

# Larval development and post-settlement metamorphosis of the barnacle *Balanus albicostatus* Pilsbry and the serpulid polychaete *Pomatoleios kraussii* Baird: Impact of a commonly used antifouling biocide, Irgarol 1051

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## Abstract

The impact of a commonly-used antifouling algicide, Irgarol 1051, on the larval development and post-settlement metamorphosis of the barnacle, *Balanus albicostatus* Pilsbry (Crustacea: Cirripedia), and the larval metamorphosis of a serpulid polychaete, *Pomatoleios kraussii* Baird, was evaluated. In the case of *B. albicostatus*, larval mortality increased with an increase in the concentration of Irgarol 1051, and there was a shift in the larval stage targeted from advanced instars to early instars. Nauplii that survived to the cyprid instar stage when reared in the presence of Irgarol 1051 showed prolonged instar and total naupliar duration when compared to the controls. The post-settlement metamorphosis of cyprids significantly varied with Irgarol concentration and also with biofilm age. One and 2-d-old untreated biofilms showed higher metamorphosis when compared to 5-d-old biofilms. However, when the biofilms that promoted cyprid metamorphosis were treated with Irgarol 1051 at low concentrations, metamorphosis rates decreased. Cyprids were prevented from metamorphosing completely by biofilms treated at the highest concentration of Irgarol 1051. Inhibition of metamorphosis was also observed in the case of competent polychaete larvae when exposed to Irgarol 1051 compared to those exposed to metamorphosis inducers such as 3-iso-butyl-1-methylxanthine (IBMX) and natural biofilms. Identification of the pathway(s) that caused the promotory biofilms to become toxic when exposed to Irgarol 1051 is discussed.

**Keywords:** *Balanus albicostatus*, *Pomatoleios kraussii*, *Irgarol 1051*, *metamorphosis*, *biofilm*

## Introduction

Structures submerged in the aquatic environment are subject to biofouling (Evans & Hoagland, 1986) which causes extensive damage. In nature, biofilms, consisting predominantly of bacteria and diatoms, begin to develop almost immediately after a surface is immersed (Wahl, 1989). These biofilms influence the settlement of many invertebrate larvae (Mihm et al. 1981; Strathmann et al. 1981; Maki et al. 1992; Pawlik, 1992; Rodrigues et al. 1993; Keough & Raimondi, 1995; Wieczorek & Todd, 1998; Hadfield & Paul, 2001; Lee & Qian, 2003). Natural microbial biofilms influence the settlement response of the cypris larvae of barnacles (Strathmann et al. 1981; Maki et al. 1988, 1990; Harder et al. 2001). Biofouling is one of the most serious problems currently faced worldwide by maritime industries (Clare, 1996; Abarzua et al. 1999; Townsin, 2003),

which costs the US Navy an estimated \$1 billion per annum (Callow & Callow, 2002). In order to solve this problem, marine coatings industries are developing long-life antifouling products. Prior to the early 1990s, organotin biocides (e.g. tributyltin (TBT) and triphenyltin) were commonly used in antifouling paints (Willemsen & Ferrari, 1993; Isensee et al. 1994; Ponasik et al. 1998; Boxall et al. 2000; Evans et al. 2000). However research during the last 25 years has demonstrated the toxic effect of TBT on both target and non-target organisms, even at very low concentrations (Bryan et al. 1986; Gibbs et al. 1988; Smith & McVeagh, 1991; Nias et al. 1993; Willemsen et al. 1998; Depledge & Billingham, 1999; Marin et al. 2000; Kobayashi & Okamura, 2002; Bhosle et al. 2004). Recently, a complete ban on the use of TBT has been approved by the International Maritime Organization and from 2008, TBT-containing paints will

not be permitted on any vessels. Copper-based paints supplemented with so-called 'booster biocides', including herbicides, have mostly replaced the organotins in antifouling paints worldwide (Readman et al. 1993; Liu et al. 1997; Turley et al. 2005). The two herbicides, Irgarol 1051 and diuron, are most commonly employed in boat paints. The herbicide Irgarol 1051 is designed to prevent the build-up of algal biofilms on ships' hulls. A consequence of this is a strong potential for contamination of coastal waters since it is moderately soluble and environmentally persistent. Degradation of Irgarol 1051 in sea- and freshwater sediment is slow, with half-lives of 100 and 200 d, respectively (Ciba Geigy, 1995; Callow & Willingham, 1996). Several studies investigating potential contamination by this compound reported its widespread occurrence in many coastal waters, lakes and marinas around the world (Readman et al. 1993; Gough et al. 1994; Dahl & Blanck, 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 sediment samples (Albanis et al. 2002).

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 & Blanck, 1996; Scarlett et al. 1999; Okamura et al. 2000; Nystrom et al. 2002; Owen et al. 2002; Macinnis-Ng & Rahlf-Peter, 2003). Accumulation of Irgarol 1051 has been shown to occur in freshwater macrophytes (Toth et al. 1996). However, studies with the marine or freshwater animals are very limited. Earlier studies have 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 was moderately toxic to crustaceans (24-h  $LC_{50} > 40 \text{ mg l}^{-1}$  for *Artemia salina*) (Okamura et al. 2000), whilst Kobayashi and Okamura (2002) reported its toxicity to sea urchin development.

