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Differential tolerance to metals among populations of the introduced bryozoan *Bugula neritina*

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Abstract Resistance to heavy metals is a potentially important trait for introduced marine organisms, facilitating their successful invasion into disturbed natural communities. We conducted laboratory and field experiments to examine differential resistance to copper (Cu) between two source populations of the introduced bryozoan *Bugula neritina*, originating from a polluted (Port Kembla Harbour, NSW, Australia) and an unpolluted (Botany Bay, NSW, Australia) environment. A laboratory toxicity test was conducted to test the relative resistance of *B. neritina* recruits from the two sources, by measuring the attachment success, survival and growth of individuals exposed to a range of Cu concentrations (0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu). Upon completion, reciprocal transplantation of the colonies to the original polluted and unpolluted locations was carried out to assess ongoing survival and growth of colonies in the field. *B. neritina* colonies originating from the polluted Port Kembla Harbour had increased resistance to Cu relative to populations from an unpolluted part of Botany Bay. There appeared to be a cost associated with increased metal tolerance. In the laboratory, Botany Bay recruits displayed significantly higher growth in control treatments and significantly poorer growth at 100 $\mu\text{g l}^{-1}$ Cu with respect to Port Kembla Harbour individuals, which showed unusually uniform and low growth irrespective of Cu concentration. No difference in attachment success or post-metamorphic survival was observed between populations. Field transplantation showed copper resistance in Port Kembla Harbour colonies constituted an advantage in polluted but not benign environments. The findings of this study provide

evidence of the benefits to invasive species of pollution tolerance and suggest that human disturbance can facilitate the establishment and spread of invasive species in marine systems.

Introduction

The distribution and abundance of organisms within many biological systems are disrupted by a range of human activities. Two of the most common disturbances affecting near shore marine ecosystems are the release of pollutants (Rygg 1985; Luoma and Phillips 1988; Cohen and Carlton 1998; Preston and Shackelford 2002), and the introduction of invasive marine pests (Carlton and Geller 1993; Cohen and Carlton 1998; Hayes et al. 2004). These two disturbances have the potential to interact and it is currently unclear whether the increased occurrence of invasive species in bays and estuaries is simply a reflection of increased rates of inoculation, or whether greater numbers of invasive organisms are taking advantage of chemically disturbed environments.

Links between the invasibility of a system and the level of disturbance experienced by that system have long been recognised (Elton 1958; Fox and Fox 1986). Both natural and anthropogenic disturbances affect biodiversity and resource availability in natural systems (Fox and Fox 1986; Ruiz et al. 1997; Levine and D'Antonio 1999), potentially increasing a communities susceptibility to invasion. Estuaries and harbours comprise some of the most disturbed marine environments, with metal pollution often a major contributor in the form of industrial waste (Apte and Day 1998; Hall et al. 1998), urban runoff (Pitt 1995), sewage discharge (Scanes 1996), biocides and wood preservatives (Weis and Weis 1992, 1996). Within such disturbed environments, greater resistance to heavy metals could be an extremely important characteristic for invading species, aiding their establishment and spread within less tolerant natural communities.

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The first stage of any invasion is the translocation of organisms outside their native range (Ruiz et al. 2000). It is at this stage in the invasion process that selection for pollution tolerance may first take place in marine systems. The attachment of invertebrates and algae on the hulls of commercial and recreational vessels (hull fouling) is recognised as an important mechanism in the transfer of introduced marine species (Ruiz et al. 2000; Minchin and Gollasch 2003; Floerl et al. 2004; Hewitt et al. 2004). Heavy metals have long been the primary biocides applied to the hulls of recreational and commercial vessels in order to prevent the establishment of fouling organisms. In the past three decades tributyl tin-based (TBT) coatings were most prevalent, though copper- (Cu) and zinc-based (Zn) coatings are again becoming increasingly common as a result of revised international regulations (I.M.O. 2001). Toxicants such as metals exert strong selective pressures for increased resistance (Klerks and Weis 1987; Levinton et al. 2003). It might be assumed that fouling organisms capable of settling and surviving on the hulls of vessels treated with metal-based antifouling biocides are individuals of the population that carry heavy metal resistant genotypes. As such, hull fouling has the potential to select and transport populations of organisms with considerable competitive advantages over 'at-risk' native populations.

Tolerance to heavy metals can occur at the level of genotype, through genetic selection, or at the level of phenotype, through physiological acclimation or modification (Lam 1996). The major mechanisms of metal tolerance are generally thought to be (a) the sequestration of metals via specific metal-binding proteins, such as metallothioneins (Klerks and Weis 1987; Klerks and Bartholomew 1991; Shirley and Sibly 1999; Long and Wang 2005) and (b) the reduced or inefficient uptake of micronutrients such as heavy metals, resulting in decreased accumulation (Klerks and Weis 1987; Daka and Hawkins 2004). Studies of marine invertebrate species have indicated that resistance to heavy metals can develop through genetic selection in as few as four generations following exposure to heavy metal pollution (Klerks and Levinton 1989). However, certain trade-offs exist between resistance and other fitness traits such as survival, growth and fecundity (Shirley and Sibly 1999; Levinton et al. 2003), which can result in the poorer performance of metal-adapted genotypes under pollution-free conditions. This can result in an equally rapid loss of resistance in metal-adapted populations once exposure ceases (Levinton et al. 2003).

Regardless of whether the variability in metal tolerance occurs at the level of genotype or phenotype it is clear that the expression of metal tolerance will be modified by the chemical, biological and physical environment (Klerks and Weis 1987; Klerks and Levinton 1989; Levinton et al. 2003). Field studies can assist us to identify the effect of differential toxicant sensitivity under ecologically realistic situations, and may enable predictions of the role of pollution tolerance in facilitating invasion.

The aim of this study was to investigate whether an introduced bryozoan, *Bugula neritina* Linnaeus, exhibits differential resistance to a heavy metal toxicant (Cu) based upon the relative levels of pollution experienced by source populations. *B. neritina* is a common fouling organisms on commercial and recreational vessels (Gordon and Mawatari 1992; Floerl et al. 2004; Wyatt et al. 2005), and has demonstrated the ability to grow on (Floerl et al. 2004) and around (Johnston and Webb 2000) surfaces treated with copper-based antifouling paints. It is recognised as national priority pest species within Australian waters, with an invasion potential classification of medium-high (Hayes et al. 2004). Marshall (2005) demonstrated that variable survival and growth can occur between different source populations of *B. neritina*, though in this case pollution was not a determining factor. For our experiment, populations of *B. neritina* were sourced from an operational commercial shipping port with a long history of heavy metal pollution, and a relatively unpolluted harbour. Our principle objective was to conduct a laboratory-based toxicity experiment to determine whether different source populations of *B. neritina* showed differential resistance to copper through the survival and growth of *B. neritina* recruits exposed to a range of copper concentrations. However, while responses to pollutants can be demonstrated in the laboratory, factors such as habitat, competition, food availability and natural disturbances can result in differing responses in the field under ecologically realistic conditions (Luoma 1996). Therefore, our second objective was to conduct a field-based determination of the benefits and/or costs of resistance in different source populations of *B. neritina* through the reciprocal transplantation of recruits to the original polluted and unpolluted source locations.

