



Low levels of copper reduce the reproductive success of a mobile invertebrate predator

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Abstract

Marine organisms that occur in urbanised bays can be exposed to low-level chronic pollution that results in sublethal changes to behavior or reproduction. The effects of low levels of copper on the reproductive success of a mobile invertebrate were assessed. Free living flatworms are common predators of bivalves and barnacles. Flatworms (*Stylochus pygmaeus*) were exposed to low levels of copper ranging from 0 to 25 $\mu\text{g L}^{-1}$ in the presence and absence of their barnacle prey (*Balanus variegatus*). Flatworms laid fewer egg batches when exposed to copper and the hatching success of the eggs was also reduced. Exposure to 25 $\mu\text{g L}^{-1}$ copper for 10 d reduced the reproductive success of flatworms by up to 80%. Results were consistent regardless of the presence or absence of prey (barnacles). Barnacles were only moderately affected by copper but exhibited major avoidance behavior (feeding inhibition) in the presence of flatworm predators. This is the first ecotoxicological study on marine flatworms. Experiments are required to quantify the effects of flatworm predator populations on sessile invertebrate community structure in the field.

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1. Introduction

Many near-shore marine ecosystems are strongly influenced by heavy metals discharged in domestic sewage, mining and industrial effluents (Mance, 1987). Often the contaminants may not result in obvious or instant mortality, however they may disrupt the physiology or behaviour of exposed organisms (Matthiessen and Law, 2002).

Behavioural responses can be an early and sensitive indicator of toxic stress (Lam et al., 2000) that may lead to deterioration in the health of the organisms and the affected ecosystems (McCahon and Pascoe, 1990; DeAngelis, 1996). Behavioural changes in controlled laboratory exposures may provide a better understanding of the possible environmental consequences of toxicants especially if behavior is disrupted at environmentally realistic toxicant concentrations (Faimali et al., 2006). The sublethal effects of heavy metal pollution include changes in feeding and reproductive behavior which may affect growth and reproductive success (e.g. Conradi and Depledge, 1998; Cebrian et al., 2003).

Copper is an essential trace metal to all marine invertebrates but it is toxic at higher concentrations (Lewis and Cave, 1982). It is a common pollutant in ports and estuaries and the harmful effects of copper on individual marine invertebrates has been widely studied and well documented (Hall et al., 1998). However, there are fewer studies of the effects of copper on multi-species test systems (Balczon and Pratt, 1994; Johnston and Keough, 2003).

Flatworms from the family Stylochidae are common pests of commercial bivalves and can cause huge economic losses (e.g. Newman et al., 1993; O'Connor and Newman, 2001). They can also reduce their prey populations alter the community structure (Skerman, 1960). Few studies have examined the reproductive and predatory behavior of marine flatworms.

Stylochids flatworms are also major predators of barnacles (Skerman, 1960) and the abundant local flatworm *Stylochus pygmaeus* preys extensively on *Balanus variegatus*, the most common barnacle in the study area. The empty barnacle shells are used as a shelter and cradle for flatworm eggs and young (personal observation but see also Merory and Newman, 2005). In our laboratory exposure study the following hypotheses were tested:

- (1) exposure to sublethal concentrations of copper does not affect the reproductive success of *S. pygmaeus* (tested in the absence of prey in Experiment 1, and the presence of prey in Experiment 2).
- (2) The feeding rate of the barnacle prey (*B. variegatus*) does not change with exposure to copper and/or flatworm predators (Experiment 2).

2. Materials and methods

2.1. Sample collection

Assemblages of sessile marine invertebrates were first allowed to develop on artificial substrata at Kurnell Pier, Botany Bay, New South Wales, Australia (33°59.92' S, 151°12.62' E). The site is well flushed with oceanic water and ambient copper concentrations are not elevated (Piola and Johnston, 2006) with less than $5 \mu\text{g L}^{-1}$ copper. Settlement plates consisting of 6×6 cm black Perspex tiles attached to the underside of a

60 × 60 cm PVC backing plate. Backing plates were suspended horizontally from the pier at a depth of 3 m below the low water for 5–7 months. Approximately 50 settlement plates were retrieved for each experiment and any flatworms found between the settlement and backing plates, and inside empty barnacle shells were collected immediately using a small paintbrush. Settlement plates were retained and brought back to the laboratory. For transport to the laboratory, each settlement plate was placed in a well-aerated transparent plastic container (15 × 8 × 8 cm) with 1 L of filtered field seawater in a dark constant-temperature room (23.5 ± 0.5 °C). After 24 h, any flatworms emerging from the assemblages were collected using a paintbrush. Sessile organisms other than *B. variegatus* were then removed from the settlement plates. Flatworms and barnacles were observed for 2 d prior to exposure to allow them to recover from collection and transport stress they were not fed during this period (Clesceri et al., 1998). Abundances of flatworms are seasonal (appearing in greater numbers in warmer months) and during this study, we recorded approximately two flatworms and 14 barnacles per 100 cm² settlement panel.

