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Environmental Sensing of Heavy Metals Through Whole Cell Microbial Biosensors: A Synthetic Biology Approach

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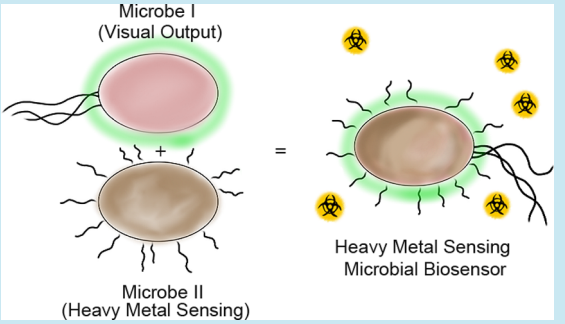
ABSTRACT: Whole cell microbial biosensors are offering an alternative means for rapid, on-site heavy metal detection. Based in microorganisms, biosensing constructs are designed and constructed to produce both qualitative and quantitative outputs in response to heavy metal ions. Previous microbial biosensors designs are focused on single-input constructs; however, development of multiplexed systems is resulting in more flexible designs. The movement of microbial biosensors from laboratory based designs toward on-site, functioning heavy metal detectors has been hindered by the toxic nature of heavy metals, along with the lack of specificity of heavy metals promoter elements. Applying a synthetic biology approach with alternative microbial chassis may increase the robustness of microbial biosensors and mitigate these issues. Before full applications are achieved, further consideration has to be made regarding the risk and regulations of whole cell microbial biosensor use in the environment. To this end, a standard framework for future whole cell microbial biosensor design and use is proposed.



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The rapid detection and quantification of pollutants in the environment is essential due to the threat they pose to ecosystems and human health. Anthropogenic activities have led to an increase in environmental contamination, resulting in a need for the constant monitoring of dangerous chemicals, compounds, and pollutants. For successful implementation of effective bioremediation strategies, rapid detection is necessary. Sites suspected of toxic compound contamination are traditionally analyzed and quantified by a number of analytical methods including atomic absorption spectroscopy, mass spectrometry, emission spectroscopy, gas chromatography, and variations of these methods.[1] However, such methods of chemical analysis are expensive, time-consuming, require transport of the sample from the site to the laboratory and provide little information on the bioavailability of the metal ions.[2,3] In addition, these technologies may not be accessible worldwide, particularly in developing countries, limiting their potential to monitor areas most likely to be contaminated due to lack of government regulatory frameworks. These factors indicate the need for devices that can continually monitor the bioavailability and concentrations of toxic compounds *in situ* in real time in areas at risk.

Biosensors technology has undergone an increased interest as a method to allow rapid detection of analytes in the environment. Biosensors are analytical devices encompassing both a biological and a transducing element for the detection of specific compounds.[4,5] These devices encompass a recognition and transducing element, with notable examples including enzymes,[6] antibodies,[7] and living cells.[8,9] Immobilized enzyme-based systems in particular have been investigated extensively due to their high specificity for detecting trace levels of compounds.[10] However, due to the expense of these types of biosensors and their sensitivity to conditions, such biosensors are not always ideal for field applications.

In homage to nature, synthetic biology has reinvigorated biosensor design in the form of whole cell microbial biosensors. Microorganisms have evolved to react to environmental fluctuations such as temperature, pH, nutrient availability, and toxic compounds. This evolutionary pressure has resulted in diverse array of regulatory elements controlling downstream signal cascades for responses to specific analytes or environmental inputs that can be utilized by synthetic biologists. Microbial biosensors are constructed through *de novo* synthesis, utilizing these naturally occurring regulatory elements to produce novel, modular gene circuits.[11,12] New developments in biosensor design have occurred, in part, due to interest through the iGEM (internationally Genetically Engineered Machine) competition. A novel modular arsenic biosensor was developed for expression in *Escherichia coli* utilizing genes from an available arsenic resistance operon.[13] Microbial biosensors have thus been developed as a means for detection of a range of heavy metals with increased specificity and sensitivity.[14−16]

Heavy metals occur naturally in a wide range of environments but are also introduced through use in industrial, domestic, agricultural, medical, and technological applications.

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Table 1. Permissible Levels of Consumption of the Most Common and Toxic Heavy Metals to Human Health[a]

metal	recommended maximum exposure	minimum current limit of detection	ref.
aluminum (Al)	100 μg/L (in water) 5 mg/day (in food)	no current limit	19
antimony (Sb)	20 μg/L	0.01 μg/L (AAS) 0.1−1 μg/L (ICP-MS)	20
arsenic (As)	10 μg/L (in water)	0.1 μg/L (ICP-MS) 2 μg/L (hydride generation or flame AAS)	21
cadmium (Cd)	3 μg/L (in water) 5 ng/m^3 (in air; annually)	μg/L (ICP-MS) μg/L (flame AAS)	22
chromium (Cr)	50 μg/L 100 μg/L	0.05−0.2 μg/L (AAS)	23
copper (Cu)	2000 μg/L	0.02−0.1 μg/L (ICP-MS) 0.3 μg/L (ICP-optical emission spectroscopy) 0.5 μg/L (flame AAS)	24
lead (Pb)	10 μg/L (in water) 0.5 μg/m^3 (in air; annually)	1 μg/L (AAS)	25
mercury (Hg)	1 μg/L (in water) 1 μg/m^3 (in air-annually)	0.05 μg/L (cold vapor AAS) 0.6 μg/L (ICP) 5 μg/L (flame AAS)	26
nickel (Ni)	70 μg/L	0.1 μg/L (ICP-MS) 0.5 μg/L (flame AAS) 10 μg/L (ICP-AES)	27
tin (Sn)	20000 μg/L (in food)	no current limit	28
uranium (U)	30 μg/L	0.01 μg/L (ICP-MS)	29

