

**Impact of Irgarol 1051 on the larval development and metamorphosis of *Balanus amphitrite* Darwin, diatom, *Amphora coffeaformis* and natural biofilm**

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**Abstract**

The effect of Irgarol 1051 on the biofilm-forming diatom, *Amphora coffeaformis*, and on natural biofilm was assessed. A reduction in the number of *A. coffeaformis* cells within a biofilm was observed after treatment with Irgarol 1051, confirming its role as an inhibitor of photosynthetic activity. The impact of this compound on the development of nauplii of *Balanus amphitrite* was evaluated through its impact on *Chaetoceros calcitrans*, which was provided as food for the larvae. A reduction in the number of cells of *C. calcitrans* was observed when treated with Irgarol 1051. When larvae of *B. amphitrite* were reared using *C. calcitrans* in the presence of Irgarol 1051, their mortality increased with an increase in the concentration of Irgarol 1051 (13% at  $1\mu\text{g L}^{-1}$  to 40% at  $1000\mu\text{g L}^{-1}$ ) compared to the control (6%). Nauplii reared in the presence of Irgarol 1051 developed more slowly (6-7 days) compared to control larvae (4-5days). Cyprid bioassay results indicated an increase in percentage metamorphosis (76%) when natural biofilms were treated with the highest concentration of Irgarol 1051, compared to untreated biofilm (28%). The enhanced rate of metamorphosis appeared to be related to an increase in bacterial numbers in the biofilm, which may have been due to lysis of diatoms caused by Irgarol 1051. *A. coffeaformis* biofilms that were grown in the presence of antibiotics showed a significant reduction in cell numbers, which on further treatment with Irgarol 1051 showed an increase in cell numbers. Thus, it can be hypothesized that *A. coffeaformis* that was subjected to stress twice may have expressed resistant genes. Furthermore, if plasmids are present in the biofilms, they may enhance transfer to the surviving cells making them more resistant to hostile conditions.

*Keywords:* Irgarol 1051, algicide, *Balanus amphitrite*, biofilm, *Amphora coffeaformis*

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## **INTRODUCTION**

Biofouling is a process of development of undesirable biological growth on structures submerged in the aquatic environment. Biofilms develop rapidly on immersed substrata (Wahl 1989), being dominated by a range of microorganisms including bacteria, fungi, and algae, especially diatoms. Such biofilms can influence the settlement of many invertebrate larvae belonging to major fouling organisms (Mihm et al. 1981; Strathmann et al. 1981; Maki et al. 1988, 1990, 1992; Pawlik 1992; Rodrigues et al. 1993; Keough and Raimondi 1995; Wieczorek and Toad 1998; Hadfield and Paul 2001; Harder et al. 2001; Khandeparker et al. 2005; Dobretsov et al. 2006).

Biofouling is a serious problem for many maritime industries (Clare 1996; Abarzua et al. 1999; Callow and Callow 2002; Townsin 2003; Schultz 2007) as well as being a vector for the spread of invasive species (Cranfield et al. 1998; Dafforn et al. 2008). In order to prevent fouling, antifouling products containing biocides such as copper and tributyltin (TBT) have been developed (Willemsen and Ferrari 1993; Isensee et al. 1994; Ponasik et al. 1998; Boxall et al. 2000; Evans et al. 2000). However, many studies identified the persistence of triorganotin compounds in seawater (Bryan et al. 1986; Gibbs et al. 1988; Smith and McVeagh 1991; Nias et al. 1993; Willemsen et al. 1998; Depledge and Billingham 1999; Marin et al. 2000; Kobayashi and Okamura 2002; Bhosle et al. 2004) and TBT was shown to cause deformation in oysters and sex changes in whelks (Ariza-Gomez et al. 2006; Limaverde et al. 2007). The use of antifouling paints containing triorganotin was prohibited from 1 January 2008 and currently most antifouling paints are based on copper (Finnie 2006) supplemented with 'booster biocides', including herbicides (Readman et al. 1993; Liu et al. 1997; Turley et al. 2005). Whilst about 18 compounds are currently used world wide as antifouling biocides, the algicide Irgarol 1051 was the first to gain prominence (Kostantinou and Albanis 2004) because of its possible adverse effects on the marine ecosystem. Irgarol

1051 is designed to prevent the build up of algal biofilms on ship hulls. The toxicity of Irgarol 1051 to algae and higher plants has been reported to be very high as it is a potent inhibitor of photosynthetic activity (Dahl and Blanck 1996; Scarlett et al. 1997; Okamura et al. 2000; Nystrom et al. 2002; Owen et al. 2002; Macinnis et al. 2003). Degradation of Irgarol 1051 in both marine and freshwater sediment is slow, with half lives of 100 and 200 days, respectively (Ciba Geigy 1995; Callow and Willingham 1996). The occurrence of this compound has been reported in many coastal waters, lakes and marinas all around the world at varying concentrations (Readman et al. 1993; Gough et al. 1994; Dahl and Blank 1996; Tolosa et al. 1996; Toth et al. 1996; Zhou et al. 1996; Liu et al. 1997, 1999; Scarlett et al. 1999; Martinez et al. 2001; Gardinali et al. 2002; Basheer et al. 2002; Lamoree et al. 2002; Sakkas et al. 2002) as well as in sediments (Albanis et al. 2002). The highest concentration ( $4.2 \mu\text{g L}^{-1}$ ) was reported in coastal waters around Singapore (Basheer et al. 2002). Toth et al. (1996) reported accumulation of Irgarol 1051 in fresh water macrophytes. Earlier studies reported that Irgarol 1051 has comparatively low toxicity towards marine bacteria and crustaceans and suspension-cultured fish cells (Okamura et al. 2000, 2002). Ecotoxicity tests revealed that Irgarol 1051 was moderately toxic to *Artemia salina* (24-h  $\text{LC}_{50} > 40 \text{ mg/L}$ ) (Okamura et al. 2000) and Kobayashi and Okamura (2002) reported toxicity to sea urchin development. Khandeparker et al. (2005) reported toxicity of Irgarol 1051 to the barnacle *Balanus albicostatus* (48h  $\text{LC}_{50}$  value for mortality of II instar was  $556 \mu\text{g L}^{-1}$ ). The present study was carried out to understand the ecotoxicological effect of Irgarol 1051 on larval development of the acorn barnacle, *Balanus amphitrite* Darwin (Cirripedia: Thoracica), which is an important component of the marine macrofouling community in Indian waters (Anil 1986, Desai 2002). The effect of the compound on *Chaetoceros calcitrans*, which is used as a food source to rear the larvae of this species, was also tested. A mono-species biofilm of *Amphora coffeaformis*, a fouling diatom which is also commonly found in natural

