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Journal Name:	British Journal of Applied Science & Technology
Manuscript Number:	2014_BJAST_13509
Title of the Manuscript:	The Effect of L-Buthionine Sulfoximine on the Cytotoxicity and Interaction of As, Cd, Hg, and Pb on MCF 7 Cell Line
Type of the Article	Short Research Article

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This journal's peer review policy states that **NO** manuscript should be rejected only on the basis of '**lack of Novelty**', provided the manuscript is scientifically robust and technically sound.

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PART 1: Review Comments

	Reviewer's comment	Author's comment (if agreed with reviewer, correct the manuscript and highlight that part in the manuscript. It is mandatory that authors should write his/her feedback here)
Compulsory REVISION comments	<p>-Please, include Figure captions for the final version (if case, final evaluation) of the Manuscript. This is compulsory to better follow the different trends of variations of the results presented. Also, verification of consistency with the text is vital.</p> <p>-Figure 3 is not referred to in the body of the text.</p> <p>Thank you.</p>	All concerns in this section have been addressed
Minor REVISION comments	<p>-As can be seen from figure 4, the dependence of TU vs lethality seems to level off (stabilize) at 60%, not 40%. Please check.</p> <p>-The addition of several recent (2012-) and illustrative References, as Authors' choice, regarding the theme approached, would be welcome.</p> <p>-Some very minor language/typing amendments: It was first thought that since the metals can interact with GSH ligand and potentially interfere with GSH measurement, the concentration of the metals used will have significant effect on the GSH levels. (page 5), The result showed that As and Cd contributed the most to the toxicity of the mixture... (page 7), or you may write: The result showed that As and Cd were the most important contributors to the toxicity of the mixture. For the caption of Table 1, please write: GSH levels, not GHS. (page 5). The same for the section Conclusion: write GSH-depleted cells. Please, use article when the situation requires, such as for cases: "Our next work will profile the level of MT..." and "The response additive model will also be considered (see Conclusions). I suggest: "The mean values of the interactive indices for all concentrations were more greater/higher than one" (page 8, the question is about a numerical value).</p>	Your suggested changes have been addressed, the corrections you indicated were addressed and the grammatically errors that were highlighted were corrected.