[a]These form the basis of the guidelines set for drinking water quality, published by the World Health Organization.[18]

Arsenic (As), cadmium (Cd), chromium (Cr), and mercury (Hg) have significant environmental impact due to toxicity and mobility.[17,18] A compressive list of common and toxic heavy metals is available in Table 1. Contamination of environments with heavy metals is considered to be a serious environmental issue due to the negative impact on human health. This is related to extended exposure and the high level of toxicity heavy metals pose to cells upon exposure.[14,18] Guidelines for exposure to heavy metals deemed toxic in soils and waterways are set at international, national, and local governing levels (Table 1). This includes guidelines from agencies such as the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), who recommend standards and provide information on the effect of dangerous heavy metals on the environment and human health.[17,18] To be successful, heavy metal microbial biosensors must meet and surpass these guidelines set (Table 1) for maximum levels of exposure.[18]

While microbial biosensor development has generally produced successful sensing pathways, a range of limitations, such as specificity and toxicity, have restricted their use. These issues have hindered progress toward the movement of microbial biosensors from laboratory based designs toward on-site, functioning heavy metal detectors. One such method to overcome these problems is the use of synthetic biology to create modular heavy metal sensing pathways for use in alternative microbial chassis. To be successful, functionality and issues with the potential risks which engineered microbes pose to the environment have to be addressed. While research is still novel, application of the synthetic biology bottom-up, standardized design process to microbial biosensor design may unlock further potential of these living sensors. This review will focus on the current state of heavy metal microbial biosensors and the impact of synthetic biology on the advancement of these designs. To continue further advancement of sensing technology, a set of standards for microbial biosensor design are suggested by the authors.

WHAT IS A MICROBIAL BIOSENSOR?

A microbial biosensor is an analytical device that incorporates one or more microorganisms to produce a measurable output resulting in qualitative or quantitative information. Unmodified bacteria have been used as biosensors in response to environmental fluctuations through changes in natural bioluminescence. *Photobacterium phosphoreum* was immobilized on a membrane connected to a photomultiplier for measurement of luminescence emitted by the bacteria in response to environmental variables such as glucose or toxic compounds including benzalkonium chloride, sodium dodecyl sulfate, and chromium(VI).[8] Bioluminescence was also initially utilized as a reporter signal through the insertion of a transposon containing a *lux* gene cassette from *Vibrio fischeri* into a naphthalene catabolic plasmid in *Pseudomonas fluorescens*.[30] Light production was induced within 15 min of exposure to naphthalene or aromatic hydrocarbons. The light bioluminescence producing *lux* operon was also introduced into *Pseudomonas fluorescens* and light production was found to be sensitive to a range of heavy metals with an increase in heavy metal ions correlated with a decrease in fluorescence.[31] The *lux* based system was also introduced in *E. coli* and used with *P. fluorescens* to screen for Zn toxicity in sludge extracted from sewerage systems.[31,32] These reporters rely on microbial inhibition to cause a decrease in fluorescence and are therefore prone to false positives in real world settings.

To increase specificity and sensitivity, modular constructs that rely on heavy metal responsive elements fused to a reporter genes were developed and expressed in bacteria.[9,33−35] The design of microbial biosensors initially involved stepwise ligation of nucleic acid sequences with known genetic functions into longer, increasingly complex, systems. These systems used

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biological gene circuits that could be expressed by a bacterial host in suitable expression vectors acting through a series of signal cascades. The presence of stimuli is detected and translated into a functional output, with the output regulated by the upstream gene fusions. Microbial biosensors are thus programmed to respond to inputs from the environment and to produce a corresponding, measurable output. Predominant microbial biosensor designs are based upon one input, two gene regulatory systems consisting of a promoter/reporter gene construct (Figure 1A). There is however promising investigation into multi-input systems, based on Boolean logic gates (Figure 1B), which are expanding the opportunities for such designs.

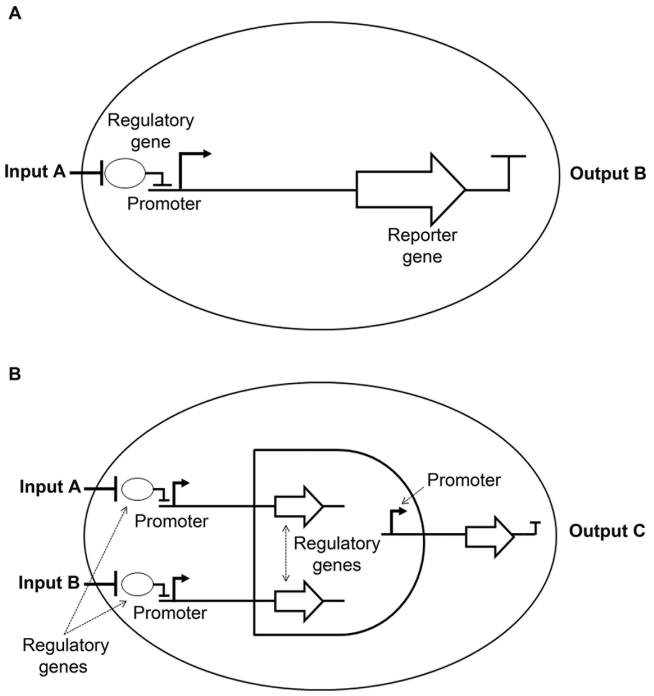

Figure 1. (A) Majority of microbial biosensors are currently single-input, modular biological pathways, encompassing a promoter and downstream reporting gene contained within an expression vector. When the bacterial cell comes in contact with the target signal, transcription is initiated downstream resulting in the expression of a predetermined output such as fluorescence. (B) Biosensor constructs are now moving toward multi-input systems based on Boolean logic gates. Multiple signals detected interact in a series of switches, transferring into a modular output.

