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## **Role of epibiotic diatoms isolated from the barnacle shell in the cyprid metamorphosis of *Balanusamphitrite***

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### **Abstract**

Barnacle shell (BSh)-associated bacteria, their culture supernatants (CS) and biofilm exopolymers (BE) are known to induce cyprid metamorphosis of *B. amphitrite*. Besides bacteria, the BSh also harbors diatoms. But, the role of their CS and BE on cyprid metamorphosis was not known and the same was evaluated using two epibiotic diatoms *Achnantheslongipes* and *Melosiranummuloides* isolated from the BSh. The CS of *M. nummuloides*, a water-borne cue, promoted cyprid metamorphosis only when isolated in the presence of BSh, whereas that of *A. longipes* inhibited. The high performance liquid chromatography (HPLC) of CS of both the diatoms indicated the dominance of arabinose, an inhibitory sugar. However, thiols were additionally present in the CS of *M. nummuloides* as indicated by fourier-transform infrared spectroscopy (FTIR) and are probably responsible for their promotory effect. The BE, a surface-bound cue, of both the diatoms facilitated metamorphosis only when isolated in the presence of BSh and were rich in N-acetyl-D-glucosamine (NAG). Thus, the interaction of NAG with the shell proteins probably provides a set of cues that facilitate cyprid metamorphosis of *B. amphitrite*.

**Keywords** Epibiotic Diatoms; *Achnantheslongipes*; *Melosiranummuloides*; Exopolymers; Cyprid; Metamorphosis

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## Introduction

Barnacles are one of the major biofoulers found in the tropics and subtropics across the globe. Their powerful adhesive property makes it very difficult to dislodge them from any substratum (Khandeparker & Anil, 2007; Maréchal & Hellio, 2011; Gohad et al., 2014). Once attached, these organisms increase the hydrodynamic drag of the vessel leading to severe financial loss in the form of fuel consumption and emission of more greenhouse gases (Abbott et al., 2000; Holm, 2012).

The barnacles have a biphasic life cycle i.e., a pelagic phase (larvae) and a benthic phase (juvenile/adult). The pelagic phase includes six naupliar instars and a cyprid instar (Desai & Anil, 2002). The nauplius I stage is non-feeding, and within 1-2 h, it molts into nauplius II stage, which feeds on phytoplankton from the water column (Desai & Anil, 2002). The nauplius II stage grows into nauplius III-VI and eventually, transforms into a morphologically distinct stage, called the cyprid (Desai & Anil, 2002; Maruzzo et al., 2012). The cyprid stage is non-feeding and its main purpose is to find out a suitable settlement site (Maruzzo et al., 2011, 2012). The cyprid bears a pair of antennules equipped with olfactory and tactile chemoreceptors for the perception of suitable water-borne and surface-bound cues, respectively from the environment (Clare & Nott, 1994; Maruzzo et al., 2011). These cues then initiate an internal signal transduction pathway resulting in the settlement and metamorphosis of cyprids (Jackson et al. 2002; Hadfield 2011). The larval settlement is defined as the process of transition from pelagic to benthic phase and can be divided into three phases: attainment of competency, attachment to a suitable substratum, and metamorphosis into juveniles which later grow into adults (Pawlik, 1992; Chen et al., 2011).

Among the barnacle species, *Balanus amphitrite* Darwin, 1854 has been used as a model organism in biofouling studies because of its wide distribution, rapid larval development and easy rearing techniques in laboratory conditions (Satuito et al., 1997; Anil et al., 2010, 2012). A vast amount of literature exists on the settlement of this species (Rittschof et al., 1984; Maki et al., 1990; Anil et al., 2001; Qian et al., 2003; Patil & Anil, 2005; Jouuchi et al., 2007; Finlay et al., 2010; Hadfield, 2011; Chen et al., 2011; Maruzzo et al., 2012). But very few studies have addressed its gregarious settlement (Khandeparker et al., 2002a, 2003, 2006; De Gregoris et al., 2012). Like any other barnacle species, juvenile and adult stages of *B. amphitrite* are gregarious in nature, i.e., they live in groups. This is essential for the barnacles because the male partner should live within the range of extensible penis for cross-fertilization (Anderson, 1994; Clare, 2011; Matsumura & Qian, 2014). The gregarious pattern of barnacle distribution is achieved during the settlement of the cyprid stage. So far, the most plausible reason for gregariousness has been attributed to chemical cues from

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adult conspecifics such as a water-borne cue (Tegtmeyer&Rittschof, 1989; Browne & Zimmer, 2001; Elbourne et al., 2008) and a tactile cue, known as settlement-inducing protein complex (SIPC) found in the shell (Matsumura et al., 1998a). It has been hypothesized that water borne cues pilot the larvae towards a parental population, while tactile cues are probably important for cyprid metamorphosis (Khandeparker et al., 2002a, 2003, 2006).