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<p><u>Optional/General</u> comments</p>	<p>The Article deals with the assessment of the effect of L-buthionine sulfoximine on the cytotoxicity and interaction of As, Cd, Hg, and Pb on MCF 7 Cell Line. In view of the minutious investigation, I recommend it for publication.</p> <p>GENERAL CHARACTERIZATION OF THE PAPER:</p> <p>The Abstract and Title are illustrative.</p> <p>The Introduction is focused on the aspects related to environmental chemical pollution, as well as on the toxic potential and health effects of the studied elements: As, Cd, Hg, Pb.</p> <p>The complex influence and toxicological effects elicited by chemical mixtures are reminded.</p> <p>Studies pointing towards various interactive effects of metal ions are reminded. Biochemical responses are followed, profiling antioxidant components such as metallothioneins or glutathione.</p> <p>It is then asserted that intracellular interaction of the protective proteins with metal ions can greatly affect the cytotoxicity of these ions.</p> <p>The level of exposure to such metallic elements influences the biosynthesis of metallothioneins and glutathione.</p> <p>It is suggested that the systematic depletion of metallothioneins and glutathione from the cells and profiling cytotoxicity of cells using multiple metal ions, could be of analytical interest, in understanding the link between the detoxifying polypeptides and cytotoxicity, in that it can lead to a better understanding of the effect of metal combination on cells could be assessed.</p> <p>The effect of depleted GSH on the cytotoxicity and interactions of As, Cd, Hg and Pb on MCF 7 cell line was determined by pre-treating the cells with L-2-amino-4-(S-butylsulfonimidoyl) butanoic acid also known as L-buthionine sulfoximine, and subsequent systematic exposure to different concentrations of both individual and combined metal ions.</p> <p>L-buthionine sulfoximine was chosen for its previously reported ability to specifically and irreversibly inhibit gamma glutamylcysteine synthetase, hence being involved in glutathione depletion.</p> <p>Nevertheless, given glutathione depletion, the cells' protection was ensured by metallothioneins. MCF 7 cell lines served for testing the induced cytotoxicity and activation of stress genes of cancer cells.</p>	<p>All the suggestions you made were considered.</p> <p>Thank you for your critical review.</p>
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	<p>Igor Pro 6.22A was employed to generate concentration-response curves and to estimate concentrations at the various response percentages. Interaction indices were calculated to assess the type of interaction among the four metals.</p> <p>The four metals were chosen in this investigation, as they are strongly correlated to environmental health; their composite mixtures have been confirmed to elicit various types of toxicological interactions.</p> <p>The Materials and methods section presents the working procedure applied for cell culture and exposure, cell viability testing by spectrofluorometry, as well as for the measurement of intracellular glutathione level in MCF7 cells.</p> <p>The Results and discussion section reveals the results obtained at the assessment of the glutathione levels in cells, in the presence of the tested metals.</p> <p>Compared to the control, cells without LBSO pretreatment exhibited increased levels of GSH after exposure to As, Cd, and Hg solutions, which was consistent with previous studies reporting increased levels of GSH in cells exposed to metals.</p> <p>The level of GSH in cells exposed to Cd and Hg increased more than 2-fold and those exposed to As increased about 1.5 fold relative to the control.</p> <p>In cells pretreated with LBSO, the levels of GSH decreased to about half of the control for all the metals used in the treatment. Cells exposed to Hg after LBSO pretreatment showed the lowest levels of glutathione.</p> <p>When compared to those without LBSO pretreatment, LBSO-treated cells exposed to Hg, Cd, and As showed about 14, 5, and 3-fold decreased levels of GSH respectively.</p> <p>Surprisingly, the same level of GSH was found in cells exposed to Pb, irrespective of LBSO pretreatment. Thus, cells exposed to Pb in both cases showed about 50% decrease in the levels of GSH, when compared to the control.</p> <p>A higher-than-control level of GSH at exposure to Pb would have been expected, since metals have the potential for eliciting GSH increase, as previously reported.</p> <p>In the present study, the influence of Pb on the level GSH was more highly expressed than the one effect expected from LBSO. Thus LBSO markedly influenced the levels of GSH on cells when exposed to the composite mixture of As, Cd, Hg, and Pb.</p> <p>The acute toxicity of individual metals in presence of LBSO was assessed. LBSO</p>	
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	<p>pretreatment on MCF 7 cells was shown to increase the toxicity of the individual metals.</p> <p>It was assessed that without glutathione protection, a relatively lower amount of each metal was able to elicit a desired response in the cells exposed to the metals. Without LBSO pretreatment, the four metals showed significantly ($P < 0.05$) different levels of toxicity towards MCF 7 cells, the trend of decrease being consistent with the EPA ranking ($Hg > Cd > As > Pb$).</p> <p>In contrast to the EPA classification, the cytotoxicity assay of cells with LBSO pretreatment showed As and Cd switching positions in the ranking, with As becoming slightly more toxic than Cd.</p> <p>Nevertheless, there were no significant differences in the toxicities of the two metals, showing similar influence on GSH.</p> <p>Hg and Pb proved the most and least toxic respectively, among the four metals, with no correlation with LBSO pretreatment.</p> <p>Each metal showed a significant percentage increase in cells mortality, at increasing concentration of the metals, pointing towards a dose-dependent cytotoxic effect of the four metals. Pb exhibited the highest range between the concentration for LC20 and that corresponding to LC80.</p>	
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	<p>Different toxic effects of the tested metals were obtained, in the absence and presence of glutathione, respectively: at 50% lethality, the toxicities of As and Hg in GSH-depleted cells were 5 and 9 times higher respectively, relative to the their toxicities in GSH-rich cells.</p> <p>The same level of GSH was observed in LBSO-pretreated cells for the metals investigated, except for lead, that showed a significantly lower toxicity, indicating the likely to occur presence of a metal-selective protective protein, which implies that, at decreased GSH amount, the level of protective proteins like metallothioneins increases, in order to keep balanced the intracellular level of total protective proteins. This assertion is supported by previous studies.</p> <p>A composite mixture of the four metals was prepared based on the EPA MCL ratio, and serial dilutions of the mixture were employed to assess concentrations at various percentage deaths of the cells.</p> <p>As predictable, the ranking for the toxicity of the metals in the composite mixture in the presence of LBSO proved consistency with the EPA ranking (Hg > As > Cd > Pb).</p> <p>The toxicities proved concentration-dependent for all the tested metals.</p> <p>Relying on LC50 data, it could be concluded that each of the four metals As, Cd, Hg, Pb in the mixture were respectively 2, 4, 2, and 48 more toxic than when they were present alone.</p> <p>The comparative study between individual and combined toxicities, aimed at estimating toxic units for each of the metals at various LCs and the concentration of each mixture component was scaled for its relative toxicity. The mean of the TU (toxic units) values of Hg and As were significantly higher, followed by Cd and Pb. It was shown that As and Cd contributed the most to the toxicity of the mixture, with equal contributions. The contribution of Hg, the second toxic metal in the mixture, was significantly lower than that of Cd and As.</p> <p>The TU values of As, Cd, and Hg were slightly higher at lower response levels and leveled up at subsequent response levels. For Hg, the TU values were erratic at lower response, before leveling at about 60% responses upwards. As and Cd values were reported as leveling off at 40% percent response upwards. In the case of Pb, the TU values were the same throughout the various response levels.</p> <p>To evaluate the type of toxicity interaction of the four metals in the composite</p>	
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	<p>mixture, the interaction of these four metals with their targets was considered, and the concentration addition model has been employed. The values for the index of interaction, considered the sum of the TU values, decreased, as the percentage response increased. The means of the interactive indices for all concentrations were greater than 1, pointing to a shift towards antagonistic interaction of the four metallic species.</p> <p>The one-sample t-test carried out on the interactive indices at different response levels showed that the interactive indices did not significantly differ from unity ($P=0.5$), proving that the interactive effect of the four metals is strongly additive. The results are discussed and compared with those previously reported.</p> <p>The prevalent factor in the present study is the suppression of the glutathione defense system, prior to exposure of the targets to the toxicants. Consequently, the targets were less capable to exhibit effective defense against the toxicity of the various metallic species mixture. Nevertheless, the various mixture components possess different degrees of potency, and they behave additively in the cells deprived of glutathione defense.</p> <p>The Conclusion Section stresses upon the main findings of this study. The mentioning of the directions for continuing this study is highly welcome.</p>	
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