



ECOLOGICAL CONSEQUENCES OF COPPER CONTAMINATION IN MACROALGAE: EFFECTS ON EPIFAUNA AND ASSOCIATED HERBIVORES

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Abstract—Many contaminants of the marine environment are able to chelate to sediments, bind within organic matrices, or be accumulated by organisms such as invertebrates and macroalgae. Marine macroalgae are recognized as effective and efficient bioaccumulators of heavy metals and are sometimes used as bioindicators. Macroalgae support abundant and diverse communities of mobile invertebrates that play key roles in temperate marine environments. However, the potential ecological consequences of the contamination of algae on associated epifauna are yet to be considered. In this study, the brown alga *Sargassum linearifolium* was experimentally spiked with copper to assess the effects of contamination on epifaunal invertebrates in both field and laboratory assays. Copper contamination greatly reduced the colonization of a variety of epifaunal taxa in the field. Laboratory assays further examined the effects of contaminated macroalgae on habitat preferences, feeding rates, survivorship, and growth in the herbivorous amphipod *Peramphithoe parmerong*. Adult *P. parmerong* were less likely to select spiked macroalgae in short-term habitat preference assays and consumed spiked algae at lower rates in feeding assays. In a longer-term (30-d) experiment, survivorship of juvenile amphipods was reduced by up to 75% by contaminated macroalgae, but no effects on the growth of survivors was observed. Heavy metal contamination of macroalgae is a widespread phenomenon that has the potential for substantial negative consequences for associated invertebrate fauna. This issue warrants further investigation by marine ecotoxicologists.

Keywords—Macroalgae Copper Epifauna Amphipods Toxicity

INTRODUCTION

Conventional aquatic toxicity testing generally assesses the toxic effects of contaminants in their dissolved state [1]. Many contaminants entering the marine environment, however, have the ability to move into various solid components of an ecosystem. For example, heavy metals may be bound within organic matrices or accumulate in sediments and macroalgae [2,3]. In this way, organisms may be affected by contaminants not only through direct exposure to metals in the dissolved state but also indirectly through the physical environments they inhabit [2] and the food they consume [4]. It is therefore essential for ecotoxicologists to examine multiple pathways by which organisms may be exposed to contaminants in order to fully understand and predict the effects of pollutants in the field. In recent years, an increasing number of toxicity tests has been published that have considered different routes through which organisms may be exposed to contaminants [2,4,5].

A likely route of exposure to metals in temperate regions is through macroalgae that are widely recognized as efficient accumulators of heavy metals such as copper, lead, and zinc [6]. Macroalgae are abundant in temperate marine environments, where they support a wide variety of invertebrates, including mollusks, annelids, and crustaceans [7]. This associated epifauna can make up the bulk of biomass and secondary production on subtidal rocky reef habitats and perform essential trophic functions as links in food webs between primary producers and higher trophic organisms such as fish [7]. As such, it is essential to assess the potential impacts that contamination of macroalgae may have on algal-associated invertebrate communities.

All species of the Phaeophyta (brown algae) contain characteristically high amounts of sulfated polysaccharides within the outer layer of their cell walls, for which heavy metals show a strong affinity [6,8]. The cell walls of the brown algae therefore display a high binding affinity for many heavy metals. While essential in trace amounts in many marine organisms, heavy metals can be highly toxic to invertebrates at high concentrations. They are a common toxicant in urban estuaries where they are introduced through sources such as storm-water runoff, industrial wastes, and marine transportation [9]. The ability of brown macroalgae to concentrate dissolved metals from surrounding waters and their widespread distribution have led to their use as bioindicators of heavy metal pollution [10] and as a mechanism of contaminant reclamation from wastewaters [6]. Consequently, much of the research into heavy metal accumulation by marine macroalgae has focused around these applied issues. However, we are not aware of any studies that have directly examined the ecological effects of this accumulation on marine epifaunal invertebrates.

Amphipods are particularly abundant in aquatic systems, including algal epifaunal communities. Many species of amphipods have been utilized to assess the potential ecological impacts of both dissolved metals [11] and metals associated with marine sediments [11,12]. As a result, the amphipods are among the best-studied marine invertebrates in the field of ecotoxicology. However, the generality of what may be learned through amphipods as model organisms will be inhibited unless all routes of exposure to metals are considered. Many species of amphipods are herbivorous, inhabiting and feeding on macroalgae, sea grasses, and their associated epiphytes [7,13]. These herbivorous organisms are likely to experience metallic contaminants through vastly different pathways than their sediment-dwelling counterparts.

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The current work examines the impacts of spiked copper concentrations in the brown alga *Sargassum linearifolium* on epifaunal invertebrates in both field and laboratory assays. Laboratory assays further examined the impacts of contamination on an abundant and indigenous herbivorous amphipod, *Peramphithoe parmerong*. Specifically we asked four main questions. First, does contamination of the brown alga *S. linearifolium* by copper affect the colonization of epifauna in the field, and does *P. parmerong* prefer uncontaminated macroalgae as habitat over copper-contaminated macroalgae? We also examined whether *P. parmerong* feeds differentially on copper-contaminated and uncontaminated macroalgae and how the survival and growth of *P. parmerong* is affected by long-term residence on contaminated macroalgae. This study is the first, of which we are aware, to examine the ecological impacts of macroalgal contamination by copper on subtidal epifaunal invertebrates.

MATERIALS AND METHODS

Study sites and organisms

Collections of algae, seawater, and invertebrates were taken from a shallow rocky reef (2–4 m deep) located within Congwong Bay, Sydney, New South Wales, Australia (33°59'24"S, 151°14'15"E). The algal community at this site is dominated by several species of brown algae, including dense beds of *Sargassum linearifolium* (Turner) C. Agardh. This brown alga provides habitat for an abundant assemblage of mobile invertebrates, including herbivorous amphipods [14]. The field colonization experiment was conducted on a similar rock platform (3 m deep) at Bare Island (33°59'29"S, 151°13'89"E), which is directly adjacent to the collection site.

