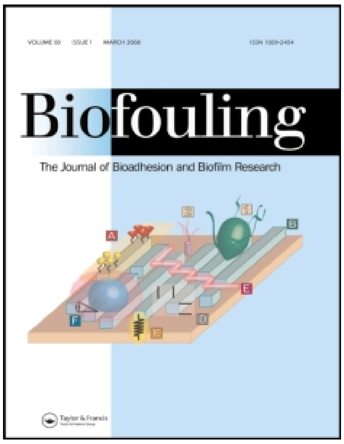

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Pulse Disturbances to the Colonization of Hard-substrates and *in situ* Determination of Copper using Diffusive Gradients in Thin-films (DGT): Quantifying Dose and Response in the Field

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Studies conducted in Port Philip Bay, Victoria, Australia are described that examined the effect of experimentally elevated copper concentrations on the recruitment of epifauna to settlement plates. Simultaneous measurement of the copper concentration using diffusive gradients in thin films (DGT) allowed direct comparisons to be made between the labile copper concentration measured at the settlement surface, and the biological effects observed. Copper concentrations created by the field dosing technique were between 20–30 $\mu g\ l^{-1}$ for the first 2 d, but then dropped considerably for the following 4 d (3 $\mu g\ l^{-1}$), and were indistinguishable from background for the final 7 d. The first 2 d of a copper pulse reduced the recruitment of barnacles, ascidians, serpulid worms, an encrusting bryozoan, and didemnid ascidians. The impacts occurred despite the copper pulse being much less than published LC_{50} values for similar species. The impacts were no longer obvious by day 7 or 14, having been obscured by either high mortality of early settlers, or large settlement events that took place after day 2. Thus the greatest impact of the pollution event occurred during the period of highest toxicant concentration. The value of this study lies in the correlation of toxicity effects with bio-available metal concentrations under realistic (natural, *in situ*) conditions.

Keywords: copper; pulse; DGT; field experiment; sessile invertebrates; recruitment

INTRODUCTION

A large number of physical, chemical and biological variables have the potential to substantially alter an organism's response to toxicant exposure from that observed under controlled laboratory conditions (Cairns, 1983; Kimball & Levin, 1985; Forbes & Forbes, 1994; Carpenter, 1996; Chapman, 2000). Accordingly, ecotoxicologists have recognized the need to develop field toxicity tests that assess impacts under ecologically realistic conditions, and that complement the existing array of laboratory and mesocosm based tests (Sprague, 1976; Kimball & Levin, 1985; Niederlehner *et al.*, 1986; Harwell & Harwell, 1989). With this in mind, a system first described by Johnston and Webb (2000) was developed to investigate the effects of transient copper pulses on the development of marine hard substratum ''fouling'' assemblages. In nature, copper pulses enter coastal waters through urban run-off, industrial, mining and metabolic wastes, antifouling paints and the corrosion of pipes (Mance, 1987; Abel, 1989; Paulson *et al.*, 1989; Depledge *et al.*, 1994; Pitt, 1995; Fabris *et al.*, 1999). To experimentally simulate such pulses, the system uses copper-spiked plaster blocks to deliver a dose of copper to a settlement plate over a relatively short time frame. The system has been subsequently used to investigate short and long-term effects of copper pulses on assemblages from several locations (Johnston & Keough, 2000; 2002; Johnston *et al.*, 2002). Although these studies have provided valuable information as to how fouling assemblages react to pulses of copper under natural conditions, there has only been limited quantification of the

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doses delivered to the settling plates by the plaster-block system (Johnston *et al*., 2002). Estimates of the copper dose *in situ* would be useful in the interpretation of the results of field experiments, and for making comparisons with the multitude of laboratory-based tests that establish dose-response relationships.

Accurate measurement of heavy metal concentrations created during conventional toxicity tests under laboratory conditions has proven difficult, and predicting aquatic toxicity using uncensored data from the literature is not recommended (Batley *et al*., 1999). Trace metal determinations in natural waters involve another degree of complexity due to the sensitivity limits of techniques, the ease of contamination, and changes in speciation of metals during the sampling process (Florence & Batley, 1980; Crompton, 1989). A growing body of evidence from laboratory studies also indicates that the toxicity of a heavy metal is related more closely to its free-ion activity than total concentration (for a critical review see Campbell, 1995). For the measurement of copper doses delivered in field experiments there is an additional difficulty. Divers are required to physically take water samples and in the process are likely to alter flow conditions and toxicant concentrations next to the experimental settlement surface. Field dosing is necessarily conducted on a small scale and the volume of water necessary for these samples might also lead to the accidental inclusion of less contaminated water and subsequent underestimations of toxicant concentrations.

