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A multiple biomarker approach to investigate the effects of copper on the marine bivalve mollusc, *Mytilus edulis*

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ABSTRACT

While copper (Cu) is considered to be an essential trace element for many organisms, overexposure to this metal can induce a wide spectrum of effects including DNA damage. Given that Cu is a highly relevant contaminant in the marine environment, we aimed to evaluate the induction of DNA strand breaks (using the comet assay) in haemocytes and concurrently also determined biological responses at higher levels of biological organisation in bivalve molluscs, *Mytilus edulis*, following exposure for 5 days to a range of environmentally realistic levels of Cu (18–56 $\mu g\,l^{-1}$). Prior to evaluation of genetic damage, the maximum tolerated concentration (MTC) was also determined, which was found to be (100 $\mu g\,l^{-1}$) above which complete mortality over the exposure period was observed. In addition to DNA damage, levels of glutathione in adductor muscle extracts, histopathological examination of various organs (*viz.*, adductor muscle, gills and digestive glands) and clearance rates as a physiological measure at individual level were also determined. Furthermore, tissue-specific accumulation and levels of Cu in water samples were also determined using ICP-MS. There was a strong concentration-dependant induction for DNA damage and total glutathione levels increased by 1.8-fold at 56 $\mu g\,l^{-1}$ Cu. Histological examination of the organs showed qualitatively distinct abnormalities. Clearance rate also showed a significant decrease compared to controls even at the lowest concentration (i.e. 18 $\mu g\,l^{-1}$; $P=0.003$). Cu levels in adductor muscle ($P=0.012$), digestive gland ($P=0.008$) and gills ($P=0.002$) were significantly higher than in the control. The multi-biomarker approach used here suggests that in some cases clear relationships exist between genotoxic and higher level effects, which could be adopted as an integrated tool to evaluate different short and long-term toxic effects of pollutants.

1. Introduction

The marine environment has long been subjected to contamination by heavy metals (e.g. As, Cd, Cr, Co, Cu, Pb, Hg and Ni), either of natural origin, such as volcanic activity, windblown dust or decaying vegetation, or from anthropogenic sources related to industrial processes and the use of antifouling paints (Rainbow, 2002). World production of copper (Cu) has increased in the last few decades and contamination by Cu has become increasingly prevalent in marine environments (IPCS, 1998), and this is likely to increase in coming decades. In relatively unpolluted coastal waters Cu concentrations are less than 5 ppb (Soegianto et al., 1999) but can reach 3 ppm in heavily polluted areas (Parry and Pipe, 2004). The increasing Cu concentrations in marine ecosystems are therefore a potential threat to living organisms. Although Cu is essential for normal functioning within organisms (e.g. being a cofactor in many enzymes), it can be toxic if present in high levels or if organisms are exposed chronically to low levels in the environment (Gaetke and Chow, 2003).

Long-term exposure of organisms to Cu is typically associated with impairment of feeding mechanisms (Nicholson, 2003), growth rates and reproduction (Fitzpatrick et al., 2008) and increased susceptibility to disease and to development of histopathological abnormalities (Zorita et al., 2006). Cu toxicity can result from its binding to DNA (Lloyd and Phillips, 1999) and proteins involved in DNA replication, transcription and repair, leading to impairment of these processes (Nor, 1987). However, Cu toxicity seems mostly to be a consequence of oxidative stress (Gaetke and Chow, 2003). Cu can directly induce oxidative stress by catalysing hydroxyl radical ($OH^{\bullet}$) production in a Fenton-like reaction leading potentially to damage to every class of biological molecule including DNA (Becker et al., 1996; Bremner, 1998; Gaetke and Chow, 2003). It is also possible that it can cause oxidative stress indirectly by binding to reduced glutathione (Friedman et al., 1989), thereby depleting a key antioxidant.

