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REVIEW ARTICLE

Engineered Whole-Cell-Based Biosensors: Sensing Environmental Heavy Metal Pollutants in Water—a Review

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Abstract

The frequent exposure and accumulation of heavy metals in organisms cause serious health issues affecting a range of organs such as the brain, liver, and reproductive organs in adults, infants, and children. Several parts of the world have high levels of heavy metals affecting millions of people, costing millions of dollars for improving the potability of water and medical treatment of the affected. Hence, water quality assessment is required to monitor the degree of heavy metal contamination in potable water. In nature, organisms respond to various environmental pollutants such as heavy metals, allowing their survival in a diverse environmental niche. With the advent of recombinant DNA technology, it is now possible to manipulate these natural bioreporters into controlled systems which either turn on or off gene expression or activity of enzymes in the presence of specific heavy metals (compound-specific biosensors) otherwise termed as whole-cell biosensors (WCBs). WCBs provide an upper hand compared to other immunosensors, enzyme-based sensors, and DNA-based sensors since microbes can be relatively easily manipulated, scaled up with relative ease, and can detect only the bioavailable heavy metals. In this review, we summarize the current knowledge of the various mechanisms of toxicity elicited by various heavy metals, thence emphasizing the need to develop heavy metal sensing platforms. Following this, the biosensor-based platforms including WCBs for detecting heavy metals developed thus far have been briefly elaborated upon, emphasizing the challenges and solutions associated with WCBs.

Keywords Toxicity · Water · Microbiology · Heavy metals · Biosensor

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Introduction

Metals that are five times denser than a molecule of water are termed heavy metals. Some of the heavy metals have biological significance as trace elements. Since worldwide industrialization, the frequent exposure and often accumulation of heavy metals in organisms cause health issues and ecological imbalances. Typically, heavy metals such as arsenic, cadmium, chromium, lead, and mercury have a specific source of origin despite being found in cosmetics, fuels, and effluents. Arsenic and lead contaminate underground potable water [1], cadmium and chromium are often infused in the workplace [2, 3], and mercury exposure occurs through dental implants (amalgams) and consumption of fish [4]. According to the WHO, the toxicity range of mercury in surface waters is as low as 0.001 mg/L, cadmium is 0.003 mg/L, while for arsenic, lead is 0.01 mg/L, and chromium is 0.05 mg/L. Beyond these levels, accumulation of heavy metal leads to a variety of toxic (arsenic, cadmium, chromium, zinc, copper, nickel), neurotoxic (lead, manganese), mutagenic (arsenic, nickel, cadmium), and carcinogenic (arsenic, cadmium, chromium) effects which result in damage to vital organs [5].

Globally, some geographic locations are inherently rich in heavy metals, while in many other locations are accumulated due to human activity. A case study in the towns of Empangeni and Richards Bay, South Africa, suggested that boron, cadmium, iron, and manganese were the major heavy metal contaminants of groundwater, while a similar study conducted in Daye, China, provides evidence that large amounts of cadmium, copper, arsenic, and lead contaminate the soil in and around mining sites [6, 7]. These reports highlight the degree of water pollution across both developing and underdeveloped countries hinting at the fact that a large portion of the global population is living under extreme health risks.

The toxicity of these heavy metals, the degree of pollution in drinking water, and the enormous costs of treatment at the population level require measures for the prevention of consumption and treatment of polluted water. Hence, water quality assessment is required to monitor the degree of heavy metal contamination in potable water. To assess the water quality, various light and mass spectroscopy-based methods have been developed thus far with extremely high sensitivity and specificity of detection [8]. However, the majority of these instruments lack direct applications in day-to-day life due to the need for specialized instrumentation depending on the source, cost, etc. Hence, developing fast, reliable, cost-effective, highly specific, and sensitive techniques is essential.

In nature, organisms respond to various stresses including environmental pollutants such as heavy metals requiring them to adapt to the same via the expression of specific genes and transcription factors in the race to survive the critical selection pressure. Hence, these genes/transcription factors expressed by the prokaryotes and eukaryotes can thus be exploited for use as biosensors to estimate the degree of water pollution. With the advent of recombinant DNA technology, it is now possible to manipulate these natural bioreporters into controlled systems which either turn on/off gene expression or activity of enzymes in the presence of specific heavy metals (compound-specific biosensors). Most organisms typically have a heavy metal-specific regulatory element from a heavy metal resistance operon expressed in response to the presence of specific heavy metals in the environment such as MerR for mercury and ArsR for arsenic [9, 10]. Further, coupling these bioreporters with a suitable readout (optical/electrochemical) allows one to construct a whole-cell biosensor (WCB) where the former functions as a bioreceptor and the latter as a transducer. WCBs developed so far can be classified into two major categories: electrochemical and optical, based on the signal transduction strategy. While electrochemical sensors function

by recording the variation in the conductance or voltage across the cell membrane, which are influenced by the physiological conditions of the microbe in response to heavy metal, the optical methods comprise the use of various inherently bioluminescent bacterial strains or organisms genetically modified with a gene cassette consisting of a metal response element and a promoterless reporter gene where the latter is expressed in the presence of a bioavailable heavy metal. Major advantages of WCBs includes their ability to detect only bioavailable heavy metals (which eventually can cause toxicity), relative ease of manipulating microbes, and rapid generation of large amounts of biomass [11].

In this review, we briefly describe the mechanisms of heavy metal toxicity and then review the traditional and modern approaches used to estimate heavy metals in drinking water with specific emphasis on the construction and working of WCBs with representative examples followed by a critical view of the current challenges in the field of WCBs.

Toxicity

A primal health effect of heavy metals is enzyme inhibition, affecting various organs such as the brain, liver, reproductive organs, and kidneys (Fig.1). A typical example includes the enzyme glutathione peroxidase, which is spatially inhibited by different metals—mercury in the brain [12], lead in the liver [13], arsenic in the testis [14], and cadmium in the bloodstream [15], to name a few. Lead is a heavy metal that causes systemic toxicity, including the brain [16–18], kidney [19], plasma [20], liver, and ovaries [13]. However, the same metal (lead) affects different enzymes such as N-acetyl-D-glucosaminidase [19], sialyltransferase [16], glutamine synthetase [17], and S-aminolevulinic acid dehydratase (ALAD) [21] as well. The above-cited examples allow one to ponder upon the observation that even though heavy metals are highly specific to the enzyme they inhibit, their effect is also influenced mainly by their location in the human body.

In addition to its adverse effects in adults, heavy metals also have adverse effects on infants and children. Infants are particularly vulnerable to neurological damage as their blood-brain barrier and the ability to repair structural damages are not completely developed, resulting in permanent neuronal development damage. Lead exposure among infants is associated with impaired intellectual [22, 23], behavioral [24], academic [25], and neurophysiological [26] functioning. Cadmium toxicity in children is associated with a reduced IQ, aberrant effects on the hypothalamus-pituitary-adrenal (HPA) axis [27], and the suppression of hypersensitivity reactions [28], while methylmercury exposure to children at the prenatal stage results in dose-dependent symptoms ranging from high blood pressure and language disorders even to the extent of mental retardation [29].