In the field, the body surface of almost all marine organisms is covered with an epibiotic biofilm comprising of microorganisms such as bacteria, diatoms, cyanobacteria, fungi and protozoans embedded in a self-produced matrix of exopolymeric substances (EPS) (Mihm et al., 1981; Wahl, 1989; Holmstrom&Kjelleberg, 1994; Kanavillil&Kurissery, 2013). These microorganisms and their secreted metabolites (EPS) in the epibiotic biofilm are known to affect the larval metamorphosis of several benthic invertebrates (Characklis& Cooksey, 1983; Armstrong et al., 2001; Wahl et al., 2012). In *B. amphitrite*, it has been reported that the epibiotic bacteria present on the outer surface of the shell (BSh) play an important role in the barnacle gregariousness by providing biochemical cues to the cyprids for settlement and metamorphosis (Khandeparker et al., 2002a, 2003, 2006). Later on, it was revealed that the bacterial community associated with the barnacle shell differs considerably from that associated with the surrounding rocks, indicating their uniqueness to the barnacle (De Gregoris et al., 2012). In addition to these bacteria, we found that *B. amphitrite* also harbors two dominant diatoms, *Achnanthes longipes* Agardh, 1824 and *Melosira nummuloides* Agardh, 1824 on the outer surface of its shell (Sahoo&Khandeparker, 2016). But their role in the gregarious settlement of this species was not known.

It is well known that the shell of *B. amphitrite* contains SIPC, which induces cyprid settlement and metamorphosis (Matsumura et al., 1998b; Dreanno et al., 2006). This SIPC probably interacts with the metabolites secreted by the epibiotic biofilm and can also modulate the cyprid metamorphosis process. A previous study has indicated that the interaction between bacteria and adult extract (contains SIPC) can alter the synthesis of bacterial cues that are responsible for the cyprid metamorphosis (Khandeparker et al., 2006). Moreover, it has been observed that the crushed barnacle shell (BSh) in the medium stimulates the growth of epibiotic bacteria and diatoms, possibly due to secretion of growth-promoting compounds from the shell (De Gregoris et al., 2012; Sahoo&Khandeparker, 2016). But, whether the BSh-diatom interaction can modulate the cyprid metamorphosis was unknown.

Keeping the above facts in mind, following questions were sought in this investigation: (1) Does the culture supernatants (CS) and biofilm exopolymers (BE) of epibiotic diatoms *A. longipes*

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and *M. nummuloides* influence the cyprid metamorphosis of *B. amphitrite* and (2) Does the BSh-diatom interaction modulate the synthesis of cues that affect cyprid metamorphosis?

## Materials and methods

### Isolation and culture of *A. longipes* and *M. nummuloides*

Few adults *B. amphitrite* were collected from the intertidal habitat of Dona Paula, Zuari estuary, Central west coast of India for isolation of diatoms, *A. longipes* and *M. nummuloides* from their epibiotic biofilm. In the laboratory, the biofilm was scraped into a petriplate containing 0.22  $\mu\text{m}$  filtered autoclaved seawater (FSW; 35 salinity). This biofilm contained the mixture of all the diatom species as well as the invertebrates. *A. longipes* and *M. nummuloides* were picked up from this mixture using a micropipette under an inverted microscope (Olympus, Japan) and their batch cultures were grown in f/2 medium (prepared in FSW) supplemented with silicate (Guillard & Ryther, 1962). It has been observed that in the batch cultures, the stationary phase of these diatoms extends from day-8 to day-14 since the day after inoculation (Sahoo & Khandeparker, 2016). During the stationary phase, diatoms are known to produce maximum EPS (Bhosle et al., 1995; Khandeparker & Bhosle, 2001), and this EPS carry a plethora of chemical cues that influence the metamorphosis of cyprids (Patil & Anil, 2005). Hence, in this investigation, EPS were isolated from the stationary phase (12 days after inoculation) of both the diatoms and their role in the cyprid metamorphosis were assessed.

For bulk isolation of EPS, batch cultures of these diatoms at an initial density of 2000 cells  $\text{ml}^{-1}$  were grown separately in polycarbonate culture flasks (7.5x7.5x10 cm, Tarsons) filled with 200 ml of f/2 medium supplemented with silicate. All the flasks were kept on a rotary shaker (Remi, India) at 80 rpm for 6 h on-off cycle to mimic the dynamic conditions which they experience in the field. The temperature was maintained at  $26 \pm 1^\circ\text{C}$  with 12:12 h cycle of dark and light ( $80 \mu\text{E m}^{-2} \text{s}^{-1}$ ). To understand the influence of barnacle shell (BSh) on the synthesis of metabolites, the diatoms were also cultured in the presence of crushed BSh (4 g in 200 ml of f/2 medium). Before use, BSh was made biofilm free by scraping with a toothbrush, autoclaved, dried and then crushed into small pieces (approximately 200-500  $\mu\text{m}$ ) as mentioned in our previous paper (Sahoo & Khandeparker, 2016). It was observed that diatoms used shell pieces as substrates for their growth. It was found that >70% cells of both the diatoms were actually found on the crushed BSh inside the culture flasks. A flask with 4g of crushed BSh in 200 ml of f/2 medium was also used as a control to know the effect of shell leachants on cyprid metamorphosis.

