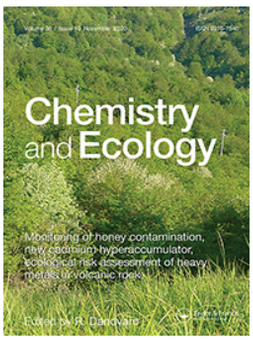

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Integrated response of the toxicity of environmentally relevant concentrations of copper in the backwater clam *Meretrix casta*

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ABSTRACT

Copper is released into the aquatic environment as a consequence of anthropogenic activities such as mining and runoffs of copper-containing pesticides and fertilisers. This may pose a threat to the health of the organisms inhabiting coastal water bodies or estuaries. The present study was therefore carried out to assess the toxicity of environmentally relevant concentrations of copper in the estuarine backwater clam *Meretrix casta*. In the experimental setup, clams were exposed to five concentrations of $CuCl_2$ (0.5, 1.0, 1.5, 2.0 and 2.5 µg/L) for 15 days in water. Biomarkers of genotoxicity (Micronucleus test and comet assay), oxidative stress (Catalase and malondialdehyde assays), acetylcholinesterase activity and condition index were employed to study the effects of copper on the clams. Genotoxicity, oxidative stress, acetylcholinesterase activity and condition index were found to be non-significant in all the concentration groups indicating that such low concentrations of copper were unable to induce any toxic response. However, using the Integrated Biomarker Response index, a concentration-dependent increase was observed indicating that the integration of these biomarker responses could give information on early signs of copper contamination in aquatic habitats. *Meretrix casta* could potentially be used as a sentinel species for monitoring copper contamination in the estuarine environment.

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Genotoxicity; Oxidative stress; acetylcholinesterase; bivalves

Introduction

Molluscs are often used as popular bioindicators of environmental pollution mainly due to their ubiquitous distribution in both terrestrial and aquatic environments around the world. They are particularly important in the functioning of ecosystems, associated with various ecological roles such as litter decomposition and contribution to large amounts of biomass at different trophic levels [1,2]. Many groups of higher animals including echinoderms, fish, birds and mammals feed on molluscs which are thus an important component of the coastal food chain.

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Copper is an essential trace element required by aquatic organisms for various metabolic functions and is also a vital component of haemocyanin in arthopods and molluscs. Copper is present naturally in the aquatic environment in minerals released from rocks or soil [3] where its concentration is relatively low. However, it may be introduced into water bodies through anthropogenic activities. This is because copper has a wide variety of industrial applications, essential for the production of wires, utensils, water pipes, fertilisers and pesticides and is also used as feed additives for livestock and poultry [4].

As a result, mining of copper ore, runoff of copper-based fertilisers and pesticides, leachates from copper pipes, etc. may have consequential effects on the aquatic environment, where its concentration can increase many fold [5–7]. Copper (free Cu^{2+} in particular) is toxic in aquatic environments at concentrations greater than 20 ppm [4]. As a result, it may accumulate in the tissues of aquatic organisms and will therefore enter the food chain [8,9]. A number of studies are available that reveal the toxic effects of copper in aquatic organisms such as fish [10–13], crustaceans [14,15] and molluscs [5,14,16,17]. These effects include DNA damage, oxidative stress, behavioural changes and physiological disturbances. In bivalves, the accumulation of copper may cause alterations in the structure of gills and the digestive gland [18,19] and may also hinder their immune activity [20,21]. Besides these effects, high concentrations of copper may even induce burrowing behaviour in bivalves as a stress response [22].

The genotoxicity of copper in aquatic organisms may occur due to its direct effect on the genome by copper ions interacting directly with nucleic acids. In addition, copper may also interact with other molecules such as water to give rise to highly reactive molecules called reactive oxygen species (ROS) that are often implicated in the cellular oxidative stress within organisms [23–25]. However, these ROS can also be produced under natural conditions by normal basal metabolism or by the influence of environmental factors. Naturally produced ROS can be easily reduced by antioxidants that are produced by the body. Conversely, the production of ROS mediated by environmental stressors build up to much higher levels and can overcome the antioxidant defences leading to a state of oxidative stress within the body. Such an imbalance may cause oxidative modification of various cellular macromolecules (such as DNA, proteins and lipids) and organelles. Therefore, these ROS can affect various cellular processes which may result in structural tissue damage or the induction of cell death. DNA is one of the key cellular components that are highly susceptible to the action of ROS [26,27].

