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Chemical cues inducing settlement and metamorphosis in the fouling oyster *Crassostrea madrasensis*

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Abstract

The Indian edible oyster *Crassostrea madrasensis* is an important fouling organism in the coastal waters of both east and west coasts of India. The oyster larvae were reared in the laboratory and their response to settlement and metamorphic inducers was tested. Natural biofilms developed under field (light) and laboratory (dark) conditions, a unispecies bacterial film, unialgal films of the diatom *Nitzschia* sp. and a polysaccharide organic film were tested for their ability to induce settlement and metamorphosis. Similarly, neurotransmitters like L- and D-dopa, dopamine, octopamine, epinephrine, norepinephrine, cAMP, db-cAMP, isobutylmethylxanthine forskolin and the amino acids lysine, arginine, phenylalanine and tyrosine were assessed for their inductive ability. Biofilms proved to be a potent source of inducers for the larvae. Unispecies bacterial films of the strain *Alteromonas* sp elicited maximum settlement and metamorphic response. Induction by the polysaccharide film of gum arabic revealed the involvement of adsorbed organic molecules on the settlement process. The neurotransmitter L-dopa was effective in inducing settlement and metamorphosis Maximum settlement and metamorphosis were observed at a concentration of 10^{-5} M of L-dopa. On exposure to catecholamines, epinephrine and norepinephrine, the larvae metamorphosed without exhibiting the characteristic settlement behavior Maximum metamorphosis was observed at a concentration of 10^{-4} M with respect to both the catecholamines tested. Lysine and arginine proved to be effective settlement cues to the larvae. The larvae were more sensitive to lysine than arginine. Results of the present study have revealed that substratum filming is an essential factor in the induction process and reveal the involvement of neuronal pathways in the induction of settlement and metamorphosis.

Keywords: Oyster larvae, settlement, metamorphosis, bacterial films, neurotransmitters, diatom films, organic film, natural biofilms and catecholamines.

1. Introduction

Materials immersed in natural waters become colonized by micro- and macrofouling organisms. The term biofouling refers to the settlement and growth of fouling organisms on man-made structures resulting in the loss of efficiency of the structures.[1] Almost any surface immersed in the sea gets covered with a conditioning film composed of dissolved organics[2], followed by a primary film comprising chiefly of bacteria and microalgae.[3] The adhesion of diatoms, fungi and cyanophytes follows adhesion by bacteria.[4] Finally, the grazers, namely, flagellate and other protozoans colonize the substratum resulting in the formation of a complex primary film.[5] The formation of microbial films on submerged surfaces has long been thought to influence the attachment of many invertebrate larvae and the development of a fouling community. Primary films can induce or inhibit settlement, depending on the organisms.[6]

Larvae of many invertebrate species are induced to settle and metamorphose after coming in contact with components associated with the substratum.[7,8] The cues may be associated with the adsorbed organic layers (proteinaceous or lipid in nature) or with exopolymeric substances secreted by the colonized bacteria.[9] Alternatively, the cues may also be products released by the microorganisms colonizing the substratum.[10] Although there is evidence that at least some of the stimuli inducing settlement and metamorphosis in the larvae may be chemical, the influence of each component of the biofilm on the process of induction of a particular invertebrate larval species has not been completely characterized.

Laboratory studies on the settlement and metamorphic behavior of a wide variety of marine invertebrates have shown to be controlled by larval sensory recognition and responsiveness to exogenous chemical and other environmental stimuli.[11–15] Even though settlement and metamorphosis of many benthic marine invertebrate larvae is known to be chemically mediated, very few natural inducers have been identified.

Settlement of invertebrate larvae results in the culmination of a complex successional sequence that begins initially with the adhesion of microbes.[15] The inductive component associated within the biofilms is poorly understood as a result of the complexity involved in isolating an inductive component.[15] In addition to studies on natural inducers of settlement and metamorphosis, artificial inducers, namely, neurotransmitters, which mimic the action of natural compounds, have been extensively investigated in many marine invertebrate species.