Synthetic biology has provided the benefits to constructing whole cell microbial biosensing systems by standardizing design and construction as well as decreasing time from design proposal to functional sensitivity and specificity testing. Methods such as, Gibson assembly,[36] Golden Gate shuffling,[37] as well as a decrease in DNA synthesis costs,[38,39] allow simultaneous multiple fragment cloning reducing time and cost of construction of sensing pathways. A range of biosensors with specificity for a broad range of anolytes such as organic pollutants,[40] toxin and spore production,[41] detection of pH change,[13] and heavy metals[42] have previously been reported. Biosensors targeting single heavy metals are the forefront in biosensor design due to consequent impact on the environment.

Development of microbial biosensors has been touted as a cost-effective, alternative that will allow monitoring environmental pollutants effectively. Robust whole cell biosensor development, especially against heavy metals, would be very beneficial in isolated locations where transportation of test samples are not feasible. Such advantages and disadvantages are listed in Table 2.

An example of a simple heavy metal biosensor design couples the *ars*R gene, characterized from *Bacillus subtilis,* to *lac*Z to produce an artificial arsenic sensing construct.[13] ArsR is an autoregulated repressor protein that binds to an arsenite sensitive promoter in the absence of arsenite.[43] Presence of arsenite ions resulted in a drop in pH (<5) due to induction of *lac*Z and production of the enzyme β-galactosidase.[13] This negative autoregulation event results in a detectable and quantifiable change in pH in the presence of arsenic.[13,43] Furthermore, arsenic detection can be recorded at levels as low as 5 μg/L, half the recommended exposure limit set by the World Health Organization (WHO) (Table 1). This finding reignited interest in microbial biosensors, with the amount/number of sensors produced over the years increasing substantially. The *ars*R system has since been linked with a number of reporter genes giving an easily detectable signal such as a color change, luminescence, or fluorescence.[44−47] A broad range of single input heavy metal biosensors have been reported (Table 3) and are showing promise.

WHOLE CELL MICROBIAL BIOSENSORS OUTPUTS

In the design of microbial biosensors for real-world applications, the manner in which the output signal will be detected is an important consideration. A wide range of outputs can currently be employed in biosensor design (Table 4). These include those which produce an easily, detectable signal; such as production of colorimetric, fluorescent, or bioluminescence proteins along with changes in the environmental pH or transfer of electrons (Table 3).

Some of the most promising outputs are currently detected through visualization. A majority of heavy metal microbial biosensors are reliant on a fluorescent and luminescence based

Table 2. Advantages and Disadvantages of Microbial Biosensors

advantages	disadvantages
1. High sensitivity measurement of biologically available fraction of contaminant	1. Real world applications limited due to laws regarding the use of genetically modified organism.
2. Continuous real-time *in situ* monitoring of the environment	2. Environmental variables such as pH, nutrient availability, temperature, water availability among others may affect the biosensor function and viability.
3. Rapid and specific detection of compounds	3. Lack of long-term genetic stability of engineered system
4. Simultaneous detection of multiple compounds and with multiplexed outputs.	4. Slow diffusion of substrates and products across cell membrane into cells
5. Reduced cost and less labor intensive compared to traditional sensing techniques	

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Table 3. Current Single-Input Microbial Biosensors Designated for the Detection of Heavy Metals

