

Contrasting effects of heavy metals and hydrocarbons on larval settlement and juvenile survival in sponges

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Received 3 August 2006; received in revised form 16 November 2006; accepted 17 November 2006

Abstract

Metals and polycyclic aromatic hydrocarbons (PAHs) contaminate sediments and waters of coastal areas threatening early stages of invertebrate development. Effects on these stages may largely determine the decline and even disappearance of invertebrate populations in polluted environments. Our study aimed to determine the possible influence of metals (Cu and Cd) and PAHs on larval settlement and consecutive survival of two widespread sponges of the Mediterranean: *Crambe crambe* and *Scopalina lophyropoda*. Larvae of both species were exposed to Cu and Cd for a short period during 1 week, and settlement and following (6 months) survival of juveniles were monitored. Short exposures to copper and cadmium at the concentrations used did not affect *C. crambe* settlement compared with SW control, and no effect on consecutive survival of juveniles was observed. In contrast, short pulses of copper and cadmium at the concentrations used enhanced *Scopalina lophyropoda* settlement and did not affect the consecutive survival of juveniles with respect to SW controls. Furthermore, experiments designed to assess the effects of short exposures to PAHs and the combined effect of contamination by Cu²⁺ and PAHs on larval settlement, were conducted during 10 days on *C. crambe* larvae. Hydrocarbons, differently than copper and cadmium, inhibited the settlement of sponge larvae to a certain extent. The synergetic negative effect of copper and hydrocarbons on *C. crambe* settlers may cause a decline of populations in areas with both sources of contamination. The present study provides the only available data on toxicity of copper, cadmium and hydrocarbon toxicants on sponge larval settlement.

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Keywords: Heavy metals; PAHs; Sponge larvae; Settlement; Juvenile survival

1. Introduction

Significant amounts of pollutants have been released to marine and estuarine environments during the last few decades. Among them, metals and PAHs are contaminating sediments and waters in coastal areas (Palanques et al., 1995; Puig et al., 1999). These contaminants may have great toxic effects and can be accumulated in organisms through the trophic chains, making them a significant threat to the sustainability and fitness of marine ecosystems (Brown and Ahsnullah, 1971; Berthet et al., 1992; Canesi et al., 1999). Copper is one of the metals most abundant in the Mediterranean sublittoral areas (Tankere and Statham, 1996) with harmful effects on marine invertebrates described (Ahsnullah and Florence, 1984; Reichelt-Brushett and Harrison, 2000; Negri and Heyward, 2001; Viant et al., 2002). The main known sources of copper are the antifouling

paints that are being used on small (under 25 m length) vessels (Adair, 1987; Claisse and Alzien, 1993), sewage discharges (Bryan et al., 1987; Vale and Harrison, 1994) and fungicides and herbicides used in coastal agricultural crops (Clark et al., 1993; Cheadle et al., 1999). Cadmium is another relevant pollutant from industrial discharge and is highly toxic to a variety of aquatic animals (e.g., Eisler, 1985; Selck et al., 1998; Au et al., 2001). On the other hand, polyaromatic hydrocarbons (PAHs) have been recognised as the main toxic component of crude oil, polluting the marine environment (Roesijadi et al., 1978; Neff, 1985; Paul et al., 1998). In particular, harbours, coastal cities and the neighbouring areas, in which urban pollution and intense marine traffic converge, are exposed to different PAH levels.

Sponges are among those invertebrates that can be frequently found in zones subjected to moderate levels of metals and hydrocarbons (Carballo et al., 1996; Carballo and Naranjo, 2002; Cebrian et al., 2003; Perez et al., 2005). Although moderate pollution may seriously affect adult sponge physiology (Olesen and Weeks, 1994; Cebrian et al., 2006), some species can survive by

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using several mechanisms for neutralising the potentially noxious metals. The main mechanisms are accumulation of a non- or less-toxic form of metals and hydrocarbons in the tissues (Feral et al., 1979; Zahn et al., 1981; Patel et al., 1985; Verdenal et al., 1990; Hansen et al., 1995; Cebrian et al., 2003; Perez et al., 2005), induction of stress proteins (HSP family) (Agell et al., 2001), and binding by metallothionein or metallothionein-like proteins (Philp, 1999; Schröder et al., 2000; Berthet et al., 2005). However, data concerning toxicity of metals on marine larvae is, in general, scarce and no studies on the effects that these contaminants may produce on early phases of the sponge life cycle (larvae and settlers), have been conducted until now.