Bearing such factors in mind, Webb and Keough (2002b) recently used the analytical technique of diffusive gradients in thin films (DGT) to quantify doses of copper delivered to settlement plates using an antifouling paint-based dosing system. DGT measures time-integrated concentrations of labile metals using diffusive gels (Davison & Zhang, 1994). The technique incorporates a cation-exchange resin layer (Chelex 100) separated from the solution of interest by an ion-permeable gel membrane of known thickness (Davison & Zhang, 1994; Zhang & Davison, 1995). The transport of metal ions to the exchange resin occurs by free diffusion through the gel membrane. Using Fick's First Law of Diffusion, the thickness of the gel membrane, and the deployment time, the concentration in the surrounding water can be calculated from the measured mass in the resin-gel. DGT can pre-concentrate metals by a factor of up to 270 over 24 h, which makes it substantially less prone to contamination concerns and analysis limits (Zhang & Davison, 1995). The final value is a time-integrated concentration for the period of deployment, which allows an estimate of average exposure in the field, in contrast to the temporal variability inherent in discrete water samples (Twiss & Moffett, 2002). DGT also measures only a labile fraction of the total metal in solution (Zhang & Davison, 1995). It is readily acknowledged that a proportion of total copper in both laboratory test solutions and natural waters may be strongly complexed, and therefore not bioavailable to flora and fauna (*e.g*. Batley & Florence, 1976; Florence, 1982; Apte & Batley, 1995). Strongly complexed copper will also be non-labile, and will not be measured by DGT units (Zhang & Davidson, 2000; Munksgard & Parry, 2003). As such, the labile fraction measured by DGT may be a better estimate of the bioavailable fraction of the metal than total metal concentrations (Apte & Batley, 1995; Campbell, 1995).

The study by Webb and Keough (2002b) showed that DGT can be used to quantify doses of copper delivered to settlement plates. However, they did not conduct concurrent surveys of epifauna to assess the ecological effects of the doses being measured. In this paper the results of field toxicity tests using the plaster block system to deliver copper to settlement plates that were deployed for 2, 7 or 14 d are reported. Simultaneously, levels of copper dosed over these periods were measured using DGT. By combining the DGT technique with field experiments, it was possible to describe the impacts of measured amounts of labile copper on the recruitment of epifaunal marine invertebrates. Such dose-response relationships have not previously been reported for field-based toxicity studies.

MATERIALS AND METHODS

Sites

Three experiments were conducted at two Piers in Port Phillip Bay, Victoria, Australia (38°S, 145°E, Figure 1). The first, Breakwater Pier, extends 300 m from the shore and is sheltered by a rocky breakwater on its southern side. The second site, Point Wilson Explosives Reserve Pier, extends 3 km from the shore without a breakwater. The assemblage of sessile invertebrates that settle on hard substrates at both sites includes polychaete worms, barnacles, bryozoans, colonial and solitary ascidians, sponges and hydroids. Public access is restricted to both piers.

Toxicant Dosing

Copper pulses were delivered to settlement plates using the dosing system first outlined in Johnston and Webb (2000), in which copper-impregnated plaster blocks are attached to the centre of settlement plates and allowed to dissolve underwater to create copper pulses. All plates received either a copper-impregnated or control plaster block at the beginning of each experiment. The copper and control plaster blocks were made according to the methods outlined in Johnston and Keough (2002). Settlement plates and the backing plates onto which they were mounted were as described in Johnston and Webb (2000). On the backing plates, the settlement plates were separated by approximately 10 cm. In these experiments, six settlement plates were attached to each backing plate using the bolts embedded in the plaster blocks (Johnston & Webb, 2000). Copper-dosed and control plates were interspersed randomly across a backing plate. The plates were suspended 3.5 m below mean low water mark.

For the three experiments, different combinations of treatments and replicate numbers were employed. These are detailed in Table I. It is worth noting that for the 2-d experiment at Breakwater Pier, two types of copper-impregnated block were used (3.2 g and 1.6 g $CuSO_4$ block^{-1}), hereafter referred to as ''high-concentration'' and ''low-concentration'', as well as the control blocks. For the other two experiments, the high-concentration copper block was used together with the control blocks.

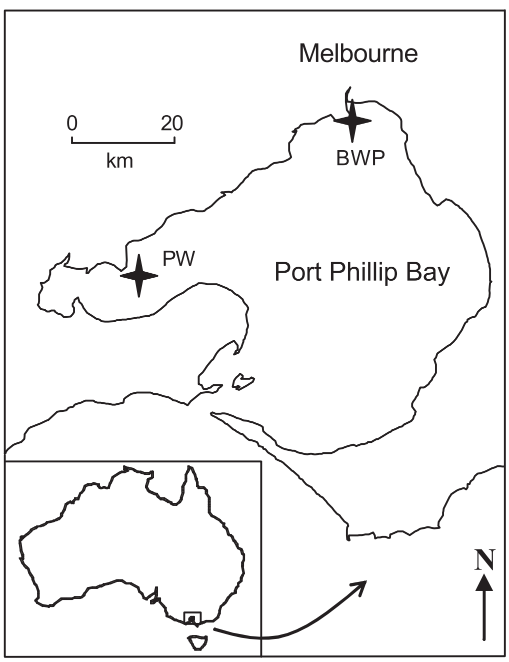

FIGURE 1 Map showing location of field sites within Port Philip Bay, Victoria, Australia. BWP = Breakwater Pier, PW = Point Wilson Explosives Reserve Pier.

Characterization of Copper Pulse

DGT units and deployment

The piston-style standard DGT deployment units, as well as the theory behind their operation have been described elsewhere (Davison & Zhang, 1994; Zhang & Davison, 1995). The deployment units, diffusive gels, metal binding gels, and membrane filters used in these experiments were as described in Webb and Keough (2002a). As with this earlier study, DGT units fitted with diffusive gels of 0.4 mm and 0.8 mm thickness were deployed simultaneously.