Choline, a bound constituent of cell membranes and precursor of the neurotransmitter acetylcholine, has been found to induce the settlement of larvae i) by acting directly on cholinergic receptors, ii) as precursor of acetylcholine and iii) by stimulating synthesis and release of catecholamines.[16,17] L-3-4-dihydroxyphenylalanine (L-dopa) and the catecholoamines, namely, dopamine, adrenaline and noradrenaline, are derivatives of tyrosine and function as neurotransmitter hormones, pigments and adhesive structural proteins. γ-aminobutyric acid, a product of glutamic acid decarboxylation, is known to hyperpolarize post-synaptic membranes by increasing membrane permeability to Cl^- ions.[18] The role of adenosine-5-cyclic monophosphate, a mediator of cellular metabolism and cell-to-cell signaling in the settlement and metamorphosis of invertebrate larvae, has been studied. Dibutyryl-cAMP, a derivative of cAMP, known to increase in intracellular cAMP levels, has been tested extensively.

This study deals with environmental cues of chemical and biological nature that influence the induction of larval settlement (substratum exploration) and the metamorphic response in the Indian edible oyster *Crassostrea madrasensis*. The response of larvae to natural biofilms developed under field (light) and laboratory (dark) conditions has been investigated. Light is an important factor of ecological significance in the aquatic environment. Biofilms developed on illuminated surfaces in natural condition could be expected to be different from those growing on surfaces devoid of light. Biofilms developed under field conditions support a greater variety of photosynthetic organisms (like the green algae and diatoms).

The larval response to individual components of biofilm was also investigated. The influence of bacterial species colonizing these biofilms was assayed individually to assess the ability of these bacteria to induce metamorphosis. Similarly, the influence of films formed by the pennate diatom *Nitzschia* sp. and their active component of diatom films was also investigated. The influence of organic films such as gum arabic and protein surface films and film of some

of the commercially available fatty acids was also investigated. The ability of different neurotransmitter and bioactive substances on the settlement and metamorphosis of oyster larvae was also investigated.

2. Materials and methods

2.1. *Terminology*

The terms 'competent', 'settlement', 'metamorphosis' and 'recruitment' need to be defined for this study. The recognition of these phases is important for researchers seeking chemical cues that may generate the behavioral (settlement) and morphogenetic (metamorphosis) response in larvae. Planktonic larvae are generally not able to metamorphose until they become mature or 'competent'. A larva is said to be 'competent' when it has entered a physiological state in which it is capable of metamorphosis when given a proper environmental stimulus. This state is often marked by a set of recognizable morphological characteristics. The time required for an young larva to become competent is species-specific. Competent pelagic larvae delay settlement and metamorphosis for variable periods of time, until they encounter a suitable substratum or environmental cue.[19-21] The ability of the competent larvae to respond to environmental cues usually coincides with the development of certain sensory receptors.[16] Several authors have defined the process of settlement and metamorphosis depending on the organism. The definition by Scheltema[7] has been followed in the present study. The term settlement is a behavioral response resulting in the termination of a pelagic, larval life and the transformation to a sessile or nonsessile sedentary life. The term metamorphosis by usage is an embryological or morphogenetic term and is aptly defined as the morphological and physiological changes that transform the individual to a new way of life. The term 'recruitment' implies the lapse of some period of time after the process of settlement and metamorphosis. Recruits have also been defined as newly settled individuals that have grown to a specific size after their settlement.[22, 23]

Sea-water media: All experiments were conducted in aged, 0.22-μm filtered, autoclaved sea water with a salinity of 34 ppt.

2.2. *Artificial sea-water media*

Experiments using artificial inducers were conducted in standard MBL sea-water media,[24] the composition of which is as follows: NaCl 423.0 mM, KCl 9.00 mM, $CaCl_2$ 9.27 mM, $MgCl_2$ 22.94 mM, $MgSO_4$ 25.50 mM, $NaHCO_3$ 2.15 mM dissolved in glass distilled water. The final pH values of all standard and modified media were adjusted to 7.4.

