

Evaluation of antifouling activity of eight commercially available organic chemicals against the early foulers marine bacteria and *Ulva* spores

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Abstract: Environmental impacts caused by tin and copper based commercial antifouling (AF) paints were proved to be detrimental to aquatic ecosystems. Therefore, a search of environmental friendly AF compounds to be used in marine paint to protect the surface of maritime developmental structures from the unwanted biofouling is a burning issue of the present time. Commercially available eight organic chemicals- allyl isothiocyanate, β -myrecene, cis-3-hexenyl acetate, citral, ethyl heptanoate, eugenol, methyl caproate, and octyl alcohol were evaluated for AF activities using both laboratory and field assays. The test chemicals were found to repel the target motile marine bacteria- *Alteromonas marina*, *Bacillus atrophaeus*, *Roseobacter gallaeciensis* and *Shewanella oneidensis* and motile spores of the green alga, *Ulva pertusa*. The bacterial and *Ulva* spore repulsion activities of the test chemicals were measured by chemotaxis and agar diffusion methods respectively. Interestingly, these test chemicals were less toxic to the test fouling species. The toxicity of the test chemicals was measured by using antibiotic assay disks against the bacteria and motility test against *Ulva* spores. Moreover, in field assay, all test chemicals showed a perfect performance of AF activity showing no fouling during the experimental period of one year. Such results and commercial as well as technical feasibility of the test chemicals firmly showed the possibility of using as alternatives of the existing toxic AF agents.

Key words: Antibiotic, Biofouling, Chemotaxis, *Ulva pertusa*, Fouling resistance
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Introduction

A biofilm starts to develop soon after a substance is submerged into the seawater. The surface becomes rapidly colonized by bacterial cells followed by the secretion of extra cellular materials (exopolysaccharide) and adsorption of other organic compounds from the surroundings comprising a structure referred to as biofilm (Cooksey and Wigglesworth-Cooksey, 1995). Bacterial biofilm changes the topography and chemistry of the surface and a number of other microorganisms including fungi, diatoms, cyanobacteria, microalgae as well as macroalgae and invertebrates that are settled and attached to form a complex biofouling (Unabia and Hadfield, 1999). Such phenomenon causes enormous damage to marine developmental structures causing huge economic losses (Callow and Callow, 2002).

Although, the use of tributyltin oxide (TBTO) and copper-oxide were proved to be the most effective by both durability and cost. They, especially, TBTO was proved to cause unacceptable environmental problems like imposex in marine invertebrates (Alzieu, 2000). Therefore, an environmentally benign solution to unwanted biofouling to substitute TBTO is urgently needed. A number of natural (Armstrong *et al.*, 2000; Viswanadh *et al.*, 2006; Bhattarai *et al.*, 2007) and synthetic (Zentz *et al.*, 2002) compounds have been tested to quantify their antifouling (AF) activities. Natural products from different marine organisms including bacteria, algae, sponges and higher invertebrates (Fusetani, 2004) have been screened for AF activities. In this present study, commercially available selected organic compounds have been tested for their AF activity using both laboratory and field assays.

A paint used in the marine surface to prevent the initial microfouling, especially bacterial, may prevent or reduce further macrofouling (Armstrong *et al.*, 2000). Environmental friendly prevention of initial bacterial microfouling can be achieved by applying the paint with AF compounds having bacterial repellent activity (negative chemotaxis). Since motile bacteria have been reported to form initial biofilm (Costerton *et al.*, 1995), and that provided chemical cues to promote further macro fouling (Wieczorek and Todd, 1997; Bhattarai *et al.*, 2006), our focus was targeted to induce negative chemotactic activity in such motile fouling bacteria to stop subsequent macrofouling. Several methods (Boyd *et al.*, 1999) have been described to measure the chemotactic behavior of bacteria. Among them the spectrophotometer based chemotaxis assay (Boyd *et al.*, 1999) has been followed in this study with minor experimental modifications. The repellent activity of the test compounds was compared with antibiotic (AB) activity against the same test bacterial strain to describe the environmental benign AF activities of the test chemicals.

Materials and Methods

Test chemicals: All the chemicals tested were directly purchased from Sigma-Aldrich (Table 1). The reasons of the selection of these test chemicals for the experiment were as follows: they showed better bioactivity than others during primary screening period, they were easily available in the market with affordable prices and they can be synthesized easily in industrial scale. Except TBTO, the other test chemicals were natural products.

