

## **Algal epibiosis on *Megabalanus tintinnabulum* and its role in segregation of *Balanus amphitrite* population**

**Ranjith E and Lidita Khandeparker\***

Council of Scientific and Industrial Research - National Institute of Oceanography, Dona Paula, Goa – 403 004, INDIA

### ABSTRACT

*Ulva* sp. and *Gracilaria* sp. were found colonizing shell surfaces of an acorn barnacle, *Megabalanus tintinnabulum*. However, this association was not noticed in the case of *Balanus amphitrite*, which was a co-inhabitant. Such a difference in intergeneric interactions with the algae was examined through laboratory experiments. For this, the influence of extracts of algae, extracts of algae - associated bacteria and natural leachants from *M. tintinnabulum* were assessed on cyprid metamorphosis of *B. amphitrite*. Extracts of algae and associated bacteria showed no effect on the metamorphosis of *B. amphitrite*. This may be attributed to absence of cue specific sugars in the exopolysaccharides (EPS) and culture supernatants (CS) of bacteria. On the other hand natural leachants of *M. tintinnabulum*, which showed presence of N-acetyl-D-glucosamine (GlcNAc), a known algal spore attractant, also inhibited metamorphosis of *B. amphitrite*. Thus, hosting specific epibionts could have important implications in the segregation of barnacle population.

*Keywords:* *Balanus amphitrite*; *Megabalanus tintinnabulum*; metamorphosis; epibiosis; cyprid; algae

\*Corresponding author. National Institute of Oceanography, Dona Paula, Goa – 403 004, India. Tel.: +91(0)832-2450 432; fax: +91(0)832-2450 615; email: [klidita@nio.org](mailto:klidita@nio.org) (Lidita Khandeparker)

## Introduction

Marine biofouling commonly refers to the undesirable growth of marine organisms on artificial structures immersed in the marine environment (Callow & Callow 2002). Mostly, it begins with the adsorption of organic molecules onto newly submerged surfaces, followed by colonization of different microfoulers such as bacteria, diatoms, fungi and settlement of macro fouling organisms which includes invertebrate larvae and algal spores (Marechal & Hellio 2011).

However, in some cases macrofoulers prefer to settle on pristine surfaces (Lejars *et al.* 2012). Algae have been reported to foul artificial substrates and living organisms as an epibiont (O'Connor & Crowe 2008). Moreover, algae have antifouling activity against various fouling organisms. When compared to other macrofoulers, barnacles are considered as one of the most successful biofoulers in the marine and estuarine environment. *Balanus (Amphibalanus) amphitrite* an acorn barnacle, serves as an important model for antifouling studies. It is euryhaline (Anil *et al.* 2010) and breeds throughout the year (Desai & Anil 2005). This species is one of the dominant components of the biofouling community in Indian waters (Desai & Anil 2005).

The cypris larva, which is the pre-settlement stage of the barnacles, has been used to study different cues influencing settlement and metamorphosis (Wieczorek *et al.* 1995; Yamamoto *et al.* 1995; Khandeparker *et al.* 2006). Microbial biofilms are generally observed to promote the settlement of macrofouling organisms. However, they also show inhibitory effect on *B. amphitrite* (Olivier *et al.* 2000; Khandeparker & Krishna kumar 2011a). Macroalgae, a source of epibiosis and their effect in host organism has been reported (O' Connor 2010). Among these mostly well understood mechanisms is production of halogenated furanones by the red algae *Delisea pulchra* (Dworjanyn *et al.* 1999). Metabolites from red algae have shown to inhibit settlement of cyprids of the barnacle *B. amphitrite* and *B. improvisus* and as well inhibit germline development of gametes of *Ulva lactuca* (Nys *et al.* 1995; Wikstrom & Pavia 2004). These secondary metabolites showed presence of furanones and dictyols - like compounds (diterpenes). Extracts from the brown alga *Sargassum tenerrimum* showed inhibitory effect on the metamorphosis of *Hydroides elegans* larvae, a polychaete living in the vicinity of *S. tenerrimum* (Lau & Qian 1997).

In the present study area i.e. intertidal region of Arambol, west coast of India (15°41'54"N 73°41'49"E) it was observed that *Megabalanus tintinnabulum* adults were covered with *Ulva* sp. and *Gracilaria* sp. on their surfaces; with no *B. amphitrite* in the vicinity. Taking above into consideration

these intergeneric interactions were evaluated by assessing the following against the metamorphosis of *B. amphitrite* cyprids.

(1) Influence of extracts of epibiotic algae (*Ulva* sp. and *Gracilaria* sp.) and the associated bacterial extracts

(2) Role of natural leachants from the adults of *M. tintinnabulum*.

(3) Influence of exo polysaccharides (EPS), bacterial culture supernatants (CS) and ethyl acetate (EA) extracts of nine bacterial strains isolated from *Ulva* sp., *Gracilaria* sp. and *Sargassum* sp..

## Materials and methods

The schematic representation of the experimental set-up is depicted in Figure 1.

### Rearing of *B. amphitrite* larvae

Adults of *B. amphitrite* were collected from the intertidal area of Dona Paula Bay located at Zuari estuary, Goa, west coast of India (15° 27.5' N, 73° 48' E) and maintained in tanks in the laboratory using a diet of *Artemia* sp.. Nauplii released by these adults were separated and mass reared in two liter glass beakers using filtered sea water of 35‰, on a diet of *Chaetoceros calcitrans*, a unicellular diatom, at a cell concentration of  $2 \times 10^5$  cells ml<sup>-1</sup>. The feed organism was replenished every day while changing the sea water. The larvae were kept at 25 (±2) °C in an incubator on a 12 hr light: 12 hr dark photoperiod. After 6-7 days the cyprids obtained were siphoned out and stored at 5 °C prior to settlement assays. Two-day old cyprids were used to carry out the metamorphosis assays.

### Preparation of adult extract

Adult barnacles (*B. amphitrite* and *M. tintinnabulum*) were collected from the field and cleaned using nylon brush and forceps to remove the epibiotic growth present on their shells. Equal weight/volume of deionized water was used and the adults were crushed using a pestle and mortar method described by Larman *et al.* (1982). The protein concentration of adult extract (AE) was determined following Lowry *et al.* (1951) method. Bovine Serum Albumin (BSA) was used as the standard. The protein concentration of 25 µg ml<sup>-1</sup> of AE was used for all the assays. The adult extract was diluted using filtered sea water which was previously autoclaved and cooled before dilution. These adult extracts of *B. amphitrite* and *M. tintinnabulum* were used as positive controls for the settlement assays.

Isolation of bacteria from the macroalgae (*Ulva* sp. & *Gracilaria* sp.) associated with *M. tintinnabulum* shells and free floating alga (*Sargassum* sp.)

Adult *M. tintinnabulum* were brought to the laboratory and rinsed with deionized water to remove dirt. The epibiotic algal films ((*Ulva* sp. and *Gracilaria* sp.) were removed from the animals using sterile forceps. *Sargassum* sp. was collected from Dias beach located at Goa, west coast of India. For bacterial isolation, known amount of algal sample was taken and homogenized using sterile homogenizer with 10ml of autoclaved filtered sea water. The samples were subsequently diluted and spread plated on Zobell Marine Agar 2216 (ZMA) and Bacto-Peptide Agar (BPA). The incubation period of ZMA was 24 hr at room temperature ( $28 \pm 2$  °C) and BPA was at 20 °C for 4 days. The bacterial colonies isolated from ZMA & BPA were maintained on ZMA and BPA slants as described by Konya *et al.* (1995). The isolated bacteria (Table I) were identified following 'Bergey's manual of systematic bacteriology (Holt *et al.* 1994) and manual for the identification of medical bacteria (Cowan 1974).

#### Bacterial growth curve and EPS production

The growth of nine different bacterial cultures (*Veillonella* sp.; *Streptococci faecalis* – isolates from *Gracilaria* sp. & *Sargassum* sp.; *Bacillus brevis*; *Campylobacter fetus* – isolated using ZMA & BPA; *Streptococci mitior*; *Campylobacter sputorum* – isolated using ZMA & BPA) was determined in Bacto-peptide medium (BPM). A 2 % (v/v) inoculum of 18h old culture grown in BPM was used and was kept at  $26 \pm 2$  °C on a shaker. Optical density (OD) of the cultures was measured at 540 nm at equal intervals to assess the bacterial growth in the culture medium. Total carbohydrate content was measured in the samples taken for OD using phenol sulphuric acid method (Bhosle & Dhople 1988).

