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Full Length Research Paper

Accumulation of heavy metals from single and mixed metal solutions by the gastropod mollusc *Tympanotonus fuscatus* linnaeus from a Niger Delta estuary: Implications for biomonitoring

Erema R. Daka[1]*, Ibarakumo Ifidi[2] and Solomon A. Braide[3]

[1]Department of Applied and Environmental Biology, [2]Institute of Geosciences and Space Technology, and [3]Institute of Pollution Studies, Rivers State University of Science and Technology, PMB 5080, Port Harcourt, Nigeria.

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The usefulness of the intertidal gastropod *Tympanotonus fuscatus* L as a biomonitor of heavy metals in tropical estuaries was assessed. The periwinkles were collected from a site in the upper Bonny Estuary, Southern Nigeria and exposed in a series of experiments either singly or binary mixtures to copper, zinc and cadmium. The accumulation of Cd was monotonic with increase in exposure concentration with a highly significant linear regression (r^2 = 0.999, p<0.001). However, no significant regressions were obtained in the accumulation of Zn (r^2 = 0.018, p=0.828) and Cu (r^2 = 0.436, p=0.125); in the case of Cu a negative relationship was apparent. Bioaccumulation factors followed the order Cu>Zn>Cd but those for Cu and Zn showed reduction as the exposure concentration increased suggesting regulation of these metals. In accumulation from binary mixtures, Cu was synergistic to Cd accumulation in combinations involving 0.05 mg/L Cu but antagonistic for 0.5 and 1.0 mg/L Cu combinations. Zn was antagonistic to Cd accumulation in mixtures with 0.05 and 1.0 mg/L Zn. Cd did not show any effect on Zn accumulation in any of the mixtures but it was antagonistic to Cu accumulation in some combinations (1.0 mg/L Cu + 0.05, 1.0 mg/L Cd). It is concluded that *T. fuscatus* is a good candidate for the biomonitoring of Cd but not for Zn and Cu. The ambient concentrations of Cu and Zn may affect the accumulation of Cd, and need to be considered in the interpretation of Cd data in *T. fuscatus*.

Key words: Bioaccumulation, bioindicator, estuary, heavy metals, interaction, periwinkle.

INTRODUCTION

The use of biomonitors in monitoring aquatic pollution began some four decades ago with studies of radionuclide abundance in marine ecosystems (Folsom et al., 1963). Analysis of organisms has obvious advantages over the use of water and sediment when determining the mechanisms and consequences of metal uptake, since tissue burdens are often a direct manifestation of biologically available metal in the environment (Bryan et al., 1985; Langston and Spence, 1994). In addition, they provide time-integrated measures of the levels of metal contamination (Rainbow and Phillips, 1993) and their ability to accumulate metals to high concentrations makes analysis relatively easy. The general acceptance of the advantages inherent in the use of biomonitors to monitor aquatic pollution has given rise to the establishment of national and international programmes employing such species in many parts of the world (Goldberg et al., 1978, 1983; Phillips, 1989).

An ideal bio-indicator (biomonitor) should satisfy certain criteria (see reviews by Phillips, 1980; Phillips and Rainbow, 1993; Langston and Spence, 1994). These include the ability to accumulate pollutants without being killed by the levels encountered in the environment; sedentary in order to be representative of the study area;

*Corresponding authors E-mail: eremadaka@yahoo.com. Tel. +234(0)8033385665.

sufficiently long-lived to allow the sampling of more than one year-class, if desired; be of reasonable size, giving adequate tissue for analysis; be easy to handle and identify, and hardy enough to survive in the laboratory to allow defecation before analysis (if desired) and laboratory studies of pollutant uptake; tolerate brackish water to allow transplantation; and the existence of a simple correlation between the pollutant content of the organism and the average pollutant concentration in the surrounding water.

A number of extrinsic (physico- chemical) and intrinsic (biological) factors (reviewed by Phillips, 1980; Phillips and Rainbow, 1993), may introduce variability in the use of biomonitors. The biological factors include differential metal-binding abilities between individuals and between tissues/organs of the same individual (Mason and Simkiss, 1983); size or age (Boyden, 1974; 1977); reproductive stage (Fowler and Oregioni, 1976) and sex (Watling and Watling, 1976). Physical and chemical parameters include variation due to microhabitat (Nielson, 1974; Roberts et al., 1986); variation in salinity (Phillips, 1976; George et al., 1978) and temperature (Fischer, 1986); metal-metal/ligand interaction (Jackim et al., 1977; George and Coombs, 1977; Daka and Hawkins, 2006).