2.2. Copper treatments

Analytical grade copper II chloride hydrous (CuCl₂ · 2H₂O) (Sigma–Aldrich USA) was used as the reference toxicant in all experiments. A 1000 mg L⁻¹ primary copper stock solution was prepared by dissolving 2.6828 g of CuCl₂ · 2H₂O in 1000 ml of Milli-Q[®] filtered water. Stock solution was stored in the refrigerator at 4 °C to prevent the reduction of copper ions in the solution. A 1000 µg L⁻¹ copper solution was prepared from this stock solution every day and diluted to obtain the sublethal experimental treatment solutions of 10 and 25 µg L⁻¹ copper. Earlier pilot studies indicated 100% flatworm mortality occurred following 24 h exposure to approximately 100 µg L⁻¹ copper. Seawater collected from Kurnell Pier used as a dilution medium was filtered through a 0.2 µm filter to minimise the reaction of copper ions with organic particles which could reduce the amount of free copper ions in the treatment solutions. The added copper chloride would therefore readily dissociate to release copper ions (Cu²⁺) that cause toxicity. Treatment solutions were replaced every 24 h. The equipment used in all experiments was acid washed in 10% nitric acid for 24 h and rinsed in Milli-Q[®] filtered water before use.

In preparation for copper concentration analysis, 50 ml sub-samples of the experimental copper treatments were taken at the commencement of and 24 h after the experiments commenced and were immediately acidified with analytical grade concentrated nitric acid (1.5 µl of acid per 1 ml of sample) and stored in a refrigerator at 4 °C according to Clesceri et al. (1998). The concentrations of copper in the stock and experimental solutions were tested independently at the NATA accredited Australian Government National Measurement Institute using an inductively coupled plasma source mass spectrometer (ICP-MS) with a detection limit of 5 µg L⁻¹.

2.3. Experimental design

In all experiments, transparent plastic containers (15 × 8 × 8 cm) were used as the experimental containers. Each container was pre-soaked in the appropriate copper solution for 24 h before the start of the experiments to ensure minimal chelation of copper ions during the exposure period. Each container was filled with 1 L of treatment solution and was continuously aerated. Exposure to treatments was maintained for 10 d. Solutions were

replaced once every 24 h and were prepared from the stock solution immediately before use. All experiments were conducted in a constant-temperature room (23 ± 0.5 °C) subjected to 11:13 light: dark cycle. Salinity (33.7–34.5 ppt), pH (7.93–7.98), temperature (23–23.5 °C) and dissolved oxygen (5.56–5.74 mg L⁻¹) were monitored daily using and YSI 556 MPS (Yellow Springs, OH, USA) water quality meter and remained constant throughout the experiments. Following 10 d exposure to copper solutions organisms were placed in clean filtered field seawater for 2 d to assess latent mortality.

In all experiments, the length and width of flatworms were measured when they were in quiescent and relaxed state. The size of opercular opening of the barnacles was measured using a pair of callipers prior to exposure to ensure that the size range of organisms in all treatments was consistent, and any changes in the response of the organisms during the experiment were not related to size differences.

2.4. *Experiment 1: Reproductive success in the absence of food*

Brooding behavior of adult flatworms and the subsequent hatching success of their eggs were observed with exposure to copper. The nominal copper concentrations used in this experiment were 0, 10 and 25 µg L⁻¹ with seven replicates for each treatment. A pair of similar size flatworms were placed in each treatment container. Flatworm larvae are planktonic immediately post-hatching and barnacles, as filter feeders are potential predators of flatworm larvae. In order to collect the flatworm larvae and calculate the hatching success of the flatworm eggs it was important not to have live barnacles inside the experimental containers. In pilot studies flatworms were not observed to consume dead barnacles nor any similar crustacean food so flatworms were not provided with a food-source in this experiment.