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

For extraction of culture supernatant, bacterial strains were grown in BPM with an initial inoculum of 2% (v/v) which was obtained by growing the bacterial strains in the same medium for 18 hr. The cultures which were inoculated in BPM were kept at 25°C at 200 rpm on a shaker. The extracts were prepared following the method as described by Khandeparker *et al.* 2002a. Briefly, after 120 hr, they were harvested by centrifuging at 20,000 g for 15 min at 4°C. Subsequently, the supernatant was filtered through sterile 0.22µm filter paper and culture supernatant was kept at 4 °C for further use. The cultures supernatants obtained using BPM were subsequently concentrated using 10,000 MW cut-off (Milipore - Amicon) filters. The concentrated samples were then analysed for protein content as

described by Lowry *et al.* (1951), uronic acid content as described by Filisetti-Cozzi & Carpita (1991) and carbohydrate content as described by Bhosle & Dhople (1988) and stored at 4 °C until further use. The larval settlement assays were carried out at a carbohydrate concentration of 25 µg ml<sup>-1</sup>.

#### Extraction of bacterial exopolymeric substances (EPS)

For the extraction of EPS, the nine different bacterial culture supernatants were treated with three volumes of absolute alcohol and left overnight at 4 °C. The precipitate was recovered by centrifuging at 5,000 g for 15 mins at 4 °C. The pellet was redissolved in sterile distilled water, and treated with DNase and RNase (1 µg/ml final concentration) for 3 hr at 37°C. The samples were subsequently dialysed using 8000 MW cut-off dialysis membrane filter at 4 °C overnight against distilled water by changing water every 2 hr. The dialysed samples were centrifuged at 5,000 g for 15 mins to remove insoluble material. The EPS thus recovered was kept at 4 °C until further use (Khandeparker *et al.* 2002a). The EPS was then analysed for proteins, carbohydrates and uronic acid content as described above.

#### Ethyl acetate extraction of bacterial culture supernatants (CS)

The bacterial culture supernatants were extracted using three equal volumes of ethyl acetate. The organic phase was collected and concentrated by using a rotary evaporator at 32±3 °C. After evaporating to dryness the precipitate was collected and kept in a vacuum desiccator. The residue was weighed and dissolved in either distilled water or dimethyl sulfoxide (DMSO). The concentrated samples were then analyzed for proteins, carbohydrates and uronic acid content. The extracts were normalized to 25 µg ml<sup>-1</sup> carbohydrate concentration and were used for further experiments.

#### Preparation of algal extracts

All three algal species were rinsed thoroughly with sterile seawater to remove associated debris. The cleaned algal samples were then surface dried by pressing it between sheets of tissue paper and air dried in the shade at 30 °C for 24 hr. Before air drying, the surface microflora was removed by washing the algal samples for 10 mins with 30% ethanol (Hellio *et al.* 2000). The dried algal samples were then suspended by stirring in 95% ethanol. After centrifuging the supernatant aqueous phase was decanted and the pellet was re-extracted five times in the same way. Subsequently, the alcoholic extracts were combined and evaporated under vacuum at 32±3 °C. The extracted samples were analysed for proteins, carbohydrates and uronic acid as described above.

## Natural leachants

*M. tintinnabulum* adults without epibiotic algae were kept in autoclaved filtered sea water for 24 hr and the leachants thus collected were characterized using HPLC and used for settlement assays.

## HPLC analysis of crude EPS, CS and EA extracts

The isolated crude EPS, CS, EA extracts, natural leachants and AE were analyzed with a Reverse Phase High Performance Liquid Chromatography (Agilent 1200) equipped with auto start kit and eluted with acetonitrile: water (85:15) at a flowrate of 1.2 ml min<sup>-1</sup> at column temperature 30 °C and 25 mins of elution time. The C18 column was used to separate the sugar components. The separated sugar components were monitored by a Refractive Index detector. Before running the sample the column was calibrated with 15 different HPLC grade sugar standards (glucose, galactose, sucrose, mannose, arabinose, GlcNAc, maltose, xylose, fructose, lactose, mannitol, sorbitol, inositol, cellubiose, melibiose) separately as well as mixed together to determine the standard curve.

## Assay protocol

The settlement assays were carried out using 24 well multiwell plates (Corning-430262). The multiwells were inoculated with different bacterial CS, EPS, ethylacetate extracts, natural leachants and algal extracts. Adult extract of *B. amphitrite* (AE-BA) and *M. tintinnabulum* (AE-MT) were used as positive controls and autoclaved filtered seawater (AFSW), 0.22 µm filtered seawater (FSW) and artificial sea water (ATSW) were used as negative controls. The single-cyprid assays were conducted in four different batches and twenty replicates (n= 4 x20). The multiwells after inoculation with bacterial extracts, CS, EPS and both AE (AE-BA and AE-MT) were incubated for 3 hr. After incubation the inoculated multiwells were rinsed with autoclaved filter seawater under laminar airflow. Subsequently, single cyprids were introduced in to each well with 2ml of autoclaved filtered seawater. The settlement assay wells were kept at 25±2 °C (12 hr light: 12 hr dark photoperiod). The settlement assays were monitored for a period of four days with an intermittent observation every day. The metamorphosed cyprids were counted at the end of each day. All settlement assays were done at carbohydrate concentration of 25 µg ml<sup>-1</sup> respectively.

The results obtained from the settlement assays in comparison with positive and negative controls divided the settlement responses into three distinct categories as described by Harder *et al.* (2002) and Khandeparker *et al.* (2006).

- (1) 'Inductive' (I): percentage of metamorphosis is greater than or equal to positive control,
- (2) 'Weakly inductive' (WI): percentage of metamorphosis is greater than negative control but lesser than positive control
- (3) 'No effect' (NE): percentage of metamorphosis is equal to or less than negative control.

### Statistical analysis

The data on settlement (%) was arcsine transformed to ensure normality and homogeneity of variances before subjecting to statistical analysis. Since all the factors met the normality assumption they were analysed using parametric statistical analysis. The influence of different bacterial EPS, CS and EA extracts in either water or DMSO on cyprid metamorphosis was evaluated using One-way analysis of variance (ANOVA) (Sokal & Rohlf 1981) and the results are presented in Table IIa-d.

## Results

### *Ulva* sp. associated bacteria

Overall, 22 bacterial strains were isolated from *Ulva* sp. using ZMA and BPA. Out of 22, two dominant strains (*C. fetus* isolated from ZMA & BPA) and one rare strain (*Veillonella* sp.) were selected (Table I). All three bacteria produced maximum amount of EPS between 48-58 hr. The influence of EPS, CS and EA extracts in DMSO and water from *Ulva* sp. associated bacteria on cyprid metamorphosis is shown in figure 2a-h. The results revealed that *Veillonella* sp. EA extract dissolved in water was significantly different with respect to both positive and negative controls on day 1 (One-way ANOVA). The extract was found to be the most inductive (95%) on *B. amphitrite* cyprids (Figure 2a) at the end of day 4 ( $p \leq 0.0001$ ). The extract from *C. fetus* isolated using ZMA (Figure 2c & d) was weakly inductive, whereas, when isolated using BPM had no effect on cyprid metamorphosis (Figure 2e & f). The trend was similar at the end of day 4. Culture supernatants were weakly inductive and the response did not change with the bacterial strain (Table III).

### *Gracilaria* sp. associated bacteria

18 bacterial strains were isolated from *Gracilaria* sp. using ZMA and BPA, out of which two dominant strains (*S. faecalis* and *S. mitior*) and a rare strain (*B. brevis*) were selected (Table I). The maximum amount of EPS production was observed between 12-48 hr. The influence of *Gracilaria* sp. associated bacteria on cyprid metamorphosis is shown in figure 3a-h. The CS and EPS of *S. faecalis*, *S. mitior* and *B. brevis* showed weakly inductive effect except EA extract dissolved in DMSO of *B. brevis*

on day 1 which showed no effect on cyprid metamorphosis (Figure 3c). The response remained similar on day 4.