Trace metals occur in almost all the coastal areas of India due numerous anthropogenic activities. As a consequence, various aquatic organisms are exposed to these metal pollutants. The aquatic organisms of concern are those that are consumed by the local people such as bivalves, crustaceans and fish. The uptake of such pollutants by these organisms could lead to adverse effects within their bodies leading to a weakened / diseased state with decreased nutritional value and could even lead to death thereby resulting in a reduced catch [28,29].

A multi-biomarker approach would provide a better understanding of the mechanisms of toxicity that occur in organisms exposed to copper. Therefore, this study was carried out to assess the genotoxicity, biochemical alterations and physiological condition in the backwater clam *Meretrix casta* exposed to low, environmentally relevant concentrations of copper.

Materials and methods

Quality assurance and quality control

The appropriate quality assurance methods of sample handling, preparation, and preservation were carried out in accordance with APHA, AWWA, WEF [30]. All chemicals used were of analytical grade procured from Himedia (Himedia, India) unless specified otherwise.

Collection and maintenance of Meretrix casta

Meretrix casta (Estuarine backwater clam) was selected for the present study as it is consumed by a majority of the Goan coastal population and also due to its availability in the Goan estuaries throughout the year. The clams (shell length 4.5 ± 0.75 cm) were collected in April 2017 from the intertidal zone from Palolem which is a pristine beach with no known industrial activity or anthropogenic stress [29,31] in Goa and transported alive to the laboratory. They were then allowed to acclimatize in aquaria containing ordinary seawater from Palolem for 30 days. The water conditions were maintained as follows: temperature 25°C, pH 7.5 and salinity 25 ppt. The water was changed once daily to reduce fecal contamination.

Experimental set-up

Clams were distributed in groups, each containing 10 individuals and were used for dose-response studies. A range of five different concentrations of $CuCl_2$ (0.5, 1, 1.5, 2 and 2.5 µg/L) were selected based on the environmental levels reported by us along the coast of Goa [29]. The clams were exposed to these doses in triplicates for a period of 15 days. The water was completely discarded and renewed daily with the respective concentration of $CuCl_2$. A group of clams was maintained in parallel without any pollutant exposure (negative control). Another group of clams was exposed to $CdCl_2$ (0.75, 1.5 and 3.0 µg/L), a well-known toxicant [32,33] to validate the various tests in the present study.

Cell viability

Prior to the comet assay and micronucleus test, the cell count and cell viability of the gill cells were checked to ensure that there were enough living cells to perform the assay employing the trypan blue dye exclusion test [34]. The samples showing more than 90% viability and a cell count of a minimum of 10^6 cells/ml were only used for the tests.

Genotoxicity tests

Micronucleus test

The micronucleus (MN) test was performed as per the protocol by Baršiene et al. [35]. The bivalves were dissected and the gills were isolated and rinsed in phosphate buffered saline (pH 7.4). A small part of the gill tissue was gently nipped with tweezers in a few drops of acetic acid-methanol mixture (1:3) and was smeared on a clean glass slide and allowed to dry. The cells were then fixed in methanol for 5 mins and then stained with 10% Giemsa.

The percentage of micronuclei (% MN) was recorded by scoring 2,000 intact cells per clam using an Olympus BX53 trinocular research microscope. Micronuclei (MN) were identified as as spherical or ovoid-shaped bodies in the cytoplasm completely separated from the main nucleus having a diameter of 1/3 - 1/20 of the main nucleus with similar colour, texture and optical features.

