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A Calcium- and Magnesium-Tolerant *Enterobacter* sp. MTT Respiratory Inhibition Assay for Rapid Screening of Mercury and Silver in Industrial River Water

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Abstract

Rapid biological screening can complement instrumental analysis by identifying water samples that warrant priority investigation. This study developed a whole-cell respiratory inhibition assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by a locally isolated bacterium, *Enterobacter* sp. strain DRY13, retained respiratory activity in the presence of Ca^{2+} and Mg^{2+} yet responded strongly to toxic metals. supported tentative placement within *Enterobacter*. Among 13 metals tested, Ag^+ and Hg^{2+} produced greater than 50% inhibition at 10 mg L^{-1} . One-phase exponential models yielded IC_{50} values of 0.139 mg L^{-1} for Ag^+ (95% CI, $0.115\text{--}0.173 \text{ mg L}^{-1}$) and 0.144 mg L^{-1} for Hg^{2+} (95% CI, $0.106\text{--}0.221 \text{ mg L}^{-1}$), with R2 values of 0.9842 and 0.999, respectively. The reported limits of detection were 0.0033 mg L^{-1} for Ag^+ and 0.0216 mg L^{-1} for Hg^{2+} ; corresponding limits of quantification were 0.0294 and 0.0386 mg L^{-1} . Most pesticides and solvent-related xenobiotics caused little inhibition at the tested concentrations, whereas sodium dodecyl sulfate was a notable interferent. River and industrial-estate samples inhibited MTT reduction, while tap water did not. Because ICP-AES measures total recoverable metal whereas whole-cell inhibition reflects the combined bioavailable toxic fraction, the assay is best interpreted as a rapid effect-based screen rather than a metal-specific quantitative method. The platform provides a simple basis for prioritizing contaminated samples for confirmatory instrumental analysis.

INTRODUCTION

Metal contamination remains a persistent water-quality concern because metallic elements do not degrade and may accumulate in sediments, suspended matter, and aquatic organisms. Their environmental significance cannot be inferred from total concentration alone: speciation, complexation, pH, redox conditions, dissolved organic matter, and competing ions determine mobility and biological availability [1–6]. Mercury is particularly hazardous because transformation and trophic transfer can amplify exposure, whereas ionic silver is strongly toxic to microorganisms and many freshwater taxa at comparatively low concentrations [7–11]. Industrial zones containing metal finishing, electrical, electronic, and foundry operations therefore require monitoring strategies that can recognize episodic releases and complex-mixture toxicity. Conventional elemental analysis provides sensitive and metal-specific measurements but requires costly instrumentation,

trained personnel, controlled laboratory conditions, and careful sample preparation. It also characterizes chemical concentration rather than the integrated biological effect of the dissolved and bioavailable fraction. Effect-based methods address a different question: whether a sample perturbs a biological process under defined exposure conditions. Whole-cell assays are particularly attractive for preliminary screening because microorganisms can be produced inexpensively, miniaturized in microplates, and challenged directly with environmental samples [12–18]. Recombinant bioreporters can offer analyte-selective signals, but constitutive metabolic assays remain useful when the intended endpoint is general toxicity rather than chemical identification [19–23].

Tetrazolium salts provide a convenient metabolic readout. Viable cells reduce MTT to a colored formazan product through cellular redox reactions involving reducing equivalents and membrane-associated or intracellular oxidoreductases [24–29].

Toxicants that disrupt membrane integrity, electron transport, enzyme function, or energy metabolism consequently decrease formazan production. This endpoint is rapid and readily quantified by absorbance, but its interpretation requires care: MTT reduction is not a direct measurement of cell number, different taxa reduce the dye through different pathways, and colored or reducing sample constituents may interfere [26–30]. Controls for abiotic dye conversion, background absorbance, and matrix effects are therefore essential.

Earlier bacterial MTT assays demonstrated practical sensitivity to toxic chemicals but could also respond to environmentally common Ca^{2+} and Mg^{2+} ions. Chelators such as EDTA may relieve this interference, yet they can simultaneously reduce the freely available fraction of the target metals and weaken the toxic response [31,32]. An organism that tolerates these major ions without requiring a chelator would make the assay more suitable for natural waters. The present study therefore screened environmental bacteria for robust MTT reduction in the presence of Ca^{2+} and Mg^{2+} , followed by evaluation against a panel of metals, pesticides, and other xenobiotics. Analytical performance was estimated for Ag^+ and Hg^{2+} , and the assay was applied to water samples from the Juru–Prai industrial area. Comparisons with established toxicity tests were used to place assay sensitivity in context, while recognizing that cross-assay IC_{50} , EC_{50} , and LC_{50} values describe different endpoints and exposure designs [33–36].