#### *Sargassum* sp. associated bacteria

28 bacterial strains were isolated from *Sargassum* sp. using ZMA and BPA, out of which two dominant strains (*C. sputorum* isolated from ZMA & BPA) and a rare strain (*S. faecalis*) (Table I). All three bacteria produced high amount of EPS with high carbohydrate content before attaining the stationary phase of growth cycle (12-24 hr). The influence of *Sargassum* sp. associated bacteria on cyprid metamorphosis is shown in figure 4a-h. On day 1, EA extract in DMSO and water from *C. sputorum* (S<sub>3</sub>-BPA) showed weekly inductive effect on cyprids and significantly different with respect to both positive and negative controls (Figure 4e). Whereas, *C. sputorum* isolated using ZMA showed no effect on cyprids (Figure 4c). Moreover, on day 4, CS and EA extract dissolved in water were inductive (One way ANOVA;  $p \leq 0.0001$ ; Table IIc) and significantly promoted metamorphosis with respect to positive and negative controls (Figure 4f).

#### Algal extracts and natural leachants

The influence of algal species and natural leachants on cyprid metamorphosis is shown in figure 5a-d. On day 1, all three extracts were inhibitory (Figure 5a). Whereas on day 4, except for *Ulva* sp. which continued to be inhibitory, extract from *Gracilaria* sp. was weakly inductive and *Sargassum* sp. was most inductive (Figure 5b). Natural leachants also showed weekly inductive effect on day 1 as well as on day 4 (Figure 5a-b).

#### HPLC analysis of crude EPS, CS and EA extracts

The HPLC analysis showed presence of six different sugars (glucose, mannitol, sorbitol, mannose, GlcNAc, and fructose) in different bacteria and adult extracts (Table IV). GlcNAc was the major constituent in the natural leachants of *M. tintinnabulum*. The same sugar group was also present in the EA extract in water of *Veillonella* sp. The percentage of GlcNAc in *Veillonella* sp. was comparatively less (56%) than that was observed in natural leachants (86%). Moreover, sorbitol (33%) and fructose (41%) were also observed in EA extract in water of *S. faecalis* (E<sub>1</sub>-ZMA). Also, sorbitol was observed in EA extract in water of *C. sputorum* (S<sub>2</sub>-ZMA (11%) and S<sub>3</sub>-BPA (28%). Glucose and mannitol were observed in EA extract in water of *S. faecalis* (S<sub>5</sub>-ZMA- 27% and 21%) and *B. amphitrite*



AE (0.97% and 0.75%) respectively (Table IV). No sugars were reported from, culture supernatants, EPS and the EA extracts that were dissolved in DMSO.

## Discussion

Extracts of algae, algae-associated bacteria and natural leachants of *M. tintinnabulum* inhibited the metamorphosis of *B. amphitrite*. When the same species are located in a particular habitat the space distribution is mostly equal. But when different species inhabited in a particular environment space distribution would not be equal and leads to interspecies competition (Wu *et al.* 1977; Bertness 1989; Gaonkar & Anil 2012; Marfenin *et al.* 2013). The biotic and abiotic factors control the distribution and abundance of the barnacles and algae, changing the settlement and survival rate. The most important abiotic factors include hydrodynamics, salinity, nutrients and light (Racco & Schaeffer 2003). In present study it evident that chemical cues play a significant role and the inhibition of cyprid metamorphosis of *B. amphitrite* was attributed to absence of cue specific sugars from algae or associated bacteria those colonized *M. tintinnabulum*. The macroalgal surfaces are frequently covered by bacteria at densities approximately  $10^7$  bacteria  $\text{cm}^{-2}$  (Armstrong *et al.* 2000; Rao *et al.* 2007; Wahl *et al.* 2010; Lachnit *et al.* 2011). Since macroalgae have high abundance of bacteria, these bacteria in algae could play a protective role by releasing secondary metabolites in to surrounding environment that help to prevent micro and macrofouling on their surfaces. Bacterial strains associated with the surface of green algae (Dobretsov *et al.* 2006), sponges (Dobretsov *et al.* 2005) and soft corals (Dobretsov & Qian 2004) have shown reduced fouling. Evelyn *et al.* (2001) reported that bacteria isolated from the surface of a tunicate prevented the settlement of barnacle cyprids.

Some seaweed extracts enhance the growth of bacteria found on their surfaces (Sakami & Sugiyama 1994). The ability of certain bacteria to take advantage of such interactions and get a protected place on the seaweed surface may provide them with an advantage over other bacteria. In such cases the bacteria themselves may have adapted responses to protect their position on the seaweed surface by means of preventing subsequent colonization by micro and macro organisms. In this study, EPS from different bacterial strains associated with the algae either showed no effect or inhibited the cyprid metamorphosis of *B. amphitrite*. These EPS showed high concentration of uronic acids when compared to proteins. EPS are known to contain non sugar components like uronic acid, sulphates, methyl esters, pyruvates, protein, nucleic acid and lipids (Sutherland 2001). HPLC results indicated absence of sugars that are otherwise reported in the bacterial EPS and have implications in cyprid

metamorphosis of *B. amphitrite* (Khandeparker *et al.* 2002b; Pagett *et al.* 2012). The absence of these sugars in EPS could be one of the reasons for no effect on the cyprid metamorphosis of *B. amphitrite*. Moreover CS also did not induce the metamorphosis.

However, the EA extracted metabolites dissolved in water showed maximum facilitation of metamorphosis on day 1 itself, where the metamorphosis rate was two-fold higher than the positive control. The EA bacterial extracts in water showed presence of different sugars like sorbitol, mannose, fructose, mannitol, glucose and GlcNAc. Since these metabolites were rich in cue specific sugars (Table IV) they could have facilitated metamorphosis (Khandeparker *et al.* 2002b). Thus, sugars play an important role in cyprid metamorphosis of *B. amphitrite*. Water is high polarity solvent for the extraction of sugars, amino acids and glycosides (Houghton & Raman 1998). Water soluble or larger insoluble macromolecules, have water soluble components that also function as cues. Clare & Matsumura (2000) reported larger insoluble proteins to induce settlement of barnacles. Ethyl acetate, a solvent of medium polarity extracts also includes alkaloids, aglycone and glycosides (Houghton & Raman 1998). Characterization of these compounds would be a step ahead in understanding the relevance of EA extracts mainly by *Veillonella* sp. of *Ulva* sp. in cyprid metamorphosis. However, when EA extract was dissolved in DMSO, it showed inhibitory effect on cyprid metamorphosis except *B. brevis* which showed no effect. DMSO is polar aprotic solvent and these extracts may include both polar and non-polar compounds.

EA extract of *S. faecalis* isolated from *Sargassum* sp. showed presence of glucose and mannitol. But when *S. faecalis* was isolated from *Gracilaria* sp. did not produce these sugars but sorbitol was produced. Earlier studies have indicated that production of different compound by the bacteria is influenced by the nutritional conditions under which they are grown and such differences regulate cyprid metamorphosis (Khandeparker *et al.* 2006). The response of cyprids also differed and depended on the nutritional conditions. EA extract of same bacteria isolated by using different media showed varying response to cyprid metamorphosis. For example, *C. fetus* and *C. sputorum* were isolated by using two different media. While ZMA isolate of *C. fetus* promoted the cyprid metamorphosis, BPA isolate of the same bacteria showed inhibitory effect on cyprid metamorphosis and vice versa. In this study the similar bacteria were isolated using different medium which was not done earlier and indicates that the nutrients conditions under which the bacteria are isolated play a significant role in the production of settlement inducers. The varying responses of these settlement inducers towards cyprid metamorphosis unravel the importance of nutrients in altering the biochemical composition of these settlement inducers. Most

inducers of colonization appear to be primary metabolites such as sugars, carbohydrates, amino acids and proteins (Steinberg *et al.* 2002). *B. amphitrite* cyprids have been shown to use glucose and mannose as important inducing settlement cues (Khandeparker & Anil 2011b; Pagett *et al.* 2012). HPLC results showed presence of glucose (Table IV) in *B. amphitrite* AE. All three algal extracts were rich in protein content (Figure 6b). The highest content was seen in *Ulva* sp. i.e 1438  $\mu\text{g ml}^{-1}$ (Figure 6b). Among the algal species, high uronic acid and carbohydrate content was observed in *Sargassum* sp. extract. Influence of different algal extracts on cyprid metamorphosis was significantly lower than the adult extracts of both *B.amphitrite* & *M. tintinnabulum*. In present study *Ulva* sp. and *Gracilaria* sp. extracts were inhibitory to a great extent followed by natural leachants. High numbers of secondary metabolites are produced by benthic marine macroalgae and these compounds showed antifouling activity against fouling organisms (Qian *et al.* 2010). In nature, this inhibitory effect could be much pronounced than the promotory effect of some of the associated bacteria. Large numbers of marine algae possess metabolites with antifouling properties (Bhadury & Wright 2004).

