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May Simultaneous Exposure to Different Heavy Metals Influence the Bioaccumulation of Each Metal by *Littorina saxatilis* (Gastropoda; Littoriniidae)

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Abstract

Laboratory assessments of toxicity and bioaccumulation of heavy metals have been concentrated on the accumulation of these metal ions when exposed singly to the test organisms. However, under the natural environmental settings, the metals are never present in isolation and may interact with each other, therefore justifying the need to study the influence of joint application of metals on accumulated levels in exposed animals. In this study, exposure of the periwinkle *Littorina saxatilis* to sublethal concentrations (equivalent to 0.1 and 0.01 of 96 h LC_{50}) of heavy metals revealed that they were bioaccumulative varying amounts, depending on the type of metal, exposure period and their concentration in the test media. While Zn and Pb ion accumulation increased steadily with exposure time, the amounts of Cu accumulated fluctuated regularly over the 30-day experimental period. The levels of Zn, Cu and Cd bioaccumulated over the 30-day experimental period were reduced by over 2-6 folds (with bioaccumulation ratio values ranging from 0.15 to 0.81) when compared to concentrations of the respective metals accumulated during single bioaccumulation studies. However, Pb concentrations accumulated during the joint action studies increased nearly 2-fold (bioaccumulation ratio range 1.36 to 2.0-fold).

Keywords: Periwinkle, filtration rate, absorption rate, Persian Gulf, metal pollution.

Introduction

It has already been recognized that bioaccumulation of heavy metals differs in different aquatic species and is influenced by water chemistry conditions (Campbell and Evans, 1991; Frazier and George, 1983). Especially marine organisms are known to be more sensitive to heavy metal contamination than fresh water animals of soft water environment (Campbell and Evans, 1991; Clark, 1992). In many cases, heavy metals occur in natural bodies of water at levels below their toxic thresholds, however, due to their non-degradable nature, such low concentrations may still pose risk of damage via uptake and subsequent bioaccumulation by organisms, which cannot effectively metabolize and excrete the absorbed metals. Several scientific observations have shown that heavy metals are bioconcentrated or bioaccumulated in one or several compartments across food webs (Bryan and Langston, 1992; Kiffney and Clement, 1993; Oyewo, 1998; Otitoloju and Don-Pedro, 2003). Metal bioaccumulation can be of importance from the public health point of view, especially when a human consumes the accumulators. Secondly, this phenomenon is now being exploited in the assessment of environmental quality, in addition to chemical surveys of water and sediment (Javanshir and Shapoori, 2011). The inclusion of bioaccumulators in a biological monitoring programmed is particularly advantageous since such organisms are known to accumulate these pollutants to levels several folds higher than the amount in the external medium, demonstrating how biological systems can render unsafe, the otherwise low and apparently safe prevalent ambient levels of persistent pollutants in ecosystems (Otitoloju and Don-Pedro, 2004). Most laboratory assessments of toxicity and bioaccumulation of heavy metals have been concentrated on the accumulation of these metal ions when exposed singly to the test organisms (Oyewo, 1998; Panigrahi and Misra, 1980; Sastry and Shukla, 1993; Wright *et al*., 1991). However, under the natural environmental settings in the field, the metals are never present in isolation and may interact with each other as a result of competition for binding sites

in the exposed animal tissues or may form complexes which may or may not be easily excreted (Otitoloju, 2002; Otitoloju, 2003; Wright and Zamuda, 1987). These interactions between the metals may lead to either a decrease or an increase in their individual uptake/excretion (Franklin *et al.,* 2002; Javanshir *et al.,* 2011). It is therefore important to establish the effects of the joint presence of the major types of heavy metals on their rate and subsequent level of bioaccumulation in aquatic organisms such as *Littorina saxatilis* (periwinkle), which plays a delicate role amongst ecosystem inhabiting the coastal areas in northern Persian Gulf. This type of study may also provide an insight into the mechanisms of antagonism, synergism or additive action, which are observed when test organisms are exposed to the toxic effects of heavy metals in joint action toxicity studies. The aim of this study is to provide simultaneous impact of different heavy metals on an intertidal grazer animal which may transfer the toxicity to the upper trophic levels.

