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PRELIMINARY DETECTION METHOD FOR HEAVY METALS BIOMONITORING VIA INHIBITIVE ASSAY OF BRAIN ACETYLCHOLINESTERASE FROM *DIODON HYSTRIX*

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Abstract: Acetylcholinesterase (AChE) is a well-known enzyme sensitive to pesticide exposure, but heavy metal sensitivity is rarely reported. Here, AChE extracted from the brain tissue of *Diodon hystrix* was exploited and used in a fast and economical way to sensitively detect the existence of heavy metals in a water sample. An inhibitive enzyme assay was conducted, and the activity of AChE was found to be sensitive (> 20% inhibition) to sub-million levels of arsenic, cadmium, nickel, and zinc and less sensitive (< 20% inhibition) to copper and lead. While exposure to argentum, cobalt, and chromium shows no significant inhibition, AChE is not sensitive to those metal ions ($p < 0.05$). Field test work has proved that the assay is suitable for preliminary detecting heavy metal contamination in the river, especially near industrial and mining sites. Secondary validation was performed using ICP-OES to identify and measure the number of elements in the sample and compare them to the inhibition level of AChE activity.

Keywords: Acetylcholinesterase, *Diodon hystrix,* inhibitive assay, heavy metal, ICP-OES.

Introduction

In the past few decades, human activities have caused the pollution of organic and inorganic compounds on a global scale. It has been determined that pollutants from industrial and agricultural activities in waterways will have potentially harmful effects on aquatic organisms and food webs (Hayat *et al.*, 2015; Nordin *et al.*, 2020). Today, heavy metal pollution is considered one of the most severe environmental problems. Heavy metals are any inorganic metal compounds that can exert their toxicity by combining with thiol groups and disulfide bonds that contribute to enzyme stability (Tamás *et al.,* 2014). Metal ions have a high affinity for the disulfide bond between two cysteine residues in any protein compound (Linsdell *et al.,* 2015). This mechanism made heavy metals very harmful to organisms, especially humans, because they can bioaccumulate in the organs, affecting biomolecular metabolism, DNA damage, and carcinogenesis (Sabullah *et al.,* 2015; Padrilah *et al.,* 2018; Basirun *et al.,* 2019). Several rivers in Malaysia have been polluted, classified as class IV and class V based on the water quality index, supported by the detected elements concentrated in the river (DOE, 2019). In addition, Mohamad *et al.* (2012) reported that various types of heavy metals pollute fish in coastal areas of Malaysia. Therefore, monitoring heavy metals in potentially polluted environments such as industrial, agricultural, and mining areas is crucial. Classical methods such as atomic absorption spectroscopy or inductively coupled plasma are costly, require well-trained technicians, complicated sample preparation, and are time-consuming due to long measurement cycles. Hence, detecting heavy metals in the environment requires simple and fast techniques.

Due to its rapid and economical method, inhibitory enzyme assays have long been used to detect toxic substances such as heavy metals (Hayat *et al.,* 2017).

Recent work to develop inhibitory enzyme assays involves using cholinesterase; ChE (such as acetylcholinesterase; AChE, and butyrylcholinesterase; BChE) to detect heavy metals contamination (Pohanka, 2015; Haris *et al.*, 2020). Generally, bioassays are not specific for particular heavy metals, but they can be used as a preliminary screening before secondary validation using a conventional method (Ahmad *et al.,* 2016). This study used an alternative source of AChE extracted from the brain tissue of inanimate *Diodon hystrix* to test their sensitivity towards metal ions and several collected water samples.

Materials and Methods

Preparation of Buffer, Chromogen, Substrate and Heavy Metal Solution

Tris-HCl buffer was prepared according to Dawson *et al.* (1969) with a slight adjustment to the pH of the buffer conducted using a few drops of 6M HCl and 6M NaOH. A sulfhydryl-sensitive chromogen 5,5-dithiobis-2-nitrobenzoic acid, DTNB (Sigma-Aldrich) was dissolved at 50 mL in the buffer to the final concentration of 0.5 mM. Acetylthiocholine iodide; ATCi (Sigma-Aldrich) were selected as the preferred substrate of the fish AChE. Deionized water was used to dissolve ATCi in 2 mM of solution. Metal ions such arsenic; As^{5+}, cadmium; Cd^{2+}, cobalt; Co^{2+}, chromium, Cr^{6+}, copper; Cu^{2+}, nickel; Ni^{2+}, lead; Pb^{2+}, silver; Ag^{2+}, and zinc; Zn^{2+} stock solutions were purchased from Merck, Darmstadt, Germany. The working solutions of 10 mg.L^{-1} for each metal ion were prepared for 10 mL using deionized water and stored in acid-washed polypropylene containers.