Excessive exposure to lead results in the massive build-up of reactive oxygen species (ROS), which is closely associated with a reduction in the number of enzymes that act as antioxidants (catalase, glutathione peroxidase, superoxide dismutase, etc.) [30]. ROS generation can affect oxidative phosphorylation [31], alter various signaling pathways resulting in aberrant gene expression, inhibit nucleic acid synthesis by introducing ssDNA breaks, sister chromatid exchange, and DNA-protein cross-linking mediated by thiols. Such cellular damage results in the apoptosis of cells [32]. Thus, it is not surprising to note that heavy metals play an influential role in the development of cancers—arsenic accelerates the development of lung, bladder, and skin cancer, and cadmium promotes cancer of the breast, esophagus, stomach, intestines, prostate, lungs, and testes. Similarly, lead plays a

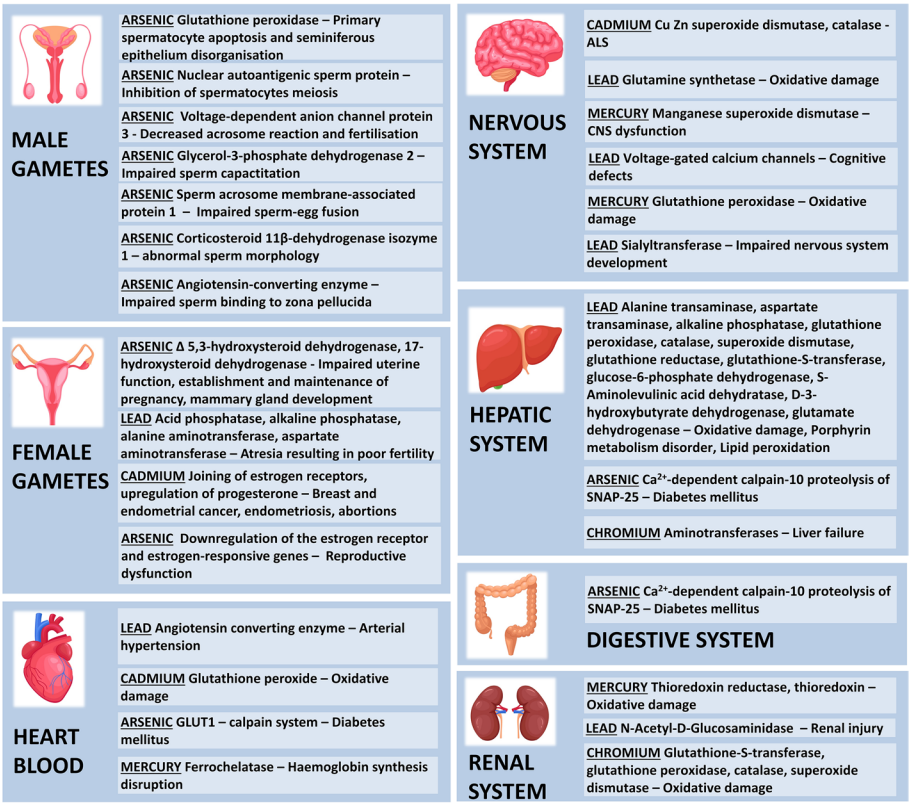

Fig. 1 Effect of heavy metals on the various human organ systems, their enzyme targets, and their biological implications. The readers are directed to the text section of Toxicity for further details and references.

supportive role in glioma, gallbladder cancer, and pancreatic cancer, and mercury lacks a causal role but is associated with renal and gastric cancers [33].

Heavy metals harm fertility as well. Among men, high concentrations of lead and cadmium in the blood result in lower sperm count and reduced motility of sperm [34, 35]. Mercury intoxication in men results in suppressed sperm motility and varied sperm morphology, in extremely high mercury concentrations—even resulting in infertility [36]. Arsenic toxicity has adverse effects on the development of the male reproductive system, decreased acrosomal reaction, decreased sperm count, and impaired spermatogenesis resulting in morphologically abnormal spermatozoa [14]. In women, cadmium intoxication has been associated with endometriosis, endometrial cancer, and breast cancer as cadmium is a metal estrogen that can join the estrogen receptors, thus stimulating it, resulting in adverse effects [37]. Arsenic hinders ovarian steroidogenesis resulting in impaired uterine function, establishment and maintenance of pregnancy, and mammary gland development, eventually resulting in reproductive dysfunction [38, 39]. Lead exposure has been associated with atresia in the ovary during the stage of folliculogenesis [40].

Various studies globally have shown that a higher arsenic concentration in groundwater is positively correlated towards spontaneous abortion, stillbirth, and higher preterm birth rates [41]. Lead and cadmium have been shown to cause the above effects in addition to

anemia and toxemia [42, 43]. A case study from Myanmar also claims that prenatal cadmium exposure resulted in lower birth weight of babies [44]. When compared to mercury, methylmercury poses a greater threat to the neuronal development of babies due to its ability to travel across the placental barrier from the affected mother, accumulating in the fetal tissues [45]. Thus, metals such as arsenic, lead, mercury, and cadmium are termed endocrine-disrupting compounds, owing largely to the adverse effect on pregnant mothers' and fetuses' health [46]. Partners of women working in stainless steel welding units where hexavalent chromium is released pose a high risk of male-mediated spontaneous abortions among pregnant women [47]. This section provides concrete evidence that heavy metals not only spatial-specifically inhibit various enzymes in adults but also have an untoward effect on infants, children, and even developing fetuses. Strong correlations have been drawn on the influence of heavy metals in the development of cancers, infertility, and prenatal health, thus providing a tangible emphasis on the need to detect these heavy metals.

Types of Biosensors for the Detection of Heavy Metals

Various chemical methods such as ICP-MS (inductively coupled plasma mass spectroscopy); SERS (surface-enhanced Raman spectroscopy) for the detection of Pb^{2+}, Hg^{2+}, and Cd^{2+}[8]; and HPLC (high-performance liquid chromatography) for Ni^{2+}, Co^{2+}, Cu^{2+}, Se^{4+}, and Cr^{6+} [48] have been exploited thus far for the detection of heavy metals. In ICP-MS, the water samples are subjected to plasma radiations, which atomize the particles and are further separated based on the m/z ratio (mass to charge) characteristic to each heavy metal, making them highly specific [49]. On the other hand, in the case of SERS, detection is done based on the Raman shift (stokes or anti-stokes) when an incident light undergoes non-Rayleigh's scattering, unique to every element [50]. Each of these methods (Table 1), despite being extremely sensitive and specific, has failed to make it to a practical application scale. This is due to the need for complex experiment setups which calls for skilled labor. In recent times, biosensors with biomolecular interfaces such as monoclonal antibodies (mAb), enzymes, metal chelating proteins, and genetically modified whole cells have been explored for in situ applications.