The test organism used in laboratory assays was the gammarid amphipod *Peramphithoe parmerong* Poore and Lowry, which constructs nests on the fronds of *S. linearifolium* and feeds on algae within and around the nest [13]. Of the readily available algal hosts in the region, *S. linearifolium* is known to be the host that *P. parmerong* most strongly prefers and the diet on which *P. parmerong* perform best [13]. This amphipod species was therefore deemed to be a suitable test species, as it is locally abundant and readily cultured and its ecology well understood.

Algal spiking procedures

Spiked macroalgae were prepared to test the effects of algal-borne copper on epifaunal invertebrates in the field and on habitat preferences, feeding, growth, and survival of *P. parmerong* in the laboratory. Algae were spiked for these experiments using a 24-h dosing procedure that was designed to mimic a transient pulse of copper, such as those that may be experienced following storm-water runoff events. All spiking vessels referred to in the following sections were soaked in a 5% nitric acid bath for 48 h and rinsed with Milli-Q® water (Billerica, MA, USA). Spiking vessels were also presoaked in the appropriate copper solution for 24 h prior to use. To spike macroalgae, a stock solution of 1,000 mg/L copper in fresh water was created by adding Cu(II)SO₄ anhydrous to Milli-Q water. This stock was stored at 4°C to prevent the reduction of copper ions in solution. When required, spiking concentrations were created by diluting the appropriate amount of stock solution with filtered seawater. A broad range of spiking concentrations were utilized for the field experiment and the habitat preference and feeding assays in order to ascertain the

range of copper burdens in algae that may impact on epifauna. Nominal spiking concentrations for these experiments were an unspiked seawater control (0 µg/L) and 50, 250, and 500 µg/L Cu(II)SO₄. While the speciation of copper in spiking solutions was not measured directly, the complexing capacity of estuarine waters ranges from 1 to 20 µg/L [15]. For the long-term (30-d) growth and survivorship assay, nominal spiking concentrations covered a smaller range of concentrations, as impacts in the initial experiments were identified at relatively low concentrations. Nominal spiking solutions were an unspiked seawater control (0 µg/L) and 25, 50, 100, 200, and 300 µg/L Cu(II)SO₄.

Algal spiking was conducted at a constant temperature of 20°C for 24 h. For the field experiment, individual algae (~10 g) clear of visible epiphytes were placed in aerated 6-L plastic aquaria (two individuals per aquaria) at the appropriate spiking concentration. For the laboratory assays, 1 g of new growth (close to apical meristem) without visible epiphytes was taken from a larger thallus and divided into seven evenly sized fragments. Each gram of algal material was then placed into 500 ml of seawater at the appropriate copper concentrations (two separate containers per concentration). In this way, 2 g (14 fragments) of algae were spiked at each concentration each time. After 24 h, algal material was removed from spiking solutions and rinsed briefly in clean seawater to remove any superficial spiking solution. Spiked algae were then rinsed briefly for a second time and added to laboratory assays or returned to the field. Following initial laboratory studies, it was observed that copper was leaching from algae spiked at the highest concentration (500 µg/L). As a result, spiking solutions were reduced, and spiked fragments were left to soak in clean seawater for 2 h before being added to subsequent assays (survivorship and growth assay).

Algal heavy metal analysis

Two complete algal individuals spiked for the field experiment and two composite samples of algal fragments spiked for the laboratory assays were reserved for heavy metal analysis from each spiking concentration. Following spiking, these algae were rinsed twice briefly in Milli-Q water to remove excess salts and spiking solutions from the surface of the alga. Samples were then placed in clean plastic zip-lock bags and stored at -4°C.

When required, samples were dried at 50°C for 12 h and then crushed into a fine powder using a metal-free mechanical homogenizer. Subsamples of 0.4 to 0.6 g of algal powder were weighed on a fine balance and added to 5 ml of distilled HNO₃, 2 ml of H₂O₂, and 3 ml of Milli-Q water in digestion vessels and microwave digested at 190°C for 20 min. After digestion, samples were made up to 30 ml using Milli-Q water and analyzed by way of inductively coupled plasma-atomic emission spectrometry (ICP-AES) at the Analytical Elemental Analysis Laboratory at the University of New South Wales, Sydney, Australia. Pasture of known copper concentrations was analyzed as a standard reference material to confirm accuracy of the ICP-AES procedure. The reference material was obtained from the Australian Soil and Plant Analysis Council (ASPAC), Werribee, Victoria, Australia (pasture; ASPAC reference no. 80). Acid blanks were also analyzed to assess the potential for sample contamination via unclean reagents.

Field colonization of copper-spiked macroalgae

A field experiment was conducted in November 2004 to assess the effect of varying copper concentrations in algal

tissues on the rates of colonization by epifaunal invertebrates. Field-collected algae were defaunated with a brief freshwater rinse (Milli-Q) and spiked with copper in the laboratory as described previously (20 replicate alga per copper concentration) and returned to the field immediately after spiking. Each alga was attached to a masonry nail by a plastic cable tie and nailed to the sandstone substrate at a haphazardly chosen position within a contiguous algal bed. Each nail was marked with fluorescent flagging tape to aid in recovery. After a period of 48 h, divers retrieved the algae, enclosing each sample in a watertight 1-L container (75 samples retrieved, out of 80). Samples were preserved in 5% formaldehyde solution.

Prior to sorting, each sample was passed through a 300- μ m sieve and rinsed in fresh water to remove formaldehyde. Samples were then sorted to major taxonomic groups (gammarid and caprellid amphipods, gastropods, copepods, polychaetes, and ostracods) using a dissecting microscope. Gammarid amphipods were further sorted to measure the response of *P. parmerong* to copper concentrations within algal tissues in the field. Invertebrate counts were converted to densities (number of animals per gram wet weight algae). Only taxonomic groups displaying abundance >0.05 organisms/g algae were formally analyzed (gammarid amphipods, copepods, gastropods, ostracods, and polychaetes).

