactans and proteins (Chamberlain 1980). The possible adhesive nature of thraustochytrid cell walls deserves attention from the point of view of primary film formation on marine surfaces. The smaller sizes of the cells attached to surfaces (Fig. 3b to d), when compared to those on pine pollen (Fig. 2a to d), suggests that the nutritional source available in the primary film could be limiting to thraustochytrids. It is likely that bacterial metabolites and polysaccharides are a source of nutrition to these protists.

"Settlement factors", belonging to the class of arthropodin proteins secreted by adult barnacles, are known to play a significant role in inducing settlement of barnacle larvae (Anil and Khandeparker 1998). In our study, thraustochytrids facilitated metamorphosis of barnacle cyprids to an even greater extent than the settlement factor. It is likely that, in nature, thraustochytrids on marine surfaces play an important role in the settlement of larvae belonging to macrofouling invertebrates. Earlier studies carried out with the larvae of *Balanus amphitrite* to evaluate the biofilm effect of bacteria have shown facilitation, inhibition and no effect depending upon the biofilm age, biofilm density and species specificity (Maki et al. 1988, 1990; Rittschof and Costlow 1989). In light of this it would be pertinent to evaluate the impact of such factors in determining the role of thraustochytrids.

Acknowledgements This paper is N.I.O. Contribution No. 3523.

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