MATERIALS AND METHODS

Reagents and test substances

Analytical-reagent-grade chemicals were used unless otherwise stated. MTT was obtained from Sigma. Plasticware and glassware were soaked in 10% HNO_3 and rinsed thoroughly with deionized water to minimize trace-metal contamination. A 10 mM MTT stock was prepared by dissolving 0.2072 g in 50 mL of 10 mM PBS (pH 7.5) and stored at 4 °C. Metal solutions were prepared from suitable analytical salts or 1 g L^{-1} atomic-absorption standards (Merck, Darmstadt, Germany). The interference panel comprised acetonitrile, ethylene glycol, ethyl acetate, ethanol, isopropanol, methanol, triethanolamine, polyethylene glycol (PEG) 400, 600, and 1000, diethylamine, acrylamide, Nonidet P-40, Triton X-100, and SDS. These substances were prepared at 2% (v/v) in deionized water and tested at 0.4% (v/v), equivalent to 4,000 mg L^{-1} for solutions treated on a mass-per-volume basis. Because this concentration greatly exceeds typical environmental levels, the panel was interpreted as a stringent interference challenge rather than an environmental exposure simulation.

Bacterial culture, and primary screening

Enterobacter sp. strain DRY13 [37] was grown in 15 mL nutrient broth in 50 mL flasks for 18 h at ambient laboratory temperature with shaking at 150 rpm. One-millilitre portions were centrifuged at $10,000 \times g$ for 10 min at room temperature. Pellets were washed once and resuspended in 10 mM PBS (pH 7.5). Preliminary screening from our laboratory culture collection shows that *Enterobacter* sp. strain DRY13 MTT-reducing activity was not appreciably inhibited by Ca^{2+} (25 mg L^{-1}) or Mg^{2+} (50 mg L^{-1}). The bacterium was then challenged with a metal panel at 10 mg L^{-1} , including Ag^+ , Hg^{2+} , Cd^{2+} , As^v , Pb^{2+} , and Cu^{2+} ; the complete screen comprised 13 metals. Each 250 μL microplate reaction contained 50 μL PBS, 75 μL of a 1% (v/v) bacterial suspension, 25 μL each of the Ca^{2+} and Mg^{2+} solutions, 50 μL metal solution or deionized-water control, and 25 μL of 10 mM MTT.

Reactions were pre-incubated for 1 h at room temperature before MTT was added. Plates were protected from light, and absorbance was measured at 550 nm with a Stat Fax 3200 microplate reader (Awareness Technology, USA). Preliminary kinetic observations indicated that color formation reached a plateau after 20 min; this incubation was used in subsequent assays. All treatments were performed in triplicate.

Calculation of inhibition and concentration–response modelling

Activity was expressed relative to the untreated control. Percent inhibition was calculated as $100 \times (\text{A}_{\text{control}} - \text{A}_{\text{sample}}) / \text{A}_{\text{control}}$, where $\text{A}_{\text{control}}$ and A_{sample} are the corresponding 550-nm absorbances after incubation. Concentration–response data for Ag^+ and Hg^{2+} were fitted by nonlinear regression in GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA). A one-phase exponential-decay model was selected from the models evaluated on the basis of goodness of fit. The IC_{50} and 95% confidence interval were obtained from the fitted curve. Precision was summarized as the coefficient of variation across replicate measurements. LOD and LOQ were calculated from blank variability and the calibration response.

Field sampling and instrumental analysis

Water samples were collected from the Juru and Derhaka rivers and the Prai Industrial Estate in Penang, Malaysia. The receiving waters collect mixed discharges from electrical, electronic, metal-processing, and foundry activities, making attribution to individual sources impracticable. Samples were collected in acid-washed HDPE bottles containing several drops of 1% (v/v) HNO_3 and transported for analysis. Before the bioassay, samples were passed through 0.45 μm syringe filters and neutralized with NaOH. Metals were measured with a PerkinElmer Optima 3000 ICP-AES. Bioassay and instrumental measurements were conducted in triplicate. Acid preservation and ICP-AES quantify an operationally defined dissolved or total-recoverable fraction, whereas neutralization and subsequent assay exposure determine the fraction capable of eliciting an acute cellular response; the two results were therefore compared qualitatively rather than treated as equivalent measurements [4–6].

