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Field assessment of effects of timing and frequency of copper pulses on settlement of sessile marine invertebrates

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Abstract $CuSO_4$-treated plaster blocks were used to create localised concentrations of copper significantly above ambient levels. Between October 1996 and March 1997 we used this system to manipulate the timing and frequency of transient copper-pollution events close to settlement plates. We assessed the impacts on the development of assemblages of sessile marine invertebrates that occur on hard substrates at Breakwater Pier in Williamstown, Victoria, Australia. Barnacle densities were reduced by up to one-third, while serpulid polychaetes were insensitive. Assemblages at different stages of development were differentially sensitive to short-term pulses. Reductions in sponge and scyphozoan polyp densities were greatest (50%) when pulse-pollution events occurred at times of high settlement. If a pulse copper-pollution event occurred in the first week of substrate becoming available for colonisation, then it had few observable impacts on recruitment for all invertebrates examined. When the first pulse occurred in the first week of the experiment there was no difference between the impacts of single or double-pulse exposures to the toxicant.

Introduction

Environmental biologists are often asked to predict the impact of a toxicant on an ecosystem or species, and in the past the majority of answers have been based on continuous exposure, single-species, laboratory-toxicity tests (Cairns 1988; Calow 1994). In the field, however, organisms rarely experience continuous exposure to constant concentrations of a toxicant in a uniform environment. In many situations, organisms experience transient pollution events, such as during rain events that create urban runoff, or industrial spills. The timing and frequency of these events are likely to vary (Beck 1996). The organisms might be exposed to sublethal levels of toxicants for short periods, and longer-lived organisms might experience repeated exposures during their lifetimes. In addition, toxicants can persist at sublethal concentrations for brief periods. In the field, organisms experience a varying environment, which can influence the relative toxicity of a chemical to an organism (Andrew et al. 1977). They are rarely found in isolation, and their interactions with other assemblage dwellers may be affected by the impact of toxicants (La Point 1995), or these surrounding organisms may mediate the impact of toxicants on the organisms of interest. While the latter two points are part of a familiar argument for field-based toxicity testing, the importance of transient exposures is a more fundamental question, relevant to ecotoxicology and to the development of theories of ecological disturbance.

A single, transient exposure to a toxicant can have complex effects. It is likely that the timing of such an exposure is critical, as most organisms show variation in sensitivity to a given toxicant among life-history stages (Weis and Weis 1989) and several studies suggest that early embryo and larval stages may be the most sensitive to copper toxicity (McKim 1977). Even within a particular life stage, there can be variation in responses. For example, larvae of marine invertebrates respond in different ways to cuprous oxide anti-fouling paints. Tubeworm and bryozoan larvae show pre-attachment mortality (Wisely 1962, 1964; Wisely and Blick 1967), barnacle larvae experience pre and post-settlement mortality (Crisp and Austin 1960), and mollusc larvae are deterred from settling on treated plates (Wisely 1963).

A transient, sublethal exposure can also have effects that are only manifest much later. For example, freshwater amphipods surviving an initial pulse exposure to the pesticide Lindane died up to 3 wk after the dose was delivered (Abel 1980), and episodic acidification in small

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streams can have long-term effects on fish communities (Baker et al. 1996). Some organisms can develop resistance to toxicants, and several studies indicate that organisms exposed to sublethal concentrations of copper become more resistant to higher concentrations (Dixon and Sprague 1981).

When there are multiple, transient exposures, or "pulse" disturbances, in the terminology of Bender et al. (1984), the relative timing of the pulses may be important. The potential importance of multiple disturbances has been recognized in other areas of ecology (Bender et al. 1984), and there are now emerging some empirical data demonstrating the complex effects of pulse disturbances that vary in their intensity and frequency (e.g. see Keough and Quinn 1998). However, to date, we know little about the effects of timing and frequency of disturbances in the form of toxicants.

Here, we describe a series of field experiments testing the effects of timing and frequency of pulses of a single toxicant on an assemblage of sessile marine invertebrates in southeastern Australia. This experimental investigation is thus directed towards answering three broad questions: (1) Are assemblages at different stages of development, differentially sensitive to short-term toxicant exposure? (2) Is there a difference between the impact of single or multiple toxicant-pulses to assemblages in the field? (3) How does the impact of repetitive pulse exposure to a toxicant differ when the interval between pulses is varied?

The toxicant chosen for this study was copper. Copper is an essential element for metabolism in most marine organisms, but at higher concentrations, laboratory tests rate it as one of the three most toxic heavy metals (Abel 1989). It is a common pollutant, entering coastal waters in many ways, including corrosion of pipes, industrial effluent or spills, metabolic wastes, mining, and deliberate release in the form of antifouling paint on boat hulls. Nationwide screening efforts in the US have identified copper as one of 18 common pollutants found in urban run-off (Pitt 1995), and it is one of several heavy metals that form a major portion of anthropogenic inputs into Port Phillip Bay, the site of this study (Phillips et al. 1992).

Copper toxicity varies with the concentration of the metal available in free-ion form (Andrew et al. 1977), which in turn is influenced by salinity, pH, the amount of organic solids present, and temperature. There has been a substantial amount of work on the toxic impact of copper on marine organisms (Lewis and Cave 1982). However, the majority of this information is based on constant concentration laboratory tests, and may be less relevant in a chemically dynamic marine system.

Materials and methods

Site

Experimental settlement plates were suspended from Breakwater Pier in Port Phillip Bay, Victoria, in southeastern Australia. There is restricted access to the Pier, which extends 300 m from the shore and includes a rocky breakwater on the southern side. Extensive biological knowledge is available for the assemblage of sessile marine invertebrates that settle on hard substrates at Breakwater Pier (e.g. Keough and Raimondi 1996), which generally includes polychaete worms, barnacles, bryozoans, colonial and solitary ascidians, sponges and hydroids.

General experimental methods

Toxicant dosing

A soft-sediment dosing system (Morrisey et al. 1996) was modified for hard-substrate assemblages. The technique uses copper-impregnated plaster blocks to create localised concentrations of copper significantly above background levels (McHugh 1996) and enables the manipulation of the timing and frequency of transient pollution events (Johnston and Webb 2000). McHugh (1996) took water samples in the field close (1 cm) to the copper plaster blocks and measured dissolved copper levels of 35 to 45 $\mu g\ l^{-1}$. Concentrations dropped to 2 $\mu g\ l^{-1}$ 8 cm from the source.

AR grade $CuSO_4$ (copper II sulphate anhydrous) was used as the reference toxicant in all experiments. This provided Cu^{2+} ions, which is one of the most toxic forms of the metal to marine organisms (Andrew et al. 1977). $CuSO_4$ was used in preference to other forms of copper as it reacts less vigorously when combined with dental plaster (McHugh 1996). Plaster blocks, containing $CuSO_4$, were attached to settlement plates. All plates received either a copper-impregnated or a control plaster block every week for the duration of the experiments. To avoid desiccation during the replacement of blocks, plates were stored in a shallow pool of local seawater. Dry weights of plaster blocks were measured before and after immersion in the field. There was little variation in the rates at which plaster blocks dissolved, with the coefficient of variation for individual treatments almost always less than 10%.

To produce the copper blocks, 1.6 g or 3.2 g of $CuSO_4$, depending on the experiment series, was completely dissolved in 13 g of deionised water and refrigerated at 4 °C for 60 min. The pH was not adjusted. Dental plaster (15 g) was refrigerated for 60 min, and then mixed with the copper solution for 15 s. The plaster was poured into a hemispherical plastic mold, 4 cm in diameter. This created plaster blocks with a concentration of 0.096 or 0.191 g $CuSO_4\ cm^{-3}$. The molds were lightly oiled with vegetable cooking oil to allow removal of blocks. A stainless steel bolt (35 × 5 mm) was placed head down into the plaster and the block was allowed to set at 30 °C until a constant weight was recorded (4 to 5 d). The same process was used to manufacture control blocks except for the addition of $CuSO_4$ and the refrigeration step.