Settlement assays: All experiments were conducted in triplicate. The standard settlement assay procedure outlined by Murthy *et al.*[25] was followed except in the case of specific changes in some assays with respect to some treatments which are mentioned below.

2.3. *Assays with natural biofilms developed under field (light) and laboratory (dark) conditions*

To generate natural biofilms, developed in field (light) conditions, the bottom halves of Petri dishes (50-mm dia) were placed in rectangular cages after covering them with a plankton net of

60-μm mesh size (to prevent larvae of other fouling invertebrates from settling on the dishes). Five-day-old natural biofilms were thus generated using glass and polystyrene Petri dishes. To generate natural biofilms, developed in laboratory (dark) conditions, the bottom halves of Petri dishes were placed in a 100-l fiber-reinforced plastic tank with provision for overflow. Raw sea water was continuously pumped into the tank. The sea-water inlet was covered with plankton netting of mesh size 60 μm to prevent larvae of other invertebrates from settling on the dishes. Five-day-old natural biofilms were thus generated using glass and polystyrene Petri dishes. A set of plates was simultaneously removed for enumeration of bacteria after staining with crystal violet. Encrusting algae and bacteria were counted using bright field microscopy. The Petri dishes were periodically removed for assay, rinsed and filled with 0.22-μm filtered sea water and the larvae released into them. Sterile Petri dishes served as controls.

2.4. *Assays with organic film*

Gum arabic was used for developing the organic film. It was developed according to the method of Kirchman *et al.*[26] About 30 g of gum arabic was dissolved in 100-ml distilled water by gentle warming and the solution was poured into sterile Petri dishes. The dishes were allowed to stand overnight, after which they were rinsed with sterile sea water. The dishes were filled with 0.22-μm filtered autoclaved sea water and used for assay. Sterile Petri dishes without gum arabic served as controls.

2.5. *Assay with bacterial films*

The bacterial strain *Alteromonas* sp. (isolated from biofilms on adult oyster shells) was grown up to early stationary phase in Zobell marine broth, harvested, washed and suspended in phosphate-buffered saline (cell concentration of 2.4×10^3 cfu/ml and pH 7.4). 10 ml of the bacterial suspension was incubated in the Petri dishes. After 24 hours, the bacterial suspension was removed using a micropipette and the dishes rinsed with sterile sea water and filled with 0.22-μm filtered sea water and the larvae released into them. Sterile Petri dishes without surface films served as controls.

2.6. *Assays using unispecies diatom films*

A pure culture of the marine diatom *Nitzschia* sp. was obtained as gift from the culture collection of Prof. V. N. Raja Rao, Center for Advanced Study in Botany, University of Madras. They were maintained in unialgal condition in culture flask containers in F/2 medium[27] at 1,500–4,500 lux (12 hD and 12 hL) at 24 + 1°C.

The diatoms were removed from the flask, concentrated by centrifugation at 1500 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 5 ml of sterile sea water and the algal concentration determined using a haemocytometer. 5 ml of this suspension was then dispensed into sterile Petri dishes and left undisturbed for 24 h for the attachment of the diatoms and for the formation of film. After 24 h, the dishes were rinsed with sterile sea water and filled with 10 ml of sterile sea water and the larvae released into them.

2.7. *Assays with protein surface films*

Bovine serum albumin (fraction V fat-free, Sigma) was dissolved at a concentration of 100 mg in 5 ml of phosphate-buffered saline (pH 7.4). 5 ml of this solution was poured into polystyrene Petri dishes (50-mm dia) and the dishes were incubated at 25°C. Prior to assays, the dishes were rinsed with sterile sea water and filled with 0.22-μm filtered, autoclaved sea water and the larvae released into them.