The gastropod mollusc *Tympanotonus fuscatus* appears to satisfy a number of criteria outlined above. The assessment of the criterion concerning correlation of metal concentrations in an organism with that in the surrounding medium requires experimental studies of accumulation from media of known concentrations. In this study, we examine the accumulation of cadmium, zinc and copper from single metal exposure solutions to test the relationship between metal concentration dosages and tissue metal levels. The net uptake of metals from binary mixtures was also determined to evaluate the nature of interactions between metals and their implications for biomonitoring using *T. fuscatus*.

MATERIALS AND METHODS

Test organism

The gastropod *T. fuscatus* belongs to the subclass Prosobranchia. They are found in intertidal locations and are widely distributed in coastal and estuarine areas in the Niger Delta. Periwinkles (*T. fuscatus*) were collected from the mangrove swamp near Eagle Island, Port Harcourt in the upper Bonny Estuary of the Niger Delta, Nigeria. Individuals were washed on site with surface water to remove mud on the shells and transported to the laboratory in plastic containers; in quantities that were not overcrowded. Individuals of similar size (32±2 mm) were used.

Exposure to single metals

Individuals were acclimatized to laboratory conditions for four days using filtered water from the site of collection in 10 L nitric acid pre-cleaned plastic containers. The water was replaced every other day to compensate for possible depletion of dissolved oxygen. The samples were then exposed to test solutions of the appropriate metal (Cu, Cd, and Zn) concentrations with concentration range: 0.01, 0.05, 0.1, 0.5, 1.0 mg/l added metal. Appropriate metal concentrations were made up by dilution of freshly prepared stocks of metal salts ($ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $CdSO_4.8H_2O$) with filtered estuarine water obtained from the site of collection of the test animals (some physicochemical parameters of the exposure water, including the concentrations of metals are shown in Table 1). The periwinkles were exposed in triplicate treatments (12 individuals per replicate) by submerging them in the metals solutions and controls (no metal added) in test chambers. These concentrations were considered sublethal based on published LC_{50} values for periwinkles (Otitoloju, 2002; Oyewo, 2003). The solutions were changed every other day to compensate for possible losses and to maintain the nominal concentrations of the metals (Daka and Hawkins, 2004, 2006).

Table 1. Physicochemical properties of dilution water used in the experiments.

Parameter	Experimental Series	
	Single metal	Mixed metals
pH	7.78	7.11
Alkalinity as HCO_3^- (mg/L)	122	94.9
Chloride (mg/L)	7090	15197
Total Dissolved Solids (mg/L)	10950	13450
Conductivity (µS/cm)	21900	26900
Zinc (mg/L)	<0.001	<0.001
Cadmium (mg/L)	<0.001	<0.001
Copper (mg/L)	<0.001	<0.001

Exposure to binary mixtures of metals

The animals were acclimatized as indicated above before exposure in triplicate (12 individuals per replicate) treatments to a combination of Cd and Zn or Cd and Cu. These were 0.05, 0.5 and 1.0 mg/L Cd in combination with 0.05, 0.5, and 1.0 mg/L Zn; 0.05, 0.5 and 1.0 mg/L Cu. In addition, individuals were also exposed singly to each metal concentration and control (no metal added).

Sample preparation and analysis

Samples of ten pooled individuals per replicate were boiled and carefully de-shelled using stainless steel needle, dried to a constant weight in an oven at 105°C overnight, and homogenized. The ground tissues were weighed and used for metal extraction through nitric acid digestion; 10 ml de-mineralized water, 10 ml of conc. HNO_3, and a few boiling chips were added to the sample in a 250 ml conical flask (Greenberg et al., 1992). The mixture was brought to slow boil and evaporated on a hot plate until digestion was complete and set aside to cool. The digest was filtered on Whatman No 1 filter paper into a 50 ml volumetric flask and made up to mark using demineralized water. Following acid digestion, all samples were analyzed for the required metals (Cu, Cd, Zn) by flame atomic absorption spectrophotometer (Perkin Elmer Analyst 100 equipped with a high sensitivity nebulizer) calibrated by successive dilution of a 1000 mg/l multi - element instrument calibration standard solution.

For each batch of elemental analysis, intra-run quality assurance standard was checked for reading deviation after every five samples. Internal blanks were used to asses any background contamination originating from sample manipulation and preparation. Blanks were processed exactly as respective regular samples.