Habitat preference assay

A preference assay tested the hypothesis that *P. parmerong* displays preference for uncontaminated algae. Adult amphipods were collected from field macroalgae and maintained in a flow-through seawater system (18°C, 35 ppt) until required. Large aerated aquaria (30 \times 15 \times 12 cm) were divided into 18 assay wells by clear Perspex® walls with mesh windows to allow for water flow. Aquaria were filled with 8 L of filtered seawater at a pH of 8.0; dissolved oxygen remained above 80% for the duration of the experiment.

The assays were run as a choice of two algal pieces, with three separate assays (control algae vs algae spiked at 50 μ g/L, control vs 250 μ g/L, and control vs 500 μ g/L). Each replicate of a treatment consisted of a single amphipod offered the choice of one fragment of algae from one of the three experimental spiking concentrations (50, 250, or 500 μ g/L) and a fragment of unspiked control algae. Algal fragments weighing 14 mg were added to wells and held in place by transparent plastic rings, with random orientation of spiked and control fragments to avoid potential bias associated with instinctual amphipod movements. An individual, haphazardly selected amphipod was placed in the center of the ring and allowed to investigate algal fragments. After 12 h, the fragment on which a nest was constructed by the amphipod was recorded. The few individuals that died during the experimental period ($<5\%$) were removed, and the replicate was run again with a new individual. Thirty replicates of each preference assay were used, and no amphipods were used in more than one trial.

Feeding assay

A no-choice feeding experiment contrasted the feeding rates of *P. parmerong* on contaminated and uncontaminated macroalgae. No-choice feeding assays are suitable for this species because once *P. parmerong* have created a nest on a given alga, individuals rarely disperse from that nest over the time scales of a feeding assay [13].

Each amphipod was placed within assay wells as described previously and given a fragment of algae from one of the three

experimental spiking concentrations or an unspiked control. Identical treatments were established without the presence of amphipods to control for mass changes in algae not associated with herbivory ($n = 12$ replicates per treatment). Algal fragments were blotted dry after spiking and weighed on a balance, with 14-mg fragments being selected for the assay. After 48 h, the silken nest material was removed from algal fragments, and the algae were blotted dry and reweighed to record mass loss (in mg).

Juvenile growth and survivorship assay

Thirty-day exposures were initiated with gravid amphipods to assess the effects of algal-borne copper on growth and survivorship of juvenile amphipods raised entirely on contaminated macroalgae. Brooding female amphipods were taken from field-collected algae (gravid individuals are easily identified by the presence of a conspicuous white mass within the brood pouch) and placed in separate 120-ml containers with filtered seawater. Females were provided with a small, unspiked fragment of algae for 48 h in order to acclimatize to test conditions. After 48 h, clean algae were removed, and females were haphazardly given algae from one of the experimental spiking concentrations ($n = 10$ per concentration). From this point on, replicates were fed a control or spiked 14-mg fragment of algae three times each week, with no removal of uneaten algae. The few females that died during the acclimation period were removed from the experiment (8–10 replicate families remained per level of the concentration treatment). At no time was food a limiting factor in any of the experimental treatments. Food was supplied in abundance and was never consumed fully prior to the addition of the next meal.

Water temperature (20°C), salinity, dissolved oxygen (80–85%), and pH (7.80–8.40) were monitored daily using an YSI 556 MPS® (Yellow Springs, OH, USA) water quality meter and remained constant throughout the experiment. Feces were removed from jars every 48 h using a pipette at the same time a 50% water change was performed. Water samples were collected from two randomly selected jars at the end of the experiment (day 30) to assess whether copper leached from spiked algae. These samples were analyzed at the Australian Government National Measurement Institute in Sydney, Australia, using ICP-AES.

Brooding *P. parmerong* release their offspring from the brood pouch over a few days, with the last juveniles ejected at the time of the females molt [13]. In order to minimize disturbance to the nest, daily counts of juveniles were not performed; rather, experimental jars were monitored daily for the presence of the females' molt, and the day on which the molt occurred was designated as day 0. Amphipods were left to feed on the respective diets for a period of 30 d from this starting date. A period of 30 d is sufficient time for *P. parmerong* to reach maturation [13].

After 14 d from the starting date, females were encouraged to evacuate the nest and were removed from the experiment and photographed using a microscope mounted camera. After 30 d, the number of surviving juveniles was recorded, and juveniles were photographed and measured. Total length measurements were taken from the base of the antennae to the end of the telson using ImageJ® image analysis software (National Institutes of Health, Bethesda, MD, USA).

Table 1. Nominal copper spiking solutions and subsequent copper concentrations within algal tissues for field and laboratory experiments (mean \pm standard error)

Nominal spiking concn.	Copper concn. in algal tissue		
	Field colonization experiment	Habitat preference and feeding assays	Survivorship and growth assay
Control	4.02 \pm 0.05	16.94 \pm 0.66	5.12 \pm 0.59
25	NA	NA	39.97 \pm 0.27
50	27.89 \pm 4.17	60.18 \pm 3.92	61.00 \pm 1.65
100	NA	NA	102.81 \pm 0.53
200	NA	NA	171.99 \pm 8.65
250	98.90 \pm 9.12	250.38 \pm 5.84	NA
300	NA	NA	224.55 \pm 0.85
500	154.42 \pm 26.95	422.51 \pm 18.80	NA

^a NA = concentrations were not used in a given experiment. Spiking concentrations expressed in $\mu\text{g/L}$, and algal tissue concentrations expressed in $\mu\text{g/g}$ (\pm standard error).