Apparatus

Four replicate settlement plates were assigned to each experimental treatment. A plate consisted of an 11 × 11 × 1 cm black Perspex sheet engraved with a 1 × 1 cm grid. The copper block was attached by its bolt through the centre of the plate, which served also to secure the plate to a larger 60 × 60 cm grey PVC backing plate. Backing plates allowed the stable submersion of multiple settlement plates. No more than 8 small experimental plates were attached to any backing plate, with the distance between the centres of neighbouring plates no less than 19 cm. To avoid possible bias caused by differential recruitment onto areas of backing plates, and as an added precaution against cross-contamination, positions on the backing plates were divided into three categories: corner, centre or edge. Treatments were spread equally across each category.

Settlement plates were attached to the underside of backing plates to minimise available light and sedimentation. A weight was attached below the backing plate to provide stability. For an illustration of the plaster block and plates experimental set-up see Johnston and Webb (2000). Water-quality data from Breakwater Pier indicated that much of the variability in pH, turbidity, temperature and salinity, which alter the toxicity of copper ions (Allen and Hansen 1996), occurred in a surface layer of less than 3 m. (J. Lewis, Australian Defence Science and Technology Organization, unpublished data). To avoid this variability in water quality, all backing plates were suspended horizontally, at a depth of 3.5 m below the low-water mark. Throughout the experiments there was little variation in salinity (range 30 to 31.5‰) and pH (range between 7.8 and 8.1) at this depth, although temperature was slightly lower in November/December experiments (ranging from 16.5 to 17.5 °C) than in February experiments (ranging from 18.5 to 19 °C) (J. Lewis, Australian Defence Science and Technology Organization, unpublished data).

Census

At the conclusion of each experiment, plates were transported in seawater to the laboratory and placed in a continuous-flow seawater system until they were examined. Plates were examined under a binocular dissecting microscope without knowing their treatment, and the position of recruits within each 1 cm^2 grid was noted. Due to the very high density of scyphozoan polyps in Experiment Series 2, the presence and position of these recruits was counted in every second square. In the 4 wk experiment, barnacles were placed in one of two size classes, large and small. Large barnacles had a test diameter of > 1.5 mm, while small barnacles, which were generally only a few days old, had a test diameter of < 1.5 mm. In the 4 wk experiment, recruits were then mapped into 0.5 cm concentric rings in order to calculate the density of recruits in each distance class from the copper source. For the 2 wk experiments, density was calculated for the entire plate, excluding the area covered by the plaster block.

Experimental designs

Experiment Series 1: exposure to 0.096 g $CuSO_4$ cm^{-3} plaster block

Three 2 wk experiments were conducted over consecutive fortnights in November/December. For each 2 wk experiment, there were three treatments: an undosed control, a copper dose in Week 1, and a copper dose in Week 2. The treatments allowed the examination of the overall short-term effect of copper doses on plates (dosed vs undosed) as well as the comparison of impacts caused by the timing of a dose.

Experiment Series 2: exposure to 0.191 g $CuSO_4$ cm^{-3} plaster block

Few significant impacts of copper pulses were observed in the short-term Experiment Series 1, so the concentration of $CuSO_4$ in plaster blocks was doubled for Experiment Series 2. This series employed the same experimental design as described for Experiment Series 1 excepting that only two 2 wk experiments were conducted over consecutive fortnights in February, and a 4 wk experiment was run concurrently. For the 4 wk experiment, we used 8 treatments: 1 undosed control, 4 single-dose and 3 multiple-dose. Single-dose treatments received a copper dose in either the

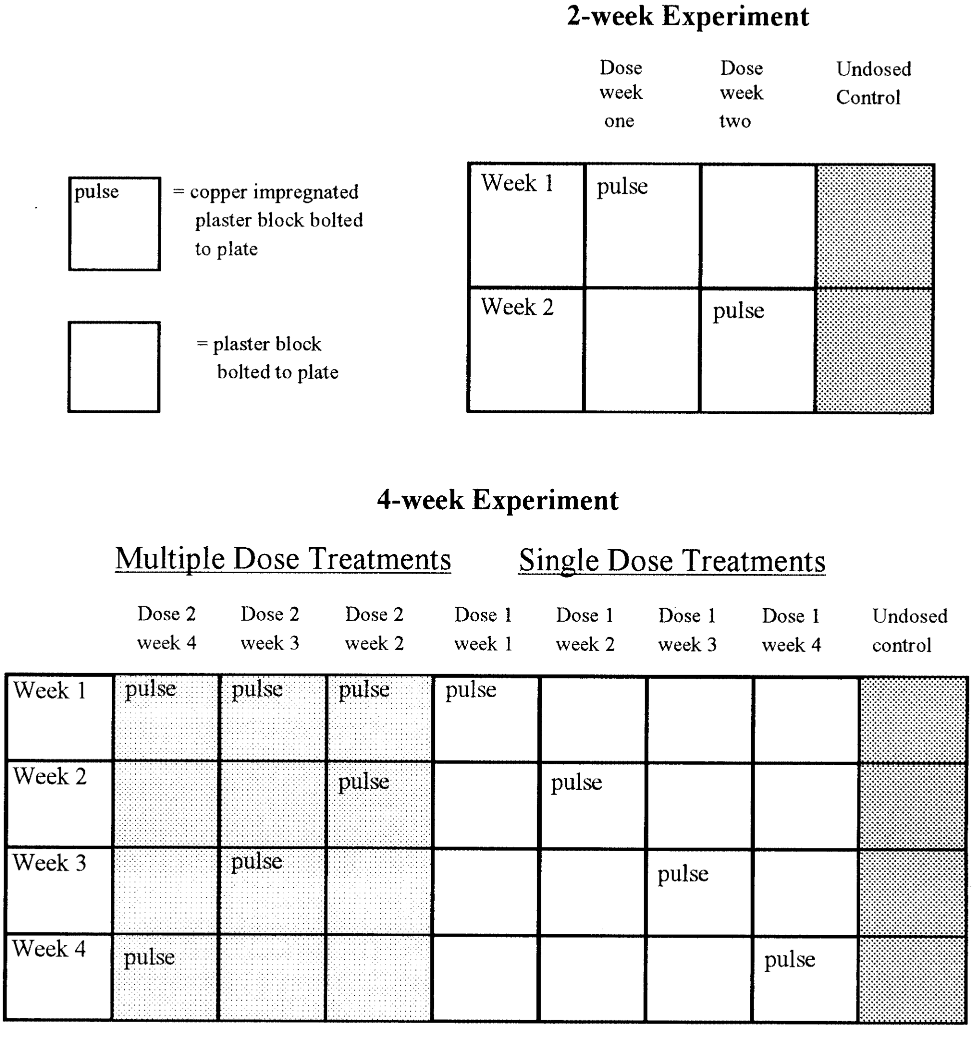


Fig. 1 Schematic representation of 2 wk and 4 wk experimental designs

first, second, third or fourth week of the experiment. All multiple-dose treatments were dosed in Week 1 and a second time in either the second, third or fourth week. Schematic representations of experimental designs are provided in Fig. 1.

Statistical analyses

All analyses were done on the raw data for each taxonomic group. For the 2 wk experiments, the density of recruits for every taxon was analysed using an analysis of variance with copper treatment as the categorical factor. A planned comparison of control against the two single-dose treatments, and a comparison of the two single-dose treatments were carried out.

For the 4 wk experiment, density of recruits for every species/group was analysed using a repeated-measures analysis, with plates as "subjects", and recruitment at different distances being the repeated measurements. The between-subjects factor was copper treatment, the within-subjects factor was distance from the copper source, and the interaction was between copper treatment and distance from the source. Two planned comparisons were then carried out. The first compared the four single-dose treatments to each other, the second compared the three multiple-dose treatments. All comparisons were carried out on the density of recruits with increasing distance from the source (within the plate) as well as on the density of recruits averaged across the plate (whole plate).

As there were many non-significant results for the 2 wk experiments, the power of these experiments to detect a 50% difference from the controls was calculated using the PowerPack computer package. The power analyses were done on the whole plate data, using the mean square variation among plates as the estimate of variation. An effect size of 50% of control values was used, as effects of this magnitude have been reported from other recruitment experiments at Williamstown (e.g. Keough and Raimondi 1996). The estimate of power for the analysis of variance was more conservative than that for the planned comparisons, and is quoted in the results.

