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Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers

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Abstract

Understanding how biomarkers relate to each other on exposure to particular contaminants in different species is key to their widespread application in environmental management. However, few studies have systematically used multiple biomarkers in more than a single species to determine the variability of sublethal effects of a particular contaminant. In this study, three marine invertebrates, the shore crab *Carcinus maenas*, the common limpet *Patella vulgata* and the blue mussel *Mytilus edulis*, were exposed over 7 days in the laboratory to environmentally realistic concentrations of the priority pollutant copper. A combination of molecular, cellular and physiological biomarkers was measured in each organism to detect the toxic effects of copper. Biomarkers included lysosomal stability (neutral red retention), neurotoxicity (acetylcholinesterase activity), metabolic impairment (total haemolymph protein), physiological status (heart rate) and induction of protective metallothionein proteins. *P. vulgata* was the most sensitive to copper with significant effects measured in all biomarkers at concentrations of 6.1 μg $Cu\,l^{-1}$. In *C. maenas*, cellular and neurotoxic endpoints were affected significantly only at 68.1 μg $Cu\,l^{-1}$. Exposure to copper also induced metallothionein production in crabs. Over a 7-day exposure period, *M. edulis* was the most tolerant species to copper with significant effects being observed at the cellular level only at 68.1 μg $Cu\,l^{-1}$. In all three species, cellular and neurotoxic pathways were more sensitive to disruption than physiological processes (protein and heart rate). Results illustrate how a suite of biomarkers applied to different sentinel species can provide a 'diagnosis of stress', whereby, effects at the molecular level can be used to interpret the level of physiological impairment of the organism.

Keywords: Biomarkers; Differential sensitivity; Copper; Heart rate; Acetylcholinesterase; Metallothionein

1. Introduction

Rapid, easy to use biomarkers which signal exposure to and, in some cases, the adverse effects of chemical contamination are recognised increasingly as a cost effective method for identifying the in situ toxic effects of pollutants on biota (Galloway et al., 2002a). The use of biomarker techniques has not, however, been incorporated widely into routine environmental monitoring probably because there is controversy

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over how biomarker responses at different levels of biological organisation should be interpreted. Much time and effort has been spent standardising individual biomarkers and defining the range of responses that can be considered 'normal' for a particular organism (Wells and Balls, 1994; Viarengo et al., 2000), which is particularly important for comparing data between laboratories. There are, however, drawbacks to this validation approach as biomarker responses are known to vary considerably with environmental factors including temperature and salinity (Hauton et al., 1998), and the age of the organism (Depledge, 1993; Sukhotin et al., 2002). For multi-biomarker studies it may, therefore, be more useful to use a suite of biomarker responses to provide a 'diagnosis of stress', whereby, effects at the molecular level can be used to interpret the level of physiological impairment of an organism (Downs et al., 2001). Therefore, to help predict the potential of contaminants to damage ecosystems, laboratory data should be obtained linking the effects of biomarkers at the biochemical, cellular and physiological level (Depledge and Fossi, 1994; Viarengo et al., 2000). In such a holistic approach to environmental assessment, standardisation of individual markers of contaminant effects may be less important than interpreting how combinations of different biomarkers reflect the integrated toxic effect of a contaminant to an organism.

As well as interpretation of biomarker responses, there remains the question of species selection (Depledge et al., 1995). It is rare to see a comparison of different biomarker responses in different organisms. Most biomarkers have been validated in only a small number of species (mainly bivalve molluscs). This is a disadvantage to their widespread application for monitoring as a chosen indicator species may occur in only a limited number of habitat types and its biomarker responses might not reflect the sensitivity of other species or functional groups within a community. It is known that species' sensitivities to different contaminants (including copper) can vary by several orders of magnitude depending on various factors including geographic distribution, taxonomic group, or functional feeding group (Brix et al., 2001).

The aim of this work was to consider the differential sensitivity of copper at environmentally realistic concentrations to three marine invertebrates from different functional feeding groups and hence with different general physiologies. The species chosen were the blue mussel *Mytilus edulis* (filter feeder), the common limpet *Patella vulgata* (grazer) and the shore crab *Carcinus maenas* (omnivore). Based on acute toxicity data (48 h LC50s), *C. maenas* was expected to be most tolerant to copper with the bivalve mollusc more tolerant than the gastropod (Spear and Pierce, 1979). The responses of the animals were determined using a suite of biomarkers chosen to detect the toxic effects of copper at the population (survival), physiological (heart rate and total haemolymph protein), cellular (lysosomal stability) and biochemical (acetylcholinesterase (AChE) activity) level as well as a specific biomarker of metal exposure (metallothionein (MT) induction).

