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Land degradation

A Near-Real-Time Ficin-Based Enzyme Assay for Biomonitoring of Heavy Metals Pollution in Waters Near the Langkawi UNESCO Kilim Karst Geoforest Park

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ABSTRACT

Near-real-time biomonitoring, especially when utilizing enzyme assays, offers exceptional sensitivity to bioavailable pollutants, yielding swift results favorable to immediate action. This approach is particularly crucial in the context of mitigating pollution in drinking water systems, safeguarding both human and animal health. This study presents an application of a previously developed enzyme assay in biomonitoring to detect pollutants, specifically heavy metals, in environmental samples from the UNESCO's Kilim Karst Geoforest Park. Utilizing the ficin dye binding assay, developed for mercury (Hg^{2+}), silver (Ag^{+}), and copper (Cu^{2+}) detection at the sub ppm level, we demonstrated its effectiveness in identifying low concentrations of these metals in marine and brackish waters. The assay provided a sensitive, rapid, and cost-effective monitoring, showing negligible inhibition (<10%) over a 6-hour field trial, indicating low pollution levels and verified using instrumental analysis. This approach enables the early detection of environmental contaminants, facilitating timely interventions and contributing to the protection of ecotourism sites by providing evidence-based data for policymaking. The simplicity and visual appeal of the enzyme assays also make them excellent educational tools, promoting environmental awareness and conservation efforts. Our findings underscore the potential of enzyme assays for widespread environmental assessment, aligning local monitoring practices with international standards and fostering global collaboration in environmental protection. This study not only contributes to our understanding of ecological health in marine and brackish waters but also highlights the importance of continuous monitoring to preserve natural habitats.

INTRODUCTION

The designation of Langkawi as a UNESCO geopark in July 2007 marked a pivotal moment in the island's evolution from a tranquil Malaysian gem to a globally recognized tourist haven. This acknowledgment by the United Nations Educational, Scientific and Cultural Organization has spurred unprecedented growth in its coastal development and tourism sectors, notably enhancing its appeal to international visitors. A prime example of this development is the Kilim Karst Geoforest Park, which has transitioned from a quiet rural area to a bustling center of tourist activity. The park, with its unique geological formations and natural landscapes, epitomizes the potential for geoparks to stimulate local economies and cultural appreciation. However, this surge in tourism has precipitated significant environmental challenges, particularly evident in the increased marine traffic

along the Kilim River and its consequent ecological ramifications. This paper aims to explore the adverse effects of heightened tourist activity, including the erosion of riverbanks and the degradation of vital mangrove ecosystems. These ecosystems are not only key to preserving biodiversity but also serve as critical barriers against coastal erosion. Moreover, the presence of heavy metals such as cadmium (Cd), cobalt (Co), lead (Pb), and zinc (Zn) in the vicinity of the Kilim Karst Geoforest Park raises concerns about the long-term sustainability of Langkawi's natural environments. These contaminants, likely emanating from boat emissions and construction-related disturbances, pose a substantial risk to the ecological equilibrium of the area [1–6].

Ecotourism, particularly water-based activities, can inadvertently elevate heavy metal concentrations in aquatic ecosystems through several interconnected pathways. The engines of boats and watercraft used in ecotourism activities release exhaust emissions into the water, which may contain heavy metals like lead, cadmium, and mercury, stemming from fuel combustion; older and poorly maintained vessels are particularly culpable in this regard. Additionally, boats often employ antifouling paints to prevent barnacle and algae growth; these paints release copper and other heavy metals as they degrade [7]. Maintenance and repair activities for these watercrafts can further introduce heavy metals directly into the water through activities such as paint scraping, engine repairs, and the replacement of metal parts.

The act of anchoring not only disturbs the sediment, resuspending heavy metals contained within but also renders them more bioavailable to the aquatic food chains. Infrastructure development to accommodate ecotourism, including the construction of docks and marinas, disturbs land and sediment, potentially releasing trapped heavy metals. The influx of tourists leads to increased wastewater and runoff, which may carry heavy metals from various sources into aquatic systems. Moreover, the use of recreational equipment, such as jet skis and motorboats, contributes to erosion and sediment resuspension, thus releasing heavy metals previously settled in the sediments. Although ecotourism is promoted as a sustainable tourism alternative, it necessitates meticulous management to mitigate its environmental impacts, including the potential elevation of heavy metal levels in water bodies [1,8–10].