biofilms was also tested against Irgarol 1051. Larval bioassays were carried out using cyprids of *B. amphitrite*; settlement was recorded on Irgarol-treated and untreated (control) biofilms.

## **MATERIALS AND METHODS**

### **Effect of Irgarol 1051 on the larval development of *B. amphitrite***

#### **Rearing of *B. amphitrite* larvae**

Adult *B. amphitrite* (= *Amphibalanus amphitrite*, see Clare and Høeg (2008) for nomenclature) were collected from the inter-tidal area of Dona Paula bay, Zuari Estuary, Goa, India. The barnacles were brought to the laboratory and exposed to air for a short duration (~30min.) and then placed in the filtered seawater, which triggered the release of larvae. The larval phase of *B. amphitrite* consists of six naupliar instars followed by a settling cyprid instar. Irgarol 1051 (2-methylthio-4-tert-butylamino-6-cyclopropylamino-s-triazine), was obtained from Ciba Specialty Chemicals, Japan. The stock solution of Irgarol 1051 was prepared (10000mg L<sup>-1</sup>) in dimethyl sulphoxide (DMSO) as described by Kobayashi and Okamura (2002). The stock solution was diluted to desired concentrations with filtered seawater. The effect of Irgarol 1051 on larval development and post-settlement metamorphosis of *B. amphitrite* was assessed using instar II nauplii. Rearing of larvae was carried out at five different concentrations of Irgarol 1051 (0.1, 1, 10, 1000, 10,000 µg L<sup>-1</sup>) at 25±1°C (12h:12h, Light: Dark cycle).

*C. calcitrans*, a unicellular diatom was used as food to rear larvae. Instar II nauplii were transferred into multiwell plates (24-well Nunclon Delta 143982) with the respective concentration of Irgarol 1051 and reared individually. The rearing wells were maintained in an incubator at 25(±1°C), and the rearing of nauplii continued until they reached the cyprid instar stage. Larval mortality and molting were recorded everyday prior to transferring the

larvae into fresh multiwells. Controls contained filtered seawater (FSW) with and without the addition of DMSO. The DMSO control was used with equivalent volumes of DMSO in filtered sea water at a final concentration of 0.001% which is the highest concentration in the test solution of Irgarol 1051 ( $1000\mu\text{g L}^{-1}$ ).

The Lethal Concentration for 50% larval mortality ( $LC_{50}$ ) was calculated at 48 h for larvae fed with *C. calcitrans* in the presence of Irgarol 1051, and for larvae maintained with Irgarol 1051 only.

The cyprid settlement assay followed the general principles described for screening antifouling compounds (Rittschof et al. 1994; Nogata et al. 2003). In order to obtain cyprids for the settlement assays, II instar nauplii were mass reared in 2L glass beakers using *C. calcitrans* as food at a cell concentration of  $1 \times 10^5$  cells  $\text{ml}^{-1}$  at  $27 \pm 1^\circ\text{C}$ . The diatom cultures were grown in f/2 medium. An antibiotic dosage of 0.3ml Crystamycin  $\text{L}^{-1}$  was used (300mg Penicillin G and 400mg Streptomycin in 4 ml distilled water) for rearing the larvae (Harmes 1986; Anil et al. 1995).

### **Development of natural biofilm**

Biofilms were developed in sterile multiwell plates (24-well Nunclon Delta 143982), which were exposed to natural seawater by immersing them with a rope tied to a weight from the jetty at Dona Paula bay, Zuary Estuary, Goa, West coast of India, for a period of two days. Non-biofilmed multiwells were used as controls. The biomass of diatoms was enumerated by chlorophyll fluorescence and direct light microscopy. Only live cells, which fluoresced red were counted when exposed to green fluorescent light. The attached bacterial density in the natural biofilm was determined using acridine orange and epifluorescence microscopy (Daley and Hobbie 1975).

### **Development of diatom biofilm**

*A. coffeaformis* was cultured in f/2 medium (Guillard 1975). When visible diatom films had formed in the culture flask, the biofilm was removed by brushing the culture flask with a sterile paint brush in a fresh medium. To this suspension, a concentrated stock solution of streptomycin and penicillin was added at a final concentration of  $2.5 \times 10^{-4}$ M streptomycin and  $1.0 \times 10^{-4}$ M penicillin as described by Harder et al. (2002). After 6h, 2 ml aliquots of the diatom suspension was inoculated into the 24 well multiwells which were gently shaken for a period of 24 hours. The multiwell plates were then rinsed three times using autoclaved filtered seawater. The films developed without antibiotics were considered as non-axenic. Axenic diatom films (ADF) and non-axenic diatom films (NADF) of *A. coffeaformis* were treated with the lowest and highest concentration of Irgarol 1051 (0.1 and  $10,000 \mu\text{g L}^{-1}$ ) Diatoms were enumerated by chlorophyll fluorescence and light microscopy.