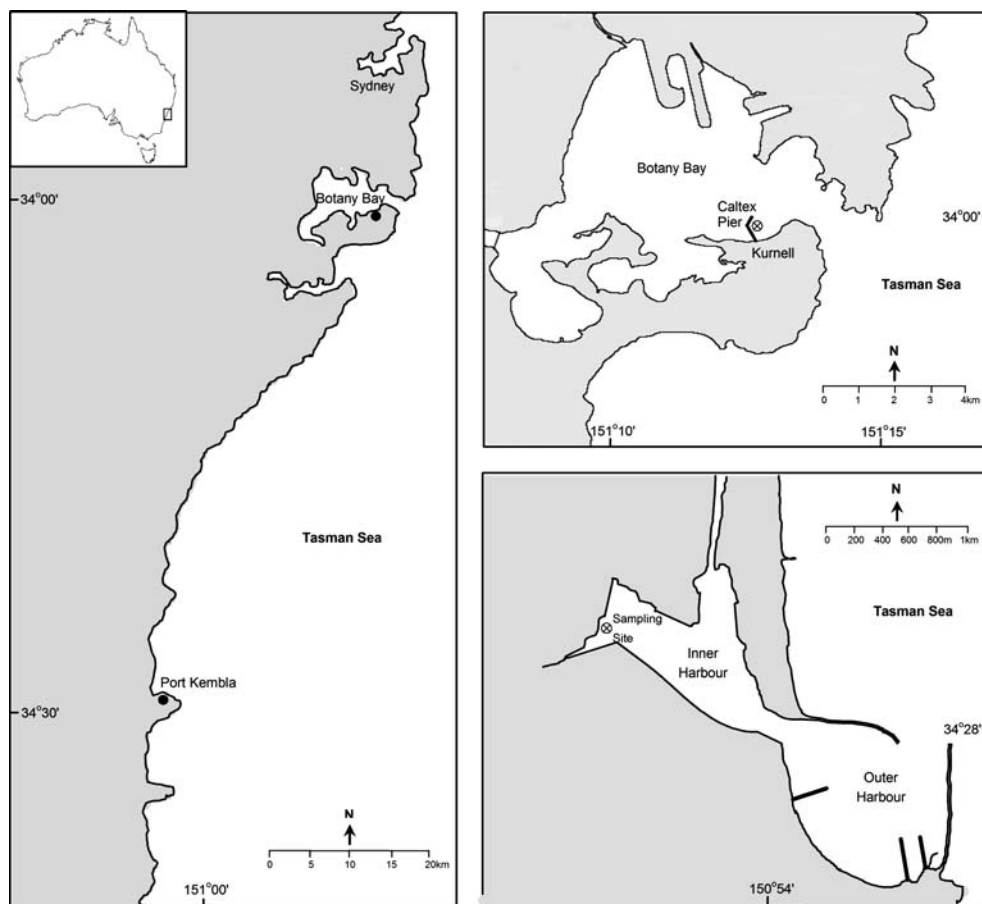
Materials and methods

Study sites

Colonies of *B. neritina* were sampled from two locations in New South Wales, Australia. The first sampling site was located at Kurnell Pier, in Botany Bay, 15 km south of Sydney (Fig. 1a). Botany Bay is a large, well flushed marine-dominated estuary, which in spite of its urbanisation has retained many natural areas, including aquatic reserves (Pollard and Pethebridge 2002). Kurnell pier extends 1.3 km from the southern shore of the bay, approximately 2 km from the bays entrance (Fig. 1b). Kurnell pier has naturally occurring sessile assemblages of sponges, ascidians, bryozoans, polychaetes, hydrozoans, anthozoans and macroalgae (Pollard and Pethebridge 2002) with background concentrations of total copper in the water less than $5 \mu\text{g l}^{-1}$ (R.F. Piola and E.L. Johnston, unpublished data).

The second sampling site, Port Kembla harbour, is a heavily modified shipping port approximately 70 km south of Sydney (Fig. 1a). The harbour provides a

Fig. 1 Map of (a) the south-east coast of Australia showing the location of study sites at (b) Botany Bay and (c) Port Kembla Harbour



variety of shipping and industrial functions (He and Morrison 2001) and has a 70-year history of industrial pollution (Moran and Grant 1989). The port has been artificially modified into an inner and outer harbour, with our sampling location situated within the inner harbour (Fig. 1c). There are several sources of heavy metal pollution in the harbour, including local catchment drainage, direct inputs of industrial effluent (from a copper smelter, steel smelter and a fertiliser manufacturing plant) and antifouling paints from commercial vessels. This pollution predominantly occurs in the inner harbour, which in conjunction with poor flushing has led to severe environmental degradation and accumulation of contaminants in the sediments in this area (He and Morrison 2001). Background concentrations of total copper in the water can reach levels greater than $13.5 \mu\text{g l}^{-1}$, more than 2.5 times the maximum ANZECC guideline recommendation of $5 \mu\text{g l}^{-1}$ (He and Morrison 2001). Sediments within the harbour also contain as much as $1,468 \text{ mg kg}^{-1}$ of copper, more than 244 times than average for the entire east coast of Australia (He and Morrison 2001), with the frequent resuspension of these sediments, through shipping movements and redevelopment, periodically elevating copper concentrations well above background levels. In addition to copper, concentrations of other metals such

as iron, lead and zinc far exceed recommended background levels outlined for water and sediments (Nelson and Johnston, unpublished data).

Bryozoan collection and maintenance

Mature colonies of *B. neritina* were collected from existing populations at Botany Bay and Port Kembla Harbour. Colonies were transported and maintained in aerated tanks of filtered seawater collected from their respective field sites. They were kept in darkness for 48 h before being induced to spawn by exposure to bright light (Wisely 1958).

Copper treatments

Analytical grade copper II chloride hydrous ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$) was used as the reference toxicant. A $1,000 \mu\text{g l}^{-1}$ Cu solution was prepared from stock solution each day and diluted with filtered seawater from Kurnell pier in order to make the three nominal experimental treatment solutions of 25, 50, and $100 \mu\text{g l}^{-1}$ Cu. Seawater was filtered through a $0.2 \mu\text{m}$ filter in order to minimise the possibility of complexation of Cu with organic particles.

All equipment was acid washed in 5% nitric acid for a minimum of 24 h and rinsed in Milli-Q filtered water prior to use.

Treatment solutions were renewed daily. Water quality (pH, salinity and dissolved oxygen) of treatment solutions was measured during the experiment using a HANNA HI-9025C pH and temperature meter and a Yeo-Kal 611 Water quality analyser. Samples of experimental Cu treatments were collected at the commencement and conclusion of the experiment and analysed for actual Cu concentrations. Samples for Cu analysis were immediately acidified with analytical grade nitric acid (1.5 µl of acid per 1 ml of sample) and refrigerated at 4°C. The concentration of copper in stock and experimental solutions was then independently tested by the Australian Government National Measurement Institute (detection limit of 5 µg l⁻¹). Actual and nominal total Cu concentrations in treatment solutions are summarised in Table 1.

Twelve-day larval toxicity test

Copper sensitivity of *B. neritina* from Botany Bay and Port Kembla was investigated by measuring the larval attachment success, post-metamorphic survival and growth of recruits exposed during a 12-day toxicity experiment conducted in September 2004. Plastic 35 mm diameter Petri dishes were acid washed then pre-soaked in the appropriate copper solution for 24 h prior to commencing the experiment. The nominal copper concentrations used were 0, 25, 50 and 100 µg l⁻¹ Cu, with 12 replicate dishes for each treatment. Concentrations of 25 and 50 µg l⁻¹ Cu represent relevant values that do exist in polluted aquatic environments (Stauber et al. 2000; Schiff et al. 2004), while the 100 µg l⁻¹ Cu concentration was primarily included to gauge maximum tolerance limits. Exposure to treatments was maintained for 12 days.

