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## Near-real-time biomonitoring of heavy metals using the xenoassay® system

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**Abstract.** Heavy metals have widespread industrial uses and have been found in increasing quantities as contaminants in all components of the biosphere. Water and sediment of rivers near industrial areas such as the Juru River in Penang and Langat River in Selangor are polluted with heavy metals. Thus, rapid and fast methods to detect the presence of heavy metals in the environment are necessary. Existing instrumental methods such as atomic absorption and emission spectrometry are very sensitive but the sole use of these instruments for heavy metal detection is extremely expensive, needs a skillful person to operate and is not amenable to near-real-time analysis. The best scenario for routine biomonitoring of heavy metals is the marriage between instrument- and bioassays. Currently, the USEPA has recognized whole cell-based bioassays such as as Polytox™ and Microtox® for the detection of heavy metals. Unfortunately these cell-based assays cannot be used as real-time or near real-time assays in the field as they require bulky incubators. Near-real-time monitoring of heavy metals giving results in less than one hour is very useful in environmental CSI (Criminal Scene Investigation) or ECSI where temporal and spatial concentrations of heavy metals in running waters are a challenge to environmentalists to pinpoint heavy metals POS (point of source) for legal purposes. Enzyme-based inhibitive assays are simple, rapid and fast and could be developed for near real-time assays. We have developed an inhibitive assay system –Xenoassay® based on proteases for the assay of heavy metals. The system could detect the heavy metals mercury, cadmium, lead, copper, zinc and silver at the sub parts per million level. Field trial near-real-time assay capability shows promising results.

**Key words:** Bioassay, Xenobiotics, Near-real-time, Heavy metals.

### Introduction

Heavy metals have widespread industrial uses and have been found in increasing quantities as contaminants in all component of the biosphere (Upreti et al, 2011). Water and sediment of rivers near industrial areas in Malaysia such as the Langat River in Selangor, Juru River in Penang, and Linggi River in Negeri Sembilan are polluted with heavy metals (Sarmani, 1989; Shukor et al., 2006, 2008; Shazili et al., 2006). Due to this, simple, rapid and fast methods to detect heavy metals in these rivers are necessary. Instrumental methods such as emission spectrometry and atomic absorption although sensitive are extremely expensive, need highly skilled persons to operate and are not amenable to near real-time analysis. Near-real-time assay is a condition where an assay for the presence of an analyte could be completed in less than an hour instead of days. The best scenario for routine biomonitoring of heavy metals is the marriage between instrument- and bioassays (Shukor et al., 2006, 2008).

Currently, the USEPA has recognized whole cell-based bioassays such as as Polytox<sup>TM</sup> and Microtox<sup>TM</sup> for the detection of heavy metals. Unfortunately these cell-based assays can not be used as real-time or near real-time assays in the field as they require bulky incubators (Masdor and Said, 2011). Near real-time monitoring of heavy metals is very useful in environmental CSI (Criminal Scene Investigation) or ECSI where temporal and spatial concentrations of heavy metals in running waters are a challenge to environmentalists to pinpoint heavy metals POS (point of source) for legal purposes (Wicke et al., 2009). Enzyme-based inhibitive assays are simple, rapid and fast and could be developed for near real-time assays. Previously we have developed a cysteine protease assay for heavy metals based on papain. The system is trademarked under the name Xenoassay. In this work we embarked upon the possibility of using this assay as a near-real-time assay for heavy metals.

## **Materials and Methods**

### ***Preparation of papain assay***

Papain assay of heavy metals was carried out according to the method of Shukor et al. (2006). Briefly, 5 · l of papain from a stock solution was added to 50 · l of 100 mM phosphate buffer, pH 6.8. The final concentration of papain was 0.1 mg/ml. Thereafter 45 · l of water sample was added and the mixture was incubated for twenty minutes at room temperature. This was followed with the addition of 50 · l of casein to the mixture. The final concentration of casein was 0.1 mg/ml. Immediately, 20 · l aliquot was withdrawn and mixed with 200 · l of Bradford dye-binding reagent and incubated for 5 minutes to get the absorbance for time zero. The remaining solution was incubated at 40 °C for 30 minutes. After the incubation period, 20 · l aliquot was again taken and treated in the same manner with the aliquot at time zero. The absorbance at 595 nm was measured using a microplate reader (Stat Fax® 3200 Microplate Reader, Awareness Technology Inc., USA).