Materials and Methods

Test Animals

Littorina saxatilis (periwinkle, Mollusca; Gastropoda, Mesogastropoda, Littorinidae) of similar sizes (shell length of 28 to 32 mm) was collected by handpicking into a bucket (12.6 L) from south Queshm island at low tide (55°42′47″ East and 26°41′55″ North). The periwinkles were always collected from the same site, in order to reduce variability in biotype. The animals were transported to the laboratory and kept in holding glass tanks (30 x 30 x 30 cm), which contained aerated lagoon water (6 L) at measured salinity that was variable depending on the time of year. Mud was collected from the same site and placed in the holding tank as substrate. Other specimens, collected as described above, were left in holding tanks with a thin layer of sediment serving as substrate and food source for five to six days to allow them to acclimatize to laboratory and experimental conditions (relative humidity: 70±2%; temperature: 26±2°C; salinity: 16‰) before using them in bioassays. The test animals were acclimatized to higher or lower salinities by diluting or concentrating the lagoon water by 2.5‰ once every 24 h; using dechlorinated tap water or seawater of known salinity strength so that after the required number of changes, the desired predetermined test salinity (16‰) for all bioassays was achieved. This approach of gradual change in salinity was adopted to allow the periwinkles, enough time to gradually adapt to the new salinity and so prevent a sudden osmotic change, which could result in stress that may distort responses to the test toxicants. *Littorina saxatilis* of similar sizes depending on shell length (28 to 32 mm) was selected for all experiments.

Test Chemicals

The heavy metals investigated in this work were obtained as metallic salts of Fisons laboratory reagents, analar grades of the following types:

a. Copper as $CuSO_4$. $5H_2O$
b. Zinc as $ZnCO_3$. $3H_2O$
c. Lead as Pb $(NO_3)_2$
d. Cadmium as $3Cd\ SO_4$. $8H_2O$

The choice of heavy metals for this study was based on the available, common and abundant metals from the results of a chemical survey of industrial effluents that empty into the Persian Gulf carried out industrial waste waters (Langston and Zhou, 1987; Omidi, 2008; Oryan, 2008).

General Bioassay Technique

Salinity of Test Media

All bioassays were carried out at salinity of 16‰. To achieve this, seawater was diluted with dechlorinated tap water to a salinity of 16 ‰ and served as test media, and also as dilution water for mixing toxicants. The animals were always acclimatized to 16 ‰. This salinity condition was used in all bioassays in order to standardize and simulate a typical brackish water medium, since changing salinity has been observed to affect toxicity. Preparation of substrate: In an attempt to simulate the natural environment of benthic test animals, sediment from the site of animal collection were used as substrates. The sediment also may be served as food source for the test animals during the bioassays.

Preparation of Test Media Including Application of Toxicant

Single Action Bioassays

A pre-determined amount of each heavy metal compound was weighed (using an oertling 30TD top loading balance) and diluted with given volume of dechlorinated tap water to obtain a stock solution of known strength. The resultant stock solution was serially diluted to obtain solutions of required concentrations.

Joint Action Bioassays

For each predetermined concentration of a mixture to be tested, the proportion of each constituent compound by weight of metallic element, dictated by the predetermined ratio of the mixture were computed and measured out into a conical flask and made up to the required test media volume by adding prepared brackish water (16‰) as diluent. The test media was then mixed thoroughly by stirring

Bioaccumulation Studies

Bioaccumulation of Heavy Metals (Zn, Pb, Cu and Cd) by *L. saxatilis* Exposed to Sublethal Concentration of Single Metal Compounds

In this series of experiments, *Littorina saxatilis* was exposed to only sub-lethal concentrations (fractions of 96 h LC_{50} values derived from experiments on acute toxicity of heavy metals against benthic animals (Otitoloju, 2001) of selected metal salts as specified below. A total of 120 test animals were exposed per sublethal concentration or control in five replicates (24 periwinkles per replicate). Each bioassay container held a thin layer of standardized sediment substrate and test media at salinity of 16 ‰. In this series of bioassays that went on for 30 days in order to investigate the rate of bioaccumulation, the semi-static bioassay procedure was always adopted to avoid drastic changes in concentration of test media via evaporation and excessive reduction in dissolved oxygen level. In this semi-static procedure each test media was changed into a fresh solution at exactly the same concentration of heavy metal salt or untreated control as the case may be, once every four days, transferring the same exposed test animals into the freshly prepared test media over a 30 days period of experimentation. At pre-determined time intervals (day 0, 4, 10, 20 and 30), four live *L. saxatilis* per replicate, making 20 per treatment including control were randomly selected, cleaned thoroughly with distilled water and placed in labeled polythene bags in which they were kept frozen awaiting digestion of the extracted whole animal tissues and analysis for test metals by Atomic Absorption Spectrophotometer (AAS), based on AAW (1995) standard methods. Sublethal concentrations under which bioaccumulation of test metals by *L. saxatilis* was investigated were as follows:

a. Zn ($ZnCO_3$. $3H_2O$) was tested at: 8.310 mg/L ($1/10^{th}$ of the 96 h LC_{50}) 0.8317 mg/l ($1/100^{th}$ of the 96 h LC_{50}) and untreated control.