The DGT units were deployed using a system based on that employed by Webb and Keough (2002b). The units were inserted through holes drilled in the settlement and backing plates so that the diffusion window was flush with the settlement surface of the plates. On each settlement plate, four DGT units were placed around the plaster block (Figure 2). Settlement plates faced downwards to reduce the accumulation of sediment on both settlement surfaces and DGT diffusion windows. DGT units were deployed at the beginning of the experimental exposures. In addition, during the Breakwater Pier 14-d experiment, DGT units were replaced after 2 and 7 d, giving 3 deployment periods of 2, 5 and 7 d (Table I). For all deployments, measurements of background copper concentrations were made by the simultaneous deployment of DGT units at the same depth, 2 m away from the backing plates. Water temperature and pH were measured at the beginning and end of each deployment (average shown in Table I). To measure contamination induced by the experimental procedures, two procedural blanks were carried to the field during each deployment and processed concurrently with deployment samples.

Upon retrieval, the DGT units were processed according to the procedures outlined in (Webb &

TABLE I Summary of experiment details. Location, start date, duration, average water temperature and pH are shown for all trials. Deployment periods and replicate numbers are shown for the copper pulse characterization studies and the recruitment studies

					Pulse characterization			Recruitment study	
Location	Start date	Total duration (d)	Avg temp (°C)	Avg pH	Deployment period (d)	Treatment replicates	Background replicates	Deployment period (d)	Treatment replicates
Breakwater Pier	01/01/2000	2	18	7.8	2	2[a, b]	2	2	8[a]
Point Wilson Pier	10/01/2000	2	19	7.6	2	3	2	2	6
Breakwater Pier	20/01/2000	14	20	7.7	2	3	2	2	6
					5	3	2	7	6
					7	3	2	14	6

[a]This experiment used two copper treatments: high concentration (3.2 g $CuSO_4$ block^{-1}) and low concentration (1.6 g $CuSO_4$ block^{-1}). Replicate numbers are for each copper concentration; [b]copper concentration over control plates was not measured in this experiment

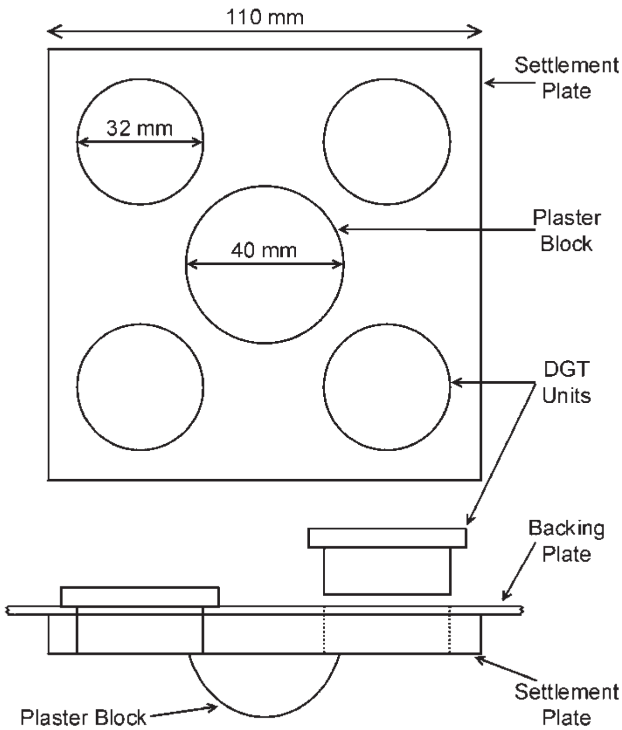

FIGURE 2 Schematic representation of the copper measurement system. Figure shows plan-view from the underside of the plate with four DGT units surrounding a central plaster block. Cross-section shows a settlement plate fixed to a backing plate with one DGT unit in place, and one removed.

Keough, 2002a). Metal concentrations within the acid eluent were measured by graphite furnace atomic absorption spectrometry (GFAAS) at the National Association of Testing Authorities (NATA) accredited commercial laboratory at the Marine and Freshwater Resources Institute (MAFRI), Victoria, Australia.

Calculations

As DGT units were effectively nested within plates, the mean mass of copper from four DGT units placed around a single plaster block was used as a single replicate value for the measure of copper over that settlement plate. An average procedural blank value was taken as a measure of contamination during each experimental process and was subtracted from all other values for that experiment. Time-averaged copper concentrations for the period of deployment can be calculated in two ways, and the results of both are presented for comparison. In the first method, calculations are based on the assumption that the actual diffusive boundary layer *in situ* is negligibly small in relation to the thickness of the diffusive layer within the DGT unit, and therefore mass transport is controlled solely by the thickness of the diffusive layer (Δg). In this case, the concentration in the bulk solution (C_b) can be calculated from the measured mass in the resin-gel (M) after a known deployment time (t) using Equation 1 (Zhang & Davison, 1995),

$$C_b = \frac{M\Delta g}{DtA} \tag{1}$$

where D is the diffusion coefficient of copper through the diffusive layer, and A is the area of gel exposed to the solution. The value of D appropriate to the temperature recorded was taken from the table of diffusion coefficients previously measured by Zhang (personal observations).

If the diffusive boundary layer *in situ* is not negligibly small in comparison to the diffusive layer thickness (Δg), Equation 1 will underestimate bulk concentrations. In this case, gels of two different diffusive layer thicknesses (Δg_1 and Δg_2) can be used and corresponding masses M_1 and M_2 measured. The concentration in bulk solution can then be calculated using Equation 2 and is considered independent of the actual diffusive boundary layer (Zhang *et al.*, 1998):

$$C_b = \frac{M_1 \cdot M_2}{D \cdot A \cdot t} \cdot \frac{\Delta g_1 - \Delta g_2}{M_2 - M_1} \tag{2}$$

Due to the practice employed in this study of deploying units of the same diffusive thickness around single plaster blocks, the results for at least two blocks had to be combined in order to provide an estimate of dose using Equation 2.

