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# Rapid Biomonitoring of Heavy Metals in Polluted Sites Using Xenoassay®-Metal

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**ABSTRACT**

Long-term water contamination, including heavy metals, may harm the reputation of the location. Because of this, it is necessary to do regular water quality checks. In light of the high expense of instrument monitoring, biomonitoring based on enzymes is rapidly gaining popularity around the world. For the purpose of this investigation, a molybdenum-reducing enzyme from the *Serratia marcescens* strain DRY6 that is sensitive to the heavy metals was used to build a quick inhibitory enzyme assay. The bacterium's molybdenum-reducing activity is extremely vulnerable to heavy metals. With the help of the established assay, heavy metals were detected in water samples collected in Malacca, Penang, and Perak. A number of sampling locations in Penang were found to be inhibiting enzyme activity, while all sampling locations in Malacca and Penang were confirmed to be free of high heavy metal concentrations. The Malaysian Department of Environment has set a maximum permissible limit for the suppression of Mo-reducing enzyme activity in Penang. Detection of heavy metals can be done in less than ten min at room temperature. The entire procedure takes less than 20 min. a first screening procedure or even a near real-time method for routine monitoring of heavy metals, assay is quick and straightforward.

## INTRODUCTION

Pollution, including pesticides, organic and inorganic compounds, and heavy metals, has risen as a result of Malaysia's extensive mining, manufacturing, and agricultural operations. There are 189 contaminated or marginally contaminated rivers within Malaysia's Department of Environment (DOE). As heavy metals can be poisonous, this is a concern. Humans, marine species, and food chains may be at risk because some toxicants have not been entirely destroyed. Hazardous to marine life are toxicants, especially heavy metals. Toxicants in polluted water must be monitored on a regular basis. Detection of heavy metals using instruments is prohibitively expensive, time consuming, and does not provide near real-time detection. Toxic compounds from industrial effluents, contaminated rivers, and other polluted places must be screened for quickly and easily. The use of inhibitive biological-based tests for mass monitoring of heavy metals in rivers is a critical option. In addition, some enzyme-based assays can yield near real-time findings that can reflect changes in heavy metal levels over time. Forensic examination of contaminants requires the screening of thousands of samples each river, and this may be done using simple methods such the use of living organisms and enzymes, which can be done by the general public and schools. To identify bioavailable metal ions rather than the less harmful water-insoluble metal sulphides, an enzyme-based bioassay provides a cheap, simple, and rapid technique. Luminescence bacteria-based tests are one type of biomonitoring system that makes use of microorganisms.such as Microtox [1], Xenoassay Light [2], and MTT-based assays [3,4]. Systems using enzymes include the use of proteases [5–10], urease [11], acetylcholinesterases [12–14] and the molybdenum-reducing enzyme [15–17].

It has been increasingly polluted as a result of Malaysia's large mining, manufacturing and agricultural activities. Pesticides are among the pollutants. More than 180 rivers in Malaysia's Department of Environment have been found to be toxic or moderately contaminated (DOE). Heavy metal poisoning is a worry because of this. Some toxicants have not been completely removed, which could put humans, marine life, and food chains at danger. Marine life is threatened by toxicants, particularly heavy metals. Regular testing of contaminated water


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for toxicants is required. It is extremely expensive, time-consuming, and does not allow near-real-time detection of heavy metals using instrumentation. Fast and easy screening of toxic chemicals from industrial effluents, contaminated rivers and other damaged areas is necessary. Large-scale monitoring of river heavy metal contamination must include inhibitory biological testing. There are enzyme-based tests that provide near-real time results that can track changes in heavy metal levels over time, as well. There are hundreds of samples each river that need to be screened for toxins, and simple procedures like the use of living organisms and enzymes can be used by both the general public and schools to conduct forensic examinations. In contrast to the water-insoluble metal sulphides, which are less dangerous and not bioavailable, an enzyme-based bioassay, for example, is a cheap, simple, and quick way of identifying bioavailable metal ions .such as Atomic Emission Spectrometry (AES) or Flow Injection Mercury System (FIMS).

Because the molybdenum-reducing enzyme is exclusively inhibited by heavy metals, it has been used for the detection of heavy metals in soil. The assay is simple and can be completed in less than five min on a regular basis. Only one molybdenum-reducing enzyme system's single element has been detected thus far, though [15–17]. Because it has a wavelength around 865 nm, molybdenum blue is an infrared-like result of molybdenum (sodium molybdate) reduction. A novel enzyme, the Mo-reducing enzyme, has been isolated but its identity is still unknown. The enzyme converts soluble molybdenum into a colloidal form that bacteria can use to counteract the toxicity of the metal [18–29]. In addition to bioremediation of molybdenum and potential recycling of molybdenum from industrial effluents, this colloidal form can be trapped in membranes such as dialysis tubing. [30,31]. We revisited our previous works with the molybdate-reducing bacterium *Serratia marcescens* strain DRY6 local isolate [32] by introducing more sampling locations for the rapid biomonitoring tool for heavy metals.