Extraction of Brain AChE from Diodon hystrix

Spot-fin Porcupinefish, scientifically known as *Diodon hystrix* was freshly obtained at the local wet market in Kota Kinabalu, Sabah. Only free diseased fish (no sign of external injuries and microbial infection) were brought for experimentation. AChE extraction was carried out based on the method developed by Khalidi *et al.* (2019) using T25 ultra-turax homogenizer, followed by ammonium sulfate precipitation and membrane desalination before affinity purification using Procainamide Sepharose Cl-6B as the matrix of chromatography column referring to the method developed by Sabullah *et al.* (2021).

Assessment of AChE Inhibition and Statistical Analysis

AChE activity could be determined using the Ellman assay, where the measurement of the activity is based on an extinction coefficient of 13.6 mM^{-1}.cm^{-1}, in which one unit of activity is defined as 1 µmole of ATC. However, this study was slightly modified using 96 wells microplate, an AChE inhibitive assay for heavy metals was carried out as explained by Ahmad *et al.* (2016), 10 µL of *D. hystrix* AChE was added to each well contained with Tris-HCl buffer (150 µL; 0.1M; pH 9.0) and 0.5 mM DTNB (20 µL), followed by the addition of heavy metals (50 µL of Ag^{2+}, As^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Cr^{6+}, Ni^{2+}, Pb^{2+} or Zn^{2+}). Distilled water (50 µL) was used for the control instead of heavy metals. ATCi (20 µL) was added to the mixture after 15 minutes of incubation at the ambient temperature. The absorbance at 405 nm was measured as suggested by (Tham *et al.,* 2009) using a microtiter plate reader (Stat Fax 3200 Microplate Reader, Awareness Technology Inc., USA) as the equation below:

$$\mathrm{En} - \left(\frac{\Delta_f - \Delta_i}{\Delta_f}\ x\ En\right) = \%\ Inhibition \qquad (1)$$

where:

En = 100% of AChE activity of control (distilled water)

Δ_i = Initial reading (0 minutes after ATCi was added) at the wavelength of 405 nm

Δ_f = Final reading (10 minutes after ATCi was added) at the wavelength of 405 nm

The means and standard deviation were calculated based on three independent experimental replicates. GraphPad Prism version 5.0 was used to analyse all data, including a comparison between groups obtained by one-way analysis of variance, ANOVA, with post hoc analysis by Tukey's test, or using a Student's t-test only for two groups. A P value of <0.05 was considered statistically significant.

Collecting of Samples (Field Trials)

Surface water was collected from different states in Malaysia. Selection criteria are based on Class I to IV rivers, including river samples from several pristine areas, agricultural areas, and heavy industrial and mining activity (Figure 1). Water samples from heavy industrial and mining activity were chosen as they were more susceptible to concentrated levels of heavy metals (Hubeny *et al.,* 2021). Moreover, the polluted locations expect a significant result of AChE inhibitory, which is affected by heavy metals. Heavy metals were assumed to be in a homogenous distribution for flowing rivers or water columns, as simulated by Pintilie *et al.* (2007). Sampling was conducted by collecting downstream at the three locations at the same river, randomly collecting around 3 to 5 km from each sampling point. Each water sample was collected in acid-washed high-density polyethylene (HDPE) bottles, followed by filtration using a 0.45 µm syringe filter. These samples were brought to the laboratory and assayed for the presence of heavy metals using the inhibitive assay as described above. Secondary validation was performed using Perkin Elmer ICP-OES (Optima 3700DV, Perkin-Elmer, USA) for identification and concentration determination. Elemental measurements were made using wavelengths (Ag^{2+}; 328.066 nm, As^{5+}; 193.691 nm, Cd^{2+}; 214.438 nm, Co^{2+}; 228.612 nm, Cr^{6+}; 267.716 nm, Cu^{2+}; 324.754 nm, Ni2+; 231.604 nm, Pb^{2+}; 220.353 nm, and Zn^{2+}; 213.856 nm) and operating parameters recommended for the spectrometer, the argon gas, purified air, and nitrogen gas were set at a pressure of 90 kPa. The pump parameter was set with a sample flow rate of 1.5 ml/min. The quality control (QC) was analysed and periodic timing of analyses was set to a frequency of one and the maximum time between QC was 30 minutes. All experiments were performed in triplicate.

