JEBAT, 2024, Vol 7, No 1, 9-14
https://doi.org/10.54987/jebat.v7i1.996

HIBISCUS PUBLISHER

JOURNAL OF ENVIRONMENTAL BIOREMEDIATION AND TOXICOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/JEBAT/index

A Near-Real-Time Ficin-Based Inhibitive Enzyme Assay for Biomonitoring Heavy Metals in Waters Near Kuah's Jetty, Langkawi

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HISTORY

Received: 28th Feb 2024
Received in revised form: 15th April 2024
Accepted: 29th July 2024

KEYWORDS

Near Real-Time
Biomonitoring
Inhibitive enzyme assay
Ficin
Heavy metals

ABSTRACT

Near real-time biomonitoring, particularly through enzyme assays, offers exceptional sensitivity to bioavailable pollutants, delivering rapid results that facilitate timely intervention. This method is crucial for reducing pollution in drinking water systems and safeguarding human and animal health. The marriage of biological and instrumental process of monitoring allows only positive samples be sent for instrumental analysis vastly increasing the frequency of monitoring and reducing cost at the same time. This study demonstrates the application of ficin as an inhibitive enzyme assay for biomonitoring, in a near real time setting, specifically targeting heavy metals in environmental samples from waters near Kuah's Jetty, Langkawi. Using the ficin dye-binding assay, designed to detect mercury silver (Ag^{+}), (Hg^{2+}), and copper (Cu^{2+}) at sub-ppm levels, we validated its ability to identify trace concentrations of these metals in waters. The assay proved to be sensitive and rapid, with minimal inhibition (<10%) during a 6-hour field trial, indicating low pollution levels, which was further corroborated through instrumental analysis. This method enables the swift identification of pollutants, prompting timely action and supporting the preservation of ecotourism sites by providing monitoring data for policy development. The simplicity and visual appeal of enzyme assays make them excellent tools for education, fostering environmental awareness and conservation efforts. Our findings underscore the potential of enzyme assays for large-scale environmental monitoring, aligning local practices with international standards and promoting global cooperation in environmental conservation. This study increases our understanding of ecological health in marine ecosystems, highlighting the need for continuous monitoring to protect natural habitats.

INTRODUCTION

Langkawi, a picturesque archipelago off the northwestern coast of Malaysia, has long been a popular tourist destination. The island's stunning natural beauty and pristine beaches have attracted visitors from around the world, fueling the growth of the tourism industry over the past few decades. However, this surge in tourism, coupled with the expansion of industrial activities, has led to a concerning increase in pollution near the island's waters. One of the primary sources of pollution in Langkawi's waters is the discharge of waste from tourism-related activities. The rapid development of hotels, resorts, and other tourist facilities has resulted in the generation of significant amounts of sewage and solid waste, much of which finds its way

into the surrounding waters. This influx of pollutants can have detrimental effects on the local marine ecosystem, potentially harming the delicate balance of the island's aquatic life. Moreover, the tourism industry's reliance on transportation has also contributed to the accumulation of pollutants in Langkawi's waters. The increasing number of boats, ferries, and other watercraft used to transport tourists has led to the release of fuel, oil, and other contaminants into the coastal waters [1–6].

The growth of industrial activities in Langkawi has also played a role in the island's water pollution. The archipelago's recognition as a UNESCO Global Geopark in 2007 has led to a surge in economic development, with the establishment of various manufacturing and processing facilities. These industries often discharge their waste directly into the surrounding waters, further exacerbating the pollution problem. To address the issue of water pollution in Langkawi, a multi-faceted approach is necessary. Local authorities must take proactive measures to regulate the discharge of waste, both from tourism and industry, and implement stricter environmental regulations to ensure the sustainability of the island's natural resources. Additionally, the tourism industry must be encouraged to adopt eco-friendly practices, such as the implementation of waste management systems and the use of renewable energy sources. By promoting environmentally responsible tourism, the negative impacts on Langkawi's waters can be mitigated, paving the way for the sustainable development of the island's tourism industry [1,7–9].

Biomonitoring through enzyme assays has emerged as a pivotal strategy in environmental management, offering a sensitive, cost-effective, and expedient means for detecting pollutants. These assays exhibit a remarkable capacity to identify low concentrations of contaminants, including heavy metals and organic compounds, enabling the timely implementation of remedial measures by authorities. Their inherent cost-efficiency and the minimal requirement for sophisticated equipment facilitate widespread and frequent environmental assessments, contributing to a comprehensive understanding of ecological health across vast areas [10–12].