Isolation of the test bacteria: The test bacteria were isolated from the sea water collected from the experimental site of Ayajin



Table - 1: The chemotactic and antibiotic activities of the test chemicals

Test chemicals	CAS No.	Chemotactic and AB ^a activity against the test bacteria			
		SCH0401	SCH0402	SCH0407	SCH0408
Allyl isothiocyanate	57-06-7	*	**	*	0
		(75)	(5)	(50)	(5)
β-Myrcene	123-35-3	0	****	*	
		(300)	(100)	(250)	*(275)
Cis-3-hexenyl acetate	3681-71-8	0	***	*	
		(350)	(500)	(550)	*(600)
Citral	5392-40-5	0	***	*	*
		325	375	(450)	(325)
Ethyl heptanoate	106-30-9	0	****	*	*
		(700)	(>1000)	(950)	(>1000)
Eugenol	97-53-0	0	***	*	0
		(90)	(1)	(25)	(50)
Methyl caproate	106-70-7	0	***	**	*
		(750)	(>1000)	(750)	(>1000)
Octyl alcohol	111-87-5	0	****	*	*
		(500)	(950)	(900)	(950)
Tributyltin oxide	56-35-9	****	**	*	**
		(0.1)	(0.1)	(0.1)	(.05)

(0) = No response, (*) = Negative less than 2 times, (**) = Negative more than 2 times, (***) = Negative more than 3 times, (****) = Negative more than 4 times
 SCH0401, *Alteromonas marina* SW-47, SCH0402, *Shewanella oneidensis* DML7, SCH0407, *Roseobacter gallaeciensis* CIP 105210^T; SCH0408, *Bacillus atrophaeus* JCM 9070

^aAB activity is given in term of minimum inhibitory concentration (MIC) value inside parenthesis
 The unit of MIC value is µg/disc

harbor, the east coast of South Korea by using the dilution method. Marine agar (Difco) prepared according to the Manufactures instructions was used to culture the bacterial colonies. The culture condition was as follows: temperature 28°C, pH 7.5 and incubation time 48 hr. Repeated streaking was done to purify the isolated colonies from the mixture until a pure culture was achieved. Bacterial motility was tested by the hanging drop method using 24 hr old broth culture in marine broth. Gram staining was tested using a gram stain kit (Yeongdong Pharmaceutical, Seoul, Korea).

Biochemical test: Starch test, hydrolysis of gelatin and casein, urase test, H₂S production test, fermentation of glucose, sucrose and lactose, indole production test, citrate test catalase test etc. were performed. Obtained data were compared with literature (Table 2) and used to support the identification of bacteria using 16S rDNA sequencing data.

DNA analysis: All the marine strains isolated were identified based on 16S ribosomal DNA (rDNA) sequence. The genomic DNA isolated from each strain was purified with a Wizard Genomic DNA Purification Kit (Promega). The primer sets 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1518R (5'-AAG GAG GTG ATC CAC CCR CA-3') were used (Giovannoni, 1999). About 1.5 kb of sequences of 16S rDNA was determined by ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied biosystem) and an automatic sequence analyzer system (model 377, Applied biosystem). The primer set used for sequence determination consisted of 518R (5'-GTA TTA CCG CGG CTG CTG-3') and 337F (5'-ACT CCT ACG GGAGGCAGC-3'). Analysis

of 16S rDNA sequence was performed using similarity rank from RDP and BLAST.

Antibiotic assay: Antibiotic assay discs (Advantec, Japan, size 8 mm in diameter) were loaded with various concentrations of the test chemicals to determine the minimum inhibitory concentration (MIC) of each test chemical against each test bacterial species. 10 µl of dimethyl sulfoxide (DMSO) was used as a solvent for each concentration of the test chemical. The discs were placed on the surface of marine agar plates that had been freshly swabbed with an overnight broth culture (20 µl, cell density- 10⁷ cells ml⁻¹) of the test strains. All the motile species isolated were used as target species to compare chemotactic and AB activity induced by each test chemical. The plates were incubated at 28°C for 48 hr. Inhibition zones developed around the discs were taken as antibiotic effects (Schulz *et al.*, 1995).