Biomonitoring through enzyme assays emerges as a powerful approach in environmental management, providing a sensitive, cost-effective, and rapid means for detecting pollutants, which greatly benefits both public awareness and authoritative action. These assays are adept at identifying low concentrations of contaminants like heavy metals and organic compounds, facilitating the early implementation of remedial measures by authorities. Their cost efficiency and the minimal requirement for sophisticated equipment enable widespread and frequent environmental assessments, contributing to a detailed understanding of ecological health across vast areas [11–14].

The swift processing of enzyme assays ensures timely interventions critical for preventing environmental degradation and safeguarding public health. Additionally, the simplicity and visual appeal of some enzyme assays serve as excellent resources for educational initiatives aimed at enhancing environmental consciousness among the community, thereby promoting active conservation efforts. The accurate data generated from these assays support evidence-based policymaking, enabling authorities to establish precise pollutant thresholds, assess the effectiveness of environmental protections, and make necessary adjustments. Moreover, enzyme activity indicators offer early warnings of ecological distress, allowing for interventions before visible damage occurs, thereby preventing long-term ecological damage [15–18]. By aligning local monitoring practices with international standards through enzyme assays, authorities can foster global collaboration in environmental protection, addressing transboundary challenges effectively.

In essence, enzyme assays for biomonitoring equip both the public and decision-makers with essential tools and knowledge for more effective environmental stewardship and public health protection. We have developed several near-real time monitoring of pollution especially heavy metals using enzymes from microorganisms and plants and utilize these assays to monitor various potential and polluted sites in Malaysia including an UNESCO site in the river Malacca [19–25].

In this study, we explore the feasibility of using the ficin dye binding assay we previously developed for mercury biomonitoring in waters from the UNESCO's Kilim Karst Geoforest Park. The ficin inhibitive assay is sensitive to Hg^{2+}, Ag^{+} and Cu^{2+} with IC50 values of 0.017 mg/L (95% C.I. from 0.016 to 0.019), 0.028 (95% C.I. from 0.022 to 0.037) and 0.023 (95% C.I. from 0.020 to 0.027) [24].

MATERIAL AND METHODS

Preparation of casein and ficin solution

Casein, procured from Sigma, was precisely measured to 2 grams and blended with 100 milliliters of deionized water. To achieve a pH level of 8.0, the solution was titrated with 5N solutions of NaOH and/or HCl. This mixture was then continuously agitated at 60°C throughout the night to ensure thorough dissolution. To separate insoluble particles, the solution was strained through multiple cheesecloth layers. Subsequent clarification was achieved by centrifuging the solution at 10,000×g at a temperature of 4°C.

The protein concentration in the resulting clear supernatant was determined via the Bradford method, employing crystalline BSA from Sigma as a reference. This prepared solution was preserved at 4°C for immediate use or frozen at -20°C for long-term storage. Ficin (from Sigma, E.C. 3.4.22.3, lot number: F4165-1ku, derived from crude dried fig tree latex, with an activity of 0.5 Units/mg) was dissolved at 4°C in a 20 mM sodium phosphate buffer with a pH of 6.77, creating a 10.0 mg/mL stock solution. From this stock, working solutions of ficin (2.0 mg/mL) and casein (10 mg/mL) were freshly prepared on a daily basis.

Ficin inhibition studies

The initiation of the positive control experiment involved combining 50 µL of ficin (0.6 mg/mL final concentration) in 20 mM phosphate buffer at a pH of 6.77, as determined from previous experiments conducted according to a Central Composite Design (CCD), with 50 µL of mercury solution to achieve a final mercury concentration of 0.040 mg/L [24]. This mixture was then incubated for 10 minutes at a temperature of 30°C. For the control setup, the mercury was substituted with an equivalent volume of the 20 mM phosphate buffer at pH 6.77. Following this substitution, 50 µL of casein solution was introduced to the mixture, resulting in a final concentration of 2.36 mg/mL, and was thoroughly mixed. An aliquot of 20 µL from this mixture was immediately combined with 200 µL of Bradford dye-binding reagent. The resulting solution was allowed to stand at ambient temperature for 5 minutes, after which the absorbance was recorded at 595 nm, marking the initial

absorbance reading. After an additional incubation period of 30 minutes, a second aliquot of 20 µL 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

Every hour for six hours, water samples were collected into acid-washed HDPE bottles, each supplemented with a few drops of 1% (v/v) HNO3, from areas adjacent to the Langkawi UNESCO Kilim Karst Geoforest Park, specifically at the coordinates 6°24'17.4"N 99°51'30.7"E (refer to **Fig. 1**). Initially, these samples underwent filtration through a 0.45 µm syringe filter 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. The necessity to power the incubator led to the use of a rented parked car as a makeshift electrical source.