Single cell gel electrophoresis (comet assay)

The comet assay was performed following the protocol of Lee and Steinert [36]. All the steps were carried out in dim light so as to prevent photo-oxidation of DNA. Gill tissue was gently minced in phosphate buffered saline (pH 7.4) and a clear cell suspension was obtained by filtering out the tissue debris using a muslin cloth. The suspension of gill cells was then mixed with 0.5% low melting agarose and placed over a layer of 1% normal melting agarose on frosted microscopic slides. Another layer of 0.5% LMA was overlaid over the cell suspension layer. The cells were then subjected to lysis by placing the slides in a lysing solution containing 10 mM Tris, 100 mM Na_2EDTA, 2.5M NaCl, 10% DMSO and 1% Triton-X at 4°C, overnight. The slides were then placed in unwinding buffer (pH 10) for 20 min for the unwinding of DNA. Electrophoresis was then performed for 30 min at 300 mA, 25 V (Biorad electrophoresis unit). Following electrophoresis, the slides were placed in neutralisation buffer (400 mM Tris base, pH 7.5) for 5 min. The cells were then visualised by placing a few drops of ethidium bromide on the gel. The slides were examined using the green filter of a fluorescence microscope (Olympus BX53). The slides were screened for random, non-overlapping cells and the % tail DNA was analyzed and recorded with the help of the comet assay software, CASP [37].

Biochemical analysis

Bivalves were dissected and their whole soft body tissues were collected and homogenised in 50 mM of Tris buffer (pH 7.4) containing 0.3M sucrose and 1 mM EDTA. This suspension was then centrifuged at 10,000 xg for 20 min at 4°C and the supernatant was collected and used for the biochemical analysis.

Catalase assay

Catalase activity was carried out based on the decrease in absorbance of the test sample by the decomposition of H_2O_2 [38]. The reaction mixture consisted of 0.1 ml of the homogenate and 13.2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0). Using a multiwell plate reader (Analytical Technologies Ltd.), the reduction in absorbance was measured at 240 nm at 25°C over 3 minutes. Total protein concentration was measured by Bradford's method [39] and was used for subsequent calculation of CAT. The activity of catalase (CAT) was expressed as µmol $H_2O_2^{-1}min^{-1}$mg protein.

Malondialdehyde assay

The malondialdehyde (MDA) assay which is used to test lipid peroxidation in the soft tissues of bivalves was carried out using a commercial kit (North West Life Science Specialities- NWK-MDA01). The assay is based on the reaction of malondialdehyde with thiobarbituric acid (TBA) forming a pink MDA-TBA_2 adduct that absorbs strongly at 532 nm. To minimize the oxidation of lipids, butylated hydroxytoluene (BHT) and EDTA were added

to the reaction mixture containing the sample homogenate. The activity of MDA was expressed as nmol $MDA^{1}min^{-1}mg$ protein.

Acetylcholinesterase assay

The acetylcholinesterase (AChE) activity was measured in the whole soft tissues of bivalves as per Ellman et al. [40] with modifications as described by Galloway et al. [41]. Sample homogenate (50 µL) was incubated with 150µl DTNB (270µM in 50 mM sodium phosphate pH 7.4) at 25°C for 5 min in microtitre plates. The reaction was initiated by the addition of 3 mM acetylthiocholine iodide and the absorbance was measured at 412 nm. The activity of AChE was expressed as nmol $thiocholine^{-1}min^{-1}mg$ protein.

Condition index

Bivalves were cleaned, dissected and the soft tissues were carefully separated from the hard shells. Both the soft tissue and the shells were placed separately in an oven (REMI) at 60°C overnight and their dry weights were determined [42]. Condition index (CI) was then calculated as follows:

$$Conditionindex = \frac{\text{Dry soft tissue weight (g)}}{\text{Dry shell weight (g)}} \times 100$$

Statistical analyses

Statistical analyses of the data were carried out using IBM SPSS 23 statistical software package. The data were tested to meet the assumptions of normality and homogeneity using Levene's test and Shapiro-Wilk's test respectively prior to subsequent analyses by linear models. A one-way ANOVA was applied to test the effect of concentration on the % MN, % Tail DNA, CAT, AChE, MDA and CI with a post hoc Dunnet's test to compare the different groups with the control group. Pearson's correlation was carried out to test the relationship between all the parameters. The Integrated Biomarker Response Index (IBR) was carried out using the protocol outlined by Devin et al. [43]. The IBR [44] is a statistical test that compares all the biomarkers with each other, providing a value that integrates all these responses at once. IBR was calculated as the mean of (k-1)! biomarker arrangements. The data were considered to be statistically significant at $p < 0.05$.