Webb and Keough (2002b) found that small errors in estimates of C_b gained using Equation 1 can compound substantially when results from two thicknesses are used in Equation 2. They suggested that results from multiple gels of the same thickness be pooled to try to reduce the effects of such errors. To gain a best estimate of copper concentration, all gels for the deployment period were pooled. This resulted in results from four or eight units of each gel thickness being used in Equation 2. To gain an estimate of the range of possible Equation 2 results, the total range of values was calculated using all possible pairing of 0.4 mm and 0.8 mm plates. Where there was no replication of gel thickness (Breakwater Pier Experiment No 1 and background estimates) the range of concentrations possible from Equation 2 was calculated given a 5% error in estimates of the concentration from Equation 1 using the methods developed by Webb and Keough (2002b). The figure of 5% was chosen as this level of precision has been seen in laboratory trials using DGT (Zhang & Davison, 2000).

Settlement Experiments

Concurrent to the dose measurement, recruitment to copper-dosed and control settlement plates was measured on plates deployed around 2 m from the

DGT deployment plates. The settlement plates were deployed using exactly the same techniques as for the DGT-measurement plates, with the exception that there were no DGT units. Numbers of plates of each treatment are detailed in Table I. For the 14-d experiment at Breakwater Pier, a total of 36 settlement plates was deployed at the beginning of the experiment. Six replicate plates of each treatment were chosen randomly and destructively sampled after 2, 7 and 14 d.

At the conclusion of each settlement experiment, plates were collected, transported and censused according to the methods in Johnston and Keough (2002). The high density of many recruits on the some plates necessitated the surveying of every second cm^2.

Analysis

To examine the effect of dose on recruitment, analyses were performed on taxonomic groups that had a minimum mean recruitment of > 5 individuals or colonies on control plates. Data were checked for normality and homogeneity of variances by visual examination of the plots of residual errors (Quinn & Keough, 2002). An analysis of variance was then conducted on the raw or transformed data for each taxonomic group with copper treatment as the categorical factor. In the 2-d Breakwater Pier experiment a planned comparison was also carried out of the control against the high-concentration copper treatment. Where there was a non-significant result, the power of experiments to detect a $\pm 50\%$ difference from control was calculated using PiFace (Lenth, 1996). This effect size was chosen as effects of this magnitude have been reported in previous copper pulse recruitment experiments at Breakwater Pier (Johnston & Keough, 2000). The relationships between copper concentration measured and the biological response was also depicted graphically.

RESULTS

Copper Measurements

Copper concentrations as calculated using Equations 1 and 2 are detailed in Table II. In general, the amounts calculated using Equation 2 were comparable to those calculated *via* Equation 1, and the range of values possible *via* Equation 2 was not large. The exception to this was the calculation for copper blocks during days 1–2 of the 14-d Breakwater Pier experiment, where the range calculated was large (15–278 $\mu g\ l^{-1}$). Over the first 2 d of immersion (2-d Breakwater Pier, 2-d Point Wilson, days 1–2 of 14-d Breakwater Pier) high-concentration copper blocks created average copper concentrations over the settlement surface of between approximately 20 and 30 $\mu g\ l^{-1}$. For the 14-d experiment, copper concentrations fell off rapidly after the first 2 d, averaging around 3 $\mu g\ l^{-1}$ for days 3–7 and being indistinguishable from background levels for days 8–14. The concentrations would not have been constant over these time periods, but are likely to have started at a higher level before dropping off. Background copper levels were uniformly low for all experiments, at around 1 $\mu g\ l^{-1}$ or less (Table II). The levels of copper found in the procedural blanks was low compared to the amount of copper accumulated in the field (average 0.3 ± 0.3 $\mu g\ l^{-1}$ for all experiments). Copper concentrations over plates fitted with

TABLE II Copper measurements for all experiments

Experiment	Days	Treatment	Dissolution of blocks (%) ± SD	Equation 1 Concentration Mean ± SD ($\mu g\ l^{-1}$)	Equation 2 Concentration ($\mu g\ l^{-1}$)	Equation 2 Range ($\mu g\ l^{-1}$)
2-d Breakwater Pier	1–2	High Conc Copper	34 ± 3	31.8 ± 2.4	26.5	23–31
		Low Conc Copper	22 ± 3	9.9 ± 2.0	6.4	5.7–7.4
		Background	-	0.8 ± 0.1	1.2	0.8–2.0
2-d Point Wilson	1–2	Copper	91 ± 11	25.8 ± 4.2	26.8	23–37
		Control	74 ± 3	6.7 ± 1.3	7.4	6–11
		Background	-	1.1 ± 0.1	1.6	1.2–2.1
14-d Breakwater Pier	1–2	Copper	38 ± 8	20.3 ± 7.8	21.8	15–278
		Control	34 ± 9	4.1 ± 0.6	7.3	6.4–8.7
		Background	-	1.0 ± 0.1	0.8	0.7–0.9
	3–7	Copper	92 ± 2	2.8 ± 1.2	3.7	2.9–5.4
		Control	87 ± 8	0.3 ± 0.0	0.6	0.5–0.7
		Background	-	0.3 ± 0.1	0.4	0.3–0.6
	8–14	Copper	100	0.4 ± 0.3	0.8	0.0–0.8
		Control	100	0.2 ± 0.1	0.5	0.3–1.3
		Background		0.3 ± 0.1	0.8	0.6–1.4