The objective of the present investigation was to understand the potential ecotoxicological effect of Irgarol 1051 on two marine organisms, an acorn barnacle *Balanus albicostatus* and a serpulid polychaete *Pomatoleios kraussii*, which form major components of the intertidal community. Experiments were undertaken to study the effect of Irgarol 1051 on the larval development of *B. albicostatus*. The effect of this compound on the post-settlement metamorphosis of competent larvae (cypris) of *B. albicostatus* and *P. kraussii* was also studied. The influence of natural biofilms exposed to different concentrations of the biocide on cypris post-settlement metamorphosis was assessed.

## Materials and methods

Irgarol 1051 (2-methylthio-4-tert-butylamino-6-cyclopropylamino-s-triazine), was obtained from Ciba Specialty Chemicals, Japan. A stock solution of Irgarol 1051 was prepared at a concentration  $10000 \text{ mg l}^{-1}$  in dimethyl sulphoxide (DMSO) as described by Kobayashi and Okamura (2002). The stock solution was used to make up a series of seawater dilutions. The first part of the manuscript deals with the effect of Irgarol 1051 on larval development and post settlement metamorphosis of the acorn barnacle, *B. albicostatus* Pilsbry (Crustacea: Cirripedia) and the second part reports the influence of the same biocide on the larval metamorphosis of *P. kraussii* Baird (Polychaeta: Serpulidae).

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

*Rearing of B. albicostatus larvae.* Adults of *B. albicostatus* were collected from the intertidal area near Seto Marine Biological Laboratory, Wakayama Prefecture, Japan. The barnacles were brought to the laboratory and exposed to air for a short time and then placed in filtered seawater, which triggered the release of larvae. The larval phase of *B. albicostatus* consists of six naupliar instars followed by a settling cypris instar. Instar II nauplii were used to carry out different rearing experiments. Rearing of larvae was carried out at four different concentrations of Irgarol 1051 (0.5, 1.0, 2.0 and  $5 \text{ mg l}^{-1}$ ), at  $27 \pm 1^\circ\text{C}$  and a 12 h: 12 h, light:dark cycle. The food used was *Chaetoceros calcitrans*, a unicellular diatom. Instar II nauplii were transferred into multiwells (24-well Nunclon Delta 143982) with the respective concentration of Irgarol 1051 with food and reared individually. The rearing wells were maintained in an incubator, and rearing of nauplii was carried out until they reached the cypris instar stage. Every day, observations of larval mortality and molting were recorded prior to transferring the larvae into fresh multiwells. Controls contained filtered seawater (FSW) with and without the addition of DMSO (the highest concentration used in the dilution series). The Lethal Concentration for larval mortality ( $LC_{50}$ ) was calculated at 48 h.

To obtain cypris larvae for settlement assays, II instar nauplii were mass reared in 2-l glass beakers with *C. calcitrans* as food at a cell concentration of  $1 \times 10^5 \text{ cells ml}^{-1}$  at  $27 \pm 1^\circ\text{C}$  and a 12 h:12 h, light:dark cycle. The diatom cultures were grown in f/2 medium. An antibiotic dosage of 0.3 ml Crystamycin  $1^{-1}$  was used (300 mg Penicillin G and 400 mg Streptomycin in 4 ml distilled water) (Harmes, 1986; Anil et al. 1995).

### Biofilm development

The sterile multiwells (24-well Nunclon Delta 143982) were used to develop biofilms by immersing in fiberglass tanks with a continuous flow of non-filtered natural seawater. Biofilms were allowed to develop in multiwells for 1, 2 and 5 d before being used in assays. Non-biofilmed multiwells were used as controls.

### Settlement assays

Cyprid settlement assays are often used in screening antifouling compounds (Rittschof et al. 1994; Nogata et al. 2003). The multiwells (24-well plates, Nunclon Delta 143982) were inoculated with different concentrations of Irgarol 1051 (0.1  $\mu\text{g}$ , 1.5  $\mu\text{g}$ , 500  $\mu\text{g}$ , 2 mg and 5 mg.  $l^{-1}$ ). Approximately 25–30 cyprids were introduced with 2 ml of each of the Irgarol 1051 concentrations made up using autoclaved filtered seawater (35‰) (Maki et al. 1990).

In addition, the multiwells with biofilms were treated with different Irgarol concentrations. These multiwells were rinsed after 3 h exposure by repeated rinsing with autoclaved filtered seawater. Approximately 25–30 cyprids were introduced with 2 ml of autoclaved filtered seawater (35‰) into each well.

Six replicates of each concentration were used for the assessments. The experiment was repeated twice using different batches of larvae ( $n=6$ , with batch as an additional factor). Filtered seawater (FSW) was used as the control. The settlement assays were monitored every 24 h for a period of 4 d and metamorphosed cyprids were counted at the end of each day. Assay wells were maintained at  $26 \pm 1^\circ\text{C}$  (12 h light:12 h dark cycle).