Sponge populations mainly rely on their larval settlement stages for maintenance (e.g., Blanquer, A., Uriz, M.J., Caujapé-Castells, J., unpublished data). Those early stages may be more sensitive to pollutants than adults, as has been reported for other invertebrates (Connor, 1972; Martin et al., 1981; reviewed in His et al., 1999). Sublethal effects of metals may have drastic repercussions at an ecological level when they alter biological processes of the organisms that indirectly affect to successive populations. For instance, a pollutant may kill half of the individuals of a species population with little or no ecological significance, whereas a pollutant that does not kill organisms but retards their development may have a considerably higher ecological impact (Moriarty, 1983). Thus, sensitivity of larvae and juveniles to low levels of pollution may largely determine a subtle decline and even disappearance of sponge populations in polluted environments. Consequently, knowledge on the pollution effects on adult sponges is not sufficient for a full understanding of its ecological influence, but effects on sponge larvae and juveniles have also to be taken in consideration in identifying hidden threats to sponge populations. Hence, our study was aimed to determine the possible influence of heavy metals (Cu and Cd) and PAHs on larval settlement and following survival of two representative sponge species of the Mediterranean (*Crambe crambe* and *Scopalina lophyropoda*).

2. Materials and methods

2.1. Larvae collection

The species selected for the study were *C. crambe* and *S. lophyropoda*, two common encrusting sponges in western Mediterranean (Boury-Esnault, 1971; Uriz et al., 1992). Both species reproduce during July–August at the study site (Blanes sublittoral, NE Spain, 41°40′37″N, 2°47′30″E), and release their parenchymella larvae to the water simultaneously (Uriz et al., 1998). Thus, larvae of both species coexist for a couple of months in the surroundings of adult populations. *C. crambe* larva is a type II parenchymellae (according to Mariani et al., 2005) with uniform dark orange to red colour. *S. lophyropoda* larva is an elongated, type I, parenchymella (according to Mariani et al., 2005), with uniform bright orange colour.

Larvae were obtained from ripe specimens of *C. crambe* and *S. lophyropoda*. Specimens of both species were collected by scuba diving at Santa Anna Point (Blanes sublittoral, NE Spain, 41°40′37″N, 2°47′30″E) a relatively unpolluted area (Pinedo,

1998; Cebrian et al., 2003), and transported to the laboratory in hermetic plastic bags. Larval release often occurred spontaneously during transport or consecutive acclimation in the laboratory.

2.2. Bioassays

2.2.1. Effects of Cu^{2+} and Cd^{2+}

Assays were carried out in sterile polystyrene culture tanks (500 ml). Five replicate tanks were used for each toxicant concentration as well as for seawater controls. Ten larvae of each species per container were used in the metal exposures and five larvae of *C. crambe* in both exposures to hydrocarbons and in synergistic exposures to Cu^{2+} and PAHs assays. The treatments used were: copper ($30 \mu g l^{-1}$) as cupric chloride ($CuCl_2$) and cadmium ($5 \mu g l^{-1}$) as cadmium chloride ($CdCl_2$). Controls consisted of 0.2 μm filtered seawater (SW) from a non-metal-polluted area (Pinedo, 1998; Cebrian et al., 2003). Metal concentrations in the seawater controls were below the threshold levels detectable by ICP-MS (see Section 2 in Cebrian et al., 2003). We selected these particular concentrations because they fit in the range of the concentrations that can be found in moderately polluted coastal waters (Cotté-Krief et al., 2002; Accornero et al., 2004).

Exposures were conducted in two steps in order to assess larval settlement and juvenile survivorship, respectively.