The growing demand for more data and faster acquisition at lower cost, size, and power has driven the research in biological and chemical sensors for environmental monitoring applications. Unlike many sensor arenas where a dominant technology has taken hold, the best choice for chemical and biological environmental monitoring remains unclear, leading to continued research across a broad range of sensor classes to find application-specific footholds. In recent years, environmental chemical contamination has increased considerably as a consequence of anthropogenic activities, with chemicals such as heavy metals, oil-based products, pesticides, fertilizers, and plastic materials becoming of global concern for their impact on aquatic and terrestrial environments [13–16].

The rapid execution of enzyme tests guarantees prompt actions essential for averting environmental deterioration and protecting public health. Moreover, the straightforwardness and aesthetic attractiveness of some enzyme tests function as valuable tools for educational programs designed to elevate environmental awareness within the community, thereby fostering proactive conservation endeavors. The precise data produced by these tests underpins evidence-based policymaking, allowing authorities to set exact pollution levels, evaluate the efficacy of environmental measures, and implement required modifications. Furthermore, enzyme activity indicators provide early alerts of ecological distress, enabling actions prior to the manifestation of apparent harm, thereby averting long-term ecological degradation [17–20]. By synchronizing local monitoring techniques with international standards using enzyme tests, authorities may promote worldwide cooperation in environmental protection, successfully tackling transboundary issues. Enzyme tests for biomonitoring provide the public and policymakers with critical tools and information for enhanced environmental management and public health safeguarding. We have created many near real-time monitoring systems for pollution, particularly heavy metals, employing enzymes derived from microbes and plants, and we use these assays to assess various prospective and contaminated locations in Malaysia [21–27] including several sites in Langkawi.

A previous study utilized the achromopeptidase dye binding assay, which is specifically designed for detecting mercury and zinc at levels below one part per million, to successfully identify trace amounts of these metals. The assay demonstrated high sensitivity, rapid results, and cost-effectiveness, with minimal inhibition (<10%) during a 6-hour field trial conducted over three consecutive days. These findings suggested low pollution levels, which were further confirmed through instrumental analysis [28]. However, the study only targets zinc, a relatively non toxic metal compared to mercury, of which the ficin assay is able to detect. In this study, we explore the feasibility of using the ficin dye binding assay [26] for a near real-time biomonitoring for the potential presence of toxic metal ions including mercury at this site.

MATERIAL AND METHODS

Preparation of casein and ficin solution

Casein, obtained from Sigma, was accurately measured to 2 grams and mixed with 100 milliliters of deionized water. The solution was titrated with 5N NaOH and/or HCl to attain a pH level of 8.0. The mixture was continuously stirred at 60°C overnight to ensure complete dissolution. The solution was tehn filtered through several layers of cheesecloth to isolate insoluble particle. Further clarification was then obtained by centrifuging the solution at 10,000×g at 4°C.

The protein concentration in the resulting clear supernatant was quantified using the dye binding Bradford method, utilizing crystalline BSA from Sigma as the benchmark standard. This prepared solution was stored at 4°C for immediate use or frozen at -20°C for future use. Ficin (from Sigma, E.C. 3.4.22.3, lot number: F4165-1ku, sourced from crude dried fig tree latex, exhibiting an activity of 0.5 Units/mg) was solubilized at 4 °C in a 20 mM sodium phosphate buffer at pH 6.77. The concentration was 10.0 mg/mL as a stock solution. Ficin fresh working solutions was prepared before use (2.0 mg/mL) and casein (10 mg/mL).

Ficin inhibition studies

The experiment was started by adding 50 µL of ficin (final concentration of 0.6 mg/mL) in a 20 mM phosphate buffer at a pH of 6.77, with 50 µL of mercury solution to attain a final mercury concentration of 0.040 mg/L. This is a positive control experiment [26]. The mixture was subsequently incubated for 10 minutes at a temperature of 30°C. In the control setup, mercury was replaced with an equivalent volume of 20 mM phosphate buffer at pH 6.77. Following this substitution, 50 µL of casein solution was incorporated into the mixture, resulting in a final concentration of 2.36 mg/mL, and was thoroughly mixed.