## **Settlement Assays**

The multiwells (24-well plates, Nunclon Delta 143982) with 2-day old natural biofilm (NBF) were inoculated with different concentrations of Irgarol 1051 (0.1, 10, 10,000  $\mu\text{g. L}^{-1}$ ). The plates were rinsed with autoclaved filtered sea water after 3 hours of exposure to the respective Irgarol 1051 concentrations. Approximately 15-20 cyprids were introduced in 2ml of autoclaved filtered seawater (35‰) into each well (Maki et al. 1990). Replicate dishes were also fixed with (2% v/v) formaldehyde for enumeration of bacterial densities. Six replicates of each of the concentrations were used for the assessment. The experiment was repeated twice using different batches of larvae (n=6, with batch as an additional factor). Assays were monitored for a period of 4 days with an intermittent observation every 24h. Controls were DMSO and FSW. Assay wells were maintained at  $26 \pm 1^\circ\text{C}$  (12h light: 12h dark cycle).

## **Statistical analysis**

The data from the settlement assays were expressed as the percentage of cyprids that underwent post-settlement metamorphosis. Data were arcsine transformed to ensure normality of means and tested for homogeneity of variance before statistical analysis. One-way analysis of variance (ANOVA) was performed to assess the influence of different concentrations of Irgarol 1051 on natural and diatom biofilms and its subsequent effect on cyprid post-settlement metamorphosis (Sokal and Rohlf 1981). The reduction in the number of diatom cells in NBF, ADF and NADF exposed to different concentrations of Irgarol 1051 was also determined using One-way ANOVA followed by Tukey's test ( $\alpha=0.05$ ). Power function regression (PFR) analyses were carried out to see the influence of Irgarol 1051 concentration (v-variable) on the developmental duration (D) as  $D=bv^m$  (where; b-intercept, m – slope).

## RESULTS

### **Influence of Irgarol on *Amphora coffeaformis*, natural biofilm and *Chaetoceros calcitrans***

The results indicated that when *A. coffeaformis* biofilms were treated with Irgarol 1051, a decrease in cell numbers was observed. By contrast, an increase in cell numbers was observed in the controls (FSW and DMSO) (one-way ANOVA,  $p \leq 0.02$ ) (Figure 1). When 2-day old natural biofilms were treated with different concentration of Irgarol 1051, there was also a reduction in the number of diatom cells with increasing concentration of Irgarol 1051. At the highest concentration ( $10,000 \mu\text{g L}^{-1}$ ), no cells were observed in the biofilm after 24h treatment (one-way ANOVA,  $p \leq 0.0004$ ) (Figure 2). The cell concentration of *C. calcitrans* in the food used to rear the larvae decreased after treatment with different concentrations of Irgarol 1051 for 24h (Figure 3) and this decrease was linear with concentration ( $Y = -2.7857x + 2.583$ ;  $r = 0.9$ ,  $p \leq 0.01$ ). The highest concentration of  $10,000 \mu\text{g L}^{-1}$  was not taken into account as there was 100% mortality of the nauplii at this concentration and all the cells of *C. calcitrans* were also dead.

### **Nauplii larvae mortality**

The mortality of naupliar larvae increased with an increase in the concentration of Irgarol 1051. At a concentration of  $10,000 \mu\text{g L}^{-1}$ , 100% mortality was observed and mortality decreased with a decrease in the concentration of Irgarol 1051 (Figure 4). In FSW, mortality was 6% and in DMSO 8% (Figure 4). The 48h  $LC_{50}$  values for mortality calculated for larvae fed with *C. calcitrans* and for starved larvae were  $1352 \mu\text{g L}^{-1}$  and  $1138 \mu\text{g L}^{-1}$ , respectively.



### **Larval development**

A prolonged duration of instar development was observed for larvae reared in the presence of Irgarol 1051, however this effect varied with the concentration and the stage of the larval instar compared to controls (Figure 5). The duration of the II instar was highest (1.8days) at  $1000\mu\text{g L}^{-1}$  of Irgarol 1051 compared to 1.4days at  $0.1\mu\text{g L}^{-1}$  and 1 day in the case of the control (FSW) (Figure 5). For other concentrations, the duration of the VI instar was longer compared to other instars. The duration was 1.7, 1.83 and 1.77 days at 0.1, 1 and  $10\mu\text{g. L}^{-1}$  of Irgarol 1051, respectively (Figure 5). The VI instar duration was 1.3 days (DMSO) and 1 day (FSW) in the controls. Instar III to V did not show much variation in response to different concentrations of Irgarol 1051, however the duration was longer than in the control. Regression analysis indicated a significant increase in the duration of II, III and VI instars (Table 1).

The total length of larval development up to the cyprid instar was longer when treated with Irgarol 1051 (Figure 6). When compared with the controls, the difference was 2-3days. The increase was significant for duration of development with Irgarol 1051 concentration (Table 1).