Data analyses

The abundance of each dominant epifaunal taxa identified in the field recolonization experiment was analyzed using a single-factor analysis of variance (ANOVA) with spiking concentration treated as a fixed factor. Planned comparisons tested for the main effect of copper concentration over the residual error from the full ANOVA model [16]. Data from the habitat preference assay were tested for a nonrandom distribution of nest construction across algal treatments (spiking concentration) by way of one-way contingency analyses for each of the three assay treatments. Mass loss recorded in the feeding assay was analyzed using a two-factor ANOVA with amphipod treatment (present or absent) and spiking concentration as fixed factors. A significant interaction between the two factors indicates an effect of spiking concentration on feeding rates [17]. For this experiment, planned comparisons tested for the main effect of spiking concentration on data from the amphipod treatments only. Main effects were tested using the residual error from the full ANOVA model [16].

Survivorship in the 30-d experiment was analyzed as a single-factor ANOVA contrasting the number of individuals surviving to 30 d across levels of the concentration treatment (fixed factor). Growth was analyzed as a nested ANOVA with concentration as a fixed factor and female (brood of offspring) as a random factor nested within concentration. Planned comparisons for these data tested for the main effect of copper concentration over the residual error from the full ANOVA model [16].

Analysis of variance and subsequent planned comparisons for all experiments were conducted using SYSTAT Version 10 (SPSS, Point Richmond, CA, USA) and Microsoft® Office Excel 2003 (Redmond, WA, USA), respectively. Contingency tables for the habitat preference assay were analyzed by way of chi-square analysis with separate contingency tables for each treatment using Microsoft Office Excel 2003. The assumptions of normality and heterogeneity of variance were tested for each variable by examining residual histograms and scatter plots of estimates versus residuals, respectively [16]. When necessary, data were log transformed to satisfy the assumptions of ANOVA.

RESULTS

Copper contents of spiked macroalgae

Algal spiking successfully increased copper concentrations in macroalgal tissues for use in both field and laboratory as-

says. Copper concentrations in algal tissues increased predictably with the concentration of the solution in which algae were soaked (Table 1). Assimilation of copper in algae spiked for the field colonization experiment appears to have been less efficient than algae spiked for laboratory assays. Only algal fronds were spiked for laboratory assays, while complete algae thalli (stipe, fronds, and holdfast) were spiked for field experiments. Holdfast and stipe tissues often have a lower affinity for copper than growing fronds [18]. This would have the effect of diluting copper concentrations when all tissues are analyzed together and explains the apparently reduced efficiency of accumulation for algae spiked for the field experiment.

Field colonization of copper-spiked macroalgae

Algal spiking strongly reduced the abundance of colonizing invertebrates. All taxonomic groups, with the exception of the polychaete worms, colonized unspiked algae to greater densities than at least one of the spiking treatments (Fig. 1 and Table 2). The total abundance of colonizers and the abundance of copepods, gammarids, and the amphipod *P. parmerong* were reduced by copper spiking regardless of treatment concentration (Figs. 1a to c and 2g, respectively, and Table 2). Gastropods and ostracods colonized algae spiked at 155 $\mu\text{g/g}$ to significantly lower densities than controls, while their colonization to algae spiked at 28 and 99 $\mu\text{g/g}$ did not differ significantly from controls (Fig. 1d and e and Table 2). Polychaetes demonstrated no response to algal spiking (Fig. 1f and Table 2).

Habitat preference assay

Peramphithoe parmerong preferred algae with low tissue copper concentrations as habitat. In all cases, *P. parmerong* nested more frequently on algae that had not been spiked with copper (Fig. 2). Nest construction across the algal spiking treatments differed significantly from a random distribution when the alternative to unspiked algae was algae spiked at 60 ($\chi^2 = 4.80$, $p = 0.028$; Fig. 2) and 250 $\mu\text{g/g}$ ($\chi^2 = 10.80$, $p = 0.001$; Fig. 2). The distribution of nesting of individuals in the assays containing algae spiked at 423 $\mu\text{g/g}$ did not differ significantly from a random distribution ($\chi^2 = 1.20$, $p = 0.273$; Fig. 2). Water samples taken from within experimental wells holding algae spiked at 423 $\mu\text{g/g}$ showed that up to 20 $\mu\text{g/L}$ copper were present in the water column, while control wells contained less than 5 $\mu\text{g/L}$ copper in the water column. This contamination of water at high spiking concentrations was prevented in further studies (growth and survival assays) by