2.8. *Assays with lipid and fatty-acid films*

Cholesterol was dissolved at a concentration of 100 mg in 5 ml of methanol. This solution was poured into polystyrene Petri dishes and methanol was allowed to evaporate. Prior to assays, the dishes were filled with 10 ml of 0.22-μm filtered sea water and the larvae released into them. Similarly, different fatty acids, viz. lauric, behonic, capric, arachidonic, myristic, stearic and palmitic acids (Sigma) were tested for their ability to induce settlement and metamorphosis. The fatty acids dissolved in methanol solution were then poured into polystyrene Petri dishes and methanol was allowed to evaporate overnight. Prior to assays, the dishes were filled with 0.22-μm filtered, autoclaved sea water and the larvae released into them.

2.9. *Amino-acid derivatives*

Different amino acids such as lysine, arginine, phenylalanine, tyrosine, tryptophan and γ-aminobutyric acid (Sigma) were tested for their ability to induce settlement and metamorphosis in oyster larvae. The compounds were prepared as a 10^{-3} M stock solution which was serially diluted to get solutions of decreasing concentrations up to 10^{-10} M. The solutions were filtered through 0.22-μm Millipore filter and 10 ml of each solution was dispensed into sterile borosilicate glass Petri dishes and the larvae were released into them.

2.10. *Tyrosine derivatives*

Neuroactive molecules such as L- and D-dopa, dopamine, octopamine, epinephrine and norepinephrine were obtained from Sigma. Test solutions were prepared by the procedure outlined by Coon *et al.*[28] The compounds were prepared initially as a 10^{-3} M stock solution. This solution was then serially diluted to obtain solution of decreasing molarity. The compounds were tested at concentrations ranging from 10^{-3} to 10^{-10} M to assess their inductive capability. Oxidation of catechol moieties was observed with L- and D-dopa in sea water at concentrations of 10^{-3} and 10^{-4}M resulting in decoloration of sea water.

Epinephrine and norepinephrine were dissolved in 0.0005 N HCl and diluted (1:8) in artificial sea water and used for assay.[28] Varying concentrations (10^{-3} to 10^{-10} M) were prepared. The larvae were exposed to these compounds for a period of one hour. After exposure, the larvae were removed, rinsed in fresh filtered sea water and then suspended in 10 ml of filtered sea water in polystyrene Petri dishes (50-mm dia). The number of larvae metamorphosing was assessed for up to 48 hours.

2.11. *Choline derivatives*

Choline Cl, acetylcholine chloride and acetylcholine iodide (Sigma) were dissolved in 0.22-μm filtered artificial sea water immediately prior to use.[29] The compounds were prepared initially

as a 10^{-3} M-stock solution. This solution was then serially diluted to obtain solutions of 10^{-3} to 10^{-10} M.

2.12. *Cyclic adenosine monophosphate and dibuteryl cyclic adenosine monophosphate*

The compounds cyclic AMP and dibutyeryl cyclic AMP (Sigma) were tested at concentrations of 2, 5, 10 μM to assess their inductive capability. The compounds, isobutylmethylxanthine and forskolin (Sigma), were also tested after dissolving in 0.22-μm filtered artificial sea water immediately prior to use.[29, 30] The compounds were tested at concentrations of 2, 5 and 10 μM to assess their inductive capability. The larvae were exposed to these compounds for a period of 30 min, removed and rinsed in fresh filtered sea water. The larvae were then suspended in 10 ml of 0.22-μm filtered, autoclaved sea water in Petri dishes. The number of larvae settling and metamorphosing was assessed up to 48 hours.

2.13. *Statistical methods*

The percentage of larvae settling and metamorphosing in triplicate test and control dishes was calculated. Student 't' test was used to compare the percentage settlement and metamorphosis in treatments against controls. One-way ANOVA[31] was used to test whether settlement and metamorphosis after different time periods differed with respect to different treatments against controls.