Isolation of culture supernatant and biofilm exopolymer from diatoms

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When the cultures reached stationary phase after 12 days of inoculation, the spent f/2 medium was first decanted into a beaker and was referred as culture supernatant (CS) (Fig.1). Subsequently, 50 ml of NaCl solution (1.5 M) was poured into the flask with attached biofilm, and all the cells were scraped by a cell scraper (BD Falcon) (Fig.1). NaCl was used to maximize the isolation of exopolymeric substances (EPS) from the biofilm as described earlier by Khandeparker&Bhosle (2001) and was referred as biofilm exopolymer (BE). Both the CS and BE were further processed by three steps: (1) sonication at 53 KHz for 20 min to disrupt the diatom clumps, (2) centrifugation at 5000 rpm and 4°C for 30 min to segregate the attached EPS from the cells and (3) filtration through a 0.2 µm polycarbonate filter paper (Millipore) to remove the bacteria (Fig.1). All the CS and BE were then concentrated by a rotary evaporator under vacuum (20°C, 60 rpm) and were used in the cyprid metamorphosis assays.

#### Preparation of adult extract

Adult extract (AE) was prepared by crushing the adults of *B. amphitrite* in a mortar using an equal weight/volume of deionized water and centrifuged as described by Larman et al. (1982). Before crushing the barnacles, the epibiotic biofilm was removed using a nylon brush. This AE was used as a positive control in the cyprid metamorphosis assays. The protein and carbohydrate content of the AE were determined using Bovine Serum Albumin (BSA) and D-glucose as standards, respectively (Lowry et al., 1951; Dubois et al., 1956). The final working concentration of protein and carbohydrate in the AE were 50 µg ml<sup>-1</sup> and 38 µg ml<sup>-1</sup>, respectively.

#### Rearing of barnacle larvae and cyprid metamorphosis assays

Mature egg sacs were collected from the *B. amphitrite* adults found in the intertidal habitat of Dona Paula (Goa, India) and carried to the laboratory in a vial containing diatom, *Chaetoceros calcitrans* f. *pumilus* Takano, 1968 as feed. As the settlement and metamorphosis of cyprids are influenced by maternal effect (Holm, 2000), the egg sacs were collected from different mother barnacles. In the laboratory, the larvae released from the egg sacs were reared in a 2 L glass beaker with filtered sea water (0.22 µm, 35 PSU) at 24±2°C and 12 h light: 12 h dark cycle. The feed was changed daily and the feed concentration was maintained at 2×10<sup>5</sup> cells ml<sup>-1</sup> (Khandeparker et al. 2002a, 2006; Khandeparker& Anil, 2011). After 5-6 days, most of the larvae got transformed into cyprids. These cyprids were siphoned out and were aged by storing them in the dark at 4°C for 2 days. It has been observed that during this aging period, cyprids store their energy and become competent (Rittschof et al., 1984; Satuito et al., 1996). Hence, two-day old cyprids were used in cyprid metamorphosis assays. Metamorphosis assay was conducted in sterile 24-well plates

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(Corning-430262, Corning, New York, USA) at  $24\pm 2^\circ\text{C}$ . The assays were conducted three times using different batches of larvae with 12 replicates in each trial ( $3\times 12=36$ ). All the assays were conducted during pre-monsoon season (March-April), and it was observed that there was no significant difference in cyprid metamorphosis between the three batches. The cyprid mortality was not observed during the assays. 50  $\mu\text{l}$  of the CS and BE of both the diatoms were inoculated into each well filled with 1 ml of FSW. AE (protein  $50\ \mu\text{g ml}^{-1}$  and carbohydrate  $38\ \mu\text{g ml}^{-1}$ ) and FSW were used as the positive and negative control, respectively. The AE was inoculated (1 ml) into each well and left for 3 h for adsorption of glycoproteins to the wells as described by Khandeparker & Anil (2011). After 3 h, the multiwells were washed thrice with FSW and were used in cyprid metamorphosis assays. Microscopic observations were carried out after 24 h (day-1) and 96 h (day-4) of cyprid inoculation into the multiwells with intermittent observations daily. Metamorphosis of cyprids to juveniles was noted down, and the average percentage metamorphosis was calculated.

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Sugar characterization of culture supernatant and biofilm exopolymer from diatoms by high performance liquid chromatography

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Sugar analyses of CS and BE isolated from the diatoms *A. longipes*, and *M. nummuloides* were carried out by a reverse phase high-performance liquid chromatography (HPLC) equipped with a refractive index detector (RID) (Agilent 1200 Series). Sugars were eluted using an Agilent Zorbax carbohydrate analysis column (4.6 mm ID x 150 mm, silica particle size  $5\ \mu\text{m}$ ) at a column temperature of  $30^\circ\text{C}$ . Acetonitrile: water (85:15) was used as a mobile phase at a flow rate of  $1.4\ \text{ml min}^{-1}$  for 35 min. The samples were run in duplicate, and the injection volume was  $10\ \mu\text{l}$  for each sample. Before the sample analysis, the instrument was calibrated with 19 different HPLC grade sugar standards (rhamnose, fucose, glucose, ribose, galactose, sucrose, mannose, arabinose, N-acetyl-D-glucosamine (NAG), maltose, xylose, fructose, lactose, mannitol, sorbitol, inositol, cellobiose, melibiose and trehalose) as described in another study (Eswaran & Khandeparker, 2014).