target heavy metal	promoter/ reporter construct	detectable output	detection range	specificity	chassis	year	ref.
arsenite and arsenate	P*ars*/*ars*R-*phi*YFP	yellow fluorescence	up to 8 μmol/L arsenite up to 25 μmol/L arsenate	–[a]	*E. coli* DH5α	2010	44
arsenic	P*ars*/*ars*R-*lacZ*	pH change	0–20 μg/L (in nonoptimized system)	–	*E. coli* JM109	2007	13
chromated copper arsenate	*ars*B-*lux*AB	bioluminescence	10 μg/L	inhibited by phosphate	*E. coli* LF20012	1997	46
arsentate	P*ars*/*ars*R-*lacZ*	pH change	<10 μg/L	bicarbonate increased biosensor response	*E. coli* JM109	2011	47
arsenite, arsenate, and copper (Cu II)	*lux*CDABE	bioluminescence decrease	arsenate 500–2000 μg/L arsenite 11000–56000 μg/L copper(II) up to 700 μg/L (*E. coli*) up to 1600 μg/L (*P. fluorescens*)	–	*E. coli* *P. fluorescens*	2002	48
arsenite	*ars*B-*lux*AB	bioluminescence increase	1000–2500 μg/L	–	*E. coli* CM1166	2002	48
arsenic	*ars*R/*lux*AB	bioluminescence	0.05–0.5 μM	Fe (II) reduced response by up to 90%	*E. coli* DH5α	2005	49
arsenite	*ars*R/*lux*A *ars*R/*lacZ*	bioluminescence pH change	8–80 μg/L above 9 μg/L	–	*E. coli* DH5α	2003	50
arsenic	*ars*R/*crt*I	red pigment color change	0.5–500 μg/L detectable to the eye at 5.0 μg/L	effected by presence of Fe (II)	*R. palustris*	2008	45
arsenic	*ars*R/ *lux*CDABE	bioluminescence	0.74–60 μg/L	–	*E. coli* species isolated from the environment	2013	51
arsenite, arsenate, and antimony	P*ars*/*ars*R-*gfp*	green fluorescence	0.1–75 μM	no significant change when exposed to multiple metals	*E. coli* DH5α	2005	52
arsenite and arsenate	*ars*R-*lux*AB	bioluminescence	0.02–0.15 μg/g (on avg)	–	*E. coli* DH5α	2007	53
cadmium lead and cadmium	*cad*A-*luc*FF	bioluminescence	0.8 μg/L	–	*B. subtilis* BR 151	2004	14, 54
	*cad*A-*luc*FF		100–800 μg/L		*S. aureus* RN4220		
			12–100 μg/L				
cadmium	*cad*R-*crt*I	red pigment production pH change	50 nM–1 mM	no significant response	*D. radiodurans*	2013	55
	*cad*R-*lacZ*		1–10 mM				
cadmium	*cad*R-*gfp*	green fluorescence	250 μM	consecutive responses to $CdCl_2$, $ZnSO_4$ and $HgCl_{12}$	*E. coli* Top 10	2013	16
cadmium and lead	*cad*A-*luc*FF	bioluminescence	3.3 nM–1 μM (cadmium)	response to antimony, zinc and tin	*B. subtilis* BR 151	1998	14
			10 nM (cadmium) 33 nM (lead)	cadmium, antimonite, zinc, tin	*S. aureus* RN4220		
cadmium and arsenic	*ars*B-*lux*AB *cad*A-*lux*AB	bioluminescence	1–10 μM cadmium (*E. coli*) 5–90 μM cadmium (*S. aureus*) 10–100 μM arsenate and 10 μM arsenite (*E. coli*) 5–10 μM arsenite (*S. aureus*)	multiple responses to varying heavy metal ions	*E. coli S. aureus*	1993	56
chromate	P*chr*/*chr*B-*gfp*	green fluorescence	100 nM	–	*E. coli O. tritici* Sbvl1	2013	15
copper	*lux*AB	bioluminescence	300 μg/L	–	*P. fluorescens* strain DF57	2001	57
arsenic and mercury	P*ars*/*ars*R-*luc*GR	bioluminescence	arsenic 10 nM–10 μM (*P. fluorescens*) 10 nM–1 μM (*E. coli*)	–	*P. fluorescens E. coli*	2001	58
	P*mer*/*mer*R-*luc*GR		mercury 100 nM–1 μM (*P. fluorescens*) 100 nM–10 μM (*E. coli*)				
nickel and cobalt	*cnr*YXH-*lux*CDABE	bioluminescence	9 μM cobalt 0.1 μM nickel	copper decreased sensitivity (Co^2+)	*R. eutopha* AE2515	2001	59
zinc and copper	*zra*P-*gfp*/*rfp*	yellow fluorescence	16 μM (zinc)	–	*E. coli* XL1-Blue	2012	60
	*cus*C-*gfp*/*rfp*		26 μM (copper)				
zinc and zinc bioavailability	*czc*R3-*gfp*c*zc*R3-*lacZ*-*gfp*	green fluorescence	5–55 μM/L	–	*P. putida* X4	2012	61

[a] –: not reported in the study.

designs (Table 3). Green fluorescent protein (GFP) has successfully been utilized in many reporting systems when fused to heavy metal promoting elements.[15,16,52] However, due to lack of stability use of alternative fluorophores have also been investigated.[72,75] Examples include *phi*YFP, a yellow fluorescent protein.[72] When merged with *ars*R, arsenate and arsenite were detected at 25 μmol/L and 8 μmol/L,[44] respectively. While these values are not the lowest detection limit reported (0.1

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Table 4. List of Common Reporter Genes Currently Available for Biosensing Constructs

gene	protein	output	ref.
bfp	blue fluorescence protein	blue fluorescence	62
*crt*I	deinoxanthin carotenoid	pigment color change	45, 63
gfp	green fluorescence protein	green fluorescence	64−66
lacZ	β-galactosidase	pH change	67−69
luc	firefly luciferase	bioluminescence	66, 70
lux	bacterial luciferase	bioluminescence	66
rfp	red fluorescence protein	red fluorescence	62, 71
yfp	yellow fluorescence protein	yellow fluorescence	72
*mtr*CABF	cytochrome proteins	electrons	73, 74

μM/L by Liao et al).[52] *phi*YFP has been revealed to be potentially more stable during pH fluctuations in the environment.[76] Incorporation of the *phi*YFP gene into construct designs has resulted in a strong emission of light in the presence of heavy metal ions. It was also hypothesized that *phi*YFP is expressed at lower levels, more rapidly than GFP production, resulting in quicker analysis.[44]

Colorimetric responses have also been explored as an output for whole cell biosensor system.[45] One such example of a colorimetric response utilizes the red pigment, deinoxanthin carotenoid, synthesized by the *crt*I gene.[55] Four potential cadmium promoters were produced via truncating the cadmium response regulator *cad*R, and fusing with *crt*I, producing a more specific biosensor capable of detecting between 50 nM to 1 mM of cadmium.[55] The biosensor is the first constructed to detect low levels of cadmium with a colorimetric output. Overall this microbial biosensor for cadmium detection is one of the most promising, so far, in development due to its simplicity of design, cost-effectiveness, use of a novel microbial chassis (*Deinococcus radiodurans*) and requiring no additional expensive technology.[55] Use of colored proteins as a reporting tool is currently one of the most promising means of heavy metal detection. Unlike previous reporting systems, such as *lacZ*, additional substrates are unnecessary for activation. Furthermore, the ease of visualization may allow a bacterial based litmus test to be developed, for rapid, on-site use.

Another promising reporter system is based on the electron transport system identified in the anaerobic, soil microorganisms genera, *Geobacter* and *Shewanella*. Such bacteria utilize a cytochrome system to allow the transfer of electrons from a viable electron donor in the environment.[77−79] This process can be converted to a readable output by providing a material based electron acceptor, such as graphite. An electrical signal, produced by the electrode associate microbe, can be detected with inexpensive equipment such as a voltmeter. Initial research into use of production of electrical current has resulted in the assembly and expression of these cytochrome systems (in particular the MtrC, MtrA, and MtrB proteins) from *Shewanella oneidensis* MR-1 into *E. coli* DH5α.[73] This construct was shown to allow *E. coli* DH5α to utilize insoluble metal oxides via the transfer of electrons. The authors query that linking of this gene cassette to promoter elements, resulting in a novel biosensing construct with an electrical output.[73] Use of this system is further discussed later in this paper.