While there is relatively good understanding of Cu toxicity and bioaccumulation in aquatic organisms from earlier studies, to our knowledge there has been no integrated study where effects at

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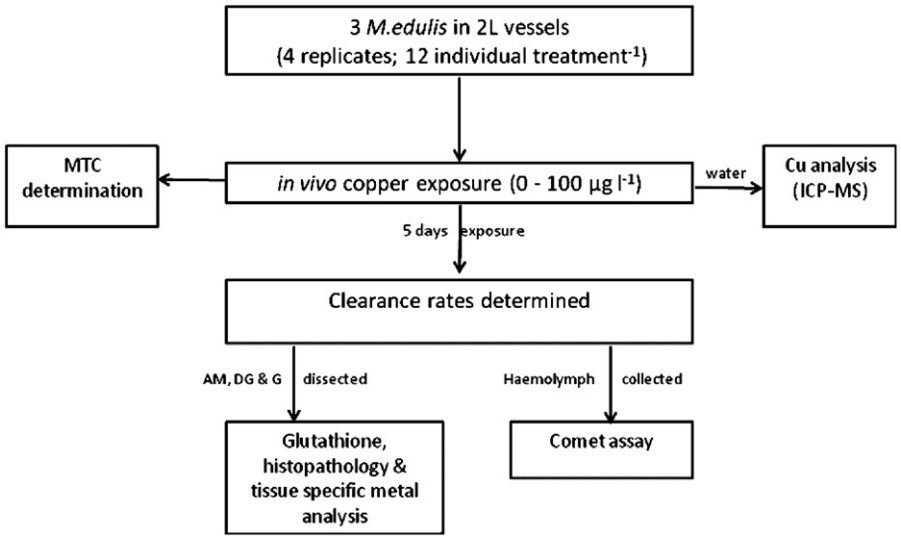

Fig. 1. Flow chart illustrating the integrated experimental design adopted in the study to evaluate genotoxicological, biochemical, structural and behavioural effects in *M. edulis*. AM=adductor muscle; DG=digestive gland; G=gills.

different levels of biological organisation have been concurrently assessed. Given this context, the objectives of this study were (a) to determine the effects of Cu exposure at different levels of biological organisation, i.e. biochemical (DNA damage and total glutathione content), tissue-specific bioaccumulation, histopathological changes and an effect at the whole organism level (clearance rate), and (b) to test for correlations between genotoxicity and the other biological responses. The aim of the latter was to assess how well these biological endpoints could be used either alone or in combination as tools to evaluate toxicological responses in the context of pollution monitoring. Among the numerous sessile organisms, bivalves are often used as sentinel organisms to study the biological effects of environmental contaminants (Livingstone et al., 2000). These are filter feeders, which are in direct contact with the contaminated compartments (sediment and water), and so can accumulate high levels of heavy metals in their soft tissues, providing a time-integrated indication of contamination with measurable cellular and physiological responses (van Duren et al., 2006). For these reasons we used the blue mussel, *Mytilus edulis*, as a model organism in the current study.

2. Materials and methods

2.1. Chemicals and animal collection

All chemicals were obtained from Sigma-Aldrich (Poole, UK) unless stated otherwise. Mussels (*Mytilus edulis*) of similar shell length (51–58 mm) were collected at low tide (January, 2008) from Port Quin, Cornwall, UK (grid reference: SW972 905), a relatively clean site (Canty et al., 2009). Although the mussels were collected at low tide, the seawater temperature at the time of collection was approximately 12 °C. After collection they were immediately transported in a cool box to the laboratory (less than 2 h), divided into three groups and placed in highly aerated tanks with filtered (<10 µm) sea water (ratio 1.5 animals l^{-1}), with a light: dark cycle of 12 h: 12 h. The mussels were kept at 15 °C and the water was changed twice weekly. They were fed with microalgae (4.6×10^9 cells l^{-1} seawater of *Isochrysis galbana*, Liquidfry Marine™, Interpet, Dorking, UK), and allowed to acclimatize for at least two weeks before use in experiments at this temperature in line with most of the studies carried out using this organism (Sheir et al., 2010; Trevisan et al., 2011). Seawater quality was checked during acclimatisation and during the experiments by measuring dissolved oxygen (95.2 ± 0.2%), pH (7.95 ± 0.03), total ammonia (0.05 ± 0.01 mg l^{-1}), temperature (15 ± 1 °C) and salinity (31.3 ± 0.12‰). Minor variations observed for different water quality parameters were considered to be within acceptable range. No spawning or mortality occurred in any of the stock tanks and the same batch of mussels was used throughout the experiment.