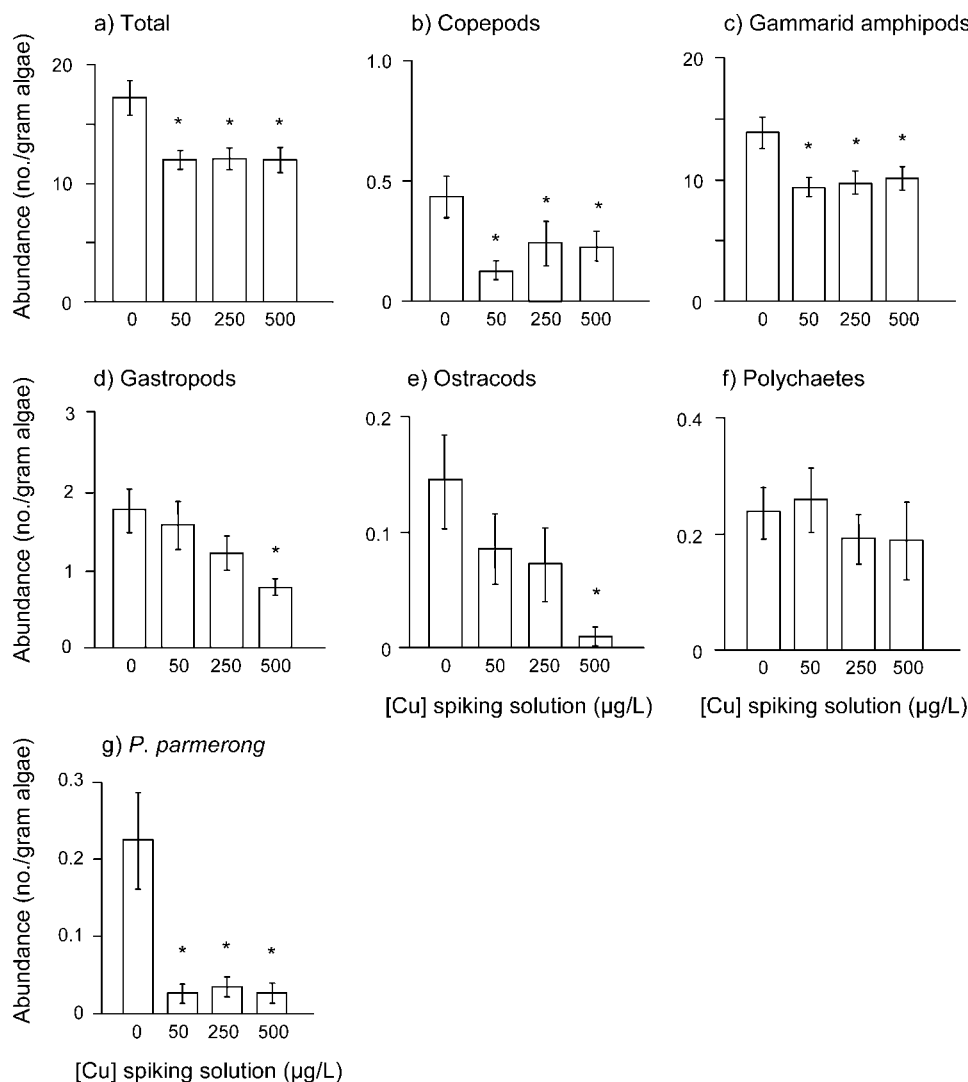


Fig. 1. Abundance of (a) total colonists, (b) copepods, (c) gammarid amphipods, (d) gastropods, (e) ostracods, (f) polychaetes, and (g) *Peramphithoe parmerong*. The data are mean abundance (\pm standard error, $n = 17$ – 20 per treatment) of individual taxonomic groups on dosed and undosed algae. Bars that are marked with * differ significantly from controls according to planned comparisons.

both reducing algal spiking concentrations and a 2-h algal depuration period in clean seawater prior to addition to experimental replicates.

Feeding assay

The copper content of macroalgae reduced the feeding rate of adult *P. parmerong*. Feeding rates were greatest on unspiked algae with reduced feeding on all spiked algae regardless of copper spiking concentration (“concentration \times amphipod present/absent” interaction $F_{3,88} = 4.21$, $p = 0.009$). Amphipods fed unspiked algae consumed approximately 4.5 mg of algae during the assay period. Amphipods fed algae spiked with copper consumed from 3 to 3.5 mg of algae in all spiking treatments (planned comparisons “control vs 60” $F_{1,88} = 17.91$, $p < 0.001$; “control vs 250” $F_{1,88} = 17.09$, $p < 0.001$; “control vs 423” $F_{1,88} = 6.65$, $p = 0.012$). Mass loss of algal tissue in the absence of herbivores was negligible (~ 0.5 mg in each treatment) and did not differ between levels of the spiking treatment ($F_{3,44} = 0.049$, $p = 0.985$). Therefore, mass loss in amphipod treatments resulted from herbivory alone.

Survivorship and growth assay

Algal-bound copper significantly reduced the number of *P. parmerong* surviving at 30 d, with mortality increasing along with copper concentrations in algal tissues (Fig. 3a; “concentration” $F_{5,49} = 4.37$, $p = 0.002$). The number of juveniles surviving per family was greater when fed on control algae than algae spiked at 103, 172, and 225 $\mu\text{g/g}$ (Fig. 3a; planned comparisons “control vs 103” $F_{1,49} = 5.75$, $p = 0.020$; “control vs 172” $F_{1,49} = 4.54$, $p = 0.038$; “control vs 225” $F_{1,49} = 16.50$, $p < 0.001$). No differences were observed between the number of juveniles surviving per family in controls and algae spiked at 40 and 61 $\mu\text{g/g}$ (Fig. 3a; planned comparisons “control vs 40” $F_{1,49} = 1.14$, $p = 0.291$; “control vs 61” $F_{1,49} = 0.54$, $p = 0.466$). No effects of algal copper content were observed on growth of *P. parmerong* at any of the spiking concentrations (Fig. 3b; concentration $F_{5,46} = 0.490$, $p = 0.776$). Measurements of female size indicated that the size of gravid amphipods initially assigned to replicates did not differ among levels of the concentration treatment (concentration $F_{5,49} = 0.40$, $p = 0.850$). Water samples taken from randomly selected jars containing spiked algae showed no leaching of

Table 2. Analyses of variance and planned comparisons for all taxa and abundant epifauna colonizing treated algae in the field experiment showing degrees of freedom (*df*), mean squares (MS), and *F* ratios (*F*). Spiking concentrations were an unspiked control and 50, 250, and 500 $\mu\text{g/L}$ (4, 28, 99, and 154 $\mu\text{g/g}$ copper in algae, respectively)

<i>Entire model</i>		Total ^a		Copepods ^a		Gammarids		Gastropods ^a	
		<i>df</i>	MS	<i>F</i>	MS	<i>F</i>	MS	<i>F</i>	MS
Concentration	3	0.576	5.313 ^b	2.596	4.511 ^b	77.067	4.572 ^b	1.580	4.127 ^b
Error	71	0.108		0.575		16.855		0.383	
		Ostracods ^a		Polychaetes ^a		<i>Peramphithoe parmerong</i>			
		MS	<i>F</i>	MS	<i>F</i>	MS	<i>F</i>		
Concentration	3	1.281	4.255 ^b	0.533	1.031	2.199	9.683 ^c		
Error	71	0.301		0.517		0.227			
<i>Planned comparisons</i>		<i>df</i>	Contrast	Total ^a		Copepods ^a		Gammarids	
				MS	<i>F</i>	MS	<i>F</i>	MS	<i>F</i>
Concentration	1	Control vs 28	1.053	9.750 ^b	6.959	12.103 ^c	182.647	10.836 ^b	
	1	Control vs 99	1.129	10.454 ^b	4.666	8.115 ^b	153.693	9.119 ^b	
	1	Control vs 154	1.350	12.500 ^c	3.116	5.419 ^d	134.074	7.955 ^b	
Error	71		0.108		0.575		16.855		
		Contrast	Gastropods ^a		Ostracods ^a		<i>Peramphithoe parmerong</i>		
			MS	<i>F</i>	MS	<i>F</i>	MS	<i>F</i>	
Concentration	1	Control vs 28	0.237	0.713	0.601	1.997	4.741	20.885 ^c	
	1	Control vs 99	0.948	2.475	0.992	3.296	3.947	17.389 ^c	
	1	Control vs 154	4.262	11.128 ^b	3.745	12.442 ^c	4.840	21.322 ^c	
Error	71		0.383		0.301		0.227		

^a Log transformed.