Table shows calculated copper concentrations and % of block dissolution that occurred during each deployment. Equation 1 concentration is the time-averaged concentration of copper ($\mu g\ l^{-1}$) around the block assuming the diffusive boundary layer is negligibly small in comparison to the diffusive gel layer (0.4 or 0.8 mm). Equation 2 concentrations are considered to be independent of the diffusive boundary layer, and are calculated using results from two diffusive gel layer thicknesses. After the 2-d Breakwater Pier experiment, "copper" in the Treatment column refers to high-concentration copper blocks control blocks (2-d Point Wilson, days 1 – 2 of 14-d Breakwater Pier) were elevated above background levels over the first 2 d of immersions (between 4 and 7 μg l^{-1}), but were indistinguishable from background levels for days 3 – 14 of the 14-d Breakwater Pier experiment. Low-concentration copper blocks (2-d Breakwater Pier) elevated copper concentrations above background over the settlement surface, but the concentration was only around one third of that created by the high-concentration blocks (Table II).

Examination of the dry weight of blocks before and after immersion shows that during the 2-d experiments, the rate of dissolution of plaster from the blocks was higher in blocks with higher copper content (Table II). However, this trend was not seen in the 14-d Breakwater Pier experiment, with similar amounts dissolving from both copper and control blocks over days 1 – 2 and days 3 – 7. By the end of the 14-d experiment, all blocks had dissolved completely (Table II).

Recruitment

The taxa that recruited to settlement plates varied among the three experiments. There was extremely sparse settlement during the 2-d experiment at Point Wilson Pier, and as such, the effects of copper pulses on recruitment could not be assessed for this experiment. The effects of copper on recruitment for the two experiments at Breakwater Pier are shown in Figures 3 and 4, and Tables III and IV.

Breakwater Pier 2-d experiment

In the 2-d experiment at Breakwater Pier, exposure to a high concentration copper pulse reduced the numbers of didemnid ascidians by 80% (Figure 3, Table III). Colonial botryllinid ascidians recruited in fewer numbers than didemnids and, although the average number of recruits on high-dose plates was reduced, there was no significant effect of exposure to copper on these recruits. The densities of newly settled ascidians were reduced by 25% by the high concentration copper pulse (Figure 3, Table III). There was a significant difference between treatments in the overall test for the barnacle *Elminius modestus*, but the comparison of control against the high concentration treatment was not significant (Figure 3, Table III). The power of both planned comparisons to detect the $\pm$ 50% difference was low (Table III). The low-concentration copper pulse did not seem to affect

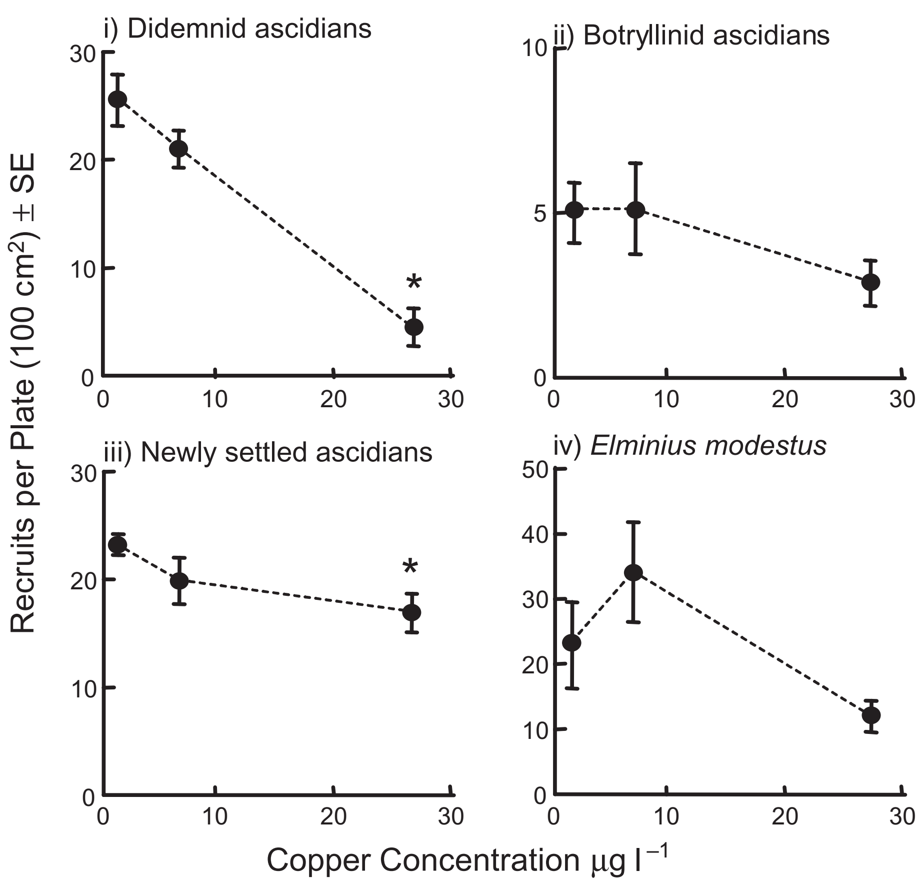

FIGURE 3 Recruits per plate (100 cm^2) of ascidians and barnacles $\pm$ 1 SE, plotted against DGT-measured copper concentrations (μg l^{-1}) calculated using Equation 2 for the Breakwater Pier 2-d settlement experiment. Abundances for control plates are shown plotted against background copper concentration, as no measurements were made over control plates for this experiment. Asterisks above the high-concentration abundance indicate a significant difference in abundance between the control and the high-concentration copper treatments.