Chemotaxis assay: The chemotaxis assay (Boyd *et al.*, 1999) was used to evaluate the bacterial repellent activity of the test compounds. A stock volume of 2% bacteriologic agar (1 ml) homogenously mixed with a test chemical dissolved in 50 µl of DMSO at the concentration of 1000 ppm was maintained at 50 °C in water bath. A series of concentrations (1000 ppm, 100 ppm, 10 ppm and 1 ppm) was prepared by diluting the mixture with 2% of agar gel (blank). The agar mixture (200 µl) was rapidly transferred to the bottom of a cuvette. A set of three replica was made for each concentration of each test chemical. A semi solid media (SSM) was prepared by mixing 18.7 g of marine broth, 2 g of bacteriologic agar and 13.4 g of synthetic sea salt (reef crystal) per liter of distilled

water. Synthetic sea salt was mixed to maintain the salinity of SSM equal to marine broth. This media let the experimental bacterial cells move freely without allowing them to settle at the bottom because of gravitational pull. All the isolated motile strains, *S. oneidensis*, *A. marina*, *R. gallaeciensis* and *B. atrophaeus* were tested separately against all test chemicals at various concentrations mentioned above. A bacterial suspension of each test species was made in SSM with cell density equal to absorbance of 0.25 at 610 nm. Cell free SSM was taken as blank during measurement of optical density (OD) of the bacterial cells. The bacterial suspension was poured into the cuvette and sealed with para film paper in a sterilized condition.

The test chemicals diffused to the target bacterial suspension from the agar gel and induced the chemotactic activity, if effective, to the test species. The bacterial cells moved away from the agar gel surface with response to the strength of the repellent activity of the test chemical. This activity of bacterial cells was recorded by a spectrophotometer indicating the change in OD of the freely suspended bacterial cells in the specially designed medium, SSM. The repulsion of bacterial cells was indicated by the decrease of OD value and vice versa.

Absorbance at 610 nm was measured at an interval of every 30 min for 5 hr using the spectrophotometer (Pharmacia Biotech). Thus obtained data were tabulated in term of change in OD, $DOD_{(t)}$ corresponding with the difference between initial OD, (OD_0) and OD at a given time, (OD_t). Three cuvettes containing only agar plugs mixed with 10 μ l of DMSO and filled with bacterial suspension in SSM served as control.

Ulva spore assays:

Preparation of spore suspension: Freshly collected *U. pertusa* thalli were carried to laboratory and washed properly with filtered sea water (0.2 μ m-filtered sea water) to remove epiphytes attached. The thalli were subjected to dry for 5-10 hr at room temperature and put inside filtered sea water for 10 min and shaken well. Zoospores produced on the surface of thalli during drought stress were, thus, harvested. The spore density was made to 30,000 per ml by dilution with filtered sea water (Fletcher, 1989). Thus made standard spore suspension was subjected for experiment

Spore motility test: Zoospore suspension of *Ulva pertusa* was treated with a series of concentrations ranging from 1000 μ gml⁻¹ to 1 μ gml⁻¹ for 2-3 min (Table 3). An aliquot of 10 μ l of treated spore suspension was taken out and observed under the light microscope to notice whether the spores were motile or not. The percentage reduction in number of motile spores was recorded in arbitrary unit.

Spore attachment assay: Bactoagar gel (made by boiling 2% agar solution in distilled water) was used to hold the test chemicals during experiment. Agar gel mixed with various concentrations (100 ppm, 10 ppm and 1 ppm) of the test chemicals were loaded on the surface of acid cleaned glass slides (2 cm X 4 cm area) and kept

for one hour to solidify perfectly. Glass slides loaded with only agar gel were served as control.

Slides were held by slide holders at a fix order and kept inside spore suspension for 5 hr in dark condition. During experimental period of 5 hr, the loaded test chemical started to diffuse out and with the response of the diffused chemical, the free swimming *Ulva* spores reacted and their settlements were affected as per the activity of the test chemical. After the incubation time was over, all slides were taken out and washed with distilled water to remove unattached and pseudo attached spores. Slides were observed under light microscope (400x) and attached spores were counted manually. The number of spores attached per square mm was calculated (Shin and Smith, 2001).