Results

Variation in recruitment over time

Recruitment onto control plates in the short-term experiments varied greatly through time. The barnacle *Elminius modestus* dominated settlement plates in November and December, with >10 times the number of recruits of any other single species or group. These barnacles were accompanied by significant numbers of solitary ascidians and moderate numbers of serpulid polychaetes. Recruitment onto control plates in February was more equally distributed between the serpulids, sponges, botryllinid ascidians, and bryozoans. There was a changeover period during 2 wk Experiment 4 (the first of Series 2), with low recruitment of both *E. modestus* and *Balanus variegatus*. Barnacle recruitment rose to moderate levels again in Experiment 5, but with a predominance of *B. variegatus*. *Cyanea capillata* polyps and several encrusting bryozoan species only settled during February. Recruitment did vary between consecutive 2 wk experiments, but greater differences existed between experiments conducted in November and those conducted in February of the following year.

Table 1 Summary of comparisons of invertebrate species responses to copper pulses in short-term (2 wk) Experiments 1 to 5, showing both comparison of control with both copper treatments (C vs Copper) and comparison of copper pulse in Week 1 with copper pulse in Week 2 (timing) (× = no effect; + = effect)

Taxon	Comparison	Short-term experiments				
		Series 1: November/December			Series 2: February	
		1	2	3	4	5
Barnacles						
Elminius modestus	C vs Copper	+	×	×		
	Timing	×	×	×		
Balanus variegatus	C vs Copper				×	×
	Timing				×	×
Polychaetes						
Serpulids	C vs Copper	×	×	×	×	×
	Timing	×	×	×	×	×
Spirorbids	C vs Copper	×	×	×	×	×
	Timing	×	×	×	×	×
Solitary ascidians		×	×	×		
Newly settled	C vs Copper	+	×	×	×	×
	Timing	×	×	×	×	×
Colonial ascidians						
Didemnids	C vs Copper	×	×	×	×	×
	Timing	×	×	×	×	+
Botryllinids	C vs Copper	×	×	×	×	×
	Timing	×	+	×	×	×
Sponges						
All pooled	C vs Copper	×	×	×	×	×
	Timing	×	×	×	×	×
Bryozoans						
All pooled	C vs Copper	+	×	+	×	×
	Timing	×	×	+	×	×
Cyanea capillata	C vs copper				×	+
	Timing				×	×

Primary space on control plates in the 4 wk experiment was dominated by large botryllinid colonies, accompanied by encrusting bryozoans and *Cyanea capillata* polyps. Large arborescent colonies of *Bugula neritina*, *B. dentata*, *B. stolonifera* and *B. flabellata* occupied secondary space above the surface of the plate.

Experiment Series 1: November/December 2 wk experiments

Three 2 wk experiments were run in consecutive periods, and they are referred to in their temporal sequence as Experiments 1, 2 and 3. Many species or species groups recruited in low numbers and with relatively high variation through space. Very few impacts of copper were recorded, and there were even fewer differences caused by the timing of a dose. Species responses are summarised in Table 1, with analytical details in Table 2. The power of experiments to detect the impact of copper on many of these invertebrates was poor, with many taxa having values < 50% (Table 2). The main exceptions were *Elminius modestus*, serpulids and newly-settled ascidians (NSA), all of which had high power.

In Experiment 1, exposure to copper in either Week 1 or 2 of the experiment reduced the density of *Elminius modestus* recruits by approximately one-third (Fig. 2). The same pattern was evident in Experiment 3, but variation in control plates was high and the trend not significant. This was probably because upon one control plate a hydroid colony grew rapidly and developed a dense covering. The presence of hydroids is the probable cause of a reduced density of barnacle recruits on this

Table 2 Probability and power values for analyses and planned comparisons of recruit densities in November/December short-term Experiments 1, 2 and 3, listing overall effect of copper on density of recruits for whole plate (*Underlined values* $P < 0.05$)

Species	All treatments	Comparisons		Power
		Control vs dosed	W1 vs W2	
Experiment 1				
Elminius modestus	0.075	0.027	0.908	78
Serpulids	0.256	0.437	0.147	63
Spirorbids	0.860	0.710	0.699	15
Newly settled ascidians	0.060	0.043	0.170	85
Didemnids	0.917	1.000	0.686	28
Botryllinids	0.736	0.562	0.615	15
Sponges	0.472	0.227	0.843	14
Pooled bryozoans	0.006	0.003	0.120	49
Experiment 2				
Elminius modestus	0.772	0.700	0.556	90
Serpulids	0.234	0.114	0.566	98
Spirorbids	0.572	0.324	0.757	37
Newly settled ascidians	0.750	0.462	0.959	85
Didemnids	0.663	0.865	0.386	20
Botryllinids	0.026	0.777	0.009	68
Sponges	0.455	0.227	0.843	45
Pooled bryozoans	0.330	0.345	0.249	13
Experiment 3				
Elminius modestus	0.145	0.061	0.640	90
Serpulids	0.242	0.576	0.117	95
Spirorbids	0.435	0.234	0.669	26
Newly settled ascidians	0.772	0.749	0.532	35
Didemnids	0.699	0.676	0.474	22
Botryllinids	0.801	0.527	0.890	45
Sponges	0.111	0.061	0.325	40
Pooled bryozoans	0.004	0.014	0.007	28

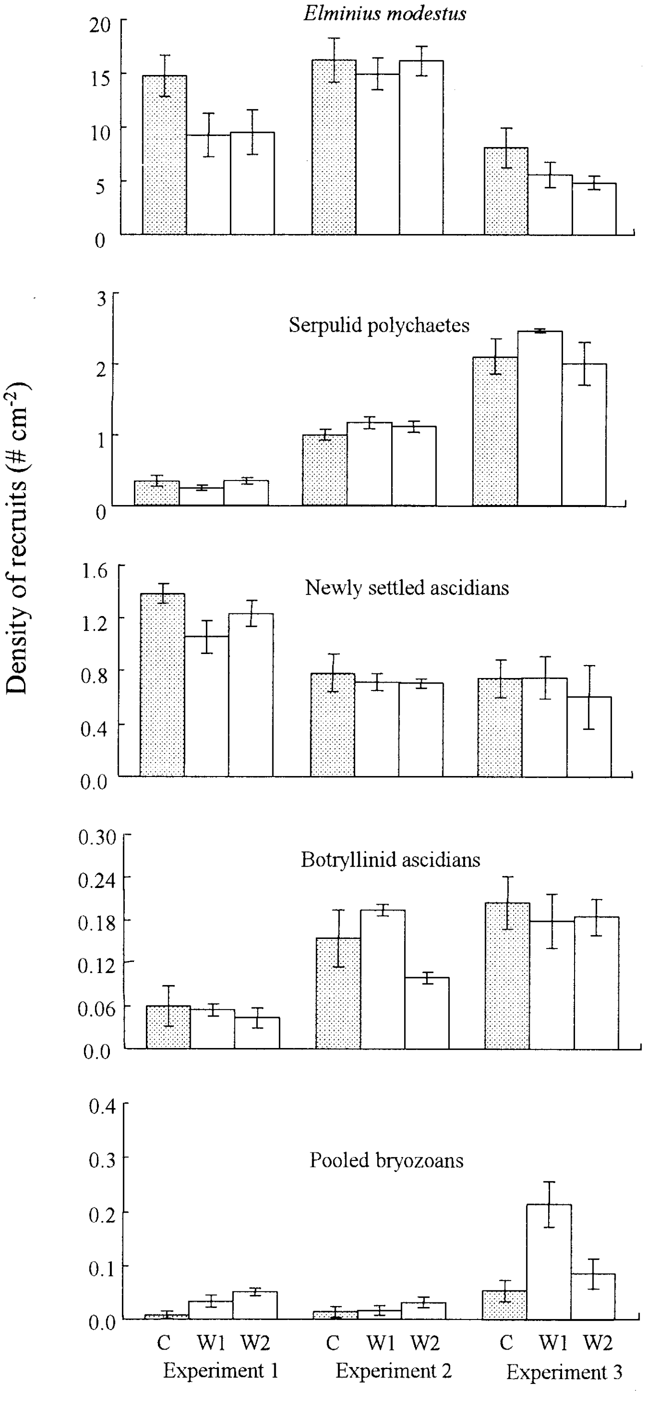


Fig. 2 Densities of recruits of selected taxa (cm^{-2}) plus standard error for November/December 2 wk Experiments 1, 2 and 3. Treatments were undosed control (*C*), copper pulse in Week 1 (W1) and copper pulse in Week 2 (W2)

plate. Analysis of the experiment excluding this plate revealed a significant difference between control and dosed plates. In Experiment 2, *E. modestus* recruitment was at its highest and copper exposure did not change the density of recruits in this experiment. In no experiment was there a difference caused by the timing of a copper dose.