A 20 µL aliquot of this mixture was immediately combined with 200 µL of Bradford dye-binding reagent. The solution was allowed to equilibrate at room temperature for 5 minutes, after

which the absorbance was recorded at 595 nm, indicating the initial absorbance value. After an additional 30-minute incubation at room temperature, a second 20 µL aliquot was extracted, mixed with the Bradford dye reagent in the same manner, and the absorbance at 595 nm was measured following a 5-minute incubation, mirroring the initial procedure.

Near real-time field trials

Water samples were collected hourly for six hours in acid-washed HDPE bottles, each containing a few drops of 1% (v/v) HNO3, from locations near Kuah's Jetty in Langkawi, specifically at the coordinates 6°24'17.4"N 99°51'30.7"E (refer to **Fig. 1**) as before [28]. Initially, these samples underwent filtration through a 0.45 µm syringe filter (Teflon) to obtain a clear filtrate. Subsequently, 50 microliters of this filtrate were assayed for mercury content using the ficin assay at a controlled temperature of 30 °C. This temperature control was achieved using a portable egg incubator (30 Watt, generic brand) powered by a DC12V to AC220V car inverter (ZTE Avid Plus, China), ensuring a stable environment of 30 ± 1 °C.

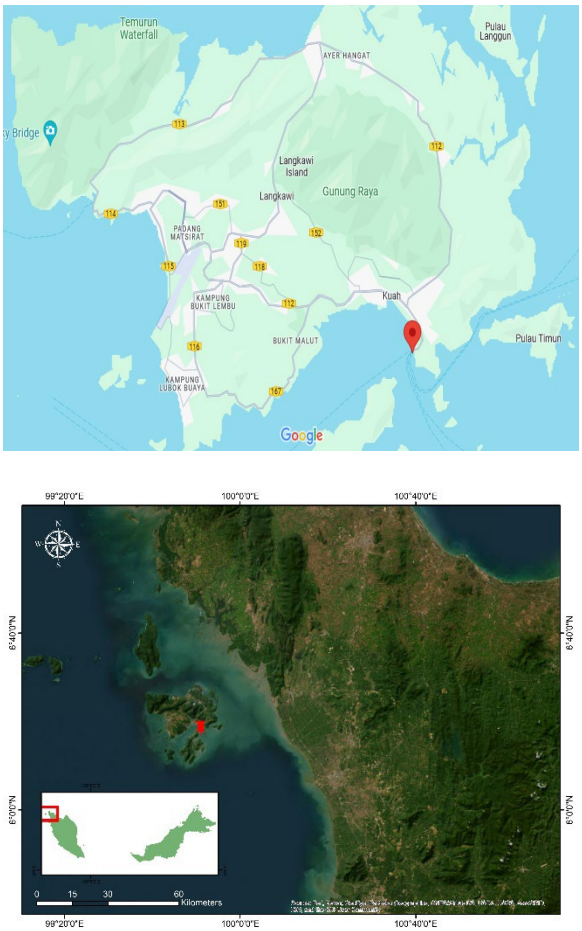

Fig. 1. Site of water sampling (indicated by red pin) at Kuah's Jetty in Langkawi, Malaysia (Source Google Earth image).

The absorbance measurements were conducted with a portable mini-spectrophotometer (Model M6+, Axiom, Germany). Post-assay, the samples were stored in a Coleman® ice cooler for preservation until they could be analyzed further in the laboratory. For detailed mercury analysis, a Perkin Elmer Flow Injection Mercury System (FIMS 400) was employed. Additionally, the concentrations of silver and copper within these samples were determined using Atomic Emission Spectrometry, specifically on a Perkin Elmer ICP OES (Optima 8300, PerkinElmer, Inc., 940 Winter Street, Waltham, MA, USA). This comprehensive approach allowed for the precise quantification of these metals, providing critical insights into the water quality near the area.

Data and Statistical Analysis

The per cent inhibition was calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{Test activity of sample} - \text{test activity of control x } 100}{\text{Test activity of control}}$$

RESULTS AND DISCUSSION

Near real-time field trials

We executed a near real-time field trial lasting six hours, with measurements recorded at hourly intervals, which exhibited negligible inhibition (under 10%) on the ficin assays employed. Instrumental investigations indicated that the concentrations of mercury, copper, and silver in the marine waters were below the maximum permitted limits (MPL) of 0.0005 mg/L, 0.0029 mg/L, and 0.050 mg/L, respectively. The criterion for substantial inhibition was set at 20%.