Results and Discussion

Heavy Metals Inhibition Studies

An elevated level of heavy metal exposure may cause harmful effects on the biological system. Moreover, heavy metals can inhibit cholinesterase enzyme activity *in vivo* or *in vitro* (Sabullah *et al.,* 2015; Basirun *et al.,* 2019). At 10 mg.L^{-1} concentration, as shown in Figure 2,

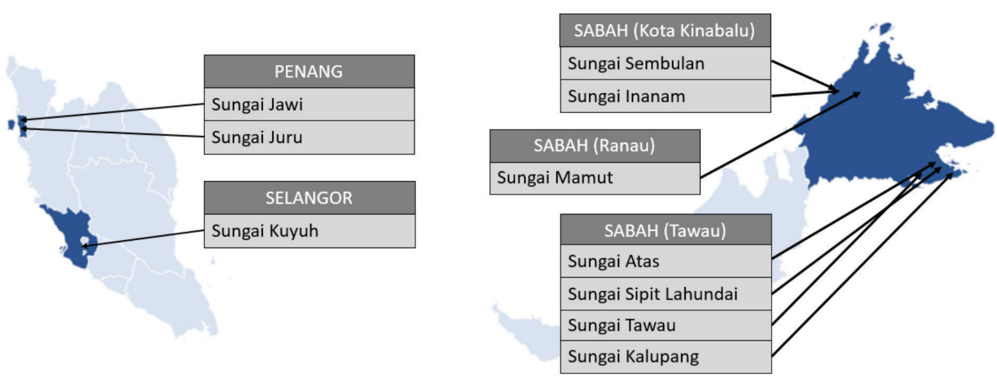

Figure 1: Sampling location of three different states coloured with dark blue. Two samples from Penang, Sungai Jawi [Class IV] and Sungai Juru [Class III], one from Selangor; Sungai Kuyuh [Class IV], 10 samples from Sabah collected from three different districts; Sungai Inanam [Class II] and Sungai Sembulan [Class III] in Kota Kinabalu district, Sungai Mamut [no classification] at Ranau district, and Sungai Tawau [Class II], Sungai Kalumpang [Class II], Sungai Atas [no classification], and Sungai Sipit Lahundai [no classification] at Tawau District. [ ] denoted as river classification based on DOE environmental report (DOE, 2019)

As^{5+}, Cd^{2+}, Ni^{2+} and Zn^{2+} severely influenced the AChE activity to 29.41, 32.35, 47.06 and 45.59 % inhibitions, respectively. Sabullah *et al.* (2015) mentioned that a 20% inhibition of AChE activity is considered significantly polluted. This means copper and leads mildly inhibited AChE activity by 8.82 and 11.76%, respectively. Other heavy metals (Ag, Co, and Cr) did not show significant enzyme inhibition, similar to tap water is also unable to lower the activity of AChE ($P > 0.05$). AChE also exists in other organs such as the liver, muscles, gills, and blood (Somnuek *et al.,* 2007). Moreover, the sensitivity of AChE from different sources towards heavy metals is different. Hayat *et al.* (2016; 2017) compared the inhibition level of *Lates calcarifer* ChE extracted from kidneys, gills, and muscles. Cu, Hg, and Pb inhibit more than 50% of gills ChE. Compared to muscle ChE, Pb is the only metal ion that inhibits 50% of activity, while kidney ChE shows less sensitives, the highest inhibition observed at 48% by Cr. The inhibitory effect of metal ions is related to the binding affinity to amino acid side chains. Proteins containing histidine residues are most likely to bind to metals, such as zinc and copper. The imidazole group of histidine provides the most potent cation-π attraction, which can interact with the nitrogen-containing cation or free metal ion of the substrate. However, Sarkarati *et al.* (1999) mentioned that the inhibition of AChE by heavy metals is due to the attraction of negative charges on the side chains of amino acids containing carboxyl groups such as glutamic acid and aspartic acid in the ChE catalytic triad, which leads to changes in the structure of the active site. Amino acids with aromatic side-chain potentially interact with metal cations in the active site or/and an allosteric site of the protein (Hu *et al.,* 1995).

Field Trials and Secondary Validation

The increasing number of heavy industries in cities such as Juru in Penang, Serdang in Selangor and Kota Kinabalu in Sabah urbanized with a vast population. Furthermore, modern industries such as agricultural products such as pesticides and fertilizer, metal-based manufacturing, electrical, electronic, and semiconductor production have driven the population's growth daily. Consequently, nearby water bodies such as the Klang and Juru rivers, including the soil and the air, are classified as heavily polluted, mainly from these industrial wastes (Zali *et al.,* 2011). In this study, we chose several places, specifically in the main cities and districts from these three states in Figure 1, with a different class of river determined by the Department of Environment Malaysia (2019). All the collected samples were analyzed using the inhibitive assay followed by secondary validation using ICP-OES.