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 (sourced from Universiti Malaya). 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 geoforest park.

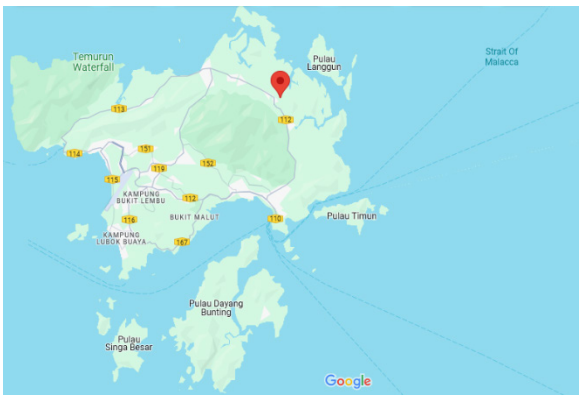

Fig. 1. Location of water sampling. (Source Google Earth image).

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 conducted a near real-time field trial over a six-hour duration, with measurements taken at hourly intervals, demonstrated minimal inhibition (less than 10%) on the ficin assays utilized. Instrumental analyses further revealed that the levels of mercury, copper, and silver in the marine/brackish waters were below the maximum permissible limits (MPL) set at 0.0005 mg/L, 0.0029 mg/L, and 0.050 mg/L, respectively. In this context, the threshold for significant inhibition was established 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 [19,20,23,26,27] and this is a second study using marine/brackish water as samples. Marine and brackish waters, expansive aquatic environments, serve as significant reservoirs where heavy metals from terrestrial sources quickly undergo dilution. Despite this rapid dispersion, elevated concentrations of heavy metals have been identified within these regions, predominantly accumulating in the sedimentary fractions.

This accumulation pattern underscores the complex dynamics of heavy metal distribution, where sediments often act as sinks for these pollutants, capturing and retaining them over time. The disparity in metal concentrations between water columns and sediments highlights the importance of comprehensive monitoring across different environmental compartments to fully assess the impact of heavy metal pollution in aquatic ecosystems [1,2,4,6]. This observation of an absence in response to ficin suggests that this area remains comparatively pristine. To protect this ecotourism destination, it will be essential to implement increased monitoring measures going forward in the future.

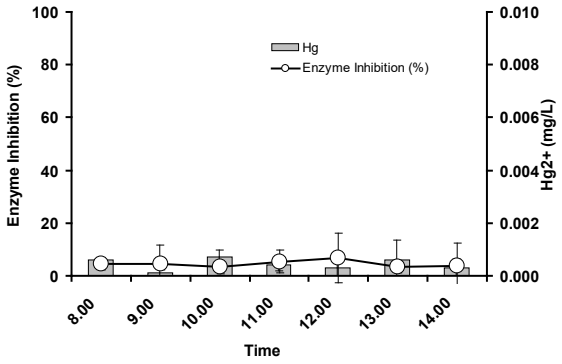

Fig. 2. Near real-time detection of mercury in the Langkawi UNESCO Kilim Karst Geoforest Park waters using the ficin inhibitive enzyme assay. Error bars represent mean ± standard deviation (n=3).

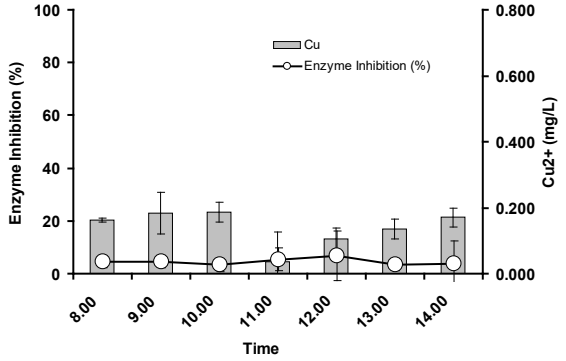

Fig. 3. Near real-time detection of copper in the Langkawi UNESCO Kilim Karst Geoforest Park waters using the ficin inhibitive enzyme assay. Error bars represent mean ± standard deviation (n=3).