### ***Enzyme inhibition studies***

Suitable volumes of heavy metals or river water samples of up to 50 · l were directly incubated with 50 · l of enzyme for 5 minutes at room temperature. The mixture was then assayed as above at room temperature. Regression curves were generated using the PRISM (Prism version 4.00 for Windows) non-linear regression analysis for four-parameter logistic equation software available from GraphPad, (GraphPad Software Inc., San Diego, CA). Means and standard errors were determined based on at least three independent experimental replicates.

### ***Field trials and near real-time assessment***

A site at the Derhaka Juru river previously reported to be polluted with heavy metals at N 05° 20.96, E 100° 24.17 (Shukor et al., 2006) was sampled every 2 hours for a period of 24 hours in December 2011 to assess near real-time capability. The determination of heavy metals in the samples was carried out using Atomic Emission Spectrometry on a Perkin Elmer Optima 3000 ICP-AES. Mercury was determined using a Perkin Elmer Flow Injection Mercury System (FIMS). All experiments were performed in triplicate.

## Results and Discussion

### Near real-time field trial

A temporal variation of heavy metals can be clearly seen in Figure 1. Instrumental analysis showed that zinc and copper exceeded the Maximum Permissible Limit (MPL) stipulated by the Department of Environment (DOE) Malaysia only at certain times. Zinc exceeded the MPL by the Malaysia DOE (3 mg/l) between 18.00 and 22.00 pm. Copper exceeded the MPL allowed by the Malaysia DOE (0.5 mg/l) approximately between 16.00-20.00 pm. All other heavy metals were below the detection level. Papain activity was inhibited in correlation to elevated levels of heavy metals measured above with about 100% inhibition discovered above 16.00 pm while other times showed a maximum inhibition of less than 50%.