The adult extract of *B. amphitrite* and *M. tintinnabulum* as well as natural leachants indicated presence of sugars (glucose, mannitol, mannose, GlcNAc) which are often used as cues by barnacles (Khandeparker *et al.* 2002b). Highest amount of GlcNAc was observed in *M. tintinnabulum* natural leachants. Barnacle molts are good source of chitin. GlcNAc is one of the important sugar components of chitin. Callow *et al.* (1981) study represented that algal spores may require carbohydrates and lectins to find appropriate surface for settlement. Relatively high amounts of mannose, glucose, and GlcNAc have been detected in algal mucilage and cell wall surfaces (Tein *et al.* 2005). The mucilage is responsible for the initial attachment of spores to the substratum (Avanzini, 1989; Bouzon *et al.* 2006). Bouzon *et al.* (2006) study also reported that these sugars are involved in the adhesion of naked spores (adhesion of rhizoids) to the substratum. This could be one of the reasons for epibiotic growth of algae on the *M. tintinnabulum* surface. The natural leachants although contained cue specific sugars could not induce the settlement of *B. amphitrite* cyprids (Figure 5a-b). This study signifies the presence of algae on the *M. tintinnabulum* surfaces as their extracts strongly inhibited cyprid metamorphosis of *B. amphitrite*. GlcNAc sugar in the natural leachants of *M. tintinnabulum* could act as a positive cue for algal epibiosis on *M. tintinnabulum* surfaces. Such intergeneric interactions of hosting a specific epibiont is reported for the first time and this could serve in segregation of barnacle population in a given habitat.

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### **Legends to Tables**

Table I Bacteria from epibiotic algae (*Ulva* sp. & *Gracilaria* sp.) associated with *Megabalanus tintinnabulum* & free living *Sargassum* sp.

Table II a-d One way ANOVA table.

Table III Summary table of the results.

Table IV Monosaccharides composition (%) of different compounds extracted from bacteria.

### **Legends to figures**

Fig. 1 Schematic representation of the experimental set-up.

Fig. 2a-h. Influence of *Ulva* sp. associated bacterial compounds on cyprid metamorphosis.

Fig. 3a-h. Influence of *Gracilaria* sp. associated bacterial compounds on cyprid metamorphosis.

Fig. 4a-h. Influence of *Sargassum* sp. associated bacterial compounds on cyprid metamorphosis.

Fig. 5a-d. Influence of algal extracts & natural leachants on cyprid metamorphosis.

Fig. 6a-d. Carbohydrates, proteins & uronic acids content of different bacterial strains and algal extracts.



Table I. Bacteria from epibiotic algae associated with *Megabalanus tintinnabulum* and free living *Sargassum* sp.

Algal samples	Associated bacteria
<i>Ulva</i> sp.	<i>Veillonella</i> sp. ( U <sub>1</sub> - ZMA) <i>Campylobacter fetus</i> ( U <sub>2</sub> - ZMA ) <i>Campylobacter fetus</i> ( U <sub>3</sub> - BPA )
<i>Gracilaria</i> sp.	<i>Streptococci faecalis</i> ( G <sub>1</sub> - ZMA) <i>Streptococci mitior</i> ( G <sub>2</sub> - ZMA) <i>Bacillus brevis</i> (G <sub>3</sub> - BPA)
<i>Sargassum</i> sp.	<i>Streptococci faecalis</i> ( S <sub>1</sub> - ZMA) <i>Campylobacter sputorum</i> ( S <sub>2</sub> - ZMA) <i>Campylobacter sputorum</i> ( S <sub>3</sub> - BPA)

ZMA – Zobell Marine Agar isolates  
 BPA – Bacto – Peptone Agar isolates

Table IIa. One way ANOVA table

<i>Ulva</i> sp. associated bacteria	Day 1						
	df	SS	MS	Fs	SS	MS	Fs
1. EPS - AE-BA	3	3021	1007	6.6***	1242	4142	2.3 NS
	1	0			7		
2. EPS - AE-MT	3	3641	1213	8.3*****	1242	4142	2.3 NS
	5	8			7		
3. EPS - AFSW	3	3536	1178	0.94 NS	303	101	0.04 NS
4. EPS - FSW	3	3536	1178	0.94 NS	1111	370	0.1 NS
	3	1000	3334	1.67 NS	2728	909	0.8NS
5. CS - AE-BA	2						
	3	1376	4589	2.3 NS	2728	909	0.8NS
6. CS - AE-MT	7						
	3	1242	4142	2.3 NS	7577	2525	1.7NS
7. CS - AFSW	7						
	3	1242	4142	2.3 NS	1404	4681	3.2*
8. CS - FSW	7				4		
	3	4233	1411	9.6	2536	8453	7.7***
9. EA extract dissolved in water - AE-BA	5	2		****	0		*
	3	4406	1468	10.3***	2536	8453	7.7***
10. EA extract dissolved in water - AE-MT	6	8		*	0		*
	3	6901	2300	19*****	2536	8453	5.9***
11. EA extract dissolved in water - AFSW	0	3			0		
	3	6901	2300	19*****	3021	1007	7.0***
12. EA extract dissolved in water - FSW	0	3			0	0	
13. EA extract dissolved in DMSO - AE-BA	3	2182	7274	4**	2667	8891	5.6***
	4				4		
14. EA extract dissolved in DMSO - AE-MT	3	2619	8732	4.9**	2667	8891	5.6***
	8				4		
	3	1697	5658	3.6**	1333	4445	2.3 NS
15. EA extract dissolved in DMSO - AFSW	4				7		
	3	1697	5658	3.6**	1374	4580	2.3 NS
16. EA extract dissolved in DMSO - FSW	4				1		

Influence of *Ulva* sp. associated bacterial extracts on metamorphosis of *B. amphitrite* cyprids on day1 and 4.

df, degree of freedom; SS, sum of the squares; MS, mean of squares; Fs, Fisher constant

\*\*\*\*  $p \leq 0.0001$  ; \*\*\*  $p \leq 0.005$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.025$ ; NS – not significant.