### Rearing of *P. kraussii* larvae

Adults of *P. kraussii*, a common serpulid species found in Japanese waters, were collected from small rocks near the Seto Marine Biological Laboratory, Japan. Obtaining gametes and raising larvae followed the method described by (Lau & Qian, 1997; Lau et al. 2002). Clean tubes of adult worms were gently broken into Petri dishes containing Millipore-filtered (0.22  $\mu\text{m}$ ) seawater, which induced the release of eggs and sperm. Newly-released oocytes and sperm were mixed and transferred to 500 ml FSW in glass beakers for fertilization. Excess sperm were removed by filtration and fertilised eggs were transferred to 2-l glass beakers containing FSW. After hatching, larvae were fed with *Isochrysis galbana* at a cell concentration of  $1 \times 10^5$  cells  $ml^{-1}$  and maintained at  $27 \pm 1^\circ\text{C}$ . The culture was aerated and maintained

in a 14 h: 10 h, light: dark cycle until the larvae reached the competent stage (6–8 d). Competency was determined by both larval morphology (Wisely, 1958; Scheltema et al. 1981; Bryan et al. 1997) and testing with 3-iso-butyl-1-methylxanthine (IBMX) as described by Qian & Pechenik (1998) and Lau et al. 2002. Approximately 20–25 larvae were transferred to  $10^{-4}$  M IBMX in filtered seawater. When approximately 90–95% of the larvae had attained competency they were concentrated by filtering through 100  $\mu\text{m}$  mesh and used to carryout the assays.

### Preparation of *P. kraussii* adult worm homogenate (adult extract)

Due to the gregarious nature of the sessile polychaete, its adult extract was used as a positive control along with IBMX as a metamorphosis inducer. The adult extract was prepared following methods described by Bryan et al. (1997). Adult worms were removed from their tubes and dipped in filtered seawater, blotted dry on a paper towel and weighed to the nearest mg. Homogenates were prepared by crushing and sonicating adults in deionized water at a concentration of 0.2 g worm  $ml^{-1}$  water. The homogenate was centrifuged ( $12,660 \times g$ ) for 10 min. The supernatant obtained was stored at  $-20^\circ\text{C}$ .

### *P. kraussii* settlement assays

The competent larvae of *P. kraussii* ( $\sim 25-30$ ) were introduced into the multiwells (6-well plates, Corning – 430343) containing 5 ml of Millipore-filtered, autoclaved seawater of salinity 35‰ with different concentrations of Irgarol 1051 ( $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$  M). The experiments were repeated twice using different batches of larvae with six replicates at each concentration ( $n=6$ , with batch as an additional factor). Larvae that had attached to the dish, produced a tube and grown tentacles were considered to have undergone normal metamorphosis. Unattached and swimming or crawling larvae were considered to be unmetamorphosed. The effect of IBMX on larval settlement and metamorphosis was tested at concentrations of at  $10^{-2}$  and  $10^{-4}$  M. Larvae were also exposed to adult extract and tested at 0.2 mg supernatant  $ml^{-1}$  seawater.

The settlement assays were monitored for a period of 4 d with an intermittent observation everyday. Assay wells were maintained at  $26 \pm 1^\circ\text{C}$  (12 h light: 12 h dark cycle). Controls were FSW (negative control) and adult extract (positive control). A 5-d-old biofilm was also assayed for its effect on larval settlement and metamorphosis.

### Statistical analysis

Data in the form of percentage larval settlement were arcsine transformed to ensure normality of means and homogeneity of variances before statistical analysis. The influence of different concentrations of Irgarol 1051 on cyprid post-settlement metamorphosis was evaluated using One-way analysis of variance (ANOVA) (Sokal & Rohlf, 1981). Similarly, the influence of different Irgarol concentrations and metamorphic inducers on metamorphosis of the competent larvae of *P. kraussii* was evaluated using One-way ANOVA. The influence of different aged biofilms (1, 2 and 5-d-old) exposed to different concentrations of Irgarol 1051 on the post-settlement metamorphosis of *B. albicostatus* cyprids was also determined using One-way ANOVA. One-way ANOVA was followed by Scheffe's test or Tukey's test ( $\alpha = 0.05$ ).

Power function regression (PFR) analyses were carried out to see the influence of Irgarol concentration (v-variable) on the instar duration and total naupliar duration (D) as  $D = bv^m$  (where, b = intercept, m = slope).

## Results

### Larval mortality

Mortality of the larvae increased with an increase in the concentration of Irgarol. At  $0.5 \text{ mg l}^{-1}$  and  $1 \text{ mg l}^{-1}$  Irgarol 1051, mortality was 45.8% and 83%, respectively. However at 2 and  $5 \text{ mg l}^{-1}$  the larvae did not survive and 100% mortality was observed. In contrast, the DMSO and FSW controls showed very low mortalities of 12.5 and 5%, respectively (see Figure 1). The 48 h  $\text{LC}_{50}$  value for larval mortality was  $0.556 \text{ mg l}^{-1}$ .

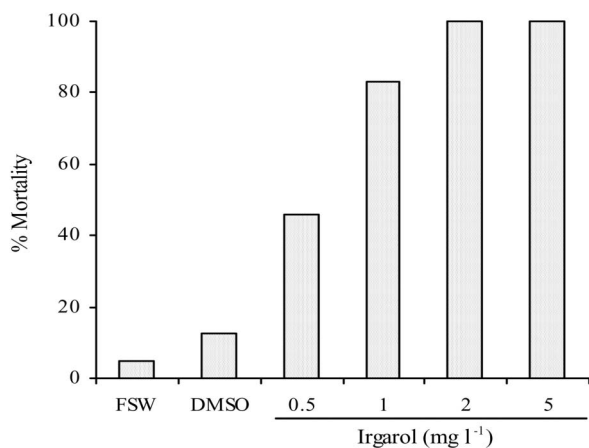


Figure 1. Percentage mortality of nauplii reared in different irgarol concentrations. Controls were reared in filtered seawater (FSW) or FSW with DMSO.

### Larval instar and total naupliar duration

The instar duration of nauplii that successfully metamorphosed to cyprids is presented in Figure 2.