Results

Data of the DNA damage, oxidative stress parameters along with AChE activity and CI induced by $CdCl_2$ (positive control) are indicated in Table 1. All concentrations of $CdCl_2$ induced significant DNA damage in the form of % MN and % Tail DNA. CAT activity and MDA levels were found to increase significantly at all concentrations of $CdCl_2$. Subsequently, AChE and CI was found to decrease significantly with increasing concentrations of $CdCl_2$.

Results of the DNA damage in the form of % MN induced by $CuCl_2$ are presented in Figure 1. The % MN did not increase significantly with increasing concentrations after 15 days of exposure. The variation across the concentration groups was also found to

Table 1. Validation of the biomarkers in the clam Meretrix casta exposed to different concentrations of CdCl2 as the positive control. Statistical differences are in relation to the negative control (NC).

Biomarker	Concentrations of $CdCl_2$			
	NC	0.75 µg/L	1.5 µg/L	3 µg/L
% MN	0.14 ± 0.05	0.25 ± 0.06*	0.47 ± 0.05***	0.75 ± 0.07***
% Tail DNA	2.57 ± 0.89	5.86 ± 0.79*	9.22 ± 0.94***	14.3 ± 1.12***
CAT	5.84 ± 0.57	8.27 ± 0.74*	11.87 ± 0.69***	15.98 ± 0.88***
MDA	3.74 ± 0.79	5.03 ± 0.81*	7.63 ± 0.91**	10.9 ± 1.05***
AChE	31.86 ± 1.45	29.35 ± 1.16*	26.87 ± 1.42***	21.72 ± 1.26***
CI	0.23 ± 0.03	0.21 ± 0.03*	0.19 ± 0.04***	0.16 ± 0.04***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

be non-significant using one-way ANOVA ($F = 2.139$, $p = 0.095$). Further, the % Tail DNA was found to increase slightly with increasing concentration, however, this increase was found to be non-significant when compared against the control using Dunnet's post hoc test (Figure 2). The overall variation between the treatment groups was also non-significant ($F = 1.781$, $p = 0.155$). The biomarkers of oxidative stress CAT and MDA (Figure 3 and Figure 4) were both non-significant compared to the control values after 15 days of exposure ($F = 0.234$, $p = 0.944$ and $F = 0.707$, $p = 0.234$ respectively). The data of AChE activity in the clams exposed to $CuCl_2$ is represented in Figure 5. No significant difference in AChE values were recorded in the soft tissues of the clams exposed to various concentrations of Cu ($F = 0.579$, $p = 0.715$). The condition index which is a measure of the physiological well-being of an organism was also relatively unaltered in the Cu-exposed bivalve groups compared to the control ($F = 0.195$, $p = 0.961$) (Figure 6).

The results of the Pearson's correlation are given in Table 2. A significant positive correlation exists between % MN and % Tail DNA ($p < 0.01$). Similarly, the activity of CAT was found to be significantly positively correlated with both % MN and % Tail DNA ($p < 0.05$ and $p < 0.01$ respectively). MDA was found to be positively correlated with % MN and CI was correlated with MDA as well as CAT.