Table IIb. One way ANOVA table

<i>Gracilaria</i> sp. associated bacteria	Day 1						
	d f	SS	Ms	Fs	SS	MS	Fs
1. EPS - AE-BA	3	2697	8992	5.5****	1454	484	2.7
		7		*	9	9	NS
2. EPS - AE-MT	3	3277	1092	6.9****	1454	484	2.7
		5	5	*	9	9	NS
3. EPS - AFSW	3	5753	1717	1.2 NS	1212	404	0.1
							NS
4. EPS - FSW	3	5753	1717	1.2 NS	1616	534	0.2
							NS
5. CS - AE-BA	3	2505	8352	4.7 ***	1808	602	4.0**
		7			6	8	
6. CS - AE-MT	3	2943	9810	5.6****	1808	602	4.0**
		2		*	6	8	
7. CS - AFSW	3	2020	6735	4.4 **	1081	360	1.9
		7			1	3	NS
8. CS - FSW	3	3071	1023	4.4**	1323	441	2.3
		6	8		6	2	NS
9. EA extract dissolved in water - AE-BA	3	3071	1023	6.6****	2051	683	4.0**
		6	8	*	1	7	
10. EA extract dissolved in water - AE-MT	3	3671	1223	8.2****	2051	683	4.0**
		7	9	*	1	7	
11. EA extract dissolved in water - AFSW	3	6466	2155	1.6NS	5961	198	0.9
						7	NS
12. EA extract dissolved in water - FSW	3	6466	2155	1.6NS	5961	198	0.9
						7	NS
13. EA extract dissolved in DMSO - AE-BA	3	3545	1118	6.8****	2465	821	4.6**
			1	*	3	7	*
14. EA extract dissolved in DMSO - AE-MT	3	3832	1277	8.0****	2465	821	4.6**
		6	5	*	3	7	*
15. EA extract dissolved in DMSO - AFSW	3	2384	7948	5.7****	4041	134	0.6
		5		*		7	NS
16. EA extract dissolved in DMSO - FSW	3	2384	7948	5.7****	2020	673	0.3
		5		*			NS

Influence of *Ulva* sp. associated bacterial extracts on metamorphosis of *B. amphitrite* cyprids on day1 and 4.

df, degree of freedom; SS, sum of the squares; MS, mean of squares; Fs, Fisher constant

\*\*\*\*  $p \leq 0.0001$  ; \*\*\*  $p \leq 0.005$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.025$ ; NS – not significant.

Table IIc. One way ANOVA table

<i>Sargassum</i> sp. associated bacteria				Day 1			
	d f	SS	Ms	Fs	SS	MS	Fs
1. EPS - AE-BA	3	2536 0	8453	5.1**	1697 4	5658	3.3*
2. EPS - AE-MT	3	3115 8	1038 6	6.5*** *	1697 4	5658	3.3*
3. EPS - AFSW	3	3536	1178	0.8 NS	3637	1212	0.5 NS
4. EPS - FSW	3	3536	1178	0.8 NS	4041	1347	0.6 NS
5. CS - AE-BA	3	1293	3233	1.6 NS	1616	538	0.5 NS
6. CS - AE-MT	3	1649 3	5497	2.8 NS	1616	538	0.5 NS
7. CS - AFSW	3	1778 3	5927	3.4*	7679	2559	1.8 NS
8. CS - FSW	3	1778 3	5927	3.4*	1454 9	4849	3.4*
9. EA extract dissolved in water - AE-BA	3	3263 5	1087 8	6.4*** *	3021 0	1007 0	8.8*** *
10. EA extract dissolved in water - AE-MT	3	3599 3	1199 7	7.2*** *	3021 0	1007 0	8.8*** *
11. EA extract dissolved in water - AFSW	3	3991 0	1330 3	9.2*** *	2778 5	9261	6.3*** *
12. EA extract dissolved in water - FSW	3	3991 0	1330 3	9.2*** *	3182 7	1060 9	7.1*** *
13. EA extract dissolved in DMSO - AE-BA	3	3799 0	1266 3	7.8*** *	3637 4	1212 4	9.2*** *
14. EA extract dissolved in DMSO - AE-MT	3	4236 5	1412 1	9.6*** *	3637 4	1212 4	9.2*** *
15. EA extract dissolved in DMSO - AFSW	3	3314 1	1104 7	8.1*** *	2788 6	9285	5.7*** *
16. EA extract dissolved in DMSO - FSW	3	3314 1	1104 7	8.1*** *	2990 7	9969	6.0*** *

Influence of *Ulva* sp. associated bacterial extracts on metamorphosis of *B. amphitrite* cyprids on day1 and 4.

df, degree of freedom; SS, sum of the squares; MS, mean of squares; Fs, Fisher constant

\*\*\*\*  $p \leq 0.0001$  ; \*\*\*  $p \leq 0.005$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.025$ ; NS – not significant.

Table IId. One way ANOVA table

<b>Algal extracts &amp; Natural leachants</b>					<b>Day 1</b>		
	<b>df</b>	<b>SS</b>	<b>Ms</b>	<b>Fs</b>	<b>SS</b>	<b>MS</b>	<b>Fs</b>
1. Algal extracts - AE-BA	3	41493	13831	17.1****	32736	10912	7.4****
2. Algal extracts - AE-MT	3	33861	11287	13.0****	32736	10912	7.4****
3. Algal extracts - AFSW	3	1481	493	0.7 NS	23037	7679	4.4***
4. Algal extracts - FSW	3	1481	493	0.7 NS	23037	7679	4.4***
5. Natural leachants - AE-BA	1	12124	12124	7.3*	3031	3031	1.8 NS
6. Natural leachants - AE-MT	1	8419	8419	4.6*	3031	3031	1.8 NS
7. Natural leachants - AFSW	1	336	336	0.2 NS	337	337	0.1 NS
8. Natural leachants - FSW	1	336	336	0.2 NS	337	337	0.1 NS

Influence of *Ulva* sp. associated bacterial extracts on metamorphosis of *B. amphitrite* cyprids on day1 and 4.

df, degree of freedom; SS, sum of the squares; MS, mean of squares; Fs, Fisher constant

\*\*\*\*  $p \leq 0.0001$  ; \*\*\*  $p \leq 0.005$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.025$ ; NS – not significant.

Table III: Summary table of the results

Bacteria & Algal extracts	EPS		CS		Ethyl acetate extract in DMSO		Ethyl acetate extract in water	
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4
<i>Ulva</i> sp. associated bacterial extracts:	WI	NE	WI	I	WI	WI	I	I
1. <i>Veillonella</i> sp. ( U <sub>1</sub> - ZMA)	WI	WI	WI	WI	WI	WI	WI	I
2. <i>Campylobacter fetus</i> ( U <sub>2</sub> - ZMA)	NE	NE	WI	WI	WI	NE	WI	NE
3. <i>Campylobacter fetus</i> ( U <sub>3</sub> - BPA)								
<i>Gracilaria</i> sp. associated bacterial extracts:	WI	WI	WI	WI	WI	NE	WI	WI
1. <i>Streptococci faecalis</i> ( G <sub>1</sub> - ZMA)	WI	WI	WI	WI	NE	WI	WI	WI
2. <i>Streptococci mitior</i> ( G <sub>2</sub> - ZMA)	WI	WI	WI	WI	NE	NE	WI	WI
3. <i>Bacillus brevis</i> ( G <sub>3</sub> - BPA)								
<i>Sargassum</i> sp. associated bacterial extracts:	WI	WI	WI	WI	WI	WI	WI	WI
1. <i>Streptococci faecalis</i> ( S <sub>1</sub> - ZMA)	WI	WI	WI	WI	NE	NE	WI	NE
2. <i>Campylobacter sputorum</i> ( S <sub>2</sub> - ZMA)	WI	WI	WI	I	WI	WI	WI	I
3. <i>Campylobacter sputorum</i> ( S <sub>3</sub> - BPA)								
<b>Algal extracts &amp; Natural leachants</b>	<b><i>Ulva</i> sp.</b>		<b><i>Gracilaria</i> sp.</b>		<b><i>Sargassum</i> sp.</b>		<b>Natural leachants</b>	
Day 1	NE		NE		WI		WI	
Day 4	NE		NE		I		WI	

\*WI – Weakly Inductive; I – Inductive; NE – No Effect or Inhibitory.

Table IV. Monosaccharides composition (%) of different compounds extracted from various bacteria

Micro organism	Compound	Glucose(%)	Mannitol(%)	Sorbitol(%)	Mannose(%)	GlcNAC(%)	Fructose(%)
<i>Veilonella sp.</i>	EA extract in water ( U <sub>1</sub> - ZMA)	×	×	33	×	56	41
<i>M. tintinnabulum</i>	Natural leachants	×	×	×	×	86	×
<i>B. amphitrite</i>	Adult extract	0.97	0.75	×	×	×	×
<i>M. tintinnabulum</i>	Adult extract	×	×	×	51	×	×
<i>S. faecalis</i>	EA extract in water ( G <sub>1</sub> - ZMA)	×	×	11	×	×	×
<i>C. sputorum</i>	EA extract in water ( S <sub>3</sub> - BPA)	×	×	26	×	×	×
<i>S. faecalis</i>	EA extract in water ( S <sub>1</sub> - ZMA)	27	21	×	×	×	×
<i>C. sputorum</i>	EA extract in water (S <sub>2</sub> - ZMA)	×	×	28	×	×	×

- No sugars were reported from the ethyl acetate extracts that were dissolved in DMSO and culture supernatants and EPS.