The variability of output signal due to bacterial growth stage is an important consideration in biosensor function. Investigation into the functionality of a chromate microbial biosensor revealed that accurate and optimal detection was reliant on the growth phase of biosensor, with levels of fluorescent varying between stages of growth, along with media used (rich vs minimal).[15] This was further supported by Sharma et al.[51] who reported that late and stationary growth phase decreased the emission of light in response to arsenic ions. Biosensors with an exponential growth phase requirement will have reduced limited application in environmental sensing.

The range of outputs investigated so far is yielding promising methods to crossover from lab based signals that can function in real world situations. In particular, those producing a visible color signal may prove most useful to on-site rapid testing methods. However, for long-term monitoring of contaminated sites, use of an electrical based system will provide a continuous means to determine levels of heavy metal ions and to transfer the signal, long distance for off-site monitoring. Currently, no method of signal transduction appears to be unaffected by environmental variables, and as such, further research is needed.

WHOLE CELL MULTIPLE INPUT MICROBIAL BIOSENSORS

Most current heavy metal microbial biosensors are constructed as single input systems. However, there are current design methods suggested for the detection of multiple inputs, which are based on the incorporation of boolean logic gates for regulated cell signaling.[80−83] Logic gates are commonly used in engineering where one logical output is produced after the detection of multiple signals. Integration of logic gates into plasmid-based sensing networks may result in multiple precise outputs in response to different inputs. Use of this design has been demonstrated via the design of a construct consisting of a two-input "AND gate" pathway.[82] The "AND gates" will only be expressed when both inputs are detected. If either input is absent then no signal will be resultant. Such circuitry has been of interest to synthetic biologists to allow the engineering of modular and orthogonal genetic logic gates for robust biologically based digital-like devices.[82]

Logic gate constructs have previously been reported for detection of a range of heavy metals and quorum signals.[80] Incorporation of multiple metal promoter elements into a logic gate design include: arsenic; mercury; copper; and zinc, into a single sensing construct in *E. coli* and *Pseudomonas syringae*, linked with a fluorescent output.[82] Double input AND gated biosensors were first generated utilizing two-input sensing pathways, utilizing two genes (*hrpR* and *hrpS*) along with the HrpL promoter element, isolated from the plant pathogen, *P. syringae*.[82−84] The gens *hrpR* and *hrpS* encode a protein complex, which activates a promoter for downstream transcription. Three individual AND gates were designed for the sensing of either: As^{3+} and Hg^{2+}; $Cu^{2+}$and the $3OC_6HSL$ quorum sensing molecule; or Zn^{2+} and Pb^{2+} or Cd^{2+}.[83] A three input cellular biosensor was then created by coupling two cell consortia containing the AND gates via quorum sensing molecules. In the first consortia, an AND logic gate utilized ArsR and MerR to active the production of $3OC_6HSL$ when both As^{3+} and Hg^{2+} are present. The second cell consortia would produce a quantifiable fluorescent output in the presence of $3OC_6HSL$ and Cu^{2+} due the interaction of LuxR and CusR with their respective promoters.[84] These genetic logic gates have the potential to function as a biological filter and an amplifier to enhance the sensing selectivity and sensitivity of cell-based biosensors. Four ribosomal binding sites with strong to very weak strengths were cloned upstream of the reporters to moderate input-output strength.[83] This system demonstrates the versatility of whole cell biosensors and the ability to

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combine different consortia through intercellular signaling. Such applications of synthetic biology toward multiplexed biosensors indicate a potential for improved biosensors.

SPECIFICITY OF HEAVY METAL BIOSENSORS

Despite the construction of a promising number of heavy metal microbial biosensors, challenges are faced in terms of both the toxicity of the heavy metal to the bacterial host and specificity of the input modules. In many cases low levels of other compounds are detected by the microbial biosensor that can result in false positives. An example is the cadmium biosensing constructs based on *cadC*, which responds to a range of ions including lead, manganese, mercury, antimony, zinc, and tin.[14,16,56] The gene *cadC* is part of the CadA cadmium resistance operon that is responsible for cadmium ion efflux from the cell. *CadC* was cloned upstream of the luciferase gene, *lucFF*, and expressed in two microbial chassis, *Staphyloccoccus aureus* RN 4220 and *Bacillus subtillus* BR151, with performance of the cadmium sensing construct assessed under identical conditions in both chassis. It was found that ions which induce luminescence of the cadmium biosensors in *S. aureus* included cadmium (10 nM), lead (33 nM), antimonite (1 nM), tin (3.3 μM), mercury (33 nM), zinc (1 μM), manganese (33 μM). The biosensor detected a minimum of 10 nM cadmium, however, once a concentration of 1 μM was reached, cell death occurred. *B. subtilis* was able to detect cadmium (3.3 nM), antimony (33 nM), zinc (1 μM), and lead (100 μM).[14] Further lack of specificity has been observed in a range of other heavy metal biosensors including a cadmium biosensor responsive to zinc, sulfate, and mercury.[16]

In terms of on-site functionality and reliability, lack of specificity to the target metal ion may result in unsafe ingestion due to inaccurate quantification.[44] It may be argued that interference results only in low levels of reporter expression, however, one of the appeals of microbial biosensors development is the promise of accurate low level heavy metal quantification. Specificity appears to be impacted by the structural similarity between heavy metal ions and other compounds. This may result in nonspecific binding at ligand binding sites on the biosensing construct.