Copper was chosen as it is a widespread contaminant and its mode of action is well understood (Hebel et al., 1997). On exposure to copper, cellular stress is often apparent due to reduced lysosomal stability at relatively low copper exposures (Svendsen and Weeks, 1997; Viarengo et al., 2000). Copper is also neurotoxic to some invertebrates. For example AChE activity, more commonly employed as a biomarker of exposure to organophosphorous (OP) pesticides, exhibits concentration-dependant inhibition on exposure to a diverse range of metals (including copper) through mechanisms independent of the OP sensitive active site (Viarengo, 1989; Najimi et al., 1997). Physiological impairment as a result of copper exposure is manifest in a number of ways including protein catabolism. Haemolymph protein content is reduced on exposure to copper in crabs (Weeks et al., 1993) and mysid shrimps (Lin and Chen, 2001). Heart rate is decreased in molluscs exposed to elevated copper (Marchán et al., 1999; Curtis et al., 2000) and increased in crustaceans (Styrishave et al., 1995; Bamber and Depledge, 1997). Finally, induction of MT after exposure to metals can be protective against the effects of metal toxicity (Roesijadi, 1996). The amount and speed of MT up-regulation might have implications for the sensitivity of a particular species to copper. Sensitivity to copper can also depend on the homeostatic regulation of its uptake, storage and excretion (Depledge and Rainbow, 1990), regulation of membrane permeability and the amount of permeable membrane to body size over which copper can absorb (Newman and Heagler, 1991).

2. Materials and methods

2.1. Sample site

Between the 5 and 19 November 2001, animals were collected from Bantham, Avon Estuary, Devon, UK. *Mytilus edulis* and *Patella vulgata* were collected directly from rocks on the shore while *Carcinus maenas* was caught using a baited dropnet. Strips of slate with a limpet attached were removed and the slate gently peeled from the limpet's foot. This method of removing *P. vulgata* from the rock avoided any internal damage which could be caused by prising limpets off directly (personal observation). Animals were transported as quickly as possible to the laboratory in buckets containing seawater from the collection site, and allowed at least 48–96 h to acclimate to laboratory conditions before being exposed to copper.

2.2. Test solution

Exposure concentrations of copper were chosen with reference to published sublethal data on biomarker responses for each invertebrate group and environmental copper concentrations. Copper was added as cupric chloride hydrate ($CuCl_2 \cdot 2H_2O$) obtained from Sigma–Aldrich (UK). A primary stock solution was prepared in distilled water. Individual test solutions were obtained by adding the appropriate volume of the primary stock to the dilution water. The copper concentration in the dilution water was measured using atomic absorption spectroscopy on a Varium SpectrAA 600. Measured concentrations were 6.1, 38.5 and 68.1 μg $Cu\,l^{-1}$. These copper concentrations are environmentally realistic and are within the limits measurable in the water column of UK estuaries (DETR, 1998).

2.3. Experimental design

M. edulis, *C. maenas* and *P. vulgata* were exposed separately to copper for 7 days at concentrations of 0, 6.1, 38.5 and 68.1 μg $Cu\,l^{-1}$. For the molluscs, there were two replicates each of eight animals per tank at each exposure concentration. *C. maenas* was exposed in four replicates each of four animals per tank to avoid overcrowding. Each tank (regardless of species) contained 18 l of 0.45 μm filtered seawater with a salinity of 30. The dilution water was changed three times per week. Tanks were maintained in a temperature controlled room (15 ± 1 °C) with a 12 h light/dark cycle and were kept continuously aerated.

Animals were fed 2 h before the water was replaced. *M. edulis* (50–60 mm shell length) were fed algal cells of *Pavlova lutheri* (200 ml of 10^6 cells per litre). *P. vulgata* (40–50 mm shell length) were placed on glass plates which had been conditioned previously in seawater for 96 h. The plates were coated with a biofilm on which the limpets grazed. The presence of faecal material throughout the exposure period in tanks containing limpets confirmed that these animals were feeding. Only green male crabs (50–70 mm carapace width) were used in the copper exposures. Crabs were fed individually on gamma-irradiated frozen cockles, *Cerastoderma edule* (Tropical Marine Centre, Chorleywood, Herts, UK), until sated.

3. Biomarkers

3.1. Survival

Mortalities were recorded daily and dead animals were discarded. The criteria of death was a lack of response of the foot for *P. vulgata*, no valve closing for *M. edulis* and a lack of ability for the claws to pinch when stimulated for *C. maenas*.

3.2. Lysosomal stability

Lysosomal stability was measured in the haemocytes of each species using the neutral red retention (NRR) assay (Lowe et al., 1995). Haemolymph, taken from *M. edulis* (adductor muscle) and *P. vulgata* (pallial artery) was added to 0.5 ml molluscan physiological saline (0.02 M Hepes, 0.4 M NaCl, 0.1 M $MgSO_4$, 0.01 M KCl and 0.01 M $CaCl_2$; pH 7.4). To obtain haemolymph from *C. maenas* a needle was inserted into the haemocoel through the arthodial membrane at the base of the third walking leg and extracted into 0.5 ml of crab physiological saline (0.5 M NaCl, 11 mM KCl, 12 mM $CaCl_2 \cdot 6H_2O$, 26 mM $MgCl_2 \cdot 6H_2O$, 45 mM $Na_2HPO_4 \cdot 12H_2O$, 45 mM Trisma-Base, 1M HCl; pH 7.4). Cysteine (50 mg ml^{-1}) was added to the crab physiological saline as an anti-coagulant (Smith and Ratcliffe, 1978). Haemolymph (40 μl) was transferred onto a clean

glass slide and the cells were allowed to adhere for 15 min in a humidity chamber before incubating for a further 15 min in neutral red dye. Cells were observed under a high powered microscope after 15, 30, 60, 120 and 150 min, and the retention of the dye within the haemocyte lysosomes was recorded. Neutral red retention times for the two replicate tanks were determined by different operators to reduce subjective bias.