Nauplii showed a prolonged instar duration developing in the presence of Irgarol, compared to the DMSO and FSW controls (Figure 2). For II–IV instar larvae, the instar duration of larvae treated with  $0.5 \text{ mg l}^{-1}$  of Irgarol was similar or slightly longer than that of the controls. Whereas, at  $1 \text{ mg l}^{-1}$  the instar duration was almost double for II–IV instars (Figure 2). In advanced instars (V and VI) the duration of the Irgarol treated larvae was longer compared to early instars, and was almost double or at times more than double when compared to the instar duration of control larvae (Figure 2). Total naupliar duration (TND) was also longer when treated with Irgarol 1051 (see Figure 3).

The DMSO and FSW control larvae showed a TND of 5.5 and 4.7 d respectively. However, in presence of Irgarol, the TND was 7.61 and 10.5 d for  $0.5$  and  $1.0 \text{ mg l}^{-1}$  Irgarol respectively (Figure 3). The power function regression (PFR) analyses indicated a significant increase in the instar duration and total naupliar duration for the Irgarol treated larvae (see Table I).

### Larval mortality at a particular instar

In the case of the FSW control, mortality was found only in the VI instar (4.1%) and in the DMSO control, in II and VI instar (4 and 8% respectively) (see Figure 4). It was observed that with an increase in the concentration of the Irgarol from  $0.5$  to  $5 \text{ mg l}^{-1}$ , the mortality of larvae in a particular instar shifted from advanced instars to early instars (Figure 4). At  $0.5 \text{ mg l}^{-1}$  Irgarol, mortality occurred only in V (8.3%) and VI (37.5%) instars. At 1 and  $2 \text{ mg l}^{-1}$  Irgarol, mortality occurred in almost all the instars, being highest in early instars (II–IV) compared to

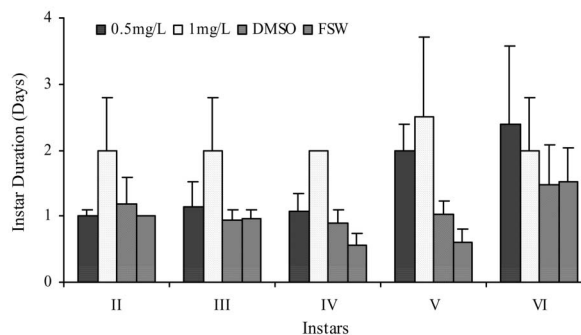


Figure 2. Variation in the naupliar instar duration of larvae reared in different irgarol concentrations. Controls were reared in filtered seawater (FSW) or FSW with DMSO. Vertical lines = SD from the mean.

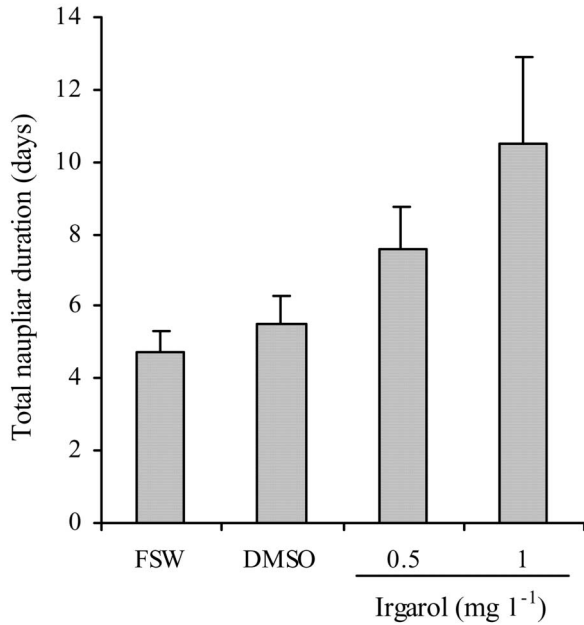


Figure 3. Total naupliar duration of the larvae reared in different irgarol concentrations. Controls were reared in filtered seawater (FSW) or FSW with DMSO. Vertical lines = SD from the mean.

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

	B	m	r	pn
II instar $p \leq 0.01$	1.127	0.2736	0.3563	61
III instar $p \leq 0.01$	0.8822	0.2341	0.3737	61
IV instar $p \leq 0.001$	0.5378	0.7175	0.808	61
V instar $p \leq 0.001$	0.5587	1.0161	0.8591	61
VI instar $p \leq 0.02$	1.3606	0.2478	0.2807	61
TND $p \leq 0.001$	4.4862	0.4472	0.8049	61

advanced instars (V and VI) (Figure 4). Whereas, in the case of  $5 \text{ mg l}^{-1}$  maximum mortality was seen in the II instar (71%) followed by III (25%) and IV (4%) instars. The larvae could not develop beyond IV instar at this concentration (Figure 4).

#### Instar duration of the dead larvae

Not all the nauplii treated with Irgarol showed mortality of the II instar, which varied with Irgarol concentration (see Figure 5). At the lowest concentration ( $0.5 \text{ mg l}^{-1}$ ) there was a gradual increase in

the instar duration of the dead larvae from II (1.09 d) to VI instar (5.3 d) (see Figure 5). At 1 and  $2 \text{ mg l}^{-1}$ , a gradual decrease in the instar duration was observed from II–VI instar. In the case of  $5 \text{ mg l}^{-1}$  Irgarol, dead larvae showed a II instar duration of 3.4 d, which decreased to 1 d for IV instar larvae (Figure 5). Larvae could not develop beyond IV instar at this concentration, indicating an adverse effect of Irgarol on larval development.