This minimal inhibition suggests the effectiveness of the ficin assay in these environmental conditions. Additionally, other near real-time studies employing enzymatic methods in riverine environments have reported varying temporal concentrations of heavy metals, indicating the potential of these bioassays for monitoring fluctuations in environmental contaminant levels [21,22,25,29,30]. This is a second investigation utilizing marine or brackish water samples. Marine and brackish waters, vast aquatic ecosystems, act as crucial reservoirs where heavy metals from land sources rapidly experience dilution. Notwithstanding this swift distribution, high levels of heavy metals have been detected in these areas, primarily accumulating in the sedimentary fractions.

This accumulation pattern highlights the intricate dynamics of heavy metal distribution, with sediments frequently serving as reservoirs for these contaminants, sequestering and preserving them over time. The variation in metal concentrations between water columns and sediments underscores the necessity of thorough monitoring across several environmental compartments to accurately evaluate the effects of heavy metal pollution in aquatic ecosystems [1,2,4,6]. The lack of reactivity to ficin indicates that this area is mostly unspoiled. Our previously monitoring system in the Kilim's Geoforest site of Langkawi using the same system shows the utility of enzyme-based inhibitive assay in a near real-time mode [31]. To safeguard this ecotourism destination, it is imperative to employ enhanced surveillance procedures moving ahead.

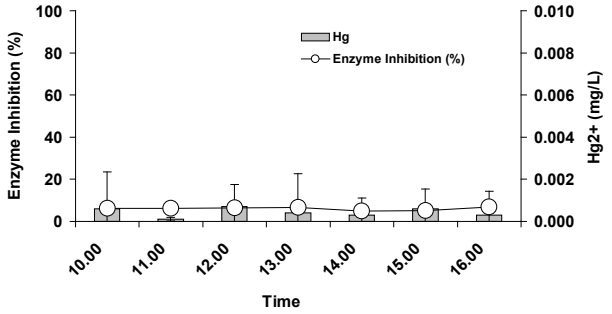

Fig. 2. Near real-time detection of mercury in the waters near Kuah's Jetty, Langkawi, using the ficin inhibitive enzyme assay. Error bars represent the mean ± standard deviation (n=3).

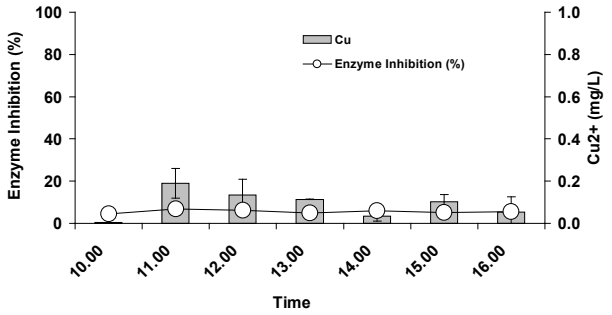

Fig. 3. Near real-time detection of copper in the waters near Kuah's Jetty, Langkawi, using the ficin inhibitive enzyme assay. Error bars represent the mean ± standard deviation (n=3).

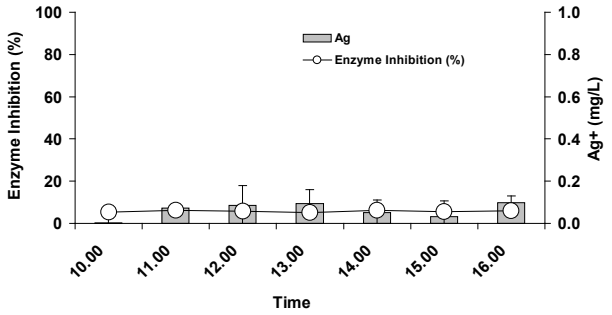

Fig. 4. Near real-time detection of silver in the waters near Kuah's Jetty, Langkawi, using the ficin inhibitive enzyme assay. Error bars represent the mean ± standard deviation (n=3).

Pollution incidents in Langkawi have increased in frequency and severity in recent years, raising substantial environmental concerns. In 2017, the Department of Environment's assessment identified Sungai Ulu Melaka as significantly polluted, with the river's water quality categorized as Class IV, signifying a severe level of contamination. This classification indicates the increasing apprehension regarding the declining condition of Langkawi's water bodies, which are essential for local ecosystems and the lives of dependent communities.