There was no overall effect of copper on serpulids and no difference between the impact of copper exposure in Weeks 1 or 2 (Fig. 2). Power was high for these polychaetes. Copper exposure did not change the density of spirorbid polychaetes, but there was low recruitment throughout experiments and the power to detect impacts was generally poor.

Exposure to copper in Weeks 1 or 2 of Experiment 1 reduced the density of solitary ascidians by 20%. There was no difference between the impact of exposure to copper in Weeks 1 or 2 (Fig. 2). There was no impact observed in Experiments 2 and 3. Exposure to a copper pulse in Week 2 reduced the density of botryllinid ascidians in Experiment 2 (Fig. 2). There were no impacts observed in Experiments 1 and 3. Didemnid ascidians recruited in low numbers (<7 per plate) in all three experiments, and no effects of copper pulses were detected. The power of all three experiments was <30% for these ascidians.

There was low recruitment of bryozoans, so species data were pooled prior to analysis. In Experiments 1 and 3, copper pulses in Weeks 1 or 2 increased the densities of bryozoan recruits. In Experiment 3, bryozoan densities were highest on plates exposed to a copper pulse in Week 1 of the experiment (Fig. 2).

Recruitment of sponges increased from a low mean of 2.5 per plate in Experiment 1, to 20 per plate in Experiment 3 (data not shown). No effects of copper pulses were detected.

Experiment Series 2: February 2 wk experiments

There were two 2 wk experiments that ran in consecutive periods, referred to in their temporal sequence as Experiments 4 and 5, and using the higher concentration of copper. Species' responses are summarised in Table 1. Analytical details are provided in Table 3. There was generally very low power to detect impacts of copper doses on recruitment of barnacles, spirorbids, solitary ascidians botryllinid ascidians, or pooled bryozoans (Table 3). In most cases, low power arose from a combination of low recruitment and high variability, but in the case of newly-settled ascidians, which recruited in relatively high numbers, the low power arises solely from high variability in recruitment. There was no impact of exposure to copper on the densities of any of these groups (Table 1).

Serpulids were abundant recruits, with an average density of 115 per control plate in Experiment 4 and more than double that number in Experiment 5. There was no overall impact of exposure to copper on densities of recruits, nor was there a difference caused by the timing of a copper dose (Fig. 3).

Table 3 Probability and power values for analyses and planned comparisons of recruit densities in November/December short-term Experiments 4 and 5, listing overall effect of copper on density of recruits for whole plate (*Underlined values $P < 0.05$*)

Species	All treatments	Comparisons		Power
		Control vs dosed	W1 vs W2	
Experiment 4				
Elminius modestus/ *Balanus variegatus*	0.715	0.966	0.426	29
Serpulids	0.247	0.455	0.136	72
Spirorbids	0.447	0.407	0.342	28
Newly settled ascidians	0.214	0.725	0.092	28
Didemnids	0.601	0.453	0.514	18
Botryllinids	0.343	0.156	0.891	67
Pooled bryozoans	0.651	0.410	0.705	20
Sponges	0.795	0.721	0.577	28
Cyanea capillata	0.434	0.821	0.251	10
Experiment 5				
Balanus variegatus	0.536	0.281	0.892	40
Serpulids	0.532	0.978	0.275	90
Spirorbids	0.593	0.370	0.652	50
Newly settled ascidians	0.664	0.439	0.663	53
Didemnids	0.054	0.134	0.043	43
Botryllinids	0.226	0.148	0.339	38
Pooled bryozoans	0.994	0.958	0.927	18
Sponges	0.204	0.210	0.191	67
Cyanea capillata	0.079	0.045	0.263	30

The settlement rates of didemnids on control plates increased from an average of 3 to 20 per plate between Experiments 4 and 5. There was no effect detected in Experiment 4, but, copper exposure in Week 2 reduced the density of didemnid recruits in Experiment 5 (Fig. 3).

Cyanea capillata, a species of scyphozoan, recruited in Experiments 4 and 5. There was no effect of copper pulses in Experiment 4. Recruitment increased by a factor of 10 in Experiment 5, and a copper dose in the first or second week reduced the density of polyps across the whole plate (Fig. 3).

Although power was reasonable, there was no impact detected in Experiments 4 or 5 on sponges.

Experiment Series 2: February 4 wk experiment

Species responses are summarised in Table 4. Probability values and associated error terms for the original analysis and all subsequent comparisons are listed in Table 5.

Timing of single doses

There was no significant difference between single-dose treatments for large, small or the sum of both size classes of *Balanus variegatus* (Fig. 4).

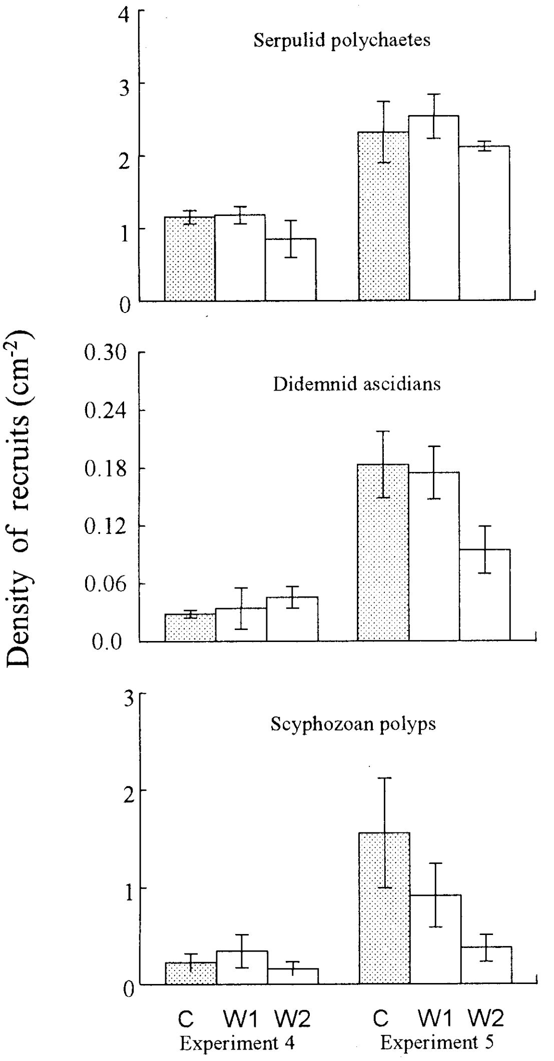


Fig. 3 Densities of recruits of selected taxa (cm^{-2}) plus standard error for January/February 2 wk Experiments 4 and 5. Treatments were undosed control (*C*), copper pulse in Week 1 (W1) and copper pulse in Week 2 (W2)

Table 4 Summary of comparisons of invertebrate species responses to copper pulses in February 4 wk experiment (× no effect; + effect)

Taxon	Analysis of plate	All treatments	Double pulses	Single pulses
Balanus variegatus				
Large	whole	+	+	×
	within	×	×	×
Small	whole	×	×	×
	within	×	×	×
Total	whole	×	×	×
	within	×	×	×
Polychaetes				
Serpulids	whole	×	×	×
	within	×	×	×
Spirorbids	whole	×	×	×
	within	×	×	×
Sabella spallanzanii	whole	×	+	×
	within	+	×	×
Bryozoans – encrusting				
W. subtorquata	whole	×	×	×
	within	×	+	×
C. pallasiana	whole	×	×	×
	within	×	×	×
All encrusting	whole	×	×	×
	within	×	×	×
Bryozoans – arborescent				
Newly settled	whole	×	×	×
	within	×	×	×
Bugula neritina	whole	×	×	×
	within	×	×	×
Bugula dentata	whole	×	+	×
	within	×	×	×
All arborescent	whole	×	×	×
	within	×	×	×
Solitary ascidians				
Newly settled	whole	×	×	×
	within	×	×	×
Solitary sp. 1 (SA1)	whole	×	×	×
	within	×	×	×
Ciona intestinalis	whole	×	×	×
	within	×	×	×
Total	whole	×	×	×
	within	×	×	×
Colonial ascidians				
Didemnids	whole	+	+	+
	within	×	×	×
Botryllinids	whole	×	×	×
	within	×	×	×
Sponges	whole	+	+	+
	within	+	×	×
Scyphozoans	whole	×	×	×
(*Cyanea capillata*)	within	×	×	×

There was no impact on the density of serpulid, spirorbid or fanworm *Sabella spallanzanii* recruits (Fig. 4).