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Fourier-transform infrared spectroscopy of culture supernatant and biofilm exopolymer from diatoms

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Functional groups of CS and BE isolated from the diatoms *A. longipes* and *M. nummuloides* were determined by micro-KBr pellet technique using fourier-transform infrared (FTIR) spectroscopy. A fine pellet was prepared by grinding lyophilized powder of CS and BE with KBr, and a spectrum was recorded using a FTIR spectrophotometer (IRAffinity-1, Shimadzu, Singapore) in the mid-infrared (mid-IR) range of  $600\text{-}4000\ \text{cm}^{-1}$ . KBr was used as a background reference.

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Data analyses

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Before statistical analysis, all the percentage data of cyprid metamorphosis were arcsine transformed. In case of zero metamorphosis in any replicate, a value of  $1/4n$  ( $n$ =number of cyprids used in one batch experiment) was added to improve the arcsine transformation (Zar, 1996). Subsequently, the data were checked for normality by Shapiro-Wilk's test and homogeneity of variances by Lavene's test to fulfill the criteria of parametric tests. In the case of the data fulfilling assumptions of parametric tests, One-way ANOVA was applied, followed by Tukey's HSD (Honest Significant Difference), and for data not fulfilling the same, Mann Whitney's  $U$ -test was performed. The Mann Whitney's  $U$ -test was done by transforming the values to ranks and was used to compare the means between two treatments (Khandeparker et al., 2002a, 2003). All the analyses were carried out using statistical software SPSS (version 16). Data were considered statistically significant when  $p < 0.05$ .

## Results

### Effects of culture supernatant and biofilm exopolymer from diatomson cyprid metamorphosis

When the cyprids were exposed to culture supernatant (CS) and biofilm exopolymer (BE) of *A. longipes*, only the BE isolated in the presence of BSh promoted metamorphosis both on day-1 and day-4 (Fig.2A-B), and the average cyprid metamorphosis was  $44 \pm 10\%$  and  $61 \pm 12\%$  on day-1 and day-4, respectively. The CS of *A. longipes* isolated in the presence of BSh also promoted metamorphosis ( $56 \pm 19\%$ ); but the response was delayed (day-4). On the other hand, both the CS and BE isolated in the absence of BSh had no effect on the cyprid metamorphosis and the metamorphosis was significantly different from AE (Mann Whitney,  $U=0.00$ ,  $Z= -1.96$ ,  $p < 0.05$ ) (Fig.2A-B).

The CS and BE of *M. nummuloides* also promoted cyprid metamorphosis both on day-1 and day-4, when isolated in the presence of BSh (Fig. 2C-D). The average cyprid metamorphosis was  $33 \pm 5\%$  and  $53 \pm 12\%$  for CS and  $42 \pm 5\%$  and  $61 \pm 7\%$  for BE on day-1 and day-4, respectively. But the CS and BE isolated in the absence of BSh had no effect on the cyprid metamorphosis and the metamorphosis was significantly different from AE both on day-1 and day-4 (Tukey's HSD,  $p < 0.05$ ).

### Sugar composition (%) of culture supernatant and biofilm exopolymer from diatoms

The presence of BSh in the culture medium of both the diatoms had no effect on the sugar composition of BE in which N-acetyl-D-glucosamine (NAG) was dominant ( $>98\%$ ) (Table 1). On the other hand, BSh influenced the qualitative and quantitative composition of sugars in the CS of both the diatoms (Table 1). *A. longipes* synthesized arabinose (34.52%), galactose (19.54%), mannose (19.07%) and ribose (17.26%) as the major sugars in the CS isolated in the absence of BSh (Table 1). But when BSh was supplemented, although the dominant sugar was arabinose (34.00%) in

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the CS, the synthesis of mannose and inositol increased, and galactose and ribose were absent (Table 1). On the other hand, in case of CS of *M. nummuloides* isolated in the absence of BSh, melibiose was the dominant sugar (48.66%); but in the CS isolated in the presence of BSh, the type of dominant sugars varied (arabinose 45.93% and ribose 32.34%) and the synthesis of trehalose and inositol increased (Table 1).

**Fourier-transform infrared spectroscopy of culture supernatant and biofilm exopolymer from diatoms**  
 FTIR spectra of the diatom CS and BE revealed a strong, broad peak at around 3200-3500 cm<sup>-1</sup> that corresponds to the stretching vibration of N-H or -OH groups (Khandeparker et al., 2002a; Stuart, 2004; Castro et al., 2014). The peaks at around 1050-1122 cm<sup>-1</sup> indicate ether linkage (C-O stretching) of carbohydrates (Khandeparker et al., 2002a; Castro et al., 2014). Apart from these, another strong band at around 1600-1650 cm<sup>-1</sup> was observed, indicating the stretching vibration of C=O and C-N of the proteins (Stuart, 2004; Lee et al., 2013; Jia et al., 2016). This suggests that the CS and BE of both the diatoms were mainly proteinaceous in nature, irrespective of the presence/absence of BSh (Fig.3A-D; Fig.4A-D).