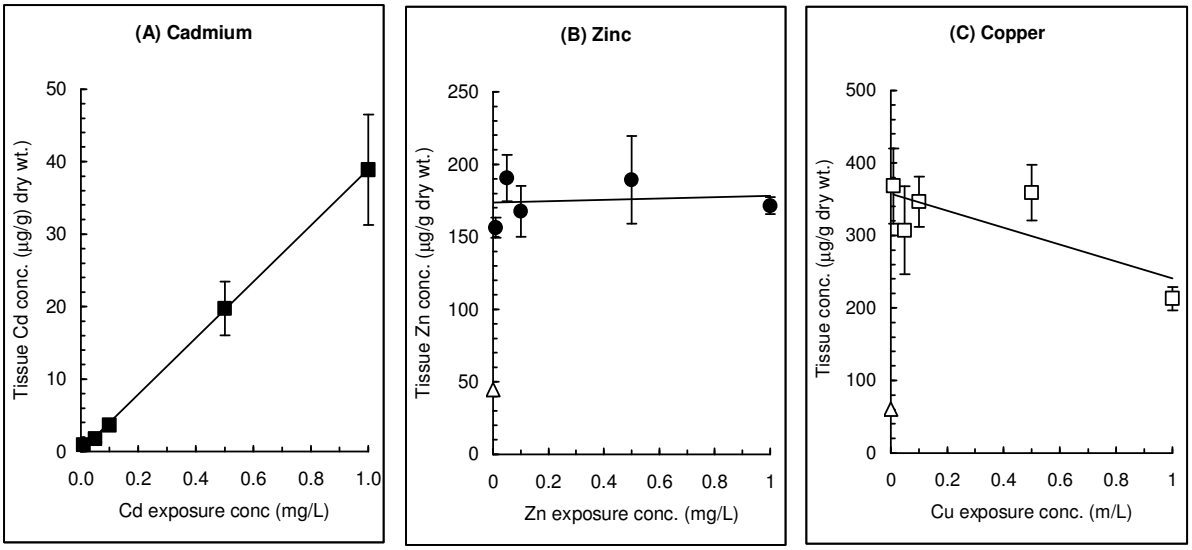

Figure 1. Patterns of accumulation of cadmium, zinc and copper in *Tympanotonus fuscatus* exposed to a range of concentrations of each metal in single metal exposures. Tissue metal concentrations are in µg/g dry weight; values are mean ± SD, n = 3. Regression equation and ANOVA in Table 1. Open triangles = control values; Cd control <0.001 µg/g.

Table 2. Regression equations, coefficients of determination and summary of regression ANOVA for accumulation of Cd, Cu and Zn by *Tympanotonus fuscatus* exposed to a range of concentrations of each metal.

Metal	Equation	R^2_{adj}	ANOVA		
			MS	F	p-value
Cd	$y = 38.88x + 0.07$	0.999	1073	7514	<0.0001
Cu	$y = -116.5x + 357$	0.463	9656	4.5	0.125
Zn	$y = 4.7x + 173$	0.018	15.82	0.056	0.828

The analytical procedure for the samples was checked by the digestion and analysis of dogfish liver certified reference material (DOLT-3). Measured values were within ±10 % of certified values.

Statistical analyses

Linear regression models were used to determine the relationship between metal accumulation and exposure concentration. Analysis of variance (ANOVA) was applied to accumulation data from binary mixtures of metals. Where ANOVA showed significant difference between treatment combinations, Bonferroni tests were used to evaluate pair-wise differences between accumulation from metal mixtures and metal alone (as control) for each exposure concentration. The analyses were performed using MINITAB R14.

RESULTS

In individual metal exposures, the accumulation of Cd from solution was monotonic with concentration over the range of exposure concentrations (Figure 1A) while those of Zn and Cu did not show linearity in accumulation with concentration (Figure 1B and 1C). The regression of tissue Cd concentrations against Cd exposure concentrations was significant ($p<0.0001$) with high coefficients of determination ($r^2 = 0.999$) (Table 1). For Cu and Zn, no significant regressions were obtained and the coefficients of determination for the regressions were low (Table 2). Bioaccumulation factors (BF) were calculated as a proportion of the tissue metal concentrations to experimental exposure concentrations. The highest BF values were obtained for Cu, followed by Zn with Cd having the least (Table 3). However, while BF values reduced remarkably with an increase in the concentrations of Cu and Zn, those of Cd were considerably similar across the range of exposure concentrations.