b. Pb ($Pb(NO_3)_2$) was tested at: 37.077 mg/L ($1/10^{th}$ of the 96 h LC_{50}) 3.7077 mg/L ($1/100^{th}$ of the 96 h LC_{50}) and untreated control.

c. Cu ($CuSO_4$.5H2O) was tested at: 3.925 mg/L ($1/10^{th}$ of the 96 h LC_{50}) 0.3925 mg/L ($1/100^{th}$ of the 96 h LC_{50}) and untreated control.

d. Cd ($3CdSO_4.8H_2O$) was tested at: 2.825 mg/l ($1/10^{th}$ of the 96 h LC_{50}) 0.2825 mg/L ($1/100^{th}$ of the 96 h LC_{50}) and untreated control.

Bioaccumulation of heavy metals by *Littorina saxatilis* when exposed to sublethal concentrations of mixtures of heavy metal compounds: For this series of experiment, a semi-static bioassay procedure was adopted. *L. saxatilis* was exposed to sublethal concentrations of equitoxic multiple mixtures of Zn, Pb, Cu and Cd. The multiple mixtures consisted of sublethal concentration of each constituent metal salt, the computation of which was based on a pre-determined fraction ($1/10^{th}$ and $1/100^{th}$) of the 96 h LC_{50} values of the test metals obtained in single action experiments after Otitoloju (2001). Details of the equitoxic metallic mixture (giving the amount of each constituent metal) to which the test animals *L. saxatilis* were exposed are given below.

a. Mixture based on $1/10^{th}$ of 96 h LC_{50} of the respective metal salt at following concentrations: 8.3170 mg Zn/L, plus 37.077 mg Pb/l, plus 3.925 mg Cu/L, plus 2.825 mg Cd/L.

b. Mixture based on $1/100^{th}$ of 96 h LC_{50} of the respective metal salt at following concentrations: 0.8317 mg Zn/l, plus 3.707 mg Pb/L plus, 0.392 mg Cu/L plus, 0.282 mg Cd/L.

c. Untreated control.

Heavy Metal Analysis of Animal Samples for Heavy Metal Content by Atomic Absorption Spectrophotometry (AAS):

Digestion of Samples

Whole animal samples (shell previously removed) of *L. saxatilis* were properly cleaned (by copiously rinsing all exposed and partially enclosed parts) with distilled water to remove debris, plankton and other external adherents before they were homogenized. A portion (10 g wet weight basis) from the homogenate of each animal was digested using a freshly prepared mixture 1:1 of hydrogen peroxide and perchloric acid. AAS Determination of heavy metals in animal samples: All digestive contents were filtered through Whatman No.1 filter paper and made up to the mark in appropriate volumetric flasks (25 ml). The heavy metal content of each sample was then determined by comparing their absorbance with those of standard AAS solutions using an Alpha-4 Cathodeon Atomic Absorption Spectrophotometer.

Data Analysis

Regression analysis (using Microsoft Excel 2000) was carried out to determine correlation co-efficient (R^2) between concentrations of test metals accumulated in the test animals *L. saxatilis* with period of exposure (Rahnama *et al*., 2010).

Bioaccumulation Ratio (BAR) was also estimated as the ratio of overall gain in metal tissue concentration after 30 days of exposure under joint accumulation studies to the overall gain in metal tissue concentration after 30 days under single accumulation studies. The aim of this computation

was to establish a relationship between concentrations of metals accumulated under joint accumulation studies to the concentration accumulated when the metals were tested singly. When, BAR= 1 indicates similar gain in metal during joint and single bioaccumulation studies (non interactive)

$$\text{Bioaccumulation ratio} = \frac{\text{Overall gain of joint accumulation studies}}{\text{Overall gain of single accumulation studies}}$$

BAR>1 indicates an increase in metal gained during joint accumulation studies (synergistic interaction).

BAR<1 indicates a decrease in metal gained during joint bioaccumulation studies (antagonistic interaction).