There was no significant difference between treatments for newly settled ascidians, solitary ascidian sp.1, *Ciona intestinalis*, or pooled solitary ascidians (Fig. 4). A copper pulse in Week 4 had a negative impact on didemnids (Fig. 5). However, no impact on the distribution of these recruits within plates was observed. There was no difference between treatments for botryllinid ascidians (Fig. 5).

There were no differences caused by the timing of a single copper exposure to *Bugula neritina*, *B. dentata*, newly settled arborescent bryozoans, the encrusting species *Watersipora subtorquata* and *Cryptosula pallasiana*, or the pooled arborescent or pooled encrusting bryozoans (Fig. 5).

Sponge densities were reduced on plates dosed in Weeks 2, 3 and 4 (Fig. 5). There was no clear impact on the distribution of these recruits within the plate. There was no effect of single doses on *Cyanea capillata* polyps or hydroids (Fig. 5).

Timing of second doses

Large *Balanus variegatus* were reduced in density on plates exposed to copper for a second time in Weeks 3 and 4. Small *Balanus variegatus* and the sum of both size

Table 5 Analysis of effect of copper pulses on density of recruits in February 4 wk experiment, showing effects of copper on recruitment for whole plate (*Whole plate*), and on recruitment patterns with distance from copper source (*Within plate*). Probabilities are shown for main tests and planned comparisons, together with between- and within-plate error terms, to allow reconstruction of full ANOVA table

Taxon	Repeated-measures analysis[a]				Planned comparisons[a]			
	Whole plate		Within plate		Single-pulse treatments		Double-pulse treatments	
	P	MS_{error}	*P*	MS_{error}	Whole *P*	Within *P*	Whole *P*	Within *P*
Balanus variegatus								
Large	0.044	0.028	0.991	0.009	0.24	0.925	0.035	0.982
Small	0.796	0.440	0.097	0.034	0.892	0.881	0.939	0.596
Total	0.582	0.909	0.110	0.079	0.707	0.202	0.279	0.882
Polychaetes								
Serpulids	0.716	3.433	0.175	0.334	0.87	0.571	0.92	0.022
Spirorbids	0.963	0.173	0.477	0.025	0.788	0.161	0.723	0.769
Sabella spallanzanii	0.098	0.163	0.016	0.013	0.282	0.463	0.019	0.145
Solitary ascidians								
Newly settled (NSA)	0.954	0.918	0.453	0.093	0.75	0.274	0.742	0.829
Solitary Sp. 1 (SA1)	0.91	0.011	0.728	0.005	0.844	0.986	0.587	0.098
Ciona intestinalis	0.873	0.013	0.439	0.007	0.445	0.631	0.926	0.233
Total	0.99	1.018	0.407	0.121	0.882	0.463	0.805	0.475
Colonial ascidians								
Didemnids	0.001	0.029	0.351	0.016	0.001	0.609	0.004	0.091
Botryllinids	0.803	0.063	0.541	0.026	0.801	0.381	0.308	0.384
All sponges	0.000	0.148	0.077	0.051	0.012	0.076	0.000	0.217
Bryozoans-arborescent								
Newly settled	0.811	0.515	0.614	0.048	0.569	0.535	0.680	0.255
Bugula neritina	0.784	0.408	0.639	0.016	0.967	0.921	1	0.818
Bugula dentata	0.169	0.017	0.67	0.009	0.385	0.593	0.043	0.874
Total	0.944	1.954	0.798	0.083	0.936	0.893	0.526	0.594
Bryozoans-encrusting								
Watersipora subtorquata	0.284	0.012	0.136	0.007	0.410	0.318	0.195	0.018
Cryptosula pallasiana	0.918	0.010	0.875	0.007	0.825	0.915	0.905	0.331
Total	0.636	0.025	0.397	0.016	0.550	0.535	0.266	0.217
Total hydroids	0.931	1.516	0.509	0.137	0.932	0.393	0.739	0.261
Cyanea capillata	0.177	4.107	0.255	0.347	0.197	0.553	0.512	0.297

[a] Planned comparisons at level of whole plate had *df* = 1,24; those within plate all had *df* = 1,144; for overall analysis, degrees of freedom were 7,24 and 42,144, respectively

classes showed no differences caused by the timing of second pulses (Fig. 4).

Serpulid densities did not differ between treatments for the whole plate, but there were differences in distribution across the plate. There was a small increase in serpulid densities close to the source on plates dosed in Week 4 (Fig. 6).

There was no difference in spirorbid density caused by the timing of second copper pulses (Fig. 4). *Sabella spallanzanii* worm densities were significantly decreased (by a factor of eight) by exposure to second copper doses in Weeks 3 and 4 compared to a second dose in Week 2 (Fig. 4). The difference in distribution across the plate, however, showed no easily interpretable pattern, and is not shown.

Newly settled ascidians, solitary ascidian sp.1, and *Ciona intestinalis* were sufficiently common to analyse and showed no difference between the impacts of second doses delivered in Weeks 2, 3 or 4 (Fig. 4). The density of didemnid recruits decreased with exposure to a second dose of copper in Week 4 (Fig. 5), but there was no difference in distribution across the plate. There was no difference between impacts of second doses delivered in Weeks 2, 3 or 4 for botryllinid recruits (Fig. 5).

There was a statistically significant difference in the distribution of *Watersipora subtorquata* recruits across the plate, with no easily interpretable pattern (Fig. 6). *Bugula dentata* was reduced in density on plates dosed for a second time with copper in Week 4 (Fig. 5). Newly settled arborescent bryozoans, *B. neritina*, and pooled arborescent bryozoans showed no difference between impacts of second doses delivered in Weeks 2, 3 or 4 (Fig. 5).

A second dose of copper, delivered in Weeks 3 or 4 of the experiment, reduced the density of sponge recruits (Fig. 5).

There was no difference caused by the timing of a second dose for *Cyanea capillata* polyps or hydroids (Fig. 5).