### **Nauplii larvae mortality and instar duration**

Observations on the mortality of larvae in a particular instar treated with Irgarol 1051 showed that at the lowest concentration ( $0.1\mu\text{g L}^{-1}$ ) mortality was observed only in II, III and IV instars, which was similar to DMSO control. However, the percentage mortality was comparatively low for the DMSO control compared to Irgarol-treated nauplii (Figure 7). Maximum mortality in a particular instar was observed at  $1000\mu\text{g. L}^{-1}$ , the highest being in IV instar (25%). At this concentration, mortality was also low compared to both lower concentrations of Irgarol 1051 and the control (Figure 7). At low concentrations ( $0.1$  and  $1\mu\text{g L}^{-1}$ ), mortality was observed only in the early instars, but with an increase in the

concentration of Irgarol 1051, higher percentage mortality was observed for all the instars. Similar results were obtained for the duration of the instars (Figure 7). At maximum Irgarol 1051 concentration ( $1000\mu\text{g. L}^{-1}$ ), longest instar duration ( $3\pm 2\text{days}$ ) was observed for VI instar nauplii before they were dead. At  $10000\mu\text{g L}^{-1}$ , where 100% mortality was observed, all the II instar larvae were dead and therefore could not develop further.

### **Larval Bioassay**

Axenic and non-axenic films of *A. coffeaformis* showed a significant difference in cell numbers after treatment with Irgarol 1051 (one-way ANOVA,  $p \leq 0.001$ ; Tukey's test,  $p \leq 0.05$ ). When ADF were treated with Irgarol 1051, a significant increase in diatom cell numbers was observed at  $0.1\mu\text{g. L}^{-1}$ , however, when the concentration was increased, cell numbers decreased, but not below the control (Figure 8).

The bioassay results indicated a significant increase in the percentage metamorphosis of cyprids when the natural biofilm was treated with  $10,000\mu\text{g L}^{-1}$  (Tukey's test;  $p=0.02$ ; Figure 9). Percentage metamorphosis was 76% at  $10,000\mu\text{g L}^{-1}$  compared to 28% for untreated biofilm. Cyprids exposed to the filtered sea water control showed higher metamorphosis (49%) compared to non-treated biofilms (Figure 9). The density of bacteria in the natural biofilm was  $1.59 \times 10^{13}$  cells.  $\text{mm}^{-2}$ . The number of bacteria increased when NBF were treated with Irgarol 1051. At 0.1, 1000 and  $10000 \mu\text{g L}^{-1}$  the density of bacteria was  $3.29 \times 10^{13}$ ,  $2.9 \times 10^{13}$  and  $3.16 \times 10^{13}$  cells  $\text{mm}^{-2}$  respectively.

### **DISCUSSION**

Irgarol 1051 is a widely used antifouling additive; however, its increased occurrence in the marine environment is of concern. Irgarol 1051 is prevalent in tropical marine ecosystems and is a potent inhibitor of coral photosynthesis at environmentally relevant concentrations

(Owen et al. 2002). Also, Irgarol 1051 is subject to restrictions in EU member states because of its environmental stability and high toxicity (Ranke and Jastorff 2000; Yebra et al. 2004). Therefore it is of interest to study the effects of this compound on marine organisms.

In the present study, at a concentration of  $0.1 \mu\text{g. L}^{-1}$ , only 8% of larvae could not complete development and reach the competent larval stage (cyprid). Mortality increased with an increase in the concentration of Irgarol 1051. At the highest concentration ( $10,000\mu\text{g. L}^{-1}$ ) 100% mortality was observed. Nauplii were able to complete their development to the cyprid instar at the lowest concentration of Irgarol 1051, however fewer reached this stage than in the control.

Earlier investigations on the effect of Irgarol 1051 on *B. albocostatus* from Japanese waters have also indicated adverse effects of this compound on larval development (Khandeparker et al. 2005). However, the 48h  $\text{LC}_{50}$  values for *B. amphitrite* were higher than recorded for *B. albicostatus*. The latter species is confined to waters around Japan and Korea (i.e. subtropical and temperate), whereas *B. amphitrite* is found in both tropical and temperate waters indicating its tolerance to a wider range of temperature and salinity. These tolerance limits may be an added advantage for *B. amphitrite* against stress. An ecological risk assessment reported the concentration of Irgarol 1051 to be around  $1.693\mu\text{g L}^{-1}$  in coastal waters of Netherlands (Hall et al. 1999), but in Singapore coastal waters, a concentration of  $4.2 \mu\text{g L}^{-1}$  Irgarol 1051 has been reported (Basheer et al. 2002). In the present investigation, mortality at a concentration of  $1\mu\text{g L}^{-1}$  (13%) was observed, which is lower than the concentrations mentioned above. The data reported here indicate that Irgarol 1051 could pose a risk to marine organisms at this concentration. At lower concentrations, although the mortality of the larvae was minimal or just above the control, the larvae showed a prolonged duration of development. The biocidal effect of copper on larval development of *Balanus amphitrite* showed that molting was a more sensitive endpoint than survival, nauplius II being

the most sensitive stage. Thus a complete larval development assay is more sensitive than assays using a particular larval stage (Qiu et al. 2005). The observations in this particular study indicated that II instar larvae are less sensitive to Irgarol 1051 as they showed lower percentage mortality compared to advanced instars. Mortality was higher for the advanced instars at the higher concentrations of Irgarol 1051 and the length of time they remained in a particular instar was also longer. Irgarol 1051 has also been reported to be toxic to another crustacean, viz. grass shrimp larvae, however the concentration required for 96h LC<sub>50</sub> was 1520 µg L<sup>-1</sup> (Key et al. 2008) which is higher than that required for the 48h LC<sub>50</sub> value for mortality of *B. amphitrite* and *B. albicostatus* larvae. Toxic effects of Irgarol 1051 to sea urchins have also been reported at low concentrations (Koabayshi and Okamura 2002; Manzo et al. 2006). Manzo et al. (2006) concluded from their study on the sea urchin *Paracentrotus lividus* that the biocidal effect of Irgarol 1051 appeared earlier and produced pre-larval arrest (embryos unable to go to larval differentiation). While studying the acute toxicity of Irgarol 1051 to *Artemia salina*, Panagoula et al. (2002) reported that the LC<sub>50</sub> value for mortality was 1620 µg L<sup>-1</sup> which is also higher than that required for *B. amphitrite* larvae.