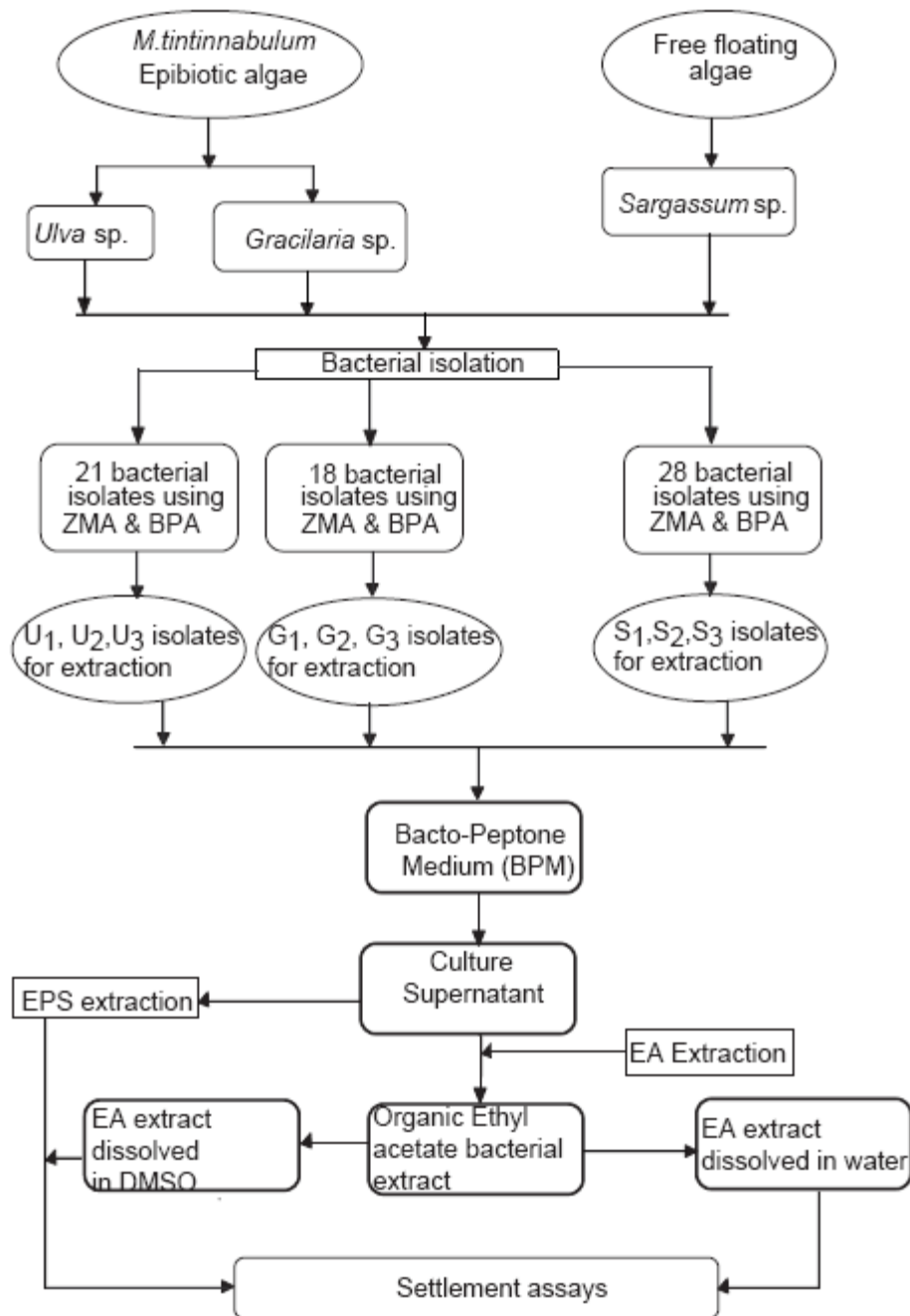


Fig. 1. Schematic representation of the experimental set-up; U-*Ulva*; G - *Gracilaria*; S-*Sargassum*; U<sub>1</sub>- *Veillonella* sp.; U<sub>2</sub>- *C. fetus*; U<sub>3</sub>- *C. fetus*; G<sub>1</sub>- *S. faecalis*; G<sub>2</sub>- *S. mitior*; G<sub>3</sub>- *B. brevis*; S<sub>1</sub>- *S. faecalis*; S<sub>2</sub>- *C. sputorum*; S<sub>3</sub>- *C. sputorum*; ZMA-Zobell Marine Agar; BPA- Bacto Peptone Agar; BPM- Bacto Peptone Medium; EPS- Exopolysaccharides; EA-Ethyl Acetate.



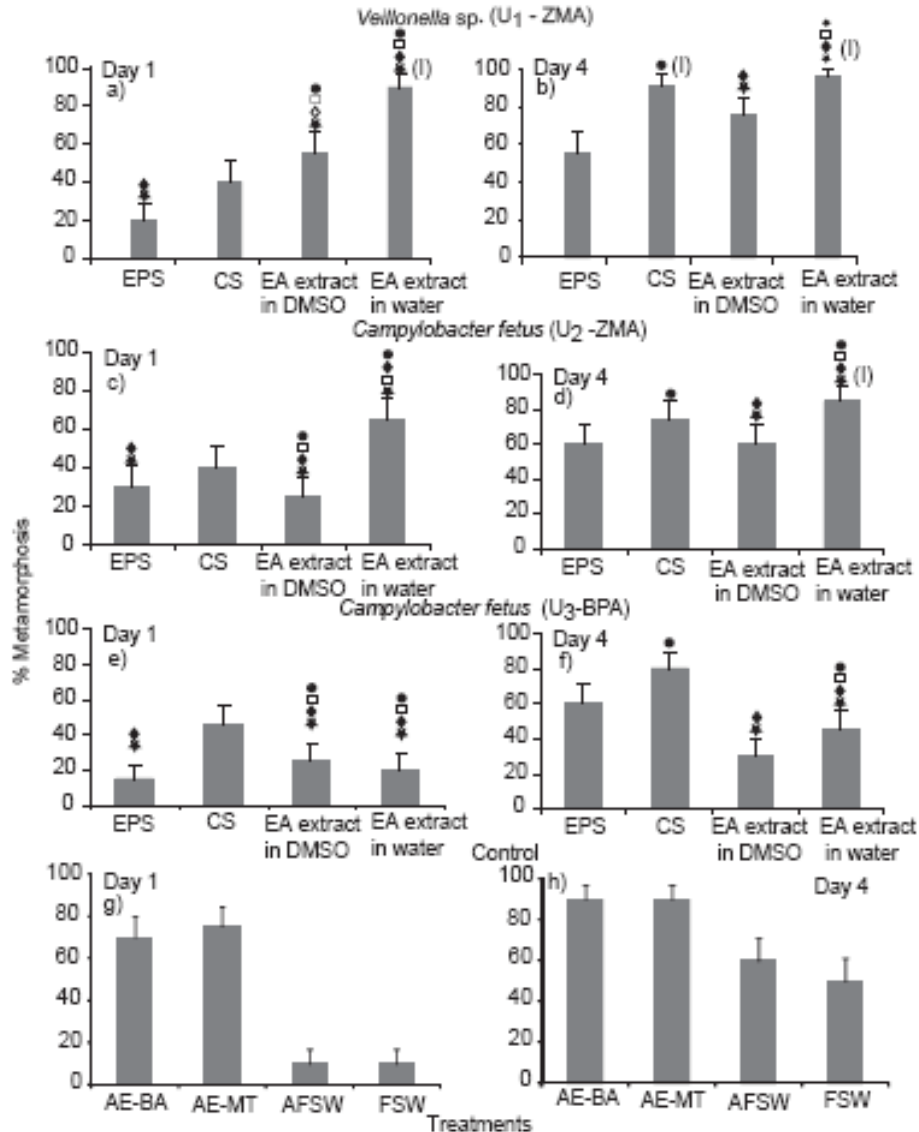


Figure. 2a-h: Influence of *Ulva* sp. associated bacterial compounds on the cyprid metamorphosis of *B. amphitrite*: EPS: Exo polysaccharides; CS : Culture supernatent; EA: Ethyl acetate; DMSO: Dimethyl sulfoxide; AE-BA: Adult extract of *B. amphitrite*; AE-MT : Adult extract of *M. tininnabulum*; AFSW: Autoclaved filtered seawater; FSW: Filtered seawater; ZMA : Zobell Marine Agar; BPA : Bacto-Peptide Agar; U - *Ulva*; I : Inductive. ◆ Significantly different with respect to AE-BA; ◻ Significantly different with respect to AFSW; ● Significantly different with respect to FSW.