Analysis of variance (ANOVA) was used to compare several treatment means between the concentrations of metals accumulated either under the single and joint accumulation studies. Additionally, the *t*-test for evaluating significant difference between two treatment means was used to compare treatment means of concentration of metals accumulated under the joint action studies to the concentration accumulated when the metals were tested singly (Sokal and Rohlf, 1995).

Results

Physico-Chemical Characteristics of Test Medium During the Bioaccumulation Studies

During the bioassays, the dissolved oxygen content of test media remained higher than 5.5 mg/L. The pH, temperature and salinity remained fairly constant at 7.8±0.2, 26±2°C and 16.0±0.2‰, respectively, throughout the duration of the experiment. Bioaccumulation of heavy metals (Zn, Pb, Cu and Cd) by *L. saxatilis* exposed to sublethal concentrations of metals in single and joint action laboratory studies.

Zinc (Zn)

Post treatment analysis of whole body tissues of *L. saxatilis* showed that animals exposed to sublethal concentrations (0.4325 mg/L and 4.325 mg/L) of Zn ions in the single action studies steadily accumulated higher quantities of the metal ions that were approximately two or four times higher than the levels accumulated in control animals, over a 30-day experimental period (Table 1).

Furthermore, the amount of Zn accumulated in the animal tissue was time (R^2= 0.818 and 0.9789 for the 0.4325 mg/L and 4.325 mg/L test media, respectively, and test medium concentration dependent. However, under the joint application studies, post treatment analysis of test animal tissues revealed that there were only minimal increment in the heavy metal content in exposed animals tissues compared to control animals (Table 1). Furthermore, the overall gain of Zn ion in the tissue of test animals after 30 days of exposure to the two test mixture concentrations (4.5885 mg/L and 45.885 mg/L) was 6.64 µg/g and 22.64 µg/g, respectively; and these values were lower than the amount (11.07 µg/g and 27.99 µg/g, respectively) of Zn accumulated by *L. saxatilis* when it was exposed to test medium containing only Zn compound at the same test concentrations (Table 4 and Figure 1). The computed bioaccumulation ratios were 0.60 and 0.81 (Table 5), thus indicating an antagonistic interaction between the metal components of the mixture in relation to Zn ion accumulation. Statistical evaluation by Analysis of variance (ANOVA) showed that there was no significant (P>0.5) differences between the concentrations of the Zn ions detected in the animal tissues collected from the various test treatments (control, 0.4325 mg/L and 4.32 mg/L) under the single or joint bioaccumulation studies. Furthermore, analysis based on the t-test for evaluating significant

Table 1. Accumulation of Zinc by *L. saxatilis* exposed to sub-lethal concentration of the test metal under single and joint bioaccumulation studies

	Mean concentrations of zinc ions in whole animal tissue ($\mu g.g^{-1}$ dry weight basis)					Overall Net gain[A]
	0 day	4 days	10 days	20 days	30 days	
Single action Untreated control	4.29	7.37	8.09	7.82	7.29	
[C]0.4325	4.29	8.42	7.44	8.93	15.36	
Net gain[B]	-	4.13	-0.98	1.49	6.43	11.07
[D]4.325	4.29	7.84	12.27	26.48	32.28	
Net gain[B]	-	3.55	4.43	14.21	5.80	27.99
Joint action Untreated control	5.62	8.26	7.71	9.26	7.73	
[C]0.4325	5.62	8.37	8.89	9.32	12.26	
Net gain[B]	-	2.75	0.52	0.43	2.94	6.64
[D]4.325	5.62	9.49	11.38	22.16	28.26	
Net gain[B]	-	3.87	1.89	10.78	6.1	22.64

Overall net gain: concentration in animal after 30 days – concentration in animal at zero day.
Net gain – difference in concentration between the immediate preceding days of harvesting, e.g. 4-0 days, etc.
$1/100^{th}$ 96 h LC_{50} values of the metal ions in the test compounds.
$1/10^{th}$ 96 h LC_{50} values of the metal ions in the test compounds.

difference between two treatment means showed that there were no significant (P>0.5) differences between the concentration of the Zn ions accumulated in the animal tissues under the single and joint bioaccumulation studies.

Lead (Pb)

With regards to lead, analysis of animal tissues exposed to sublethal concentrations (3.9635 mg/L and 39.63 mg/L) of Pb ions in the single action studies revealed that the animals accumulated measurable quantities of the metal that were approximately three-times above the levels accumulated by control animals, over the 30-day experimental period (Table 2).

