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Yeast Inhibitive Assay for Anionic Heavy Metals: A review

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ABSTRACT

One of the most common types of pollution that has a negative impact on the biotic community in aquatic habitats is heavy metal poisoning of the water. Both essential and non-essential heavy metals can be toxic to living things if their concentrations are too high for their bioavailability. Although the toxicity of heavy metals, and especially anionic metal ions, is better known than that of cationic metal ions, it is just as toxic, if not more so. The focus of this review is on the usefulness of eukaryotic organisms like yeast, *Saccharomyces cerevisiae*, for toxicity assessment because they can be easily maintained and developed in controlled circumstances, thereby avoiding variability issues that arise when employing more complex organisms. Recent research has shown that the majority of cellular MTT reduction occurs outside of the mitochondrial inner membrane, and that this reduction is dependent on NADH and NADPH but is resistant to respiratory chain inhibitors.

INTRODUCTION

As Malaysia's civilisation progresses, the level of heavy metal pollution in the environment continues to rise due to fast industrialisation, urbanisation, agricultural advancement, and other modern-era activities [1]. Heavy metal poisoning of water is one of the most common types of pollution that has a negative impact on the biotic community in aquatic habitats. Heavy metals, both essential and non-essential, have the potential to be hazardous to living organisms if their concentration exceeds a particular bio-available threshold. Water quality is one of the most critical environmental challenges associated with sustainable development, particularly in terms of ensuring national drinking water safety [2–4]. Adilah et al. [5] concluded that nearby mining activity is the main source of pollution in the Jemberau River and Chini River, both of which are classified as Class III (water supply requires significant treatment) because the heavy metal concentration in the water samples is marginally greater than the National Water Quality Standard permissible range. Nevertheless, another study found that anthropogenic activities such as livestock rearing, and oil palm planting are major contributors to low-level heavy metal contamination in the Linggi River [6]. According to the Environmental Quality Report (2013), the percentage of conformity for chromium was just 62 percent, while arsenic was 54 percent for municipal water supply [7].

Arsenic— chemistry, uses and pollution

Arsenic undergoes a natural cycle at the earth's surface, where it is transformed from arsenic sulfides into arsenic trioxide by the weathering of rocks [8]. Even more concerning is the fact that arsenic can exist in either organic or inorganic molecules in water, and that it can exist in numerous oxidation states [9–11]. Redox mechanisms, precipitation, sorption, and dissolution all play a role in limiting the mobility of inorganic arsenic compounds in a contaminated aquatic and sediment environment [12–14]. The ferric iron phase is well-known to be crucial for the sorption of dissolved arsenate in oxic groundwater [15–18]. Microbial activity, including detoxifying and metabolic pathways, is generally responsible for the reduction of arsenate to arsenite during the transition from aerobic to anoxic pore fluids. The relationship between calcium and bicarbonate with arsenic is hypothesized as a byproduct of biological activity in the aquifers [19–22].

As^{3-} (arsine), As° (arsenic), As^{3+} (arsenite), and As^{5+} (arsenate) are the four most common oxidation states of arsenic [12–14]. Arsenic is typically found in a soil environment in two oxidation states, As^{3+} (arsenite) and As^{5+} (arsenate), and in the air as a combination of the two oxidation states. Arsenate, one of two oxidation states, is the primary species linked to arsenic contaminations in soil; its chemical formula, AsO_4^{3-}, is strikingly

similar to that of phosphate [19–22]. Arsenic's industrial applications include lead-acid batteries for automobiles, semiconductors, and light-emitting diodes [23].

Possible inhibition of oxidative phosphorylation by arsenate. Due to its importance in human and metazoan energy metabolism, this is a cause for worry [19–22]. Many enzymes, particularly those involved in respiration, contain reactive sulfur atoms, and arsenite, the most poisonous and soluble form of arsenic, can interact with these atoms [24,25]. In addition, it is well-known that the toxicity of soluble inorganic arsenic is typically higher than that of the organic form [19–22].

Arsine, in contrast to arsenate and arsenite, is commonly found in the environment at low concentrations but in the form of very hazardous gases such as $(CH_3)_3$ and H_3As [19–22]. However, arsenic concentrations in seawater can reach 2.6 µg/L while those in freshwater are typically around 0.4 µg/L. Arsenic levels in geothermal water in Japan varied from 1.8 to 6.4 mg/L [26], whereas in New Zealand they reached as high as 8.5 mg/L due to the country's strong thermal activity [27]. Drinking water wells in Jessore, Bangladesh, were analyzed and found to contain arsenic at concentrations as high as 225 mg/L, which is the main health concern in Bangladesh [16]. Suspended particulate matter in Malaysia has been shown to contain heavy metals including arsenic and lead, and the primary sources of pollution have been determined to be the use of automobiles and the combustion of biomass.