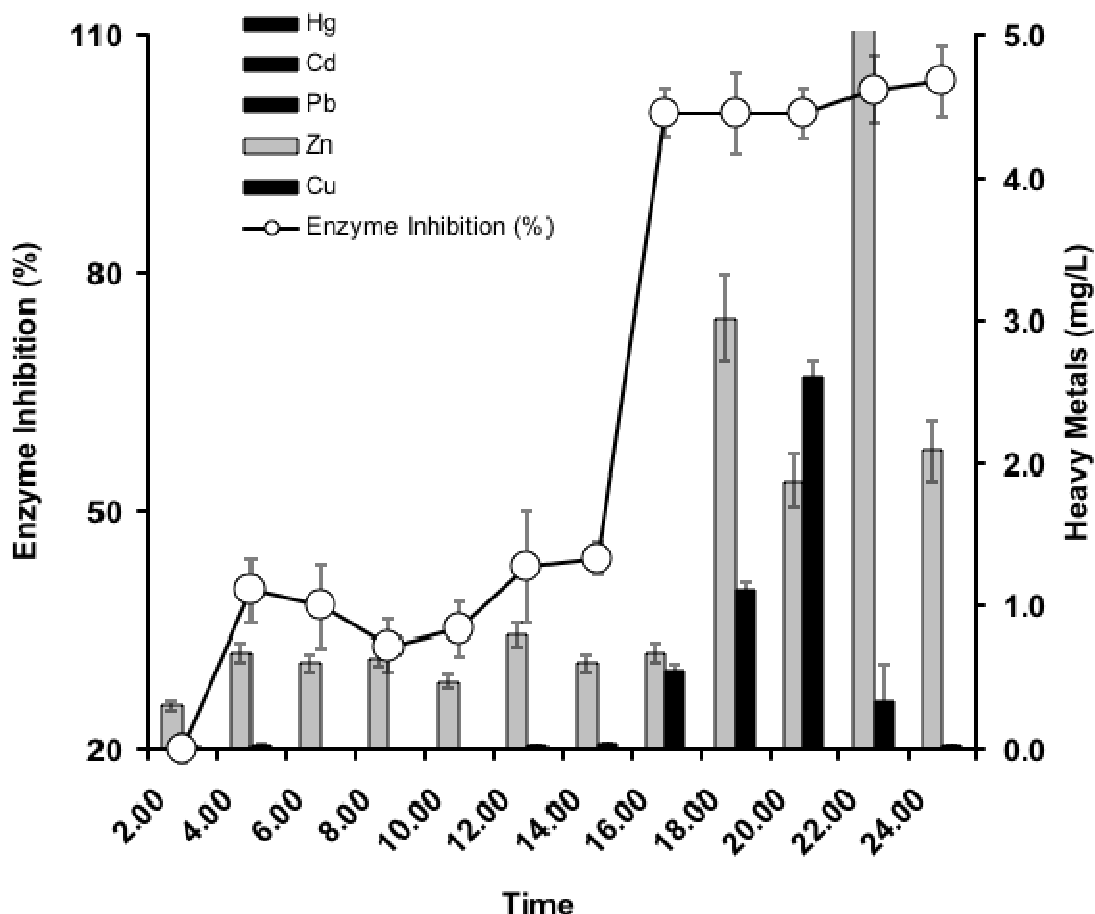


Figure 1. Temporal inhibition of enzyme activity and heavy metals concentration from a river water sample. Values are mean  $\pm$  standard error of the mean (n=3).

The Bradford casein dye-binding assay was used to indicate the inhibition of papain by heavy metals. The basis for the protein assay using casein as a substrate relies on the inability of the Bradford casein dye-binding reagent to stain polypeptide with less than a molecular weight of 2 kDa (Shukor et al., 2006, 2008). In the presence of enzyme, the substrate casein would be degraded and the degradation product is not stained by the Bradford reagent, thus the solution remains brown in color. The undigested casein would be stained blue by the Bradford casein dye-binding reagent. The studies on the optimization of papain showed that this method requires much less enzyme and a lower temperature optimum than all of the other previous proteases. The assay has an optimum temperature at 40 °C but it could also be carried out at room temperature making this assay amenable for near real-time application. The papain assay showed concentration that caused 50% inhibition or IC<sub>50</sub> for zinc, cadmium, copper, mercury and lead at 2.11, 1.0, 0.10, 0.39, 2.16 mg L<sup>-1</sup>, respectively, while the LOQ (Limits of Quantitation) are 0.2, 0.004, 0.1, 0.05, 0.1 mg L<sup>-1</sup>, respectively (Shukor et al., 2006, 2008). The IC<sub>50</sub> and LOQ values for zinc and copper could be directly used as a legal indicator of water toxicity since the values are within the MPL (maximum permissible limit) range while the rest of the heavy metals could also be an indicator for heavy metals toxicity in the same league as the other assays.

The elevated level of zinc found in the near real-time field trial work is solely responsible for the inhibition of enzyme activity of at least 50% at all measuring period. Since galvanised metal work are located in this area, it is suspected that these types of industries are responsible for the elevated zinc level in this area. Zinc can cause DNA aberrations especially at a high concentration such as those recorded in this study. The temporal level of zinc in the waters ranging from below to elevated level of the MPL for zinc highlight the problem in heavy metals sampling in running water bodies. Often unscrupulous industrial operators release heavy metal pollution into rivers during heavy raining or at night to evade detection by enforcement agencies. Temporal variation of heavy metals in river and running water is often seen in running water (Wicke et al., 2009) but previous works have shown that even sedimentary samples have large variations in terms of spatial and temporal concentration of heavy metals (Birch et al., 2001). Application of real-time or near real-time monitoring of heavy metals are almost nonexistent since instrumental and biological-based assays such as urease and Microtox™ require bulky instrument or take too long (> 2 hour) per assay. Near-real-time biomonitoring is an exciting and a recent trend as realtime or near-realtime biomonitoring of heavy metals using commercial inhibitive assay systems are almost nonexistent as the majority of the present assay systems takes too long. Most of the available system can be defined as batch system monitoring as they need to be transported to the laboratory (Jung et al., 1995; Hsieh et al., 2004). The longer the sample is kept, the less is the bioavailability of heavy metals as they are prone to adsorption to walls of sample containers and in the case of mercury and arsenic, evaporation into the atmosphere. System such as Microtox™ has been demonstrated to be affected by sample age (Birch et al., 2001). Heavy metals such as mercury and arsenic could be loss via evaporation during transport or be irreversibly attached to sampling container walls- all reducing the availability of the heavy metals (Shukor et al., 2006).

In conclusion, the Xenoassay® system based on papain has been successfully used in near real-time assay of heavy metals and the results validated by instrumental analysis. A temporal variation of the heavy metal zinc was discovered. The development this assay would allow a temporal analysis of heavy metals pollution to be carried out and legal action towards the culprit responsible for the pollution be carried out.

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