3. Results

3.1. *Assays with natural biofilms developed under field (light) and laboratory (dark) conditions*

Bacteria were the dominant group of microorganisms in the multispecies biofilms. In general, four types of bacterial colonies (*Alteromonas* group, *Flexibacter/flavobacterium* group, *Serratia* sp., and *Pseudomonas* sp.) were found to be dominant in all the biofilms developed. Among the microalgae, pennate diatoms (*Nitzschia* sp. and *Navicula* sp.) were dominant with the appearance of blue-green algal mats on 5-day-old biofilms developed under field (light) conditions. In comparison, biofilms developed in the laboratory in dark were characterized by a few diatom species and the absence of blue-green algal mats. The 5-day-old biofilms developed under both field (light) and laboratory (dark) conditions were characterized by heavy entrapment of sediment particles. The percentage of oyster larvae settling and metamorphosing in response to biofilms was significantly higher, compared to controls (Table I) in both the biofilms developed under field (light) ($P < 0.0001$) and laboratory (dark) ($P < 0.0001$) conditions.

Comparison of settlement and metamorphosis on biofilms developed under light and dark conditions showed no significant difference ($P < 0.0001$). These results revealed that substratum filming is a prerequisite for induction of settlement and metamorphosis whereas qualitative changes in the biofilm composition (as a consequence of development under light or dark conditions) did not significantly alter the ability of the biofilms to induce settlement and metamorphic response in larvae.

Table I
Induction of settlement and metamorphosis in *Crassostrea madrasensis* larvae in response to different treatments

Treatments	% Settlement*	% Metamorphosis*
Sea-water control	12.6 ± 1.1	5 5 ± 0.4
Fatty-acid film (lauric acid)	37.2 ± 0.4	21.4 ± 0.8
Fatty-acid film (cholesterol)	46.2 ± 1.2	31.2 ± 0.8
Fatty-acid film (behonic)	38.7 ± 1.7	17.5 ± 0.8
Fatty-acid film (arachidonic)	39 0 ± 0.6	25.0 ± 0.6
Fatty-acid film (myristic)	39 0 ± 0.6	24.4 ± 0.8
Fatty-acid film (stearic)	37.4 ± 2.9	21.4 ± 2 9
Fatty-acid film (palmitic acid)	38.4 ± 2 1	24.0 ± 1.2
Organic film (gum arabic)	54.0 ± 0 2	27.5 ± 0.6
Protein film (bovine serum albumin)	56.5 ± 0.9	35 1 ± 3 4
Diatom films of *Nitzschia* sp.	74 2 ± 0.2	48 2 ± 0.8
Five-day-old biofilms, lab (dark) conditions	79.5 ± 0.5	42.7 ± 0.8
Five-day-old biofilms, field (light) conditions	84.5 ± 2.5	65.1 ± 2.3
Bacterial film of species *Alteromonas* sp	90.4 ± 0.7	67.6 ± 0.6

*Percentage settlement and metamorphosis represent the fraction of the total larval population that settled and metamorphosed.

3.2. *Assays with organic film*

Settlement behavior was initiated within 15 min of exposure to this substratum. Percentage settlement and metamorphosis on organic film-coated substrata were significantly higher ($P < 0.0001$) compared to sea-water controls (Table I).

3.3. *Assay with bacterial films*

Competent larvae of *C. madrasensis* settled and metamorphosed in response to *Alteromonas* sp. biofilms. Significant differences in percentage settlement ($P < 0.0001$) and metamorphosis ($P < 0.0001$) in response to the bacterial film compared to sterile control Petri dishes were observed (Table I).

3.4. *Assays using diatom films*

C. madrasensis larvae were induced to settle and metamorphose in response to the diatom films tested (Table I). Significant differences were observed in the number of larvae settling and metamorphosing in response to diatom films compared to sterile Petri dish ($P < 0.0001$).

3.5. *Assays with protein surface films*

Protein surface films were offered to the larvae to assess their ability to induce settlement and metamorphosis in oyster larvae. The protein bovine serum albumin proved to be a potential inducer of settlement and metamorphosis (Table I). Significant differences in the percentage

settlement ($P < 0.0001$) and metamorphosis ($P < 0.0001$) were observed with respect to sterile control Petri dishes.