Heavy metals were found in both surface and groundwater and built up along the shore, especially in proximity to urban areas. The highest level of arsenic was reported near Port Klang with levels far above the maximum permissible limit for sediment [28]. Natural heavy metal deposits, particularly in abandoned tin mine ponds and gold mining regions, were also a cause for concern. Multiple freshwater and marine species tested positive for the heavy metals, indicating that persistent exposure to arsenic and mercury may pose a concern to some populations and their biomonitoring is important [29].

Chromium— chemistry, uses and pollution

Sodium and potassium dichromate, which are employed in the chrome industry for the manufacturing of antiseptics and the manufacture of pigments and colours, are good sources of Cr (VI). In solution, heavy metals can exist as cations with positive charges or anions with negative charges. Some metal ions have several oxidation states, which influences their toxicity. Several anionic metal ions, including chromate, molybdate, and arsenate, are hazardous. In nature, chromium is found in the third oxidation state as a cation (Cr^{3+}) [Cr (III)] and the sixth, Cr (VI), as anions [30].

Chromium (III) is a mineral that exists naturally. Chromium is a metal that can be found in a variety of different states in nature, including as a solid, liquid, or gas, in places like rocks (ores), animals, plants, and soil. Some sources place chromium's abundance in the earth's crust as high as the sixth most abundant transition metal [31–35]. Although chromium compounds are not likely to enter groundwater due to their strong binding to the soil, they are quite persistent in aquatic sediments. Some of its soluble forms are employed in wood treatments. It is used in the production of textiles, electroplating, leather tanning, metal finishing, chromate preparation, metal protective coatings (electroplating), magnetic tapes, paints, cement, paper, rubber, and composition floor covering, among many other applications [36]. Thus, the discharge of industrial effluent into the environment is a possible source of chromium to drinking water contamination. Chromium (VI) compounds are soluble in water, forming $HCrO^{4-}$ and $Cr_2O_7^{2-}$ ions at pH 1–6, and CrO_4^{2-} ions at pH > 6. The effect of chromium compounds on living things is determined by the chromium's oxidation state, solubility, and mode of entry into the body [37]. In contrast to chromium (III), which is both important to human health and less harmful than chromium (VI) compounds, which are recognized carcinogens [38].

Cr (VI) is highly toxic and can be found in many types of industrial fluids; exposure to it can result in severe diarrhoea, vomiting, lung congestion, and liver and kidney damage. Furthermore, breathing at high amounts can lead to nasal irritation, nose ulcers, a runny nose, and breathing issues like asthma, coughing, shortness of breath, or wheezing. The EPA sets the safe level of lead in water at 0.1 parts per billion. Meanwhile, bottled water cannot have more than 1 mg/l (1 ppm) of lead, as stated by the FDA. Heavy industrial locations on the western coast of Malaysia tend to have higher than average chromium levels [28].

Molybdenum— chemistry, uses and pollution

Mo is an essential trace element for all living things, especially nitrogen-fixing plant enzymes. At low concentrations, it is essential, but at higher concentrations, it becomes hazardous, thus it's important to find ways to get rid of it. While the maximum concentration of Mo in drinking water is 0.07 mg/L, the maximum concentration of Mo in water consumed by cattle is 0.5 ppm (general guidelines) [39]. This is because molybdenum is very toxic to spermatogenesis in mammals in general and ruminants in particular, causing scouring and death at concentrations as low as a few parts per million. Mo concentrations above 100 mg/L had a deleterious effect on mouse testes, as evidenced by alterations in the oxidative stress-related enzymes superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GPx) [40]. This is likely molybdenum's mechanism of toxicity. Molybdenum can exist in oxidation levels from -2 to +6, with +4 and +6 being the most stable.

Hexavalent molybdenum oxyanion molybdate (+VI) is the most water-soluble molybdenum salt [41]. The predominating ionic species of Mo (+VI) reported to be present in solution at pH > 2, were $Mo_7O_{24}^{6+}$ (pH 2–7) and MoO_4^{2-} (pH > 4) [42]. Dissolved molybdenum (VI) compounds are found in biological systems as the molybdate ion at physiological pH $[MoO_4]^{2-}$ and sodium molybdate dihydrate is the gold standard for toxicology tests [43]. Although molybdenum is rarely found in significant concentrations in the environment, discharges from industrial operations can create high concentrations of Mo, which could pose a risk for water or soil contamination if released into the environment [44].

