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A Near-Real-Time Protease-Based Enzyme Assay for Biomonitoring of Copper Pollution in Sg. Kuyoh

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ABSTRACT
Sg. Kuyoh is a small river that runs close to the Serdang Industrial Area, where the previous work reported a copper-contaminated sediment with a high copper (Cu) concentration. An inhibitory enzyme assay based on the protease papain was used as a near-real-time biomonitoring tool for copper pollution in Sg. Kuyoh. Water samples were collected at two-hour intervals periodically from 8.00 AM to 10.00 PM. Inhibitory enzyme assay was carried out using the casein-Coomassie dye-binding protease assay reaction. Hg was determined by Flow Injection Mercury System. Ag and Cu were determined by ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy). Copper exhibited clear temporal variation with values between ~0.25 and 0.87 mg/L with the highest values at 8:00 PM and 10:00 PM. Enzyme activity displayed the opposite trend, decreasing from approximately 60% in the morning to below 20% at night, consistent with inhibition by bioavailable Cu. In contrast, Ag and Hg remained at low concentrations that did not contribute to the inhibition pattern. All Cu measurements were higher than the conservative Cu value of 0.020 mg/L as written in the Malaysian National Water Quality Standards. The results support the suitability of protease-based inhibitive assay as a rapid, low-cost field screening method for temporal Cu pollution detection in Sg. Kuyoh.

Keywords: Near-real-time monitoring; Protease assay; Copper; Sg. Kuyoh; biomonitoring; inhibitive enzyme assay
SDG Keywords: SDG 6 Clean water and sanitation; SDG 12 Responsible consumption and production; SDG 9 Industry, innovation and infrastructure

INTRODUCTION

Heavy metal pollution is still one of the major environmental concerns in urban and industrial river systems. This is because metal ions are usually invisible and odorless, persistent, non-biodegradable, and bioaccumulate in aquatic organisms and sediments. Copper, mercury, silver, lead, cadmium and other metals pollute rivers through irresponsible industrial discharge, from metal or car workshops runoff, metal-processing activities and, to a certain extent, urban drainage. Of these metals, copper is an essential trace element, but high Cu concentrations can be toxic for aquatic organisms. This toxicity is due to Cu binding to proteins, interfering with the activity of enzymes, and affecting biological membranes (Bakhasha et al., 2024). The case of Sg. Kuyoh is rather worrying, especially it is the source for some of the freshwater fish sold in the wet market, and it is also very near the Serdang Industrial Area. Yazdani et al. (2009) reported Cu-polluted sediment from Sg. Kuyoh in the Serdang Industrial Area, with Cu concentration in sediment reaching 348 ug/g. This elevated level suggests that the river has been affected by substantial Cu input. What this means is that the sediment may be a reservoir for remobilized Cu under changing environmental conditions, and hence, a fast monitoring method is needed to detect the temporal variation of bioavailable Cu in the water column.

Conventional instrumental methods like atomic absorption spectroscopy, ICP-OES and other related metal analysis platforms are accurate and sensitive, but they require laboratory infrastructure, trained personnel, sample transportation and relatively high running costs, limiting their usefulness for high-frequency field monitoring. In contrast, enzyme inhibition assays are rapid with near-real-time capability (Shukor et al., 2013), inexpensive, and can respond to bioavailable toxicants. Therefore, inhibitive enzyme assays can be used as first-line screening tools, and only positive samples are sent for instrumental validation. Previous work on enzyme-based monitoring has shown that inhibitive assays can be used for rapid or near-real-time monitoring of metals in industrial and aquatic environments. The assays include molybdenum-reducing enzyme assays (Bakar et al., 2019), crude plant protease assays (Gunasekaran et al., 2013; Halmi et al., 2015), papain (Shukor et al., 2006), bromelain (Shukor et al., 2008), ficin (Uba et al., 2023) assays and acetylcholinesterase-based assays (Abdulrasheed & Ahmad, 2017; Asri et al., 2017).

Protease-based assays are particularly useful because they are based on the inhibition of casein hydrolysis by proteases due to metal inhibition of the protease. For instance, the papain-casein dye binding (PCDB) assay works by hydrolyzing casein in the presence (test) and absence of metals (control). The unhydrolyzed casein due to papain inhibition by heavy metals

would show low protein content as measured using the Coomassie dye binding or Bradford assay. Heavy metals such as mercury, silver, and copper exhibited sigmoidal inhibition curves with IC_{50} and LOD values well within the range of the maximum permissible limit of heavy metals in Malaysian waters (Shukor et al., 2006).