The amount of Pb accumulated in the animal tissue was also found to be time (R^2= 0.8859 and 0.9715 for 3.9635 mg/L and 39.635 mg/L test media, respectively) and test medium concentration dependent. Similar observation was also recorded under the joint action studies (Table 2). In the presence of other metals, *i*.e. under joint action studies, the total amount of Pb ion accumulated in animals exposed to each of the two sublethal concentrations (4.5885 mg/L and 45.885 mg/L) of metal mixtures was 287.95 µg/g and 459.56 µg/g respectively (Table 2); and these accumulated values were approximately two times and 1.4 times higher than the amounts of (144.05 µg/g and 339.03 µg/g, respectively) Pb ion accumulated under the single action studies (Table 5, Figure 2). The computed bioaccumulation ratios were 2.0 and 1.36 (Table 5), thus indicating a synergistic interaction between the metal components of the mixture in relation to the Pb ion accumulation. Statistical evaluation by Analysis of Variance (ANOVA) showed that there were significant (P<0.5) differences between the

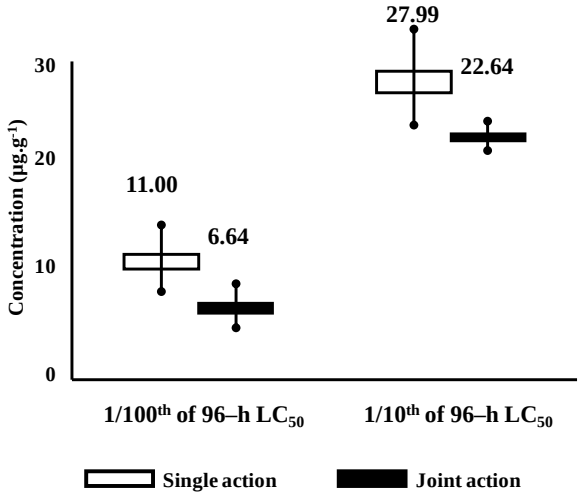

Figure 1. figure of the overall gain in heavy metal (Zn ions) by *L. saxatilis* exposed to the test metals under single and joint bioaccumulation studies

Table 2. Accumulation of Lead by *L. saxatilis* exposed to sub-lethal concentrations of the test metal under single and joint bioaccumulation studies

	Overall Net gain[A]					
	0 day	4 days	10 days	20 days	30 days	
Single action Untreated control	29.90	29.19	49.84	58.64	60.55	
[C]3.9635	29.90	31,54	39.54	160.75	173.95	
Net gain[B]	-	1.64	8.00	121.21	13.20	144.07
[D]39.635	29.90	80.79	130.55	203.43	368.93	
Net gain[B]	-	50.89	49.76	72.88	160.50	339.03
Joint action Untreated control	28.75	28.88	46,31	60.12	61.20	
[C]3.9635	28.75	34.60	63.22	220.41	316.70	
Net gain[B]	-	5.85	28.62	157.19	96.29	287.95
[D]39.635	28.75	110.64	196.76	360.17	488.31	
Net gain[B]	-	81.89	82.12	163.41	128.14	459.56

Mean concentrations of Lead ions in whole animal tissue (µg.g^{-1} dry weight basis)
Overall net gain: concentration in animal after 30 days – concentration in animal at zero day.
Net gain – difference in concentration between the immediate preceding days of harvesting, e.g. 4-0 days, etc.
1/100th 96 h LC_{50} values of the metal ions in the test compounds.
1/10th 96 h LC_{50} values of the metal ions in the test compounds.

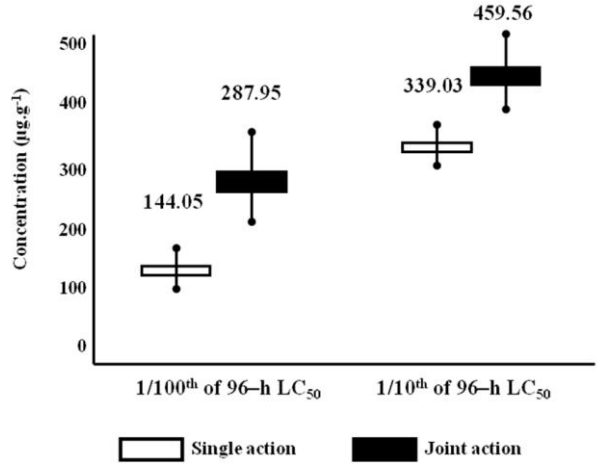

Figure 2. figure of the overall gain in heavy metal (Pb ions) by *L. saxatilis* exposed to the test metals under single and joint bioaccumulation studies.