Molybdenum is a highly unsafe heavy metal, and its contamination has been documented in places like Terengganu, Malaysia [45], Tokyo Bay, Tyrol in Austria and in the Black Sea, where molybdenum concentration achieves worrying concentrations [46]. Furthermore, sewage sludge contamination is a major source of molybdenum pollution on Earth, which poses serious health risks. [46]. The extensive use of molybdenum in several industrial applications—including as an alloying agent, anti-freeze component of automotive engines, corrosion-resistant steel section, and molybdenum disulphide lubricant—is the primary cause of these pollutions.

Spent oil lubricants, particularly those with a typical molybdenum sulphide-based oil lubricant's molybdenum content of 0.5% to 5%, are a major source of molybdenum pollution that

often goes unnoticed. Oil lubricant molybdenum disulfide is oxidized to molybdenum trioxide (MoO_3), which then dissolves in water to generate the extremely soluble molybdate anions. A summary of the toxicity of anionic heavy metals on various test organisms is shown in **Table 1** while the chemical structure of the anionic heavy metals used in this study is shown in **Fig. 1**.

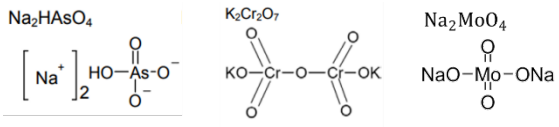

Fig. 1. Chemical structure of sodium arsenate, potassium dichromate and sodium molybdate used in this study.

Toxicity of anionic heavy metals

Table 1. The toxicity effects of anionic heavy metals on various test organisms.

Anionic heavy metals	Concen-tration	Subject	Duration of exposure	Observed disorders/ Response	Ref
Molybdate MoO_4^{2-}	50 mg/kg	Adult male rat	60 days	Induced testicular damage and decreased sperm count and sperm motility.	[47]
	10 mM	Human embryonic kidney (HEK293) and hepatoma liver (HepG2) cells	24 hours	triggered toxicity by interfering with signalling pathways dependent on reactive oxygen species and phosphorylation and, consequently, gene expression.	[48]
Chromate $Cr_2O_7^{2-}$	20 mg/kg	Female albino rats	5 months	Caused abnormal levels of sex hormones; a significant increase in FSH and LH serum levels and a significant decrease in progesterone and oestradiol serum levels.	[49]
	30 mg/kg	Wistar albino male rats	28 days	Induced hepatoxicity related to oxidative stress, inhibition of antioxidant enzymes, lipid peroxidation and structural liver tissue injury.	[50]
	100 µM	Cerebellar granule neurons	48 hours	Dichromate ion act as a neurotoxic agent that causes oxidative stress by enhanced reactive oxygen species (ROS) production.	[51]
Arsenate AsO_4^{3-}	5 mg/kg	Male Swiss albino mice	1 week	Neurotoxicity was induced by elevating oxidative stress markers such as lipid peroxidation, inducible nitric oxide synthase, and nitric oxide while simultaneously reducing antioxidant enzyme and non-enzymatic marker levels.	[52]
	10 mg/kg	Male mice	4 weeks	Reduced avoidance memory retention by causing deleterious effects on learning and memory functions.	[53]

Microorganisms and enzymes as rapid toxicity assay for anionic heavy metals

Heavy metals are hazardous to both human and environmental health. Because of their toxicity and potential for bioaccumulation, these chemicals should be subjected to compulsory surveillance. As a result, there is an urgent need to assess the toxicity of these compounds as well as the danger of exposure to these contaminants [54]. Even though the application of conventional methods for the detection of toxic compounds using instrumental tests such as Atomic Absorption Spectrometry (AAS), High-Performance Liquid Chromatography (HPLC), and Gas Chromatography (GC) provides high sensitivity and accuracy, they come with limitations like time-consuming, expensive, and require special training. Therefore, to cut the cost of instrumental analysis, low-cost biomonitoring systems using enzymes and microorganisms have been intensely researched. The use of biomonitoring systems as preliminary screening tools can be more effective in detecting toxicants as only positive samples are sent for instrumental analysis [55].