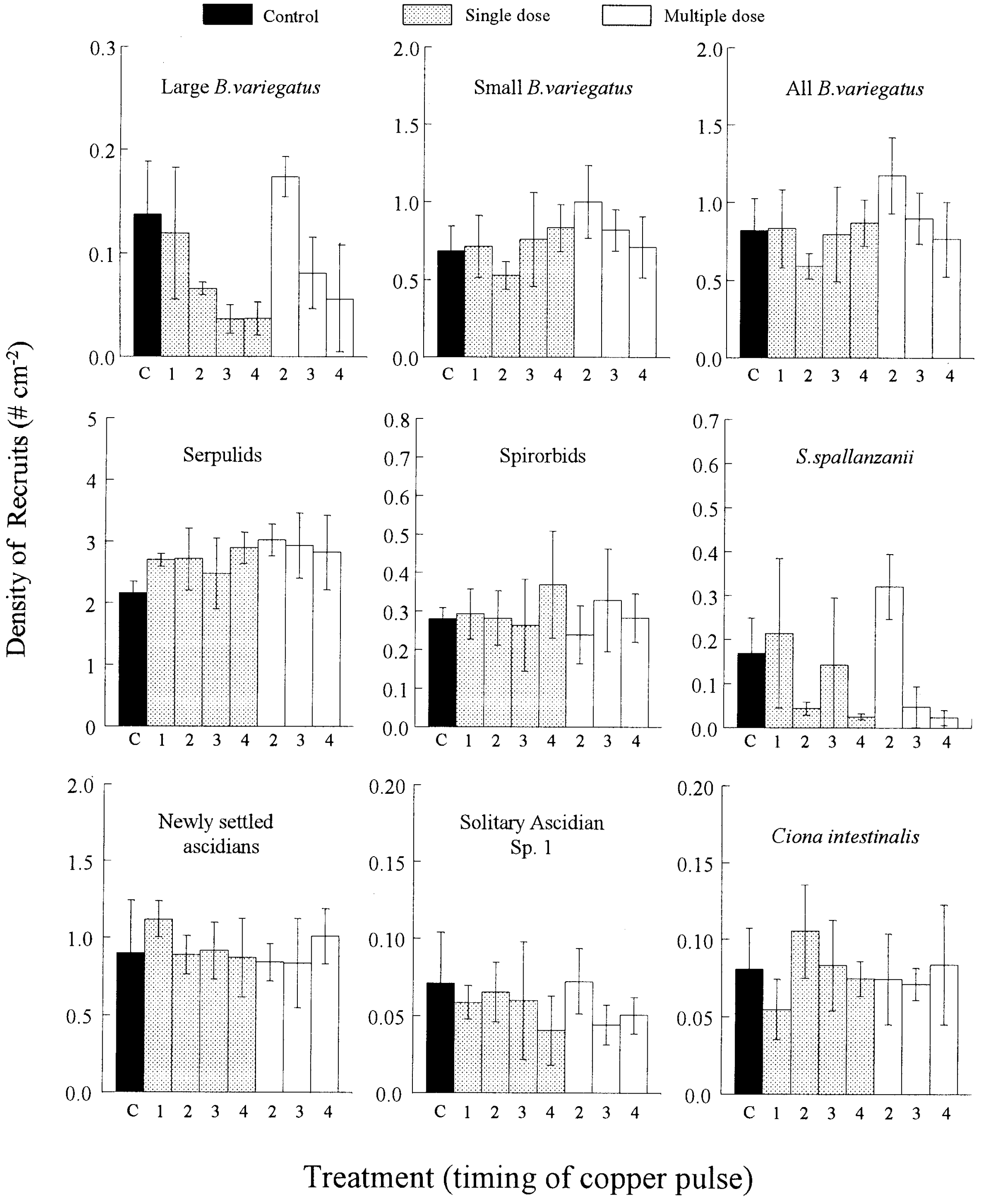
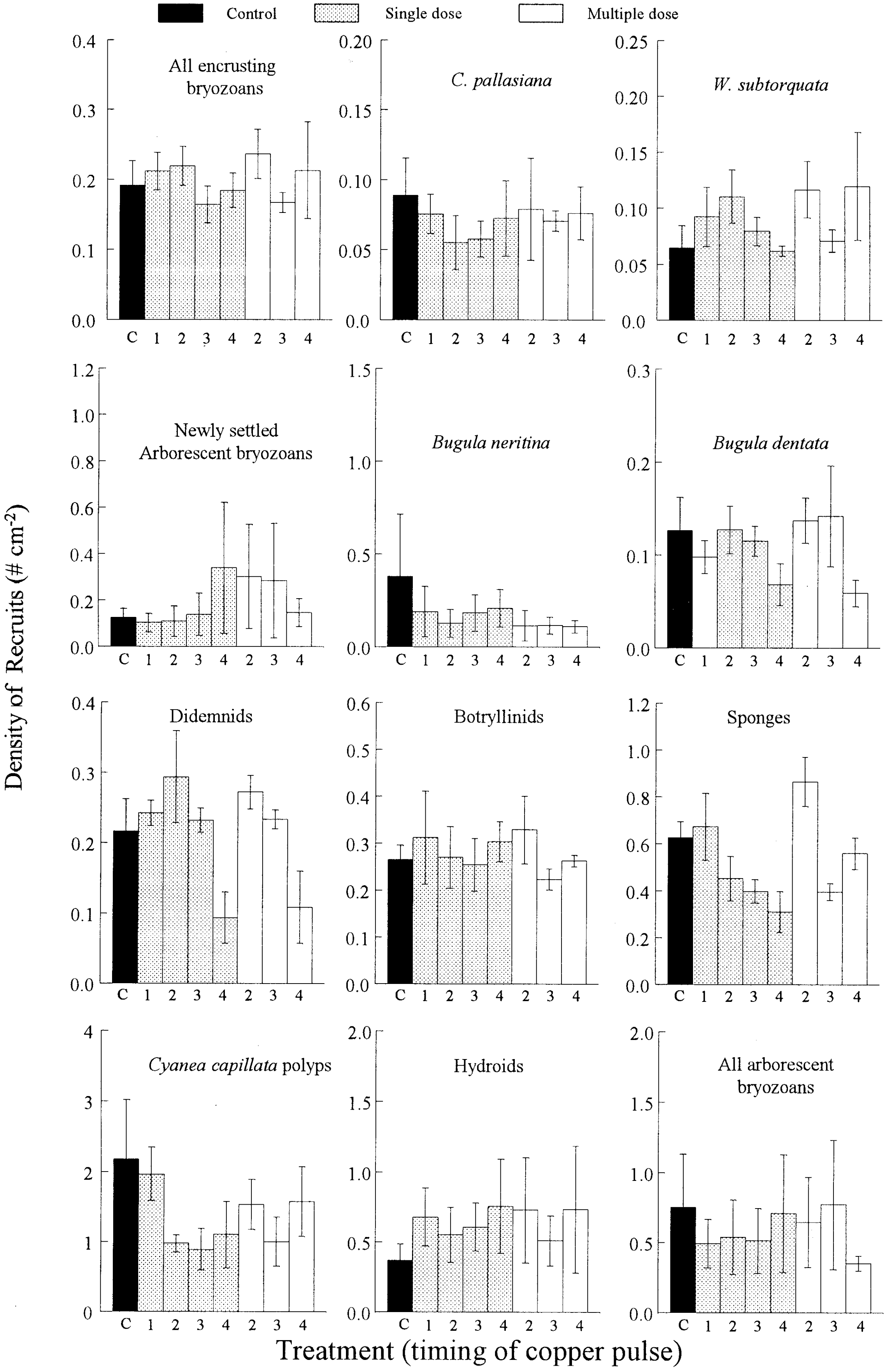


Fig. 4 Densities of recruits of barnacles, polychaetes and solitary ascidians (cm^{-2}) ±SE for February 4 wk experiment. Week in which pulse for single-dose treatments or second pulse for double-dose treatments was delivered is indicated by numbers below graphs (C = control). Standard error bars (based on all plates and distances) are *not* those used for *planned comparisons*, which used between- and within-subjects terms from analysis of variance (*B. variegatus* = *Balanus variegatus*; *S. spallanzanii* = *Sabella spallanzanii*)

Discussion

The timing of pulse-pollution events can influence the nature and extent of toxicant impacts on an assemblage. Exposure to copper pulses produced a suite of responses among invertebrate assemblage recruits. The responses not only varied considerably among species, but also through time. Direct impacts on populations could be detected at both short and longer time scales.

On short time scales, we assessed the impacts of copper exposure in the first 2 wk of a substratum being exposed to recruitment, and compared effects of copper doses in different weeks. These copper pulses are likely to act on individual populations of invertebrates, because there was abundant settlement space for the duration of the experiment and few observable competitive


Control
Single dose
Multiple dose
All encrusting bryozoans
C. pallasiana
W. subtorquata
Newly settled Arborescent bryozoans
Bugula neritina
Bugula dentata
Didemnids
Botryllinids
Sponges
Cyanea capillata polyps
Hydroids
All arborescent bryozoans
Density of Recruits (# cm-2)
Treatment (timing of copper pulse)
C 1 2 3 4 2 3 4

◄

Fig. 5 Densities of recruits of bryozoans, colonial ascidians, sponges, scyphozoan polyps and hydroids (cm^{-2}) ±SE for February 4 wk experiment. Week in which pulse for single-dose treatments or second pulse for double-dose treatments was delivered is indicated by numbers below graphs (*C* = control). Standard error bars (based on all plates and distances) are *not* those used for *planned comparisons*, which used between- and within-subjects terms from analysis of variance (*C. pallasiana* = *Cryptosula pallasiana*; *W. subtorquata* = *Watersipora subtorquata*)

interactions within the assemblage. The initial recruits to a surface at this study site also do not exert a strong influence on settlement (Keough 1998).