The experiments carried out with diatoms indicated that Irgarol 1051 significantly influenced the growth of phytoplankton, which concurs with its known effect as an inhibitor of photosynthesis (Dahl and Blanck 1996). Cell numbers of *C. calcitrans*, provided for larval development, decreased with increasing concentrations of Irgarol 1051. The 48h LC<sub>50</sub> values for the mortality of larvae that developed in the presence of food exposed to Irgarol 1051 and Irgarol 1051 alone, indicated that 210µg L<sup>-1</sup> of extra Irgarol 1051 was needed to attain the same mortality as that when Irgarol 1051-treated food was provided. Thus, Irgarol 1051 affects the food on which the larvae develop exhibiting an indirect effect on their development. Biocidal effects of Irgarol 1051 showed inhibitory effects on zoospores of *Enteromorpha intestinalis* at a concentration as low as 0.1 µg L<sup>-1</sup> (Scarlett et al. 1997).

Inhibitory effects were also reported for *Fucus serratus* (Braithwaite and Fletcher 2005). In the present study, it was observed that the cell numbers in both the monospecies biofilms of *A. coffeaformis* and the natural biofilms decreased after Irgarol 1051 treatment. Key et al. (2008), while working with larvae of grass shrimp indicated that cholesterol levels in the larvae exposed to Irgarol 1051 decreased and they suggested that cholesterol was necessary for crustaceans to synthesize the molting hormone, ecdysteroid. However, crustaceans are not able to synthesize cholesterol and must obtain it from the diet (Key and Fulton 1993). The reduced cholesterol levels in larvae may have been caused by exposing them to Irgarol 1051, thus interfering with the hepatopancreatic function and/or with feeding. The lengthening of the instar duration of the larvae fed in presence of Irgarol 1051 in the present study can also be attributed to changes in larval physiology that resulted from an inadequate diet.

A significant increase in the percentage metamorphosis of cyprids of *B. amphitrite* was observed when the natural biofilms were treated with 10,000 $\mu\text{g L}^{-1}$  Irgarol 1051. In the case of *B. albicostatus*, cyprids were prevented from metamorphosing when natural biofilms were treated with higher concentration (5mg L<sup>-1</sup>) of Irgarol 1051 (Khandeparker et al. 2005). In the present study, the number of diatom cells decreased significantly when NBF was treated with 10,000 $\mu\text{g L}^{-1}$  Irgarol 1051. Bacterial density in natural biofilm was 1.59x 10<sup>13</sup> cells. mm<sup>-2</sup>. It is a known fact that settlement of barnacle cyprids, like most other larvae, is usually facilitated on a substratum that is covered by a well-developed biofilm (Crisp 1974; 1984; Clare et al. 1992). Several studies have addressed the importance of bacterial films on the settlement and metamorphosis of *B. amphitrite* cyprids (Anil and Khandeparker 1998; Khandeparker et al. 2002, 2003, 2006; Maki et al. 1988; Avelin Mary et al. 1993; Neal and Yule 1994; Lau et al. 2003). Biofilms have varying effect (inhibitory/inductive/no effect) on the settlement of *B. amphitrite* cyprids (Olivier et al. 2000; Wieczorek et al. 1995). Biofilms provide specific chemical cues that are identified by larvae and which indicate preferred

ecological conditions (Unabia and Hadfield 1999). For a chemical cue to be detected by larvae it must be either expressed on the surface of the substratum or released as a waterborne cue (Hadfield and Scheuer 1985; Tamburri et al. 1992). In the case of *B. amphitrite* cyprids, the waterborne and surface associated cues from bacteria function differentially in mediating their metamorphosis (Khandeparker et al. 2006). In the present study, an increase in percentage metamorphosis of *B. amphitrite* cyprids exposed to natural biofilm treated with the highest concentration of Irgarol 1051, indicated that removal of the diatom component from the natural biofilm changed an inhibitory biofilm into one that promoted settlement. In addition, bacterial numbers in the natural biofilm increased after treatment with Irgarol 1051. Thus, it can be hypothesized that Irgarol 1051 caused the lysis of diatom cells, thereby contributing to increased numbers of bacteria in the biofilm. According to Cole (1982), stressed algae enhance bacterial production. In the present study, this increased production resulted in enhanced metamorphosis of the fouling organism, however, the species composition of the bacteria needs further elucidation.