In the first step, experiments were conducted for 7 days to assess whether low concentrations of Cu^{2+} and Cd^{2+} affected larval settlement. We quantified settlement by directly counting the number of settlers per tank at each time point. We counted as a settler the stage at which larvae were irreversibly attached to the substratum to initiate the metamorphosis. We performed separate experiments instead of running all the treatments together in a sole experiment to avoid no interpretable interaction terms in the MANOVA analysis due to the high number of factors (Von Ende, 1993). Settlement in our controls occurred from 1 to 7 days after the release as has been reported for larvae of the two species (*C. crambe* and *S. lophyropoda*) in the field (Uriz, 1982; Uriz et al., 1998, 2001; Maldonado and Uriz, 1999), which indicates that our experiments had appropriate conditions.

In a second step, the water of controls and treatments of the previous experiment was changed by clean (non-filtered) seawater and juvenile survivorship was monitored during 6 months.

2.2.2. Effects of PAHs and the synergistic effects of Cu^{2+} and PAHs

Experiments designed to assess the effects of low levels of polycyclic aromatic hydrocarbons (PAH-mix, consisting of Benzo(a)pireno:Benzo(e)pireno:Benzo(a)antraceno:Criseño:Benzo(b)fluoranteno:Benzo(j)fluoranteno:Benzo(k)fluoranteno, in a proportion 10:2:2:2:2:2:2 $\mu g l^{-1}$ in acetonitril) and the combined effect of Cu^{2+} and PAHs on larval settlement lasted 10 days. Two concentrations of PAHs were used: 500 and 1000 $ng l^{-1}$. Filtered seawater (SW) (0.2 μm pore diameter) was used as a control. PAH concentrations in the seawater control were below the threshold levels detectable by gas chromatography coupled to mass spectrometry in the electron

Table 1
Two-way MANOVA results of treatment (copper, cadmium and seawater) and species (*Crambe crambe* and *Scopalina lophyropoda*) as factors

	Wilks value	F	Effect d.f.	Error d.f.	p
First step					
Species	0.109	20.97	7	18	0.000
Treatment	0.159	3.88	14	36	0.000
Species × treatment	0.200	3.17	14	36	0.003
Second step					
Species	0.341	4.979	7	18	0.003
Treatment	0.617	0.702	14	36	0.758
Species × treatment	0.409	1.449	14	36	0.181

impact mode (GC–MS–EI) (Solé et al., 2000). The synergistic effect of copper and PAHs was assessed by comparing four treatments: copper ($30 \mu\text{g l}^{-1}$), hydrocarbons (500 ng l^{-1} of PAHs) and a mixing of the two ($30 \mu\text{g l}^{-1}$ of copper and 500 ng l^{-1} of PAHs). A $0.2 \mu\text{m}$ filtered seawater (SW) was used as a control.

In the first-step experiments, water was changed daily (in both treatments and controls) to minimise changes in the concentration of pollutants with time due to metal absorption on the container walls (Batley et al., 1999). In the second-step experiment, the seawater that was unfiltered in order to supply some natural food to the sponges was changed twice a week. Temperature ($20\text{--}22^\circ\text{C}$) and the day/night cycle were similar to that of the sponge habitat. The concentrations referred to in the experiments are nominal concentrations, not concentrations available to the organisms, which was not determined.

2.3. Data treatment

The number of settlers at each observation time was standardised with respect to the initial number of larvae (time = 0). Differences between treatments were analysed by means of multivariate analysis of variance (MANOVA), since data do not meet the assumptions for ANOVA (i.e., sphericity Mauchly's test, Von Ende, 1993).

Two-way MANOVA was used to analyse the effects of metals on larval settlement, with species (*S. lophyropoda* and *C. crambe*) and treatment (30 Cu and $5 \mu\text{g l}^{-1} \text{ Cd}$) as factors. The results of the short-term and long-term experiments were

analysed separately. As the MANOVAs detected a significant effect of the species, one-way MANOVA was then performed for each species separately. Similarly one-way MANOVA was used to examine the effects of PAHs and copper + PAHs (combined effects) on *C. crambe* larvae. One-way ANOVAs were also used to analyse differences on larval settlement for treatment at each observation time. The Tukey test was used for post-hoc comparisons.

Data met the assumptions of normality and homogeneity of variances as assessed by the Kolmogorov–Smirnov and Bartlett tests, respectively. All the analyses were performed using Statistica 6.0 package.