## MATERIALS AND METHODS

### *Serratia marcescens* strain DRY6 growth and maintenance

Isolation and characterization of the bacterium was previously reported [18]. As previously described, the bacterium was grown and maintained in a low phosphate medium enriched with molybdenum as before [18]. The composition (w/v%) of the medium (pH 7.0) is as follows: $Na_2MoO_4.2H_2O$ (0.484), NaCl (0.5), $(NH_4)_2.SO_4$ (0.30), glucose (1.0), $MgSO_{4.7}H_2O$ (0.05) and $Na_2HPO_4$ (0.05), yeast extract (0.05). This mixture was supplemented with 1.5 percent agar to keep the bacterium alive. Molybdenum-reducing properties of this bacterium were preserved by the low phosphate media.

### Molybdenum-reducing enzyme assay

NADH and 12-phosphomolybdate are used as electron donors and acceptors in this study's enzyme assay. In a short time, 12-phosphomolybdate is turned into molybdenum blue by the enzyme. As an overview, 50 mM of phosphomolybdic acid, or 12-phoshomolybdate, (sodium phosphomolybdate hydrate, Sigma, St. Louis, USA) was produced as an initial stock solution in 10 mM of phosphate buffer pH 5.8 and added to a 1-milliliter enzyme reaction mixture at a final concentration of 3-milliliters [33,34]. Finally, NADH is added to bring the final concentration up to 2.5mM. The reaction was started by adding 50 microliters of a crude molybdenum-reducing enzyme fraction (1 mg/mL final protein) to the mixture. The entire volume of the reaction was 1 ml. After a one-minute incubation time at ambient temperature, the absorbance was measured at 865 nm. When it comes to determining molybdenum reductase activity, 1 nmole molybdenum blue produced per minute at room temperature is considered one unit of activity. In order to measure molybdenum blue, the extinction coefficient at 865 nm is 16.7 $mM^{-1}cm^{-1}$ at 865 nm. If the absorbance at 865 nm increased by 1.00 units per minute per mg of protein, then a one-milliliter assay mixture containing 60 nmole of Mo-blue would be produced [34].

### Preparation of crude enzyme

The sole difference between the high and low phosphate media was the phosphate concentration, which was increased to 100 mM to prevent the creation of molybdenum blue while still retaining the bacterium's Mo-reducing activity for large-scale growth [34]. Because cell harvesting is hindered by the development of molybdenum blue, the phosphate concentration must be increased. Molybdate was still present in the media. There were five liters of culture medium for the bacterium to develop in a series of 250 mL conical flasks. For 48 h, growth was induced at 30°C using a 120-rpm orbital shaker (Yihder, Taiwan).

The Coomassie dye-binding method was used to measure protein using crystalline BSA as a reference. Every experiment described here was performed at 4 degrees Celsius unless otherwise noted. Centrifugation at 10,000 ×g for 10 min resulted in the collection of cells. At least one 50 mM Tris.Cl wash was performed on the cell pellet. Tris-buffered Cl buffer pH 7.5 (made at 4 ºC) was resuspended in a minimal volume of the same buffer and resuspended. We prepared the pellet by mixing it with 10 mL of the aforementioned Tris-HCl solution. Buffer in which 0.1 mM dithiothreitol has been added. To achieve a sonication period of at least 20 min, the cells were sonicated for one minute on an ice bath and cooled for four min afterward. After centrifuging the sonicated fraction at 10,000 ×g for 20 min, the supernatant becomes the crude enzyme fraction which was transferred and kept at -80 ºC. The crude enzyme maximal activity was at pH of 6.0 and temperatures of between 25 and 35 ºC (unpublished results).

### Real water samples monitoring works

Polycarbonate containers were used to collect river water samples from several locations in Penang, Perak River, and Malacca River. The samples were transported to the laboratory in cooled containers. The samples were taken between 2016 and 2017. pH values ranged from 6.25 to 7.25 for all of the water samples tested. The water samples were filtered through Teflon membrane filter (0.45 □m). For rapid bioassay works, suitable volumes of heavy metals or river water samples of up to 50 □l were first incubated for 5 min at room temperature with 50 □l of crude enzyme preparation. The mixture using a Perkin Elmer Optima ICP OES (Optima 8300) from the agriculture faculty of UPM. Mercury was determined using a Perkin Elmer Flow Injection Mercury System (FIMS). All experiments were performed in triplicate.