3.3. Acetylcholinesterase (AChE)

Measurement of AChE activity in haemolymph was preformed in triplicate using the cholorimetric method of Ellman et al. (1961), with acetylthiocholine iodide (ATCI) as a substrate and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) as chromogenic reagent as described by Galloway et al. (2002b). The concentrations of substrate required to saturate the enzyme, determined experimentally, were 1.5 mM ATCI for *P. vulgata*, and 3 mM ATCI for *M. edulis* and *C. maenas*. Each haemolymph sample or buffer blank (50 μl) was incubated with 150 μM DTNB, 270 μM in 50 μM sodium phosphate, pH 4 at 25 °C for 5 min to allow for the non-specific reaction between DTNB and haemolymph. Enzyme activity was recorded over 5 min after addition of the appropriate concentration of ATCI. AChE activity was expressed as specific activity (nm substrate hydrolysed min^{-1} mg^{-1} protein). AChE activity was detectable in the haemolymph of all three invertebrates but baseline levels varied by several orders of magnitude as indicated by different levels measured in control animals (Table 1). This difference might be relevant for the role of AChE in different species.

3.4. Haemolymph total protein (HTP)

The total protein in the haemolymph was determined spectrophotometrically using a commercial kit (BioRad™) with bovine serum albumin as a standard.

Table 1
Baseline levels of AChE activity (μmol ACTC min^{-1} mg^{-1} protein) in the haemolymph of three invertebrates

Species	*n*	Mean (±1 S.D.)
Carcinus maenas	13	0.0097 ± 0.0056
Mytilus edulis	16	1.339 ± 3.997
Patella vulgata	12	332.5 ± 240.1

3.5. Heart rate

Heart rate was recorded using the non-invasive computer aided physiological monitor (CAPMON) system for 1 h after exposure to copper for 7 days (Depledge and Andersen, 1990). An infra-red sensor was glued (Locite 314) to the shell/carapace of each animal over the position of the heart. As *P. vulgata* has a ridged shell, the shell was gently filed down using a Dremel™ before the sensor was attached. This procedure did not affect the heart rate of the limpet after a 1 h acclimation period (personal observation).

3.6. Metallothionein (MT) induction

MT quantification was performed on the midgut gland of *C. maenas* and, for ease of sampling, on the whole body tissues of *M. edulis* and *P. vulgata*. The samples (which had been stored previously at −80 °C) were ground to a fine powder under liquid nitrogen. A known weight of sample was dissolved in an ice cold solution of 1 mM DTT (dithiothreitol) and 1 mM PMSF (phenylmethylsulphonylfluoride). The solution was ultracentrifuged at 55,000 rpm for 70 min then MT was purified by extraction with ethanol and chloroform at 4 °C. The MT pellet was resuspended in 1 mM EDTA buffer (pH, 7.4) and reacted with 0.43 mM DTNB in 0.2 M phosphate buffer. Direct quantification of MT was determined spectrophotometrically at 412 nm using glutathione (GSH) as a reference standard (Viarengo et al., 1995; Pedersen et al., 1997).

3.7. Statistical analyses

The acute toxicity of copper to *P. vulgata* was calculated using the moving average angle method. For each species, the effect of copper on each biomarker was evaluated separately. For *M. edulis* and *C. maenas*, data were tested for normality (Shapiro-Wilks) and for equality of variances (Bartlett's test) before using analysis of variance techniques. If data did not conform to these assumptions, they were $\log_{10}$ transformed. If the assumptions for ANOVA were still not met, data were analysed using Kruskal–Wallis analysis of variance by ranks, followed by Mann–Whitney tests to highlight which treatments were different. Due to mortality of *P. vulgata* at higher concentrations, Students *t*-tests, assuming either equal or unequal

variances, were carried out to detect differences between the control and animals exposed to 6.1 μg $Cu\,l^{-1}$.

