Biosensors and Bioelectronics 191 (2021) 113359

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Microbial whole-cell biosensors: Current applications, challenges, and future perspectives

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ARTICLE INFO

Keywords:
Microbial whole-cell biosensor
Living cells
Synthetic biology
Reporter protein
Environmental and agricultural monitoring
Biomedical diagnostics

ABSTRACT

Microbial Whole-Cell Biosensors (MWCBs) have seen rapid development with the arrival of 21st century biological and technological capabilities. They consist of microbial species which produce, or limit the production of, a reporter protein in the presence of a target analyte. The quantifiable signal from the reporter protein can be used to determine the bioavailable levels of the target analyte in a variety of sample types at a significantly lower cost than most widely used and well-established analytical instrumentation. Furthermore, the versatile and robust nature of MWCBs shows great potential for their use in otherwise unavailable settings and environments. While MWCBs have been developed for use in biomedical, environmental, and agricultural monitoring, they still face various challenges before they can transition from the laboratory into industrialized settings like their enzyme-based counterparts. In this comprehensive and critical review, we describe the underlying working principles of MWCBs, highlight developments for their use in a variety of fields, detail challenges and current efforts to address them, and discuss exciting implementations of MWCBs helping redefine what is thought to be possible with this expeditiously evolving technology.

1. Introduction

The first microbial cell-based biosensors developed in the late 1970s coupled the known fermentation of organic compounds, production of electroactive metabolites, or change in respiratory activity of the cells with an electrochemical transducer capable of measuring these phenomena (Turner et al., 1987). While these early cell-based biosensors presented an exciting and cost-effective alternative to enzyme-based biosensors, they could not compete with the high selectivity of enzyme-based biosensors of the time. Indeed, enzyme-based electrodes dominated the biosensing and bioelectronics field from their inception, but microbial cell-based biosensors soon radically evolved thanks to the arrival of recombinant DNA technologies. With new molecular tools at their disposal, investigators began engineering microorganisms to house foreign proteins of interest that produce a unique and quantifiable signal in response to a target analyte instead of relying on the microbes' naturally occurring reactions to said analytes.

In the years since, rapid advancements in the fields of microbial physiology and synthetic biology have resulted in the development of a variety of microbial cell-based biosensors. For the purposes of this review, it is important to clarify the distinction between Microbial Surface-Display Biosensors (MSDBs) and the subject of this work, Microbial Whole-Cell Biosensors (MWCBs). Despite the fact they both employ microbes for their construction, there exist fundamental differences in their underlying working mechanisms and sensing functionalities which distinguish them as two different classes of biosensor.

In its most basic form, a biosensor is defined as an analytical device which incorporates a biological sensing element integrated or closely associated with a transducer (Pandey and Malhotra, 2019). In MSDBs, microorganisms are typically engineered such that foreign proteins of interest are transported and immobilized onto the microbial cell surface by genetically fusing them onto the anchoring motifs of outer membrane

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https://doi.org/10.1016/j.bios.2021.113359
Received 23 March 2021; Received in revised form 13 May 2021; Accepted 15 May 2021
Available online 23 May 2021

proteins. The activity of these surface-displayed proteins, i.e., inhibition rate or catalytic activity, in response to a target analyte is then measured as a means of quantifying levels of a target analyte. As such, in the case of MSDBs, the surface-displayed enzyme is the biological sensing element whose activity is closely associated with a transducer, while the microbes serve as the matrix onto which they are immobilized (Han et al., 2018). The field of MSDBs development is growing rapidly, and various biosensors have been developed for the detection of an array of molecules of interest such as paraoxon (Han and Liu, 2017), formate (Liu et al., 2016), glutamate (Song et al., 2015), organophosphates (Tang et al. 2014a, 2014b), and monosaccharides (Li et al., 2012; Liang et al. 2012, 2013). Notably, given the stability and lack of complex purification processes of surface-displayed enzymes, MSDBs have also demonstrated great potential for their use in biofuel cells (Fan et al., 2015; Feng et al., 2016; Lang et al., 2014). A comprehensive review on the working mechanisms, applications, and shortcomings of MSDBs can be found elsewhere (Han et al., 2018).