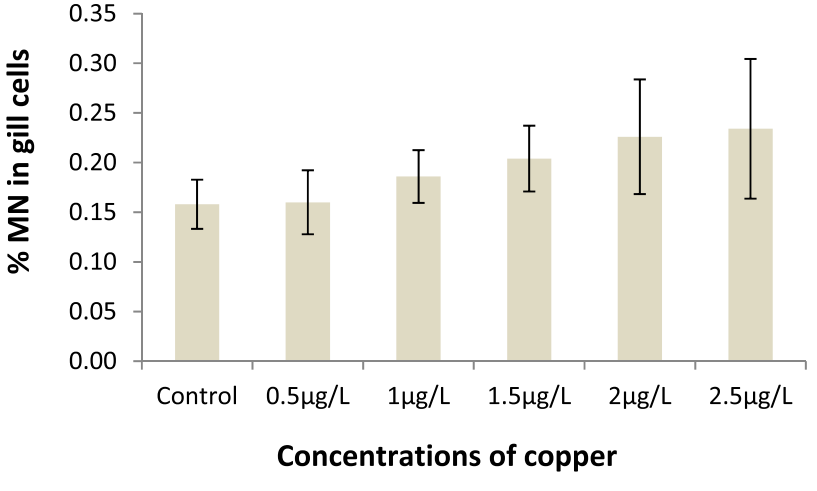

Figure 1. MN (%) in the gill cells of M. casta exposed to various concentrations of CuCl2. Data are expressed as mean ± SD.

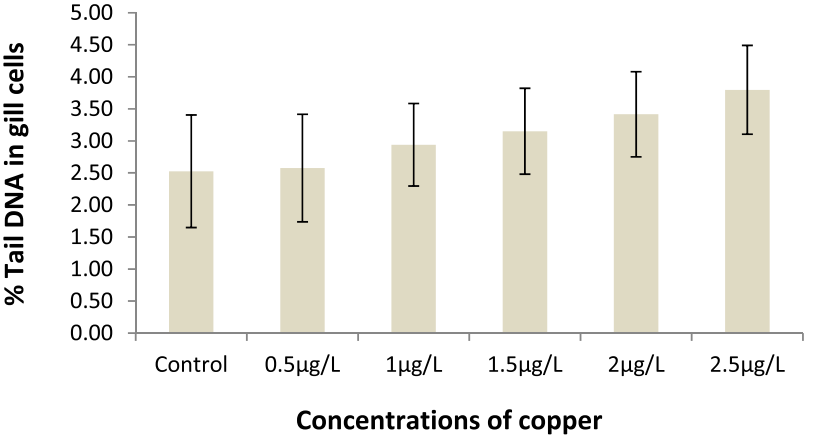

Figure 2. Tail DNA (%) in the gill cells of M. casta exposed to various concentrations of CuCl2. Data are expressed as mean ± SD.

The values of the IBR index are plotted in Figure 7. as the mean of three different biomarker arrangements. Although significant differences were not observed within individual biomarkers, the IBR was found to increase with increasing concentration of $CuCl_2$ with the highest response being seen at the highest concentration group.

Discussion

In the present study, $CuCl_2$ did not induce significant toxic effects in the clam *M. casta* with any of the concentrations studied. Although DNA damage in the form of % tail DNA was seen to increase, it was found to be non-significant. This is in agreement with the studies of Vernon and Jha [45] in which they could not find significant DNA damage (% tail DNA) in

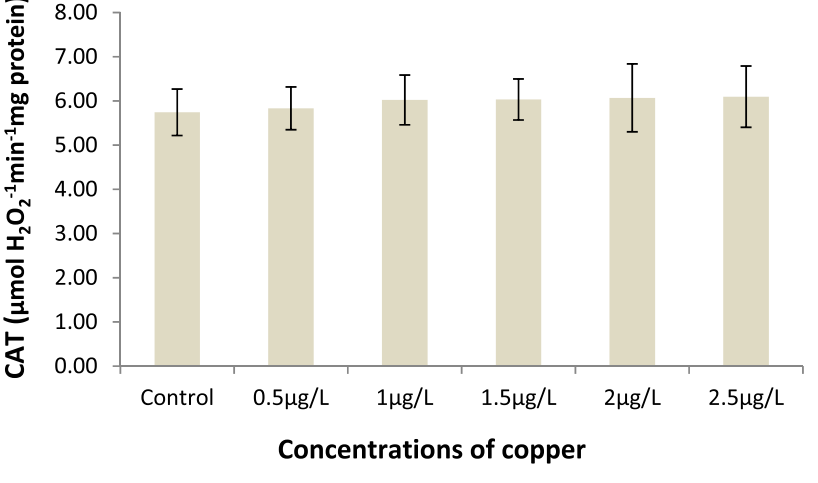

Figure 3. CAT in the soft tissues of M. casta exposed to various concentrations of CuCl2. Data are expressed as mean ± SD.