At present, the utilization of bioindicators or bioassays that make use of microorganisms and components of cells such as enzymes can provide a rapid, low-cost, and simpler method for the detection of toxic pollutants [56]. Commonly used test methods for determining the toxicity of chemicals and effluents include bioassays, which rely on assessing the reaction of organisms exposed to pollutants, relative to a control. [44]. Due to their strong tolerance for sub-optimal circumstances in terms of temperature and pH, microorganisms offer a more practical approach to toxicity testing. Also, they have higher sensitivity due to their simple morphology and large surface area about their small size when compared to larger and more complex organisms that require a longer time to give results [57].

Various studies regarding heavy metals toxicity tests using bacteria have been done [58,59] and this includes the commercial Microtox™ assay (**Table 2**). Meanwhile, an enzyme such as acetylcholinesterase has been proven sensitive for toxicity testing of heavy metals [60]. It is also possible to rapidly screen environmental samples for hazardous metals with an electrochemical linked assay based on the enzymes urease and glutamate dehydrogenase [61].

Table 2. Comparison of toxicity values obtained for some anionic heavy metals using microorganisms and enzymes as rapid toxicity tests.

IC_{50}, EC_{50} or LC_{50} (mg/L)				
Anionic heavy metals	Acetylcholi-nesterase	*Tetrahymena* sp.	*Daphnia magna*	Microtox ™
solybdate MoO_4^{2-}	26,492 [a]	-	2847.5 [a] 367.8 [c]	-
dichromate $Cr_2O_7^{2-}$	0.632 [a]	-	0.29 [a]	12.4 [d]
arsenate AsO_4^{3-}	-	1420 [b]	-	821 [d]

Note: [a][34], [b][36], [c][18], [d][37].

Rapid toxicity test

Baker's yeast (*Saccharomyces cerevisiae*) as a simple and rapid toxicity assay for anionic heavy metals

Eukaryotes, such as yeast, and *Saccharomyces cerevisiae,* offer great potential for toxicity assessment since they are easy to maintain and develop under controlled circumstances, avoiding variability issues that arise when employing more complex organisms [62,63]. Heavy metals have been researched in particular for their ability to inhibit yeast respiratory metabolism. Chromate, or the reduced form Cr (III), may operate at many places in the mitochondrion to restrict respiration and cause petite mutants by inhibiting mitochondrial protein synthesis in yeast. Unlike prokaryotic bioindicator organisms like *V. fischeri*, yeasts are eukaryotic, making them a better proxy for human biological

responses to pollution [64]. Furthermore, 45 per cent of yeast proteins have at least a portion of their primary amino-acid sequence in common with a human protein. In addition [65] advocated using yeast as an alternate organism to investigate the acute toxicity of pharmaceuticals and environmental contaminants as a first screening approach. Among various yeast strains available, [66] was among the earlier studies that make use of commercially available dry Baker's yeast as the test microorganism in developing a toxicity assay for heavy metals. Due to these demonstrations of the advantages of a yeast-based assay, various studies of heavy metals toxicity utilizing yeast have been done as shown in **Table 3** but these are mostly for cationic heavy metals with the exception of chromate and arsenate where the sensitivity needs further enhancement before it can be used for biomonitoring works.

A fast preview of prospective toxicity levels can be obtained using yeast-based tests for early screening of xenobiotics and environmental samples where large levels of contamination are predicted, as was recently observed [67]. The commercially available GreenScreen bioassay, which consists of genetically modified yeast cells that become progressively luminous when exposed to high levels of genotoxic chemicals, is one example. This bioassay has been proposed as suitable for aquatic environmental toxicity monitoring as it can simultaneously measure general non-specific toxicity besides measuring genotoxicity [68].

Table 3. A summary of the application of yeast (*Saccharomyces cerevisiae*) as rapid toxicity assay.

Metal ions detected	Toxicity value (mg/L)	Yeast strain	System use	Detection	Reference
Potassium dichromate	IC_{50}: 19.35	NCYC 2939	Fluorescent	Resazurin/ Alamar Blue	[69]
Arsenic trioxide	EC_{50}: 187.2	Baker's yeast	Conductometric	-	[64]
Pb^{2+}	EC_{50}: 558.1				
Hg^{2+}	EC_{50}: 110.1				
Cu^{2+}	EC_{50}: 5.6	Baker's yeast	Colorimetric	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)	[66]
Hg^{2+}	EC_{50}: 0.8				
Zn^{2+}	EC_{50}: 19.5				
Ag^{+}	EC_{50}: 6.3				
Cu	EC_{50}: 78.8	Baker's yeast	Colorimetric	INT	[70]
Cr	EC_{50}: 12.3				
Hg	EC_{50}: 101				
Zn	EC_{50}: 162.8				
Cd^{2+}	EC_{50}: 0.000185 *	Baker's yeast	Colorimetric	Triphenyl tetrazolium chloride (TTC)	[71]
Cr^{6+}	EC_{50}: 2.5	Baker's yeast	Turbidity	-	[72]
Cu^{2+}	EC_{50}: 2.1				
Hg^{2+}	EC_{50}: 3				