Two identical treatment series were prepared, with one series of treatment dishes inoculated with larvae of *B. neritina* from Botany Bay, and the second with larvae from Port Kembla Harbour. Between 8 and 15 larvae were added to each treatment dish. At no time during the experiments did the organism loading exceed the specified ASTM 1192-97 guidelines of 0.5–0.8 g organism l⁻¹ (ASTM 1999). Following inoculation of larvae,

all treatments were kept in darkness for 24 h to encourage settlement, after which time they were subjected to a shaded 12:12 light:dark cycle. The experiment was conducted at a constant temperature of 21 ± 0.4°C.

After 24 h the proportion of attached larvae was observed in each container using a dissecting microscope. Attachment was defined as a larva that had attached to the surface of the container and initiated metamorphosis into the ancestrula. The location of individual larvae within the container was mapped for reference. Survival was recorded at the completion of the laboratory exposure (12 days). Survival was defined as successful metamorphosis into an ancestrula with a primary orifice present. A zooid was deemed to be dead if it appeared empty or if only a brown-body (Brusca and Brusca 1990) was visible. Growth of settled larvae was recorded at 3, 6, 9 and 12 days, and was recorded by counting the number of new zooids budded from each ancestrula, with a bud being counted as a new zooid once a primary orifice had developed. A pilot study indicated that larvae of *B. neritina* can develop to fully feeding settled individuals between 1 and 2 days after attachment and metamorphosis. Therefore, from day 2 onwards a food source (the microalgae *Isochrysis galbana*) was included as a component of each treatment solution at a concentration of 10⁵ cells ml⁻¹.

Laboratory-to-field transfer

To assess the post-exposure survival and growth of Botany Bay and Port Kembla *B. neritina* colonies exposed to copper, a reciprocal transplant experiment was conducted in October 2004 immediately after the laboratory exposure period (12 days). Half the Petri dishes from each of the experimental treatments for the Botany Bay and Port Kembla *B. neritina* treatment series were transplanted to Botany Bay, while the remaining half from each treatment series were transplanted to Port Kembla harbour. Petri dishes were randomly arranged onto a PVC backing plate (50×50×0.5 cm) and attached using silicon glue. The backing plate was suspended in the water column at a depth of 2 m below low water mark with treatment dishes on the underside to minimise available light and sedimentation. The temperature, salinity, pH and dissolved oxygen concentrations were measured at each site at the time of deployment using a Yeo-Kal 611 Water quality analyser. Dishes were retrieved from the field after 33 days and transported back to the laboratory and maintained in a recirculating seawater system prior to census.

Colonies derived from experimental larvae were distinguished from colonies growing from field recruitment using the mapped positions recorded during the laboratory experiments. The number of colonies surviving in each dish was recorded as a percentage of the colonies present in the dish at the time of field deployment. The growth of *B. neritina* colonies was assessed by counting the number of bifurcations along three separate branch

Table 1 Nominal and measured copper concentrations for copper treatments used during the 12-day *Bugula neritina* toxicity experiment (± 1SE, n=2)

Nominal copper (µg l ⁻¹)	Measured copper (µg l ⁻¹)
0	< 5
25	24.5 ± 3.5
50	47.0 ± 5.0
100	97.0 ± 13.0

Lowest detection limit for analysis was 5 µg l⁻¹

lengths within any one colony. This number was then averaged to get a mean value for that colony. Bifurcations have been found to be a reliable estimate of colony size (Keough and Chernoff 1987).

Water quality parameters recorded at Botany Bay at the time of deployment were 8.21 for pH, 7.3 mg l⁻¹ for dissolved oxygen content, 34.31 for salinity and 18.01°C for temperature, while physico-chemical readings at Port Kembla were 8.17 for pH, 6.1 for dissolved oxygen content, 34.37 for salinity and 20.12°C for temperature.

Recruitment experiment

Results from the laboratory-to-field transplant experiment (outlined above) indicated that large numbers of recruiting organisms at Botany Bay, relative to Port Kembla Harbour, had the effect of obscuring any post-Cu-exposure survival and growth patterns for the *B. neritina* transplants at this site. We therefore conducted a field-based aging experiment, over November and December 2004, to investigate the effect that natural recruitment had on the survival and growth of *B. neritina* colonies. Petri dishes containing filtered seawater were inoculated with 10–20 larvae from *B. neritina* colonies collected at Kurnell pier. After 24 h the number of attached larvae was recorded in each container and the location of individual larvae within the container was mapped for reference. Containers were assigned to either caged, uncaged or cage-control treatments groups, with seven replicates in each treatment. Petri dishes from the caged treatments were placed inside individual cages consisting of a 100 ml plastic jar with large holes cut into the sides and lids that were covered with 250 µm mesh. These cages were designed to reduce recruitment of larvae within the caged area but still allow food and water to pass through. Cage-controls consisted of jars identical to those used for cage treatments, except the lids were removed. Uncaged treatment dishes had no caged covering. All treatment dishes were randomly arranged onto a PVC backing plate (50×50×0.5 cm) and attached using silicon glue. Deployment of the backing plate at Kurnell pier was identical to the method described for the laboratory-to-field transfer experiment, with treatments retrieved after 34 days. In addition to recording the survival and growth of experimental *B. neritina* colonies, the amount of recruitment to each treatment container was quantified using image analysis software (Image-Pro Express v4.01, Media Cybernetics, Silver Spring, MD, USA) and expressed as the percentage of available space occupied by sessile invertebrates.

Data analysis

Differences in the percent attachment of larvae and the survival of recruits in the 12-day laboratory exposure were analysed using two-factor analysis of variance

(ANOVA) with the Source of the Larvae and Cu treatment being fixed factors. Tukey's post hoc tests were conducted on significant results. Repeated measures ANOVA was used to test for the effects of source location and copper on the growth of recruits during the 12-day laboratory exposure, with the Source of Recruits and Cu treatment being the between-subjects effects and Time the repeated effect. Planned comparisons were carried out on significant results to determine differences between Botany Bay and Port Kembla recruits, with all planned comparisons tested against the error term for the main test of Source (Quinn and Keough 2002). For the laboratory-to-field transfer a two-factor ANOVA was used to test for variation in the post-metamorphic survival and growth of colonies transplanted to Botany Bay and Port Kembla, with Source of the Colonies and Cu treatment as fixed factors. Tukey's post hoc tests were performed on significant results. All data were assessed for homogeneity of variance and normality using plots of residuals vs means and descriptive statistics and when required analysis was performed on square-root transformed data. If present, outliers were removed and the analysis repeated to test for possible changes in significance.

Results

Larval attachment success and survival

The proportion of *B. neritina* larvae from Botany Bay and Port Kembla Harbour attaching to Petri dishes after 24 h ranged from 32 to 82% across all Cu treatment (Fig. 2). There was a significant decrease in the attachment of larvae at 100 µg l⁻¹ Cu compared to control, 25 and 50 µg l⁻¹ Cu treatments (Table 2, Fig. 2). The source of the adult colonies had no impact on the attachment success of larvae (Table 2, Fig. 2). Post-metamorphic survival of *B. neritina* recruits was assessed at day 12. There was a significant decrease in the survival of recruits in 100 µg l⁻¹ Cu compared to control, 25 and 50 µg l⁻¹ Cu treatments (Table 3, Fig. 3). Again, the source of the adult *B. neritina* colonies had no impact on the recruit survival irrespective of Cu treatment.