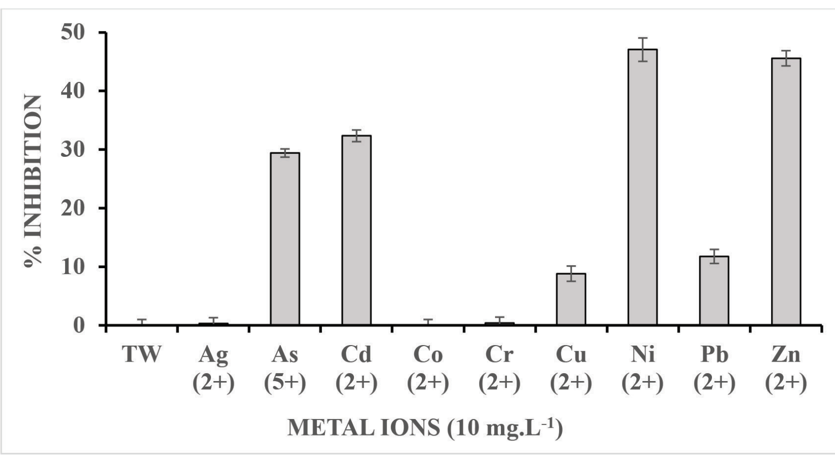

Figure 2: Percentage inhibition of *D. hystrix* AChE activity after incubation in 10 $mg.L^{-1}$ of metal ions. Each bar represents the mean ± standard deviation of triplicates data (n=3). TW = Tap Water

The field trial result (Figure 2) exhibited that sample from high population associated with the high industrial area, Sungai Penang and Sungai Jawi, both from Penang and Sungai Kuyuh from Selangor inhibit more than half (higher than the dotted line) of AChE activity, and JP3 and JPW3 completely inhibit the activity. In the state of Sabah, although Kota Kinabalu is one of the most developed industrial with rapid urbanization area, the main river, such as Sungai Inanam and Sungai Sembulan, the water sample inhibits less than 20% of AChE activity. Conversely, samples from Sungai Mamut in Ranau District inhibit almost 80% of activity. In comparison, other samples collected from Semporna District, such as Sungai Kalupang, Sungai Atas, and Sungai Pagagau considered unaffected.

Based on Environmental Quality Report (2019), Sungai Inanam, Sungai Sembulan and Sungai Kalupang were classified as class II rivers, while Sungai Atas and Sungai pagagau have no records. According to Sabullah *et al.* (2015), tested river waters that expressed more significant inhibition than 20% can be specified as polluted river samples and denoted with a dash straight horizontal line in Figure 3. Meanwhile, the ChE inhibited for more than half the actual activity percentage can be considered toxic and denoted with a thick horizontal line (Shukor *et al.,* 2013).

All the samples that inhibit more than 50% of AChE activity were continued for secondary validation using ICP-OES for the determination concentration for all the test elements in the inhibition study. Considering the multiple elements that will be detected, the changes in AChE activity are related to the synergistic effect due to various metal ions. The synergistic effect is the result of the interaction of two or more processes, and its effect is greater than the cumulative effect produced when these processes are used alone.

Table 1 shows all the nine elements with different concentrations detected at the part per million level followed by conversion to part per billion level (ppb). Both river samples from Penang, JWP, and JP show no detection of Co. Additionally, no Cr in all JP samples, especially JP3, shows no presence of Cd. The synergistic effect here indicated that more variation of elements in the river samples highly inhibits the activity of AChE. However, the synergistic effect of Sungai Kuyuh and Sungai Mamut exhibited different situations where, although

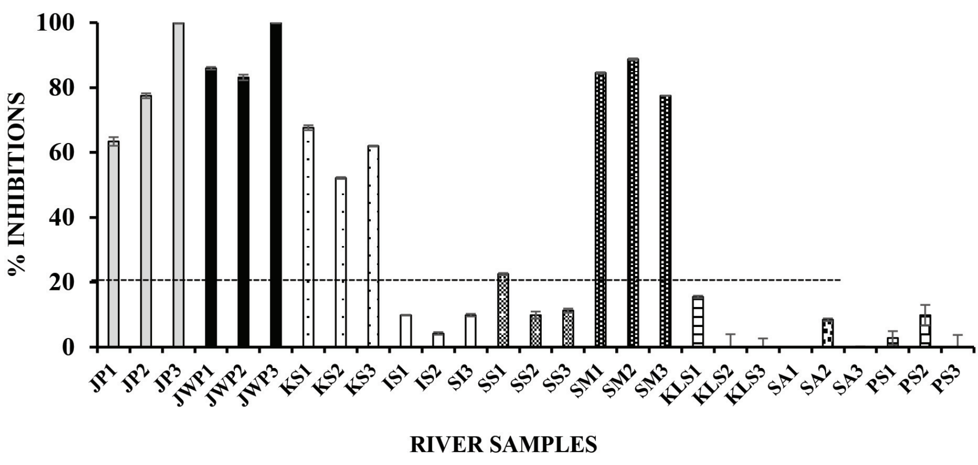

Figure 3: The inhibition profile of purified AChE from *Diodon hystrix* AChE when tested with rivers sample, JP, JWP, KS, IS, SSS, SM, KLS, SA and PS stands for Sungai Juru, Sungai Jawi, Sungai Kuyuh, Sungai Inanam, Sungai Sembulan, Sungai Mamut, Sungai Kalumpang, Sungai Atas and Sungai Pegagau, respectively. All values were represented in mean ± standard deviation (SD) and n is a triplicate of the data. Dash line denoted as more than 20% inhibition considered as polluted

the average of all metal ions in both samples is much higher, the inhibition level is still lower compared to Sungai Jawi. This situation could be related to the main concepts of synergistic effect, concentration effect, and independent action, which enhance the adverse effect of the biological system, or caused less negative effects than individual toxicants, as mentioned in inhibition studies.