Cd accumulation from binary mixtures with Cu showed interactions ranging form enhanced accumulation to depressed accumulation, depending on the interacting pairs of metals/concentrations (Figure 2). A general pattern of response was observed at the three Cd concentrations. However, in interactions with 0.05 mg/L Cd, enhanced net uptake was found with 0.05 mg/L Cu and 0.5 mg/L Cu, the earlier giving more enhancement than

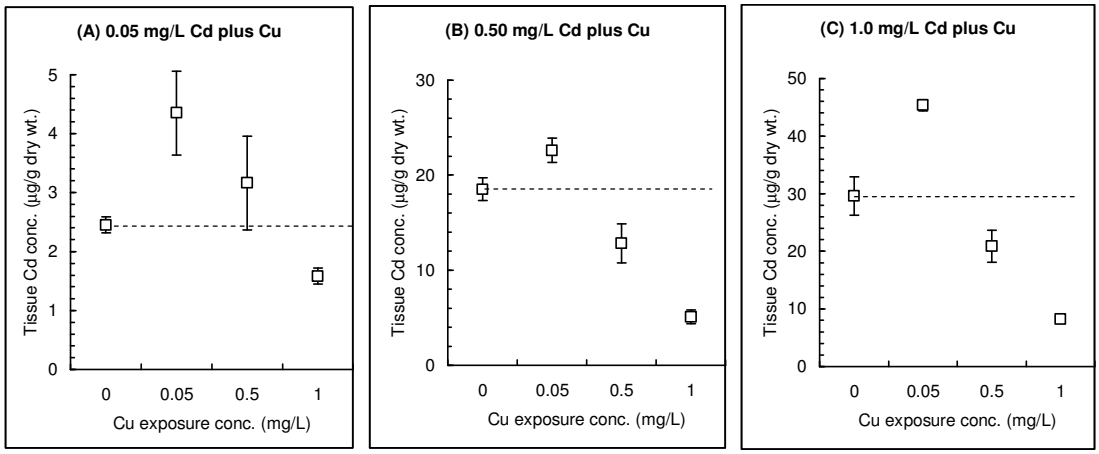

Figure 2. Accumulation of cadmium in *Tympanotonus fuscatus* exposed to cadmium (0.05 mg/L, 0.5 mg/L, 1.0 mg/L) and different concentrations of copper. Tissue Cd concentrations are in µg/g dry weight; values are mean ± SD, n = 3.

Table 3. Bioaccumulation Factors for *Tympanotonus fuscatus* exposed to various concentrations of heavy metals.

Exposure conc.(mg/L)	Bioaccumulation Factor		
	Cu	Zn	Cd
0.01	36828	15636	92
0.05	6144	3814	35
0.10	3465	1676	37
0.50	719	379	40
1.00	213	171	39

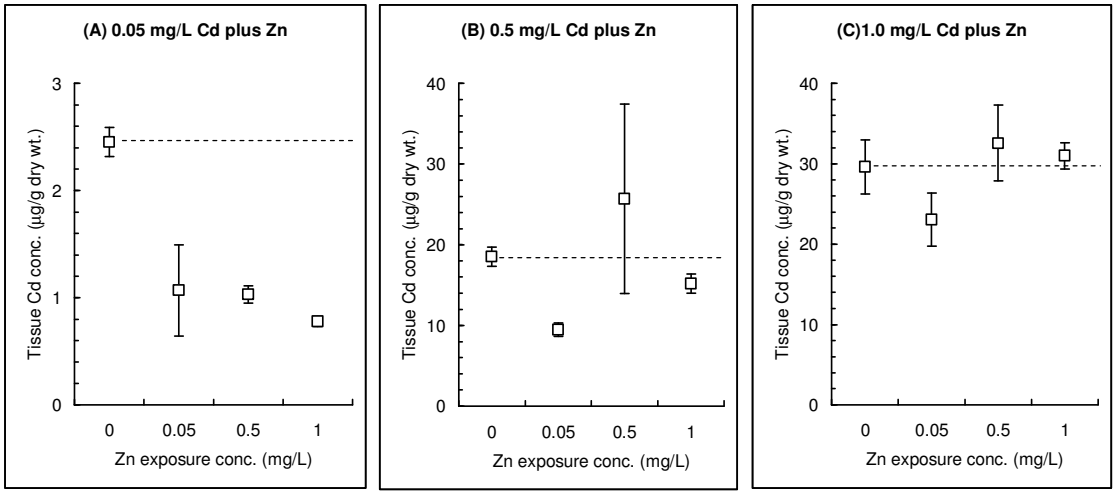

Figure 3. Accumulation of cadmium in *Tympanotonus fuscatus* exposed to cadmium (0.05 mg/L, 0.5 mg/L, 1.0 mg/L) and different concentrations of zinc. Tissue Cd concentrations are in µg/g dry weight; values are mean ± SD, n = 3.

the latter (Figure 2A). At 0.5 and 1.0 mg/L Cd, enhanced accumulations were found in combinations with 0.05 mg/L Cu, while reduced accumulations were obtained with 0.5 mg/L Cu and 1.0 mg/L Cu (Figure 2B and 2C). ANOVA and Bonferroni tests show that accumulations in all combinations of Cu with 0.5 mg/L Cd and 1.0 mg/L Cd were significantly different from accumulation from exposure to Cd alone (Table 4).