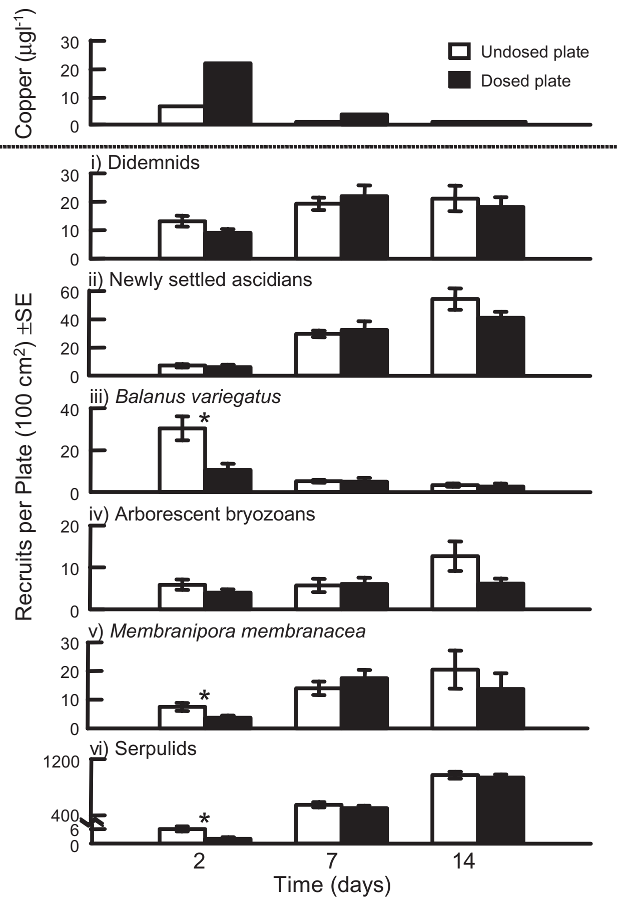

FIGURE 4 Breakwater Pier 14-d experiment. Top bar graph shows DGT-measured copper concentrations ($\mu g\ l^{-1}$) calculated using Equation 2 for 3 immersion periods (days 1–2, 3–7, and 8–14) on dosed plates (filled bars) and control plates (open bars). Lower bar graphs show recruits per plate (100 cm^2) of taxa $\pm$ 1 SE censused after 2, 7 and 14 d deployment. Asterisks indicate significant effects of copper treatment for that deployment period.

recruitment, with similar numbers settling on control and low-concentration plates (Figure 3).

Breakwater Pier 14-d experiment

Didemnid ascidians, newly settled ascidians, the barnacle *Balanus variegatus*, pooled arborescent bryozoans, the encrusting bryozoan *Membranipora membranacea*, and pooled serpulid worms settled in sufficient numbers to warrant analysis.

Days 1–2 The numbers of recruits were reduced significantly for *B. variegatus*, *M. membranacea* and the Serpulids (Figure 4, Table IV). Recruitment of didemnid ascidians was reduced on copper dosed plates, but not significantly despite the analysis having high power to detect a $\pm$ 50% difference from control means (Figure 4, Table IV). There was no observed impact of copper on newly settled ascidians or arborescent bryozoans (Figure 4, Table IV).

Days 3–7 and 7–14 For the final two immersion periods there were no significant effects of treatment on any of the taxonomic groups (Figure 4, Table IV). Between days 2 and 7, there was a large spat fall of serpulids and their abundance rose from a mean of five per control plate to over 500 per control plate during this period (Figure 4). Simultaneously there was a large reduction in the number of live barnacles on both control and copper plates (Figure 4). Many empty barnacle shells were observed on both control and copper dosed plates.

DISCUSSION

Use of DGT to Characterize Copper Pulses

Copper is a common pollutant that enters coastal waters through urban run-off, industrial, mining and metabolic wastes, antifouling paints, and the corrosion of pipes (Mance, 1987; Abel, 1989; Paulson *et al.*, 1989; Depledge *et al.*, 1994; Pitt, 1995; Fabris *et al.*, 1999). The use of DGT for the *in situ* measurement of labile copper during field toxicity tests has for the first time allowed a comparison of the biological impacts of copper and the magnitude of the labile copper pulse being created at the settlement surface. As stated above, the labile fraction of copper is more likely to represent the bioavailable fraction. The experimental treatment was successful in increasing the concentration of copper at the settlement surfaces well above background. Background levels of copper measured in this study were similar to those previously reported by Phillips *et al*. (1992) from Hobson's Bay, close to Breakwater Pier (0.8–2 $\mu g\ l^{-1}$ total copper). In polluted sites near spills, marinas or industrial runoff, total copper concentrations have been measured in the range $\sim 6\ \mu g\ l^{-1}$ (Paulson *et al.*, 1989), 10–100 $\mu g\ l^{-1}$ (Stauber *et al.*, 2000), and extremes of several hundred $\mu g\ l^{-1}$ (Stiff, 1971), although older measurements (pre-1980) of Cu concentrations have generally been found to overestimate true concentrations (Batley *et al.*, 1999). The average concentrations achieved by experimental manipulation of between 20–30 $\mu g\ l^{-1}$ were approximately 20–30 times greater than ambient levels and within the lower range of values that have been measured at grossly ''polluted'' sites. It should also be remembered that concentrations were probably higher than this during the early part of the 2-d deployment period. These values are within the lower end of the range used for laboratory-based toxicity tests (*e.g*. Conradi & Depledge, 1998; Bellas *et al*., 2001).