Immunosensors (mAb)

Immunosensors rely on the use of antibodies specific to heavy metals as a bioreceptor coupled with a suitable readout such as SPR to detect the interaction. Zhu et al. have raised monoclonal antibodies (mAb) against chelated Cd^{2+} ions (Cd^{2+} - 1-(4-isothiocyanobenzyl) ethylene diamine N,N,N′,N′-tetra acetic acid (ITCBE)) using Cd^{2+} coupled protein carrier (keyhole limpet hemocyanin, KLH) as an immunogen, utilizing the hybridoma technology. The immunosensor developed with the raised anti-Cd^{2+} mAbs has shown to be effective (despite being chelated with a linker and a protein carrier) in ELISA tests with a recovery of between 91 and 105% in tap water fortified with Cd^{2+} ions, thus proving the applicability of the sensor for real-time sample analysis [55]. On similar lines, mAb against chelated - Hg^{2+} [56], polyclonal antibodies against chelated - Cd^{2+} [57] have been developed, which can in the future be considered for developing sensor platforms. For instance, mAb against Pb^{2+} has been immobilized onto a fiber optic-based system to detect Pb^{2+} using a localized surface plasmon resonance (LSPR)-based strategy [58]. However, the high cost of mAbs

Table 1 Advantages and limitations of the various chemical methods adopted for the detection of heavy metals.

S. no.	Method	Advantages	Limitations	Ref.
1	SERS	1. Fingerprint vibrational spectrum—identification without separation 2. Ability to multiplex	1. Complex instrumentation 2. The requirement of a metal surface	[51]
2	ICP/MS	1. Extremely high sensitivity, selectivity, accuracy	1. Laborious 2. Expensive instrumentation 3. Prolonged analysis time	[52]
3	HPLC	1. High sensitivity 2. Rapid detection 3. Versatile techniques based on the metal to be detected	1. Skilled labor 2. Complex instrumentation	[53]
4	Biosensors	1. High specificity, accuracy, precision, reproducibility, and repeatability 2. Rapid detection 3. Extremely low limit of detection (pM concentrations)	1. Poor reproducibility, stability	[54]

production using hybridoma and their mass production in real sample analysis seems to impose limitations.

Protein-Based Sensors

A protein-based sensor, as the name suggests, utilizes a protein/peptide as the primary heavy metal sensing component. The detection strategy using enzymes, by and large, involves a conformational change in enzymes due to enzyme inhibition by heavy metals, which are studied using LSPR [59], fluorescence [60], and electrochemical techniques such as amperometry [61, 62] and conductometry [63]. Biorecognition elements include (not limited to) acetylcholine esterase for Hg^{2+} [61]; alkaline phosphatase for Hg^{2+}, Cd^{2+}, Ag^{+}, Zn^{2+}, and Cu^{2+} [62]; tyrosinase for Cr^{3+} [64], glucose oxidase for Hg^{2+} [65], acid phosphatase for Hg^{2+}, Cu^{2+}, and Cr^{2+}; [60] and urease for Cd^{2+} and Pb^{2+} [59, 66]. In addition to this, multi-enzyme sensors using invertase/mutase/glucose oxidase to detect Hg^{2+} using amperometry [67] and conductometry [63] have been developed.

Apart from typical enzymes, the uses of isozymes and apoenzymes are also particularly attractive options. For instance, the enzyme glutathione S-transferases (GST)-Theta-2-2 from the bovine liver has been exploited for Zn^{2+} detection, while recombinant GST with $(His)_6$ tag has been reported for the detection of Cd^{2+} [68]. Apoenzymes are activated only in the presence of a cofactor (heavy metal) as in the case of alkaline phosphatase, activated by Zn^{2+}, which has been used for the detection of Zn^{2+} with an inbuilt microfluidic system by Ikuo Satoh as early as 1990 [69]. In addition to enzymes, low-molecular-weight cysteine-rich peptides such as metallothionein and its plant-derived counterpart phytochelatins (PCs) also find a place in protein-based sensors for the detection of heavy metals given their inherent affinity to metals such as Zn^{2+} and Cd^{2+}. By exploiting this, a paper disc-based electrochemical sensor for As^{3+} and Hg^{2+} [70] and an LSPR-based sensor for cadmium, zinc, and nickel have been developed [71]. Given the higher affinity of metal binding by phytochelatins (PCs) [72], a synthetic PC-based capacitive biosensor has been developed by Bontidean et al. Even though the sensor lacks selectivity (highest sensitivity for Zn^{2+}), the work showcases the potential of PCs to be used as heavy metal sensing elements [73].

Although enzyme-based sensors are relatively specific and sensitive, they have limited on-site applications due to their stability issues and poor reproducibility as they are affected by adverse environments such as temperature, pH, salt concentration, and inhibitors ensuing in the reduction of enzyme activity. On the other hand, non-enzymatic sensors provide an upper hand as they typically lack the biological component (enzyme) and thus provide a longer shelf-life while simultaneously allowing reusability of the sensor, thence enhancing its robustness.

DNA-Based Sensors

DNA-based sensors typically use electrochemical transduction methods where the sensing interface is ssDNA (similar to an aptamer), having a high affinity to heavy metals. Effective targets for metal ions in DNA include the negatively charged oxygen atoms in the phosphate backbone, nitrogen as part of nucleobases, the keto groups of the exocyclic rings, and the hydroxyl from the deoxyribose [74]. Effective immobilization of the DNA probe is essential for the sensor to achieve high sensitivity. Physical adsorption, covalent binding, self-assembled monolayers (SAM), etc. are a few methods reported for the same [75].

The physisorption process is a physical method that exploits the natural ability of DNA to bind to a positively charged substrate with a high affinity for DNA. However, one of the major drawbacks of the method is that the reaction is reversible, thus accounting for its low stability [76]. Oliveira et al. have fabricated a glassy carbon electrode modified with dsDNA probe by multi-layer physical adsorption to detect Pb^{2+}, based on its ability to cause a change in conformation of the probe as it binds to the adenine residues in DNA [77].

Covalent binding or chemical adsorption is the formation of an irreversible chemical bond between the DNA and the substrate resulting in high stability [76]. By exploiting the above principle, ssDNA has been immobilized onto a stationary mercury film electrode to detect Pb^{2+} and Cd^{2+} in water, based on their ability to bind with the immobilized DNA. A major advantage of the devised sensor is its ability to specifically detect only lead and cadmium even in the presence of potential heavy metal interferents such as nickel, cobalt, and zinc, as much as tenfold higher [78].