Table 3. Accumulation of copper by *L. saxatilis* exposed to sub-lethal concentrations of the test metal under single and joint bioaccumulation studies

	Overall Net Gain[A]					
	0 day	4 days	10 days	20 days	30 days	
Single action Untreated control	11.04	14.63	17.62	10.69	6.55	
[C]0.1021	11.04	12.96	13.05	7.65	11.48	
Net gain[B]	-	1.92	0.09	-5.4	3.83	0.44
[D]1.021	11.04	21.69	18.89	12.39	18.27	
Net gain[B]	-	10.65	-2.80	-6.50	5.88	7.33
Joint action Untreated control	10.02	14.12	16.93	10.22	6.44	
[C]0.1021	10.02	9.01	14.96	8.29	10.64	
Net gain[B]	-	-1.01	5.95	-6.67	2.35	0.62
[D]1.0206	10.02	8.17	12.26	15.43	11.10	
Net gain[B]	-	-1.85	4.09	3.17	-4.33	1.08

Overall net gain: concentration in animal after 30 days – concentration in animal at zero day.
Net gain – difference in concentration between the immediate preceding days of harvesting, e.g. 4-0 days, etc.
$1/100^{th}$ 96 h LC_{50} values of the metal ions in the test compounds.
$1/10^{th}$ 96 h LC_{50} values of the metal ions in the test compounds.

Table 4. Accumulation of cadmium by *L. saxatilis* exposed to sub-lethal concentrations of the test metal under single and joint bioaccumulation studies

	Overall Net gain[A]					
	0 day	4 days	10 days	20 days	30 days	
Single action						
Untreated control	0.02	0.02	0.03	0.06	0.08	
[C]0.0904	0.02	0.03	0.11	0.32	0.58	
Net gain[B]	-	0.01	0.08	0.21	0.26	0.56
[D]0.904	0.02	0.26	0.91	1.22	1.26	
Net gain[B]	-	0.24	0.65	0.31	0.04	1.24
Joint action						
Untreated control	0.020	0.025	0.033	0.054	0.076	
[C]0.0904	0.02	0.07	0,09	0.12	0.31	
Net gain[B]	-	0.05	0.02	0.03	0.19	0.29
[D]0.0904	0.020	0.024	0.360	0.380	0.810	
Net gain[B]	-	0.004	0.336	0.020	0.430	0.79

Overall net gain: concentration in animal after 30 days – concentration in animal at zero day.
Net gain – difference in concentration between the immediate preceding days of harvesting, e.g. 4-0 days, etc.
$1/100^{th}$ 96 h LC_{50} values of the metal ions in the test compounds.
$1/10^{th}$ 96 h LC_{50} values of the metal ions in the test compounds.

concentrations of the Pb ions detected in the animal tissues collected from the various test treatments (control, 3.9635 mg/L and 39.635 mg/L) under the single or joint bioaccumulation studies. Furthermore, analysis based on the *t*-test for evaluating significant difference between two treatment means showed that there were no significant (P>0.5) differences between the concentration of the Pb ions accumulated in the animal tissues under the single and joint bioaccumulation studies.

Copper (Cu)

Post treatment analysis of whole body tissues of *L. saxatilis* showed that the animals exposed to sublethal concentrations (0.1021 mg/L and 1.0206 mg/L) of the Cu compound accumulated measurable quantities of the metal ions that were approximately two or three higher than the level accumulated by animals in untreated control media (Table 3). Furthermore, at the end of the 30-day exposure period, the amount of Cu accumulated by the exposed animals fluctuated significantly over the 30-day exposure period and there were no significant positive correlations between the amount of Cu accumulated by the animals with respect to time (R^2= 0.1351 and 0.3986 for 0.1021 mg/L and 1.021 mg/L test media, respectively) and test medium concentration. Similar observation was also recorded under the joint action studies (Table 3). In the presence of other metals, *i.e.* under joint action studies, the total amount of Cu ion accumulated in animals exposed to each of the two sublethal concentrations (4.5885 mg/L and 45.885 mg/L) of metal mixtures were 0.62 µg/g and 1.08 µg/g, respectively, compared to 0.44 µg/g and 7.23 µg/g respectively of Pb ion accumulated by animals exposed to corresponding concentrations under the single action studies (Table 5, Figure 3).