4. Results

4.1. Patella vulgata

There was a significant effect of Cu on the survival of *P. vulgata* with a 7-day LC50 of 16.8 (CL 11.1–23.1) μg $Cu\,l^{-1}$. All limpets exposed to concentrations of 38.5 and 68.1 μg $Cu\,l^{-1}$ died within 7 days with no mortality in the controls. A significant reduction in the retention time of neutral red dye in the haemocytes of *P. vulgata* was observed at 6.1 μg $Cu\,l^{-1}$ compared with the control (Students *t*-test, $P < 0.05$) (Fig. 1). AChE activity in *P. vulgata* was significantly increased at 6.1 μg $Cu\,l^{-1}$ compared with the control (Student's *t*-test, $P < 0.05$) (Fig. 1). Increased AChE activity in Cu-exposed limpets occurred in concert with significantly lower HTP content in animals exposed to 6.1 μg $Cu\,l^{-1}$ compared with the control animals (Student's *t*-test, $P < 0.05$) (Fig. 1). The heart rate of *P. vulgata* was significantly lower in animals exposed to 6.1 μg $Cu\,l^{-1}$ compared with the control (Students *t*-test, $P < 0.05$) (Fig. 1). There was also a significant decrease in the MT content of *P. vulgata* at 6.1 μg $Cu\,l^{-1}$ compared with the control (Students *t*-test, $P < 0.05$).

4.2. Carcinus maenas

There was no significant mortality of *C. maenas* over the range of Cu exposures used in the study. Reduction in the neutral red retention time of blood cells was observed in crabs exposed to 68.1 μg $Cu\,l^{-1}$ compared with crabs in the controls and other Cu exposures (One-way ANOVA, $F_{3,48} = 9.67$, $P < 0.05$) (Fig. 2). There was no effect on HTP in the copper exposed crabs but AChE activity in *C. maenas* was significantly inhibited compared with the control at the 68.1 μg $Cu\,l^{-1}$ (Kruskal–Wallis, $P < 0.05$) (Fig. 2). A significant increase in heart rate was observed for *C. maenas* at 6.1 μg $Cu\,l^{-1}$ compared with the control but not at 38.5 and 68.1 μg $Cu\,l^{-1}$ (one-way ANOVA, $F_{3,47} = 4.65$, $P < 0.05$) (Fig. 2). A significant induction of MT was observed in the mid-gut gland of

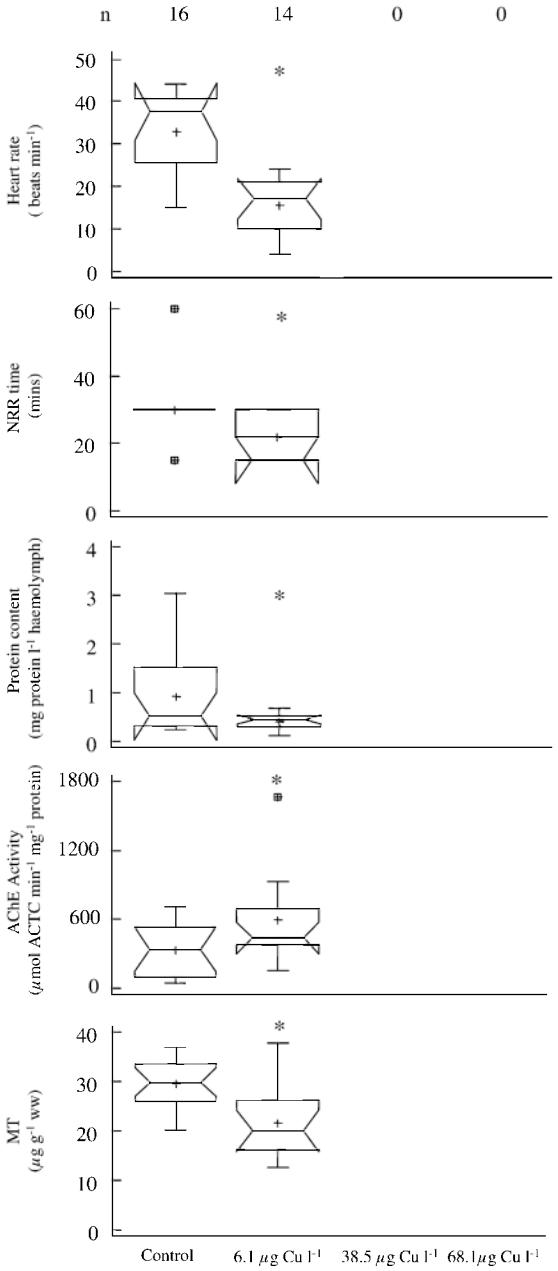

Fig. 1. The biomarker responses of *Patella vulgata* at different copper exposures. *n* is the number of animals surviving after 7 days and * indicates a significant difference at the 5% level. The box and whisker plots show the entire range of the data measured, with 75% of the data falling within the box plot area. The mean of the data is indicated by (+) and the median by the solid line across each box (—). Square boxes (□) and (+) outside of the box plots indicate data points that are considered outliers.