Digital photographs of the flatworms and location of the egg batches in each container were taken every 24 h using Olympus compound microscope (SZX-ILLK200) connected to Pixelink (PL-A 642) with a magnification of 40×. The number of eggs laid from each pair of flatworms was counted from the photographs using Image-Pro Express 4.0.1 software. Flatworm larvae were collected by pouring all the 1-day-old solutions from each treatment container into a 1.25 L sample jar and preserved at 7% formalin. New copper solutions were then poured to each treatment container. All the preserved solutions from the same treatment were combined and poured through a 10 µm sieve, rinsed with Milli-Q[®] filtered water and made up to a 500 ml sample solution. Flatworm larvae in each sample solution were counted by sub-sampling 20 ml of the sample solution five times. The number of flatworm larvae present in each treatment was estimated by multiplying the average number of larvae present in the 20 ml sub-sample by 25. Hatching success of flatworm eggs was calculated as: (number of larvae/number of eggs) × 100%.

The number of egg batches laid and the time taken for the flatworms to lay the first egg batch was recorded. Flatworm activity was recorded every 4–6 h from when the first egg batch was observed until larvae were observed hatching from the eggs. Flatworms were considered to be actively “brooding” if their body covered any part of an egg batch.

2.5. *Experiment 2: Reproductive success with food provided*

In order to ensure that the response of flatworms and the timing of egg laying were not influenced by the absence of food, barnacles were placed in each treatment con-

tainer as a food source in this experiment. A settlement plate with 3–5 barnacles (4 ± 0.3 SE) and a pair of similar size flatworms were placed in each treatment container. Containers holding a settlement plate with barnacles and no flatworms were used as barnacle mortality controls. The nominal copper concentrations used in this experiment were 0, 10 and $25 \mu\text{g L}^{-1}$, with four replicates for each treatment and three replicates for each barnacle mortality control at each copper concentration. During the 10 d exposure period treatment solutions were replaced daily and barnacles were fed three times with 5 ml of hatched artemia cysts to ensure that any mortality of barnacles was not a result of starvation. The total number of egg batches laid and the time taken for the flatworms to lay the first egg batch was recorded. Flatworm activity was recorded every four to 6 h from when the first egg batch was observed until larvae were observed hatching from the eggs. Flatworms were considered to be actively “brooding” if their body covered any part of an egg batch. The feeding rate and mortality of barnacles in all treatments was recorded every 4–6 h for the entire 10 d observation period. Feeding rate of barnacles was recorded as the number of beats of cirri in 1 min.

2.6. Data analysis

All statistical analysis was completed in Statistical Package for Social Sciences (SPSS version 11.5) with all data assessed for homogeneity of variance and normality using plots of residuals versus means and descriptive statistics. In each experiment, barnacle size was tested using a one-factor analysis of variance (ANOVA) with the presence of flatworms as a fixed factor. Flatworm length was tested using a one-factor ANOVA with copper treatments as the categorical factor. The number of egg batches laid, time spent brooding (averaged the observation period) and the hatching success of flatworm eggs in all treatments were tested separately using a one-factor ANOVA with copper treatment as the categorical factor. The feeding rate of barnacles (cirri beats per minute averaged over the 10 d period) in Experiment 2 was analyzed using a two-factor ANOVA with copper treatments and presence of flatworms as fixed factors. Planned comparison tests were conducted on all significant results to determine the differences between controls and individual copper treatments. Planned comparisons used the error term from the main test of copper treatment (Quinn and Keough, 2002). Planned comparisons are conducted when specific post hoc tests are required. They are usually more powerful than procedures that test every treatment combination (e.g. Tukeys). In ecotoxicology studies such as this, the tests of interest are usually a comparison of the control treatment against individual toxicant concentrations.

3. Results

3.1. Reproductive success of flatworms

Copper reduced the reproductive success of flatworms; approximately 40% fewer egg batches were laid by exposed flatworms (ANOVA $P = 0.006$; Fig. 1a). Planned comparisons showed that this effect was evident at $25 \mu\text{g L}^{-1}$ Cu ($P = 0.007$) but not at $10 \mu\text{g L}^{-1}$ Cu ($P = 0.763$). Hatching success was highly consistent among replicates. In control treatments, about $92 \pm 0.4\%$ of eggs hatched (Fig. 1b). Hatching success of flatworm eggs was