Growth

Post-metamorphic growth was assessed as the change in the average number of live zooids per colony. There was a significant interaction between Time, Population Source and Cu treatment, with Botany Bay colonies growing increasing larger than Port Kembla colonies in 0 µg l⁻¹ Cu from day 3 onwards (Table 4, Fig. 4a). This was in direct contrast to growth observed in the 100 µg l⁻¹ Cu treatments where colonies originating from Port Kembla contained significantly more viable zooids than those from Botany Bay following day 6 (Table 4, Fig. 4d). No differences in growth were

Fig. 2 Effects of 0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu exposure on the attachment success of *Bugula neritina* larvae from Botany Bay and Port Kembla Harbour after 24 h. Bars represent mean (\pm 1SE). Different letters represent significant differences and lines represent no difference in Tukey's post hoc comparisons ($\alpha = 0.05$)

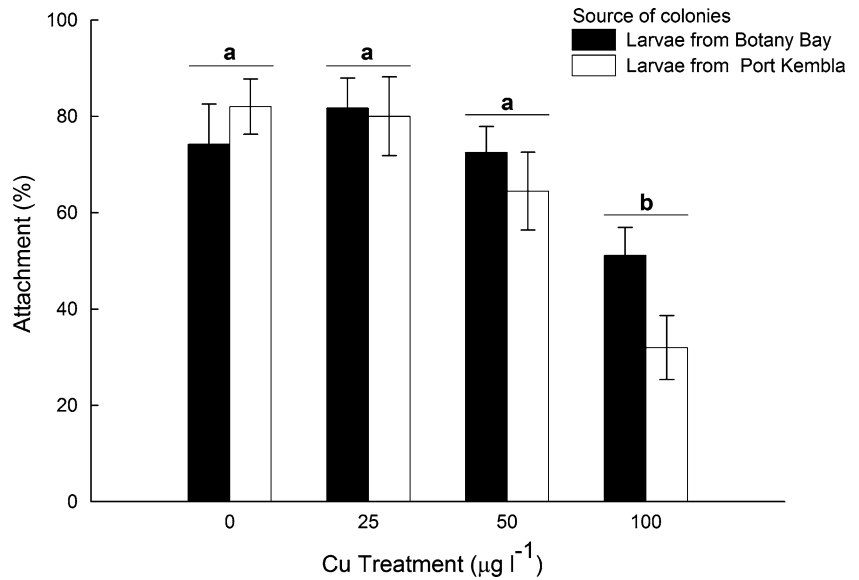


Table 2 Analysis of variance of the attachment success of *Bugula neritina* larvae from Botany Bay and Port Kembla Harbour exposed to 0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu in the laboratory for 24 h

Main test	df	MS	F	P
Source	1	0.063	1.152	0.286
Cu treatment	3	0.713	13.043	<i>0.000</i>
Source×Cu treatment	3	0.070	1.285	0.285
Error	84	0.055		

P-values in italics indicate significant differences at $\alpha = 0.050$
P = 0.000 denotes values < 0.001

Table 3 Analysis of variance of the survival of *Bugula neritina* recruits from Botany Bay and Port Kembla Harbour exposed to 0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu in the laboratory for 12 days

Main test	df	MS	F	P
Source	1	0.051	0.950	0.333
Cu treatment	3	1.639	30.275	<i>0.000</i>
Source×Cu treatment	3	0.007	0.128	0.943
Error	84	0.054		

P-values in italics indicate significant differences at $\alpha = 0.050$
P = 0.000 denotes values < 0.001

observed between Botany Bay and Port Kembla colonies in 25 and 50 $\mu\text{g l}^{-1}$ Cu treatments (Table 4, Fig. 4b, c).

Laboratory-to-field transfer

Survival

Survival rates of *B. neritina* colonies transplanted to Botany Bay and Port Kembla were calculated as a proportion of the number of colonies transplanted alive. A significant Source by Cu Treatment interaction was observed in *B. neritina* colonies transplanted to Port Kembla Harbour (Table 5, Fig. 5a). Relatively little

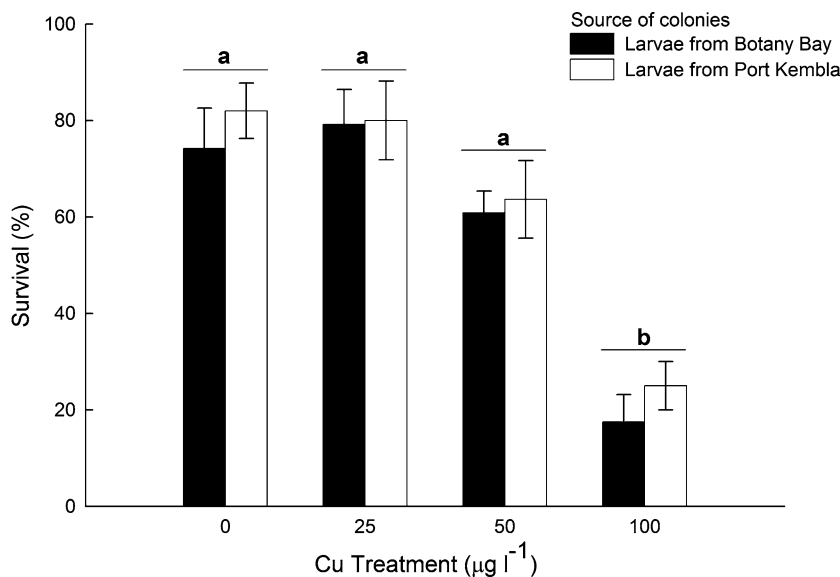
mortality was recorded for colonies originating from Port Kembla, with 98, 86 and 85% survival in control, 25 and 50 $\mu\text{g l}^{-1}$ Cu treatments, respectively (Fig. 5a). This was in contrast to the pattern observed for colonies originating from Botany Bay, where significantly increasing mortality was observed with increasing Cu concentrations (Fig. 5a). A 100% mortality was recorded for all colonies from the 100 $\mu\text{g l}^{-1}$ Cu treatment irrespective of their origin (Fig. 5a).

Considerably higher mortality was observed in all colonies transplanted to Botany Bay relative to colonies transplanted to Port Kembla Harbour (Fig. 5b). Significantly higher mortality was recorded for colonies pre-exposed to 100 $\mu\text{g l}^{-1}$ Cu compared to control, 25 and 50 $\mu\text{g l}^{-1}$ Cu treatments (Table 5, Fig. 5b), with 90 and 100% mortality observed in Port Kembla and Botany Bay colonies, respectively. The high variability observed in the survival of *B. neritina* recruits transplanted to Botany Bay relative to Port Kembla may be explained by the very different patterns of recruitment (and the associated competition for space and resources) observed at these sites. Aside from several barnacles and small colonies of the bryozoan *Bugula flabellata*, experimental dishes at Port Kembla showed little-to-no recruitment during field deployment. This is in contrast to the dishes transplanted to Botany Bay, which had nearly all available space occupied by recruiting organisms, primarily barnacles (*Balanus variegates*), colonial ascidians (*Botrylloides leachii* and *Diplosoma listerianum*) and bryozoans (*Watersipora subtorquata*), within the 33-day deployment period.