RESULTS

Metal screening and concentration–response behaviour

Ag^+ and Hg^{2+} were the only metals in the 13-metal screen that suppressed MTT reduction by more than 50% at 10 mg L^{-1} (Fig. 2). Mercury produced almost complete loss of activity, while Ag^+ reduced absorbance to near background. Cd^{2+} caused a smaller decrease; the remaining tested elements retained substantial activity under the screening conditions. These results identify Ag^+ and Hg^{2+} as the principal assay-responsive metals in this panel, but they do not establish chemical specificity because the endpoint reports respiratory inhibition and because each metal was initially evaluated at a single concentration.

The Ag^+ and Hg^{2+} concentration–response profiles were described by one-phase exponential-decay models (Fig. 3). The estimated Ag^+ IC_{50} was 0.139 mg L^{-1} (95% CI, 0.115–0.173 mg L^{-1} ; $R^2 = 0.9842$), and the Hg^{2+} IC_{50} was 0.144 mg L^{-1} (95% CI, 0.106–0.221 mg L^{-1} ; $R^2 = 0.999$). Replicate CVs across the metal experiments ranged from 2% to 15%. The reported LODs were 0.0033 mg L^{-1} for Ag^+ and 0.0216 mg L^{-1} for Hg^{2+} ; the reported LOQs were 0.0294 and 0.0386 mg L^{-1} , respectively. The source data show a steep early response to Hg^{2+} followed by a residual activity plateau.

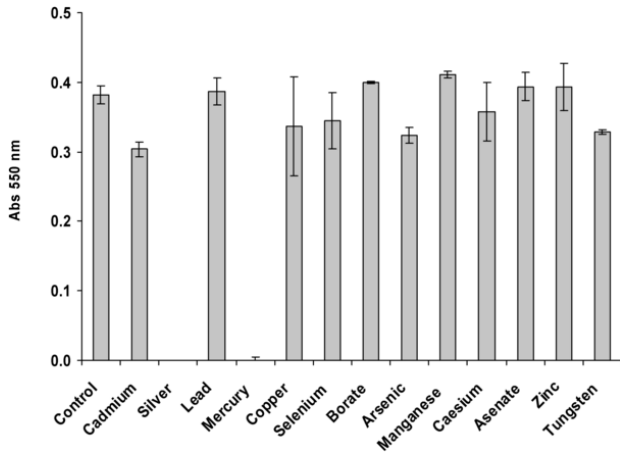


Fig. 2. Effect of the 13-metal screening panel on MTT reduction by *Enterobacter* sp. strain DRY13. Bars show mean absorbance at 550 nm $\pm$ standard error ($n = 3$). Ag^+ and Hg^{2+} produced the strongest inhibition at the screening concentration.

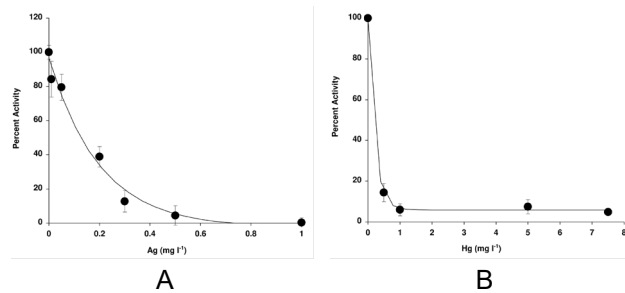


Fig. 3. Concentration-dependent inhibition of MTT reduction by (A) Ag^+ and (B) Hg^{2+} . Points show mean relative activity $\pm$ standard error ($n = 3$); lines are the original fitted curves. Model R^2 values were 0.9842 for Ag^+ and 0.999 for Hg^{2+} .

Interference by xenobiotics and pesticides

Among 13 non-pesticide xenobiotics, SDS was the dominant interferent and nearly abolished MTT activity at 0.4% (**Fig. 4**). Its reported IC_{50} was 20 mg L^{-1} . Acrylamide and Triton X-100 also reduced activity, although less markedly, while several PEG grades produced values close to or slightly above the untreated control. The pesticide panel did not differ significantly from the control overall (one-way ANOVA, $F_{7,16} = 0.0894$, $P > 0.05$) at 4 mg L^{-1} . These results support resistance to the tested pesticide challenge, but the strong SDS response demonstrates that the assay is not intrinsically metal-specific.