#### Settlement assays

When cyprids were exposed to different concentrations of Irgarol 1051 there was a significant difference in post-settlement metamorphosis (One-way ANOVA,  $p \leq 0.001$ ; Scheffe's test,  $p \leq 0.05$ ) when compared to FSW (see Figure 6a). At lower Irgarol concentrations, metamorphosis was similar ( $0.1$ – $500 \mu\text{g l}^{-1}$ ), although less than in FSW. A significant inhibition in metamorphosis was evident at  $2 \text{ mg l}^{-1}$  Irgarol ( $p = 0.011$ , Tukey's test; Figure 6a) and total inhibition of metamorphosis was observed at  $5 \text{ mg l}^{-1}$  ( $p = 0.009$ , Tukey's test; Figure 6a) at the end of day 1. A similar trend was observed at the end of day 4. One-d-old biofilm promoted the post-settlement metamorphosis of cyprids (Figure 6b). However, when 1-d-old biofilm was treated with Irgarol 1051, there was a decrease in metamorphosis of cyprids. A significant inhibition was observed at  $2 \text{ mg l}^{-1}$  ( $p = 0.038$ , Tukey's test; Figure 6b) and at  $5 \text{ mg l}^{-1}$  complete inhibition was observed ( $p = 0.0102$ , Tukey's test; Figure 6b). Similar observations were made for 2-d-old biofilms (Figure 6c). Whereas, 5-d-old biofilm showed a slightly lower percentage of cyprid metamorphosis compared to 1 and 2-d-old biofilms (Figure 6d) at the end of day 1. Cyprids did not settle for 3 days when exposed to 5-d-old biofilm treated with  $2 \text{ mg l}^{-1}$  ( $p = 0.010$ , Tukey's test; Figure 6d), and at  $5 \text{ mg l}^{-1}$  Irgarol complete inhibition was observed ( $p = 0.0102$ ; Figure 6d). As a general trend, cyprids exposed to biofilms of different ages treated with low concentrations of Irgarol ( $0.1$ ,  $1.5$  and  $500 \mu\text{g l}^{-1}$ ) showed similar metamorphosis to control cyprids and at higher concentrations either inhibition of metamorphosis or no metamorphosis was observed (see Figure 6b–d) on days 1 and 4.

#### *Pomatoleios kraussii*

Competent larvae of *P. kraussii* showed an inhibition of metamorphosis when exposed to different concentrations of Irgarol 1051 and this effect was significantly different from the metamorphosis inducer, IBMX that promoted metamorphosis at  $10^{-2}$  M (one-way ANOVA,  $p \leq 0.001$ ; Scheffe's test,  $p \leq 0.05$ ) (see Figure 7). When compared to IBMX ( $10^{-2}$  M) the adult extract and 5-d-old biofilm

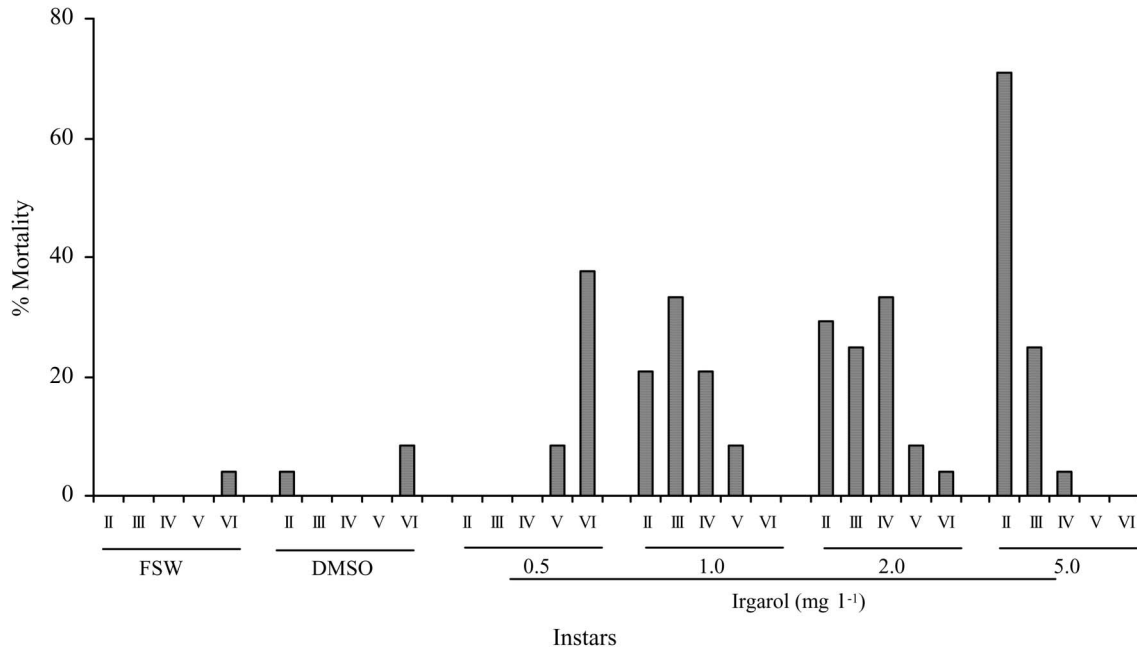


Figure 4. Percentage mortality of nauplii in different instars reared in different irgarol concentrations. Controls were reared in filtered seawater (FSW) or FSW with DMSO.