The weak absorption peaks at around 2800-3000 cm<sup>-1</sup> and 2500-2650 cm<sup>-1</sup> are attributed to the C-H and S-H stretching of aliphatic hydrocarbons and thiols, respectively (Stuart, 2004; Pretsch et al., 2009; Castro et al., 2014; Jia et al., 2016). These groups of compounds were synthesized by *A. longipes* in the CS and BE isolated in the absence of BSh; but in the presence of BSh, these were not detected (Fig.3A-D). Similarly, in case of CS of *M. nummuloides*, aliphatic hydrocarbons and thiols were also synthesized in the absence of BSh; but in the presence of BSh, only thiols were detected (Fig.4A, C). In contrast, the presence/absence of BSh did not reveal any variation in the BE of *M. nummuloides* (Fig.4B, D).

## Discussion

The present study evaluated the role of CS and BE isolated from the barnacle shell-associated diatoms, *A. longipes* and *M. nummuloides* on the cyprid metamorphosis of *B. amphitrite*. To the best of our knowledge, this is the first report that demonstrates the role of epibiotic diatoms in the gregarious settlement of barnacles. Previously, the role of benthic diatoms isolated either from the sediment or glass biofilm in the settlement of *B. amphitrite* was known (Patil & Anil, 2005; Jouuchi et al., 2007).

The CS of both the diatoms, *A. longipes* and *M. nummuloides* isolated in the absence of BSh inhibited cyprid metamorphosis. FTIR spectroscopy indicated the presence of aliphatic hydrocarbons in the CS of both the diatoms. These aliphatic hydrocarbons were probably responsible for the lack

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of promotion in cyprid metamorphosis. Aliphatic hydrocarbons do not possess hydroxyl groups which are important for cyprid metamorphosis, as these groups interact with the polar groups of cypris temporary adhesive (CTA) (Yule & Walker, 1987; Neal & Yule, 1996; Khandeparker & Anil, 2011). In the case of cnidarian *Hydractinia echinata*, aliphatic hydrocarbons have been reported to inhibit the metamorphosis of larvae to juveniles (Chicu & Berking, 1997). Another probable reason for inhibition in metamorphosis in case of *A. longipes* could be that the CS isolated in the absence of BSh was rich in arabinose and ribose. These sugars, although possess hydroxyl groups, may not be effective enough for their stronger binding with the polar groups of cyprids; because these are pentose sugars and contain less number of hydroxyl groups than hexose sugars (Neal & Yule, 1996). The inhibitory effect of arabinose and ribose on cyprid metamorphosis has also been reported in previous studies (Khandeparker et al., 2003; Khandeparker & Anil, 2011).

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However, when the cyprids were exposed to the CS of *M. nummuloides* isolated in the presence of BSh, the metamorphosis was promoted. FTIR spectroscopy revealed the presence of thiols in the CS. This suggests that thiols were probably responsible for their promotory effect on cyprid metamorphosis. As there is no supporting literature available on the role of thiols in the larval metamorphosis, this needs further validation. The sugar composition of the CS of *M. nummuloides* isolated in the presence of BSh revealed higher concentrations of trehalose and inositol, although it was dominated by arabinose and ribose (inhibitory sugars) (Khandeparker et al., 2003; Khandeparker & Anil, 2011). Thus in addition to thiols, the sugars such as trehalose and inositol might also be responsible for promotion in cyprid metamorphosis. This is because trehalose and inositol are hexose sugars and possess more hydroxyl groups than the pentose sugars (arabinose and ribose). Moreover, trehalose is a disaccharide consisting of two glucose (hexose) units and contains even more hydroxyl groups (–OH) than a monosaccharide. In one study, it has been reported that trehalose secreted by coral-associated zooxanthellae attracted the coral larvae (Hagedorn et al., 2015).

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On the other hand, the CS of *A. longipes* isolated in the presence of BSh showed inhibitory effect on cyprid metamorphosis on day-1. Sugar analysis of this CS revealed that although arabinose was the dominant sugar; mannose and inositol were also present in significant quantity. Thus, it can be suggested that the inhibition in cyprid metamorphosis on day-1 could be due to arabinose, which is known to be inhibitory (Neal & Yule, 1996; Khandeparker & Anil, 2011). But on day-4, the CS of *A. longipes* promoted metamorphosis, the reasons for this time-dependent response could be either due to (1) the degradation of arabinose by cyprid-associated bacteria and/or (2) the overriding effect of mannose and inositol. Besides this, the age of cyprids, which is known to influence the

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metamorphosis process, i.e., increase in metamorphosis in old cyprids due to depletion in energy reserve (Rittschof et al., 1984; Romero et al., 2013) cannot be ruled out. Mannose and inositol are hexose sugars, which possess more hydroxyl groups than arabinose causing a stronger binding with the polar groups of cyprid antennules and are hence considered as promotory sugars. In previous studies, mannose has been known to promote the cyprid metamorphosis of *B. amphitrite* (Khandeparker et al., 2002b, 2003) and is a part of glycan moiety of the settlement-inducing protein complex (SIPC), a known inductive cue for the cyprid metamorphosis (Pagett et al., 2012). The other sugar, inositol is thought to be a component of the internal signal transduction pathway that facilitates the cyprid metamorphosis (Clare, 1996).