The problem of heavy metal pollution in Langkawi's aquatic ecosystems—rivers, lakes, and marine waters—has been thoroughly researched and recorded. Heavy metal concentrations in these water bodies exhibit considerable variability, both geographically and temporally, highlighting the dynamic nature of pollution in these ecosystems. The concentrations of contaminants vary over time due to variables including industrial discharge, agricultural runoff, and seasonal variations. This diversity is a difficulty for uniform monitoring and efficient environmental management.

Moreover, the pollution of sediments in Langkawi's aquatic systems exhibits a comparable pattern, characterized by geographical and temporal variations in heavy metal concentrations. Sediment analysis is crucial, as sediments frequently serve as both a reservoir and a source of pollutants, discharging toxins into the water column under specific conditions. The intricate patterns of environmental contamination in water and sediments underscore the complicated nature of pollution, necessitating ongoing monitoring and customized treatments to successfully address the root causes. The escalating pollution levels and the presence of heavy metals in Langkawi's water systems highlight the pressing necessity for enhanced pollution control methods, superior waste management practices, and more stringent environmental legislation to protect the natural environment and human health [32]. Addressing these fluctuations necessitates the development of rapid detection methodologies capable of tracking changes in heavy metal concentrations, a crucial aspect of environmental forensics.

Conventional techniques for identifying heavy metals have primarily utilized batch processing of water and sediment samples. The samples are gathered from the field, transported to a laboratory, and analyzed, a procedure that is both time-intensive and inadequate for capturing real-time variations in pollutant levels. The inherent delay in this method frequently results in the failure to swiftly detect fast fluctuations in pollution levels caused by numerous environmental or anthropogenic variables. This delay can impede prompt response and the efficient handling of pollution incidents. In light of these constraints, there has been a notable transition towards real-time or near real-time monitoring methods that provide more instantaneous insights into the presence and quantity of contaminants.

A new technique entails employing bioassays that utilize the inherent characteristics of plants, microbes, and enzymatic responses to identify pollutants. These bioassays offer a rapid and effective method for evaluating environmental quality, facilitating ongoing monitoring and expedited reactions to pollution incidents. These strategies leverage biological systems that respond to environmental stressors, yielding expedited results and facilitating continuous, localized environmental evaluations without the necessity for extensive sample transportation and analysis [16,20,33–36].

The improvements in bioassay technology are promising, presenting substantial potential for enhancing the precision and efficacy of pollution monitoring and enabling more effective environmental management techniques. Enzyme assays provide swift findings, with the complete procedure from sampling to detection attainable in less than an hour through portable spectrophotometry, rendering them suitable for on-site analysis. The necessity for the implementation of quick, near real-time biomonitoring in drinking water systems is complex, largely focused on protecting public health. This monitoring facilitates the prompt identification of biological and chemical contaminants, serving as an essential early warning system to avert exposure to hazardous compounds and reduce health risks. This prompt response adheres to rigorous regulatory criteria, guaranteeing that water quality stays within safe consumption thresholds and enabling water utilities to implement immediate remedial measures as necessary. Furthermore, it improves operational efficiency by facilitating real-time modifications to

water treatment processes, hence improving resource utilization and minimizing expenses related to over-treatment or urgent pollutant extraction. The transparency and immediacy of near real-time biomonitoring are crucial for sustaining public trust in the drinking water supply, ensuring consumers of its safety. Furthermore, it facilitates flexibility to fluctuating environmental conditions that may impact water quality, such as meteorological occurrences or industrial incidents, and aids in averting any infrastructure damage resulting from contaminants [37–39]. In summary, quick biomonitoring is crucial for the ongoing guarantee of drinking water safety and quality, highlighting its importance in public health protection, regulatory adherence, and the effective functioning of water treatment systems.

Our research has demonstrated the effectiveness of enzyme-based assays in identifying temporal fluctuations of heavy metal concentrations in aquatic environments located in industrial areas [21,24,27,28,40]. The application of the ficin test for mercury monitoring in marine environments exemplifies a unique method and acts as a first proof of the technique's potential. Subsequent study will build upon this foundation by pinpointing further sampling locations and executing comprehensive field experiments. This directive reaffirms the efficacy of enzyme assays for environmental monitoring and paves the way for the expanded application and advancement of real-time detection systems that can markedly improve our responsiveness to environmental toxins.