In this study, a near real-time PCDB assay was used to monitor a single-day preliminary exercise for measuring Cu pollution in Sg. Kuyoh with the objectives of determining the temporal pattern of Cu concentration, comparing the enzyme inhibition with Cu variation, assessing the contribution of Ag and Hg to the inhibition observed, and discussing the significance of the findings in relation to Malaysian DOE water quality standards.

MATERIALS AND METHODS

Study area and sampling site

Field monitoring was done at Sg. Kuyoh, near Serdang Industrial Area, Selangor, Malaysia in early 2019. The river was chosen for sediment analysis because sediment analysis in this area previously identified increased Cu contamination, suggesting that the site may be receiving metal inputs from nearby industrial and urban activities. The sampling point was selected in a river section where there is a likelihood of receiving drainage or discharge influences from the industrial area.

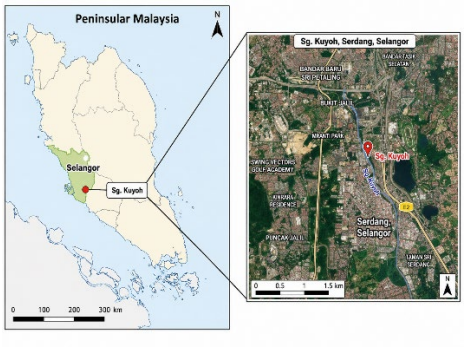


Figure 1. Location map of Sg. Kuyoh, Serdang, Selangor, Peninsular Malaysia. The inset shows the enlarged study area and surrounding urban features based on Google Earth satellite imagery. Source: Adapted from Google Earth, accessed 7 July 2025.

Periodic water sampling

Water samples were collected periodically at two-hour intervals for one day of monitoring. 8:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00 and 22:00 were the sampling times. The enzyme assay included a control sample with deionized water as a comparison. River water was collected in clean acid-washed polypropylene tubes or bottles at each sampling time. Polypropylene containers were used to minimize metal adsorption and contamination during handling of the samples. For instrumental metal analysis, preserved water samples were filtered through a 0.45 um membrane filter, as necessary, to remove suspended particles, and a few drops of conc nitric acid were added. For inhibitive enzyme assay using the papain-casein dye binding (PCDB) assay. The dissolved metals were determined in the filtrate. Prior to use all glassware and plasticware used in the preparation of samples were acid washed and rinsed with deionized water.

Preparation of protease assay reagents

The inhibitive assay of protease was based on casein-Coomassie dye-binding method (Shukor et al., 2006). Papain (SIGMA, E.C. 3.4.22.2, crude dried papaya latex. 0.5 Units/mg) was dissolved (100 mg) in 10 mL of a 50 mM sodium phosphate pH 6.5 solution at 4 °C to prepare a stock solution of 10 mg/mL. Solution aliquoted to 1 mL aliquots and stored at -20°C. The protease substrate was casein and residual casein after enzymatic digestion was determined by Coomassie Brilliant Blue G-250 reagent. Casein stock solution was prepared by dissolving casein in deionized water and adjusting the pH to ~8.0 to aid solubilization. The mixture was stirred overnight at 60 o C. Insoluble material was removed by filtration through several layers of cheesecloth and centrifugation at 10,000 × g for 10 to 15 min. The supernatant was the clear stock solution of casein. The protein concentration was measured using the Bradford method with BSA as a standard. Fresh working solutions of papain (2.0 mg/mL) and casein (0.3 mg/mL) were prepared daily. The Coomassie dye-binding reagent was prepared by dissolving Coomassie Brilliant Blue G-250 in ethanol and phosphoric acid and diluting to the required volume with deionized water. The reagent was filtered and preserved in a dark bottle. Alternatively commercial Bradford reagent may be used.

PCDB near-real-time enzyme assay

Samples of the river water were assayed by a protease-based inhibitory enzyme assay. The principle of the assay is that the bioavailable metal ions inhibit the casein hydrolysis catalyzed by protease. Without inhibitors, protease hydrolyzes casein to give lower residual protein and lower absorbance after addition of Coomassie dye. The presence of inhibitory metal ions, e.g., Cu, Hg, or Ag, reduces casein hydrolysis, producing more residual casein, inadvertently giving a stronger blue with Coomassie dye. This phenomenon indicates a lower protease activity.