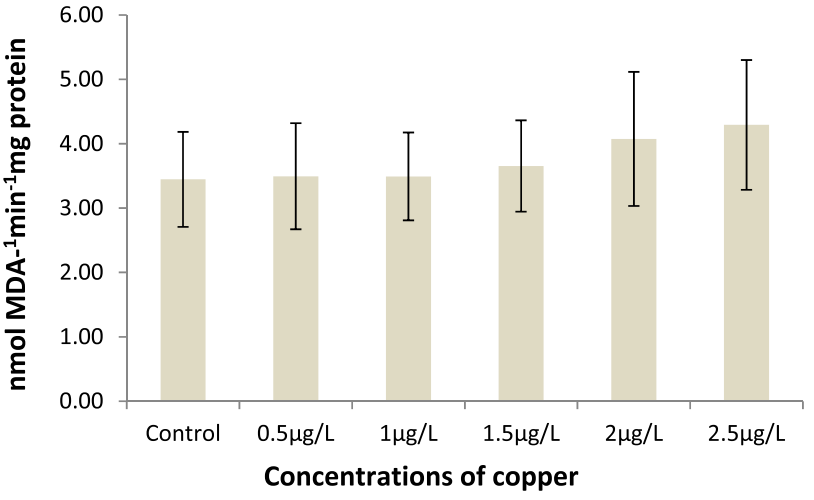

Figure 4. MDA in the soft tissues of M. casta exposed to various concentrations of CuCl2. Data are expressed as mean ± SD.

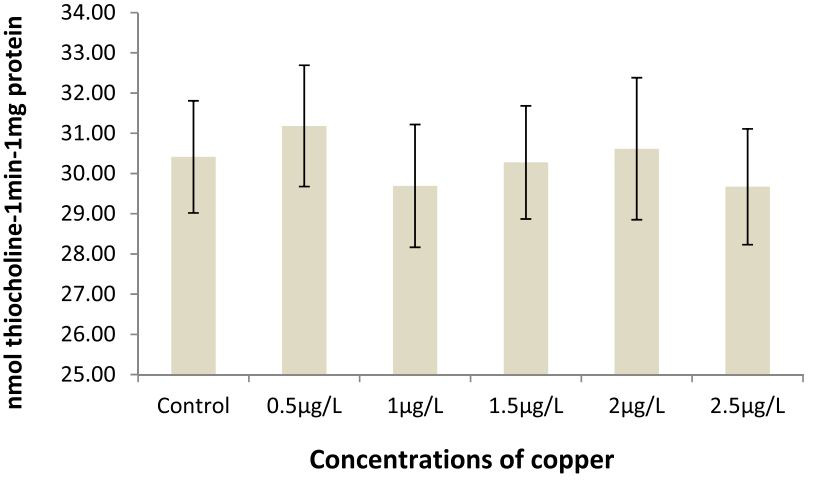

Figure 5. AChE in the soft tissues of M. casta exposed to various concentrations of CuCl2. Data are expressed as mean ± SD.

Table 2. Pearson's correlation between the % MN, % Tail DNA, CAT, MDA, AChE and CI.

	% MN	% Tail DNA	CAT	MDA	AChE	CI
% MN	-					
% Tail DNA	0.56**	-				
CAT	0.48*	0.61**	-			
MDA	0.52**	0.22	0.47**	-		
AChE	-0.01	0.01	0.33	-0.19	-	
CI	0.16	0.05	0.51**	0.44*	-0.11	-