2.2. Experimental design

The overall experimental design is presented in Fig. 1. *M. edulis* individuals were exposed to 0, 18, 32, 56 or 100 µg l^{-1} copper in 2 l glass beakers (3 animals $beaker^{-1}$), with each exposure being carried out in quadruplicate (i.e. in total 12 individuals were analysed for each exposure concentration). The concentration range of Cu was based on an earlier *in vivo* study using bivalve molluscs (Bolognesi et al., 1999), which was slightly modified to fit the semi-logarithmic scale, widely used in ecotoxicological studies (Jha et al., 2000; Canty et al., 2009). A primary stock solution of Cu in distilled water was prepared using $CuSO_4.5H_2O$ (99% purity), and this was serially diluted to obtain the appropriate concentration for each exposure. To avoid the confounding effects of general toxicity, the maximum tolerated concentration (MTC) was determined as the highest concentration that elicits a specific toxic effect, mortalities being the final arbiter (Hutchinson et al., 2009). Exposure of *M. edulis* specimens to Cu was carried out over 5 days, with removal of mussels at the end of exposure for estimation of the DNA damage and histopathology. The 5 day exposure period matched that used in the earlier study (Bolognesi et al., 1999) where each treatment was renewed daily. Animals were not fed during the experiment.

2.3. Sample preparation for biochemical and DNA damage analysis

2.3.1. Collection of haemolymph samples

To access the adductor muscle the valves were pried apart approximately midway towards the posterior from the byssus using a fixed blade knife. Haemolymph was withdrawn via the posterior adductor muscle using a 1 ml sterile syringe fitted with a 21 gauge needle. Samples were diluted with an equal volume of physiological saline (0.02 M HEPES, pH 7.4 containing 0.4 M NaCl, 0.1 M $MgSO_4$, 0.01 M KCl and 0.01 M $CaCl_2$) and centrifuged at 800 g_{av} for 2 min to remove the haemocytes (Al-Subiai et al., 2009). The samples were then placed on ice until analysis.

2.3.2. Preparation of adductor muscle extract

The posterior adductor muscles from two mussels (about 0.2 g wet weight) were dissected out and were homogenised using the method of Al-Subiai et al. (2009) by grinding with a mortar and pestle using with acid-washed sand (0.50 g) and ice-cold extraction buffer at a ratio of 1:3 (w/v). The extraction buffer was 20 mM Tris-chloride, pH 7.6, containing 0.15 M KCl, 0.5 M sucrose and 1 mM EDTA, and freshly supplemented with 1 mM DTT and 100 µl protease inhibitor cocktail (Sigma P2714; reconstituted according to manufacturer's instructions). The crude homogenate was centrifuged for 35 min (10,500 g_{av}. at 4 °C) after which the supernatant was removed and stored at −80 °C until use. Adductor muscles from pairs of animals were pooled and used for the preparation of extracts. Hence, over the four replicate beakers six extracts were prepared, and the total glutathione contents of these were determined.

2.4. Determination of DNA strand breaks using the comet assay

Induction of DNA strand breaks using haemocytes was determined as described elsewhere (Jha et al., 2005). Briefly, the final cell-agarose suspension was spread over pre-coated slide with 1% normal melting point agarose (NMA). The slides were covered with chilled lysis solution, pH 10.0, and kept at 4 °C for