SAMs refer to spontaneously formed ordered molecular assemblies as in the case of affinity-based interaction between SH-modified DNA and a gold electrode surface (thiol-Au interaction) [79]. The detection of Cr^{3+} in the picomolar range has been developed based on the unique property of chromium to undergo underpotential deposition onto the ssDNA probes (immobilized based on SAMs) owing to its extremely low limit of detection [80].

These DNA-based sensors find limited application as only a few ions have an affinity to DNA, thus limiting the range of metals detected. Also, the chemistry of interactions between DNA and heavy metals remains largely controversial and widely remains under critical exploration.

Whole-Cell Biosensors

As mentioned earlier, WCBs consist of two typical components, namely the metal-responsive element and a readout. The following section provides a brief overview of the different parameters to be taken into consideration when constructing a whole-cell biosensor.

Choice of Metal Regulatory Element

According to the Antibacterial Biocide & Metal Resistance Genes Database (BacMet), as of March 2018, 420 experimentally verified metal resistance genes for over 20 metals have been identified. They are classified into seven major families of cytosolic metal-responsive transcriptional regulators in bacteria, namely MerR, CsoR-RcnR, NikR, DtxR, Fur, CopY, and ArsR/SmtB [81, 82]. All the families consist of the above-mentioned metalloregulatory protein, which when bound to the effector metal results in an allosteric switch mediated by either co-repression (DtxR, NikR, Fur), activation (MerR), or de-repression (ArsR/SmtB, CsoR-RcnR, CopY) which promotes the expression of its downstream structural gene coding for either the transport of the metal into the cell, efflux pumps, or other enzymes for the reduction of the metal to a non-toxic form [9].

The MerR and ArsR/SmtB families are the most abundant and well-studied metal regulators. They have an affinity to most of the common heavy metals, typically via the thiol groups present on their cysteine residues in their active sites. The Ars operon belonging to the ArsR/SmtB family involved in the detoxification of arsenic consists of two critical

components, namely the As(III) responsive repressor and As(III) efflux permease encoded by ArsR and ArsB, respectively. While the former functions as a trans-acting repressor controlling transcription by either binding to the operator region repressing transcription of the Ars operon in the absence of arsenite or dissociating from the DNA upon binding to arsenite thence mediating transcription of the Ars operon, the latter (ArsB) effluxes the reduced arsenic either via the influence of a proton motive force or via ArsA-dependent ATP hydrolysis. It is interesting to note that arsenic does not require specialized transporters for its influx into cells while efflux pumps are necessary, the opposite is true in the case of mercury. The Mer operon codes for a periplasmic transport protein (MerP) that orchestrates Hg^{2+} influx into cells, followed by an inner membrane transporter (MerT) and, finally, a mercuric reductase (MerA) which reduces the Hg^{2+} to volatile Hg^{0} in an NADPH-dependent manner. The key regulatory component of the inducible Mer operon system is MerR which controls the transcription of the operon by controlling the distance between the −10 and −35 regions of the promoter in a Hg^{2+}-dependent fashion. The major differences between the two families have been detailed in Table 2.

In addition to these regulatory genes, a class of metal-binding proteins called Metallothioneins are also used for sensing Cd^{2+} (CdMT) and Cu^{2+} (CuMT) [86]. Similar to metalloregulators, the expression of genes encoding for these proteins is induced by the exposure of cells to these metals and thence has been exploited for the detection of copper and cadmium in *Tetrahymena thermophila* [87, 88]. Apart from the well-established known metalloregulators, several groups have also identified specific genes which are upregulated in response to heavy metals without the need for a regulatory element. In which case, a much simpler design cassette consisting of the promoter for the gene cloned upstream of a promoterless reporter gene. Examples of such genes include the cadmium responsive genes SEO1, DR_0659, and *gro*EL each of whose promoters has been used to develop WCBs to detect bioavailable cadmium [89–91].

Heavy Metal Sensing Gene Cassette Expression

Invariably, WCBs rely on the need for cloning either a metalloregulator or a promoter for binding of the metalloregulator or a promoterless reporter gene or in most cases all—into the host organism via plasmids. This being the case, properties of the cassette that could influence the sensitivity of the WCB include the copy number of the plasmid and whether the plasmid is integrated into the genome as a single copy or is maintained as an episome. In the case of GolS expression from *Salmonella* to *E.coli*, it was found that the chromosomal integration results in the prevention of leaky expression since the former is found in a single copy while the latter is found as multiple copies due to expression via a plasmid resulting in leaky expression and thence compromised sensitivity [11]. Contrary to the above-mentioned results, a study by Cayron et al. has reported that the chromosome integration of the metal regulatory cassette (C35A-RcnR/P*rcnA*) compromises the sensitivity of the WCB to respond to heavy metals (Ni). In which case, compensation in terms of deletion of the Ni-efflux pump (*rcnA)* and introduction of genes encoding Ni-uptake systems was found to yield higher sensitivity [92]. However, in the case of bioluminescent bacteria, it has been reported that irrespective of the expression of the regulatory protein as a plasmid or as a single chromosomal insertion did not influence the sensitivity of the sensor [93].

In addition, the overall design of the expression system in terms of whether the whole cassette is expressed via one plasmid or via two plasmids where one encodes for the

Table 2 Comparison of MerR and ArsR/SmtB metal regulatory families [83–85]

Component	MerR family	ArsR/SmtB family
Representative regulators	CueR, MerR, GolS, ZntR, PbrR	AioF, ArsR, AztR, BxmR, CadC, CmtR, CzrA, KmtR, NmtR, SmtR, ZiaR
Major heavy metal targets	Cu^{2+}, Hg^{2+}, Au^{+}, Zn^{2+}, Pb^{2+}	As^{3+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}
Mode of action	Transcriptional activation/repression	Transcriptional repression
Metal-binding site	C-terminal	Homologs of α3N, α5
DNA binding domain	N-terminal	Same as the metal-binding site
Allosteric regulation	Twist in the conformation of transcriptional regulator bound promoter region upon metal binding	Reduction in DNA binding affinity of the complex (transcriptional regulator + metal) upon metal binding
Critical control feature	Distance between −35 and −10 regions of the promoter	Sufficient binding of metal to regulator via appropriate coordination chemistry

metalloregulator and the other for the metalloregulator binding promoter upstream of the reporter has also been reported to have a major influence on the WCB performance. In the latter case of using two different plasmids, it has been detailed that the use of a high copy number plasmid for expressing only the metal regulatory protein while a medium copy number plasmid expressing the promoter for the regulatory protein-coupled with the promoterless reporter gene significantly reduced leaky expression of the reporter in the absence of the heavy metal [93]. Another strategy to reduce leaky expression of the metalloregulator is to clone it under the control of its own promoter instead of a constitutive promoter thence mediating the development of a WCB with a well-controlled positive feedback loop-like system [11]. All of the considerations stated above have been combined by Kim et al. for the development of a highly specific and sensitive WCB to detect cadmium and lead under a T7 signal amplification feedback loop for enhanced reporter gene (GFP) expression [94].