About 10 mL of surface river water samples were first filtered through a 0.45 µm syringe filter (Teflon) to obtain a clear filtrate. 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). This ensures a stable environment of 40 ± 1 °C, which is the optimum reaction of the papain assay (Shukor et al., 2006). The reaction mixture was prepared by the addition of 5 µL of papain from the stock solution to 20 µL of 100 mM phosphate buffer pH 6.5 followed by the addition of 50 µL of the river water sample in an Eppendorf tube. Deionized water was used as a control instead of heavy metals. The final volume was adjusted to 100 µL with deionized water, and the mixture was incubated for 20 min at room temperature. After the incubation period, 50 µL of casein from a stock solution of 0.3 mg/mL was added and mixed well. Time zero absorbance was determined by removing an aliquot of 20 µL and combining it with 200 µL of Bradford dye-binding reagent and incubating for 5 min at room temperature.

After an incubation period 10 min at 40 °C, again a 20 µL aliquot of this reaction mixture was taken and mixed with 200 µL of Bradford dye-binding reagent and this mixture was further incubated for 5 min. The absorbance measurements were conducted with a portable mini-spectrophotometer (Model M6+, Axiom, Germany) with a fixed filter at 600 nm. Post-assay, the samples were stored in a Coleman® ice cooler for preservation until they could be analyzed further in the laboratory. A control reaction was set up using deionized water instead of river water, and the control was considered to be 100% enzyme activity. Percentage enzyme activity in each water sample was determined relative to the control, and the percentage inhibition was

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calculated using the formula 100 – percentage enzyme activity. All assays were performed in triplicate and the results were expressed as mean +/- standard deviation.

Determination of mercury by Flow Injection Mercury System

Mercury concentration in the preserved water samples was determined using a Flow Injection Mercury System. The method was selected because the FIMS is suitable for the determination of trace Hg in environmental water samples. Water samples were acid preserved and prepared for analysis as described in the FIMS operating procedure. The instrument method involved reducing mercury in the sample chemically to elemental mercury vapor. The mercury vapor was transported into the absorption cell by the carrier gas, and the Hg was measured by cold vapor atomic absorption. Calibration standards were prepared from certified Hg stock solution, and acidified deionized water was used as the diluent. Reagent blank and calibration standards were run prior to the samples. Quality control samples were incorporated to assess instrument stability and recovery. Mercury concentrations were in mg/L.

Determination of copper and silver by ICP-OES

Copper and silver concentrations were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Water samples were acid-preserved, filtered and, if necessary, diluted appropriately prior to introduction into the ICP-OES system. Calibration standards for Cu and Ag were prepared in acidified deionized water from certified single-element or multi-element standards. The ICP-OES was calibrated with a blank and a series of working standards within the expected concentration range of the samples. The sample was measured after the instrument had been stabilized. Analytical wavelengths for Cu and Ag were selected based on ICP-OES method and checked for spectral interference. A reagent blank, calibration verification standard, and replicate samples were used to perform quality control. Copper and silver concentrations are in mg/L. To evaluate whether temporal variation in Cu was linked to enzyme inhibition, we directly compared Cu data to protease enzyme activity. We used Ag data to test for the possibility that Ag is involved in the observed inhibition pattern. Where possible, field blanks and reagent blanks were included. The enzyme assay was performed in triplicates. Instrumental calibration was verified using standards and blanks before analysis of samples.

Data analysis

The plot of Cu concentration and enzyme activity against sampling time indicate temporal Cu level. Both Ag and Hg concentrations were tabulated together to determine whether these metals could contribute to enzyme inhibition. Data was presented as mean ±standard deviation of triplicates.

RESULTS

Temporal variation of Cu and enzyme activity

The copper concentration in Sg. Kuyoh was found to vary considerably over the sampling period. From the near-real-time profile, Cu was found to be at about 0.35 mg/L at 8:00 AM, and rose to a level of about 0.47 mg/L at 10:00 AM. Then it reduced to about 0.38 mg/L at 12.00 PM, 0.27 mg/L at 2.00 PM and 0.25 mg/L at 4.00 PM. Moderate increase at 6:00 PM (Cu ~0.31 mg/L). The highest Cu concentrations were observed at night, with sudden increases to about 0.69 mg/L at 8:00 PM and 0.87 mg/L at 10:00 PM. The protease assay showed a distinct inhibition response in the same period. Enzyme activity was approximately 59% at 8:00 AM, 50% at 10:00 AM, 44% at 12:00 PM, 56% at 2:00 PM, 47% at 4:00 PM and 35% at 6:00 PM. The most pronounced inhibition was observed at 8:00 PM and 10:00 PM, when the enzyme activity decreased to about 20 and 16%, respectively. The control maintained 100% enzyme activity. It can be seen that high level of Cu was associated with strong inhibition of enzymes.