Cadmium accumulation in solutions of 0.05 mg/L Cd in combinations with 0.05, 0.5 and 1.0 mg/L Zn showed a progressive reduction with increase in Zn (Figure 3A) and there were significant reductions between all Zn + 0.05

Table 4. Summary of Analysis of variance (ANOVA) to test for significant differences in Cd accumulation by *Tympanotonus fuscatus* exposed to combinations of Cd and Cu or Cd and Zn. Bonferroni tests show pair-wise differences between Cd+Cu or Cd+Zn combinations against Cd alone.

	ANOVA				Bonferroni		
Treatment	**df**	**AdjMS**	**F**	**p-value**	**0.05 mg/L**	**0.5 mg/L**	**1.0 mg/L**
0.05 mg/L Cd + Cu	3	4.1	14	0.002	**	ns	ns
0.5 mg/L Cd + Cu	3	172.9	89.7	<0.001	*	**	***
1.0 mg.L Cd + Cu	3	728.6	145.4	<0.001	***	**	***
0.05 mg/L Cd + Zn	3	1.72	33.1	<0.001	***	***	***
0.5 mg/L Cd + Zn	3	137.5	3.9	0.055			
1.0 mg.L Cd + Zn	3	157.5	4.45	0.041	ns	ns	ns

* = p<0.05, ** = p<0.01, *** = p<0.001, ns = not significant.

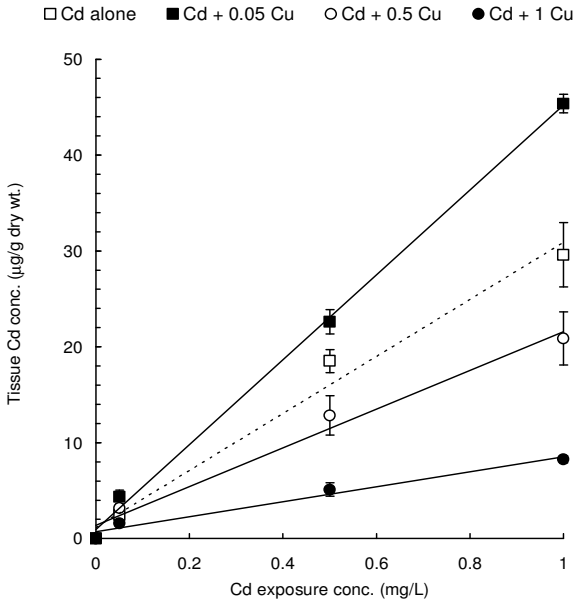

Figure 4. Regressions of cadmium accumulation in *Tympanotonus fuscatus* exposed to individual metal solutions and from solutions containing binary mixtures of cadmium and copper. Regression equations and ANOVA in Table 3.

mg/L Cd combinations compared to values in Cd alone (p<0.01, Table 4). Reduced accumulations were also found in combinations of 0.5 mg/L Cd with Zn (Figure 3B), but these were not significantly different from Cd alone (Table 4). Similarly, accumulations from 1.0 mg/L Cd with Zn did not produce any significant differences at any combination, although a tendency for enhanced accumulation was observe in the combinations with 0.5 and 1.0 mg/L Zn (Figure 3C).

Figure 4 and 5 show that the linearity of the regression of Cd accumulation over exposure concentration is affected by the addition of Cu or Zn. The addition of 0.05 mg/L Cu gave a regression coefficient higher than that of Cd alone in solution, while the addition of 0.5 mg/L Cu and and 1.0 mg/L Cu gave lower regression coefficients; 1.0 mg/L Cu produced a higher depression of the slope than 0.5 m/L Cu (Figure 4 and Table 5). The addition of 0.05 mg/L Zn produced a higher depression of the regression slope of Cd accumulation than 1.0 mg/L Zn (Figure 5 and Table 5). Although the regression coefficient obtained by the addition of 0.5 mg/L Zn was higher than that of Cd alone, the coefficient of determination was the lowest and regression had the weakest significance.

There was a tendency for Cu accumulation to be reduced in Cd+Cu mixed solutions; significant reductions in Cu accumulation were observed in combinations of 0.05 mg/L Cu with 0.5 mg/L Cd compared to 0.05 mg/L Cu alone (Figure 6A and Table 6), and all combinations of 1.0 mg/L Cu with 0.05, 0.5 and 1.0 mg/L Cd compared to accumulation from 1.0 mg/L Cu only (Figure 6C and Table 6). The exposure of the winkles to Cd+Zn did not produce any significant differences in the accumulation of Zn for any of the metal combinations (Figure 7 and Table 6).