^b $0.01 > p > 0.001$.

^c $p < 0.001$.

^d $0.05 > p > 0.01$.

copper from algal tissues in any spiking treatments (mean concentrations of copper in treatments ranged from <5 to $7.5 \mu\text{g/L}$).

DISCUSSION

While macroalgae are known to readily accumulate heavy metals from solution, the ecological consequences of this for

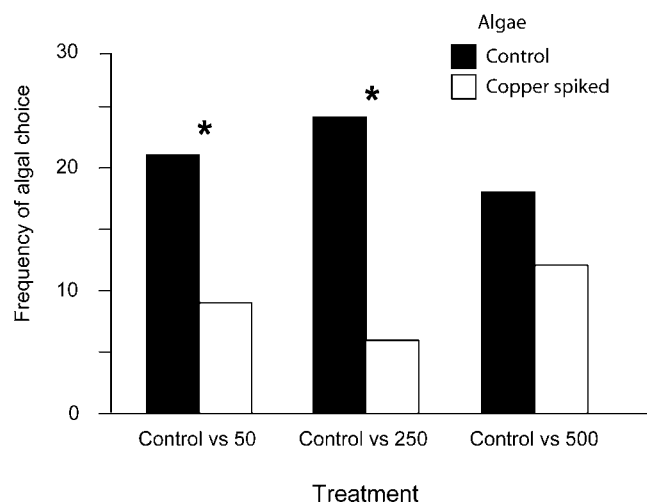


Fig. 2. Preferences displayed by *Peramphithoe parmerong* for control or spiked macroalgae. Data are frequencies of choice ($n = 30$ replicates per choice treatment). Treatments marked with * show a distribution of habitat preferences that differs significantly from the random.

invertebrates that inhabit algae and consume their tissues have not been considered. The current work highlights the need to further consider this pathway of exposure to heavy metals. More generally, these results add to gathering evidence that the negative impacts of heavy metal contaminants may be realized indirectly through habitat and food [2,4]. Waterborne contaminants are often introduced through pulse pollution events and may fluctuate in concentration in the water column through time [19]. The accumulation of heavy metals within marine macroalgae may represent a more persistent pressure on epifaunal invertebrate communities. Algae from highly contaminated regions have been found to have copper burdens of greater than $300 \mu\text{g/g}$ [20,21], which is comparable to the concentrations achieved in the highest spiking treatment in these experiments ($500 \mu\text{g/L}$). Therefore, algal contamination must be considered a potentially important toxicant exposure pathway for epifaunal invertebrates, and the impacts identified here may be indicative of impacts on field populations.

Effects of algal contamination

Previous research has found low abundances of epifauna on contaminated macroalgae close to pollution sources [22]; however, this is the first study known to us that has directly related epifaunal colonization to experimentally enhanced metal concentrations in algal tissues. In the present work, uncontaminated macroalgae were colonized by significantly greater abundances of almost all the common epifaunal groups. Highly mobile taxa such as copepods and gammarid amphipods avoided spiked macroalgae regardless of the actual dose; however, slower-moving gastropods demonstrated a dose-response re-