Spore germination assay: Spore suspension (1 ml, spore density- 1000 cells μ l⁻¹) was treated with a series of concentrations (1000 μ gml⁻¹, 100 μ gml⁻¹, 10 μ gml⁻¹, 1 μ gml⁻¹) of the test chemicals in triplicate in 24 wells plate for 6 hr in dark condition. After the treatment period was over, all the plates were incubated at 18°C (Petit *et al.*, 2004) with a 16hr: 8hr, light: dark cycle for 6 days. After 6 day of incubation, the bottom of each well was observed under light microscope (200x) and the germinated spores were counted. Spore suspension without test chemical treatment was taken as negative control and commercial TBTO treated was taken as positive control.

Field experiment: Each test chemical was mixed with resin based paint and sprayed on the surface of PVC panels (size, 10X10 cm²). The constituents of paint included vinyl resin dissolved in methyl ethyl ketone (MEK) in 30% of concentration. The content of the test chemical was 10% in the paint. The thickness of paint film was ranged between 100-150 μ m. The painted panels were air dried sufficiently prior to use. The completely dried panels were tied on the PVC pipes and kept vertically in sea of Ayajin harbor, the east coast of South Korea (38° 12' 17" North and 128° 28' 29" East) on October, 2004. Ten replica were tested for each test chemical. The test chemicals released from the paint and the AF activities of the released chemicals were indicated in the form of fouling coverage values observed periodically. The fouling coverage was monitored monthly for the period of one year. American standard test method (ASTM, 1987) was followed to evaluate the fouling coverage of the test panels. The coverage percentage of both micro and macro fouler on the surface of the test panels was measured by using a quadrature net of size 10X10 sq cm. The identification of fouling species was carried out (Yamaji, 1969).

Results and Discussion

Bacteria identification: Among several isolated bacterial colonies, only four were found to be motile. The motile bacterial species were selected as the test species to compare the repellent activity and AB activity of each test chemical against each test species. The identified bacterial species were as follows- *Shewanella oneidensis* DML7 (97.91%), *Alteromonas marina* SW-47 (99.51%), *Roseobacter gallaeciensis* CIP 105210^T, (97.07%) and *Bacillus atrophaeus* JCM 9070, (99.93). The percent values given in parenthesis are



Table - 2: Morphological and physiological characteristic of the isolated test bacteria with their similarity rank to the closest species and the number of sequenced base pairs

Strains	SCH0401	SCH0402	SCH0407	SCH0408
Colony color	White	Orange	White	Brown
Cell shape	Rod	Rod	Rod	Rod
Motility	+	+	+	+
Gram stain	-ve	-ve	-ve	+ve
Catalase	+	+	+	+
Gelatinase	+	+	-	+
Oxidase	+	+	+	+
Urase	-	-	-	+
Starch	+	-	-	+
Casein	+	-	-	+
H ₂ S production	-	+	-	-
Indole test	-	-	-	-
L-lactate	-	+	-	-
Succinate	-	+	-	-
Fumarate	-	+	+	+
D-mannitol	-	-	+	+
Glycerol	-	-	+	+
Sucrose	-	-	+	+
D-fructose	-	-	+	+
D-glucose	-	-	+	+
D-Galactose	-	+	+	+
D-maltose	-	-	+	+
Citrate	-	-	-	+
Sequenced base pair	1500	1512	1435	1552
Similarity (%)	97.91	99.51	99.07	99.93
Gene bank accession number	AY881234	AY881235	AY881240	AY881241
References ^a	Yoon et al. (2003)	Venkateswaran et al. (1999)	Ruiz-Ponte et al. (1998)	Nakamura (1989)

SCH0401, *Alteromonas marina* SW-47; SCH0402, *Shewanella oneidensis* DML7, SCH0407, *Roseobacter gallaeciensis* CIP 105210^T, SCH0408, *Bacillus atrophaeus* JCM 9070,

^aReferences were used to support the bacterial identification by comparing biochemical and morphological characteristics of the identified bacteria from the published literature

the similarity rank to the closest species. The identification was based on the sequence data of 16S rDNA followed by morphological and physiological characteristics (Table 2).