Growth

A significant Source by Cu Treatment interaction was observed in the growth of colonies transplanted to Port Kembla (Table 5, Fig. 6a). High growth rates were observed for Port Kembla colonies grown in control,

Fig. 3 Effects of 0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu exposure on the survival of *Bugula neritina* recruits from Botany Bay and Port Kembla Harbour after 12 days. Bars represent the mean ($\pm 1\text{SE}$). Different letters represent significant differences and lines represent no difference in Tukey's post hoc comparisons ($\alpha = 0.05$)



25 and 50 $\mu\text{g l}^{-1}$ Cu treatments (Fig. 6a). Similarly high growth rates were also observed for control and 25 $\mu\text{g l}^{-1}$ Cu Botany Bay colonies; though this was followed by a dramatic decrease in growth for colonies pre-exposed to 50 $\mu\text{g l}^{-1}$ Cu (Fig. 6a). No readily iden-

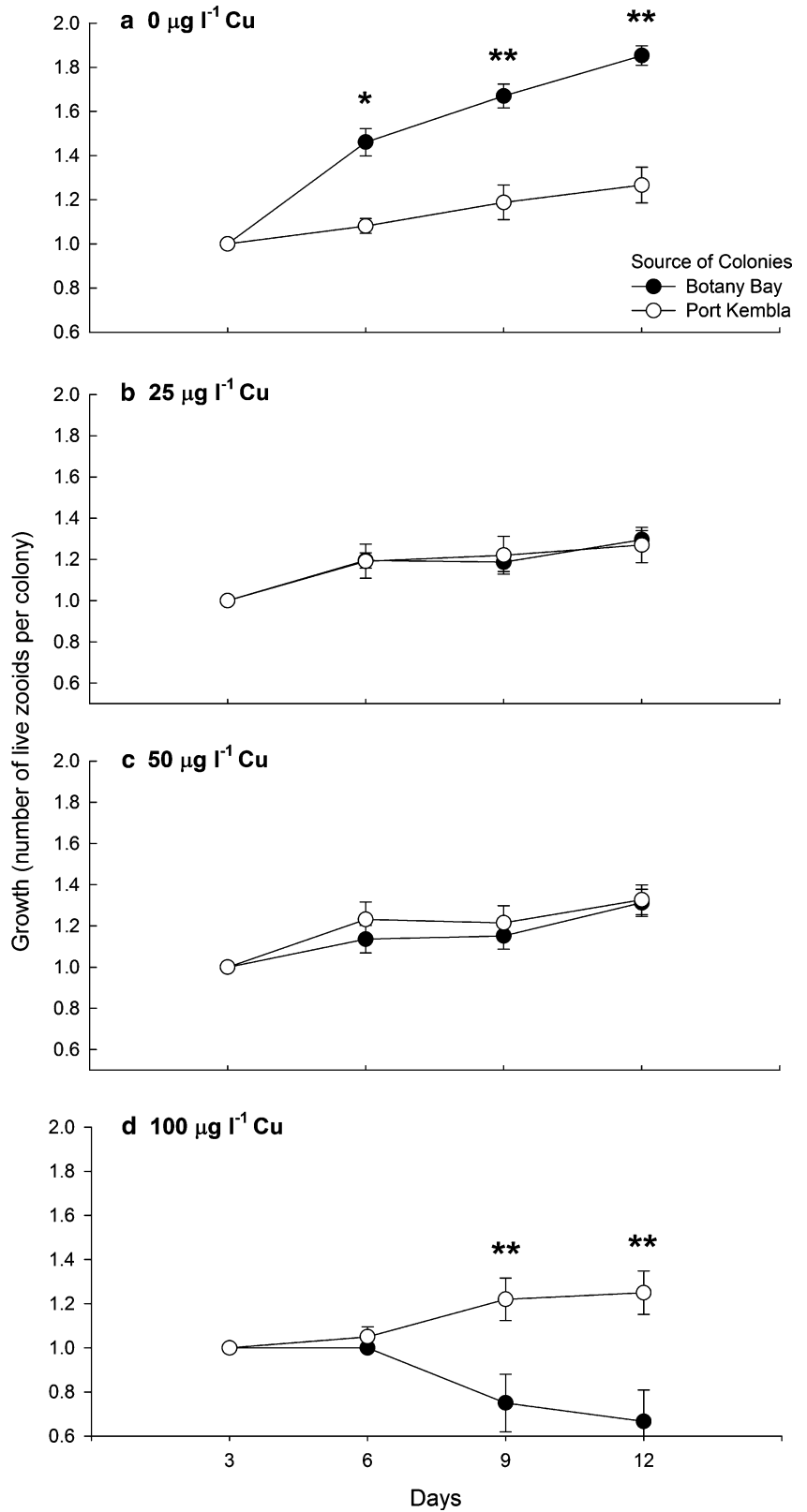
tifiable patterns of growth were observed in *B. neritina* recruits transplanted to Botany Bay. There was no significant difference in growth observed between source populations from Botany Bay and Port Kembla Harbour (Table 5, Fig. 6b).

Table 4 Summary of (a) Repeated Measures Analysis for the growth of *Bugula neritina* recruits from Botany Bay and Port Kembla Harbour exposed to 0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu in the laboratory for 12 d, and (b) planned comparisons of source population within each copper and time combination

(a) Main test	Analysis			
	<i>df</i>	MS	<i>F</i>	<i>P</i>
Between subjects				
Source	1	0.013	0.081	0.777
Cu treatment	3	2.032	12.230	0.000
Source×Cu treatment	3	2.027	12.196	0.000
Error	84	0.166		
Within subjects				
Time	2	0.304	13.941	0.000
Time×source	2	0.039	1.797	0.169
Time×Cu treatment	6	0.127	5.810	0.000
Time×source×Cu treatment	6	0.156	7.176	0.000
Error	168	0.022		
(b) Botany Bay vs Pt Kembla	Planned comparison			
	<i>df</i>	MS	<i>F</i>	<i>P</i>
0 $\mu\text{g l}^{-1}$ Cu				
Day 6	1, 84	0.788	4.747	0.032
Day 9	1, 84	1.266	7.627	0.007
Day 12	1, 84	1.879	11.319	0.001
25 $\mu\text{g l}^{-1}$ Cu				
Day 6	1, 84	0.000	0.000	1.000
Day 9	1, 84	0.007	0.042	0.838
Day 12	1, 84	0.004	0.024	0.877
50 $\mu\text{g l}^{-1}$ Cu				
Day 6	1, 84	0.055	0.331	0.566
Day 9	1, 84	0.024	0.145	0.705
Day 12	1, 84	0.001	0.006	0.938
100 $\mu\text{g l}^{-1}$ Cu				
Day 6	1, 84	0.014	0.084	0.772
Day 9	1, 84	1.205	7.259	0.009
Day 12	1, 84	1.856	11.181	0.001

All planned comparisons were tested against the error term for the main test of Source. *P*-values in italics indicate significant differences at $\alpha = 0.050$. *P* = 0.000 denotes values < 0.001

Fig. 4 Effects of (a) 0, (b) 25, (c) 50 and (d) 100 $\mu\text{g l}^{-1}$ Cu exposure on the growth of *Bugula neritina* recruits from Botany Bay and Port Kembla Harbour over 12 days. Values represent the mean (\pm 1SE). Asterisk represent significant differences based on planned comparisons, with *representing $P < 0.05$ and **representing $P < 0.01$



Recruitment experiment

There was considerably less (> 50%) recruitment to caged dishes than uncaged and cage-control dishes.