CONCLUSION

In this study, biomonitoring using enzyme assays, particularly the ficin dye-binding assay, has proven to be a highly effective tool for the detection of contaminants in the waters adjacent to Kuah's Jetty, Langkawi. The ficin-based enzyme assay exhibited remarkable sensitivity in identifying trace levels of heavy metals, such as mercury, silver, and copper, at sub-ppm quantities. This sensitivity facilitates the early identification of pollutants, offering essential data for timely environmental management and intervention. Timely detection of pollutants is essential for averting more environmental deterioration and safeguarding the well-being of both the local populace and visitors to the region. The straightforwardness, affordability, and ease of application of enzyme tests render them an optimal alternative for undertaking comprehensive and widespread environmental evaluations. They provide an effective alternative to conventional laboratory procedures, which frequently necessitate prolonged sample processing durations and may overlook real-time variations in pollutant levels. Enzyme assays provide continuous monitoring of environmental quality without the necessity for intricate and expensive equipment, rendering them appropriate for routine surveillance in pollution-prone regions. Our data indicate that the vicinity of Kuah’s Jetty is mostly unpolluted, as demonstrated by the minimal inhibition (<10%) recorded during the experiments. This indicates that the water quality in this location remains within acceptable contamination limits. The study underscores the necessity of continuous monitoring to identify any potential fluctuations in pollution levels throughout time. Regular evaluations are crucial for maintaining the biological integrity of the region, especially considering its significance as a principal ecotourism destination in Langkawi. The data produced from these assessments can guide policymaking, provide a scientific basis for the formulation of specific environmental protection plans and legislation. Moreover, the study enhances public awareness by illustrating the significance of monitoring and safeguarding natural water bodies. The study seeks to motivate local authorities and the people to engage actively in environmental protection, promoting a culture of responsibility for sustaining ecological balance. This study highlights the essential importance of enzyme tests in environmental management. They offer a quick, precise, and accessible approach for assessing environmental quality, crucial for preserving ecological integrity and public health. As environmental concerns escalate, such inventive and pragmatic solutions will become increasingly essential for safeguarding our natural resources for future generations.

ACKNOWLEDGEMENT

This research is funded under the Fundamental Research Grant Scheme (FRGS/1/2017/TK05/UPM/02/11) by the Ministry of Higher Education (MOHE) Malaysia.

REFERENCES

1. Mohamed B. Effects of tourism activities and development on the physical environment of Kilim River, Langkawi, Malaysia. Malays For. 2012 Jan 1;75:81–6.
2. Tajam J, Kamal mohd lias. Marine Environmental Risk Assessment of Sungai Kilim, Langkawi, Malaysia: Heavy Metal Enrichment Factors in Sediments as Assessment Indexes. Int J Oceanogr. 2013 Jan 1;2013.
3. Tyler L. Trouble in paradise: Langkawi struggles to hold onto Unesco geopark status [Internet]. South China Morning Post. 2014 [cited 2024 Apr 6]. Available from: https://www.scmp.com/magazines/post-magazine/article/1577608/langkawi-victim-its-own-success
4. Idris SMM. Mangrove forest in Kilim Geo Park under threat [Internet]. Free Malaysia Today. 2016 [cited 2024 Apr 6]. Available from: https://www.freemalaysiatoday.com/category/opinion/2016/05/27/mangrove-forest-in-kilim-geo-park-under-threat/
5. Lee LM. Langkawi getting trashed [Internet]. R.AGE. 2016 [cited 2024 Apr 6]. Available from: https://www.rage.com.my/langkawi-getting-trashed/
6. Halim M, Ahmad A, Abd Rahman M, Mat Amin Z, Abdul Khanan MF, Musliman I, et al. Land use/land cover mapping for conservation of UNESCO Global Geopark using object and pixel-based approaches. IOP Conf Ser Earth Environ Sci. 2018 Jul 31;169:012075.
7. Chowdhury A, Maiti SK. Assessing the ecological health risk in a conserved mangrove ecosystem due to heavy metal pollution: A case study from Sundarbans Biosphere Reserve, India. Hum Ecol Risk Assess Int J. 2016 Oct 2;22(7):1519–41.
8. Haeruddin H, Ayuningrum D, Oktaviani J. Maximum Tolerable Intake of Mangrove Oyster (Crassostrea rhizophorae) from the Tapak River, Semarang City, Indonesia, which Contains Cd and Pb Metals: J Pengolah Has Perikan Indones. 2022 Dec 15;25(3):418–27.
9. Sabdono A, Ayuningrum D, Sabdaningsih A. First Evidence of Microplastics Presence in Corals of Jepara Coastal Waters, Java Sea: A Comparison Among Habitats Receiving Different Degrees of Sedimentations. Pol J Environ Stud. 2022 Jan 28;31(1):825–32.
10. Mirzavand S, Aeini M, Mirzaei Najafgholi H, Moazen-Rezemehale H. Identification and Characterization of Bacterial Strains Capable of Degrading Atrazine and Metribuzin Herbicides in Sugarcane Fields. Sugar Tech. 2023 Sep 11;
11. Bohórquez-Herrera J, Matías I, Castañeda C. Impact of different environmental pollution processes on bacterial key-indicators in tropical rivers: Scoping review. FEMS Microbiol Lett. 2023 Sep 27;370.
12. Ahmad NI, Noh MFM, Mahiyuddin WRW, Jaafar H, Ishak I, Azmi WNFW, et al. The mercury levels in crustaceans and cephalopods from Peninsular Malaysia. Environ Sci Pollut Res. 2015;22(17):12960–74.
13. Amaro F, Turkewitz AP, Martín-González A, Gutiérrez JC. Whole-cell biosensors for detection of heavy metal ions in environmental samples based on metallothionein promoters from Tetrahymena thermophila. Microb Biotechnol. 2011;4(4):513–22.