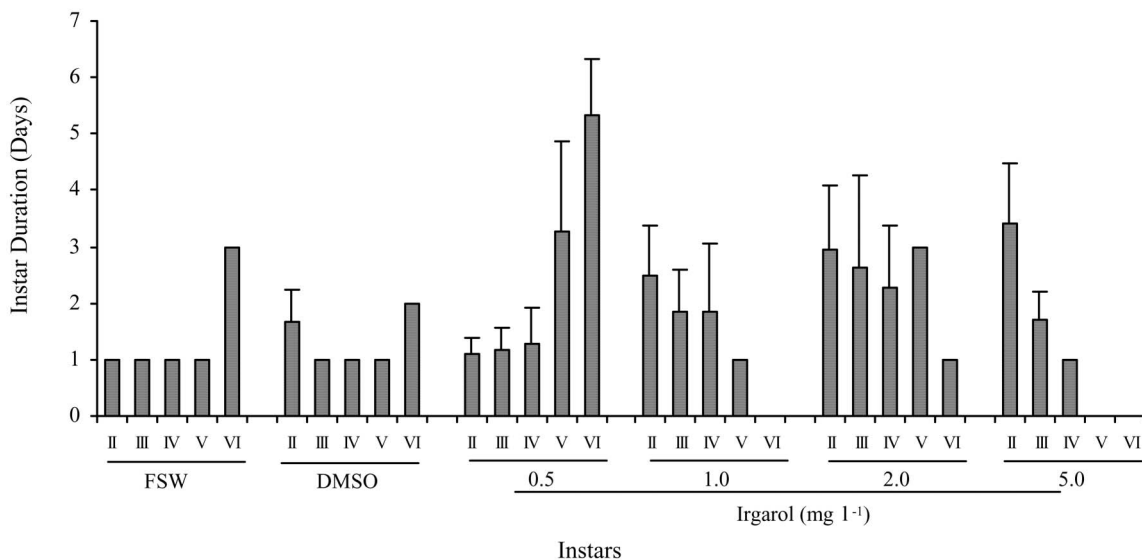


Figure 5. Naupliar instar duration (II–VI instar) of larvae reared under different irgarol concentrations, DMSO and FSW. Vertical lines = SD from the mean.

showed a similar and reduced effect. The effect of competent larvae exposed to Irgarol 1051 was lower than these settlement cues (Figure 7).

**Discussion**

Irgarol 1051 is prevalent in tropical marine ecosystems and is a potent inhibitor of coral photosynthesis

at environmentally relevant concentrations (Owen et al. 2002). However, the effect of this compound on marine invertebrate development is not well understood. In the present experiment, *B. albicostatus* larvae were reared using different concentrations of Irgarol 1051. At 0.5 mg l<sup>-1</sup> only 50% of the larvae could metamorphose successfully into cyprids while at lower concentrations larval development was