DISCUSSION

The accumulation profiles of the Zn and from single metal solutions did not indicate any concentration-dependent relationship. In the case of Cu, there was a generally negative relationship between net uptake and concentration which was not significant. This shows that *T. fuscatus* is not a good candidate for the monitoring of these two metals in aqueous media. On the other hand, the accumulation of Cd from single metal exposures gave a positive concentration dependent relationship. This implies that *T. fuscatus* is good bioindicator of ambient Cd levels in solution.

The different accumulation patterns between Zn and Cu on the one hand and Cd on the other may be related to the fact that Zn and Cu are essential for metabolic activities. For example, carbonic anhydrase, carboxy pe-

Table 5. Regression equations, coefficients of determination and summary of regression ANOVA for accumulation of Cd by *Tympanotonus fuscatus* exposed singly or to binary mixtures of Cd and Cu or Cd and Zn.

Metal Combination	Equation	R^2_{adj}	ANOVA		
			MS	F	p-value
Cd alone	$y = 29.74x + 1.12$	0.976	576	124	0.007
Cd + Cu					
Cd + 0.05 Cu	$y = 44.28x + 0.93$	0.997	1278	1001	0.0009
Cd + 0.5 Cu	$y = 20.19x + 1.39$	0.973	265	110	0.008
Cd + 1.0 Cu	$y = 7.83x + 0.70$	0.962	39	77	0.012
Cd + Zn					
Cd + 0.05 Zn	$y = 22.73x - 0.41$	0.986	336	228	0.004
Cd + 0.5 Zn	$y = 34.5x + 1.46$	0.880	775	23	0.04
Cd + 1.0 Zn	$y = 31.33x - 0.41$	0.999	640	3957	0.0002

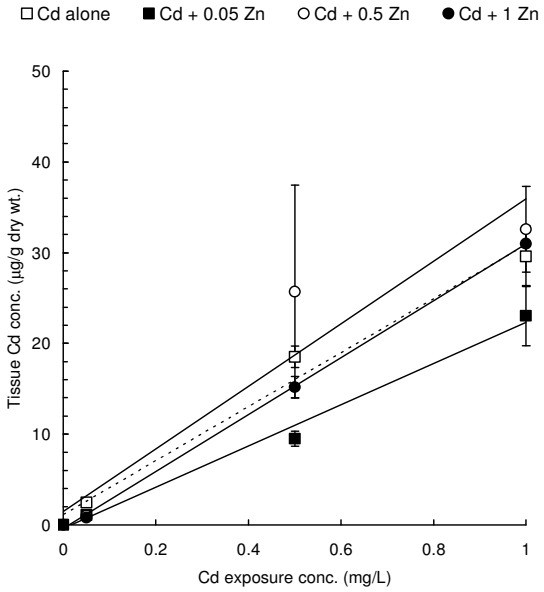

Figure 5. Regressions of cadmium accumulation in *Tympanotonus fuscatus* exposed to individual metal solutions and from solutions containing binary mixtures of cadmium and zinc. Regression equations and ANOVA in Table 3.

peptidase A and B and several dehydrogenases contain zinc, while haemocyanin contain copper.

Conversely, Cd is considered a non-essential metal because it has no known biological function (Bryan, 1984). Accumulation patterns for essential metals include regulation of body metal concentration, accumulation without excretion, and accumulation with some excretion either from the metabolically active pool or from the detoxified store (Depledge and Rainbow, 1990; Rainbow, 2002). In the littoral crustacean *Palaemon elegans*, the body concentration of Zn does not show any change over an increasing range of dissolved Zn exposures until a threshold external dissolved availability is reached. Although new Zn is enter-ing the body in significant amounts at all exposures but an equivalent amount of Zn is excreted to match the rate of Zn uptake. When the rate of Zn uptake from solution exceeds the rate of excretion, the body concentration of Zn then rises above the regulated values. There also appears to be regulation of Cu in the same species over a wide range of Cu availabilities until regulation breaks down. Then an increase in body Cu concentration follows any further increase in dissolved Cu exposure concentra-tion, with a pattern reflective of accumulation with some excretion. Some gastropod molluscs are known to be capable of regulating the levels of certain metals especi-ally those essential for metabolic activities such as Cu, Zn, Mn and Fe (Bryan et al., 1983; Webb, 1990). The progressive reduction of the BF with increasing exposure concentra-tions (Table 3) show that both Cu and Zn are regulated in *T. fuscatus*. The accumulation patterns sug-gest that the uptake of these metals take place with excretion, with the rate of excretion adjusted to match the uptake to keep the tissue concentration to keep the tissue concentrations within the levels required for metabolism. It is also likely especially with respect to Cu which had a negative tissue to exposure concentration relationship that changes in membrane characteristics were induced to reduce the influx of the metal at the higher concentra-tions.