Antibiotic activity: This test indicated the toxic strength of the test chemicals against the target marine bacteria. The MIC value of the test agents against the target strains were various (Table 1). The commercial AF agent, TBTO, showed its MIC value always equal or less than 0.1 µg disk⁻¹ against the all test strains. The two test chemicals, allyl isothiocyanate and eugenol showed MIC values in a range of 5-90 µg disk⁻¹ for various test species. Interestingly, methyl caproate, octyl alcohol and ethyl heptanoate showed MIC values always more than 500 µg disk⁻¹ against all test strains. Similarly, the other test chemicals, β-myrcene, cis 3 hexenyl acetate and citral showed MIC values in the range of 100 to 500 µg disk⁻¹.

Chemotaxis assay: This assay showed the chemotactic activity induced by the test chemicals against the target bacteria. The various strength of repulsion activity indicated the respective AF potency of the test chemicals. All test chemicals induced negative chemotactic activity in *S. oneidensis* and *R. gallaeciensis*. β myrcene, ethyl heptanoate and octyl alcohol induced the highest quantity of negative

chemotactic activity in *S. oneidensis* at 0.1% level of concentration. The decrease in OD of the test bacterial cells was more than 4 times when compared to control. Similarly, cis-3-hexenyl acetate, citral, eugenol and methyl caproate showed negative chemotactic activity with reduction in OD of the test bacterial cells by more than 3 times at 0.1% level of concentration. However, same chemical did not induce negative chemotactic activity to all test bacteria. The test chemicals were less effective to *A. marina*. Only, allyl isothiocyanate showed weak negative chemotactic activity against *A. marina*. Similarly, allyl isothiocyanate and eugenol did not show any type of chemotactic activity in *B. atrophaeus*. But, the other test chemicals induced weak negative chemotactic activity in *B. atrophaeus* showing less than two times reduction in change in OD of the test bacterial cells at the end of five hr long experimental period (Table 1).

Ulva spore assay :

Motility test: This test quantified the toxic effects of the test chemicals against the motile reproductive spores of ubiquitous fouling marine green alga, *Ulva pertusa*. The effects of the test chemicals on the motility of spores at various concentrations ranging from 1000 µgml⁻¹ to 1 µgml⁻¹ are given in Table 3. β myrcene, citral, ethyl heptanoate,