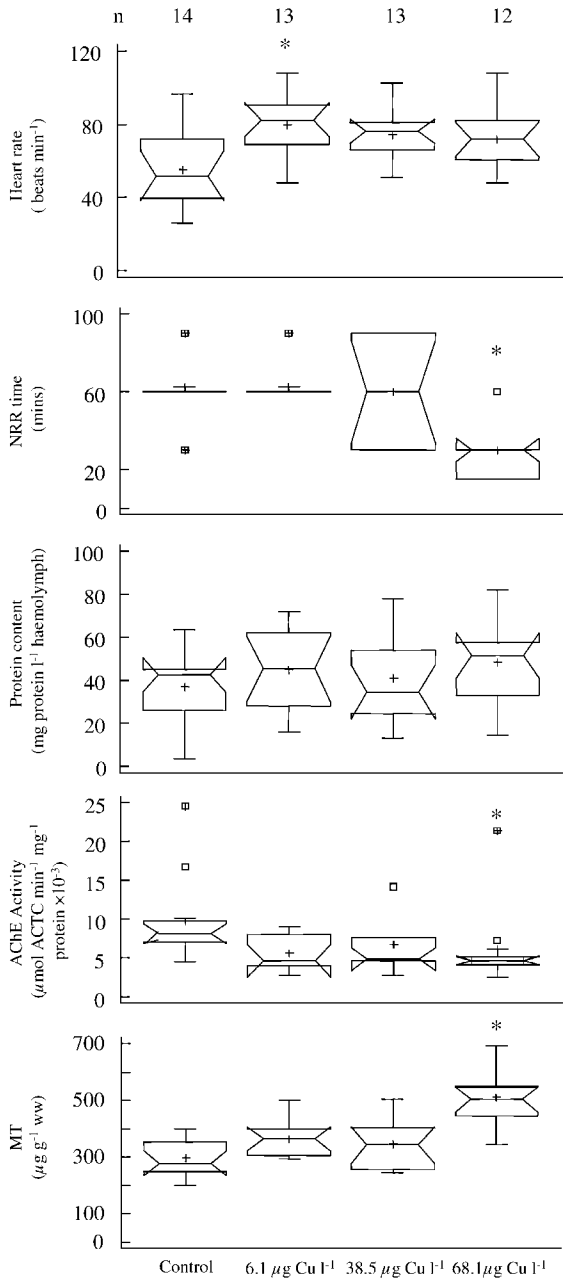

Fig. 2. The biomarker responses of *Carcinas maenas* at different copper exposures. *n* is the number of animals surviving after 7 days and * indicates a significant difference at the 5% level. The box and whisker plots show the entire range of the data measured, with 75% of the data falling within the box plot area. The mean of the data is indicated by (+) and the median by the solid line across each box (—). Square boxes (□) and (+) outside of the box plots indicate data points that are considered outliers.

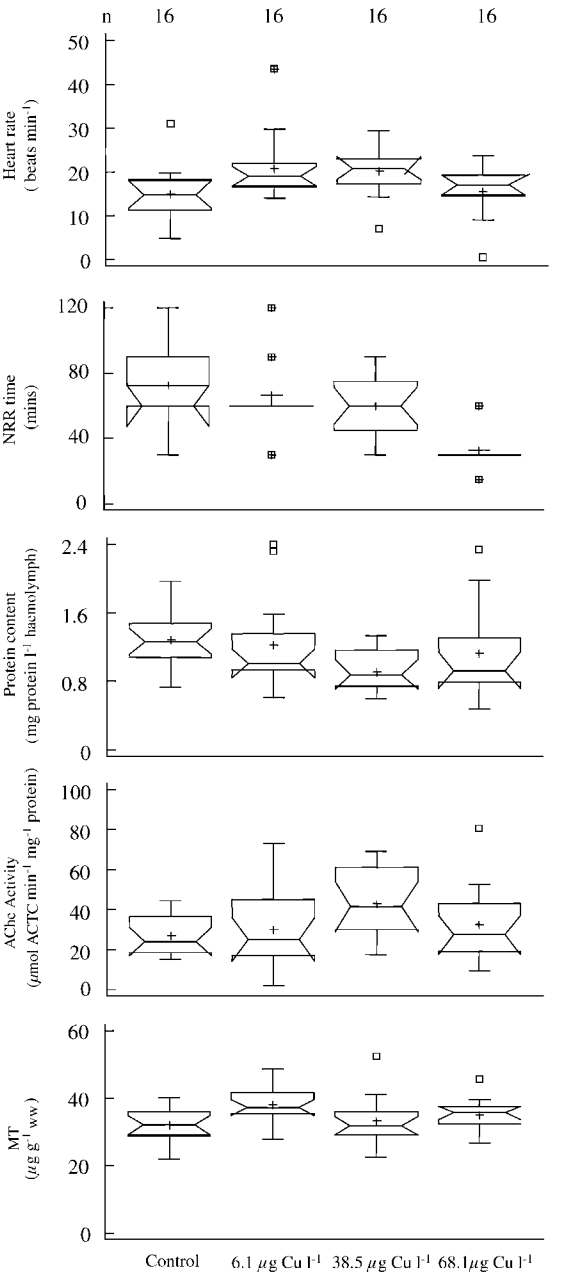

Fig. 3. The biomarker responses of *Mytilus edulis* at different copper exposures. *n* is the number of animals surviving after 7 days and * indicates a significant difference at the 5% level. The box and whisker plots show the entire range of the data measured, with 75% of the data falling within the box plot area. The mean of the data is indicated by (+) and the median by the solid line across each box (—). Square boxes (□) and (+) outside of the box plots indicate data points that are considered outliers.