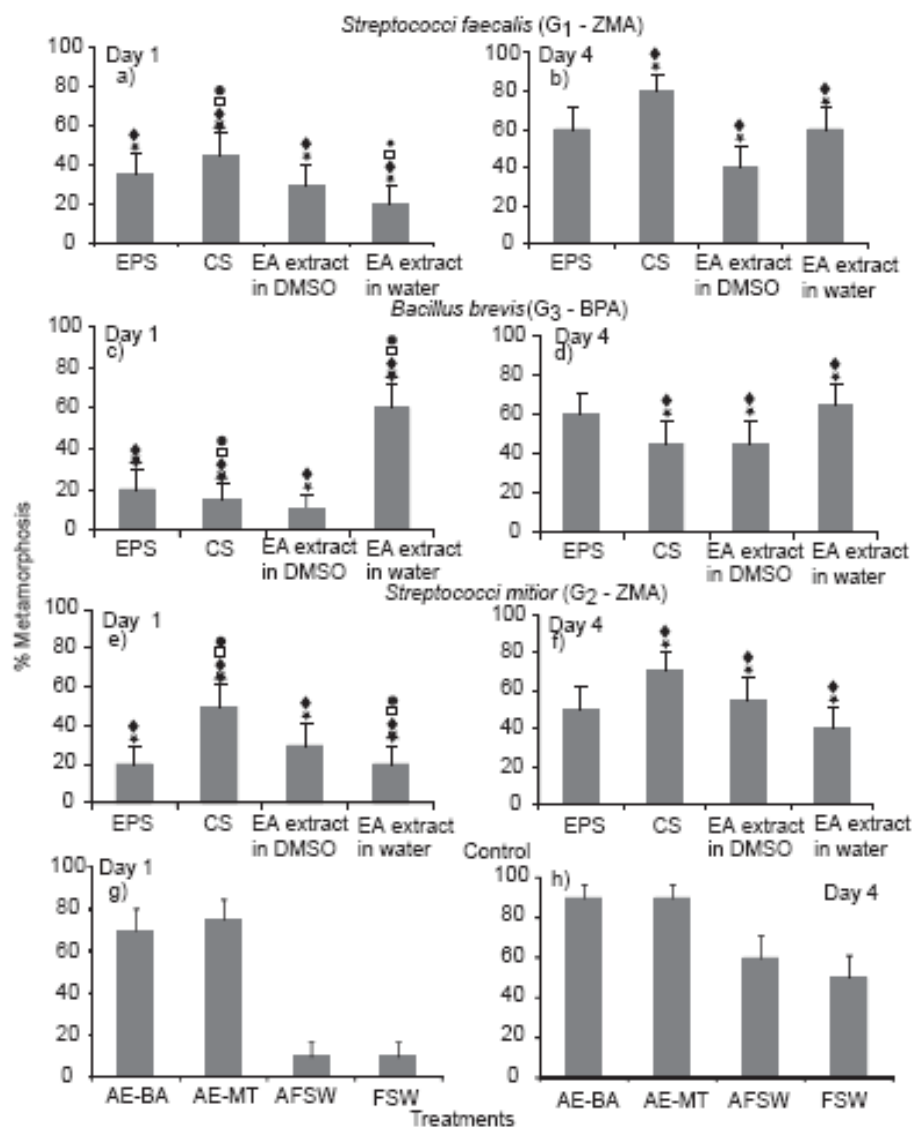


Figure. 3a-h: Influence of *Gracilaria* sp. associated bacterial compounds on the cyprid metamorphosis of *B. amphitrite*: EPS: Exo polysaccharides; CS : Culture supernatant; EA: Ethyl acetate; DMSO: Dimethyl sulfoxide; AE-BA: Adult extract of *B. amphitrite*; AE-MT : Adult extract of *M. tintinnabulum*; AFSW: Autoclaved filtered seawater; FSW: Filtered seawater; ZMA : Zobell Marine Agar; BPA : Bacto-Peptone Agar; G - *Gracilaria*; I : Inductive; ◆ Significantly different with respect to AE-BA; ◻ Significantly different with respect to AFSW; ● Significantly different with respect to FSW.

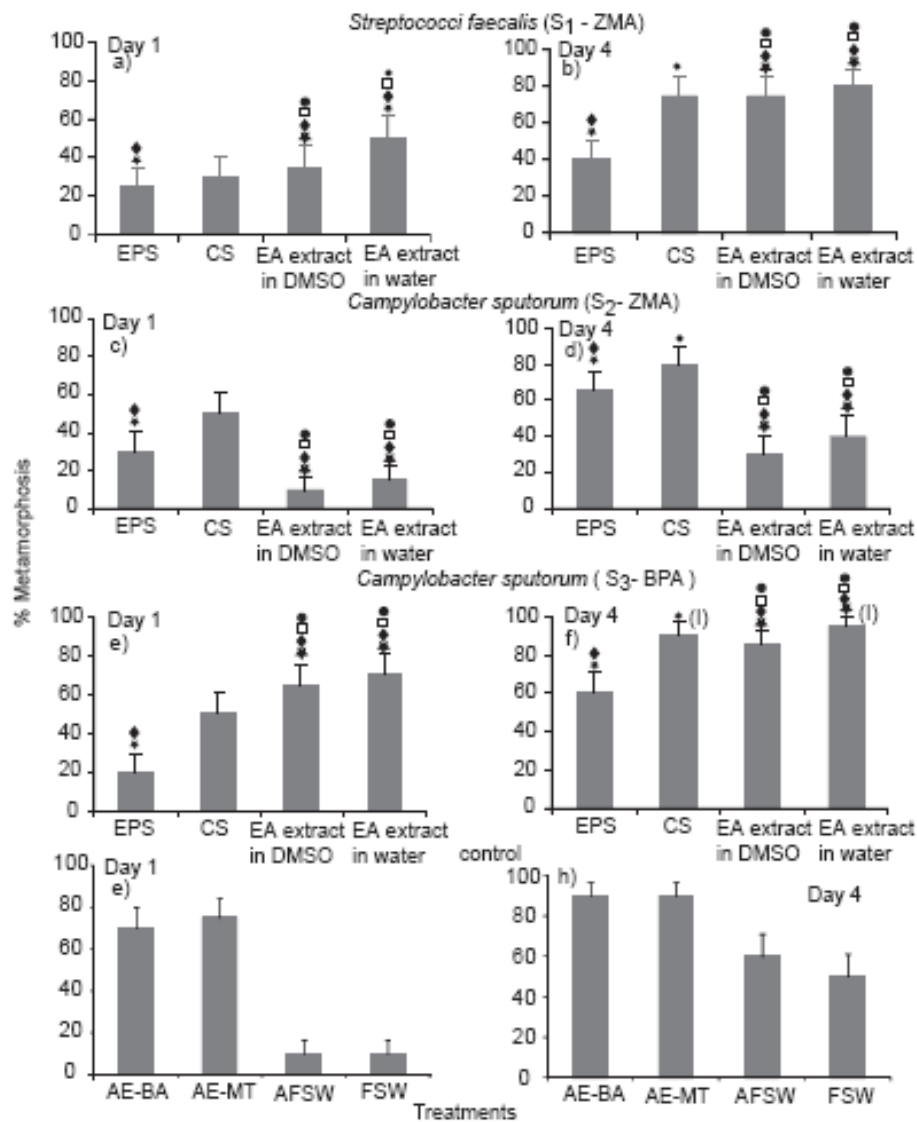


Figure. 4a-h: Influence of *Sargassum* sp. associated bacterial compounds on the cyprid metamorphosis of *B. amphitrite*: EPS: Exo polysaccharides; CS : Culture supernatant; EA: Ethyl acetate; DMSO: Dimethyl sulfoxide; AE-BA: Adult extract of *B. amphitrite*; AE-MT : Adult extract of *M. tintinnabulum*; AFSW: Autoclaved filtered seawater; FSW: Filtered seawater; ZMA : Zobell Marine Agar; BPA : Bacto-Peptone Agar; S - *Sargassum* ; I : Inductive. \* Significantly different with respect to AE-BA; ♦ Significantly different with respect AE-MT; □ Significantly different with respect to AFSW; ● Significantly different with respect to FSW.