** $p < 0.01$, * $p < 0.05$.

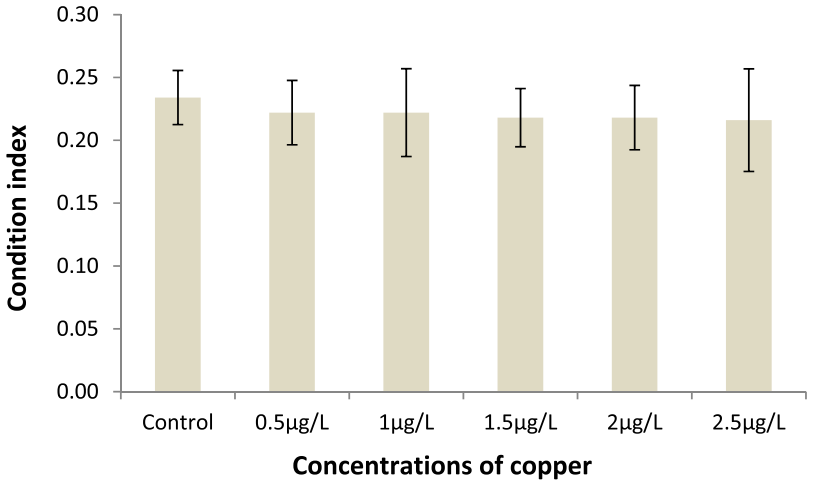

Figure 6. Condition index of M. casta exposed to various concentrations of CuCl2. Data are expressed as mean ± SD.

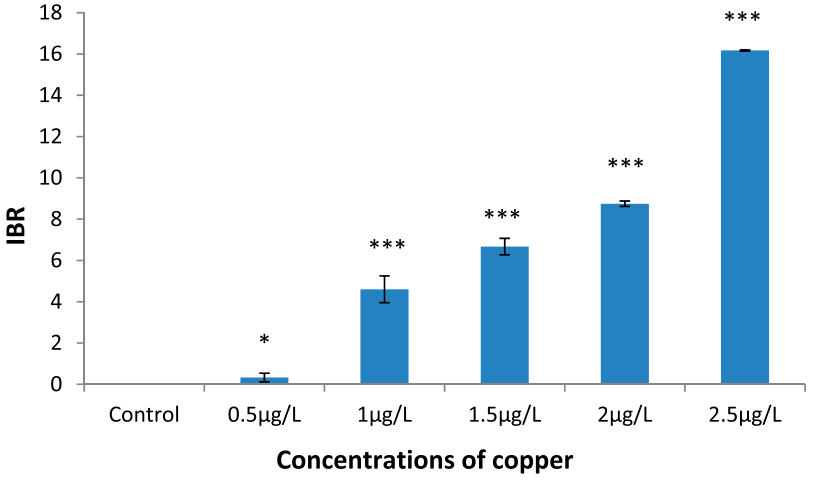

Figure 7. Integrated Biomarker Response in M. casta exposed to various concentrations of CuCl2. Data are expressed as mean ± SD (* $p < 0.05$, *** $p < 0.001$).

the bivalves *Mytilus galloprovincialis* and *Dreissena polymorpha* exposed to 18 µg/L of copper. Similarly, in another study, DNA in the hemolymph of the mussel *Perna perna* exposed to 12.5 µg/L of copper, showed no significant damage when measured by the comet assay [46]. The % of MN was also found to be non-significant compared to the control for all the concentrations of copper in the present study. This observation finds similarity with that of Vernon and Jha [45] in which the % MN was found to be non-significant in the gill cells of the bivalves *Mytilus galloprovincialis* and *Dreissena polymorpha* exposed to 18 µg/L of copper, but increased at higher concentrations. Our observation complemented by the above reports infers that copper (in the studied concentrations) is not capable of inducing significant DNA damage in bivalves.

Further, catalase activity was also found to be non-significant compared to the control. This observation is on par with that of Zhang et al. [47] in which they did not observe a significant difference in catalase activity of the bivalve *Chlamys farreri* exposed to 2.68 µg/L of copper. In a similar study by Xu et al. [48], catalase activity was found to be non-significant in the mussel *Mytilus coruscus* exposed to 2 µg/L of $CuCl_2$ for a period of 6 days, but subsequently increased significantly after 12 days of exposure. A possible reason for non-significant CAT activity may be due to the inability of these concentrations of copper to induce the formation of ROS in the tissues of the clams. Additionally, the level of ROS produced might not have been able to overcome the antioxidant defenses.