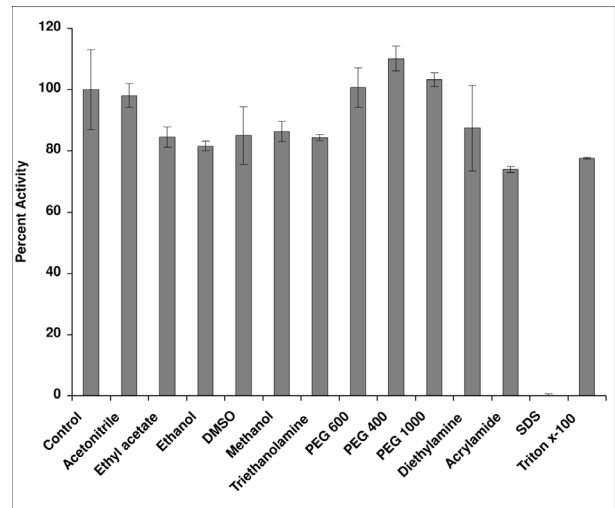


Fig. 4. Effect of selected solvents, polymers, surfactants, and related xenobiotics on MTT reduction by strain DRY13. Activity is expressed relative to the untreated control (mean $\pm$ standard error, $n = 3$). SDS was the major interferent at the tested challenge concentration.

Field-sample response and ICP-AES measurements

Samples from the Prai industrial estate and the Juru and Derhaka rivers inhibited bacterial MTT reduction, whereas tap water did not (**Fig. 5**). Relative activity was approximately 75% for sample 1, 78% for sample 2, 100% for tap water, and 37% for sample 4. ICP-AES detected Hg^{2+} in the three environmental samples at 16.670–21.593 mg L^{-1} , with lower concentrations of Cu^{2+} , Zn^{2+} , Cd^{2+} , Ag^+ , and Pb^{2+} (**Table 2**). The strongest inhibition occurred in sample 4, which also contained the highest measured Hg^{2+} and Pb concentrations and detectable Cd^{2+} and Ag^+ . Samples 1 and 2 nevertheless retained more activity than would be anticipated from the single-metal Hg^{2+} curve. This divergence is scientifically plausible because the instrumental result and biological endpoint describe different fractions, and because sample neutralization may promote precipitation or complexation. Matrix constituents may additionally antagonize or potentiate toxicity [2–6,40–42].

Table 2. Metal concentrations in field samples measured by ICP-AES (mean $\pm$ standard error, $n = 3$; mg L^{-1}).

Metal	Sample 1 Prai Industrial Estate	Sample 2 Sungai Juru	Sample 3 Tap water	Sample 4 Sungai Derhaka
Hg^{2+}	18.460 ± 0.775	16.670 ± 0.224	n.d.	21.593 ± 0.112
Cu^{2+}	0.133 ± 0.001	0.003 ± 0.003	n.d.	0.005 ± 0.001
Zn^{2+}	0.390 ± 0.015	0.027 ± 0.002	n.d.	0.029 ± 0.000
Cd^{2+}	n.d.	n.d.	n.d.	0.005 ± 0.003
Ag^+	n.d.	n.d.	n.d.	0.012 ± 0.001
Pb^{2+}	0.152 ± 0.035	0.152 ± 0.035	n.d.	0.243 ± 0.001

Note: n.d., not detected.

Table 1. Comparative sensitivity of selected toxicity assays to Hg^{2+} and Ag^+ .

Metal	Immobilized urease	Papain	Bromelain	Microtox 15 min	Daphnia magna 96 h	Rainbow trout 96 h	<i>R. meliloti</i> MTT	This study 95% CI
Hg^{2+}	0.33 ± 0.021	0.24–0.62	0.13–0.16	0.003 ± 0.002	0.0052–0.21	0.033–0.21	0.0159 ± 0.004	0.106–0.221
Ag^+	Not determined	0.33–0.49	No inhibition	0.008 ± 0.001	1.930	0.05	Not determined	0.115–0.173

Note: Units are mg L^{-1} . Source comparisons: urease [33], papain [34], bromelain [35], aquatic organisms [36], and the earlier bacterial MTT assay [31,32].

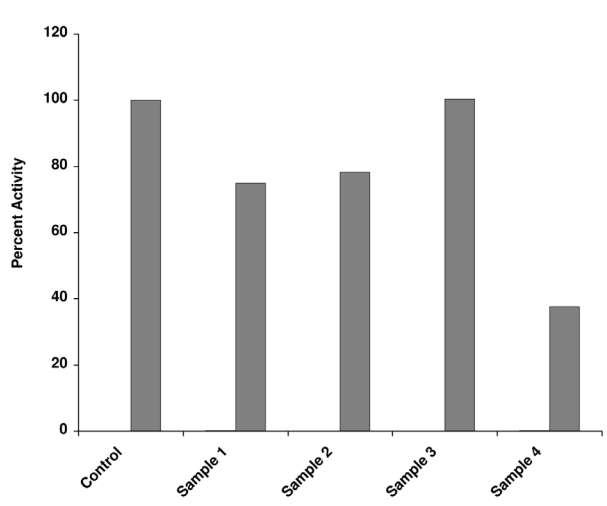


Fig. 5. Relative MTT-reducing activity after exposure to field samples. Sample 3 was tap water. Bars show mean activity relative to the control (n = 3).