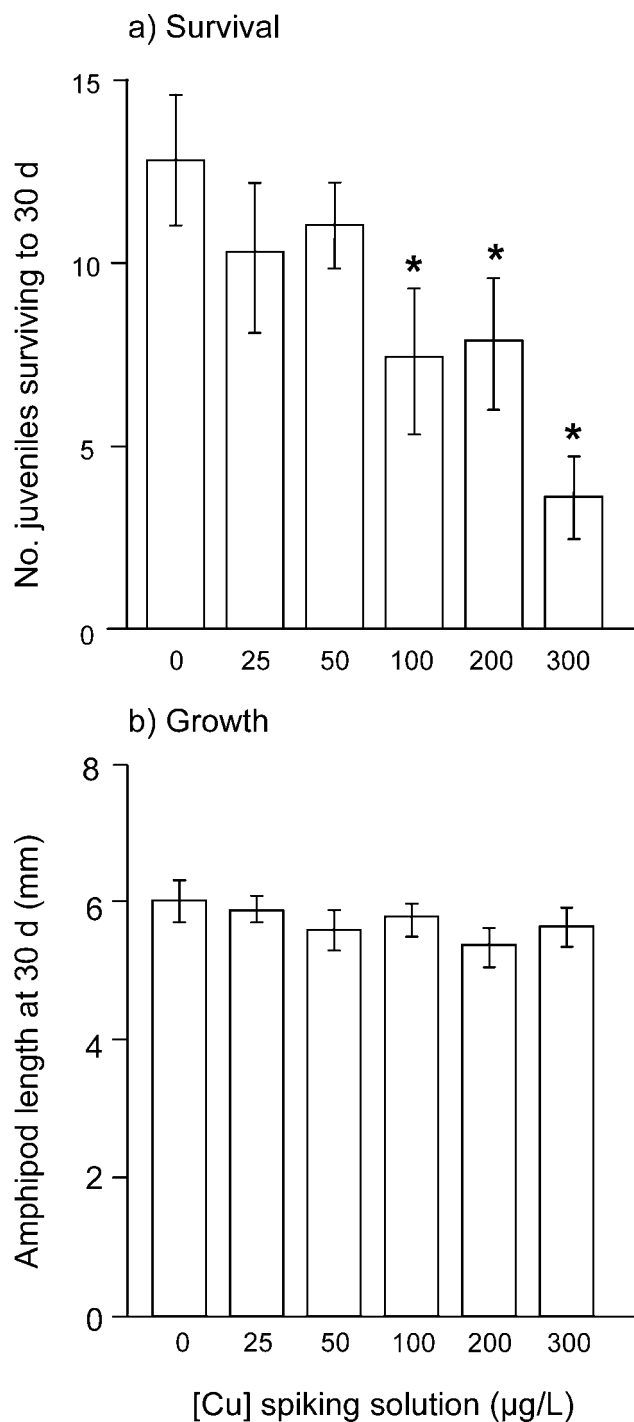


Fig. 3. (a) Survivorship and (b) growth of *Peramphithoe parmerong* raised on spiked algal diets for 30 d. Survivorship data are mean number of individuals surviving from a single brood (counts \pm standard error, $n = 8-10$ per treatment). Growth data are mean size of amphipods at 30 d (mm \pm among family standard error, $n = 8-10$ per treatment). Survivorship in treatments marked with * differed significantly from survivorship of controls according to planned comparisons.

lationship. This result suggests that the relative dispersal abilities of the organisms under consideration may play an important role in mediating their responses to surface-bound contaminants. It has been shown that turnover rates of gastropods on algal habitats are substantially lower than for amphipods [23]. For these relatively slow-moving organisms, the ener-

getic costs and risk of predation involved in searching for new habitat may outweigh the negative effects of remaining on contaminated algae. It may be that it is only at high concentrations that the negative effects of remaining on contaminated macroalgae outweigh the negative effects of moving between habitat patches. Alternatively, rapidly dispersing taxa such as amphipods may attempt to avoid contaminated algae altogether, as costs and risks of rapid dispersal are comparatively low. Generally, however, the results of the field experiment demonstrate that even a relatively low concentration of copper in macroalgal tissues (28 $\mu\text{g/g}$) is likely to have widespread impacts on the colonization of many different groups of epifaunal invertebrates.

The only group analyzed that was unaffected by copper contamination was the polychaete worms. Evidence suggests that the polychaete worms are among the more tolerant of invertebrate taxa to heavy metals [24,25]. Polychaetes have been shown to develop resistance to sediments contaminated with heavy metals [24]. Polychaetes in sessile invertebrate assemblages are also more tolerant of copper pulses than many co-occurring species, with chemically disturbed assemblages being characteristically dominated by serpulids [25].

The herbivorous amphipod *P. parmerong* displayed reduced colonization to contaminated macroalgae and was used in laboratory assays to further examine the mechanisms that may explain these patterns. In laboratory assays, *P. parmerong* nested preferentially on unspiked algae. Avoidance of contaminants accumulated in environmental components is a widespread phenomenon. Reduced burrowing in the infaunal amphipods *Corophium volutator* [26], *Rhepoxynius abronius*, and *Eohaustorius sencillus* has been noted in contaminated sediments [27], while the amphipod *Heterophoxus videns* prefers uncontaminated sediments [28]. Behavioral avoidance of habitat-bound contaminants are likely to enforce inefficient and prolonged searching and foraging patterns on organisms in the field and may impact indirectly on survivorship (through increased risk of predation).

The second behavioral impact identified was that of reduced feeding by *P. parmerong* when offered spiked macroalgae. Such a reduction in feeding rate has the potential to result in negative impacts on amphipod fitness. The results presented here show that reductions in feeding on algae may be induced at concentrations as low as 60 $\mu\text{g/g}$ copper, which is a relatively low concentration when compared to the concentrations at which diets are avoided in some species of semiterrestrial and terrestrial crustaceans. The talitrid amphipods *Orchestia gammarellus* and *Orchestia mediterranea* demonstrate significantly reduced grazing on copper-enriched diets of stranded kelp at a threshold concentration of 688 $\mu\text{g/g}$ copper [29], while the terrestrial isopod *Porcellio scaber* shows reduced feeding on maple leaves spiked at 1,200 $\mu\text{g/g}$ [30]. Comparisons between semiterrestrial, terrestrial and the subtidal amphipods such as *P. parmerong* should be made cautiously. For instance, terrestrial crustaceans are much more reliant on their diets for their essential complement of copper than marine crustaceans [29] and may therefore be expected to respond differently to copper spiking treatments.

The relative palatability of a potential host algal species plays an important role in habitat preferences displayed by *P. parmerong* [13]. In the present work, reduced amphipod feeding on spiked algal diets matched the lower tendency for amphipods to inhabit that alga in our habitat preference assay. For organisms that both feed on and inhabit macroalgae, adult

habitat preferences may also be closely linked to juvenile performance on that same alga, and negative consequences often exist for an organism that remains on poor-quality hosts [13].

The results of the 30-d exposure show that algal-borne copper can significantly reduce the number of juvenile amphipods that survive to maturity. Survival of amphipods at a spiking concentration of 300 $\mu\text{g/L}$ (a copper burden in algal tissues of 225 $\mu\text{g/g}$) was approximately 25% of that in controls. The lowest spiking concentration at which survivorship was affected was 100 $\mu\text{g/L}$ (103 $\mu\text{g/g}$ copper in algae), where survivorship was approximately 60% of that in controls. Somewhat surprisingly, while survivorship was markedly affected by copper spiking, the sublethal measure of growth was unaffected at any of the spiking levels. It is well documented that juvenile amphipods are generally more sensitive to heavy metals than adults of the same species [12]. For example, the concentration of cadmium lethal to 50% (LC50) of juvenile *Leptocheirus plumulosus* (an estuarine amphipod) in 96-h exposures is 360 $\mu\text{g/L}$, while adults of the same species have an LC50 of 880 $\mu\text{g/L}$ [31]. In the present study, the opportunity exists for strong selection by copper-rich diets for those more resistant individuals at the juvenile stage. Tolerant individuals would then be expected to perform relatively well in the later stages of the 30-d exposure. A second explanation is that detoxification of accumulated metals in surviving amphipods prevented toxic effects manifesting (reduced growth) [32].