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As observed in CS, the cyprid metamorphosis of *B. amphitrite* was also inhibited, when they were exposed to BE of both the diatoms isolated in the absence of BSh. On the other hand, it was promoted when the cyprids were exposed to BE of both the diatoms isolated in the presence of BSh. Sugar analysis revealed that N-acetyl-D-glucosamine (NAG) was the dominant sugar irrespective of the presence/absence of BSh. These results suggest that NAG alone has no role in the cyprid metamorphosis, whereas its interaction with the shell proteins probably provides a set of chemical cues that facilitate the cyprid metamorphosis. Previously, NAG has been known to play an important role in the attachment of fouling diatoms (Bahulikar & Kroth, 2008; Khodse & Bhosle, 2010). The BE of *M. nummuloides* isolated in the presence of BSh showed promotory effect despite the presence of aliphatic hydrocarbons (known inhibitors of metamorphosis), which were absent in the BE of *A. longipes*. Thus, it seems that the promotory cues in the BE of *M. nummuloides* could have masked the inhibitory effect of aliphatic hydrocarbons.

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The promotory effect of CS and BE of *M. nummuloides* isolated in the presence of BSh indicates that both water-borne, as well as surface-bound cues, are equally important for cyprid metamorphosis. This corroborates one previous study in which both leachants (water-borne) as well as surface-bound cues of bacterial exopolymers have been known to promote the cyprid metamorphosis (Khandeparker et al., 2002a). Besides barnacle, the involvement of both water-borne and surface-bound cues have also been reported in the larval metamorphosis of other marine invertebrates such as oyster and mussel (Bao et al., 2007; Vasquez et al., 2013).

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Thus far, SIPC is the only known biochemical cue responsible for the gregariousness of *B. amphitrite* (Matsumura et al., 1998a, c; Dreanno et al., 2006). Biochemically, this is a glycoprotein, and it has been proved that the protein part of SIPC is also as important as the carbohydrate part in inducing cyprid metamorphosis (Matsumura et al., 1998a; Petrone et al., 2015). Moreover, biofilm proteins are known to play an important role in the cyprid metamorphosis process (Khandeparker &

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Kumar, 2011). In the present study, FTIR spectral data revealed the proteinaceous nature of CS and BE of both the diatoms irrespective of the presence/absence of BSh. Thus, in addition to the effect of carbohydrates, the observed differences in cyprid metamorphosis could also be due to the quantity and quality of proteins (amino acid composition) in the exopolymers of diatoms. This is because side chains of certain amino acids can probably interact with the receptors found on the antennular surface of cyprids, thus affecting metamorphosis (Mishra & Kitamura, 2000). Also, it is possible that the glycoproteins may have some specific 3-dimensional folding structures and their functional groups may not be available for interaction with the polar groups of cyprid antennules. Characterization of these glycoproteins/proteins and their role in the cyprid metamorphosis could provide a deeper insight into the mechanisms of cyprid metamorphosis and is a step ahead.

### **Conclusions**

The present study has revealed that the epibiotic diatoms such as *A. longipes* and *M. nummuloides* growing on the BSh play an important role in the gregariousness settlement of barnacles. The biofilm exopolymers (BE), which are surface-bound cues of each of these diatoms, promoted cyprid metamorphosis only when isolated in the presence of barnacle shell and were rich in N-acetyl-D-glucosamine (NAG) sugar. Thus, the interaction of NAG with the shell proteins probably provides a set of chemical cues that facilitate the settlement and metamorphosis of cyprids.

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### **Conflict of Interest**

The authors declare that they have no conflict of interest.

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## Figure legends

**Fig.1** Schematic diagram illustrating the isolation of culture supernatants (CS) and biofilm exopolymers (BE) from the batch cultures of *Achnantheslongipes* and *Melosiranummuloides* both in the absence and presence of barnacle shell (BSh). MWCO refers to molecular weight cut-off

**Fig.2** Average cyprid metamorphosis (%) in response to culture supernatants (CS) and biofilm exopolymers (BE) isolated from stationary phase (12 days) batch cultures of *Achnantheslongipes* on (A) day-1 and (B) day-4 and *Melosiranummuloides* (C) day-1 and (D) day-4. The values indicate average  $\pm$  SE (n=36). AE (Adult Extract) is positive control, and FSW (Filtered Autoclaved Sea Water) is a negative control. [\* indicates significant difference ( $p < 0.05$ ) with respect to positive control]. BSh in the figure indicates barnacle shell (+ presence, - absence)