Individual taxa could be grouped into those affected and those unaffected by copper dosing. The tests on unaffected species varied in power. For some, such as serpulids, power was very high, so we are confident that they are unaffected by copper pulses of this magnitude and over this time scale. A range of other taxa recruited in low numbers and with relatively high variation through space, resulting in low power. It would be unwise to give much weight to those non-significant results. The invertebrates that recruited in high numbers and with relatively low variation through space in short-term experiments were *Elminius modestus*, serpulids, newly settled ascidians, and polyps of the scyphozoan *Cyanea capillata*. Our discussion is therefore restricted to the impacts of copper exposure on these groups.

Recruitment of *Elminius modestus* was reduced by copper, but was not affected by the timing of the pulse. *E. modestus* are rapid colonisers of available space and, unlike many other invertebrates, including serpulids, they do not respond to the presence of bioorganic films (biofilms) (Keough and Raimondi 1996). *E. modestus* larvae, if present, could therefore be expected to settle onto plates immediately upon immersion. There is no evidence that barnacle larvae are able to detect copper ions and therefore avoid settling in areas of high concentrations, but exposure to copper in the laboratory can kill or injure barnacles both pre- and post-settlement (Crisp and Austin 1960). A dose of copper in Week 2 has the potential to kill more organisms post-settlement than a dose of copper in Week 1. In this case, we would expect to see a difference in recruitment caused by a timing of a dose. Our results indicate that copper exposure is killing barnacles pre-settlement.

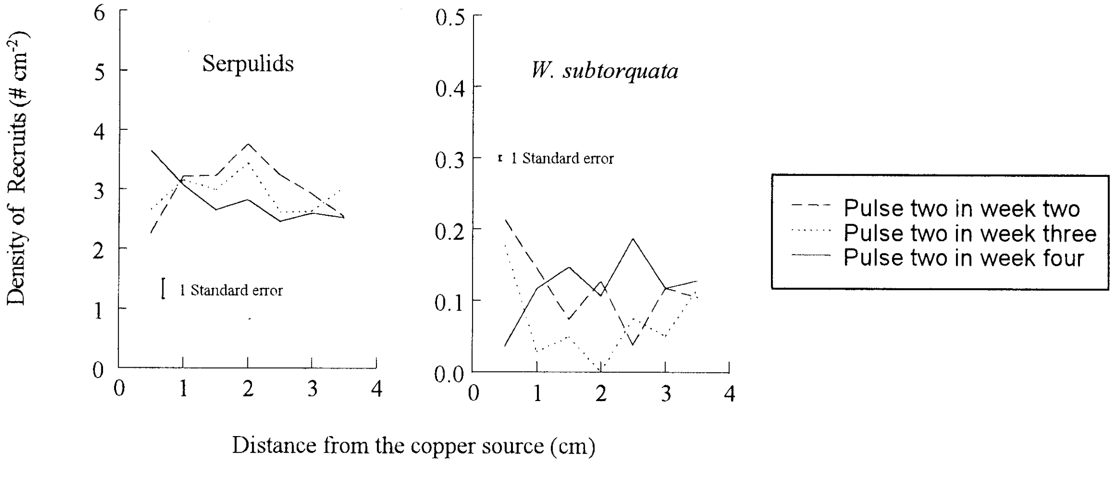
In Experiment 2, which had the highest settlement rate of *Elminius modestus* (an average of 16 cm^{-2}), there was no impact of a pulse exposure to copper in either Weeks 1 or 2 of the experiment. At times of high settlement, the impact of a pulse pollution event may be evident for a shorter period of time before settlement onto unoccupied space obscures it. Alternatively, conditions in the water during these weeks may have combined to modify the copper ion concentrations and reduce the toxicity of copper to *E. modestus*.

The timing of a pulse did influence polyps of the schyphozoan *Cyanea capillata*. Copper exposure in Week 2 of the experiment reduced the density of polyps compared to copper exposure in Week 1. Planulae of other *Cyanea* species settle preferentially onto hydrophobic surfaces (Brewer 1984). As a biofilm develops, a hard surface becomes more hydrophobic (Mihm et al. 1981), so if planulae in our study act similarly to those described elsewhere, we would expect higher settlement rates onto older settlement plates. A dose in Week 1 would occur at a time when few polyps were present, and a pulse in Week 2 would affect settling planulae, reducing overall numbers.

Serpulid densities were not affected by exposure to copper in either week of the short-term experiments. These results are consistent with previous field work on copper toxicity to serpulids (McHugh 1996), but not with laboratory work on other species, where *Galeolaria caespitosa* larvae were found to be sensitive to copper exposure (Wisely and Blick 1967).

Overall densities of newly-settled ascidians were slightly reduced by a copper pulse in Weeks 1 or 2 in

Fig. 6 Distribution of serpulid polychaetes and *Watersipora subtorquata* in February 4 wk experiment for double-pulse treatments. Mean density (cm^{-2}) of recruits with distance (cm) from source. Plates received second copper pulse in Weeks 2, 3 or 4. Error bars are derived from plates × distance error term, which was used to compare distributions between treatments



only one of the short-term experiments. Newly settled ascidians have few obvious diagnostic taxonomic features, and it is not possible to identify many of the species until they are a few weeks old, so this group includes several species. While pooling species into higher taxanomic groups may not obscure a pollutant signal (Warwick 1988), it is possible that some individual species were affected within this composite group.

The timing of a pulse had more obvious effects in longer-term experiments. Although a dose of copper appeared to have no impact on *Balanus variegatus* in 2 wk experiments, the longer-term experiment showed a greater negative impact of the later doses. This might occur because older barnacles are more sensitive or because effects of exposure take more than 2 wk to manifest (e.g. Abel 1980).

Copper doses in later weeks of the 4 wk experiment reduced the densities of sponge recruits. If sponge larvae and young recruits are equally susceptible to copper exposure, then these results can be explained by post-recruitment death (later doses having the potential to kill all the recruits that have settled in previous weeks). Many toxicity tests, however, have indicated that larvae of marine organisms are the most sensitive to toxicant exposure (McKim 1977). Examination of sponge recruitment onto short-term experiment control plates showed increasing sponge recruitment in the later weeks of the long-term experiment. Therefore, the most probable explanation consistent with increased sensitivity of larvae, is that copper pulses in later weeks had a greater impact on the density of sponges because they occurred at times of high settlement. These two explanations can only be resolved by assessing the sensitivity of different life-history stages of these sponges.

Serpulids were not affected by copper exposure except for a slight increase in densities close to the source on plates dosed for a second time in Week 4. These results give credence to the earlier hypothesis that serpulids remain unaffected by copper exposure. Cumulative impact can result from sequential stresses over time (Canadian Environmental Assessment Research Council 1988, as cited in Niederlehner and Cairns 1992; and see Keough and Quinn 1998). However, in contrast to the marked effects of the timing of a pulse, exposure to single and multiple pulses had the same effects (Johnston 1997). Our first dose was always during the first week of immersion of a substratum, and this dose could have corresponded to a period when few recruits were present. Species responding to the presence of a biofilm would be expected to settle in lower numbers in the first week. Alternatively, the high natural mortality of recruits in the first week after settlement that has been documented for other sessile marine invertebrates (see Hunt and Scheibling 1997 for a review) may obscure the impact of a initial copper pulse. Answering our third question, as to how the impact of repetitive pulse exposure to a toxicant differs when the interval between pulses is varied, would require that the first pulse be administered at a different time.