Recruitment to caged, cage-control and uncaged treatment dishes was 32, 83 and 100%, respectively (Table 6, Fig. 7a). The barnacle *B. variegates* and the colonial ascidians *B. leachii* and *D. listerianum* dominated

Table 5 Analysis of variance for the survival and growth of *Bugula neritina* recruits from Botany Bay and Port Kembla Harbour transplanted to the field at (a) Port Kembla and (b) Botany Bay

Transplant location	Survival				Growth			
	<i>df</i>	MS	<i>F</i>	<i>P</i>	<i>df</i>	MS	<i>F</i>	<i>P</i>
(a) Port Kembla								
Source	1	0.215	9.152	<i>0.005</i>	1	5.694	14.276	<i>0.001</i>
Cu treatment	3	1.833	78.044	<i>0.000</i>	3	54.548	136.756	<i>0.000</i>
Source×Cu treatment	3	0.073	3.088	<i>0.039</i>	3	2.556	6.408	<i>0.001</i>
Error	37	0.023			37	0.399		
(b) Botany Bay								
Source	1	0.052	1.200	0.281	1	9.072	4.899	<i>0.033</i>
Cu treatment	3	0.497	11.426	<i>0.000</i>	3	22.042	11.902	<i>0.000</i>
Source×Cu treatment	3	0.019	0.440	0.726	3	1.366	0.738	0.536
Error	36	0.043			36	1.852		

Recruits had previously been exposed to 0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu in the laboratory for 12 days

P-values in italics indicate significant differences at $\alpha=0.050$

P=0.000 denotes values <0.001

Fig. 5 Survival of *Bugula neritina* colonies transplanted to (a) Port Kembla Harbour and (b) Botany Bay following 12-day exposure to 0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu in the laboratory. Values represent the mean ($\pm 1\text{SE}$). Different letters represent significant differences and lines represent no difference in Tukey's post hoc comparisons ($\alpha=0.05$)

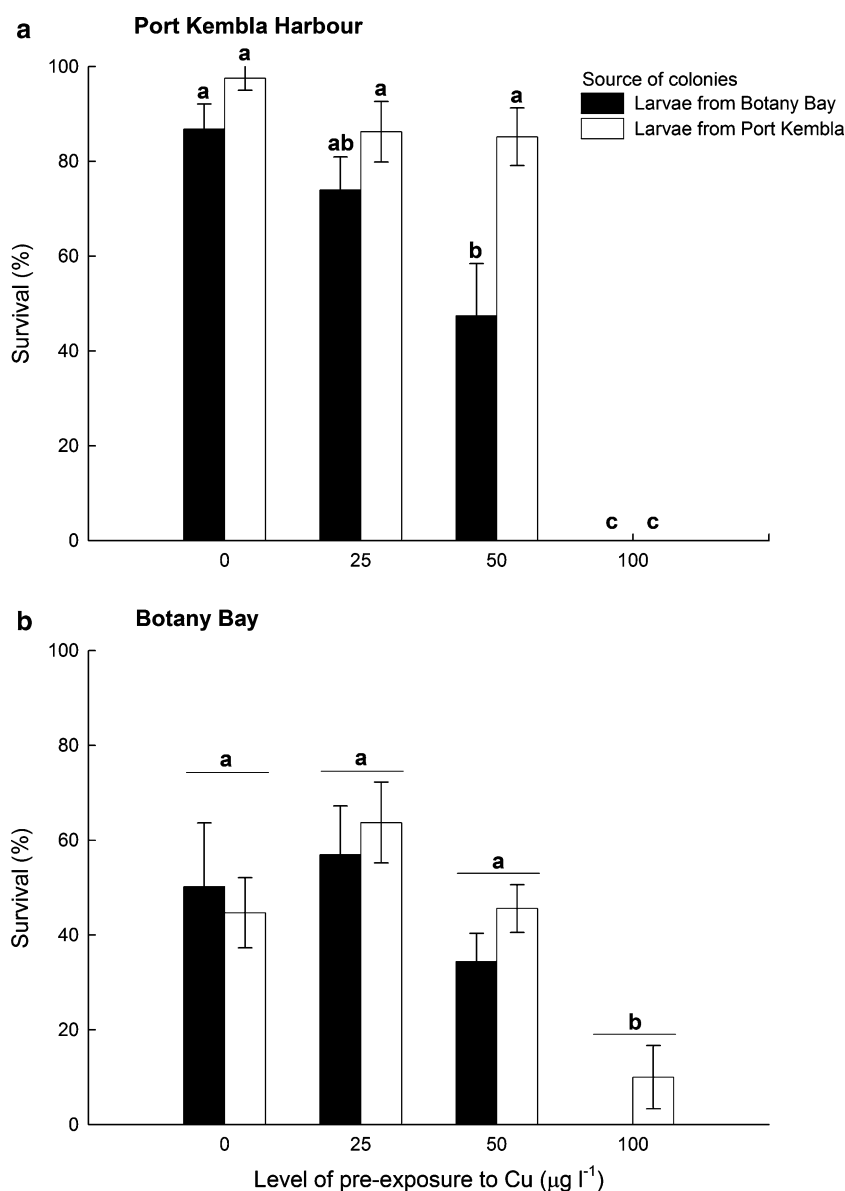
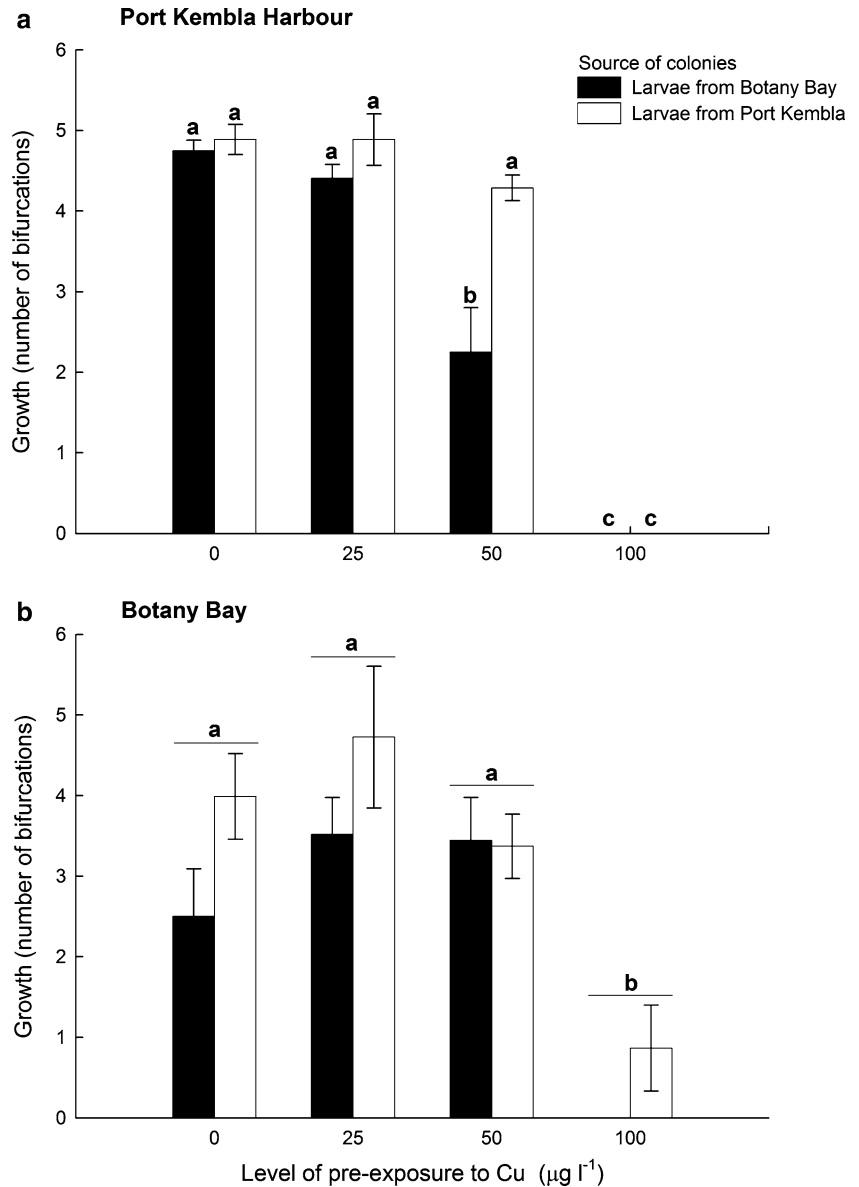


Fig. 6 Growth of *Bugula neritina* colonies transplanted to (a) Port Kembla Harbour and (b) Botany Bay following 12-day exposure to 0, 25, 50 and 100 $\mu\text{g l}^{-1}$ Cu in the laboratory. Values represent the mean (\pm 1SE). Different letters represent significant differences and lines represent no difference in Tukey's post hoc comparisons ($\alpha=0.05$)



available space on uncaged and control dishes, and polyps of the moon jelly *Aurelia aurita* were the major occupiers of free space in caged dishes.