In some cases, however, it has been reported that there is no interference by heavy metal ions. A novel chromium GFP biosensor developed by Branco[15] et al. based upon putative *chr* promoter and *chrB* regulator, isolated from *Ochrobactrum tritici* 5bvl1, reported very minor interference from previously reported analytes.[15,85] Testing was performed by exposure to water samples from a Portuguese river containing variable concentrations of chlorides, magnesium, selenium, and barium, also contaminated with chromium ranging 1−10 μM, which resulted in no effect on GFP expression.[15]

OVERCOMING THE TOXIC NATURE OF HEAVY METALS

Additional challenges of microbial biosensor design are that of the toxic nature of metals to the microbial chassis. Although, bacteria have evolved internal mechanisms to deal with increased levels of heavy metals high concentrations found in nature may prove toxic and consequently inhibit the biosensing construct or result in death of the biosensor entirely.[44] Cadmium biosensors, in particular, have reported susceptibility to cadmium toxicity.[55,86] While we are more interested in the lower levels of heavy metal concentrations, a durable sensor is still desirable to avoid failure due to increased levels of heavy metal contaminants.

To overcome chassis susceptibility, biosensing systems may utilize genetic resistance mechanisms to increase heavy metal tolerance. An example of this is a biosensing construct involved the amalgamation of *znt*A gene to *lac*Z expression.[86] The *znt*A mutant has previously been shown to increase resistance to cadmium and zinc ions via the encoding of an ATPase drive efflux pump. Exposure to low levels of cadmium (50−1000 nM in seawater, 5.34 μM in soil) resulted in a detectable pH change and exposure to <10 μM cadmium resulted in a decrease of β-galactosidase expression and signal due to cell death.[86] Similar results have been shown in alternative cadmium biosensors.[55]

Currently, the majority of biosensing constructs are expressed in variants of *E. coli* (Table 3). Use of alternate bacterial or yeast species may result in more robust biosensor less affected by environmental variables. Previous studies have noted that the ability to tolerate heavy metals is also, in part, dependent on the cell wall of the microbe, in terms of Gram-positive or negative.[87,88]

EXPANSION OF MICROBIAL CHASSIS FOR HEAVY METAL BIOSENSORS

Microbial chassis have intrinsically different resistance levels to heavy metals. This may be taken advantage of for construction of whole cell biosensors when chassis with higher heavy metal tolerances are used. While a goal of synthetic biology is to have transferable modules across chassis, currently expression levels of various constructs can differ greatly between microbial chassis.[14] One such example is of a cadmium biosensing construct was expressed in both *S. aureus* RN4220 and *B. subtilis* BR151. It was found that overall *S. aureus* was more sensitive with a higher induction coefficient to lower levels of other heavy metals (lead and antimony). However, in terms of sensitivity to cadmium only, *B. subtilis* proved superior under the same environmental conditions.[14] A uranium biosensor created in *Caulobacter crescentus*, experienced no interference from nitrate, lead, and chromium with minimal interference by cadmium.[42] In addition, this biosensor showed a high tolerance to cadmium (48 μM), uranyl, and uranium. These examples highlight the need to properly test and characterize each sensing construct within different microbial chassis. A number of biosensors are utilizing alternative microbial chassis with success, as seen with the use *Deinococcus radiodurans*[55] *Pseudomonas putida*,[61] and *Rhodopseudomonas palustris*,[45] which contain intrinsic resistance to certain heavy metals. However, comparison of microbial biosensing constructs is currently under-studied with the aforementioned results indicating that this is a necessary step in optimizing microbial biosensors even with the application of synthetic biology.

To further overcome chassis sensitivity, the isolation of heavy metal tolerant bacteria isolated from contaminated environments is being explored.[51] Thirty-nine *E. coli* strains were isolated from water samples from locations in India with varying levels of arsenic contamination. The *E. coli* strains were screened with exposure to arsenic by minimum inhibitory concentration tests (MIC) to determine intrinsic resistance before transformation with arsenic biosensing constructs.[51] This resulted in a microbial biosensor detecting arsenic as low as 60 μg/L with very minimal responses (recorded as light emission) for mercury, cadmium, zinc, and lead in comparison to arsenite and arsenate.[51] A previous study found 14 isolates resistant to arsenic were also resistant to copper, cobalt, lead,

nickel, molybdenum, chromium, selenium, antimony, tin, and silver (50000 μg/L), suggesting that general resistance mechanisms may be useful for microbial biosensors against a range of contaminants.[89] Use of microbes isolated from these sites may result in a more robust biosensor. However, a minority of metals in high enough concentrations are still proving to be toxic to biosensing constructs (cadmium, mercury, tellurium, and zinc).[89]

MICROBIAL BIOSENSORS IN ACTION: FROM LAB TO FIELD

There has been a plethora of research regarding the use of microbial biosensors as an alternative means for environmental monitoring; however, movement from lab to field has been hindered. This is resultant from a range of acknowledged problems associated with microbial biosensor technology (Table 2) including unreliable output response to inputs under environmental conditions. This is partly due to the unpredictability of natural environmental fluctuations including responses to the resident microbial communities, change in pH and nutrient availability. Few biosensors presented in the literature have been tested for reliability in field settings. Some of these problems have been overcome with production of commercialized, compartmentalized biosensors. Trademarked under different names including MicroTox, ToxAlert, and Biotox such toxicity tests are based upon the production of luminescence by *Vibrio fischeri* exposed to compounds of interest.[90] Exposure is within a confined, environment preventing accidental release. While advantageous for detection of toxins and pollutants, such tests have not been refined for heavy metal detection.[90−92]

Along with previously discussed problems including specificity, toxicity, and choice of output are problems associated with accidental release or gene transfer from genetically modified microbes (GMMs). Overcoming previous issues associated with recombinant DNA technology, synthetic biology is producing a range of novel solutions for management of biological risk, which are likely to be applied to microbial biosensor design in the future.