Variability of results

An additional feature of our tests was variation between experiments using the same copper-dosing technique. Laboratory toxicity tests generally expose organisms to a constant concentration of copper in a uniform environment. An important element of the work reported here was to examine the impact of copper exposure in a real-world situation. At Breakwater Pier, changes in water flow associated with wave action and tides would alter the rates of dispersion of copper ions and the rate at which plaster blocks dissolve. Furthermore, changes in salinity, organic content, temperature and pH all affect the concentrations of copper ions in the water (Allen and Hansen 1996). Through such mechanisms, the concentrations of the most toxic form of copper will fluctuate and may only occasionally reach the level necessary to kill or deter larvae. This may explain the variable impacts of copper pulses on *Elminius modestus*, colonial ascidians and bryozoans. The toxicity of copper pulses may therefore depend on the field conditions at the time of the pulse and not only on the timing of the experimental doses relative to the successional stage of the fauna. The work done so far has been restricted to one field season and one site. Results, therefore, cannot be readily extrapolated.

As evidenced by the response of sponges to the timing of copper doses, variations in copper toxicity can also be explained by variations in recruitment rates between weeks. Most of the invertebrates described here show extremely patchy recruitment (Keough 1983), and the effects of the timing of pulse-pollution events on sessile marine invertebrates must be examined in the context of the natural fluctuations in settlement rates.

There was no consistent response to copper pulses in the field at the level of the whole assemblage, nor did particular taxonomic groups respond in a characteristic way. Responses varied greatly among species and to a lesser extent through time. It is not possible therefore to make general statements regarding the overall composition of the assemblage under different dosing regimes. However, in respect of our original questions; (1) Assemblages at different stages of development are differentially sensitive to short term pulses of a toxicant; (2) if the initial pulse occurs in the first week of the experiment, or when substrate first becomes available, there will be little difference between the impacts of single- or double-pulse exposures to a toxicant; (3) given the above, to investigate how the impact of repetitive pulse exposure to a toxicant differs when the interval between pulses is varied, would require that our first copper pulse be administered after the first week.

As Beck (1996) noted, the more we succeed in restricting the release of contaminants into the environment, the more likely we are to have to deal with transient disturbance events that are the consequences of failure in our infrastructures of pollution control. In studying the responses of marine animals to levels of pollutants, researchers have focused on mortality effects following short-term exposures at high concentrations in the laboratory. The variability of toxicant impacts and species responses illustrated in this study are an aspect of reality not represented well by such laboratory tests. Extending ecotoxicological studies, such as have been reported here, to time frames that allow the examination of succession in hard-substrate invertebrate assemblages would provide a more comprehensive understanding of the effects of the timing and frequency of pulse-pollution events.

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References

Abel PD (1980) Toxicity of gamma-hexachlorocyclohexane (Lindane) to *Gammurus pulex*: mortality in relation to concentration and duration of exposure. Freshwat Biol 10: 251–259

Abel PD (ed) (1989) Water pollution biology. Ellis Horwood Limited, Chichester, England

Allen HE, Hansen DJ (1996) The importance of trace metal speciation to water quality criteria. Wat Envir Res 68: 42–54

Andrew RW, Biesinger KE, Glass GE (1977) Effects of inorganic complexing on the toxicity of copper to *Daphnia magna*. Wat Res 11: 309–315

Baker JP, Van Sickle J, Gagen CJ, Carline RF, Baldigo BP, Bath DW, Simonin HA, Wigington PJJ (1996) Episodic acidification of small streams in the Northeastern United States: effects on fish populations. Ecol Applics 6: 422–437

Beck MB (1996) Transient pollution events: acute risks to the aquatic environment. Water Sci Technol 33: 1–15

Bender EA, Case TJ, Gilpin ME (1984) Perturbation experiments in community ecology: theory and practice. Ecology 65: 1–13

Brewer RH (1984) The influence of orientation, roughness, and wettability of solid surfaces on the behavior and attachment of planulae of *Cyanea* (Cnidaria: Scyphozoa). Biol Bull mar biol Lab, Woods Hole 166: 11–21

Cairns J (1988) What constitutes field validation of predictions based on laboratory evidence? In: Adams WJ, Chapman GA, Landis WG (eds) Aquatic toxicology and hazard assessment. Vol. 10. American Society for Testing and Materials, Philadelphia, pp 361–368

Calow P (ed) (1994) Handbook of ecotoxicology. Vol. 2. Blackwell Scientific Publications, Oxford

Crisp DJ, Austin AP (1960) The action of copper in antifouling paints. Ann appl Biol 48: 787–799

Dixon DG, Sprague JB (1981) Acclimation to copper by rainbow trout (*Salmo gairdneri*) a modifying factor in toxicity. Can J Fish aquat Sciences 38: 880–888

Hunt HL, Scheibling RE (1997) Role of early post-settlement mortality in recruitment of benthic marine invertebrates. Mar Ecol Prog Ser 155: 269–301

Johnston EL (1997) Effects of the timing and frequency of copper pulses on a sessile marine invertebrate assemblage. BSc (Hons) thesis. University of Melbourne, Melbourne

Johnston EL, Webb JA (2000) Novel techniques for field assessment of copper toxicity on fouling assemblages. Biofouling 15: 165–173

Keough MJ (1983) Patterns of recruitment of sessile invertebrates in two subtidal habitats. J exp mar Biol Ecol 66: 213–245

Keough MJ (1998) Responses of settling invertebrate larvae to the presence of established recruits. J exp mar Biol Ecol 231: 1–19

Keough MJ, Quinn GP (1998) Effects of periodic disturbances from trampling on rocky intertidal algal beds. Ecol Applics 8: 141–161

Keough MJ, Raimondi PT (1996) Responses of settling invertebrate larvae to biofilms: a comparison of the effects of local and "foreign" films at three sites. J exp mar Biol Ecol 207: 59–78

La Point TW (1995) Signs and measurements of ecotoxicity in the aquatic environment. In: Hoffman DJ, Rattner BA, Burton GA Jr, Cairns J Jr (eds) Handbook of ecotoxicology. 1st edn. Lewis Publishers, Boca Raton, pp 13–24

Lewis AG, Cave WR (1982) The biological importance of copper in oceans and estuaries. Oceanogr mar Biol A Rev 20: 471–695

McHugh J (1996) The effects of copper on early life stages of sessile marine invertebrates. BSc (Hons) thesis. University of Melbourne, Melbourne

McKim JM (1977) Evaluation of tests with early life stages of fish for predicting long-term toxicity. J Fish Res Bd Can 34: 459–472

Mihm JW, Banta WC, Loeb GI (1981) Effects of adsorbed organic and primary fouling films on bryozoan settlement. J exp mar Biol Ecol 54: 167–179

Morrisey DJ, Underwood AJ, Howitt L (1996) Effects of copper on the faunas of marine soft-sediments: an experimental field study. Mar Biol 125: 199–213

Niederlehner BR, Cairns J (1992) Community response to cumulative toxic impact: effects of acclimation on zinc tolerance to *Aufwuchs*. Can J Fish aquat Sciences 49: 2155–2163

Phillips DJH, Richardson BJ, Murray AP, Fabris JG (1992) Trace metals, organochlorines and hydrocarbons in Port Phillip Bay, Victoria: a historical review. Mar Pollut Bull 25: 200–217

Pitt RE (1995) Effects of urban runoff on aquatic biota. In: Hoffman DJ, Rattner BA, Burton GA Jr, Cairns J Jr (eds) Handbook of ecotoxicology. Lewis Publishers, Boca Raton, pp 609–630

Warwick RM (1988) The level of taxonomic discrimination required to detect pollution effects on marine benthic communities. Mar Pollut Bull 19: 259–268

Weis JS, Weis P (1989) Tolerance and stress in a polluted environment: the case of the mummichog. BioSci 39: 89–95

Wisely B (1962) Effects of antifouling paint on a bryozoan larva. Nature, Lond 192: 543–544

Wisely B (1963) Detection and avoidance of a cuprous oxide antifouling paint by bivalve and gastropod larvae (Mollusca). Aust J mar Freshwat Res 14: 60–69

Wisely B (1964) Effects of antifouling paints on attaching larvae of two spirorbid tubeworms (Serpulidae: Polychaeta). Aust J mar Freshwat Res 15: 172–179

Wisely B, Blick RAP (1967) Mortality of marine invertebrate larvae in mercury, copper and zinc solutions. Aust J mar Freshwat Res 18: 63–72