Malondialdehyde, which is formed as a result of oxidative damage to lipids, was non-significant in the soft tissues of *M casta* exposed to all concentrations of $CuCl_2$ in the present study. Low MDA levels could be due to the low concentrations of ROS which were unable to react with lipids. This observation is supported by a number of studies on bivalves exposed to copper. For example, Zhang et al. [47] observed non-significant levels of MDA in the bivalve *Chlamys farreri* exposed to 2.68 µg/L of copper. A similar observation was reported by Pytharopoulou et al. [49] in which *Mytilus galloprovincialis* exposed to various concentrations of $CuCl_2$ (10-40 µg/L) did not induce any significant alterations in MDA levels. Further, high MDA levels have also been associated with significant DNA damage due to the formation of DNA adducts [50].

Acetylcholinesterase (AChE) inhibition is a well-established biomarker of neurotoxic effects and commonly used in aquatic biomonitoring. The exposure of copper did not significantly alter the activity of AChE in the present study. This observation finds similarity with that of Moncaleano-Niño et al. [51] in which they reported a non-significant alteration of cholinesterase activity in the oyster *Saccostrea sp*. exposed to various concentrations of copper for a duration of 96 h. In another study conducted by Bonnard et al. [22], the alteration in AChE activity in the bivalve *Scrobicularia plana* exposed to 25 µg/L of copper was found to be non-significant. However, at higher concentrations of copper, significant inhibition of AChE has been reported [22,52].

A decrease in condition index usually indicates the deteriorating health of the organism as a consequence of exposure to toxic substances. However, in the present study, the physiological CI of the clams remained unaltered at all the concentrations of $CuCl_2$. This observation could possibly be compared with the studies of Pampanin et al. [53] in which the CI values were not correlated with the accumulation of Cu in the soft tissues of native and transplanted *M. galloprovincialis* in the canals of Venice. Our findings can also be compared with that of Osuna-Martínez et al. [54] in which they did not observe any significant correlation between CI values and Cu accumulation in the oysters *Crassostrea gigas* and *Crassostrea corteziensis* under laboratory conditions.

The results of the Pearson's correlation test indicate a significant relationship between % MN as well as % Tail DNA. Although both the tests measure DNA damage, % Tail DNA reflects the damage done to DNA soon after exposure in the form of DNA strand breaks whereas % MN reflects damage done to the DNA which is accumulated over a period of time and may be difficult to repair [55]. Therefore, short term damage may convert to long term damage with prolonged exposure to a particular DNA damaging agent [56]. Further, a significant relationship was also observed between % tail DNA and CAT activity. This could be due to the presence of reactive oxygen species which is formed

as a result of Cu exposure and is ameliorated by CAT. However, ROS can also affect DNA when the level of ROS increases above the level of detoxification.

The result of the IBR shows a significant increase in the biomarker responses in each exposure group and reached a peak at the highest concentration of Cu. Although significant variation was not observed within the test biomarkers, the combined effects of these biomarkers showed a significant concentration-dependent increase. The present study focused on toxic effects of relatively low concentrations of copper compared to other studies conducted by other researchers which used much higher concentrations. Therefore, the IBR could be used as a statistical model in bivalves to give early warning signs of copper contamination prevailing in water bodies.

Conclusion

The present study revealed copper (at low concentrations upto 2.5µg/L) was unable to induce significant alterations in DNA damage, oxidative stress, AChE and condition index in the backwater clam *Meretrix casta*. On the other hand, the integrated biomarker response index showed a concentration –dependent increase which could potentially be used to give information on early signs of copper contamination at a particular site. The DNA damage parameters showed a positive association with each other, whereas the % tail DNA was associated with CAT activity, indicating that the mechanism of Cu genotoxicity could probably be due to oxidative damage. In conclusion, *Meretrix casta* could potentially be used as a sentinel species for monitoring contamination of coastal or estuarine water bodies by copper.

Disclosure statement

No potential conflict of interest was reported by the authors.

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