3. Results

3.1. Effect of short pulses of Cu^{2+} and Cd^{2+} on settlement and juvenile survival

The effect of metals on larval settlement was different between species ($p < 0.01$; Table 1). Copper and cadmium did not affect larval settlement in *C. crambe* ($p = 0.187$; Fig. 1A) but both metals enhanced larval settlement in *S. lophyropoda* ($p < 0.05$; Fig. 1B) (Table 2). During the first 3 days of the experiment, larvae of *S. lophyropoda* behaved similarly in treatments and seawater control (Tukey $p > 0.05$), but from day 4 on, Cu^{2+} treatment significantly enhanced settlement (Tukey $p < 0.01$). Cd^{2+} also significantly enhanced settlement from day 5 on. At the end of the experiment, the percentage of settlers was significantly different between treatments themselves and between

Table 2
One-way MANOVA results of the effects of cadmium and copper on each species at the first- and second-step experiment respectively, and of PAHs and combined effects (copper + HAPS) on *C. crambe*

	Wilks λ -value	F	Effect d.f.	Error d.f.	p
Heavy metals					
First step					
<i>Crambe crambe</i> treatment	0.114	1.678	14	12	0.187
<i>Scopalina lophyropoda</i> treatment	0.037	3.591	14	12	0.016
Second step					
<i>Crambe crambe</i> treatment	0.469	0.394	14	12	0.950
<i>Scopalina lophyropoda</i> treatment	0.192	1.494	12	14	0.235
PAHs treatment	0.197	1.463	12	14	0.246
Synergy treatment	0.059	3.031	18	31.60	0.003

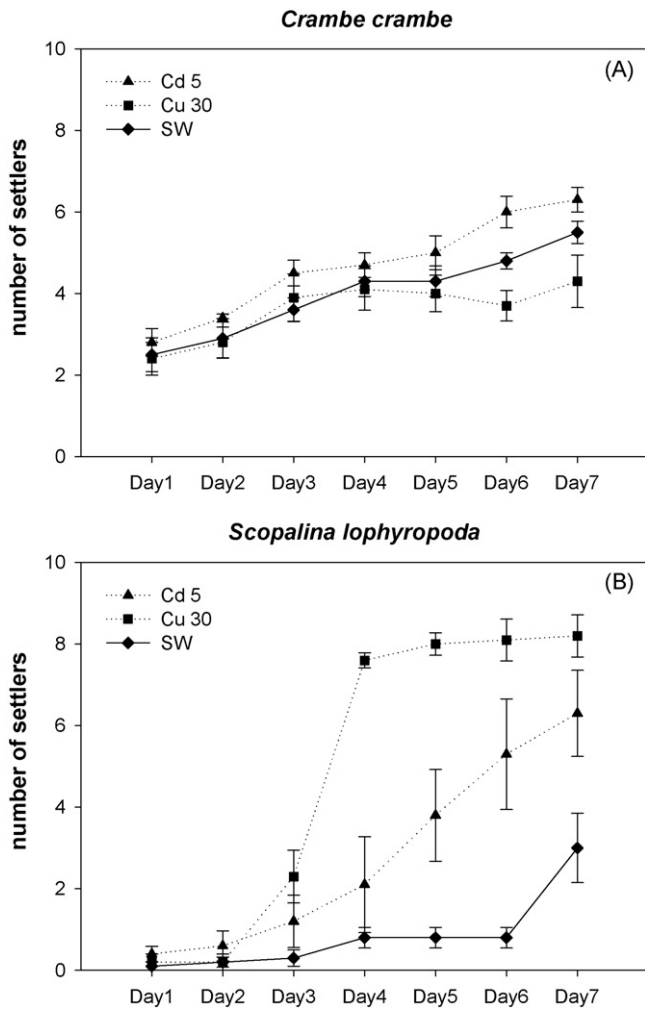


Fig. 1. Settlement of *Crambe crambe* (A) and *Scopalina lophyropoda* (B) larvae treated with copper ($30 \mu\text{g l}^{-1}$) and cadmium ($5 \mu\text{g l}^{-1}$) as a function of time. Vertical bars are standard errors. Values are from five replicate tanks per treatment with 10 larvae per tank.

each treatment and seawater controls, being the highest in the Cu^{2+} treatment (Tukey $p < 0.05$; Fig. 1B).