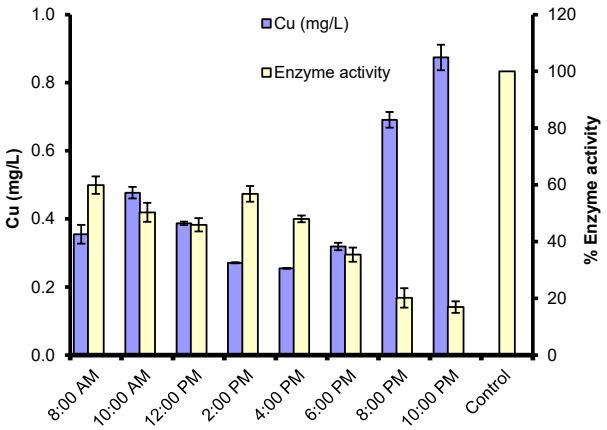


Figure 1. Near-real-time temporal pattern of Cu concentration and protease enzyme activity in Sg. Kuyoh from 8:00 AM until 10:00 PM. Bioavailable Cu inhibited the protease assay as indicated by the marked increase in copper at 8:00 PM and 10:00 PM and the sharp decrease in percentage enzyme activity.

Silver and mercury concentrations

Ag and Hg were detected at much lower concentrations than Cu throughout the sampling period. The concentrations of Ag ranged from 0.001205 to 0.007993 mg/L and Hg from 0.0000 to 0.0007 mg/L. These concentrations were less than Cu and did not follow the pattern of sharp inhibition at 8:00 PM and 10:00 PM.

Table 1. Concentrations of silver and mercury measured during near-real-time monitoring of Sg.

Time	Ag (mg/L)		SD	Hg (mg/L)		SD
8:00 AM	0.001205	±	0.0006	0.0005	±	0.0000
10:00 AM	0.007510	±	0.0008	0.0007	±	0.0000
12:00 PM	0.007409	±	0.0005	0.0000	±	0.0000
2:00 PM	0.001896	±	0.0002	0.0007	±	0.0001
4:00 PM	0.007368	±	0.0005	0.0002	±	0.0001
6:00 PM	0.007993	±	0.0000	0.0004	±	0.0001
8:00 PM	0.003860	±	0.0009	0.0001	±	0.0001
10:00 PM	0.006114	±	0.0002	0.0000	±	0.0001
Control	0.000000	±	0.0000	0.0000	±	0.0000

DISCUSSION

The Kuyoh River catchment lies within the state of Selangor and Federal Territories of Kuala Lumpur. Kuyoh River is a tributary of Klang River, 12.5 km length of major river and covers an area of 83 sq. km (**Figure 1**).The tributaries of Kuyoh River are: Anak Sungai Kuyoh, Sungai Jinma, Sungai Pati Liat, Sungai Midah, Sungai Rekah, and Sungai Bohol, which complete the whole catchment area and connect to the lower Klang River basin. The 8.4 km of the main river, which is from upstream at Universiti Putra Malaysia, Serdang down to the National Sport Complex, Bukit Jalil is classified as class IV (Hashim & Teo, 2014), and includes three sub tributaries namely Anak Sungai Kuyoh, Sungai Jimna and Sungai Pati Liat is the main areas of several studies on water quality and environmental monitoring including biomonitoring (Gunasekaran et al., 2014; Hashim & Teo, 2014; Yazdani et al., 2009). Near real-time monitoring of Sg. Kuyoh

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revealed Cu as the dominant metal associated with protease inhibition. The National Water Quality Standards for Malaysia have been published by the Department of Environment Malaysia, which lists water classes and uses. Class I is conservation and sensitive aquatic species, Class IIA is conventional water treatment and sensitive aquatic species, Class IIB is recreational use with body contact, Class III is extensive treatment and tolerant fisheries, Class IV is irrigation and Class V is none of the above (DOE, 2026). For the conservative river-water assessment in this work, the values of Cu 0.020 mg/L, Hg 0.001 mg/L, and Ag 0.050 mg/L were applied. All measured Cu concentrations in Sg. Kuyoh in the present study were above the conservative Cu value of 0.020 mg/L, ranging from about 0.25 to 0.87 mg/L. The highest Cu values (8:00PM, 0.69 mg/L and 10:00PM, 0.87 mg/L) were about 35- and 44-fold higher than this value respectively. This confirms that the Cu pollution in the sampled water was not marginal but severe during the monitoring period.