Design and construction of novel biological pathways must take into consideration a number of factors: gene flow between modified organisms and the resident microbial community, stability of the construct, and the potential for dangerous mutation. To combat such concerns synthetic microbes must have both biological and physical constraints to decrease chance of accidental spread. A major concern is that of horizontal gene transfer from GMMs to the environment. One such example is of kill-switch systems employed.[93,94]

Due to the potential for mutations that could inhibit and render kill switch safety systems ineffective, multiple safety measures will be required to be implemented. Furthermore, it has previously been indicated that free DNA in the environment may persist over time and has the potential to be taken up by resident bacteria.[95] A recent method which has been developed with potential to counter such risks is emerging in a field known as orthogonal biology or xenobiology.[96] Orthogonal biology relies upon the creation of completely novel nucleic acid system and sequences, which do not interact with a microbe's natural machinery. Orthogonal biology makes use of XNAs (Xeno Nucleic Acids), which produce a different backbone to DNA through formation of a double helix[96] becoming "invisible" to DNA polymerases and thus natural biological systems. Production of unnatural molecules not recognized by natural host machinery avoids the chance of gene transfer and spread through the environment.

Further safeguards include the tracking GMMs in the environment. This is made possible via the inclusions of synthetic watermarks as demonstrated by Gibson et al.[97] The idea of incorporation of genetic markers is not new;[98] however, the method is now presented as a means to identify the cell as synthetic. Watermarks have been included in pathway designs at intragenic sites of a synthetically constructed genome of *Mycoplasma genitalium*.[99] Sequences are designed to cause little interference with natural host machinery or amino acid and to only act as encrypted identification tags.[98] This approach may prove useful when tracking GMMs within a mixed community environment.

In parallel with internal safety mechanisms, is the use of physical confinement as an additional level of security. Production of a biosensor combined with microbial fuel cell technology (MFC) is a potential novel means for a functional on-site biosensing while physically removing GMMs from the environment.[74] Transcription of proteins responsible for outer membrane cytochrome (OMC) formation (MtrA, MtrB, and MtrF), facilitated electron transfer from the microbe *Shewanella oneidensis*.[74] Electron transfer was driven by an L-arabinose promoter after providing a stimulus and lactate as a carbon source. An electrical current was produced indicating that such an organism may prove a model organism for further biosensing applications.[100,101] Such a system provides a potential manner of containment of GMMs, while providing real-time environment analysis results. It is considered that both physical containment along with inbuilt molecular safety are both necessary as it is often speculated that no system is fail-safe.[94] As pointed out, predominant use of synthetic microbes already produced are contained within enclosed facilities.[102]

Along with the associated risks of accidental release is the transportation and activation of biosensors to the site of testing. Several microbial biosensing studies have investigated the effect of transport and freeze-drying of the cells. The microbial biosensor was reconstituted on site, a day prior to use with no detectable negative effect on output and resulted in accurate quantification.[14] This method has more recently been repeated with an arsenic biosensing construct via air-drying and freeze-drying, with no adverse effects noted.[49] However, previous investigation does indicate that freeze-drying may result in increased long-term efficiency.[49] An additional factor to be addressed is the conditions necessary for activation of the microbial biosensor. This is highlighted in a study that utilized the *cadR* gene fused to a GFP reporter in an *E. coli* chassis.[16] This simple design was optimized for laboratory conditions and while demonstrated to be a viable biosensor the need to incubate the sample at 30 °C for 4 h shows that not all biosensors are practical for real-world applications.

Few biosensors have been extensively tested in real-world conditions. The first large-scale field trial involving a microbial biosensor was in 2005 testing an arsenic construct. Based in *E. coli* DH5α (*ars*R-*lux*AB) with the presence of arsenite resulted in downstream production of luciferase.[102] Groundwater samples from 194 locations in Vietnam (from known arsenic contaminated sections of Red and Mekong rivers) were collected and stored. Optimal biosensing conditions were found to include the addition of HNO_3 resulting in acidification and the addition of the bacterial suspension (1:1 volume) before a final addition of n-decanal to neutralize the final solution.[102] Quantified values found a linear proportion of

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Table 5. Proposed Standard of Criteria in Regard to Novel Microbial Biosensor Construction in Keeping with Synthetic Biology Framework

criteria	description
1. Specifictiy	ensure rigorous testing of functional biosensors in the presence of a range of stimuli and conditions
2. Detection levels	Stimuli detection levels must be at or below reasonable exposure levels as set by the world health organization (WHO) or equivalent
3. Reproducible results	Over time and between samples the quantitative result must stay at set detection limits of criteria 2
4. Ease of use	The method used for recording the biosensor response should be relatively simple to detect and quantify without the need for expensive on sight equipment
5. Longevity	The microbial biosensor must maintain function for the period of time required for monitoring
6. Rapid response	Response times for stimuli detection should be rapid; <1 h
7. Inbuilt safety switches	Methods should be employed to prevent gene transfer, uptake of foreign DNA, or potentially harmful mutations
8. Risk-free application and detection	Minimal risk must be associated with testing of contaminated sites with no potential harm to the user or environment
9. Ease of assembly	The biosensor should be easily transferable to contaminated site locations and useable with minimal training

luciferase in response to arsenite (0−75 μg/L). Cross-analysis of the biosensor values to atomic absorption spectroscopy (AAS) found that the reported levels of arsenic contamination had resulted in only 8% false-negatives. This is reportedly lower than the same value reported by chemical tests. This field-trial boasts a means in which a microbial biosensor may be employed as a rapid screening technique.[102] The optimized protocol allows for accurate contaminant quantification; however, the additional chemicals needed does hinder the ease of process. Despite this, in comparison to alternative chemical and technological methods available, this research is a step forward toward reliable real-world assays.