The survival trend with time of the *C. crambe* and *S. lophyropoda* juveniles during the long-term experiment is represented in Fig. 2A and B. The multivariate analysis of variance indicated no significant differences between species and treatment (MANOVA, $p = 0.11$, 0.69 , and 0.31 , respectively) and no significant interaction term ($p = 0.55$).

When the species were analysed separately, no significant differences ($p = 0.95$) between treatment and seawater control were found for *C. crambe* juveniles (Table 2). The percentage of *C. crambe* survivors was more or less constant from day 12 to the end of the experiment (6 months later) (Fig. 2A). As for *S. lophyropoda*, juveniles showed no significantly different survival between treatments and control ($p = 0.23$; Table 2) from day 8 to the end of the experiment (Fig. 2B). The percentage of survivors ranged from 50% to 60% in the treatments and from 50% to 77% in the controls after 1 month. After 6 months, however, the percentage decreased to ca. 10–20% in both treatment and control.

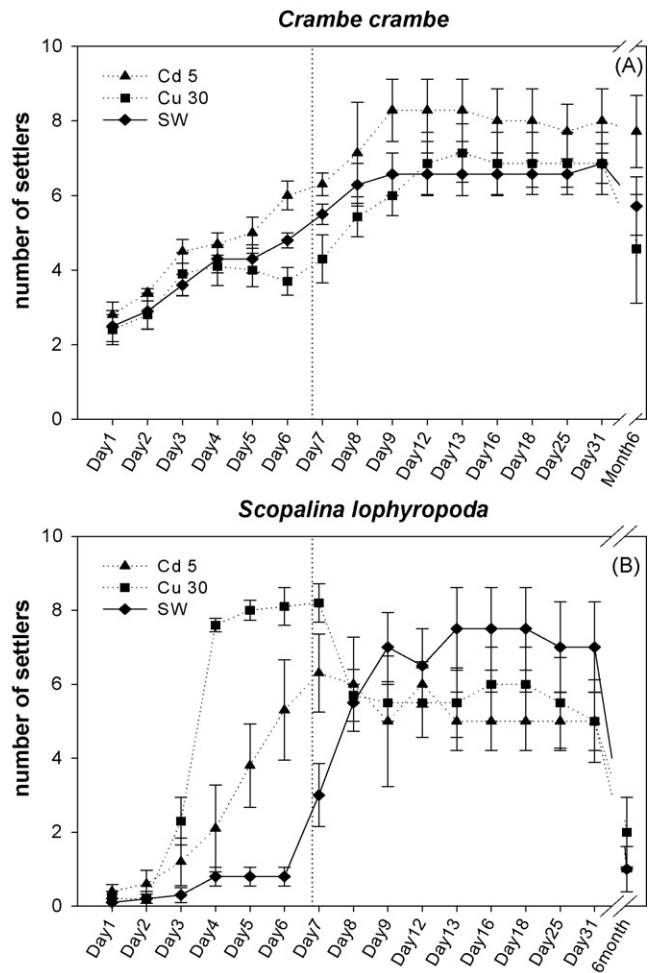


Fig. 2. Survival of *Crambe crambe* (A) and *Scopalina lophyropoda* (B) juveniles, previously incubated under Cu and Cd (concentrations as in Fig. 1), during the long term experiment. Vertical dashed lines separate the metal exposure period (first-step experiment) from that of maintenance in clean seawater (second-step experiment). Vertical bars are standard errors. Values are from five replicate tanks per treatment with 10 larvae per tank.

3.2. PAHs

No significant effect of PAHs on settlement of *C. crambe* larvae was detected in the MANOVA ($p = 0.24$; Table 2). Nevertheless, when the treatments were analysed for differences at day 10, one-way ANOVA revealed a significantly higher percentage of settlers in SW control (65%) than under the two PAH concentrations (25% and 30% in 500 and 1000 ng l^{-1} , respectively) (Fig. 3).