Specificity

Cysteine residues are the critical players in determining the specificity of the metal regulators to heavy metals. Since different heavy metals have varied coordination affinities to a specific set of cysteines, mutations in cysteine residues allow one to fine-tune the specificity of the metalloregulators. This allows for multiplex detection using a single metalloregulator as well as reducing the specificity of the metalloregulator to only one heavy metal thence rendering them extremely robust. This principle has thus been exploited to improve the specificity of the metal-responsive transcriptional regulator—GolS. Cerminati et al. have mutated S77 to cysteine thence allowing for multiplex detection of Hg, Pb, and Cd ions [11]. While in this study, effective estimation of specific heavy metals was not reported, in a different study, bioavailable cadmium, lead, and arsenic from a mixture of the same have been estimated with the use of two sensor sets coupled with a binary linear regression model for eliminating the effect of interference [95]. In addition to multiplex detection where one metalloregulator detects multiple heavy metals, the same concept has also been extended to mutating the metal regulator initially specific to multiple heavy metals to respond to only one. A classic example of the same is a single-point mutation of RcnR (specific to nickel and cobalt) at C35A yielding exceptional specificity to only nickel while eliminating its interaction with cobalt [92].

Electrochemical Readout

Despite being highly sensitive, electrochemical methods that measure the variation in potential or conductance in cells in their native form (without mediators) suffer the major limitation of being extremely slow in terms of the electron transfer rate between the electrode and the cell. Hence, various artificial mediators have been utilized to enhance the rate of electron transfer by essentially replacing the oxygen to aid in the shuttling of electrons [96]. These mediators are classified based on their solubility as lipophilic and hydrophilic mediators. While the lipophilic mediators (menadione (MD) and 2,3,5,6-tetramethyl phenylene diamine (2,3,5,6-TMPD)) can actively cross the microbial cell membrane, they are not suitable for aqueous systems due to their sparse solubility [97]. On the other hand, the hydrophilic mediators (ferricyanide) are highly suitable for aqueous samples and thus have been combined by multiple groups for the development of mediator-based WCBs to detect Cu^{2+}, Cd^{2+}, Ni^{2+}, and Pb^{2+} [98, 99]. Unlike other WCBs, which are genetically

manipulated, the developed sensors are based on assessing the acute toxicity in the cells in response to the heavy metals [100–102]. Gao et al. immobilized *Saccharomyces cerevisiae* upon the introduction of the metal ions, which results in the overall inhibition of metabolic processes, which is further amplified by the mediators—menadione (lipophilic) and ferricyanide (hydrophilic) [98].

Unlike the conventional approaches of using a reporter gene, Webster et al. have explored the possibility of using a bioelectrochemical system using genetically engineered *Shewanella oneidensis* to detect arsenic. The design is based on introducing an arsenic-sensitive promoter for effective expression of MtrB (essential for reduction reaction at the electrode), thus yielding an arsenic concentration-dependent current response [103]. Another such innovative conductometric approach has been attempted using the algae *Chlorella vulgaris* to detect Cd^{2+} and Zn^{2+} in water samples. Since the algae constitutively express alkaline phosphatase on its cell wall, heavy metal-mediated enzyme inhibition has been recorded based on conductometry and correlated with the concentration of heavy metal in the sample with a detection limit of 10 ppb [104].

Substrate Dependence—Optical Readout

One of the most widely adopted enzymes is the bacterial luciferase (*lux*) which yields bioluminescence upon the addition of the substrate luciferin. The *lux* operon consists of five genes (*luxABCDE*), out of which *luxAB* encodes for luciferase, which mediates oxidation of its substrate (long-chain fatty aldehyde—myristyl aldehyde) to produce light, while the other genes (*luxCDE*) function to regenerate the substrate [105]. Thence, for the fabrication of a WCB, the *luxAB* gene alone can be expressed, in which case the long-chain aldehyde is added along with the substrate. Otherwise, the whole gene cassette *luxCDBAE* can be expressed owing to spontaneous fluorescence upon addition of substrate, excluding the need for intervention [106]. Since the end application of the sensors is for on-field application, it is essential to consider the stability of the bioluminescence encoding genes. For instance, while the bioluminescent gene encoded by *Vibrio fischeri* is not stable at temperatures above 30°C, a suitable alternative is that expressed by *Photorhabdus luminescens* [93].

Another class of luciferase is the single polypeptide *luc* expressed in firefly, which is expressed in the presence of the substrate D-luciferin. Since these genes are expressed spontaneously in the presence of the substrate, it is possible to monitor the expression of the metal regulatory gene continuously, thus aiding in the indirect estimation of the heavy metals [88, 95, 107].

LacZ operon in *E. coli* encodes for the enzyme β-galactosidase (β-gal), which mediates the hydrolysis of the substrate X-gal in the presence of IPTG (isopropylthio-β-galactoside) as an inducer to obtain a blue-colored product. Since β-gal is expressed endogenously by cells, there is a significant background activity that makes the detection unreliable to an extent. Owing to background noise, only a few WCBs have been developed with LacZ as a reporter [90, 108, 109]. While the *luxAB* and LacZ systems fall under the substrate-dependent expression systems, the prime consideration, in this case, is the permeability of the substrate across the cell (influenced by the cell type and the substrate), failing which requires the need for cellular lysis.

In contrast to the above cases, the expression of the green fluorescence protein (GFP) is independent of the substrate but dependent upon the wavelength of the external source of light provided the protein is folded properly [110]. Thus, gene products such as GFP

can be readily detected based on fluorescence microscopy intensity. However, there is a significant lag in establishing a stable fluorescence in the cells due to time invested in the processing and maturation of GFP since it is a multistep process requiring a longer analysis time. Out of all the available reporter systems, this is currently the most popular reporter system for WCBs, largely owing to the simplicity associated with its construction [11, 111, 112]. When considering visual detection, as expected for field applications, RFP has been reported to be superior to GFP owing to the visible color change associated with it [113].

Amaro et al. have demonstrated the difference between the substrate-based luciferase readout and the GFP-based assay on the metallothionein-based *Tetrahymena* WCB for the detection of Cd^{2+} where they claim that for the same metal regulatory gene cassette, the GFP-based assay has a higher tolerance range for Cd^{2+} up to 15 μM since it is expressed as an extrachromosomal high copy number plasmid unlike luciferase which is a single copy chromosomal insertion and also has greater practical applicability since no exogenous substrate is required [87]. However, luciferase-based assays provide an upper hand in terms of a lower limit of detection (5 nM) and lower response time [88].