14. Lopez-Roldan R a, Kazlauskaite L a b, Ribo J c, Riva MC c, González S a, Cortina JL a d. Evaluation of an automated luminescent bacteria assay for in situ aquatic toxicity determination. Sci Total Environ. 2012;440:307–13.
15. Osimitz TG, Droege W, Driver JH. Human Risk Assessment for Nonylphenol. Hum Ecol Risk Assess Int J. 2015 Oct 3;21(7):1903–19.
16. Prabhakaran K, Nagarajan R, Merlin Franco F, Anand Kumar A. Biomonitoring of Malaysian aquatic environments: A review of status and prospects. Ecohydrol Hydrobiol. 2017;17(2):134–47.
17. Perić L a, Petrović S b. Acetylcholinesterase activity in the gills of mussels (Mytilus galloprovincialis) from the north-eastern Adriatic coast. Fresenius Environ Bull. 2011;20(11):2855–60.
18. Lionetto MG, Caricato R, Calisi A, Giordano ME, Schettino T. Acetylcholinesterase as a biomarker in environmental and occupational medicine: New insights and future perspectives. BioMed Res Int. 2013;2013.
19. Hayat NM, Shamaan NA, Shukor MY, Sabullah MK, Syed MA, Khalid A, et al. Cholinesterase-based biosensor using *Lates calcarifer* (Asian Seabass) brain for detection of heavy metals. J Chem Pharm Sci. 2015;8(2):376–81.
20. de Souza PR, de Souza KS, de Assis CRD, de Araújo MC, Silva KCC, de Fátima Xavier da Silva J, et al. Acetylcholinesterase of mangrove oyster Crassostrea rhizophorae: A highly thermostable enzyme with promising features for estuarine biomonitoring. Aquat Toxicol. 2018;197:109–21.
21. Halmi MIE, Johari WLW, Amir S, Sulaiman R, Azlina A, Shukor MY, et al. Monitoring of heavy metals level in fish using *Photobacterium* sp. strain MIE. Bioremediation Sci Technol Res. 2013;1(1):19–22.
22. Halmi MIE, Jirangon H, Johari WLW, Abdul Rachman AR, Shukor MY, Syed MA. Comparison of Microtox and Xenoassay light as a near real time river monitoring assay for heavy metals. Sci World J. 2014;2014.
23. Shukor M, Masdor N, Halmi M, Kamaruddin K, Syed M. Near real-time biomonitoring of heavy metals using the Xenoassay® system. In: Proceedings of The Annual International Conference, Syiah Kuala University-Life Sciences & Engineering Chapter. Universitas Syiah Kuala, Indonesia: Universitas Syiah Kuala; 2013.
24. Shukor MY, Anuar N, Halmi MIE, Masdor NA. Near real-time inhibitive assay for heavy metals using achromopeptidase. Indian J Biotechnol. 2014;13(3):398–403.
25. Sabullah MK, Sulaiman MR, Shukor MS, Yusof MT, Johari WLW, Shukor MY, et al. Heavy metals biomonitoring via inhibitive assay of acetylcholinesterase from *Periophthalmodon schlosseri*. Rendiconti Lincei. 2015;26(2):151–8.
26. Uba G, Manogaran M, Gunasekaran B, Halmi MIE, Shukor MYA. Improvement of Ficin-Based Inhibitive Enzyme Assay for Toxic Metals Using Response Surface Methodology and Its Application for Near Real-Time Monitoring of Mercury in Marine Waters. Int J Environ Res Public Health. 2020 Jan;17(22):8585.