DISCUSSION

The principal outcome is the selection of a bacterium that combines strong MTT reduction with tolerance to the Ca^{2+} and Mg^{2+} concentrations used in the screen. That property addresses a practical weakness of earlier bacterial MTT methods, in which common divalent ions suppressed the endpoint unless a chelator was added [31,32]. Avoiding EDTA is advantageous because complexation changes free-metal activity and can mask toxicity. The resulting assay retains the operational simplicity that makes whole-cell platforms useful: culture preparation is straightforward, the endpoint is measured in a standard microplate, and the response develops within 20 min after MTT addition.

Ag^+ and Hg^{2+} produced the dominant responses. Both metals disrupt bacterial physiology through multiple mechanisms rather than a single molecular target. Hg^{2+} binds thiol-containing proteins, perturbs redox homeostasis, and impairs membranes and enzymes; Ag^+ ions interact with cellular envelopes, respiratory proteins, and nucleic acids [7–11]. A respiratory dye is therefore well positioned to capture their aggregate effect. The similarity of the fitted IC_{50} values does not imply identical mechanisms, and comparison with Microtox, Daphnia, trout, or enzyme assays must remain descriptive. Those systems differ in exposure duration, medium composition, test species, chemical form, and endpoint [33–36]. Non-overlap of two 95% confidence intervals can indicate a difference, but overlap alone is not a formal test of equivalence [39].

The interference experiment refines the intended use. Minimal response to the pesticide panel at the tested concentration suggests that these compounds were not major confounders under the assay conditions. SDS, by contrast, nearly abolished activity, consistent with membrane disruption by an anionic surfactant. The assay should therefore be described as preferentially responsive to Ag^+ and Hg^{2+} within the tested metal panel, not as chemically selective for those metals. In an unknown sample, a positive signal may arise from surfactants, other untested toxicants, extreme salinity, pH excursions, or mixtures. Appropriate sample blanks, pH and conductivity measurements, dilution series, and confirmatory chemical analysis are necessary.

The field trial shows why effect-based and instrumental measurements are complementary. ICP-AES revealed exceptionally high total concentrations of Hg^{2+} in the three environmental samples, yet the biological response was not proportional to the single-metal calibration. Acid preservation retains metals for instrumental determination; subsequent neutralization can alter speciation and solubility before biological exposure. Organic ligands and suspended or colloidal phases can further reduce the freely available fraction. Conversely, mixture interactions may enhance toxicity. Thus, a bacterial assay cannot be used to back-calculate an Hg^{2+} concentration from a river-water inhibition value without matrix-matched calibration and speciation data [2–6, 40–42]. Its uses is as prescreening where samples exceeding a predefined inhibition threshold are prioritized for confirmatory ICP-based measurement and source investigation. Several limitations should guide future validation. Further studies are being carried out to get independent-day precision, recovery, robustness to pH and salinity. In addition, future studies will use certified reference materials, spiked matrix controls, or formal correlation analysis.

CONCLUSION

Enterobacter sp. strain DRY13 supported a rapid MTT respiratory-inhibition assay that remained functional in the presence of Ca^{2+} and Mg^{2+} and responded strongly to Ag^+ and Hg^{2+} . The assay produced sub-milligram-per-litre IC_{50} estimates and differentiated contaminated field samples from tap water. Its main value is as a low-cost effect-based screen for prioritizing samples, not as a substitute for metal-specific analysis. The pronounced response to SDS and the divergence between total ICP-AES concentrations and biological inhibition underscore the need for matrix controls and confirmatory instrumental measurement. With expanded validation across water chemistries, culture batches, and independent sampling campaigns, the bacterium can be used as a preliminary surveillance of industrially affected waters.

DECLARATION OF COMPETING INTEREST

The authors declare no competing financial interests or personal relationships.

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ETHICS APPROVAL

Not applicable

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DATA AVAILABILITY STATEMENT

The raw dataset supporting the findings of this study are openly available in Figshare at
<https://doi.org/10.6084/m9.figshare.33793243>

AUTHORS' CONTRIBUTION

The conceptualization of the study was led by M.Y.S. Work and data analysis was conducted by F.A. with the remaining authors contributing to the review and editing.

AI USAGE DECLARATION

During the preparation of this work, the authors used generative AI technologies solely for language editing and refinement. All of the authors reviewed and edited the content. The authors take full responsibility for the final content of the publication.

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