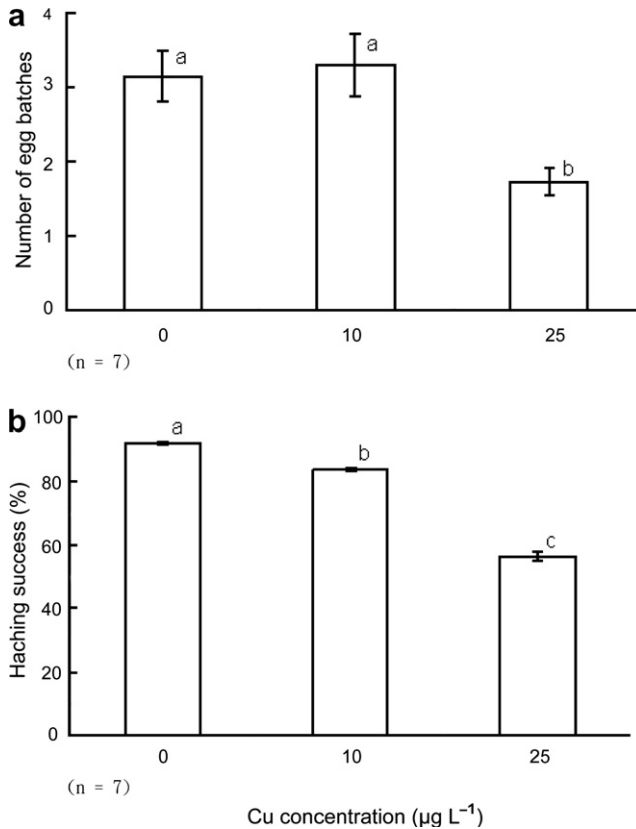


Fig. 1. Effects of 0, 10 and 25 $\mu\text{g L}^{-1}$ Cu treatment on (a) the number of flatworm egg batches laid and (b) the hatching success of flatworm eggs in Experiment 1. Error bars represent mean (± 1 SE). Number of replicates for each treatment is provided in parentheses. Letters indicate significant difference at $\alpha = 0.050$.

reduced by 10% at 10 $\mu\text{g L}^{-1}$ Cu and 40% at 25 $\mu\text{g L}^{-1}$ Cu compared to that in the controls (ANOVA and planned comparisons, $P < 0.001$; Fig. 1b).

Flatworms were able to endure protracted fasting and fasting did not appear to affect *S. pygmaeus* reproductive behavior. In Experiments 1 (flatworms unfed) and 2 (flatworms fed), copper exposure caused no differences in the time taken for the flatworms to lay the first batch of eggs (5 days) (Experiment 1: ANOVA, $P = 0.303$; Experiment 2: ANOVA; $P = 0.142$). Copper caused no difference in the time spent brooding egg batches (Experiment 1: ANOVA, $P = 0.296$; Experiment 2: ANOVA; $P = 0.698$). Flatworms were observed brooding eggs for more than 90% of the time.

3.2. Predation rate of *S. pygmaeus*

No barnacles were observed to die in the mortality controls in Experiment 2 so any mortality of barnacle was therefore due to the presence of the flatworm predators. Flatworms consumed an average of one barnacle over the experimental period (0.5 barnacles per flatworm 10 d^{-1}) and this was not affected by exposure to 10 or 25 $\mu\text{g L}^{-1}$ Cu in (Kruskal–Wallis, $P = 0.735$; Fig. 2).

3.3. Feeding rate of *B. variegatus*

Feeding rate of barnacles was reduced by both the presence of flatworms and exposure to copper. However, the presence of flatworm predators had a much larger effect than copper exposure (Fig. 3). Barnacle feeding rate was reduced by <10% when exposed to copper, but it was reduced by >30% by the presence of flatworm predators. In the presence of flatworms, barnacle feeding rate was significantly reduced in the 10 and 25 $\mu\text{g L}^{-1}$ Cu treatments when compared to that in the control (planned comparison $P < 0.001$). However, in the absence of flatworm predators, this effect was only observed at 25 $\mu\text{g L}^{-1}$ Cu (planned comparison $P < 0.001$).