The enzyme response is in accord with the same interpretation. Protease activity was lower than the control at all sampling times, with the strongest inhibition at the highest Cu concentration. The enzyme activity decreased to about 20% and 16% at 8:00 PM and 10:00 PM, respectively, while Cu sharply increased. The inverse correlation supports the use of the protease assay as a rapid biological signal for bioavailable Cu. Copper and other heavy metals can interfere with proteins and enzymes by binding to functional groups such as thiol, imidazole, and carboxyl groups, and this is one reason enzyme assays can respond rapidly to bioavailable metal toxicity. Ag and Hg on the other hand were found in much lower concentrations. Ag ranged between 0.001205 and 0.007993 mg/L, which is below the conservative value of Ag of 0.050 mg/L. Hg ranged from 0.0000 to 0.0007 mg/L, which is below the conservative value of Hg of 0.001 mg/L. Thus, despite the fact that Ag and Hg are known inhibitors of many enzymes, it was unlikely that their concentrations in this sampling event were responsible for the large drop in protease activity.

The most consistent inhibition profile was with Cu being the major toxicant. The most important environmental finding is the temporal pattern. Sampling the site only in the morning or afternoon would have missed the highest Cu event. This is consistent with the rationale of earlier Malaysian biomonitoring studies, where enzyme assays were suggested as instrumental monitoring is expensive, slow and not suitable for near-real-time detection. In the study of the Malacca River UNESCO site, enzyme assays were reported to be useful for indication of temporal variation of heavy metal levels and for support of forensic analysis of pollution sources (Gunasekaran et al., 2014). The same idea is very strong for Sg. Kuyoh. The night-time Cu pulse could be episodic discharge, resuspension of contaminated sediment or changes in flow and drainage input from nearby industrial areas. To account for matrix effects on the PCDB assay in the future, water quality parameters of the river after filtration, which is the requirement of the PCDB assay, will be carried out.

The Sg. Kuyoh Sg. result is also consistent with the historical sediment record. Yazdani et al. (2009) reported 348 ug/g Cu in sediment from Sg. Kuyoh - Serdang Industrial Area section. This sediment load indicates that the river is not only receiving Cu in the water column but may also have a long-term reservoir of Cu in the sediment. Under appropriate hydrological or chemical conditions, sediment-associated Cu could be remobilized into the water column. The present near-real-time result therefore provides biological and instrumental support that Cu pollution in Sg. Kuyoh is still environmentally relevant.

In addition, the Juru Industrial Estate has a history of repeated, near-real-time monitoring, which is important. In several real water samples, this study found significant inhibition and recommended frequent monitoring to distinguish between a one-time event and a continuing industrial source (Halmi, et al., 2015). This statement is directly applicable to Sg. Kuyoh as the strongest Cu signal was found to occur only at night. A single grab sample would not suffice to describe the real pollution pattern. The results also support the practical role of biomonitoring as a first screening system, but not a substitute of instrumental analysis. Previous Malaysian studies suggested a combined approach where biological assays are used as pre-screening methods and positive samples are confirmed by Atomic Emission Spectrometry, ICP-OES or Flow Injection Mercury System (Halmi et al., 2015). This strategy is suitable for Sg. Kuyoh, since the protease assay can quickly identify suspicious sampling times and the instrumental analysis can confirm which metal is responsible. Such a system may reduce the monitoring cost while increasing the temporal coverage. To date, several enzyme-based inhibitory assays have been conducted in various locations in Malaysia (Table 2), and show promising results as a rival candidate for other near-real-time or semi-near-real-time assays like the Microtox™ assay.

Monitoring of toxicants in water bodies is important in time and space as the levels of pollutants vary with discharge events, rainfall, disturbance of sediments, tidal mixing and land use activities. Heavy metals and organic pollutants are not evenly distributed; they may be accumulated in the sediments, increased near points of effluents, and decreased downstream by dilution or settling. Spatial and temporal variations of non-residual heavy metals in stream sediments of the Suquia River system (Gaiero et al., 1997) indicate that the pollution status may be misrepresented by single-time sampling. Similarly, Khan et al. (2017) reported spatio-temporal variation of physicochemical parameters and heavy metals in Ramganga River and emphasized the requirement of repeated multi-site monitoring. Monitoring of toxicants is also important for human health risk assessment. The PAHs in water, sediments and blood cockles along the Klang Strait could potentially cause exposure risks from seafood consumption and environmental contact as shown by Sany et al. (Sany et al., 2014). Therefore, temporal and spatial monitoring provides more robust evidence for pollution control, source apportionment and public health protection.