Sungai Jawi has been listed as one of the most polluted rivers in Penang. Fast-paced industrial factories, livestock plantations, and neighbouring municipalities surround Sungai Juru. The unethical disposal of waste from the nearest manufactory and inconspicuous pig farming activity has resulted in an obvious change in the river water in terms of colour, smell, and appearance (Kasnoon, 2013; Persatuan Pengguna Penang, 2019). In conjunction with the unlawful waste removal, Sungai Jawi has noted the high concentrations of metal ions, especially silver, exceeding the maximum permissible limit for Class III set by the Malaysian Department of Environment. Hence, a drastic attempt to monitor the river quality is essential with the series of field surveys in the recovery effort by the authority.

Following the polluted river records by the Penang authority, Sungai Jawi and Sungai Juru were classified as Class IV and Class III in the water quality index, respectively. The high concentrations of heavy metals, including Ag and Cd, surpassed the Malaysian Department of Environment's maximum permissible limit of heavy metals for Class III. Sungai Juru has been identified as polluted since the 1970s, with intense manufacturing activities releasing toxic wastes and proximate residential area discharging wastewater into the nearby drainage waterway and, at last, to the river (Kasnoon, 2013). Mamut lake sample shows the high inhibition of AChE activity of *D. hystrix* brain. The preliminary semi-quantitative data obtained were tallied with the ICP-OES analysis, which highlighted the extremely high concentration of numerous metal ions such as cadmium, nickel, lead, and zinc beyond the Malaysian standard of national water quality. From 1975 until 1999s, Mamut lake was actively exploited as an open quarry mine site for valuable minerals, including Cu, Ag, and gold (van der Ent & Edraki, 2018). From the vigorous mining, Mamut has since experienced environmental degradation with the production of acid mine drainage, resulting in very acidic pH water conditions (Jopony & Tongkul, 2009; Low *et al.,* 2020).

Therefore, proactive plans are obligatory to ensure no overwhelming drawbacks are encountered soon. River water samples from Sungai Kuyuh exhibited half inhibition towards AChE activity, and ICP-OES data showed a low concentration of tested heavy metals, as in Table 1. Sungai Kuyuh is classified within the Class III river and is surrounded by multiple development industries, and a dense population resides close to the river area. The faulty waste dumping in the river causes deterioration in quality, accompanied by the emancipation of noxious substances as industrial by-products (Sreenivasan *et al.,* 2012).

Sungai Sembulan and Sungai Inanam showed low inhibition towards AChE activity, corresponding to the minute amount of heavy metals analysed using ICP-OES. However, both rivers were slightly contaminated after domestic wastes from industries, livestock plantations, and household buildings reached the nearby water source (Ladoni, 2016; DOE, 2020; Khalidi *et al.,* 2020). Another contributing factor to the contamination of Sungai Sembulan and Sungai Inanam was the vigorous exploitation of logging activity. Both rivers were classified within Class III and Class II from the Department of Environment report on water quality status in 2016. As reported by DOE, Sungai Kalumpang was categorized in Class II, which signified relatively clean rivers for domestic usages, such as recreational activities, fishing, and farming.

The ICP-OES analysis showed a low amount of heavy metals in the rivers sampled. The river's water, particularly Sungai Kalumpang was collected and treated in the water treatment plant before being distributed house-to-house through a pipeline for everyday use. Sungai Atas,

Table 1: Quantifying metal ions concentration in river samples from Peninsular and Sabah areas using ICP-OES. N/A = Not Available, N/D = Not Detected