*expressed in %, compared to control (100%)

Advantages of MTT assay and its application

MTT assay is initially developed based on the ability of the bacterium *Rhizobium meliloti* to reduce a water-soluble tetrazolium dye, MTT (3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) that results in a color change from pale yellow to insoluble purple-blue formazan. As toxic compounds inhibit reduction of the dye, lower color intensity indicates less reduction of MTT-formazan and so higher inhibition from a toxic compound. This assay offers a simple, fast, and inexpensive method as it does not require special equipment or training to run, but its sensitivity compares favorably to Microtox™ and Polytox™ microbial assays [73]. A *Bacillus* sp-based MTT assay was also developed and tested to be sensitive toward toxic response [58]. Tetrazolium salts can be used to detect dehydrogenase activity or other enzyme systems where redox equivalents are produced. Therefore, MTT assay is beneficial for testing cell proliferation and cell viability and is also used for cytotoxicity tests [74]. Cell viability can be thought of as the percentage of total cells that are alive and able to grow, divide, and interact with their environment, or it can be thought of as the number of total cells divided by the number of total cells that have died [75].

Active mitochondria in living cells cleave the tetrazolium ring, resulting in the formation of formazan. Therefore, the number of living cells is directly correlated with the amount of formazan produced [76]. A cell's ability to convert MTT into formazan is lost when it dies. MTT is a positively charged compound that can easily enter living eukaryotic cells, allowing us to use the resulting color change as a marker of only the surviving cells [77]. As demonstrated in, succinate dehydrogenase, a component of mitochondrial complex II, is responsible for the conversion of MTT to formazan (**Fig. 2**).

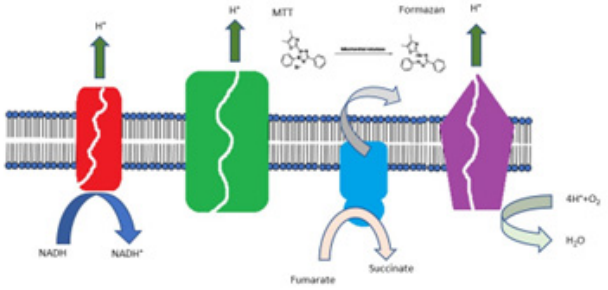

Fig. 2. One of the sites of MTT reduction at the mitochondrial respiratory chain.

Because of its low cost, ease of use, and speed, the MTT dye-reduction system is a popular choice [78]. No studies or data have been reported on the application of MTT assay for measuring the inhibition of heavy metals on Baker's yeast activity. Adding the fact that an inadequate study was done regarding this, an attempt to develop a yeast inhibitive assay using MTT as an indicator should be conducted to obtain a highly sensitive toxicity assay.

Optimization method for improving the sensitivity of toxicity assay

The use of approach one-factor-at-a-time (OFAT) is often used in optimization works of analytical chemistry by changing one significant parameter at one time [79]. The main drawback of this method is that it does not reveal the full effects of the parameter on the response by neglecting the interactive component of the factors involved [80]. Another notable disadvantage of this one-factor optimization is that higher amounts of experiments are required to conduct the research, which resulted in increased time and costs. Additional reagents and consumables are also needed [81]. Despite that, the OFAT optimization method is a useful and powerful technique for understanding microbial regulation of parameters such as carbon, nitrogen and phosphorus sources [82]. Statistical and machine learning techniques to improve sensitivity can include the response Surface Method (RSM) and Artificial Neural networks [83–88].

CONCLUSION

The toxicity of cationic heavy metals such as arsenic, molybdenum and chromium are not addressed as intensively as cationic heavy metals. The toxicity of cationic heavy metals is on par with cationic heavy metals. Rapid bioassay using microorganisms such as yeast can allow the marriage between

bioassay and instrumental methods. This can allow for more routine and rapid screening of heavy metals from the environment. The yeast bioassay system for cationic heavy metals is only partially complete and further studies are needed to be done.

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