Alternatively, MWCBs involve the assimilation of molecular recognition of analytes with signal generating proteins to create a sensing microbe. The integration of the sensing microbes with a transducer element converts the recognition event into a measurable output for the detection of a target molecule(s). The engineered microbes typically harbor modified genomic or plasmid DNA with genes for sensing proteins that regulate the production of a reporter protein in response to a target analyte. Thus, in MWCBs, the whole microbial cells operate as the biological sensing elements that generate a quantifiable signal, as opposed to functioning as matrices for protein immobilization. Furthermore, by taking advantage of the sensitive molecular recognition between proteins and a target ligand, MWCBs allow for the selective determination of the bioavailable analyte, or class of analytes, in a dose-dependent manner (Struss et al., 2010). Providing physiologically relevant data on the bioavailability of a target analyte is a key functional feature of MWCBs, seeing as how the target analyte must first interact with the living microbes to induce a response. Moreover, as opposed to other protein-based sensors, MWCBs are typically inexpensive to prepare and store, can withstand variations in temperature or pH, and can be conveniently engineered for miniaturization, high-throughput analysis, and multi-plexing purposes (Struss et al., 2010).

MWCB's are generally categorized into two different types, according to the mode of expression of the reporter protein: constitutive or inducible (Gu et al., 2004). Constitutive expression systems were the earliest versions of MWCBs and involve the constitutive expression of the reporter at high basal levels. The intensity of the signal generated by the MWCBs decreases in direct proportion to the toxicity of the sample being analyzed as a result of either decreased metabolic activity or cell death. It should be stressed that constitutive MWCBs are non-selective and will respond to any type of substance that is toxic to the cells, therefore their utility is somewhat limited as they cannot be used to determine the type of toxin present (Ramanathan et al., 1997a). However, constitutive MWCBs have been successfully employed for toxin detection in environmental monitoring, such as in the commercially available Microtox assay (Bulich and Isenberg, 1981), and more recently for the detection of urinary tract infections (Reyes et al., 2020). These techniques can assess the relative level of toxicity of a given water, soil, or sediment sample, and presence of pathogens in urine samples, based on the changes in light production of living luminescent bacteria.

Ultimately the desire for an analyte specific MWCB, along with rapidly increasing knowledge on the fundamentals of microbial gene regulation, spurred the creation of inducible MWCBs. Briefly, in microbial gene regulation, there are DNA sequence elements and regulatory protein elements that work in tandem to regulate the expression of genes in the microbial genome. Crucial to the transcription of a given gene is the binding of RNA polymerase to what is referred to as the promoter region, an essential DNA sequence element. Typically, regulatory proteins bind onto promoter regions to regulate RNA polymerase activity. Some regulatory proteins facilitate the binding of RNA polymerase to a promoter region, thereby up-regulating the expression of a gene, while others inhibit said binding to down-regulate gene expression. Microorganisms have evolved a remarkable network of DNA sequence and regulatory protein elements that respond to specific cues from their environment to help ensure their survival. With the proper combination of promoter elements and regulatory proteins, these networks can be exploited to engineer inducible MWCBs that regulate the expression of a reporter gene in response to a target analyte (Daunert et al., 2000).

Early inducible MWCBs took advantage of the known resistance certain bacterial strains had evolved to a variety of metals and metalloids, helping establish the framework for the development of inducible MWCBs (Ramanathan et al. 1997b, 1998; Scott et al., 1997). Briefly, MWCBs for the sensitive detection of antimonite and arsenite were engineered based on the *ars* operon present in certain strains of *Escherichia coli*. Plasmids were constructed such that they carried a section of the *ars* operon containing the *arsR* regulatory protein and part of the *arsD* gene fused to the reporter gene. In the absence of antimonite and arsenite, the ArsR regulatory protein is bound to the *ars* promoter and inhibits transcription of the reporter. Arsenite and antimonite induce expression of the reporter by binding to ArsR and causing its release from the *ars* promoter, thus allowing transcription of the reporter to occur. In order to highlight the versatility of inducible MWCBs, both an electrochemical MWCB using the *lacZ* reporter (Fig. 1) and a luminescent MWCB using the *luxA* and *luxB* reporters were developed, based on the same expression system previously described.

In the years since, knowledge on tightly regulated genetic circuitry has greatly expanded and, as such, the MWCB field has focused less on constitutive expression systems and more on the development of inducible expression systems. The original MWCBs for metal and metalloid detection which demonstrated the working principle underlying inducible expression systems left room for improvement, and there have been many variations on their design which will be discussed further into this review. However, the general framework behind the construction of inducible expression systems remains largely the same: constitutive basal expression of a regulatory protein, which binds specifically to a target analyte, creates a protein-analyte complex which initiates expression of the reporter via an inducible promoter. Thus, in the absence of the target analyte the reporter is expressed at low basal levels, while in its presence the reporter is expressed in a dose-dependent manner. Depending on the nature of the promoter, inducible expression systems can be either negatively or positively regulated. In negative regulation, the regulatory protein is bound to the promoter region and inhibits expression of the reporter gene. In the presence of the analyte, the analyte binds to the regulatory protein, which in turn releases the promoter and enables the expression of the reporter. In positive regulation, the analyte-regulatory protein complex binds to the promoter and initiates expression of the reporter protein (O'Connor et al., 2015; Raut et al., 2012). A visual representation of the underlying working principles of constitutive and inducible MWCBs can be seen in Fig. 2..