The computed bioaccumulation ratios were 1.41 and 0.15 (Table 5), thus indicating varying

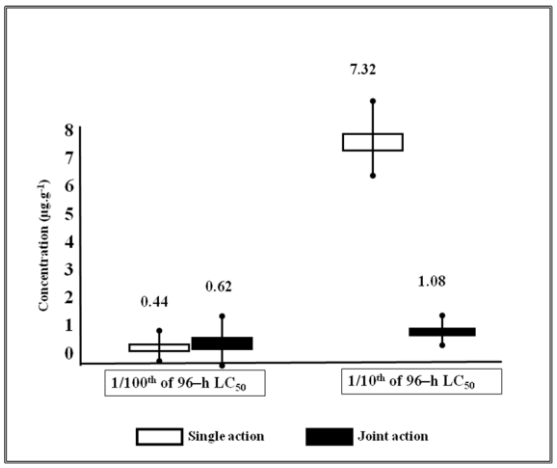

Figure 3. Figure of the overall gain in heavy metal (Cu ions) by *L. saxatilis* exposed to the test metals under single and joint bioaccumulation studies.

Table 5. Comparison of the overall gain in heavy metals by *L. saxatilis* exposed to the metals under single and joint bioaccumulation studies in laboratory bioassays

Treatment/Metal	Overall gain* of metal in single bioaccumulation studies (µg.g^{-1})	Overall gain* of metal in joint bioaccumulation studies (µg.g^{-1})	BAR**
1/100th of 96-h LC_{50}			
Zn	11.07	6.64	0.60
Pb	144.05	287.95	2.00
Cu	0.44	0.62	1.41
Cd	0.56	0.29	0.52
1/100th of 96-h LC_{50}			
Zn	27.99	22.64	0.81
Pb	339.03	459.56	1.36
Cu	7.23	1.08	0.15
Cd	1.24	0.79	0.65

A. Overall gain : concentration in animal after 30 days – concentration in animal at zero day
B. Bioaccumulation ratio (BAR).
BAR = 1: indicates similar gain in metal during joint and single bioaccumulation studies (additive)
BAR > 1: indicates an increase in metal gained during joint bioaccumulation studies (synergism)
BAR< 1: indicates a decrease in metal gained during joint bioaccumulation studies (antagonism)

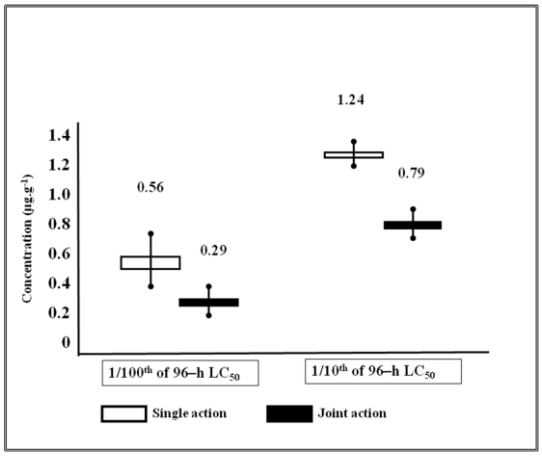

Figure 4. Figure of the overall gain in heavy metal (Cd ions) by *L. saxatilis* exposed to the test metals under single and joint bioaccumulation studies.

interactions at different levels of Cu concentrations in the mixture. Statistical evaluation by Analysis of Variance (ANOVA) showed that there were no significant (P>0.5) differences between the concentrations of the Cu ions detected in the animal tissues collected from the various test treatments (control, 0.1021 mg/L and 1.021 mg/L) under the single or joint bioaccumulation studies. Furthermore, analysis based on the t-test for evaluating significant difference between two treatment means showed that there were no significant (P>0.5) differences between the concentrations of the Cu ions accumulated in the animal tissues under the single and joint bioaccumulation studies.