Table 2
Copper exposure at which concentration significant dose dependant effects on biomarker responses were observed in each species

	NRR ($\mu g\ l^{-1}$)	AChE activity ($\mu g\ l^{-1}$)	HTP ($\mu g\ l^{-1}$)	Heart rate ($\mu g\ l^{-1}$)	MT ($\mu g\ l^{-1}$)	Rank stress index
P. vulgata	6.1 ↓	6.1 ↓	6.1 ↓	6.1 ↑	6.1 ↓	5/5
C. maenas	68.1 ↓	68.1 ↓	>68.1	>68.1	68.1 ↑	3/5
M. edulis	68.1 ↓	>68.1	68.1[a]	68.1[a]	>68.1	1/5

↓: significant decrease in response; ↑: significant increase in response at the 95% confidence level.
[a] Effects observed at 38.5 $\mu g\ Cu\ l^{-1}$ but not at 68.1 $\mu g\ Cu\ l^{-1}$.

C. maenas at 68.1 $\mu g\ Cu\ l^{-1}$ compared with the controls (Kruskal–Wallis, $P < 0.05$) (Fig. 2).

4.3. Mytilus edulis

There was no significant mortality of *M. edulis* over the range of Cu exposures used in this study. Significant reduction in the neutral red retention time of cells from *M. edulis* occurred only at 68.1 $\mu g\ Cu\ l^{-1}$ (one-way ANOVA, $F_{3,57} = 13.94$, $P < 0.05$) (Fig. 3). A significant decrease in HTP was observed at 38.5 $\mu g\ Cu\ l^{-1}$ compared with the control (one-way ANOVA, $F_{3,56} = 2.99$, $P < 0.05$) but there was no effect of copper on the AChE activity of *M. edulis* (Fig. 3). A significant increase in the heart rate of *M. edulis* was observed at 38.5 $\mu g\ Cu\ l^{-1}$ compared with the control (Kruskal–Wallis, $P < 0.05$) (Fig. 3). There was no induction of MT in *M. edulis* over the exposure range tested.

The copper concentrations giving a statistically significant dose dependant effect for each biomarker response in *P. vulgata*, *C. maenas* and *M. edulis* are summarised in Table 2.

5. Discussion

Under laboratory conditions, inter-species differences in sensitivity to environmentally relevant copper concentrations were reflected in the biomarker responses of the organisms. The order of relative sensitivity to copper was *P. vulgata*> *C. maenas*> *M. edulis*. This does not reflect acute toxicity data where *M. edulis* was more sensitive to copper than *C. maenas* (Spear and Pierce, 1979).

P. vulgata was highly sensitive to copper with no survival at 38.5 and 68.1 $\mu g\ Cu\ l^{-1}$. The available mortality data for *P. vulgata* exposed to copper confirm present findings. For example limpets maintained on rocks taken directly from the field showed 100% mortality after 7 days exposure to 100 $\mu g\ Cu\ l^{-1}$ (Marchán et al., 1999). Sublethal biomarker responses at all levels of biological organisation also reflected the sensitivity of *P. vulgata* to copper (Table 2). The biomarkers used in this study were chosen to link the presumed pathway of response to copper. Initial effects on cell membrane stability lead to changes in physiological processes (through inhibition of enzymes and protein breakdown), and these metabolic effects are apparent ultimately at the whole organism level in the form of changes in heart rate (and presumably oxygen consumption). In addition, the animals would be expected to attempt to detoxify the copper at the biochemical level through up-regulation of protective MT proteins.

Membrane permeability was severely affected by copper in *P. vulgata* as indicated by a reduction in NRR time in the haemocytes of limpets exposed to 6.1 $\mu g\ Cu\ l^{-1}$. One of the main modes of toxic action of copper is to reduce membrane permeability and, therefore, lysosomal stability is a particularly sensitive endpoint for demonstrating the effects of this metal as shown in several invertebrate species (Svendsen and Weeks, 1997; Ringwood et al., 1998; Viarengo et al., 2000).

Copper did not inhibit AChE activity in *P. vulgata*. Indeed, there was a significant increase in AChE activity at 6.1 $\mu g\ Cu\ l^{-1}$. In view of the high activity of AChE in *P. vulgata* haemolymph compared with that of *M. edulis* and *C. maenas* (Table 1), it is possible to speculate that the haemolymph enzyme may function in ways unrelated to cholinergic neurotransmission. Stress-induced alterations in cholinesterase transcription and morphogenic growth-factor-like activity have been described in both vertebrate (Sternfeld et al., 2000; Soreq and Seidman, 2001) and invertebrate models (Srivatsan, 1999), raising the possibility of a

physiological role for the increased AChE activity. Alternatively, neurotoxic effects may be masked by the net effect of copper on metabolic homeostasis. There was severe catabolism of protein in limpet haemolymph evident from significantly reduced HTP at 6.1 μg Cu l^{-1}. Problems of standardising AChE activity against a parameter which is itself variable, such as total protein, have been discussed by Radenac et al. (1998). In this case, apparent increase in mid-winter AChE levels was postulated to be a result of structural protein loss in less active mussels at low water temperatures while metabolically active enzyme levels were maintained (Radenac et al., 1998). Other parameters to which AChE could be standardised include body size or biomass but these endpoints are also variable and seasonal effects on growth and reproduction have been associated with apparent changes in AChE activity in bivalve molluscs (Escartin and Porte, 1997). Standardisation of AChE activity to HTP is, therefore, justifiable but caution must be observed when making assumptions about effects on AChE activity without first confirming the status of total protein levels.