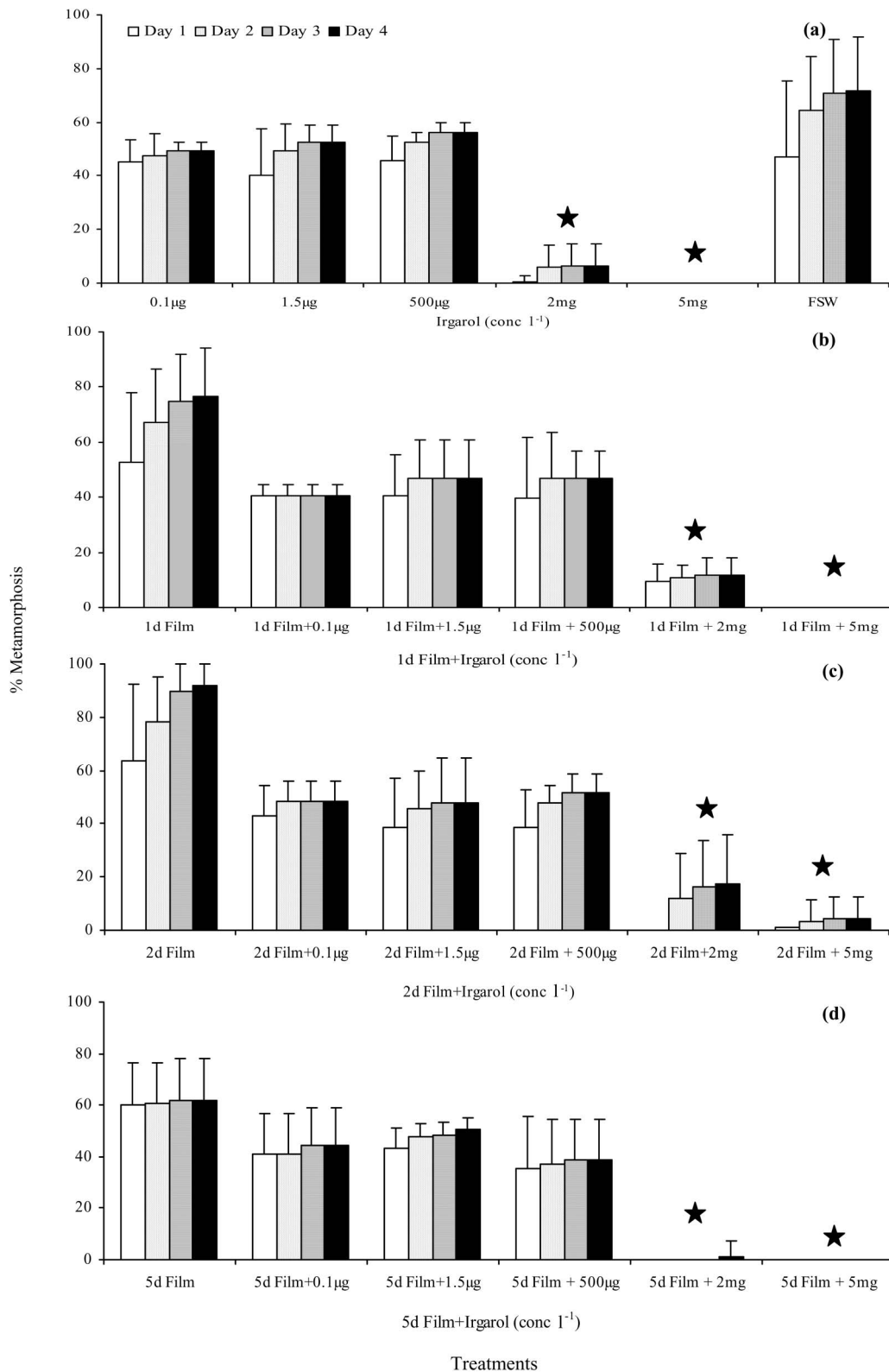


Figure 6. Percentage metamorphosis of *B. albicostatus* cyprids (a) exposed to different concentration of irgarol 1051; (b) exposed to different concentration of irgarol 1051 in presence of 1-d-old biofilm; (c) exposed to different concentration of irgarol 1051 in the presence of 2-d-old biofilm; (d) exposed to different concentration of irgarol 1051 in presence of 5-d-old biofilm. Vertical lines = SD from the mean. \* = significantly different data according to One-way ANOVA ( $p \leq 0.001$ ), followed by Tukey's test ( $p \leq 0.05$ ).

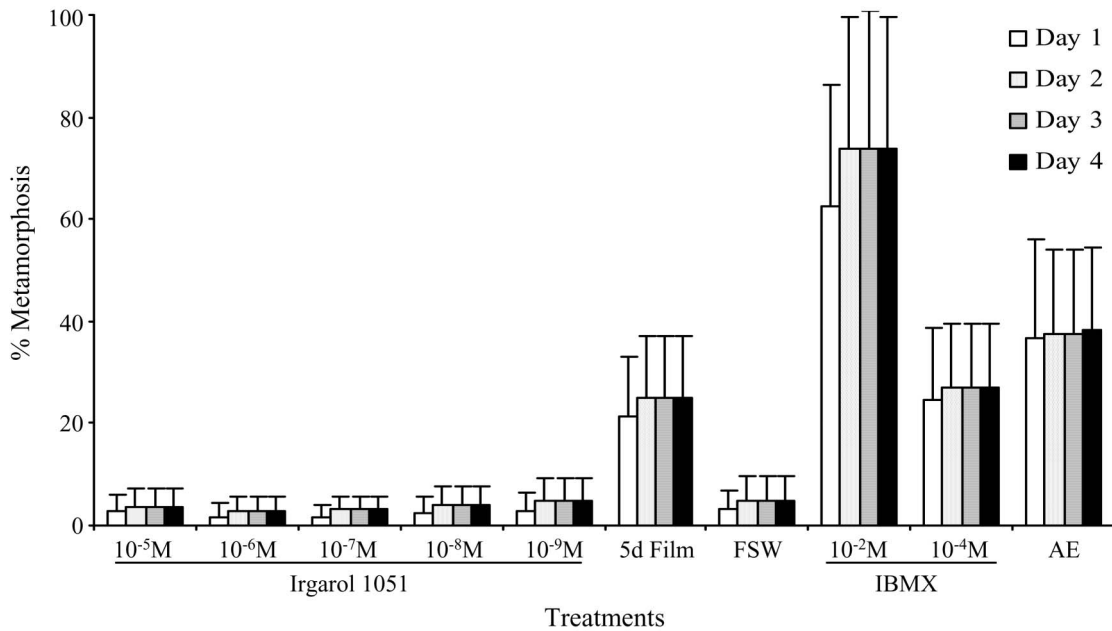


Figure 7. Percentage metamorphosis of *P. kraussii* larvae. Vertical lines = SD from the mean.

similar to that of the controls. The results indicated that mortality increased with an increase in the concentration of Irgarol 1051. At higher concentrations tested (2 and 5 mg l<sup>-1</sup>) larvae could not develop and 100% mortality was observed. Earlier investigations on the effect of long-term changes (3 months) in temperature and salinity on Irgarol 1051 release rates from antifouling paints indicated that temperature significantly increased the rate of release from 0.4–2.0 µg cm<sup>-2</sup> d<sup>-1</sup> (Technical Report; CEFAS, 2001). When incorporated on to marine antifouling coatings, Irgarol 1051 is expected to leach into water at a rate of 2.5–16 µg cm<sup>-2</sup> d<sup>-1</sup> (Ciba Specialty Chemicals, 2001). Based on an Irgarol ecological risk assessment (Hall et al. 1999), the range of Irgarol concentrations in estuarine/coastal waters may be as high as 1693 ng l<sup>-1</sup>. The present study reveals that at this concentration it is not harmful to the growth and development of *B. albicostatus*. However, it would be toxic if the concentration in the marine environment increased as a consequence of faster leaching rates or more boats using paint containing Irgarol 1051.