There was little variability in the initial 2-d copper pulse created at two sites and on three occasions. The similarity of pulse concentrations created at two times at Breakwater Pier is not surprising since environmental conditions during both trials were similar and the same quantity of the high concentra-

TABLE III Results of ANOVA on the recruitment of selected taxa during the Breakwater Pier 2-d experiment

Taxon	Test	df	MS	P	Power (%)
Didemnid Ascidians	Treatment	2	978.000	< **0.001**	
	Control *vs* High Concentration	1	1764.000	< **0.001**	
	Error	21	26.952		
Botryllinid Ascidians $Log_{10}(x+1)$	Treatment	2	0.081	0.211	
	Control *vs* High Concentration	1	0.136	0.118	42
	Error	21	0.049		
Newly Settled Ascidians	Treatment	2	81.375	**0.033**	
	Control *vs* High Concentration	1	162.562	**0.010**	
	Error	21	20.155		
Elminius modestus	Treatment	2	979.125	**0.035**	
	Control *vs* High Concentration	1	473.062	0.182	23
	Error	21	248.274		

Probabilities are shown for the test of main effect (Treatment), and the planned comparison (control *vs* high concentration (3.2 g $CuSO_4$) block), together with the value for Mean Squares to allow reconstruction of full ANOVA table. Significant *P* values ($P < 0.05$) are highlighted in bold. Power values (in % terms) for the planned comparison are included for non-significant results

TABLE IV Results of ANOVA on the effect of copper pulses on the density of selected taxa in the Breakwater Pier 14-d experiment after different immersion periods (2, 7 and 14 d)

	2-d immersion			7-d immersion			14-d immersion		
Taxon	MS	P	Power	MS	P	Power	MS	P	Power
Didemnid ascidians ($Log_{10}(x+1)$)	0.072	0.090	99	0.004	0.659	99	0.006	0.789	60
Error	0.020			0.020			0.075		
Newly settled ascidians ($Log_{10}(x+1)$)	0.022	0.495	83	< 0.001	0.904	99	0.036	0.157	63
Error	0.044			0.021			0.016		
Balanus variegates($\sqrt{}$)	16.039	**0.003**		0.339	0.514	41	0.817	0.319	25
Error	1.011			0.742			0.742		
Arborescent bryozoans ($Log_{10}(x+1)$)	0.045	0.240	86	0.003	0.825	60	0.156	0.161	42
Error	0.029			0.062			0.068		
Membranipora membranacea ($Log_{10}(x+1)$)	0.200	**0.014**		0.032	0.381	98	0.104	0.353	28
Error	0.022			0.038			0.110		
Serpulids ($Log_{10}(x+1)$)	0.156	**0.018**		0.004	0.320	99	0.001	0.592	100
Error	0.021			0.004			0.002		

Probabilities, error terms and Mean Squares are shown for the test of control *vs* copper (df = 1,10). Significant *P* values ($P < 0.05$) are highlighted in bold. Power values (in % terms) for the ANOVA are included for non-significant results

tion plaster block dissolved during both 2-d periods. At the more exposed site of Point Wilson Explosives Reserve Pier, however, more than 90% of the high concentration copper block had dissolved within 2 d. It is likely that although a greater amount of copper was released by the field dosing technique during this period, it was also dispersed to a greater extent, so that there was no great increase in the time-integrated copper concentrations measured at the settlement surface. This is likely to reflect the increased rate of dispersion of any pulse pollution event occurring at high energy sites.

Two copper dose levels were used in the first Breakwater Pier trial. The low concentration plaster block, which was spiked with half the initial $CuSO_4$ of the high concentration, created a 2-d time-integrated copper pulse of only one third that of the high concentration pulse for the same trial. The difference can be attributed to the slower rate of dissolution of the low concentration plaster block, and indicates that the amount of copper added to plaster blocks cannot by itself ensure direct control over the toxicant dose.

Measurement of the copper dose over 14 d immersion confirmed expectations that this field dosing system creates a clear pulse pollution event. High average concentrations in the first 2 d of the trial were reduced by close to an order of magnitude over the following 5 d, and were indistinguishable from background in the second week.

Control values for settlement plates with un-spiked plaster blocks during the first 2 d of immersion at both Point Wilson and Breakwater Pier were less than dosed plates, but were also above background concentrations. This indicates some cross-contamination of treatments across a backing plate. Greater separation of settlement plates would reduce the risk of such cross-contamination, but a number of studies have already been performed that examine the biological effects generated by the dosing system (Johnston & Webb, 2000; Johnston & Keough, 2002; Johnston *et al*., 2002), and the results of this study allow a useful interpretation of these results. Moreover, a recent synthesis of available saltwater acute copper toxicity data carried out for an Ecological Risk Assessment (Hall *et al*., 1998) used data for 57 saltwater species, and determined a 10th percentile value (represents a no-effect concentration that protects 90% of the test species) of 6.3 $\mu g\ l^{-1}$. The majority of species unprotected at this concentration consisted of diatoms and microalgae. It is unlikely, therefore, that copper from cross-contamination on control plates was sufficient to cause acute toxicity to recruiting sessile invertebrates during the first 2 d of block immersion. However deterrence or sublethal effects cannot be ruled out. The results of Hall *et al*. (1998) also explain the lack of effect seen from the low-concentration copper blocks; the concentrations generated were not high enough to affect the settlement of the epifauna.