Heart rate is used widely as a measure of metabolic activity and oxygen consumption in marine invertebrates (Depledge and Andersen, 1990; Bamber and Depledge, 1997). Reduced heart rate (bradycardia) appears to be a characteristic response to copper exposure in marine molluscs (Marchán et al., 1999; Curtis et al., 2000; de Pirro et al., 2001). In the present study, limpets exposed to 6.1 μg Cu l^{-1} were under severe metabolic stress as indicated by significant bradycardia. This decrease in heart rate supports the adverse responses indicated by the cellular (NRR) and molecular (AChE) biomarkers and suggests the limpets were in very poor condition. Progressively increasing bradycardia has been observed previously in *P. vulgata* on exposure to 100 μg Cu l^{-1} resulting in death within 7 days (Marchán et al., 1999). Marchán et al. (1999), however, observed no effect on heart rate in *P. vulgata* at 10 μg Cu l^{-1}. In the present study, the higher temperature (15 compared with 10 °C) may have increased the metabolism, uptake and toxicity of copper in *P. vulgata* and lower salinity (30 compared with 33) may have increased the bioavailability of the free metal ion thus increasing the sensitivity of *P. vulgata* to copper (Hall and Anderson, 1995). Other physiological parameters such as pedal mucus production (apparently related to limpet movement and activity) were affected by just 6 h exposure to 10 μg Cu l^{-1} (Davies, 1992).

Although elevated MT levels have been measured in *P. vulgata* obtained from copper and cadmium contaminated sites in the field (Noël-Lambot et al., 1980), MT was not induced by copper exposure in the present study. Instead, significant inhibition of MT was observed in *P. vulgata* exposed to 6.1 μg Cu l^{-1}. This response is consistent with the severe protein catabolism observed in the haemolymph of the limpets. Induction of MT might have been expected to increase the tolerance of limpets to copper by increasing their capacity to store metal ions (Roesijadi, 1996).

The shore crab *C. maenas* demonstrated intermediate species sensitivity to copper with three of the five biomarkers showing a dose–dependant response (Table 2). No mortality occurred but three of the biomarkers measured (NRR, MT and AChE activity) were significantly altered at 68.1 μg Cu l^{-1}, reflecting signs of stress. Copper concentrations at which biomarker effects are measurable in *C. maenas* occur in the water column of UK estuaries (DETR, 1998). The NRR assay has been used previously in field trials with *C. maenas* to demonstrate differences between polluted and non-polluted sites (Wedderburn et al., 1998; Astley et al., 1999). In this study, baseline NRR times were similar to those reported previously (Wedderburn et al., 1998) and the assay was sensitive enough to detect cellular stress at 68.1 μg Cu l^{-1}. In crabs, inhibition of AChE activity has been characterised in response to exposure to OP pesticides (Lundebye et al., 1997), but the present study also showed evidence for a neurotoxic effect of copper with inhibition of AChE at high copper exposures (68.1 μg Cu l^{-1}). Trace metals (particularly copper) inhibit AChE activity in some invertebrates (Bocquené et al., 1990; Najimi et al., 1997; Hamza-Chaffai et al., 1998), presumably through binding to protein SH residues (Viarengo, 1989). There was no apparent physiological impairment due to copper as indicated by the stability of protein metabolism and heart rate. Heart rate and HTP are both affected in crabs exposed to copper but only at exposures greater than 100 μg Cu l^{-1} (Weeks et al., 1993; Bamber and Depledge, 1997; Lundebye and Depledge, 1998). Lack of effect on physiological processes at higher copper concentrations may be due to the protective response of MT, which was significantly, induced at 68.1 μg

$Cu\,l^{-1}$. The pathway of copper toxicity is clearly apparent in *C. maenas* with biochemical and cellular responses affected by lower copper exposures than those known to affect major physiological processes (Table 2).