Harder et al. (2002) mimicked the patchiness and variability of natural biofilms by mixing bacteria and diatoms at different ratios in a study of larval settlement of *Hydroides elegans*. He reported that mixed diatom-bacteria biofilms showed higher percentages of settlement than the monospecies biofilms. In case of the bryozoan, *Bugula neretina* quantitative and qualitative changes in bacteria and diatom taxa also affected larval settlement (Dahms et al. 2004). In a preliminary study conducted to assess the influence of monospecies diatom films of *A. coffeaformi* cultured under axenic and non-axenic conditions, it was revealed that when the diatoms were treated with antibiotics there was a significant decrease in the number of diatom cells. Earlier studies have reported that addition of natural marine bacteria to exponentially growing diatoms resulted in either growth stimulation or inhibition depending on diatom species and growth phase (Grossart et al. 2005). Bacteria

affect the growth of marine diatoms and vice versa. It is also well known that marine bacteria can express high activities of hydrolytic enzymes (Martinez et al. 1996) leading to increased algal death and lysis once the algae are stressed. (Cole 1982). Thus, it can be hypothesized that antibiotics indirectly control diatom cell numbers by targeting bacteria. When these ADF were further treated with Irgarol 1051, a significant increase in cell numbers was observed at  $0.1\mu\text{g. L}^{-1}$ , however when the concentration was increased, cell numbers decreased, but did not reach a lower density than the controls. Antibiotics retard bacterial proliferation by interfering with the production of components needed for cell division (Levy 1998), which bacteria frequently overcome by expressing resistance genes. It can be thus hypothesized that these genes could be carried by plasmids thereby enhancing the chances of survival in hostile conditions and they may also provide protection in other stress conditions. Further studies will be conducted to test this hypothesis.

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### **Caption for Table**

Table 1. Power function regression values for instar and total naupliar duration ( $D=bv^m$ ), for the influence of Irgarol (V) on the instar and (TND) total naupliar duration (D) of the larvae. n – number of observations.

### **Captions for figures**

Figure 1. Variation in the cell number of *Amphora coffeaformis* in biofilm treated with Irgarol 1051. Before Treatment- Cell numbers without Irgarol 1051 treatment; After Treatment - Cell numbers after Irgarol 1051 treatment.

Figure 2. Variation in the cell number of diatoms in natural biofilms treated with Irgarol 1051. Vertical lines indicate standard deviation from the mean and are shown as positive. Before Treatment- Cell numbers without Irgarol 1051 treatment; After Treatment - Cell numbers after Irgarol 1051 treatment.

Figure 3. Cell concentration of *Chaetoceros calcitrans* the food, treated with different concentrations of Irgarol 1051 for 24h. Vertical lines indicate standard deviation from the mean and are shown as positive.

Figure 4. Percentage mortality of *Balanus amphitrite* larvae reared in different concentrations of Irgarol 1051. Controls were filtered sea water (FSW) and DMSO. Vertical lines indicate standard deviation from the mean and are shown as positive.

Figure 5. Naupliar instar duration of the larvae reared in different concentrations of Irgarol 1051 and control. Vertical lines indicate standard deviation from the mean and are shown as positive.

Figure 6. Total naupliar duration of the larvae reared at different concentrations of Irgarol 1051, filtered sea water and DMSO (control). Vertical lines indicate standard deviation from the mean and are shown as positive.

Figure 7. Instar duration and percentage mortality of the dead larvae treated with Irgarol 1051. Vertical lines indicate standard deviation from the mean and are shown as positive for instar duration and negative for % mortality.

Figure 8. Variation in the cell numbers of *Amphora coffeaformis* axenic and non-axenic biofilm treated with highest and lowest concentration of Irgarol. Vertical lines indicate standard deviation from the mean and are shown as positive.

Figure 9. Percentage metamorphosis of *Balanus amphitrite* cyprids at different concentrations of Irgarol 1051 in the presence of 2 day old natural biofilm. Vertical lines indicate standard deviation from the mean and are shown as positive.

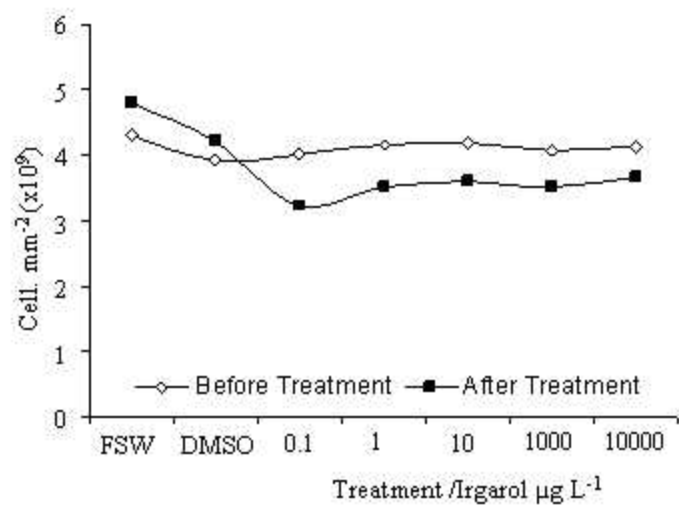


Fig. 1.