The choice of regulatory protein and promoter controlling the expression of the reporter is of utmost importance to the detection limit, sensitivity, response time, and selectivity of the MWCB to a target analyte. Even so, special care must be taken to select a reporter protein which best serves the intended use of the MWCB. The signal from the selected reporter protein is detected by coupling to a transducer element for optical (i.e., colorimetric, fluorescent, bioluminescent, chemiluminescent) or electrochemical analysis. Advancements in recombinant DNA technologies have yielded an arsenal of reporter proteins sourced from the genetic code of a variety of organisms for use in MWCBs. A comprehensive list of reporters typically employed in MWCBs, their detection methods, advantages, and limitations, can be found in Table 1. More detailed descriptions of the origin, mechanism of action, and profiling of these reporters can be found in elsewhere (Daunert et al., 2000; Lopreside et al., 2019).

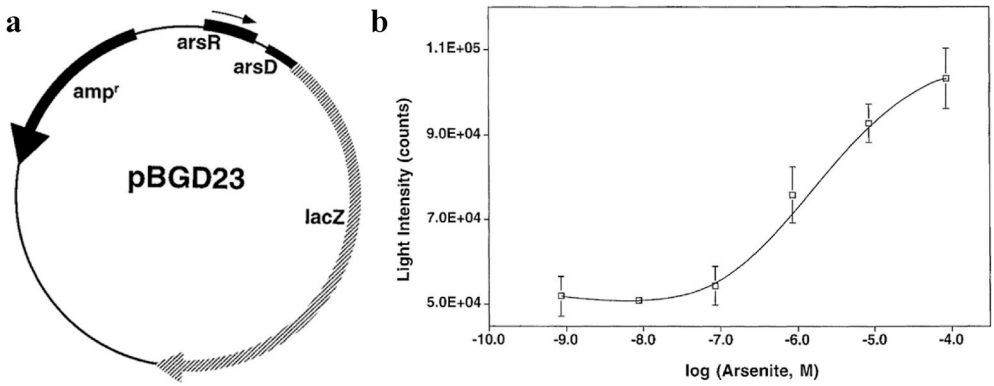

Fig. 1. **(a)** Plasmid map of the vector pBGD23 employed by the inducible MWCB for arsenite detection carrying genes for the arsR regulatory protein controlling expression of the β-galactosidase (lacZ) reporter protein. **(b)** Dose-response curve of the inducible MWCB for arsenite detection based on the chemiluminescent signal generated from β-galactosidase expression. Adapted from Scott et al., (1997) and Ramanathan et al., (1998).

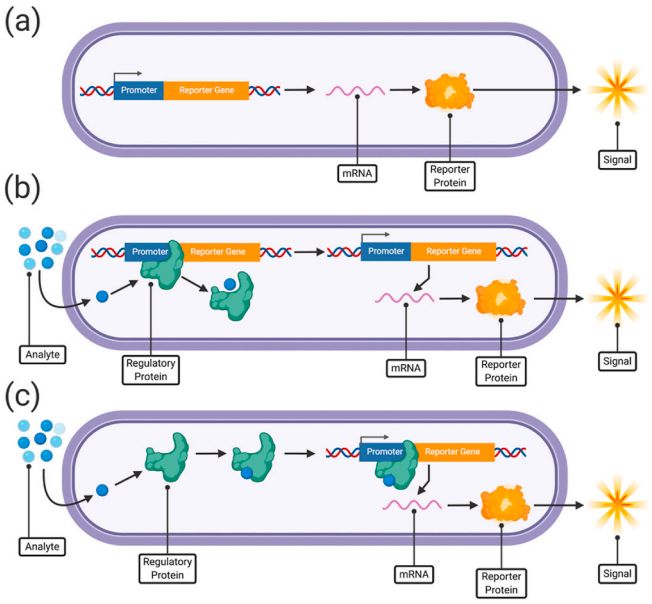

Fig. 2. Types of Microbial Whole-Cell Biosensors based on reporter expression. **(a)** Constitutive MWCB with no regulation of reporter expression. The presence of toxic substances reduces metabolic activity and/or causes cell death, resulting in a decrease in reporter signal. **(b)** Inducible MWCB with negative regulation of the reporter. The regulatory protein inhibits expression of the reporter via the promoter. Binding of the analyte to the regulatory protein releases it from the promoter, thus inducing expression of the reporter. **(c)** Inducible MWCB with positive regulation of the reporter. The regulatory protein - analyte complex binds specifically to the promoter, inducing expression of the reporter.