Reducing field recruitment dramatically increased the survival and growth of *B. neritina* recruits for the short-term duration of this experiment. The 20% mortality observed for recruits from the caged treatment was significantly lower than the 56 and 70% mortality recorded for the cage-control and uncaged treatments, respectively (Table 6, Fig. 7b). This pattern was repeated for the growth of *B. neritina* colonies, with significantly increased growth observed for caged recruits relative to uncaged colonies (Table 6, Fig. 7c).

Discussion

Results demonstrate that *B. neritina* colonies originating from the polluted Port Kembla Harbour have increased

resistance to Cu relative to populations from the relatively unpolluted Botany Bay, but that this resistance does not constitute an advantage outside of polluted environments. Laboratory exposure to a range of Cu treatments showed no difference in attachment of larvae and the subsequent survival of recruits between source populations from Port Kembla and Botany Bay. The growth of Botany Bay recruits however, was noticeably affected by copper exposure, with dramatically higher growth in control treatments and significantly poorer growth at 100 $\mu\text{g l}^{-1}$ Cu, which contrasted markedly with the conspicuously uniform growth of Port Kembla colonies irrespective of Cu concentration. This difference between Port Kembla and Botany Bay populations suggests a possible trade-off exists between Cu-resistance and growth in Cu-resistance genotypes, as evidenced by the lower growth rates of Cu-resistant control treatment colonies relative to the non-resistant control populations.

Table 6 Analysis of variance for the caging experiment, including (a) the percent cover of sessile invertebrates on experimental surfaces, and the corresponding (b) survival and (c) growth of *Bugula neritina* recruits in each treatment

Main test	Analysis			
	<i>df</i>	MS	<i>F</i>	<i>P</i>
(a) % Cover sessile invertebrates				
Cage treatment	2	36.506	193.591	<i>0.000^a</i>
Error	18	0.189		
(b) Survival of <i>Bugula neritina</i>				
Cage treatment	2	0.455	8.125	<i>0.003</i>
Error	18	0.056		
(c) Growth of <i>Bugula neritina</i>				
Cage treatment	2	11.313	6.492	<i>0.008</i>
Error	18	1.743		

Treatments comprised of caged, uncaged and cage-control
P-values in italics indicate significant differences at $\alpha=0.050$

P=0.000 denotes values <0.001

^aSquare-root transformed

Resistance to copper appeared to be closely related to the relative levels of pollution experienced by the source populations. Colonies from the heavily polluted Port Kembla Harbour exhibited increased Cu-resistance relative to those from the relatively unpolluted Botany Bay site. Pollutants, such as heavy metals, produce very powerful selective forces on both the targeted pests and non-target organisms, generally favouring genotypes that increase the resistance of local populations (Kim et al. 2003; Levinton et al. 2003). *B. neritina* is a cosmopolitan fouling species, which has most likely been introduced to ports, harbours and estuaries worldwide via hull-fouling on commercial and recreational vessels (Gordon and Mawatari 1992; Floerl et al. 2004; Wyatt et al. 2005). Given the majority of modern vessels are treated with antifouling coatings, of which copper is often the primary biocide, coupled with the fact that many *B. neritina* populations likely originate from ports and harbours already polluted with metals (Paulson et al. 1989; Weis and Weis 1992; Pitt 1995; Weis and Weis 1996; Apte and Day 1998; Hall et al. 1998), we speculate that resistance to copper in introduced populations of *B. neritina* is likely to have been selected for prior to their introduction to new locations.

The inheritance of heavy metal resistance has been shown for a range of invertebrate organisms, including annelid worms (Bryan and Hummerstone 1971; Grant et al. 1989; Klerks and Levinton 1989; Klerks and Bartholomew 1991), molluscs (Hoare et al. 1995), crustaceans (Brown 1976) and insects (Shirley and Sibly 1999). Resistance to metals can occur at the level of genotype, through genetic selection, or phenotype, through modified physiological and biochemical mechanisms (Lam 1996). Metallothionein is a cysteine-rich metal-binding protein (Kägi and Kojima 1987) that can sequester

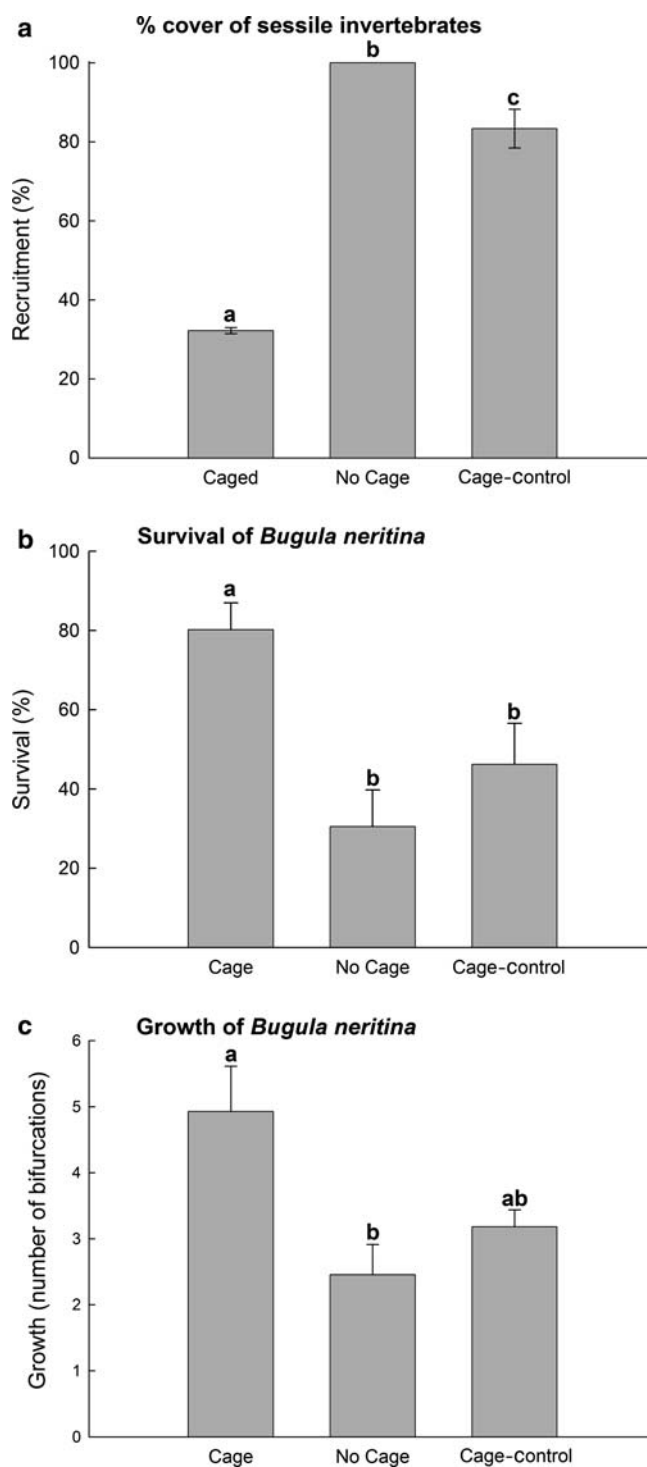


Fig. 7 Effect of recruitment of sessile invertebrates on the survival and growth of *Bugula neritina* colonies transplanted to Botany Bay, showing (a) the percent cover of sessile invertebrate fauna on experimental surfaces in three treatments (caged, uncaged and cage-control), and the subsequent (b) survival and (c) growth of *B. neritina* colonies in each treatment. Bars represent the mean ($\pm 1SE$). Different letters represent significant differences in Turkey's post hoc comparisons ($\alpha=0.05$)