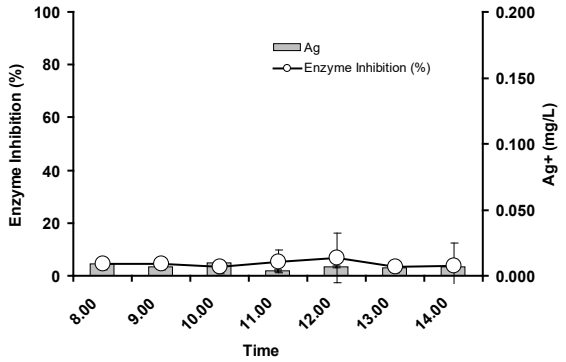

Fig. 4. Near real-time detection of silver in the Langkawi UNESCO Kilim Karst Geoforest Park waters using the ficin inhibitive enzyme assay. Error bars represent mean ± standard deviation (n=3).

Pollution incidents in Langkawi have escalated, with Sg Ulu Melaka being identified as contaminated and categorized as Class IV in 2017, as per the Department of Environment's report. Variations in the concentrations of heavy metals in aquatic environments, including rivers and marine waters, are well-documented phenomena, highlighting the dynamic nature of these pollutants. Sediments, too, exhibit spatial and temporal variability in heavy metal content, underscoring the complex patterns of environmental contamination [28]. Addressing these fluctuations necessitates the development of rapid detection methodologies capable of tracking changes in heavy metal concentrations, a crucial aspect of environmental forensics.

Traditional methods for detecting heavy metals have predominantly relied on batch processing of samples, which must be collected and transported to a laboratory for analysis [29–31]. This process is time-consuming and may not capture the immediate changes in pollutant levels. As a response to these limitations, there has been a shift towards real-time or near real-time monitoring techniques. Innovations in bioassays utilizing plants, microorganisms, and enzymatic reactions have emerged as promising solutions for immediate environmental assessment [32–34]. Enzyme assays, in particular, offer rapid results, with the entire process from sampling to detection being achievable in under an hour using portable spectrophotometry, making them ideal for on-site analysis.

The imperative for implementing rapid, near-real-time biomonitoring in drinking water systems is multifaceted, primarily centered around safeguarding public health. Such monitoring enables immediate detection of biological and chemical contaminants, acting as a crucial early warning system to prevent exposure to harmful substances and mitigate health risks. This rapid response aligns with stringent regulatory standards, ensuring that water quality remains within safe consumption limits and allowing water utilities to take swift corrective actions as needed.

Additionally, it enhances operational efficiency by enabling real-time adjustments to water treatment processes, thereby optimizing resource use and reducing costs associated with over-treatment or emergency contaminant removal. The transparency and immediacy of near-real-time biomonitoring also play a vital role in maintaining public confidence in the drinking water supply, reassuring consumers about the safety of their water. Moreover, it allows for the adaptation to changing environmental conditions that could affect water quality, such as weather events or industrial accidents, and helps prevent potential infrastructure damage caused by contaminants. Overall, rapid biomonitoring is essential for continuous assurance of drinking water safety and quality, underscoring its significance in public health protection, regulatory compliance, and the efficient operation of water treatment systems.

Our work has shown the efficacy of enzyme-based assays in detecting temporal variations of heavy metal concentrations in water bodies situated in industrial regions. Specifically, the use of the ficin assay for mercury monitoring in marine environments represents an innovative approach and serves as a preliminary demonstration of this technique's potential. Future research will expand on this groundwork by identifying additional sites for sampling and conducting extensive field trials. This direction not only reaffirms the viability of enzyme assays for environmental monitoring but also sets the stage for broader application and development of real-time detection systems that can significantly enhance our responsiveness to environmental pollutants.

CONCLUSION

In this study, biomonitoring through enzyme assays, particularly the ficin dye binding assay, has proven to be an invaluable tool in environmental management for detecting pollutants in the UNESCO's Kilim Karst Geoforest Park. Demonstrating sensitivity to low concentrations of heavy metals such as mercury, silver, and copper, these assays enable the early detection of contaminants, facilitating prompt remedial actions. The cost-effectiveness and ease of application of enzyme assays make them ideal for widespread environmental assessments, contributing significantly to our understanding of ecological health. Moreover, the negligible inhibition observed in our assays suggests that the area under study remains relatively unpolluted, highlighting the importance of ongoing monitoring to preserve this ecotourism site. The data derived from these assessments not only can inform policymaking authorities but also enhance public awareness and conservation efforts. This study underscores the critical role of enzyme assays in environmental stewardship, offering a rapid, accurate, and accessible means to safeguard both ecological integrity and public health.

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