It was observed that nauplii that developed successfully showed prolonged instar duration in the presence of Irgarol. Similarly, this was reflected in the total naupliar duration of the larvae, some larvae taking almost double the duration to reach the cyprid instar in presence of Irgarol 1051. Recently, the toxic effect of copper on larval development of *Balanus amphitrite* was studied and it was shown that molting was a more sensitive endpoint than survival, nauplius II being the most

sensitive naupliar stage. However, a complete larval development assay was more sensitive than assays using a particular larval stage (Qiu et al. 2004). Prolongation of larval development may increase the chance of larval dispersal from the settlement habitat thereby increasing the threats of larval mortality for example by predation or dispersal and may therefore indirectly result in the control of fouling by this species. Predation, dispersal, availability of suitable food, changes in the physical environmental factors are considered as important factors for larval mortality in marine invertebrates (Thorson, 1950; Crisp, 1976; Olson & Olson, 1989; Dattesh & Anil, 2002).

The present study showed that larval mortality of particular instars indicated a shift in mortality from advanced instars to early instars with an increase in Irgarol concentration. At the lowest concentration, mortality was seen only in advanced instars, whereas at the highest concentration, mortality was observed only in early instars, II instar being the most sensitive and at this concentration larvae could not develop beyond IV instar. Observations on the instar duration of dead larvae also indicated similar results. In the present investigation naupliar development was studied in the presence of algal food as well as Irgarol 1051. The effect of the herbicide targeting the algal food and thereby indirectly affecting larval development cannot be ruled out, since at the lowest concentration of the biocide, mortality was seen only in advanced instars. This may indicate that the quality or quantity of food available after treatment with Irgarol may not be sufficient for the sustenance



of advanced instar larvae. This topic warrants further study.

The effect of Irgarol 1051 on larval metamorphosis of marine invertebrates is not well documented. When cyprids were exposed to different concentrations of Irgarol 1051 ( $0.1-5 \text{ mg l}^{-1}$ ), there was a significant difference in the post-settlement metamorphosis. One and 2-d-old biofilms produced higher metamorphosis compared to 5-d-old biofilms, indicating a difference in post-settlement metamorphosis with age of the film. Barnacle cyprids, like most other larvae, prefer to settle on substrata that possess a well-developed biofilm (Crisp, 1984; Clare et al. 1992). Films composed of individual strains of bacteria can have varying effects on larval settlement of fouling organisms such as barnacles, bryozoans, polychaetes and oysters (Kirchman et al. 1982; Weiner et al. 1985, 1989; Maki et al. 1988, 1989; Szewzyk et al. 1991; Holmström et al. 1992; Avelin Mary et al. 1993; Neal & Yule, 1994; O'Connor & Richardson, 1996; Khandeparker et al. 2002, 2003; Lee & Qian, 2003). The effect generated by bacterial strains, whether stimulatory or inhibitory for larval settlement, is amplified with the age of the film (Maki et al. 1989; Holmström et al. 1992). It has been shown earlier (Keough & Raimondi, 1995) that recruitment rates varied significantly with age of the film. Polychaete species showed a positive relationship whereas the barnacle *Balanus variegates* recruited in greater numbers onto less filmed surfaces. In order to mimic the potential variability and patchiness of natural biofilms, the influence of bacteria and diatoms mixed at different ratios was assessed on the larval settlement of *Hydroides elegans* (Harder et al. 2002). Most of the treatments showed higher percentages of settlement on mixed diatom-bacteria biofilms than on monospecies biofilms. Qualitative and quantitative changes in bacterial and diatom taxon composition and density also affected larval settlement of the bryozoan *Bugula neretina* (Dahms et al. 2004).

A lower rate of post-settlement metamorphosis was observed when the biofilms were treated with Irgarol 1051. Since Irgarol is a herbicide that is a potent inhibitor of photosynthetic activity, the reduction in metamorphosis rates can be attributed to its toxic effect on diatoms, which resulted in a lower concentration of diatoms in the biofilm. However at high concentration ( $2 \text{ mg l}^{-1}$ ), post settlement metamorphosis was 10–15%, and complete inhibition was observed when the films were exposed to  $5 \text{ mg l}^{-1}$  of Irgarol. When exposed to extreme environments or a range of stresses, organisms tend to respond to stress at the cellular level by expressing stress proteins and organic pollutants (Sanders, 1990). In nature, micro-organisms en-

counter adverse conditions frequently and thus need adaptational strategies to survive. When reacting to different stress factors, cells may change the rate of synthesis of certain cell components (Nepple & Bachofen, 1997), which could also be one of the reasons for the strong inhibition produced by the biocide-treated biofilms. In addition, the bioconcentration of Irgarol 1051 in biofilms cannot be ruled out. In the case of oyster larvae, it was found that settlement and metamorphosis was inhibited due to bioconcentration of TBT within biofilms (Labare et al. 1997).

Inhibition of metamorphosis was also observed for competent polychaete larvae when exposed to Irgarol 1051 compared to those exposed to IBMX and biofilm. Kobayashi and Okamura (2002) reported that at moderate concentration ( $1 \text{ mg l}^{-1}$ ), sea urchin eggs showed retarded cleavage and development whilst at a high concentration of Irgarol ( $10 \text{ mg l}^{-1}$ ), many sea urchin eggs were mostly unfertilized and later showed induced cytolysis. The development of polychaete larvae in the presence of this biocide needs further evaluation.

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