### Calculation for percent of inhibition

The percent inhibition was calculated according to the subsequent formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$


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## RESULTS AND DISCUSSION

Although the Mo-reducing enzyme has been isolated, its identity has not yet been established. Soluble molybdenum is made less harmful by the enzyme's ability to turn it into colloidal molybdenum. Molybdenum bioremediation and industrial effluent molybdenum recycling could benefit from this colloidal form of molybdenum, which can be contained in membranes like dialysis tubing. The crude enzyme preparations from the Mo-reducing bacteria have been previously utilized as a rapid biomonitoring tool for heavy metals [32,35]. However, despite the fact that the enzyme that reduces Mo has been isolated, its identity is still a mystery. The enzyme's capacity to convert soluble molybdenum into colloidal molybdenum reduces its toxicity. Membranes such as dialysis tubing could be used to store colloidal molybdenum for use in bioremediation and industrial wastewater recycling.

### Evaluation of developed assay

After that, water samples from the Malacca River were used to evaluate the newly devised assay. Enzyme inhibition was detected in less than 10% of the water samples analyzed. These water samples did not contain heavy metals at levels over the maximum permitted limit, according to heavy metal analysis. There were no higher levels of hazardous heavy metals found in the Malacca River, according to the study's findings (**Table 1**). This was also found in our previous sampling work [32] indicating that this area is not prone to heavy metal pollution.

The results for Perak River also indicated that the river was free of elevated concentration of toxic heavy metals (**Table 2**). This was also found in our previous sampling work [32] indicating that this area is not prone to heavy metal pollution. The inhibition of Mo-reducing enzyme activity in several areas in Penang (**Table 3**) was also found in our previous sampling work [32] indicating this area is prone to heavy metal pollution. The levels of heavy metals were found to be higher than the Maximum Permissible Limit as stipulated by the Malaysia Department of Environment [36]. These areas are large drains from several industries surrounding the Juru areas, which are notorious for their polluted discharge effluents [37,38].

**Table 1.** Table of percentage activity (%) the Mo-reducing enzyme and heavy metal contents from Sg. Melaka.

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| Sample | GPS location | Percentage enzyme activity (%) | Concentration of heavy metals presence in the samples (mg/L) | | | |
|---|---|---|---|---|---|---|
| | | | $Cu^{2+}$ | $Hg^{2+}$ | $Cr^{6+}$ | $Ag^{+}$ |
| Malacca 1 | N $2^0$ 12.466' E $102^0$ 15.096' | 94.32 | n.d. | n.d. | n.d. | n.d. |
| Malacca 2 | N $2^0$ 12.414' E $102^0$ 15.075' | 94.91 | n.d. | n.d. | n.d. | n.d. |
| Malacca 3 | N $2^0$ 12.388' E $102^0$ 15.022' | 90.00 | n.d. | n.d. | n.d. | n.d. |
| Malacca 4 | N $2^0$ 12.361' E $102^0$ 15.056' | 94.56 | n.d. | n.d. | n.d. | n.d. |
| Malacca 5 | N $2^0$ 12.360' E $102^0$ 15.061' | 91.19 | n.d. | n.d. | n.d. | n.d. |
| Malacca 6 | N $2^0$ 12.283' E $102^0$ 15.078' | 93.18 | n.d. | n.d. | n.d. | n.d. |
| Malacca 7 | N $2^0$ 12.257' E $102^0$ 15.084' | 90.00 | n.d. | n.d. | n.d. | n.d. |
| Malacca 8 | N $2^0$ 12.214' E $102^0$ 15.096' | 92.53 | n.d. | n.d. | n.d. | n.d. |

[2] n.d. = not detected

**Table 2.** Table of percentage activity (%) the Mo-reducing enzyme and heavy metal contents from various area in Penang.