3.6. *Assays with lipid and fatty-acid films*

Cholesterol and all the fatty acids tested (lauric, behonic, capric, arachidonic, myristic, stearic and palmitic acids) proved to be potential inducers of settlement and metamorphosis in oyster larvae (Table I). Significant differences in the percentage settlement and metamorphosis were observed with respect to fatty acid and cholesterol surface films, compared to sterile control Petri dishes.

3.7. *Effect of amino-acid derivatives on settlement and metamorphosis*

Of the different types of amino acids tested, only lysine and arginine induced increased levels of settlement and metamorphosis. Lysine (with a second amino group placed at the α-position on its aliphatic chain) evoked the maximum settlement and metamorphic response followed by arginine (which contains a guanidium group and is strongly positively charged at pH 8.0). The amino acids, phenylalanine and tyrosine, exhibited low levels of settlement and metamorphosis (Table II). All the other amino acids evoked comparatively low levels of settlement and metamorphic response. The amino-acid derivative GABA did not elicit any settlement behavior at any of the concentrations of the compound tested.

3.8. *Effect of choline derivatives on the settlement and metamorphosis*

The compounds, choline Cl, acetylcholine Cl and acetylcholine iodide, did not induce settlement and metamorphosis in the larvae at any of the concentrations of compound tested (Table II).

3.9. *Effect of tyrosine derivatives on the settlement and metamorphosis*

L-dopa was observed to induce significant settlement and metamorphosis only at 10^{-5} M, and not at other (lower or higher) concentrations tested. Prolonged exposure of larvae at higher concentrations (10^{-3} and 10^{-4} M) of L-dopa caused mortality of the larvae. At lower concentrations (10^{-6} to 10^{-10} M) no significant difference in the percentage settlement and metamorphosis with controls was observed (Table II). D-dopa, (the isomer of L-dopa), dopamine and octopamine did not induce settlement or metamorphosis in oyster larvae. Exposure to D-dopa at concentrations of 10^{-3} to 10^{-4} M was found to be toxic to the larvae with effects similar to those observed with L-dopa.

The larvae metamorphosed in response to the neurotransmitters epinephrine (EPI) and norepinephrine (NE) in a concentration-dependent manner. When exposed to EPI and NE, the larvae did not exhibit the stereotypical search and crawl behavior. The larvae sank to the bottom within minutes and metamorphosed. Maximum induction was observed at concentration of 10^{-4} M EPI and NE. The number of larvae metamorphosing was found to be higher with EPI than with NE (Table II).

Table II

Effects of selected compounds tested against settlement and metamorphosis of the Indian edible oyster *C. madrasensis*