Non-essential metals may be accumulated without excretion or with some excretion (Rainbow, 2002). Much of the Cd accumulated by aquatic invertebrates is bound to metallothionein in the cytosol of the organ predominan-tly used for accumulated Cd storage (Langston and Zhou, 1987; Rainbow, 2002). The similarities of the BFs across the range of concentrations (Table 3) also suggest that Cd accumulation in the periwinkles was without much excretion, but this requires confirmation with radiotracer studies. Similar results were obtained for *Littorina saxailis* from the Isle of Man (Daka, 1996).

The interactions obtained in the accumulation of Cd in mixed solutions suggested synergistic effects at the low-

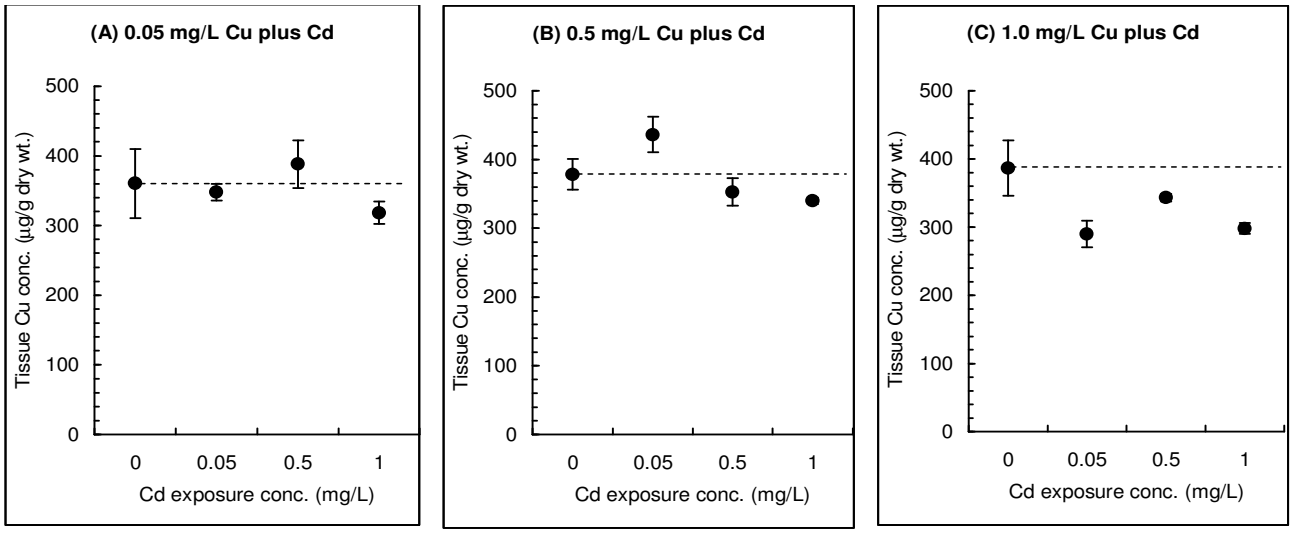

Figure 6. Concentrations of copper in *Tympanotonus fuscatus* exposed to copper (0.05 mg/L, 0.5 mg/L, 1.0 mg/L) and different concentrations of cadmium. Tissue Cu concentrations are in µg/g dry weight; values are mean ± SD, n = 3.

Table 6. Summary of Analysis of variance (ANOVA) to test for significant differences in Zn and Cu accumulation by *Tympanotonus fuscatus* exposed to combinations of Cd with Zn or Cu. Bonferroni tests show pair-wise differences between Zn+Cd or Cu+Cd combinations against Zn or Cu alone.

	ANOVA				Bonferroni		
Treatment	**df**	**AdjMS**	**F**	**p-value**	**0.05 mg/L**	**0.5 mg/L**	**1.0 mg/L**
0.05 mg/L Cu + Cd	3	10847	11.8	0.003	ns	*	ns
0.5 mg/L Cu + Cd	3	1336	2.57	0.127			
1.0 mg/L Cu + Cd	3	4343	8.62	0.007	*	ns	**
0.05 mg/L Zn + Cd	3	1810	1.62	0.261			
0.5 mg/L Zn + Cd	3	6813	0.99	0.443			
1.0 mg.L Cu + Cd	3	971.6	5.21	0.028			