Sample Code	Class#	Heavy Metals Concentration (ppb) ± Standard Deviation								
		Ag	As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Tap water	N/A	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
JWP 1	IV	4.355*	8.739± 0.001	0.662*	N/D	2.424± 0.001	3.700± 0.001	1.367± 0.001	1.056± 0.004	12.06*
JWP 2	IV	4.197± 0.001	4.899± 0.002	0.443± 0.001	N/D	2.275± 0.001	3.315± 0.001	0.692± 0.001	3.393± 0.001	11.185 ±0.001
JWP 3	IV	4.445*	12.986± 0.009	0.489± 0.001	N/D	2.327± 0.001	3.164± 0.001	1.128± 0.001	4.992± 0.003	11.031*
JP 1	III	3.872*	11.362 ± 0.005	0.450± 0.001	N/D	N/D	1.123± 0.001	33.794 ±0.001	4.200± 0.002	17.546*
JP 2	III	3.798± 0.001	9.576± 0.007	0.227± 0.001	N/D	N/D	0.704± 0.001	34.634 ±0.001	5.404± 0.002	15.342*
JP 3	III	3.671*	10.309± 0.013	N/D	N/D	N/D	0.861± 0.001	34.165 ±0.001	5.296± 0.001	17.268 ± 0.001
SM 1	N/A	4.555*	8.845± 0.005	4.170± 0.001	202.95± 0.002	4.603*	2348.191± 0.022	492.673 ±0.004	26.178± 0.004	1593.5± 0.009
SM 2	N/A	4.800*	11.856± 0.002	3.696± 0.001	194.965± 0.001	5.093± 0.001	2346.743± 0.012	479.963 ±0.002	24.129± 0.001	1537.8± 0.01
SM 3	N/A	4.739*	13.948 ± 0.003	3.790*	174.52± 0.001	5.116± 0.003	2158.358± 0.024	432.297 ±0.003	27.617± 0.002	1393.7± 0.011
SK 1	III	4.220*	20.125 ± 0.002	0.549*	N/D	2.324± 0.002	6.525± 0.001	6.298 ±0.004	1.717± 0.001	36.543± 0.001
SK 2	III	4.345*	41.34± 0.010	0.993*	N/D	2.492*	4.160*	2.520± 0.001	2.474± 0.002	29.558± 0.001
SK 3	III	3.695*	40.593± 0.002	0.726± 0.001	0.092*	5.669± 0.001	17.666± 0.001	9.079± 0.001	21.028± 0.003	333.38± 0.004

Code: JWP = Sungai Jawi, JP = Sungai Juru, KS = Sungai Kuyuh, SM = Sungai Mamut

#Classification of river based on Environmental Quality Report, 2019

*Standard deviation is too small

Sungai Pegagau and Sungai Kalupang from Semporna exhibited low inhibition with almost 90% remaining activity of *D. hystrix* AChE. The heavy metals analysis using ICP-OES also suggested a very low concentration of toxicants in the water composition. This situation is supported as all listed rivers in both areas were underdeveloped and located moderately distant from downtown. The Malaysian Department of Environment provided no report on these rivers' quality status; hence, based on the present study, these rivers can be classified as clean rivers similar to Class I river.

Conclusion

D. hystrix AChE was proved to be sensitive towards several metal ions at ascending order of $Co^{2+} < Ag^{2+} = Cr^{6+} < Cu^{2+} < Pb^{2+} < As^{5+} < Cd^{2+} < Zn^{2+} < Ni^{2+}$. Preliminary screening on various river samples shows different sensitivity in which the inhibition of AChE is associated with the amount and variety number of heavy metals in the sample, which has been validated using ICP-OES. This study proved the ability of *D. hystrix* AChE as alternative source for biosensor kit development. Future study is recommended to test with other toxicant such as pesticides, detergents and drugs.

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References

Ahmad, S. A., Sabullah, M. K., Basirun, A. A., Khalid, A., Yasid, N. A., Iqbal, I. M., Shamaan, N. A., Syed, M. A., & Shukor, M. Y. (2016). Evaluation of cholinesterase from the muscle and blood of anabas testudineus as detection of metal ions. *Fresenius Environmental Bulletin*, *25*(10), 4253-4260.

Aidil, M. S., Sabullah, M. K., Halmi, M. I. E., Sulaiman, R., Shukor, M. S., Shukor, M. Y., Shaharuddin, N. A., Syed, M. A., & Syahir, A. (2013). Assay for heavy metals using an inhibitive assay based on the acetylcholinesterase from pangasius hypophthalmus (Sauvage, 1878). *Fresenius Environmental Bulletin*, *22*(12), 3572-3576.

Basirun, A. A., Ahmad, S. A., Yasid, N. A., Sabullah, M. K., Daud, H. M., Sha'arani, S., Khalid, A., & Shukor, M. Y. (2019). Toxicological effects and behavioural and biochemical responses of Oreochromis mossambicus gills and its cholinesterase to copper: A biomarker application. *International Journal of Environmental Science and Technology*, *16*(2), 887-898. https://doi.org/10.1007/s13762-018-1711-1

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1969). *Data for Biochemical Research, 37*(8), A490. Oxford, UK: Clarendon Press.

Department of Environment, Malaysia; DOE. (2019). *Malaysia Environmental Quality Report*.

Department of Environment, Malaysia; DOE. (2020). *Laporan Siasatan Notification Pollution Event (NPE) di Sungai Sembulan, Kota Kinabalu, Sabah – ENVIRO-MUSEUM*. Enviro Knowledge Management Center. https://enviro2.doe.gov.my/emuseum/Kursuscollection/laporan-siasatan-notification-pollution-event-npe-di-sungai-sembulan-kota-kinabalu-sabah

Haris, A., Nordin, N., Mustapa, N. A., Sani, S. A., Shukor, M. Y., & Sabullah, M. K. (2020). Profiling of cholinesterase extracted from the brain tissue of diodon hystrix and its inhibition reaction towards carbamates. *Pertanika Journal of Science and Technology*, *28*(Special Issue 2), 95-106.