Compound	Concentration (M)	Settlement *	Metamorphosis*
AMINO-ACID DERIVATIVES			
Sea-water controls		13.6±0.0	5.5±0 4
GABA	10^{-3} to 10^{-10}	11.7±0.8 to 13.6±0.0	6.5±0 9 to 5.5±0.4
Lysine	10^{-3} to 10^{-10}	63.8 ±1.3	36.4±5.7
Arginine	10^{-3}	53.5±0 9	35.5±0.9
	10^{-4}	53.5±0.9	35.5±0.9
	10^{-5} to 10^{-10}	42.9±0.8 to 27.3±0.8	27.2 ±0.8 to 13 3± 0.4
Phenylalanine	10^{-3} to 10^{-10}	11.9± 0.4 to 9.7± 0.7	4.5 ±0.4 to 5.5 ±0.4
Tyrosine	10^{-3}	25.0±0.3	12.0±1 1
	10^{-4} to 10^{-10}	10.0±1.1 to 7.4±0.4	5.5±0.4 to 5.1± 0.4
CHOLINE DERIVATIVES			
Sea-water controls		13.6±0.0	5.5±0.4
Choline chloride	10^{-3} to 10^{-10}	10.0±1.2 to 13.6 to 0.0	5.2±0.4 to 5.1±0.4
Acetylcholine chloride	10^{-3} to 10^{-10}	6.5±0.4 to 13.6±0.0	5.2±0.4 to 5.5±0.4
Acetylcholine iodide	10^{-3} to 10^{-10}	7.4±0.9 to 13.6±0.0	5.1±0 4 to 5.5±0.4
TYROSINE DERIVATIVES			
Sea-water controls		13.6±0.0	5.5±0.4
L-dopa	10^{-3}, 10^{-4}	#	#
	10^{-5}	51.2±0.6	28.5±1.9
	10^{-6} to 10^{-10}	11.6±0.7 to 13.6±0.0	5 8±0.5 to 5.5±0.4
D-dopa	10^{-3},	#	#
	10^{-4}	17.2±0.9	14.9±0.7
	10^{-5} to 10^{-10}	13.0±0.3 to 13.6±0.0	5.2±0.4 to 5.5±0.4
Dopamine	10^{-3} to 10^{-10}	8.6±0.4 to 13.6±0.0	5.5±0.4 to 5.5±0.4
Octopamine	10^{-3} to 10^{-10}	8.7±0.0 to 13.6±0.0	5.8±0.8 to 5.5±0.4
Epinephrine	10^{-3}	—	17.9±0.3*
	10^{-4}	—	86.3±0.0*
	10^{-5}	—	62 6±0.7*
	10^{-6}	—	20.1±1.2*
Norepinephrine	10^{-3}	—	18.4±0.6*
	10^{-4}	—	60.1±0.6*
	10^{-5}	—	37.9±0.6*
	10^{-6}	—	21.6±0.4*
COMPOUNDS INFLUENCING INTRACELLULAR cAMP			
Sea-water controls		13.6±0.0	5.5±0.4
Db-cAMP	10^{-3} to 10^{-10}	—	—
cAMP	10^{-3} to 10^{-10}	—	—
IBMX	2–10 10^{-6}	#	#
Forskolin	2–10 10^{-6}	7.8±0.0 to 13.6±0.0	3.5±0.4 to 5.5±0.4

—no effect, *percentage of larvae induced to metamorphose without settlement behavior, # toxic or inhibitory effects

GABA (γ-aminobutyric acid); L-dopa l-dihydroxyphenylalanine; D-dopa d-dihydroxyphenylalanine cAMP 3`5`-cyclic adenosine monophosphate; db-cAMP dibutyrl cAMP; IBMX isobutylmethylxanthine.

*Percentage settlement and metamorphosis represent the fraction of the total larval population that settled and metamorphosed

3.10. *Effect of cAMP and db-cAMP on settlement and metamorphosis*

The compounds, cAMP and db-cAMP, did not induce settlement behavior and metamorphosis in the larvae of *C. madrasensis* at all concentrations tested (Table II). The compounds isobutylxanthine and forskolin also did not induce settlement behavior and metamorphosis in the larvae of *C. madrasensis* at any of the concentrations tested.

4. Discussion

Settling larvae are known to respond to several factors such as surface-associated features, e.g. texture, contour, roughness, wettability, microbial biomass/density and organic substances. Simulated laboratory experiments help us in gaining insight into the conditions preferred by larvae for settling on a particular substratum.

The results of the present study demonstrated that pediveliger larvae of the oyster *C. madrasensis* are induced to settle and metamorphose in response to a variety of environmental stimuli such as provided by natural biofilms. Even though field(light)-developed biofilms displayed a greater variety of algae and other protozoans, settlement and metamorphic rate were significantly different from those on laboratory(dark)-developed biofilms. It can be inferred that settlement and metamorphosis of *C. madrasensis* larvae may be more dependent on the species of microbes present rather than on the microbial biomass.

Results show that an organic film formed by gum arabic (a polysaccharide) was capable of inducing settlement and metamorphosis, though at a lower level when compared to other treatments tested. The results suggest that macromolecular organic compound comprising the 'conditioning' film[2] may also trigger settlement and metamorphosis and may constitute an important source of natural inducer in the environment. The low percentage of settlement and metamorphosis on organic films, when compared to natural biofilms, unispecies bacterial films and diatom films may suggest a lower concentration of the inducing moiety.