In addition to the conventional reporter systems, the introduction of genes such as *phzM* and *phzS* under the control of a MerR promoter in *P. aeruginosa* can yield a red-colored compound (pyocyanin) in the presence of its substrate and Hg^{2+}, which has been used for the detection of Hg^{2+} [114]. Along similar lines, Joe et al. have coupled the *crtI* reporter gene under the control of a cadmium-inducible gene, wherein the presence of cadmium mediates the conversion of the colorless substrate phytoene to a visible red color as a result of carotene synthesis [91]. Similar to GFP, the monomeric red fluorescent protein (RFP) has been modified by the inclusion of a cysteine residue at its 199th position, promoting its dual role not only as a reporter system but also as a heavy metal sensor mediated by the interaction of the thiol group of cysteine with mercury [115]. With the advent of synthetic biology, several complex genetic circuit-based signal amplification systems have also been developed based on quorum sensing, the use of GFP mutants, and the T7 feedback loop [94, 116, 117].

Switch On vs Switch Off—Optical Readout

"Switch-on" bioreporters are genetically manipulated by introducing a reporter gene sequence linked to a regulatory region that responds to an environmental cue (in this case is a heavy metal-responsive transcriptional regulator). In such a system, the reporter gene is transcribed and translated allowing visual luminescence detection, either via colorimetry, fluorescence, or bioluminescence. The intensity of fluorescence/bioluminescence, in this case, becomes a quantitative indicator of the concentration of metal ions present in the sample. On the other hand, the switch-off type of system (similar overall construction) expresses a threshold intensity of light but diminishes upon the presence of the particular analyte as it intercepts the general metabolism of the cell [118].

A larger portion of the WCBs developed thus far are based on the switch-on type since they rely on the activation of the metal regulatory genes in the presence of the target heavy metal. However, WCBs that function by analyzing the heavy metal-mediated cytotoxicity (bioluminescence inhibition assay) can be considered classic examples of the switch-off type sensor. In the case of natural self-luminescing bacteria such as *Anabaena torulosa* and *Vibrio campbellii*, and synthetically modified bioluminescent bacteria, the amount of luminescence emitted by these bacteria is a direct indication of their metabolic state. Thence, heavy metal-mediated inhibition of enzymatic activity yields lower fluorescence and thus

has been exploited for the estimation of copper, cadmium, lead, mercury, and zinc without the need for inclusion of metal regulatory genes [119–122]. A particularly important consideration in the case of inherently non-luminescent microbes is to make sure the reporter expressing plasmid is of high copy number to ensure maximum background bioluminescence and thence better sensitivity in response to the heavy metal. Various optical WCBs are being developed to detect heavy metals over the last few decades (Table 3).

In addition to the above-discussed approaches, a few attractive approaches for heavy metal detection include the study of electrophysiological changes of cardiac cells in response to heavy metals. Even though the study focused on addressing the toxicity effect of the heavy metals on cells, this system of detection combined with the introduction of a metal-responsive element is a possible fool-proof method for detecting heavy metals in real-world water samples [131]. Another innovative perspective is the construction of a logic gate-based heavy metal-responsive genetic circuit in *E. coli* consisting of a triple input-based AND gate (the basic digital ON-OFF logic gate) for the detection of arsenic, mercury, and copper [132].

Challenges

In order to critically evaluate the challenges associated with the use of WCBs at a commercial level, it is paramount to define the typical characteristics of a WCB. From a practical point of view, an ideal WCB is expected to (i) report the presence of heavy metals both qualitatively and quantitatively, (ii) the population of which could be controlled artificially, (iii) not alter the ecology of the site, and (iv) withstand environmental stresses.

The ability of the WCB to actively take up heavy metals from the surroundings is a prime factor essential for its applicability as a quantitative sensor platform. Transporters and channels are the typical structures through which metal ions access entry into cells. A typical WCB with high sensitivity is required to have metal ion-specific uptake mechanisms and require active transporters with a higher affinity for heavy metals on the extracellular side. Such properties would allow the uptake of metal ions even at low concentrations. While metals such as Ni^{2+}, Cd^{2+}, and Cr^{6+} can diffuse across the cell membrane, other metals either have a transporter for the import into the cell or for both imports as well as export outside the cell [133]. For instance, the uptake of Hg^{2+} within cells is mediated by the transporters MerP (into the cytoplasm) and MerT (into the nucleus) with no specialized transporters for its export in *E. coli* as the reduced form of mercury can freely diffuse across the membrane [134]. On the other hand, in the case of Pb^{2+}, the uptake is promoted by PbrD, while its efflux is controlled by the expression of *pbrABC* in *R. metallidurans* [135]. Thence, manipulating the Pb sensing WCB to maintain a fine balance between promoting influx and limiting the efflux of lead is pertinent for exploiting this mechanism for heavy metal sensing. Certain reports also claim that the use of ciliates such as *Tetrahymena thermophila* is advantageous since they lack a cell wall during their vegetative stage allowing for easier uptake of heavy metals into the cell thence improving the WCBs sensitivity [86].

In general, selectivity, sensitivity, reproducibility, and recovery are the typical characteristics of a biosensor for real sample sensing applications. Particularly, the sensitivity and selectivity of sensors for heavy metals are of prime importance because even the presence of as low as 0.001 mg/mL of mercury, lead, or arsenic in water is considered to be toxic. Thus, the sensors should have an extremely low limit of detection to precisely gauge the

Table 3 A representative list of optical readout-based genetically engineered WCBs developed for the detection of heavy metals.

S. no	Metal(s) detected	Regulatory gene	Regulatory gene family	Reporter system	Manipulated host organism	Limit of detection (LOD)	Ref.
1	Zinc	*zntR*	MerR	GFP	*Escherichia coli*	-	[111]
2	Mercury, lead, cadmium	*golS* (S77C)	MerR	GFP	*Escherichia coli*	Hg = 4.4 nM Cd = 283.9 nM Pb = 39.6 nM	[11]
3	Copper	*cueR*	MerR	GFP	*Pseudomonas putida*	Cu = 1 mg/mL	[112]
4	Copper	*zntA*	MerR	GFP	*Escherichia coli*	Cu = 5 µM	[123]
5	Cadmium	*cadC* in combination with T7 signal amplification	MerR	GFP	*Escherichia coli*	Cd = 3 µM	[94]
6	Mercury	*merR*	MerR	GFP mutant	*Escherichia coli*	Hg = 1.3 nmol/L	[117]
7	Cadmium, mercury, zinc, lead, copper, silver	*merR*, *zntR*, *cueR*, *pbrR*, *cadR*	MerR	Lux	*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*	Hg = 0.03 µg/L Cd = 1.8 µg/L Pb = 33 µg/L Zn = 1626 µg/L Cu = 24 µg/L Ag = 340 µg/L	[93]
8	Mercury	*merR*	MerR	RFP	*Escherichia coli*	Hg = 50 nM	[113]
9	Mercury	*merR* in combination with a positive feedback loop of *luxR* and *luxI*	MerR	RFP	*Escherichia coli*		[116]
10	Mercury	*merR*	MerR	RFP (pyocyanin)	*Pseudomonas aeruginosa*	Hg = 10 nM	[114]
11	Arsenic	*arsR1*	ArsR/SmtB	GFP	*Escherichia coli*	As = 0.01 µM	[124]
12	Cadmium	*cadR*	ArsR/SmtB	GFP	*Escherichia coli*	-	[125]
13	Arsenic, lead, cadmium	*cadC*, *zntR*, *arsR*	ArsR/SmtB	Luc	*Escherichia coli*	-	[95]
14	Arsenic	*arsR1*, *arsR2*	ArsR/SmtB	LacZ	*Pseudomonas putida*	As = 8 ppb	[108]