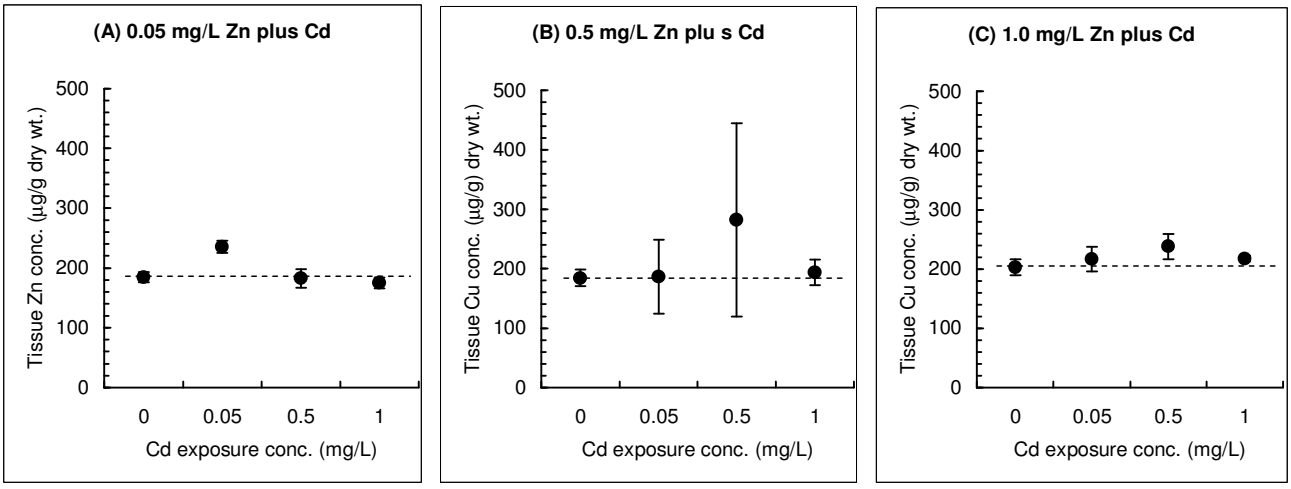

Figure 7. Concentrations of zinc in *Tympanotonus fuscatus* exposed to zinc (0.05 mg/L, 0.5 mg/L, 1.0 mg/L) and different concentrations of cadmium. Tissue Zn concentrations are in µg/g dry weight; values are mean ± SD, n = 3.

est Cu concentration (0.05 mg/L Cu) in combination with all Cd concentrations. However, at higher concentrations, Cu produced antagonistic effects on Cd accumulation. Daka and Hawkins (2006) have similarly reported that the accumulation of Zn by *L. saxatilis* from Zn and Cu combined solutions, indicated concentration-dependent interactions, switching from synergism at low concentrations of Cu to antagonism at high concentration of Cu. They suggested that mechanisms primarily targeted at reducing the influx of Cu rather than competition for ligands, resulted in a reduced accumulation of Zn. Looking at the accumulation pattern for Cu alone (Figure 1C) and that for the Cd + Cu mixtures (Figure 2), there is a trend indicative of a similar effect in *T. fuscatus*, whereby the regulation of Cu also led to antagonistic effects on Cd. The synergistic effects observed in combi-nations with the lowest Cu concentration (0.05 mg/L) might be as a result of the increased production of non-specific ligands. Non-specific ligands capable of binding a wide variety of metals are produced by basophil cells of the digestive gland and the nephrocytes of the kidney which occur at specific sites (Mason and Simkiss, 1983).

As a result of their chemical affinities, Cd and Zn may share similar uptake pathways into organisms (Rainbow, 1997). Zn was mostly antagonistic to Cd accumulation (highly variable data for 0.5 mg/L + Cd combinations gave putative synergism). However, in contrast to Cu, Zn did not depress Cd accumulation in proportion to the concentration of Zn in solution. On the other hand, the concomitant dosage of Cd and Zn did not show clear effects on the accumulation of Zn in any of the combinations. It has been similarly observed that in many molluscan species, exposure to the non-essential metal Cd had no effect on Zn accumulation; whereas exposure to essential metal Zn had an antagonistic effect on Cd accumulation (Amiard-Triquet and Amiard, 1998; Daka and Hawkins, 2006). However, Ahsanullah et al. (1981) reported for the decapod crustacean *Callianassa australliensis*, that exposure to a mixture of Zn and Cd, increased the bioaccumulation of both elements.

We conclude that *T. fuscatus* may be used as a suitable biomonitor of Cd in Niger Delta estuaries, but not for Zn and Cu. Also, the presence of the essential metals Cu and Zn may have repercussions in the interpretation of Cd data in *T. fuscatus* and should be taken into consideration in biomonitoring programmes involving this species.

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