2. Established applications of microbial whole-cell biosensors

For the last quarter century, MWCBs have gained popularity in a wide range of applications, as they offer a simple and cost-effective alternative to assess analytes of interest. From the environmental monitoring of metallic trace elements (MTEs) in contaminated soil, polychlorinated biphenyls (PCBs) levels in wastewater, quorum sensing molecules in disease states, microbial communities of healthy soils, to organic pollutants in industrial effluents, MWCBs have been developed to address an extensive list of pressing issues. Herein, we will highlight said work.

2.1. Microbial whole-cell biosensors for environmental and agricultural monitoring

The environment is a complex mixture of physical, chemical, and biotic factors locked in an eternal balancing act, where changes in one parameter can greatly affect others. It is of crucial importance to the overall well-being of humanity that crops are efficiently and continuously grown. Thus, it is paramount to monitor components that affect the environment and agriculture, such as nutrients, MTEs, and organic toxic compounds. A variety of MWCBs have been developed and applied for monitoring of environmental and agricultural systems, with the majority being based on fluorescent, bioluminescent, or electrochemical reporter proteins.

Application of MWCBs in environmental and agricultural monitoring is currently focused on generating relevant sensors for analytes of interest. As the toolkit of MWCBs grows, so will the ways in which they are applied to address current needs in monitoring. A list of MWCBs for analytes relevant to environmental and agricultural monitoring can be found in Table 2.

2.1.1. Microbial whole-cell biosensors for micronutrient monitoring

In recent years, food supply production has struggled to meet the demand of an ever-growing world population (Tripathi et al., 2019). The availability of cultivatable land is shrinking in proportion to sharp increases in population, highlighting the need for more efficient crop production. To meet this existential food crisis, some investigators have turned to the optimization of soil conditions to maximize crop yields. Efficient crop production depends on micronutrients such as nitrogen (N), phosphorus (P), and carbon (C) compounds, MTEs such as zinc (Zn^{2+}) and copper (Cu^{2+}), and beneficial microorganisms such as nitrogen fixers and decomposers, among others. While chemical analysis has long been the standard technique for the assessment of soil components, it does not yield information on the bioavailability of these components. As such, from the onset of synthetic biology, agricultural scientists have been consistently optimizing and deploying MWCBs to monitor these essential nutrients.

Early MWCBs for soil monitoring were based on constitutive, naturally luminescent bacteria such as the *Aliivibrio fischeri* employed in the Microtox assay previously described. As recombinant DNA technologies advanced, a growing list of luminescence- and fluorescence-based biosensors were developed for soil monitoring of bioavailable nutrients. MWCBs for the monitoring of Carbon (C), Nitrogen (N), and Phosphorus (P) have been developed to monitor the triple nutrients in rhizosphere and bulk soil (Cardemil et al., 2010; Koch et al., 2001; Kragelund et al., 1997; Muñoz-Martín et al., 2014; Roca and Olsson, 2001). The reporter was placed under the control of starvation regulons such that upon starvation of either carbon, nitrogen or phosphorus, an increase in the

Table 1
Microbial whole-cell biosensor reporters.