FRAMEWORK FOR FUTURE BIOSENSOR DESIGN

Despite the challenges outlined, the widespread development of optimized, functional microbial biosensors is well in progress. However, to date there has been little movement toward the development of a standard framework to aid development of optimal microbial biosensing constructs. The secondary wave of biosensor designs should focus on movement of microbial biosensors out of the 'proof of concept' phase and into novel sensors with field potential. To move the large assortment of available microbial biosensors toward real world applications, a set of criteria or 'standards' is required to aid the process. By setting guidelines for construction of microbial biosensors, moving from laboratory to field trials could be increased, saving time and money. As such, a standard framework is proposed in this review for future designs and construction of microbial biosensors (outlined in Table 5). While such criteria have partially met during novel biosensor construction, few biosensing designs meet the whole standard.

RISK AND REGULATION OF GENETICALLY MODIFIED MICROBES (GMMS)

The goal of synthetic biology is to link technological output to real world applications, including production of medically relevant compounds[103,104] and synthetic biofuels.[105] With the advancement of synthetic biology toward commercialization, discussions of risks and regulations are necessary. Synthetic biology now faces similar hurdles with which research in biotechnology and genetically modified organisms (GMOs) has dealt. Talk of biotechnology risks and regulation may be linked back to the Asilomar conference in the mid 70s, which incited discussion of recombinant DNA technology resulting in a range of recommendations for risk management.[106] Recommendations still applicable include the necessity for both physical and biological barriers (including choice of a fastidious host and nontransmissible vectors).[106] It was also pointed out that self-regulation of laboratories and appropriate national laboratory standards met is important. Constant reassessment and education would also allow careful monitoring of potential risks.[106]

In terms of biosecurity, the monitoring and screening of the synthesis of DNA have been deemed necessary to ensure that accountability with some external monitoring already in place.[107] The Massachusetts Institute of Technology (MIT) is currently leading movement toward an open-source type style of research providing functional gene sequences via the registry of standard biological parts.[11] This method of obtaining gene sequences is highly self-regulated; however, biosecurity fears are low due to the minimal risk factor associated with sequences obtainable. The idea behind the Biobrick registry, and indeed the synthetic community, is to allow freedom of information and open source parts available to those interested in ethical, scientific advancement. Parts are freely available and thus avoids potential future advancements limited via patents.[108] Intellectual property (IP) laws are set in place to legally protect the rights of the creator to their creative work. Such rights include copyrights, trademarks, and patents. While such laws have been developed to protect the owner, such roles may prove to have a detrimental impact upon synthetic biology, hindering scientific research and development.[109] The synthetic biology community tends to push toward an open-source approach to molecular information. Allowing accessible DNA sequences is a corner stone. Analysis has revealed that there is an increase of patents based in synthetic biology, driven by new companies and leading universities, predominantly in the U.S.[110] It is also hypothesized that the number of patents can be used to determine the commercial appeal of the research.

While such measures are still upheld today, fear of genetically modified organisms and products has the power to halt the synthetic biology community. Similar framework derived from GM laws may in theory help regulate synthetic biology; however, just as synthetic biology has advanced, so must these laws.[101] With increased knowledge on function, a more flexible set of rules that incorporates self-regulation may result in quicker advancement in this field and with technological achievements. To crack down and highly regulate synthetic biology, as has been shown in the past, may prove highly disadvantageous for the field and the applications for the general public. In a synthetic biology driven world, communication of risks should be transparent, with embedded safety measures and reliability. Furthermore, maintaining positive public relationships is integral to mitigate fear. Thus, a pro-active approach may result in a mutually beneficial future for synthetic biology processes.

Microbial biosensors face social fear that may only be alleviated with implementation of a broad range of inbuilt safety mechanisms along with open-communication between the scientific community and the public.[111] The implementation of an international regulatory standard that is agreed upon by the scientific community, public, and politicians may alleviate this fear. Alongside this it may be suggested that evidence based risk assessment of synthetic biology research is on a case-by-case basis. Only through communication and an understanding of the science behind synthetic biology can we continue to move forward with development of biosensors. In either case, investigation of regulatory framework indicates that updated policies are necessary,[111] which has previously been discussed among the synthetic biology network.

CONCLUSIONS

The use of whole-cell microbial biosensors as a novel method for the detection of heavy metals is proving to be a viable alternative to current chemical and physical methods of detection. There are several limitations to biosensor design, such as interference by environmental compounds and the toxic nature of heavy metals to the microbial chassis. Many research groups have begun to look toward the use of alternate microbial chassis for these heavy metal sensing pathways, resulting in increasingly robust biosensors. Furthermore, many current biosensor designs are now leading to increasingly specific sensors, far surpassing the recommended exposure guidelines set by the world health organization. This is in part due to the synthetic biology work process, which is heralding in the potential for new opportunities in biosensor development, such as the construction of multiplexed systems via the use of Boolean logic gates. What is currently needed is increased research into alternate sensing pathways and novel microbial chassis. This may aid the movement of microbial biosensors from laboratory based experiments to working, field based sensors. Such developments require the implementation of safeguards to avoid accidental gene transfer or release. Methods currently showing promise include the use of synthetic XNA, "watermarks" to allow tracking of genetically modified microbes, and enclosed containment devices. To aid this development a series of criteria may provide a standardized framework in which future biosensing constructs can be designed around. This will ensure continued movement forward of heavy metal whole cell microbial biosensor designs.

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Notes

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