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Table 2. Selected biomonitoring studies using inhibitive assays for heavy metal detection, with emphasis on Malaysian waters and industrially influenced sites.

Study location	Assay system	Main target metals	Main biomonitoring finding	Instrumental validation	Reference
Juru Industrial Estate, Penang	Molybdenum-reducing enzyme from Serratia sp. strain DRY5	Hg, Cu, Ag, Cd	Several water samples caused strong enzyme inhibition; the assay was proposed as a first screening method for large-scale monitoring.	AES and FIMS	(Halmi et al., 2015)
Malacca River, UNESCO heritage site	Molybdenum-reducing enzyme from Serratia sp. strain DRY6	Hg, Cu, Ag, Cr	One sampling location inhibited enzyme activity, but instrumental metal concentrations were below the Malaysian permissible limit.	AES and FIMS	(Uba, et al., 2023)
Juru/Prai industrial discharge area	Garlic protease, casein-Coomassie assay	Cu	Near-real-time monitoring detected temporal Cu variation, with high Cu during specific sampling periods.	Instrumental metal analysis	(Halmi et al., 2016)
Port Klang, Straits of Malacca	Ficin protease optimized by RSM	Hg, Ag, Cu	RSM improved assay sensitivity; 24 h near-real-time monitoring showed no Hg pollution at the sampled site.	Instrumental analysis	(Uba et al., 2020)
Waters near Langkawi Kilim Karst Geoforest Park	Ficin-based enzyme assay	Hg, Ag, Cu	Low inhibition below 10% during field monitoring, suggesting low heavy metal pollution.	Instrumental analysis	(Uba et al., 2023)
Kuah Jetty, Langkawi	Achromopeptidase-based assay	Zn	Low inhibition below 10% during field monitoring, suggesting low Zn pollution.	Instrumental analysis	(Uba et al., 2023)
Industrial effluent sites in Malaysia	AChE from Periophthalmodon schlosseri	Cu, Hg, Cr, As	Field trial showed agreement between AChE inhibition and instrumental detection of polluted industrial effluents.	ICP-OES and FIMS	(Sabullah et al., 2015)
Sg. Kuyoh, Serdang Industrial Area	Protease-based inhibitive assay	Cu, with Ag and Hg comparison	Cu exceeded the conservative DOE value at all sampling times; strongest inhibition coincided with 8:00 PM and 10:00 PM Cu peaks.	Hg by FIMS; Ag and Cu by ICP-OES	Present study

CONCLUSION

The protease-based inhibitive enzyme assay successfully detected temporal variation in Cu pollution in Sg. Kuyoh. Copper concentration increased sharply at 8:00 PM and 10:00 PM, while enzyme activity decreased to about 20% and 16%, respectively. Ag and Hg were present only at low concentrations and did not explain the strong inhibition trend. All Cu values exceeded the conservative Malaysian DOE value of 0.020 mg/L, indicating environmentally significant Cu contamination. The results indicate that the assay is suitable as a rapid, low-cost, near-real-time screening method for detecting Cu pollution events in Sg. Kuyoh, especially in areas influenced by industrial activity. Instrumental confirmation remains necessary for regulatory reporting, but the assay can greatly improve the detection of episodic pollution events that may be missed by conventional low-frequency sampling.

CONFLICT OF INTERESTS

The authors have no competing financial interests or personal relationships to disclose.

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ETHICS STATEMENT

Not Applicable

AUTHORS' CONTRIBUTIONS

GU carried out the work and wrote the paper; MM did the analysis and wrote the paper; MIEH did the analysis and edited the galleyproof; and MKS managed the project and contributed to the analysis.

DATA AND MATERIALS AVAILABILITY

Data available upon request

AI STATEMENT

The authors used generative AI technologies for the purpose of language editing and refinement. All authors reviewed and edited the content, and the authors are responsible for the contents of the publication.

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Abbreviations:

AChE	Acetylcholinesterase
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
PCDB	Papain-Casein Dye Binding Assay

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