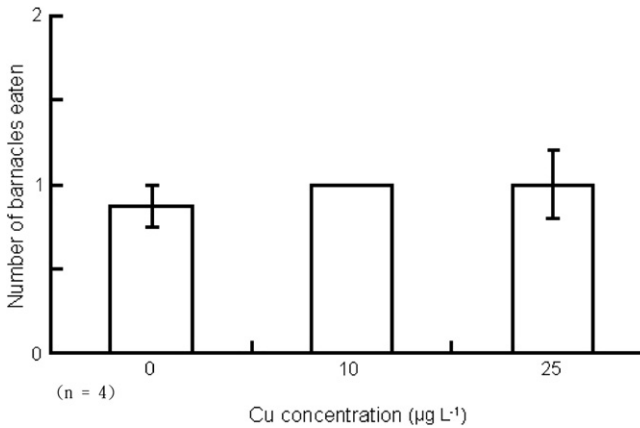


Fig. 2. Effects of 0, 25 and 50 $\mu\text{g L}^{-1}$ Cu treatments on the number of barnacles eaten by flatworms in Experiment 2. Error bars represent mean (± 1 SE). Number of replicates for each treatment is provided in parentheses.

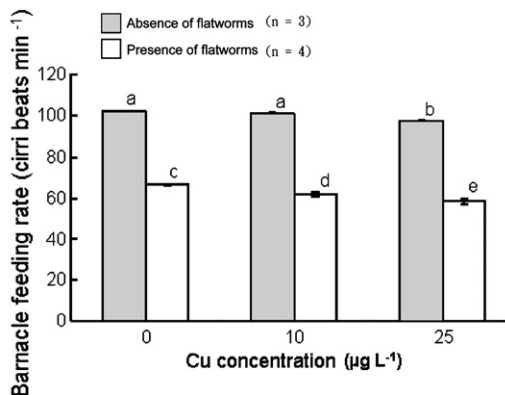


Fig. 3. Effects of 0, 10 and 25 $\mu\text{g L}^{-1}$ Cu treatments on the feeding rate of barnacles in the presence and absence of flatworm predators in Experiment 2. Error bars represent mean (± 1 SE). Number of replicates for each treatment is provided in parentheses. Letters indicate significant difference at $\alpha = 0.050$.

3.4. Size of barnacles and flatworms

Length of flatworm was used as an indicator of overall flatworm size. The average length of flatworms collected and tested in Experiment 1 was 12.7 ± 0.14 mm; Experiment 2 was 11.6 ± 0.46 mm and Experiment 3 was 16.4 ± 0.63 mm. Flatworm length was similar across treatments within each experiment: Experiment 1 (ANOVA, $P = 0.27$) and Experiment 2 (ANOVA, $P = 0.06$).

Operculum length was used as an indicator of overall barnacle size. Barnacle size was consistent between treatments (ANOVA, $P = 0.31$). There were 4 ± 0.3 barnacles (6.8 ± 0.2 mm) in each treatment and 4 ± 0.17 barnacles (6.5 ± 0.25 mm) in each barnacle mortality control.

3.5. Chemical analysis

The nominal and measured copper concentrations taken at the commencement of and 24 h after the experiment are shown in Table 1. All filtered field seawater samples contained $<5 \mu\text{g L}^{-1}$ copper. Since all treatment solutions were prepared and changed every 24 h from a 1000 mg L^{-1} stock solution and the measured copper concentrations of the treatment solutions from the experiment are close to their nominal values, we have confidence in the accuracy of our treatment solutions.

4. Discussion

Low levels of metal pollution had a major impact on the reproductive success of the mobile flatworm, *S. pygmaeus*. The barnacle prey, *Balanus variegates*, reduced its feeding rate in the presence of a predator but was only slightly affected by exposure to the pollutant. Copper concentrations used in this study fall into the lowest range of those typically used in laboratory toxicity tests and they reflect realistic concentrations experienced by marine assemblages in polluted harbours (Stauber et al., 2000) or close to vessels coated with copper antifouling paints. The differential sensitivity of a predator and prey observed in this study warrants field experiments investigating the likely structural changes to the marine invertebrate community in areas of low level heavy metal pollution.