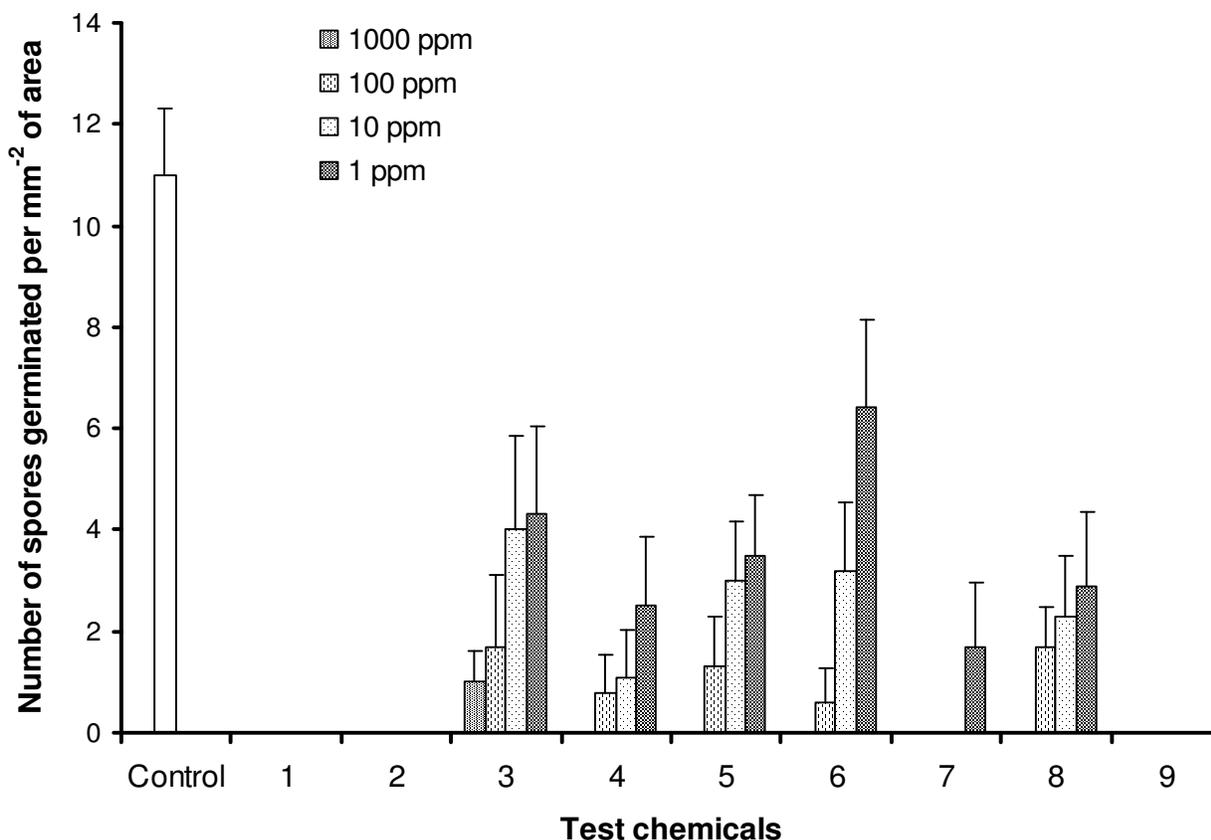


Fig. 1: The effects of the test chemicals on *Ulva* spore germination

1 = allyl isothiocyanate, 2 = β -myrcene, 3 = cis-3-hexenyl acetate, 4 = citral, 5 = ethyl heptanoate; 6 = eugenol, 7 = methyl caproate, 8 = octyl alcohol, 9 = TBTO

methyl caproate and octyl alcohol showed no effect in motility of the treated spores at lower than $50 \mu\text{gml}^{-1}$ level of concentration. Similarly, allyl isothiocyanate, cis 3 hexenyl acetate and eugenol showed no hindrance in motility of the test spores at $1 \mu\text{gml}^{-1}$ level of concentration. The commercial TBTO showed very high degree of toxic effect even at $1 \mu\text{gml}^{-1}$ level of concentration showing reduction in spore motility by 75% to 94%.

Spore attachment assay: This assay showed the attachment activity of motile spores against the test chemicals. The number of spores attached per square mm area of agar gel with response to various concentrations of the test chemicals is given here (Table 3). Commercially used AF agent, tributyltin oxide (TBTO), showed the highest activity of reducing spore attachment by 91.5% in average when compared to control (blank). Therefore, TBTO was used as a reference to compare the activity of the other test chemicals. Methyl caproate and β -myrcene showed more than 80% decrease in spore attachment than control. The other four test chemicals, eugenol, ethyl heptanoate, cis-3-hexenyl acetate and citral showed more than 70% decrease in spore attachment. And, the remaining two compounds namely, octyl alcohol and allyl isothiocyanate showed 68.7% and 47.8% of decrease in spore attachment respectively.

Spore germination assay: This assay demonstrated the activity of *Ulva* spore germination against the effects of the test chemicals at various concentrations. All test chemicals inhibited the *Ulva* spores germination with various strengths (Fig. 1). The percentage of reduction of spore germination also indicated the AF potency of the test chemicals. Allyl isothiocyanate, β -myrcene and TBTO inhibited the *Ulva* spores germination completely at all concentrations examined. Similarly, the complete inhibition was observed for citral, ethyl heptanoate, eugenol and octyl alcohol at 1000mgml^{-1} level of concentration. The lower degree of test concentration of these four compounds showed the various strengths of reduction in spore germination. Methyl caproate inhibited spore germination at more than $1 \mu\text{gml}^{-1}$ level of concentration. Similarly, cis 3 hexenyl acetate inhibited the spore germination at $1000 \mu\text{gml}^{-1}$, $100 \mu\text{gml}^{-1}$, $10 \mu\text{gml}^{-1}$ and $1 \mu\text{gml}^{-1}$ of concentration by 90%, 84%, 64% and 60% respectively.