M. edulis was relatively insensitive to copper compared with *P. vulgata* and *C. maenas* as reflected in both survival and in the biomarker responses (Table 2). Copper did affect *M. edulis* at the cellular level with a significantly reduced NRR at 68.1 μg $Cu\,l^{-1}$. Previously, copper has been found to affect NRR in *M. edulis* at exposures as low as 40 μg $Cu\,l^{-1}$ (Viarengo et al., 2000). The NRR assay was the only biomarker affected significantly in all three species over the exposure range used in this study, suggesting that regardless of species, membrane stability is one of the first parameters to be disrupted by copper exposure. This is probably because lysosomal stability is a general indicator of organism stress and will reflect both direct copper toxicity (i.e. copper that has been taken up by haemolymph cells) and indirect copper toxicity (for example in *M. edulis*, where feeding was reduced, the effect of copper on NRR is likely to reflect oxidative stress through lack of food rather than direct copper toxicity). There was no inhibitory effect on AChE activity, heart rate, or MT induction up to 68.1 μg $Cu\,l^{-1}$ over 7 days. In mussels, a relationship between neurotoxicity and heart rate has been established with copper stimulating the cholinergenic nerves to the heart, however, this response was noted to occur at copper concentrations higher than those used in this study (Curtis et al., 2000). Although filtering activity was not measured experimentally in this study, it was apparent from the concentration of algae remaining after 2 h (and from observations of closed valves) for mussels exposed to 38.5 and 68.1 μg $Cu\,l^{-1}$, that they had reduced feeding rates. This lack of feeding would presumably reduce the uptake of copper by *M. edulis* (Davenport and Manley, 1978). The effects of copper on physiological processes in mussels are usually reported for animals that have been exposed to either higher copper concentrations (>100 μg $Cu\,l^{-1}$) or for longer periods (>7 days) (Curtis et al., 2000). Seven days is unlikely to have been long enough for MT to have been induced in mussels (Géret et al., 2002). Interestingly, slight physiological stress was indicated by a reduction in HTP and an increase in heart rate in mussels exposed to 38.2 μg $Cu\,l^{-1}$ compared with the control but not compared with mussels exposed to other copper concentrations.

The high sensitivity of *P. vulgata* to copper in this study is particularly surprising in view of the well-documented tolerance of this species to extreme environmental conditions (salinity, desiccation) (Jones and Baxter, 1985). In addition, *P. vulgata* have been found to be relatively tolerant to other contaminants (organophosphate pesticides) under similar exposure conditions as described for the present study (Browne et al., 2003). Sensitivity to copper can vary for many reasons, for example if the permeable area over which Cu can absorb is high. A number of other factors could have contributed to a reduced tolerance in limpets, for example, uptake of copper via food. Food supply is thought to be a major route of uptake for metals in invertebrates (Bryan, 1984; Depledge and Rainbow, 1990). While the conditions for exposure for all three species were standardised, the biofilm supplied as a food for *P. vulgata* may have been accumulating copper. Furthermore, as copper tolerance varies with season, it may be that *P. vulgata* were in poor condition when these experiments were conducted (November) post-spawning in September and October. Internal regulation of copper is not as well characterised for *P. vulgata* as for other invertebrates although there is evidence to suggest that a MT metal binding capacity for *P. vulgata* of 3.4 g-atoms mol^{-1} is lower than for oysters or mussels restricting the ability of limpets to regulate metals (Howard and Nickless, 1977).

Whatever the reasons for such low copper tolerance in *P. vulgata*, it is of some concern due to the well documented importance of this species to the functioning of rocky shore communities for example in *Fucus* recruitment (Hawkins and Hartnoll, 1983). Mass mortality of *P. vulgata*, due to oil dispersants used after the Torrey Canyon Spill, resulted in excessive algal growth on rocky shores (Southwood and Southwood, 1978). In view of this evidence, it is surprising that *P. vulgata* has not been considered previously as an indicator species for pollution events. The relative difficulty of collecting and maintaining limpets in aquaria may be a factor. Indicator species such as *M. edulis* and *C. maenas* have been demonstrated to be useful in biomarker studies for highlighting the effects of metal contamination. The apparent sensitivity of *P. vulgata* to copper in the laboratory suggests that this species may be useful for highlighting the more

subtle sublethal effects of contaminants in the field resulting in the protection of a wider range of species.

In summary, *P. vulgata* was the most sensitive species to copper with effects at all levels of biological organisation at measured copper concentrations of 6.1 μg $Cu\,l^{-1}$. In *C. maenas*, significant cellular and neurotoxic endpoints were apparent at 68.1 μg $Cu\,l^{-1}$. Copper also induced metallothionein production in crabs which may be conferring some tolerance to this metal. Over a 7-day exposure period, *M. edulis* was apparently the most tolerant species to copper with effects only being observed at the cellular level and only at 68.1 μg $Cu\,l^{-1}$. In all species, copper appeared to follow a similar mode of action with cellular and neurotoxic pathways being most sensitive to disruption by copper and physiological processes (protein and heart rate) being affected only under extreme copper stress.

In conclusion, this study illustrates how the prudent use of combinations of biomarkers can integrate overall physiological status with specific, molecular effects, providing a 'diagnosis of stress' for the organism. Understanding how biomarkers relate to each other on exposure to particular contaminants and how these responses vary between species is key to interpreting the effects of biomarkers in the field. In addition, if *P. vulgata* proves equally sensitive to other contaminants, inclusion of this species in routine biological surveys using biomarkers may ensure protection of a greater range of species than is currently used.

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