In general, there was a good correlation between the values for copper concentrations calculated using the two equations. This indicates that mass transport in these trials was predominantly controlled by the thickness of the diffusive gel layer, and ambient water flow conditions at the two sites were sufficient to ensure the width of the actual diffusive boundary layer was negligibly small. This confirms previous field applications of DGT made in open waters (Davison & Zhang, 1994; Zhang & Davison, 1995). Field measurements in marinas under low flow conditions have encountered some problems with diffusive boundary layers and the use of thicker diffusive gel layers has been recommended under these conditions (Webb & Keough, 2002b). The total range of estimates calculated from the Equation 2 were used to provide an estimate of experimental error. This range was occasionally large (*e.g*. 14-d experiment copper dose measurements for the first 2 d), but on the whole the range of values calculated was quite narrow. To estimate the possible range of values in this study, results from four replicate gels of each thickness were pooled prior to calculation of copper concentration *via* Equation 2. The quality of the results confirms the hypothesis of Webb and Keough (2002b) that pooling larger numbers of replicate gels would reduce the error associated with the use of Equation 2.

Settlement Experiments

The 2-d pulse pollution event of $20-30\ \mu g\ l^{-1}$ copper caused major reductions (often more than 50%) in the recruitment of invertebrate taxa from a number of phyla. Conventional LC_{50} toxicity tests expose organisms to a constant concentration of a toxicant in a uniform environment for a period of up to 4 d. Lang *et al*. (1980) using *B. improvisus* nauplii reported a 24 h LC_{50} for Cu of 200 $\mu g\ l^{-1}$ (total copper); a more recent study on *B. amphitrite* nauplii reported a 24 h LC_{50} value of 480 $\mu g\ l^{-1}$ (total copper) (Sasikumar *et al*., 1995). In the present study, a 48-h average concentration of just over 20 $\mu g\ l^{-1}$ (labile copper) was enough to reduce *B. variegatus* recruitment in the field by 70%. The recruitment ''assay'' performed in the present study also had a very different endpoint to an LC_{50} determination, and it is plausible that lower concentrations of copper are required to prevent settlement by cyprids than to kill nauplii. It is also possible that barnacle nauplii and cyprids are differentially sensitive to the effects of copper. This question could only be answered by very brief ecotoxicological investigations, as the cyprid stage of a barnacle's life history is very short. The results of conventional toxicity tests often provide the basis for regulatory decisions, including the setting of water quality guidelines. The discrepancy found here between field and laboratory toxicity test values confirms the need for safety application factors and highlights the value of multiple test endpoints (*e.g*. in this case measuring settlement inhibition as well as the survival of larvae). Studies such as this, that examine different, and more ecologically relevant, endpoints, can only serve to improve the ecological interpretation of dose-response data.

Of the taxonomic groups analysed after 2 d of immersion, only botryllinid ascidians showed no sensitivity to the measured copper pulse. The power to detect an impact on this group was poor and further trials are necessary before its susceptibility can be defined. Didemnid ascidians and newly settled ascidians (too young to identify further) showed variable responses to copper pulses, in terms of statistical significance. It should be noted, however, that the two groups appeared to be reacting to the copper pulse in qualitatively similar ways between the two experiments. For newly settled ascidians, an impact was detected in the 2-d experiment despite there only being a small effect of copper. A larger effect size in the first 2 d of the 14-d experiment was not significant due to the low power of the test. The validity of the non-significant results is therefore questionable. For didemnid ascidians, detection of an impact correlated with high settlement and a large effect size of copper. Where no impact was detected, high power to detect a $\pm$ 50% effect size was available, but a smaller effect size was seen.

The major reductions in recruitment, evident from pulse copper pollution events after 2 d, were no longer detectable at day 7 or 14. Through either mass mortalities, as occurred for *B. variegatus*, or mass recruitment events, as occurred for the serpulid polychaetes, previous impacts were completely obscured. Thus, small-scale copper pollution events lasting 2 to 7 d are likely to cause a disturbance to the early development of an invertebrate assemblage only for the period that there is a high load of toxicants in the system. This disturbance may only be detectable at a fine temporal scale and represents a true ''pulse'' disturbance to the system (Bender *et al.*, 1984). Longer-term field experiments that measure the impact of pulse disturbances on more established assemblages have previously detected press disturbances to community structure when toxicants reduce the densities of adult organisms and dominant space occupiers (Johnston & Webb, 2000; Johnston & Keough, 2002; Johnston *et al.*, 2002).

CONCLUSION

The combined use of a toxicant dosing system and *in situ* toxicant measurements have enabled the examination of the biological impact of the bioavailable fraction of a toxicant under field conditions. It was demonstrated that considerable changes in early assemblage development can be caused by copper concentrations in the range of 20 – 30 μg l^{-1}. The *in situ* measurements also clearly demonstrated that the toxicant was delivered as a pulse, and that natural recruitment and mortality following the pulse can mask the previously seen effects of copper. Importantly, the *in situ* measurements indicated noticeable biological effects at copper concentrations around an order of magnitude lower than LC_{50} estimates, and thus highlight the need for the further development of such tests.

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