Table 3 (continued)

S. no	Metal(s) detected	Regulatory gene	Regulatory gene family	Reporter system	Manipulated host organism	Limit of detection (LOD)	Ref.
15	Cadmium	*MTT1*, *MTT5*	CdMT	GFP	*Tetrahymena thermophila*	Cd = 8.9 µM	[87]
16	Cadmium	*MTT1*, *MTT5*	CdMT	Luc	*Tetrahymena thermophila*	Cd = 5 nM	[88]
17	Nickel	*rcnR* (C35A)	CsoR/RcnR	Lux	*Escherichia coli*	Ni = 80 nM	[92]
19	Cadmium	*SEO1* promoter		GFP	*Hansenula polymorpha*	Cd = 1 µM	[89]
20	Arsenic, mercury	*SEO1* promoter		GFP	*Saccharomyces cerevisiae*	As, Hg = 1 µM	[89]
21	Cadmium	*groEL* promoter		LacZ	*Escherichia coli*	Cd = 0.2 µg/mL	[90]
22	Mercury	*mCherry* (L199C)		mCherry	*Escherichia coli*	Hg = 1 µmol/L	[115]
23	Cadmium	Cd-inducible gene DR_0659		Carotene synthesis—*crtI*	*Deinococcus radiodurans*	Cd = 50 nM	[91]
24	Mercury, zinc, copper, cadmium	Reduced luminescence due to cytotoxicity of heavy metals		Lux	*Acinetobacter baylyi Tox 2*	-	[122]
25	Zinc, cadmium, iron, copper	Reduced luminescence due to cytotoxicity of heavy metals		Lux	*Acinetobacter* sp. *DF4*	Zn = 5 mg/L Cd = 30 mg/L Fe = 40 mg/L Cu = 40 mg/L	[121]
26	Copper, cadmium, lead	Photosynthetic electron transport inhibition		Self-luminescent	*Anabaena torulosa*	Cu = 1.41 µg/L Cd = 0.25 µg/L Pb = 0.5 µg/L	[120]
27	Zinc	Reduced luminescence due to cytotoxicity of heavy metals		Self-luminescent	*Vibrio campbellii*	Zn = 0.97 mg/L	[119]
28	Antimony	*arsR*	ArsR/SmtB	GFP	*Escherichia coli*	Sb = 0.25 µM	[126]
29	Uranium	*urcA*		GFP	*Caulobacter crescentus*	U = 4.2 µM	[127]

Table 3 (continued)

S. no	Metal(s) detected	Regulatory gene	Regulatory gene family	Reporter system	Manipulated host organism	Limit of detection (LOD)	Ref.
30	Uranium	Two component system—UzcRS, UrpRS		GFP	*Caulobacter crescentus*	U = 1 µM	[128]
31	Copper	*cueR*	MerR	GFP	*Escherichia coli*		[129]
32	Copper, zinc, nickel	*coaR*, *nrsR*		Lux	*Synechocystis* sp.	Co = 0.3 µM Zn = 1 µM Ni = 0.2 µM	[130]

wwsafety of water samples, failing which may result in extreme health threats. It is important to note that a particular metal regulatory region is not highly specific to only one heavy metal, as in the case of MerR, which is reactive to not only mercury but also lead and cadmium. This compromised specificity results in overestimation in a co-polluted environmental sample thence compromising the analysis specificity in actual samples [95]. Since the metal regulatory elements are biological components, like all other expressible genes may undergo saturation at high metal concentrations, resulting in an underestimation of heavy metals in water samples. A possible solution is to moderate the concentration of the cells utilized for sensing by using an array with different cell concentrations and choosing the one with the highest sensitivity as the number of heavy metals varies on a sample-to-sample basis. Other constituents of the sample may act as interferants and inhibitors of the metal regulatory gene, resulting in false-negative results. With respect to the reporter system, the turnover of the reporter should be optimized such that it is moderate in comparison with a stable expression which results in compromising the sensitivity of the sensor.

The naturally evolved regulatory genes in microbes are restricted to only a few metals. This limits the number of heavy metals that can be detected using WCBs, thus ruling out the detection of other extremely toxic metals irrespective of their higher concentrations. Thence, particular focus should be on developing recognition elements covering a range of metals with similar affinity without compromising sensitivity. The advent of synthetic biology provides a viable alternative as genetic modifications are workable to be fine-tuned to selectively detect a broader range of heavy metals, unlike its natural counterparts.

Particularly, since living cells that are continuously dividing are being used, controlling and maintaining their growth rate such that the number of cells in the electrode surface is neither a limiting factor nor an overpowering factor for detection is paramount. Particularly maintaining the growth rate of the microbes is essential since it tends to cause complex reproducibility issues. Encapsulation of microbes is an attractive option to overcome a part of the problem while improving the sensor's shelf life. Typically, encapsulation of the microbes within hydrogels based on alginate has been widely adopted for environmental sensors [123, 136]. The alginate shell acts as a physical barrier preventing the escape of the microbes into the environment and protects the microbes from the harsh environmental stresses without compromising the entry of metals into the hydrogel due to its mesoporous structure [137]. In addition to the conventional hydrogels, encapsulation of microbes within artificial liposomes like lipids vesicles via integration with a microfluidics-based platform has also been reported [138]. One such study for lactate sensing with GFP as a reporter system using *E. coli* has reported that encapsulation of bacteria has improved not only its shelf life but also yielded a 60% higher sensor response in comparison to the non-encapsulated sensor [139].