**Fig.3** Fourier-transform infrared (FTIR) spectroscopy of culture supernatants (CS) and biofilm exopolymers (BE) of *Achnantheslongipes* cultured (A-B) in the absence of barnacle shell (BSh) and (C-D) in the presence of barnacle shell (BSh)

**Fig.4** Fourier-transform infrared (FTIR) spectroscopy of culture supernatants (CS) and biofilm exopolymers (BE) of *Melosiranummuloides* cultured (A-B) in the absence of barnacle shell (BSh) and (C-D) in the presence of barnacle shell (BSh)

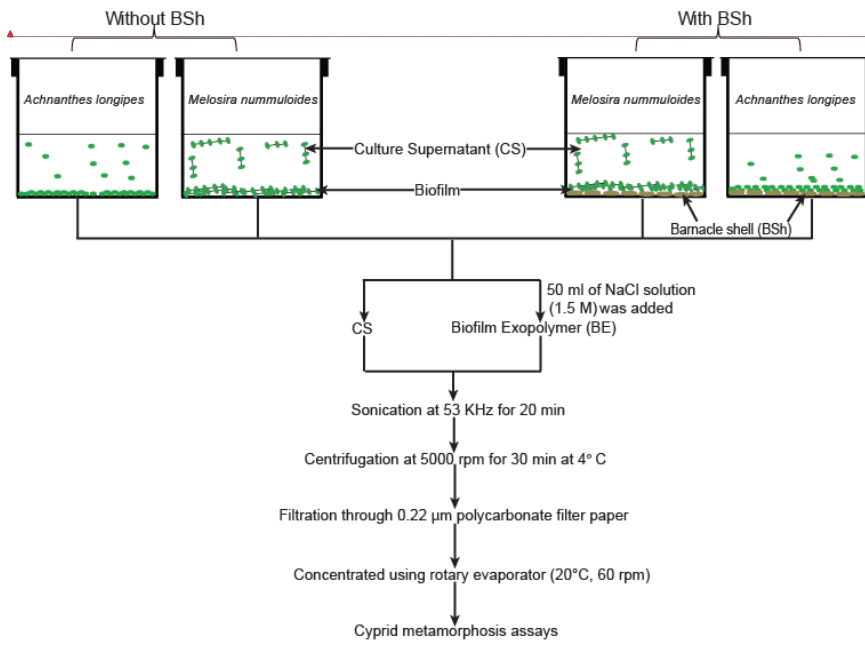
## Table legend

**Table 1** Composition (%) of sugars in the culture supernatants (CS) and biofilm exopolymers (BE) isolated from stationary phase (12 days) batch cultures of *Achnantheslongipes* and *Melosiranummuloides*