3.3. Synergy

The time course of the % of settlers of *C. crambe* under PAHs (500 ng l^{-1}), Cu ($30 \mu\text{g l}^{-1}$) and a mixing of the two treatments, along the experiment (10 days) is represented in Fig. 4. The multivariate analysis of variance MANOVA indicated significant differences between treatments ($p < 0.01$; Table 2). Settlement was similar in all treatments until day 7 of the experiment. However, from day 9 on, the percentage of settlers increased in SW

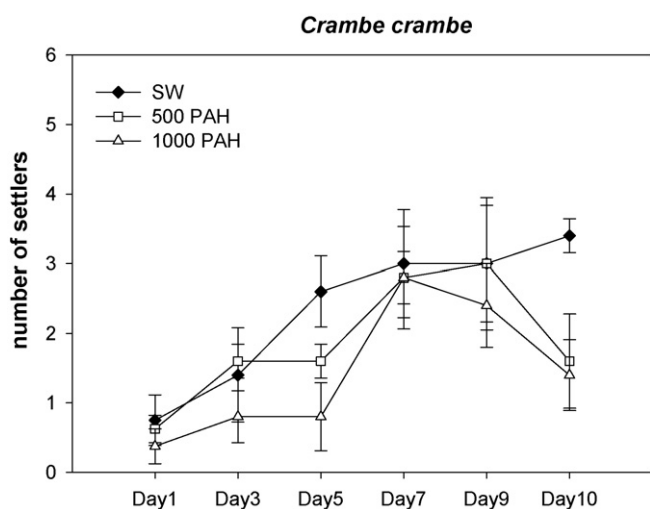


Fig. 3. Time courses of settlement of *Crambe crambe* larvae during exposure to 500 and 1000 ng g⁻¹ of PAHs and in SW control. Vertical bars are standard errors. Values are from five replicate tanks per treatment with five larvae per tank.

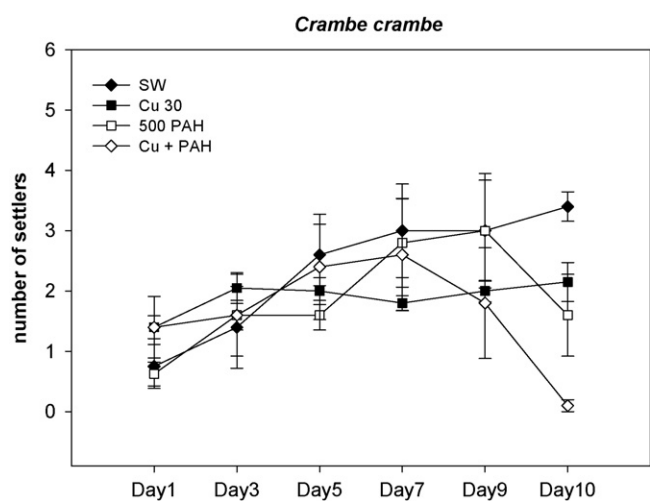


Fig. 4. Time courses of settlement of *Crambe crambe* larvae during exposure to 30 µg g⁻¹ Cu, 500 ng g⁻¹ of PAHs, a mixture of 30 µg g⁻¹ Cu and 500 ng g⁻¹ of PAHs, and SW control. Vertical bars are standard errors. Values are from five replicate tanks per treatment with five larvae per tank.

control, was constant in the Cu treatment, decreased moderately in PAH treatment, and decreased drastically in the mixing treatment (Cu and PAHs) (Fig. 4). One-way ANOVA on day 10 revealed significant differences among treatments. Posterior Tukey tests indicated that the percentage of settlers was significantly higher in SW control and lower in the mixing treatment, while there were no differences between Cu and PAHs, pointing to a synergistic effect of these two pollutants.

4. Discussion

This study approaches for the first time the effects of short exposures to low concentrations of heavy metals and PAHs on sponge larval settlement, and analyses whether these short

exposures have any consequence on the consecutive survival of juveniles.