metals and protect against metal toxicity (Roesijadi 1992), and is the most important protein involved in heavy metal resistance (Shirley and Sibly 1999). One possible genetic mechanism of resistance is the production of multiple copies of resistance gene(s) which results in excess amounts of metallothionein being produced (Mokdad et al. 1987; Lange et al. 1990). As an example of phenotypic level resistance, evidence exists for the transfer of heavy metal resistance through inheritance of maternal metallothioneins by embryos (Roesijadi et al. 1982; Ohtake et al. 1983; Hoare et al. 1995), and the induction of metallothionein through bioaccumulation and sublethal exposure to heavy metals (Roesijadi and Fellingham 1987; Viarengo 1989; Long and Wang 2005). Comprehensive breeding experiments would need to be conducted to determine the exact role that genotype and/or phenotype play in heavy metal resistance in populations of *B. neritina*.

Results from our laboratory toxicity experiment on *B. neritina* are comparable to other heavy metal resistance studies on invertebrates that show non-resistant genotypes perform worse than metal-resistant genotypes in metal-polluted environments, but out-perform them in unpolluted environments (Bryan and Hummerstone 1973; Shirley and Sibly 1999). One explanation for the differential resistance among our *B. neritina* populations may be the trade-offs that exist between resistance to heavy metals and overall fitness. Our laboratory toxicity experiment demonstrated that relatively Cu-sensitive *B. neritina* colonies from Botany Bay displayed a wide range of growth dependent upon the level of Cu exposure. This is in contrast to the growth in Port Kembla colonies, which was conspicuously uniform across all Cu concentrations—and considerably lower than that of Botany Bay colonies in unpolluted conditions. These findings suggest that increased Cu-resistance is exacting some toll upon Port Kembla colonies and that a loss of resistance translates to increased fitness in Botany Bay populations growing in an unpolluted environment. Laboratory studies on the fruit fly *Drosophila melanogaster* have demonstrated that flies reared on a medium polluted by Cd evolved resistance to the metal and showed higher survival and fecundity when exposed to this polluted environment (Shirley and Sibly 1999). However, resistant lines paid a fitness cost in unpolluted environments, with reduced fecundity and weight relative to non-resistant flies, which would likely translate to a rapid loss of the resistant genotype in unpolluted environments. Similar resistance-related fitness costs have been observed in annelid worms, with Cd-resistant genotypes from polluted sediments displaying very slow somatic growth relative to worms from cleaner sites (Levinton et al. 2003). While this study focused on populations of *B. neritina* from only two sites, examination of populations from additional polluted and unpolluted sites may reveal a broader range of Cu-resistance within this species, with the potential for varied fitness costs being associated with each.

Several probable mechanisms exist which may explain these observed fitness costs associated with heavy metal resistance in unpolluted environments. Detoxification mechanisms may divert energy from other fitness traits, such as growth or reproduction (Sibly and Calow 1989). For example, while the aforementioned duplication of the metallothionein-production gene(s) may increase the fitness and survival in polluted environments, it may incur fitness costs in unpolluted environments where production of excess metallothionein provides no benefit (Shirley and Sibly 1999). Another explanation for observed fitness costs may be that certain individuals within a population may be less efficient at trace metal uptake and utilisation, resulting in micronutrient deficiencies in unpolluted environments (Wright 1986; Harper et al. 1997). Given that hull fouling is such an intensely selective mode of introduction for *B. neritina*, favouring Cu-resistant genotypes, one would predict that once that selective pressure is removed (through the transfer of individuals into unpolluted environments) a rapid loss of resistance would follow within that population. While studies of benthic annelid worms have shown that the evolution of resistance to heavy metals can occur in as little as one to four generations following initial exposure (Klerks and Levinton 1989), resistance in the same populations can be lost in as little as nine generations once the heavy metal exposure ceases (Levinton et al. 2003). While resistance to heavy metals would be advantageous (and perhaps essential) to the survival of *B. neritina* colonies in Port Kembla, due to the ongoing occurrence of heavy metal pollution in the area, the relative absence of metal pollution in Botany Bay may have resulted in a rapid loss of resistance at this location, in exchange for improved survival and fitness.

Strong patterns of resistance observed in the laboratory translated into only minor differences when colonies were transplanted to the field. A decrease in the survival and growth of Botany Bay colonies relative to Port Kembla recruits only became readily apparent in recruits that had been pre-exposed to $50 \mu\text{g l}^{-1}$ Cu. Within any population one would expect to find a range of natural tolerances. It is highly likely that the stressful nature of our laboratory toxicity experiment effectively selected for the most robust Botany Bay recruits, resulting in the surprisingly good performance of the surviving genotypes when transplanted to a polluted environment. Despite this, delayed mortality and decreased growth was more pronounced in Botany Bay recruits transplanted to Port Kembla. This was most likely a result of ongoing stress from the polluted conditions of Port Kembla, irrecoverable damage sustained from the toxicant exposure in the laboratory, or a combination of both.

Resistance to heavy metals appeared to have no positive or negative effect on the survival and growth of *B. neritina* colonies transplanted to the unpolluted Botany Bay site. There was higher mortality and lower growth of all recruits transplanted to Botany Bay relative to those transplanted to Port Kembla Harbour. Previous

research has found lower settlement rates and species diversity at Port Kembla Harbour relative to similar unpolluted environments (Moran and Grant 1989; Clark and Johnston, manuscript in preparation). Competition for space is a major factor affecting the distribution and abundance of sessile marine organisms (Russ 1982; Buss 1986), with the level of recruitment to an area often influencing the survival and growth of juveniles and adults within that area (Connell 1985; Underwood and Keough 1986). We attribute the increased mortality and lower growth of our experimental *B. neritina* at Botany Bay to the intense competition pressure exerted by the greater number of recruiting organisms. This assessment is supported by our own recruitment–exclusion caging experiment, which demonstrated that competition for space is a primary determinant in the early-stage survival and growth of *B. neritina* populations at this location. It is interesting to note that in this unpolluted environment the metal-resistant recruits originating from Port Kembla performed on par with non-resistant recruits from Botany Bay. This raises the question of the true extent of resistance-related fitness costs in a real-world environment, and supports the findings of several botanical studies on resistance that have failed to find any evidence of fitness costs associated with copper resistance in the Common Monkeyflower *Mimulus guttatus* growing in unpolluted environments (Macnair and Watkins 1983; Harper et al. 1997). Stressful environments, such as those affected by toxicants or pollution, have potentially detrimental effects on community structure and health through decreased species diversity, increased dominance by ‘weedy’ r-strategists, and decreases in the size, growth and fecundity of individuals (Odum 1985). *B. neritina* is a classic r-strategist, which through the development of resistance, is able to survive and grow in stressful polluted environments. In unpolluted environments the species is only capable of occupying space in recently disturbed areas and is rapidly overgrown by better competitors (Johnston and Keough 2002). Under benign conditions, overwhelming competition-induced mortality may obscure any differential growth or survival attributes that different populations of *B. neritina* may exhibit.

To our knowledge this is the first study to clearly demonstrate differential heavy metal resistance in populations of an invasive marine organism. The findings of this study provide evidence of the benefits to invasive species of pollution tolerance and suggest that human disturbance may play a role in facilitating the establishment and spread of invasive species in marine systems.

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