Larvae of the oyster *C. madrasensis* settled and metamorphosed in response to monospecific bacterial films (*Alteromonas* sp.) at substantially high percentages compared to natural bio and diatom films. The data indicate that in the natural environment, bacterial films may induce settlement of *C. madrasensis*. It is probable that bacterial films release an inducer of settlement and metamorphosis or alternatively the inducing moiety is associated with the cell surface or exopolymer of the bacteria. Earlier studies have investigated the possible role of ammonia,[32] a byproduct of protein catabolism and L-dopa, a melanin precursor[33] secreted by some marine bacteria, on oyster settlement and metamorphosis.

Induction of settlement and metamorphosis by diatom films indicates that diatom films may also be involved in the induction process in the natural environment. Induction by protein and lipid surface films reveals the involvement of these compounds in the induction of settlement and metamorphosis other than those already documented for bacterial films and natural biofilms.

Induction by fatty-acid surface films suggests the involvement of signal transduction pathways. Free fatty acids are known to perturb membrane and affect the signal transduction path-

ways.[34] Similar results have been observed for the polycheate worm *Phragmatopoma lapidosa californica* which has been induced to settle in response to free fatty acids.[29]

The present study demonstrates that oyster larvae can be induced to metamorphose by short exposures to millimolar concentrations of L-dopa and the catecholamines, epinephrine and norepinephrine. This confirms earlier reports on the induction of settlement and metamorphosis of *Crassostrea virginica*[35] and *Crassostrea gigas*[36] species by L-dopa. While externally applied L-dopa effectively induces settlement and metamorphosis, externally applied dopamine and octopamine were found to be ineffective. Similar results were observed in the case of the larvae of the pacific oyster *Crassostrea gigas* by Fitt *et al.*[36] who postulated that externally applied L-dopa was converted into dopamine within the larval body which then reacted with specific dopaminergic receptors to effect the behavioral response. Induction by L-dopa may possibly be attributed to the specificity of the compound to an unidentified L-dopa receptor available on the larval chemosensory membrane. Similar results have been obtained with the polycheate *Hydroides ezoensis* which initiated metamorphosis upon exposure to L-dopa.

Settlement behavior of L-dopa-treated larvae was similar to that observed with natural bio and bacterial films. Induction by L-dopa suggests that it may be possible that under natural conditions, L- or L-dopa mimetic molecules may act as environmental cues to induce settlement. In natural environments, the source of L- or L-dopa mimetic molecules may be bacterial films or adult oysters.[38] L-dopa and its oxidation product (melanin pigment) have been isolated from several marine bacteria.[38]

Larvae of *C. madrasensis* are also induced to metamorphose on exposure to the catecholamines, epinephrine and norepinephrine. Induction by EPI and NE results in metamorphosis without inducing behavioral searching phase of settlement. The larvae, on exposure to these compounds, cease swimming, and immediately sink to the bottom and metamorphose, without attaching to the substratum. Similar induction by EPI and NE at a concentration of 10^{-4} M has been reported for *C. gigas* and *C. virginica* by Coon *et al.*[32] However, larvae of *C. virginica* were less consistent in their response to EPI, which was attributed to the involvement of species-specific factors.[38]

5. Conclusion

In conclusion, substratum filming is an important factor in the settlement and metamorphosis of the oyster larvae. Bacteria appeared to be a potential inducer of settlement and metamorphosis. Induction by protein and lipid surface films reveals the involvement of a parallel mechanism. Induction by free fatty acids suggests the possible involvement of a signal transduction pathway in the induction process.

Screening with a variety of neurotransmitter substances revealed the specificity of these substances to induce settlement and metamorphosis. The compounds which were consistent in their effects were L-dopa, epinephrine and norepinephrine. Induction by L-dopa suggests the involvement of dopaminergic receptors in the settlement and metamorphosis of oyster larvae. The involvement of cAMP as a second messenger was also investigated by adding exogenous cAMP, dibutyryl-cAMP and adenylate cyclase activator forskolin. No significant increase in

settlement and metamorphosis was observed in these treatments, negating the role of this pathway in the induction process.

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