Cadmium (Cd)

Post treatment analysis of whole body tissues of *L. saxatilis* showed that there were little differences between the amount of Cd accumulated by the animals exposed to the treated media (0.0904 mg/L and 0.904 mg/L) and the control animals (Table 4). Although the concentration of Cd accumulated by the test animals was not substantial, there were significant and positive correlations between the amount of Cd accumulated by *L. saxatilis* with time of exposure (R^2= 0.966 and 0.9712 for 0.0904 mg/L and 0.904 mg/L test media, respectively). Similar observation was also recorded under the joint action studies (Table 4). In the presence of other metals i.e. under joint action studies, the total amount of Cd ion accumulated in animals exposed to each of the two sublethal concentrations (4.5885 mg/L and 45.885 mg/L) of metal mixtures were 0.29 µg/g and 0.79 µg/g respectively; and these values were approximately three times and two times lower than the amounts of 0.56 µg/g and 1.24 µg/g of Cd accumulated by *L. saxatilis* when exposed to test medium containing only Cd compound at corresponding concentrations (Table 5 and Figure 4).

The computed bioaccumulation ratios were 0.52 and 0.64 (Table 5), thus indicating an antagonistic interaction between the metal components of the mixture in relation to the Cd accumulation.

Discussion

In the single action studies, the exposure of *L. saxatilis* to sublethal concentrations of Zn, Pb or Cu resulted in a steady increase in the body tissue concentration of the metals to levels that were two to six times higher than in control animals. The observation of increased concentration of these metals in animals exposed to sublethal concentrations of the metals in single action laboratory studies is well established (Baron, 1995; Bryan and Langston, 1992; Oyewo, 1998). The bioaccumulation of heavy metals in animal tissues occurs as a result of competing rates of chemical uptake and excretion. Furthermore, the synthesis of low-molecular weight proteins, *e.g.* metallothionein which form complexes with the metal ions, as well as the formation of encapsulated metal granules in tissues of exposed animals have also been reported to be responsible for metal accumulation in some aquatic mollusk (Clark, 1992; George, 1989; Langston and Zhou, 1987). It must however be noted that the exposure of *L. saxatilis* to the sublethal concentrations (3.92 mg/l and 0.392 mg/l) of Cu revealed that the amount of Cu accumulated in the tissues of the exposed animals fluctuated significantly over the experimental period. Furthermore, exposure of the test animals to sublethal concentration of Cadmium resulted in minimal changes in the body tissue concentration of Cadmium in the exposed animals over the 30-day experimental period, indicating the ability of the animal to excrete Cadmium rapidly before it builds up at the test concentration. The exposure of the animals to the test sublethal concentrations of heavy metals (Zn, Pb, Cu and Cd) mixtures, in order to demonstrate the influence of the metals on the individual rate and pattern of bioaccumulation revealed that there were interactions between the constituent metals when

applied jointly against *L. saxatilis*. These interactions resulted either an increase or decrease on overall concentrations of the metals accumulated in the animal tissues at steady state when compared to results obtained in the single action studies. For instance, the concentrations of Zn, Cu and Cd ions accumulated by the periwinkle were found to be about two-seven times lower than the concentrations of the same metals in exposed animals during the single actions studies, indicating an antagonistic interaction between the metals. Interestingly, this observation of antagonistic interactions between the test metals is in conformity with an observation of reduction in toxicity of these metals when applied in mixtures against *L. saxatilis* (Otitoloju, 2001). However, with regards to Pb ion accumulation, the concentration of the metal accumulated by *L. saxatilis* over the 30-day experimental period was found to be higher (about two times) than the concentration of the metal accumulated over a similar period when the animal was exposed to Pb acting singly. This observation also corroborated the results of previous work, who reported an enhancement in the toxicity of Pb compound against *L. saxatilis* when applied jointly with Zn, Cu ad Cd compounds (Otitoloju, 2001). The mechanism(s) responsible for the enhanced or reduced bioaccumulation of the test metals when applied jointly against *L. saxatilis* is therefore suspected to be as a result of interference with penetrability of the *L. saxatilis*, competition for binding site or the formation of metal complexes which may be easier (or not) to excrete. This should therefore merit future investigations in order to establish more accurately the mechanism (s) responsible for the antagonistic interactions between these metals when tested against *L. saxatilis*. The practical significance of this joint bioaccumulation study is that it provides an important exploratory bioassay which could assist in understanding the mechanism (s) responsible for the type of interactions existing between heavy metal types when applied jointly in toxicity studies. Furthermore, results of metal interactions and the subsequent reduction on the concentration of the metals bioaccumulated in exposed animals may be useful in the management of metal contaminated water bodies in a similar way in which such observation of antagonistic interaction between mercury (Hg) and selenium (Se) was previously exploited on the treatment of mercury contaminated fishes in Swedish Lakes (Paulsson and Lundbergh, 1991).

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