The next major concern of using WCBs is their associated environmental risk. Considering that microorganisms are genetically modified, it is essential to be mindful of the type of modifications done on the microbes. The environmental risks associated with WCBs include the (i) imbalance in the local ecology and (ii) the threat of spreading to other locations leading to the threat of evolution into an undesirable organism. The modifications should be rendered ecologically and clinically harmless if, by chance, the organisms escape into the environment. Several genetic containment systems are being developed which could be adopted to limit the growth of the microbes used in the WCB. One such prospective containment system is based on toxin-antitoxin systems (TAs). TAs are a pair of autoregulatory genes, one of which produces a toxic protein (toxin), and the other produces a protein that inhibits the toxin (antitoxin). TA proteins form a complex and repress their operon. The expression of the TAs genes is a function

of the metabolic state of the cell [140]. TAs hold a high potential value because of their ability to kill or induce dormancy. Various toxins inhibit different targets such as cell membrane depolarization, mRNA cleavage (endoribonuclease activity), inhibition of translation, and inhibition of gyrase [141]. TA systems are implicated in plasmid maintenance within a bacterial population. Those plasmids with functional TAs tend to be maintained in the population longer than those that do not have. This phenomenon of plasmid maintenance is referred to as plasmid addiction. Any cell that loses the TAs encoding plasmid will be left with acute loss of antitoxin resulting in increased toxin activity and thereby induction of cell death/stasis [142]. Using the properties of antitoxin and toxins, one can design new genetic circuits to conditionally cause an antitoxin-dominant state (growth permissible) or toxin-dominant state (growth impermissible) [143]. TAs are potent in the containment of bacteria, but for purposes such as in WCB, we need to know when we should contain them. The power of control is artificial and bacteria itself.

For example, one could use an inducer (assuming inducible promoter upstream of TA genes) to control the growth of bacteria. The ability to regulate the TA genes' expression gives the possibility to control the population growth of bacteria. Further, the exploitation of natural genetic systems such as quorum sensing allows population density control. Bacteria with quorum sensing systems secrete compounds whose concentration is an indication of the number of bacteria within that closed environment [144]. Based on the type of quorum sensing, the bacteria elicit different responses which include stress responses, production of a modulator, and other strategies to protect the population.

One can use quorum sensing mechanisms to engineer bacteria that will grow to a population density and stop further growth [145–147]. Synthetic systems based on quorum sensing coupled with TAs could be of high value for addressing the environmental risks of using WCB (Figure 2). Optimization could be carried out based on the expression and growth regulation such that the WCBs could control their population within the location without the need for human intervention.

There is a lot of scope to further improve the speed and specificity of the WCBs by applying the versatile tools recombinant DNA technology has to offer, given that microbes are highly adaptable as per specific needs. However, when biosensors are fabricated with whole cells, other factors such as shelf life and dosage dependence are also critical parameters to be considered and optimized to be deemed as an ideal whole-cell biosensor. The linearity of dosage-dependent response is also a necessary criterion for an ideal whole-cell biosensor as quantification of heavy metals in environmental samples is essential to classify them as polluted or safe based on various guidelines (WHO, USEPA). The immobilization of the cells on the electrode surface should be optimized such that they are covalently and irreversibly bound to the electrode to prevent the washing-off of the cells from the electrode when subjected to real sample analysis.

The ultimate aim of developing WCBs is to use them in a real-world scenario for water quality analysis. Considering practical usage, these sensors should be economically feasible to procure and perform analysis. Practical usage includes the need for eliminating high technical requirements both in terms of instrumentation and the need for skilled personnel to perform the analysis and interpret the results. Finally, while the above conditions are particularly desirable for a WCB, the critical feature that restricts WCBs to only laboratories is their inability to function effectively in a complex real-world environment. The environment largely influences the functioning of living cells, and thus selection of microbes and the type of genetic modification has to be meticulously considered keeping the above considerations in mind.

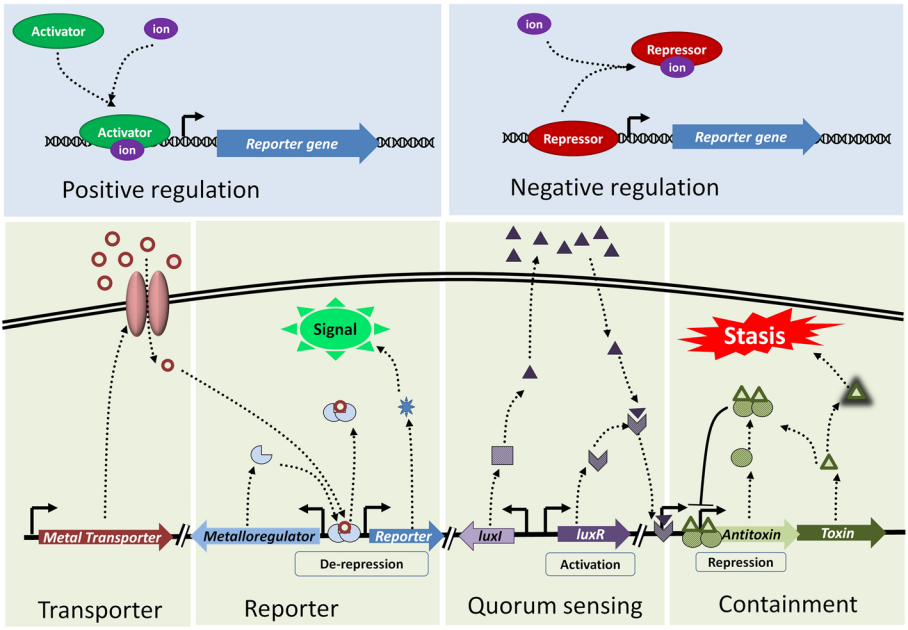

Fig. 2 Components of an ideal whole-cell biosensor. A whole-cell biosensor should consist of genes encoding for a metal ion transporter, reporter system (sensor, metal resistance gene; transducer, optical reporter gene) which is activated in the presence of the heavy metal via positive or negative regulation, quorum sensing element, and TA system for containment of the synthetic construct.

Conclusion

Taking into consideration the previously discussed challenges, an ideal whole-cell biosensor for the detection of heavy metals in water should not only consist of a metal sensing element coupled with a receptor. It should be made of four synthetic components: a metal ion transporter, reporter platform, quorum sensing element, and a containment system (Fig. 2). While the gene coding for the metal ion-specific transporter facilitates the entry of heavy metals into the cell, the reporter system acts as a sensor-transducer element. The metal resistance genes derived from bacteria function as the metalloregulatory element activating the optical reporter gene either via positive or negative regulation through activators and repressors, respectively. In addition, the inclusion of the quorum sensing element and TA system ensures the minimization of environmental threats associated with the use of synthetic constructs.

The future directions include miniaturization of these devices at a screen-printed electrode level with the incorporation of microelectronics and an integrated microfluidics setup to facilitate in situ analysis. These can further be expanded to an array of WCBs, each sensor element specific to a metal allowing simultaneous identification and quantification of all heavy metals in a sample. Riboswitches and DNAzyme-based synthetic genetic circuitry platforms in living cells hold immense potential in improving the selectivity and range of metals that can be detected due to their exceptional specificity. Further efforts should focus on better cell immobilization techniques and integration to create smart sensors consisting of a sensing interface, sampler, detector, and logic

circuits to make sense of the information and just give the exact quantity of the heavy metal in the sample as a portable setup.

Author Contribution SK performed the literature search and data analysis. BCMR and SK drafted and critically revised the work.

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Declarations

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