27. Rahman MFA, Manogaran M, Shukor MY. Rapid Biomonitoring of Heavy Metals in Polluted Sites Using Xenoassay®-Metal. J Environ Microbiol Toxicol. 2022 Jul 31;10(1):4–8.
28. Uba G, Manogaran M, Gunasekaran B, Halmi M, Shukor Y. A Near real-time Achromopeptidase-Based Enzyme Assay for Biomonitoring of Zinc Pollution in Kuah's Jetty in Langkawi. J Environ Microbiol Toxicol. 2023 Dec 31;11:29–34.
29. Shukor MY, Tham LG, Halmi MIE, Khalid I, Begum G, Syed MA. Development of an inhibitive assay using commercial *Electrophorus electricus* acetylcholinesterase for heavy metal detection. J Environ Biol. 2013;34(5):967–70.
30. Effendi Halmi M, Gunasekaran B, Shukor M. An inhibitive determination method for biomonitoring of heavy metals using ficin; a cysteine plant protease. In: International Agriculture Congress. 04 Oct 2016 - 06 Oct 2016, Hotel Bangi-Putrajaya; 2016.
31. Uba G, Manogaran M, Gunasekaran B, Halmi MIE, Sabullah MK, Shukor MY. A Near real-time Ficin-Based Enzyme Assay for Biomonitoring of Heavy Metals Pollution in Waters Near the Langkawi UNESCO Kilim Karst Geoforest Park. Bull Environ Sci Sustain Manag E-ISSN 2716-5353. 2023 Dec 31;7(2):15–9.
32. Birch GF, Taylor SE, Matthai C. Small-scale spatial and temporal variance in the concentration of heavy metals in aquatic sediments: A review and some new concepts. Environ Pollut. 2001;113(3):357–72.
33. Bhakta JN, Lahiri S, Bhuiyna FA, Rokunuzzaaman M, Ohonishi K, Iwasaki K, et al. Profiling of heavy metal(loid)-resistant bacterial community structure by metagenomic-DNA fingerprinting using PCR–DGGE for monitoring and bioremediation of contaminated environment. Energy Ecol Environ. 2018;3(2):102–9.
34. Wang F, Zhang J, Sun X, Zhang Z, He X, Zhao Y. Study on intelligent designs of heavy metal online monitoring system for water. Chem Eng Trans. 2015;46:349–54.
35. Raissy M, Ansari M. Health risk assessment of mercury and arsenic associated with consumption of fish from the Persian Gulf. Environ Monit Assess. 2014;186(2):1235–40.
36. Verma N, Kaur H, Kumar S. Whole cell based electrochemical biosensor for monitoring lead ions in milk. Biotechnology. 2011;10(3):259–66.
37. Bhat SA, Singh J, Singh K, Vig AP. Genotoxicity monitoring of industrial wastes using plant bioassays and management through vermitechnology: A review. Agric Nat Resour. 2017 Oct 1;51(5):325–37.
38. Eom H, Ashun E, Toor UA, Oh SE. A solid-phase direct contact bioassay using sulfur-oxidizing bacteria (SOB) to evaluate toxicity of soil contaminated with heavy metals. Sens Actuators B Chem. 2020 Feb 15;305:127510.
39. Polyakova G, Pashenova N, Senashova V, Podolyak N, Kudryasheva N. Pine Stands as Bioindicators: Justification for Air Toxicity Monitoring in an Industrial Metropolis. Environments. 2020 Apr;7(4):28.
40. Halmi MIE, Khayat ME, Gunasekaran B, Masdor NA, Rahman MFA. Near real-time biomonitoring of copper from an industrial complex effluent discharge site using a plant protease inhibitive assay. Bioremediation Sci Technol Res. 2016 Jul 31;4(1):10–3.