Hayat, N. M., Ahmad, S. A., Shamaan, N. A., Jlah, M. K. S., Shukor, M. Y. A., Syed, M. A., Khalild, A., Khalil, K. A., & Dahalan, F. A. (2017). Characterisation of cholinesterase from kidney tissue of Asian seabass (Lates calcarifer) and its inhibition in presence of metal ions. *Journal of Environmental Biology*, *38*(3), 383-388. Scopus. https://doi.org/10.22438/jeb/38/3/MRN-987

Hayat, N. M., Shamaan, N. A., Shukor, M. Y., Sabullah, M. K., Syed, M. A., Khalid, A., Dahalan, F. A., Khalil, K. A., & Ahmad, S. A. (2015). Cholinesterase-based biosensor using *Lates calcarifer* (Asian Seabass) brain for detection of heavy metals. *Journal of Chemical and Pharmaceutical Sciences*, *8*(2), 376-381.

Hayat, N. M., Shamaan, N. A., Sabullah, M. K., Shukor, M. Y., Syed, M. A., Khalid, A., Dahalan, F. A., & Ahmad, S. A. (2016). The use of Lates calcarifer as a biomarker for heavy metals detection. *Rendiconti Lincei*, *27*(3), 463-472. https://doi.org/10.1007/s12210-015-0501-7

Hu, P., Sorensen, C., & Gross, M. L. (1995). Influences of peptide side chains on the metal ion binding site in metal ion-cationized peptides: Participation of aromatic rings in metal chelation. *Journal of the American Society for Mass Spectrometry*, *6*(11), 1079-1085. https://doi.org/10.1016/1044-0305(95)00549-8

Hubeny, J., Harnisz, M., Korzeniewska, E., Buta, M., Zieliński, W., Rolbiecki, D., Giebułtowicz, J., Nałęcz-Jawecki, G., & Płaza, G. (2021). Industrialization as a source of heavy metals and antibiotics which can enhance the antibiotic resistance in wastewater, sewage sludge and river water. *PLOS ONE*, *16*(6), e0252691. https://doi.org/10.1371/journal.pone.0252691

Jopony, M., & Tongkul, F. (2009). Acid mine drainages at Mammut Copper Mine, Sabah, Malaysia. *Borneo Science*, 83-94.

Kasnoon, K. (2013a). Nasib sungai kita di tangan kita. *Astro Awani*. https://upm.edu.my/newspaper/nasib_sungai_kita_di_tangan_kita_sendiri-13857

Kasnoon, K. (2013b). 5 sungai dikenal pasti paling tercemar di negara ini. *Astro Awani*. www.astroawani.com/berita-malaysia/5-sungai-dikenal-pasti-paling-tercemar-di-negara-ini-23535

Khalidi, S. A. M., Sabullah, M. K., Sani, S. A., Ahmad, S. A., Shukor, M. Y., Jaafar, 'I N. M., & Gunasekaran, B. (2019). Acetylcholinesterase from the brain of *Monopterus albus* as detection of metal ions. *Journal of Physics: Conference Series*, *1358*, 012028. https://doi.org/10.1088/1742-6596/1358/1/012028

Khalidi, S. A. M., Sabullah, M. K., Sani, S. A., Shukor, M. Y. A., Basirun, A. A., Gafar, A. A., Jaafar, 'Izazy Nur Mohd & Nordin, N. (2020). Acetylcholine receptor-based biosensor derived from AsianSwamp Eel, Monopterus Albus for heavy metals biomonitoring. *Pertanika Journal of Science and Technology*, *28*(S2). https://doi.org/10.47836/pjst.28.s2.07

Ladoni, A. M. H. (2016). The sources of pollution in the Likas and Inanam River Basin in Kota Kinabalu, Sabah, Malaysia. *Jurnal Pendidikan Sains Sosial dan Kemanusiaan*, *2*(1), 89-106.

Linsdell, P. (2015). Metal bridges to probe membrane ion channel structure and function. *Biomolecule Concepts*, *6*(3), 191-203.

Low, Y. Y., Chin, G. J. W. L., Joseph, C. G., Musta, B., & Rodrigues, K. F. (2020). Bacterial diversity of the abandoned mamut copper mine in Sabah, Malaysia and its correlation with copper contamination. *Malaysian Journal of Microbiology*. https://doi.org/10.21161/mjm.190610

Mohamad, A., Azlan, A., Abd. Shukor, M. Y., S., M. Z., Halmi, M. I. E., & Razman, M. R. (2012). Heavy metals (mercury, arsenic, cadmium, plumbum) in selected marine fish and shellfish along the Straits of Malacca. *International Food Research Journal*, *19*(1), 135-140.