4.1. Reproductive success of *S. pygmaeus*

Copper reduced the reproductive success of flatworms. There were fewer eggs laid when flatworms were exposed to low levels of copper, and the likelihood that any one of those

Table 1

Nominal and measured total copper concentrations ($\mu\text{g L}^{-1}$) at the commencement of and 24 h after the experiments began (± 1 SE, $n = 2$)

Nominal concentration	Measured concentration	
	0 h	24 h
0	<5	<5
10	13 ± 1	13 ± 0
25	23 ± 3	24 ± 2

Lowest detection limit for analysis was $5 \mu\text{g L}^{-1}$.

eggs would hatch into a young larva was lowered. Copper was clearly reducing the capacity of flatworms to produce eggs. However, the mechanism by which the hatching success was affected is not clear from this study. The early life stages of many organisms are more sensitive to pollutants and are often used in toxicity tests (Mora, 2003). The toxic effects of copper on adult health may have resulted in the laying of poor quality eggs, but eggs may have also been directly affected by copper exposure, and the newly hatched larvae may also have been directly affected. This situation mimics toxicant exposure in the field where adults, eggs and larvae are likely to be simultaneously exposed.

Exposure to copper negatively affects the reproductive success of other marine organisms, such as sponges (Cebrian et al., 2003) amphipods (Conradi and Depledge, 1998) and polychaetes (Xie et al., 2005). However the flatworms used in this study appear to be more sensitive than other marine invertebrates. Toxic effects of copper on the survival, growth and development of barnacles have been widely studied (e.g. Pyefinch and Mott, 1948; Koryakova and Korn, 1993). It is apparent that barnacles are capable of tolerating quite high copper concentrations (e.g. 24 h LC50 for copper for adult *Balanus balanoides* and *B. crenatus* was 320 and 1350 $\mu\text{g L}^{-1}$, respectively, Pyefinch and Mott, 1948). Flatworms have no shell to cover their fragile body, and they rely heavily on diffusion directly across the epidermis for respiration and excretion of waste products. Hence, they may be generally more susceptible to changes in water quality than their barnacle prey, which are protected by both a calcium carbonate external skeleton and a thick integument.

The response of flatworms to copper exposure was the same in the presence and absence of food (barnacles). The predation rate of *S. pygmaeus* in this study (0.5 barnacle consumed per worm 10 d^{-1}) was relatively low when compared to the field observations of other stylochids. For example, *Stylochus tauricus* consumed five to ten *Balanus improvisus* in a month (Murina et al., 1995). A low predation rate may be due to the relatively small size of *S. pygmaeus* compared to other stylochids (Merory and Newman, 2005) or a result of reduced energy expenditure of flatworms in well-controlled laboratory conditions. *S. pygmaeus* also spent more than 90% of its time “brooding” egg batches. Chintala and Kennedy (1993) found that flatworms are able to maintain a high reproductive effort by using stored energy to produce eggs despite reductions in available food. It may be that *S. pygmaeus* invests time in mating and brooding rather than foraging, when a mate is available.

4.2. Predator avoidance

Predator-avoidance behaviour has been widely studied in terrestrial and aquatic ecosystems (e.g. Nelson et al., 2004; Peacor, 2002). Such behaviour has both direct and indirect effects upon the reacting prey such as reduction of survival and growth. Predator-avoidance behaviour (feeding inhibition) was clearly shown by *B. variegatus* in response to the presence of flatworms in this study. The presence of flatworms reduced the feeding rate of barnacles to a much greater extent than exposure to low levels of copper. *B. variegatus* reduced its encounter rate with its flatworm predator by closing opercular valves. Such a defense mechanism was noted in Hurley (1976) which examined the establishment of *B. pacificus* in the presence of its flatworm predator, *Stylochus tripartitus*. The mechanism by which *B. variegatus* identified the presence of *S. pygmaeus* is not known. However, chemical cues may be involved (Hagen et al., 2002). This behavioral response is presumably performed to reduce the predation risk but it comes at the cost of reduced foraging

time and hence potentially reduced growth, reproduction and development of the prey species (Lefcort et al., 1999).

5. Conclusion

This study provides new information on the impacts of low concentrations of copper in sea water on the reproductive success of a mobile invertebrate predator. Copper had obvious detrimental effects on flatworms, reducing their reproductive success by up to 80%. It has been suggested that long-term exposure of organisms to elevated copper concentrations might have serious consequences (such as local extinction) through effects on reproduction and growth (Conradi and Depledge, 1998). If concentrations used in this study were to occur in coastal waters in a similarly uncomplexed chemical form, decreased reproductive success in *S. pygmaeus* would ultimately lead to a decline in flatworm population size in the area exposed. Within a couple of generations this may result in complete recruitment failure as the density of mature flatworms drops below a critical encounter threshold (Russell and Fowler, 2002). Patterns of population change in barnacles would need to be examined over a longer period and under field conditions in order to determine how the barnacle beds are replenished in the presence and absence of flatworms.

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