While the field experiment and habitat and feeding assays in combination have established that amphipods are able to detect and avoid heavy metals in macroalgae, in a more realistic field setting it may not be possible for organisms to disperse the distances required to escape metal contamination. In regions with a long history of chemical pollution, algal contamination may occur across spatial scales great enough to force dispersing epifauna onto habitats and diets they may otherwise avoid. It is therefore of concern in the current work that the survivorship of juveniles raised on contaminated diets was significantly reduced by copper concentrations that may be expected in the field. Field surveys of algal contamination across both small (within patch) and large (e.g., between patches/bays) spatial scales are required to assess the degree to which field populations are likely to be affected by macroalgal contamination and how they may respond in field settings.

One surprising result of the laboratory assays was that algae spiked at the highest concentration (423 $\mu\text{g/g}$) appeared to be relatively well tolerated in the habitat preference assay despite reduced colonization to those algae in the field and reduced feeding on that algal spiking treatment. Water samples taken from jars containing algal fragments spiked at 423 $\mu\text{g/g}$ returned waterborne copper concentrations of approximately 20 $\mu\text{g/L}$ copper, while water in containers holding control algae returned waterborne concentrations of <5 $\mu\text{g/L}$ copper. Some leaching of copper is therefore occurring from algae spiked in very high spiking solutions after the algal fragment is removed from solutions and placed in clean seawater. The lowest observable effect concentration for mortality in *P. parmerong* after 24-h exposures to aqueous copper is 100 $\mu\text{g/L}$ (B. Cumbo, University of New South Wales, Sydney, Australia, personal communication). While the leached concentrations of copper are not sufficiently high to result in mortality, they may have been sufficient to affect amphipod behavior, resulting in poor habitat selection in these assays. Retention of accumulated metals has been shown to be high for a range of metals in the brown alga *Laminaria digitata* [33], while retention of metals

in the green alga *Ulva lactuca* can be relatively poor [34]. If spiked macroalgae are to be used for toxicity testing, a sufficient period of depuration must be allowed before spiked tissues are used in experiments. In the current study, a period of 2 h appears to have been sufficient; however, further research is required to assess the rates at which metals are released by various algal species and the effect this has on test organisms.

Implications for ecotoxicological testing

The occurrence of contaminated macroalgae in urbanized and industrialized regions [20,22] indicates a potentially common route of metal exposure and one that warrants further consideration by ecotoxicologists. Despite the findings of laboratory-based tests that suggest that heavy metals are a toxicant of primary concern, few studies have found direct effects of heavy metals in the field, except under extreme circumstances [2]. If researchers are aiming for an ecologically relevant toxicological test, then contaminants must be presented in forms and at concentrations that are likely to be encountered in the field. Because of the process of bioaccumulation, marine organisms are likely to be exposed to accumulated contaminants (through numerous pathways) at much higher concentrations than metals in the dissolved state.

However, some aspects of utilizing spiked macroalgae in toxicological testing should be considered. While we are arguing that the results obtained here are a result of accumulated metals within algal tissues, an alternative explanation may be that the copper soaking technique damaged the algal material and that organisms are responding to damage to algal diets. Marine algae are relatively insensitive to high concentrations of heavy metals in the water column [35]. Furthermore, previous studies have found algae of the genus *Sargassum* to be among the most resistant of algae to heavy metals as a result of the detoxifying effects of binding metals within cell wall polysaccharides [36]. Toxic effects of metals on sublethal measures generally may take a week to manifest in macroalgae [36], and the effects of 24-h exposures to metallic ions on macroalgae are unclear. No significant leaching of phlorotannins or algal decay was observed in any of the spiking treatments, and algal fragments remained viable for the duration of the laboratory assays and field experiment. We are therefore confident in concluding that impacts identified were a result of metal accumulation in algae rather than damage to the algae.

The results presented here demonstrate the need for careful selection of endpoints when conducting ecotoxicological testing, with close consideration of the test organism. In the current study, amphipod behavior and survivorship were affected by copper, yet no significant impact on growth was observed. Amphipods store excess copper accumulated from their diets in granules (rendering them nontoxic) that are then gradually excreted [8]. Therefore, if the test organism is capable of detoxifying unrequired metals at a rate that matches the intake of excess metals, then it is possible for substantial accumulation to occur without the onset of toxicity [8]. Consequently, if one does not know the specific rate at which the test species detoxifies the test toxicant, then it is essential that endpoints are considered that do not require the accumulation of toxic amounts of heavy metals to eventuate.

It is unclear how behavioral measures such as habitat preferences, feeding rates, and field colonization are affected by the accumulation of metabolically available metals within amphipods. These behavioral impacts have the potential to occur

in an organism that demonstrates no adverse physical response to a toxicant, and by not considering these measures, one may greatly overestimate the point at which a contaminant becomes toxic. Furthermore, these behavioral impacts are more likely to be realized under field conditions where exposure to contaminants may be common, but exposure to levels of contaminants capable of producing physical responses may be relatively rare. It is therefore essential that endpoints be carefully selected [37] or, alternatively, that a variety of endpoints are measured simultaneously [38].

Finally, many ecotoxicologists promote the use of field-based toxicant exposure experiments [39]. The main argument for this is to enhance the realism of a toxicity test by allowing for the known and unknown ecological, biological, and chemical processes that operate within a natural system to occur unobstructed [40]. These field tests also often allow for the simultaneous assessment of toxicant effects on a range of organisms with different life histories, behavioral patterns, and biology and often result in surprising results best tested in the laboratory. Field experimentation may not always be possible, but, where it is, laboratory and field experimentation can be viewed as complementary rather than alternative approaches in the field of ecotoxicology [39].

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