| Sample | locations | Percentage enzyme activity (%) | Concentrations of Heavy metal (mg/L) Ag | Cu | Hg | Cr |
|---|---|---|---|---|---|---|
| Penang 1 | N05º20.447 E100º 26.403' | 92.12 | n.d | n.d | n.d. | n.d. |
| Penang 2 | N05º20.665' E100º26.364' | 93.44 | n.d | n.d | n.d. | n.d. |
| Penang 3 | N05º20.601' E100º26.427' | 91.79 | n.d | n.d | n.d. | n.d. |
| Penang 4 | N05º20.640' E100º 26.470' | 25.99 | 0.56±0.04 | 0.45±0.05 | 0.012±0.01 | 0.36±0.09 |
| Penang 5 | N05º18.947' E100º26.348' | 16.02 | 0.25±0.02 | 0.74±0.03 | 0.033±0.08 | 0.68±0.07 |
| Penang 6 | N 05º20.96, E 100º24.17' | 9.43 | 0.21±0.04 | 4.02±0.04 | n.d. | n.d. |
| Penang 7 | N 05º20.96, E 100º17.25' | 16.67 | 0.11±0.06 | 0.94±0.08 | 0.019±0.05 | n.d. |

[2] n.d. = not detected

**Table 3.** Table of percentage activity (%) the Mo-reducing enzyme and heavy metal contents from Sg. Perak.

| Sample | GPS location | Percentage enzyme activity (%) | Concentration of heavy metals presence in the samples (mg/L) | | | |
|---|---|---|---|---|---|---|
| | | | $Cu^{2+}$ | $Hg^{2+}$ | $Cr^{6+}$ | $Ag^{+}$ |
| PR 1 | N$04^0$ 30.685 E $100^0$ 55.583 | 91.72 | n.d. | n.d. | n.d. | n.d. |
| PR 2 | N$04^0$ 30.804 E $100^0$ 55.555 | 94.44 | n.d. | n.d. | n.d. | n.d. |
| PR 3 | N$04^0$ 30.901 E $100^0$ 55.569 | 93.45 | n.d. | n.d. | n.d. | n.d. |
| PR 4 | N$04^0$ 31.970 E $100^0$ 55.586 | 90.98 | n.d. | n.d. | n.d. | n.d. |
| PR 5 | N$04^0$ 31.077 E $100^0$ 55.637 | 94.57 | n.d. | n.d. | n.d. | n.d. |
| PR 6 | N$04^0$ 30.685 E $100^0$ 55.583 | 90.44 | n.d. | n.d. | n.d. | n.d. |
| PR 7 | 4°46'22.73"N 100°56'40.73"E | 93.92 | n.d. | n.d. | n.d. | n.d. |
| PR 8 | 4°46'23.80"N 100°56'40.05"E | 97.93 | n.d. | n.d. | n.d. | n.d. |
| PR 9 | 4°46'40.24"N 100°56'42.88"E | 95.37 | n.d. | n.d. | n.d. | n.d. |
| PR 10 | 4°45'53.73"N 100°57'24.74"E | 94.67 | n.d. | n.d. | n.d. | n.d. |
| PR 11 | 4°45'16.20"N 100°57'33.67"E | 94.08 | n.d. | n.d. | n.d. | n.d. |
| PR 12 | 4°35'54.32"N 100°52'57.74"E | 93.20 | n.d. | n.d. | n.d. | n.d. |
| PR 13 | 4°35'41.69"N 100°52'58.76"E | 92.13 | n.d. | n.d. | n.d. | n.d. |
| PR 14 | 4°30'22.33"N 100°55'29.90"E | 91.30 | n.d. | n.d. | n.d. | n.d. |
| PR 15 | 4°30'13.03"N 100°55'30.67"E | 99.44 | n.d. | n.d. | n.d. | n.d. |
| PR 16 | 4°29'10.47"N 100°54'48.27"E | 91.12 | n.d. | n.d. | n.d. | n.d. |
| PR 17 | 4°29'9.67"N 100°54'47.18"E | 97.51 | n.d. | n.d. | n.d. | n.d. |
| PR 18 | 4°29'9.40"N 100°54'49.01"E | 99.51 | n.d. | n.d. | n.d. | n.d. |

[2] n.d. = not detected


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## CONCLUSION

A molybdenum-reducing enzyme-based test for heavy metals that is both sensitive and quick was utilized to rapidly monitor heavy metals in Penang, Perak and Malacca as part of a continuing work on rapid biomonitoring of water bodies and industrial effluent areas in Malaysia. The enzyme converts relatively colourless and soluble phosphomolybdate to molybdenum blue using NADH as the electron-donating substrate. In the presence of toxic metal ions, the conversion is inhibited. Nearly all of the samples show the absence of elevated level of heavy metals and reflected in the biomonitoring results. Similar to our previous results, several sites in Penang from the Juru industrial estate were found to contain elevated heavy metals concentration that should be an alarm for authorities monitoring heavy metals to take action and carry out more routine monitoring, hopefully involving rapid biomonitoring tools like the one developed in this study.

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