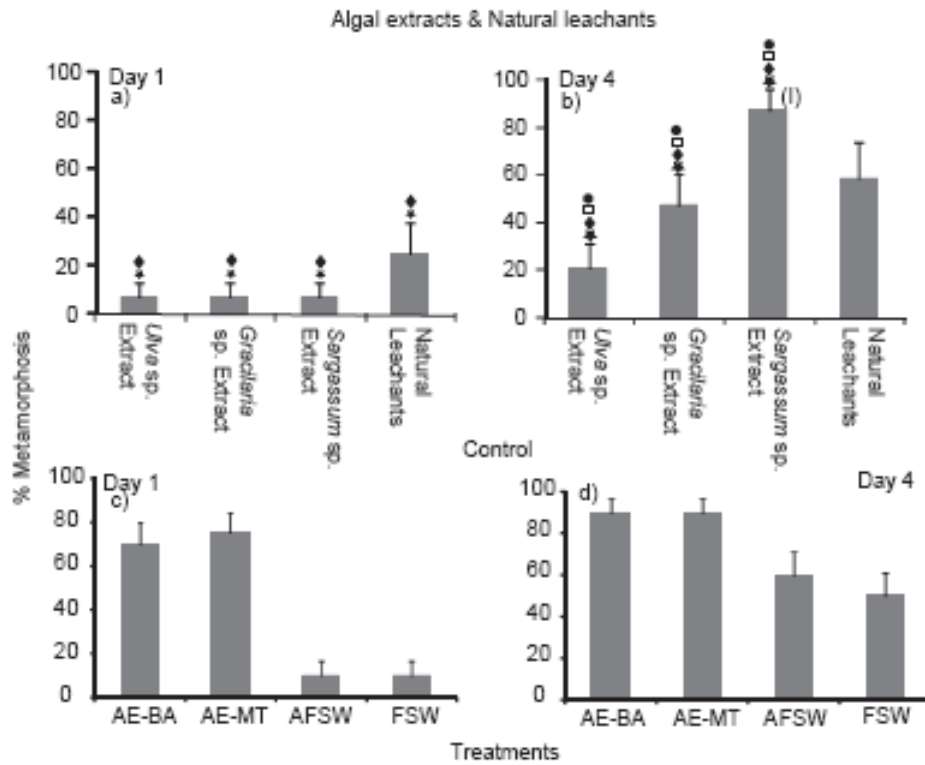


Figure. 5a-d: Influence of algal extracts and Natural leachants on the cyprid metamorphosis of *B. amphitrite*; AE-BA : Adult extract of *B. amphitrite*; AE-MT : Adult extract of *M. tintinnabulum*; AFSW: Autoclaved filtered seawater; FSW: Filtered seawater; \* Significantly different with respect to AE-BA; # Significantly different with respect to AE-MT; □ Significantly different with respect to AFSW; + Significantly different with respect to FSW; I : Inductive.

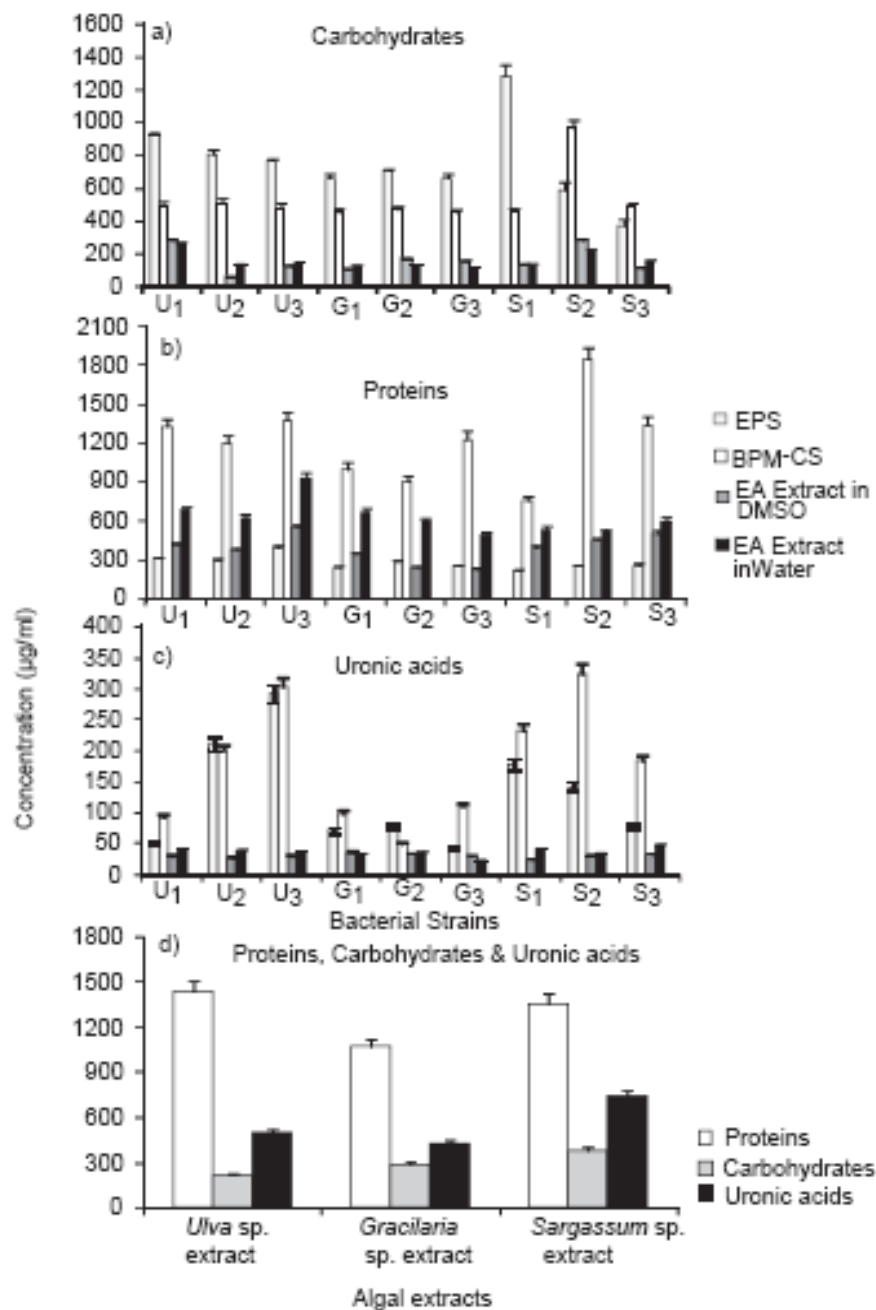


Figure. 6a-d: Proteins, Carbohydrates & uronic acids content of different bacterial strain & algal extracts; U<sub>1</sub>- *Veillonella* sp. ; U<sub>2</sub> - *C. fetus* (ZMA) ; U<sub>3</sub> - *C. fetus* (BPA) ; G<sub>1</sub>- *S. faecalis* ; G<sub>2</sub> - *S. mitior* ; G<sub>3</sub> - *B. brevis* ; S<sub>1</sub> - *S. faecalis* ; S<sub>2</sub> - *C. sputorum* (ZMA); S<sub>3</sub> - *C. sputorum* (BPA).