Nordin, N., Cletus, R. R., Sabullah, M. K., Khalidi S. A. M., Abdulla, R., & Ahmad, S. A. (2020). Cholinesterase from the liver of diodon hystrix for detection of metal ions. *Pertanika Journal of Science and Technology*, *28*(Special Issue 2), 107-119.

Padrilah, S. N., Sabullah, M. K., Shukor, M. Y. A., Yasid, N. A., Shamaan, N. A., & Ahmad, S. A. (2018). Toxicity effects of fish histopathology on copper accumulation. *Pertanika Journal of Tropical Agricultural Science*, *41*(2), 519-540.

Persatuan Pengguna Penang. (2019). Pencemaran air dari parit-parit JPS di daerah Seberang Perai Selatan, sampai bila. https://consumer.org.my/ms/pencemaran-air-dari-parit-parit-jps-di-daerah-seberang-perai-selatan-sampai-bila-2/

Pintilie, S., Brinza, L., Betianu, C., Pavel, L., Ungureanu, F., & Gavrilescu, M. (2007). Modelling and simulation of heavy metals transport in water and sediments. *Environmental Engineering and Management Journal*, *6*, 153-161. https://doi.org/10.30638/eemj.2007.021

Pohanka, M. (2015). Biosensors containing acetylcholinesterase and butyrylcholinesterase as recognition tools for detection of various compounds. *Chemical Papers*, *69*(1), 4-16. https://doi.org/10.2478/s11696-014-0542-x

Sabullah, M. K., Nordin, N., Wahid, D. N. A., Khalidi, S. A. M., Abdulla, R., Jawan, R.,

& Shukor, M. Y. (2021). Synthetisation of an affinity matrix (Procainamide Sepharose Cl-6b) for brain cholinesterase purification and separation source from Monopterus albus. *Journal of Physics: Conference Series*, *1882*(1), 012093. https://doi.org/10.1088/1742-6596/1882/1/012093

Sabullah, M. K., Sulaiman, M. R., Shukor, M. S., Yusof, M. T., Johari, W. L. W., Shukor, M. Y., & Syahir, A. (2015). Heavy metals biomonitoring via inhibitive assay of acetylcholinesterase from *Periophthalmodon schlosseri*. *Rendiconti Lincei*, *26*(2), 151-158. https://doi.org/10.1007/s12210-014-0359-0

Sabullah, M. K., Sulaiman, M. R., Shukor, M. Y. A., Shamaan, N. A., Khalid, A., & Ahmad, S. A. (2015). In vitro and in vivo effects of *Puntius javanicus* cholinesterase by copper. *Fresenius Environmental Bulletin*, *24*(12B), 4615-4621.

Sarkarati, B., Cokuğraş, A. N., & Tezcan, E. F. (1999). Inhibition kinetics of human serum butyrylcholinesterase by Cd2+, Zn2+ and Al3+: Comparison of the effects of metal ions on cholinesterases. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology & Endocrinology*, *122*(2), 181-190. https://doi.org/10.1016/s0742-8413(98)10102-0

Shukor, Y., Tham, L., Effendi Halmi, M., Khalid, I. K. B., Begum, G., & Syed, M. (2013). Development of an inhibitive assay using commercial *Electrophorus electricus* acetylcholinesterase for heavy metal detection. *Journal of Environmental Biology/Academy of Environmental Biology, India*, *34*, 967-970.

Somnuek, C., Cheevaporn, V., Saengkul, C., & Beamish, F. W. H. (2007). [No title found]. *ScienceAsia*, *33*(3), 301. https://doi.org/10.2306/scienceasia1513-1874.2007.33.301

Sreenivasan, J., Govindan, M., Chinnasami M., & Kadiresu, I. (2012). Solid waste management in Malaysia – A move towards sustainability. https://www.intechopen.com/chapters/40529

Tamás, M. J., Sharma, S. K., Ibstedt, S., Jacobson, T., & Christen, P. (2014). Heavy metals and metalloids as a cause for protein misfolding and aggregation. *Biomolecules*, *4*(1), 252-267. https://doi.org/10.3390/biom4010252

Tham, L., Perumal, N., Syed, M., Shamaan, N. A., & Shukor, Y. (2009). Assessment of *Clarias batrachus* as a source of acetylcholinesterase (AChE) for the detection of insecticides. *Journal of Environmental Biology/Academy of Environmental Biology, India*, *30*, 135-138.

van der Ent, A., & Edraki, M. (2018). Environmental geochemistry of the abandoned Mamut Copper Mine (Sabah) Malaysia. *Environmental Geochemistry and Health*, *40*(1), 189-207. https://doi.org/10.1007/s10653-016-9892-3

Zali, M. A., Retnam, A., & Juahir, H. (2011). Spatial characterization of water quality using Principal Component Analysis Approach at Juru River Basin, Malaysia. *World Applied Sciences Journal*, *14*, 55-59.