Field assay: This assay showed the AF activities of the test chemicals in field experiment. The control panel was attached by a number of micro and macro foulers. The micro fouler included various species of micro algae including *Desmidium* sp, *Fragilaria* sp, *Gamphonema* sp, *Licmophora* sp, *Navicula* sp, *Nitzschia* sp, *Stephanopyxis* sp and

Table - 3: The effect of the test chemicals on *Ulva* spore motility and attachment at various concentrations*

Test chemicals	Average number of spores attached per sq mm (n=10)		
	284±10.7		
	Test chemical concentration		
	100 ppm	10 ppm	1 ppm
Control (blank)			
TBTO	24±3.7 ++++	37±3.7 ++++	44±9 +++
Methyl caproate	39±2 +	56±9.2 -	77.3±4.6 -
β-Myrcene	55±7.1 +	88±11.9 -	97±4.5 -
Eugenol	57±8.3 ++	74±6.5 +	79±1.3 -
Ethyl heptanoate	65±6.9 ++	70±9.7 -	96±5.3 -
Cis-3-hexenyl acetate	68±5.8 ++	148±9 -	164±7.6 -
Citral	83±14 ++	129±23.6 -	140±6.7 -
Octyl alcohol	89±12.7 -	108±11.3 -	120±16.2 -
Allyl isothiocyanate	136±21 +++	148±30 +	164±27.6 -

*The concentration indicated the content of the test chemicals in agar gel during spore attachment assay and in spore suspension during spore motility assay. Very strong response- ++++ = 100-95%, Strong- +++ = 94-75%, Moderate- ++ = 74-50%, Weak- + = 49-25% and No response- = 24-0%

Striatella sp etc. Similarly, wide ranges of macro fouler were observed. The macro fouler consisted to both soft and hard fouler. The attached soft fouler were sea weeds including different species of green, brown and red algae, and slimes. The hard fouler attached were tunicates and barnacles. With these fouling organisms, the average value of fouling coverage on the surface of control test panel was 93%. But, the test chemicals showed perfect results of AF activity containing no fouling on the surface of test panels.

This present study was based on the strategy of searching AF compounds that would make the marine developmental surface clean by repelling early biofilm forming marine bacteria and algal spores followed by decreasing the settlement of macrofoulers without killing fouling organisms (Armstrong *et al.*, 2000). The test chemicals showed the various strengths of AF activity during different laboratory assays described here. However, some of the test compounds, especially, allyl isothiocyanate and eugenol, were comparatively toxic to the test bacterial species and *Ulva* spores. Moreover, these compounds were the constituents of edible vegetables and spices (Srivastava, 2003). Therefore, it might be nontoxic to the non target marine organisms; though, researches with detail toxicity profiles of the test chemicals are yet to be completed. In addition, in one year

long field assay the test chemicals completely inhibited the settlement and attachment of fouling organisms. From these observations it can be summarized that a composite strategy of AF activities may be applied to get such perfect AF activities. Though, it is yet to be confirmed, it was presumed that the various AF activities (antibiotic and repellent to both bacterial and *Ulva* spores) of the test chemicals were effective together to get the anticipated AF activity. Either these chemicals influenced the quorum sensing system of bacteria (Costerton *et al.*, 1995; Stickler *et al.*, 1998) that regulated biofilm formation and motility (Lindum *et al.*, 1998) or they made the loose attachment of fouling organisms on the test surfaces, which were cleaned, shortly, by natural current of moving water. Moreover, the durability and efficiency of the AF paints are determined by the achieved AF release rate (Shin *et al.*, 2001; Shin and Smith, 2002).

Thus, in conclusion, the test compounds induced better negative chemotactic activity to the early fouling motile marine bacteria with weaker antibacterial activities than the existing toxic AF agent, TBTO. Such results clearly showed the high potency of the test chemicals to stop the formation of pioneer bacterial biofilm that will ultimately lead to the decrease of settlement and attachment of further macrofoulers. In addition, the test chemicals were natural products from different plants. All the chemicals tested were cheap and affordable by consumers as well as easily synthesizable. And finally, all the test compounds were easily soluble in solvent based paints without changing the physical and chemical properties. All these points are prerequisite for the commercial success of the proposed AF compounds. All mentioned criteria were met by these test chemicals. Consequently, these test chemicals showed their potential for the future candidates to substitute the existing toxic TBTO. However, selection of a proper binder to get the controlled rate of AF compounds from the paint, durability and life of paint, biodegradability and toxicity profiles of the test chemicals are the other important issues to be researched well before applying the paint commercially.

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