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Fig.1

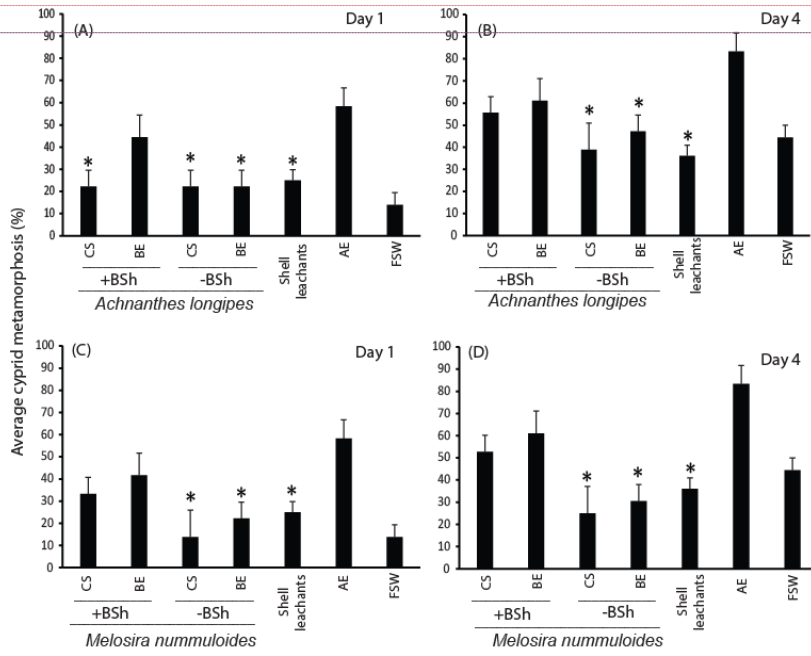


Fig. 2

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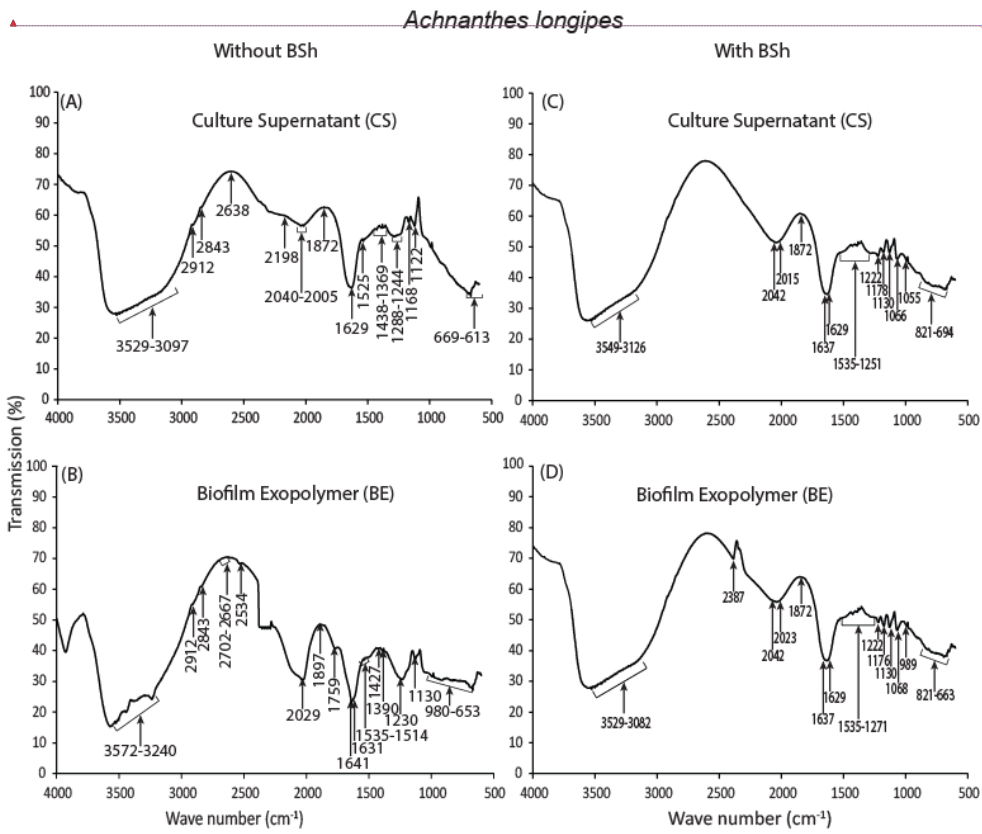


Fig.3

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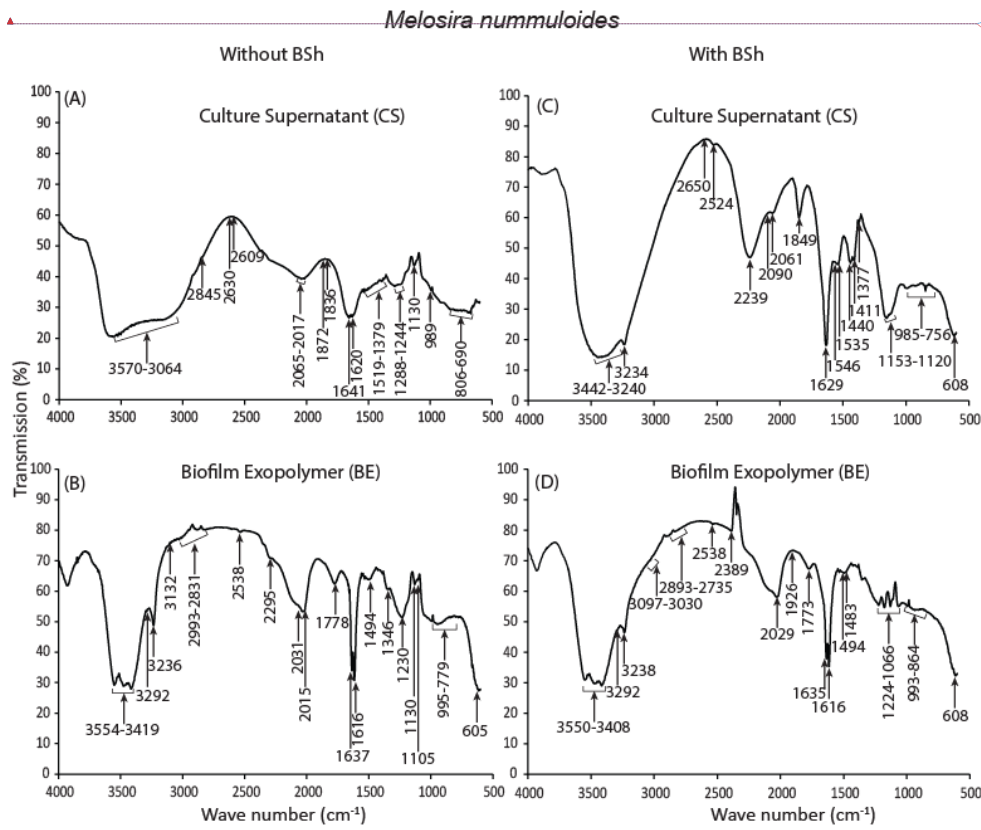


Fig. 4

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**Table 1** Composition (%) of sugars in the culture supernatants (CS) and biofilm exopolymers (BE) isolated from stationary phase (12 days) batch cultures of *Achmanthes longipes* and *Melosira nummuloides*

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