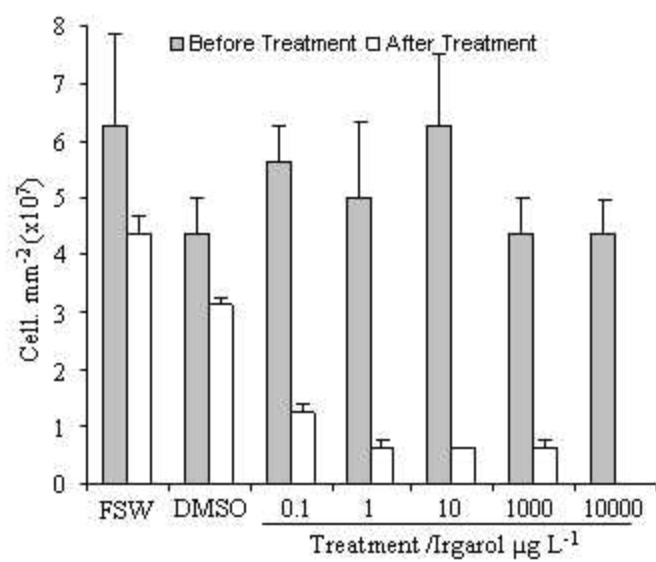


Fig 2

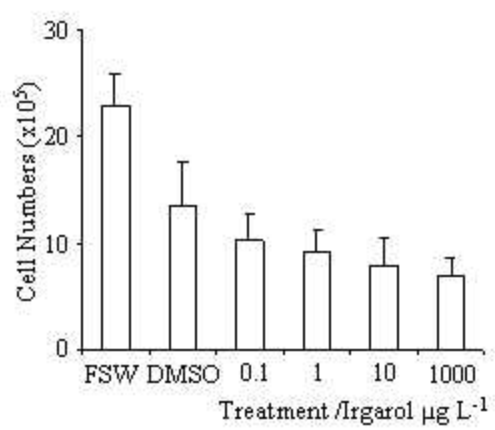


Fig. 3

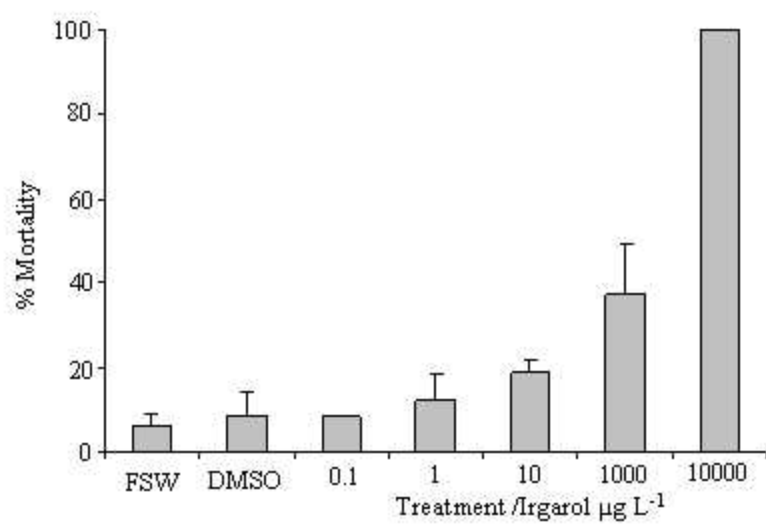


Fig. 4



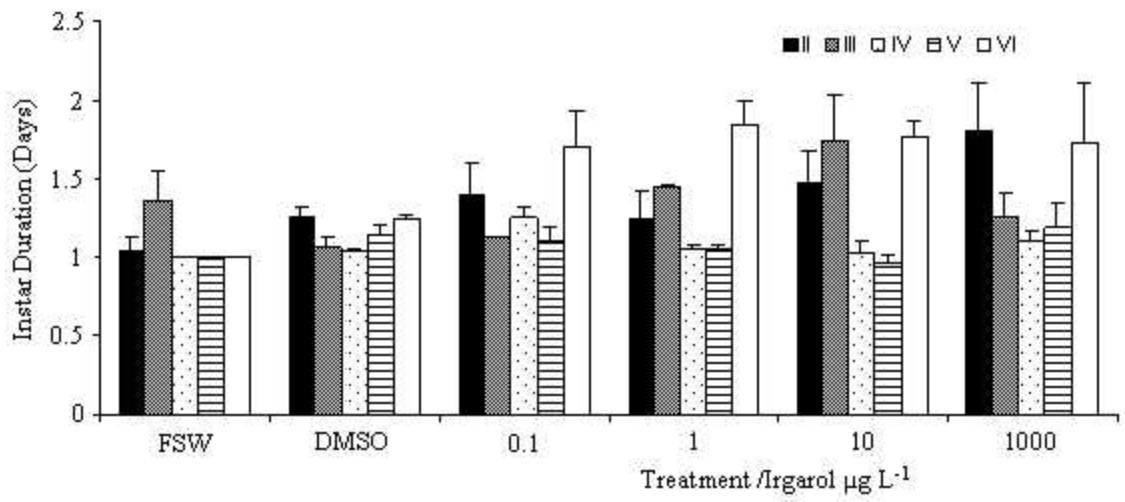


Fig. 5

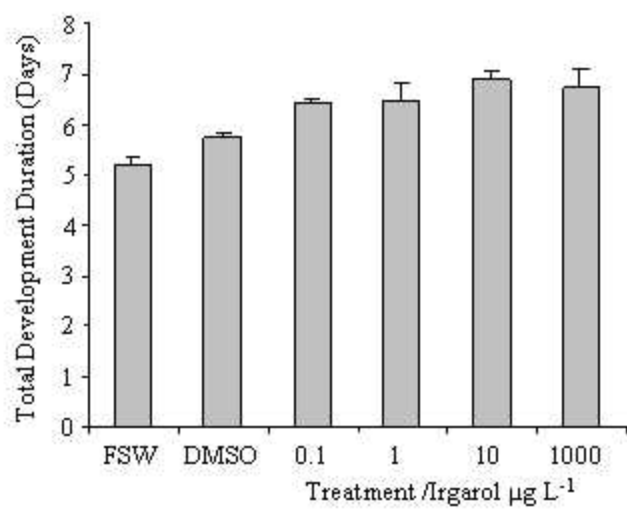


Fig. 6

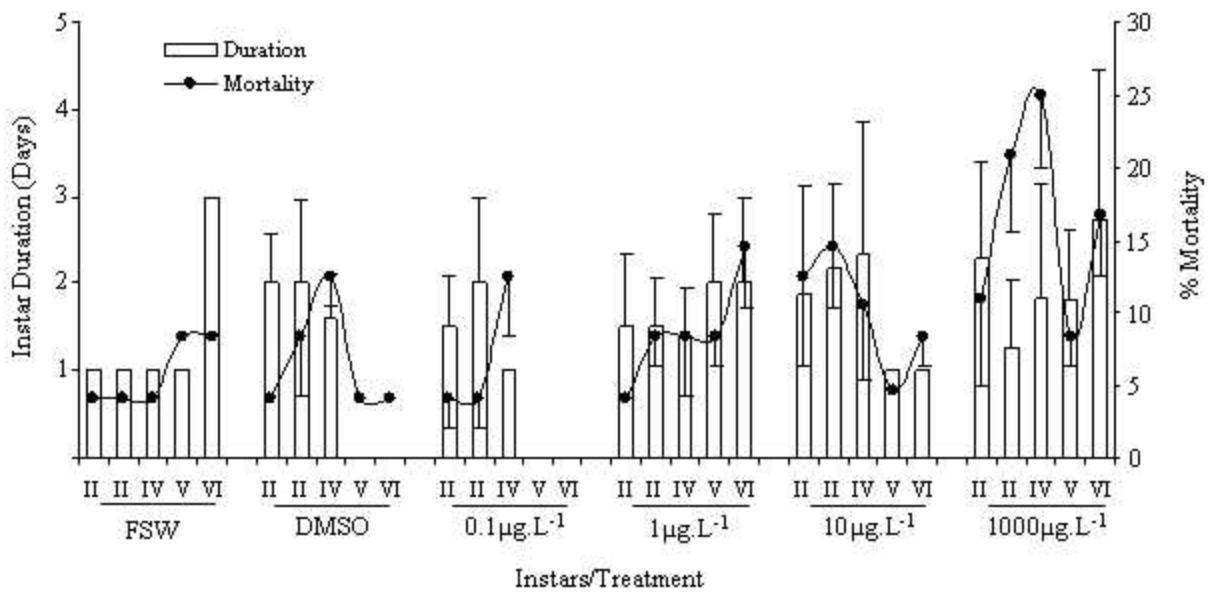


Fig. 7

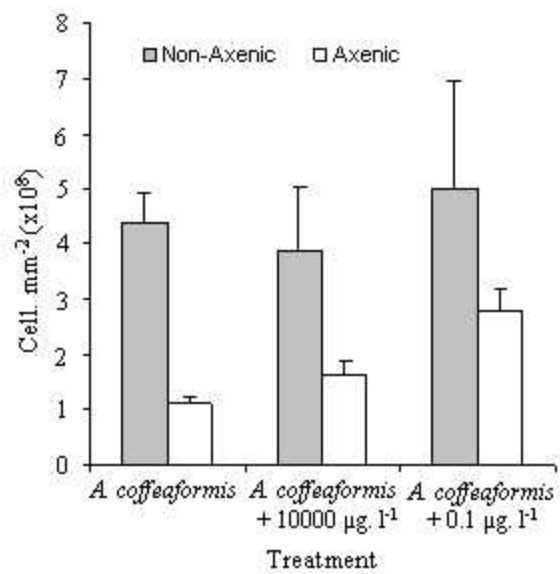


Fig. 8

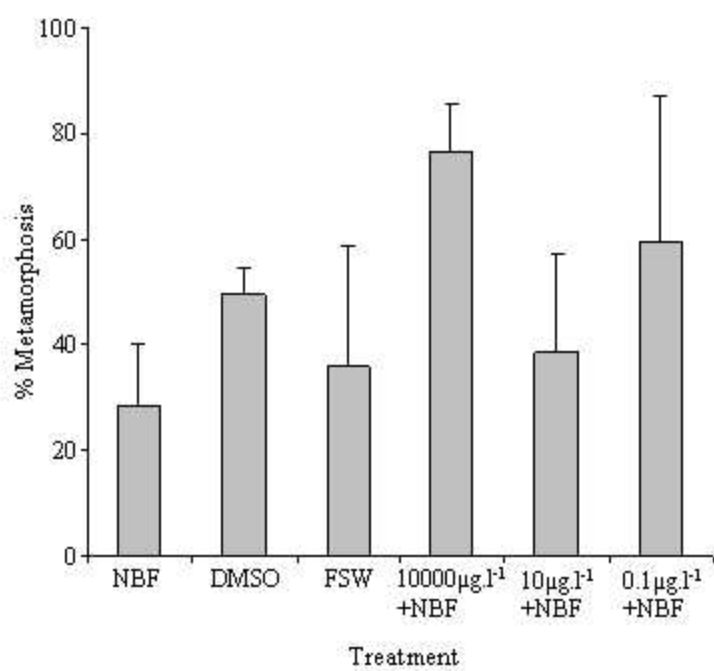


Fig 9

Table 1. Power function regression values for instar and total naupliar duration ( $D=bv^m$ ), for the influence of Irgarol (V) on the instar and (TND) total naupliar duration (D) of the larvae. n – number of observations; ns, not significant

	b	m	r	n	p
II instar	1.065	0.15	0.252	117	≤0.01
III instar	1.08	0.122	0.23	118	≤0.02
IV instar	1.01	0.01	0.02	118	Ns
V instar	1.07	-0.06	0.13	118	Ns
VI instar	1.12	0.26	0.38	118	≤0.001
TND	5.51	0.13	0.53	118	≤0.001