The effects of metals (Cu and Cd) on larval settlement vary depending on the sponge species: low concentrations of copper and cadmium at the concentration assayed did not affect *C. crambe* settlement, but enhanced *S. lophyropoda* settlement, compared with SW controls. These results may seem at first glance surprising, since early developmental stages of invertebrates have been reported to be more responsive to toxicants than adults (His et al., 1999). Accordingly, there are some reports of harmful effects of Cu and Cd on invertebrate larvae (Wu et al., 1997; Reichelt-Brushett and Harrison, 2000), and metamorphosis (Negri and Heyward, 2001), although the concentrations assayed in the cited studies were much higher than those used in our experiments. On the other hand, the enhancement of *S. lophyropoda* settlement is in agreement with that of previous studies reporting hormesis (positive effects of toxicants at low concentrations) for metals (Stebbing, 1982; Beiras and His, 1994). For example, Ng and Keough (2003) found that exposure to 100 µg l⁻¹ of copper accelerated bryozoan larval attachment. Furthermore, like for *C. crambe* larvae, there are also many other examples of no significant noxious effects of Cu and Cd on larval settlement, metamorphosis and post-metamorphic stages of invertebrates (e.g., Pechenick et al., 2001; Bellas et al., 2001; Blidberg, 2004). As for the possible mechanisms involved in the observed induction of settlement, it has been reported that bivalent metals such as Cu and Cd may penetrate larval cells via transition channels (Gill and Epple, 1992), leading to increased of intracellular Ca²⁺ release (Marchi et al., 2004). Since an extracellular supply of calcium induces cell aggregation in sponges (Philp, 1997), the reaggregation and recognition processes of cells, involved in larval settlement, are promoted in presence of Cu and Cd (Cebrian, E. and Uriz, M.J, unpublished data).

The early settlement of *S. lophyropoda* larvae induced under Cu and Cd might favour juvenile survival, since the length of the larval period is shortened and larval reserves may be redirected to juvenile maintenance. Accordingly, it has been shown that juveniles originated from short-lived larvae survived better and grew faster than long-lived larvae (Maldonado and Young, 1999).

It has been reported that stresses experienced by larvae under low concentrations of pollutants may affect juvenile performance in a variety of taxonomic groups (reviewed by Pechenick et al., 1998). Our results showed, for both species, no significant differences in survival between settlers coming from larvae previously treated with low concentrations of Cu or Cd and those maintained in SW control. Thus, we can conclude that the low concentrations of copper and cadmium used did not exert any effect on consecutive survival and growth of the sponge juveniles.

The present results are in accordance with those of Pechenick et al. (2001), where individuals surviving even the most toxic treatment, once transferred to clean seawater, performed as well after metamorphosis as control individuals. PAHs, in the concentrations used, seem to exert a negative effect on larvae of *Crambe crambe* by inhibiting settlement to a certain extent, although long-term experiments are needed in order to better assess potential impacts on juvenile and adult survival. Rinkevich and Loya

(1977) who pioneered short-term studies on the effects of oil pollution on coral larvae reported that concentrations of 1.5 and 10 $\mu\text{g l}^{-1}$ of crude oil also significantly reduced settlement success.

Our results indicate that settlement of *C. crambe* larvae is more sensitive to hydrocarbons than to copper and cadmium pollution, and that there is a synergetic negative effect of copper and hydrocarbons on juveniles. Consequently, *C. crambe* populations inhabiting copper- and hydrocarbon-contaminated places are expected to experience a combination of negative effects such as a decrease of recruitment and low reproduction rates as has been reported by Cebrian et al. (2003).

To summarise, low concentrations of copper and cadmium at the concentrations assayed did not affect *C. crambe* settlement compared with SW control neither exerted any effect on the consecutive survival of juveniles. In contrast, hydrocarbons seem to inhibit settlement to a certain extent. The synergistic negative effect of copper and hydrocarbons on *C. crambe* settlers may represent an important risk for populations in areas with both sources of contamination. On the other hand, low concentrations of copper and cadmium at the concentration used enhanced *S. lophyropoda* settlement but did not affect the consecutive survival of juveniles.

The present study provides the only available data on the toxicity of copper, cadmium and hydrocarbon toxicants on sponge larval settlement. Nevertheless, our *in vitro* experiments need to be confirmed by further *in situ* approaches, since environmental variables may influence in several ways and strengths the impacts of these pollutants on sponge populations.

Acknowledgements

We thank R. Martí and G. Agell for helping in larval collection and experimental procedures. This research was partially funded by grants from the CE (SPONGES project COOP-CT-205-017800) and the CICYT (Spain) (INTERGEN, CTM2004-05265/MAR).

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