Reporter protein	Gene	Catalyzed reaction	Detection method	Quantitative Values	Advantages	Disadvantages
Chloramphenicol (CM) acetyltransferase	*cat*	CM + acetyl CoA → 3-acetyl CM + CoA 3-acetyl CM ⇌ 1-acetyl CM 1-acetyl CM + acetyl CoA → 1,3-diacetyl CM + CoA	FL RI	Kcat = 600 S^{-1} (CAT III) (Shaw and Leslie, 1991)	No endogenous activity	Radioisotope/ fluorescent labeling, requires substrate and cofactor addition, narrow linear range.
β -galactosidase	*lacZ*	Hydrolysis of β -galactosides	FL CL EC CR	Kcat = 600 ± 11 S^{-1} (ONPG) Kcat = 90 ± 1 S^{-1} (PNPG) Km = 0.12 ± .01 mM (ONPG) Km = 0.040 ± .002 mM (PNPG) (Huber et al., 2003)	Numerous detection methods, high stability at various temperatures (Asraf and Gunasekaran, 2010), detection by naked eye, applicable in anaerobic environment	Exogenous substrate requirement.
Bacterial luciferase	*lux*	$FMNH_2$ + R-CHO + O_2 → FMN + H_2O + RCOOH + light	BL	$t_{1/2}$ = ~240 min (*P.luminescens*) Waidmann et al. (2011) QY = 0.10	Rapid response, high signal to noise ratio, no endogenous activity	Oxygen requirement, thermal lability
Firefly luciferase	*Fluc*	D-luciferin + O_2 + ATP → oxyluciferin + AMP + Pi + light	BL	QY = 0.48 (*Photinus pyralis*), 0.43 (*Luciola cruciate & Luciola mingrelica*) at pH 8.0 (Niwa et al., 2010) $t_{1/2}$ = ~3h (Thorne et al., 2010)	High signal to noise ratio, no endogenous activity	Exogenous substrate, oxygen and ATP requirement
Renilla luciferase	*Rluc*	Coelenterazine + O_2 → coelenteramide + CO_2 + light	BL	QY = 0.10 $t_{1/2}$ = ~4.5h (Thorne et al., 2010)	High signal to noise ratio	Exogenous substrate and oxygen requirement
NanoLuc luciferase	*Nluc*	Furimazine + O_2 → Furimamide + CO_2 + light	BL	150-fold brighter than firefly luciferase, highly stable, >15 h at 37 °C in culture medium (Hall et al., 2012)	High intensity, no thermal lability, ATP independent reaction	Exogenous substrate and oxygen requirement
Aequorin	*aequorin*	Coelenterazine + O_2 + Ca^{2+} → coelenteramide + CO_2 + light	BL	QY = 0.15 (Daunert et al., 2000)	High signal to noise ratio, no endogenous activity	Exogenous substrate and Ca^{2+} requirement
Green fluorescent protein (GFP)	*avGFP*	Post translational formation of an internal chromophore	FL	QY = 0.80 (GFP), 0.93 (mTurquoise2) (Goedhart et al., 2012) ε = 55,000 $M^{-1}cm^{-1}$ (EGFP) ε = 84,000 $M^{-1}cm^{-1}$ (EGFP) (McRae et al., 2005)	No substrate requirement, high stability, numerous mutated versions available such as CFP, BFP, YFP etc.	Background fluorescence, slow maturation, low sensitivity
Red fluorescent protein (DsRed)	*DsRed*	Post translational formation of an internal chromophore	FL	DsRed requires >48 h to reach >90% of maximal fluorescence ε = 75,000 $M^{-1}cm^{-1}$ QY = 0.7 (Baird et al., 2000)	No substrate requirement, high stability, low background fluorescence, wide selection of mutated DsRed proteins available such as mCherry, and mBanana	Slow maturation, moderate sensitivity
Uroporphyrinogen III methyltransferase	*cobA*	Urogen III + SAM → precorrin-2 precorrin-2 → sirohydrochlorin precorrin-2 + SAM → trimethylpyrrocorphin	FL	Km = 52 nM (uroporphyrinogen III) Blanche et al. (1991)	No substrate requirement, auto-fluorescent	Endogenous activity
Catechol 2,3-dioxygenase or Metapyrocatechase (MPC)	xylE	Catechol + O2 → 2-hydroxymuconate semialdehyde	CR	Km = 1.87 μM (catechol) Km = 7.5 μM (O2) Kcat = 278 S-1 (Ishida et al., 2002)	Instrument-free detection, detection by naked eye	Exogenous substrate requirement
Chromoprotein (amilCP, CjBlue, eforRed, aeBlue)	*amilCP, eforRed, aeBlue*	Post translational formation of an internal chromophore	CR	ε = 66,700 $M^{-1}cm^{-1}$ (CjBlue) Chan et al. (2006) Maturation $t_{1/2}$ ~ 24 min (aeBlue) and 54 min (amilCP) Liljeruhm et al. (2018)	Instrument-free detection, detection by naked eye, No substrate requirement	Oligomer formation

SAM= S-adenosyl-L-methionine, ONPG= Ortho-Nitrophenyl-β-galactoside, PNPG= Para-Nitrophenyl-β-galactoside, Kcat = Catalytic constant, Km = Michaelis-Menten constant, QY = Quantum Yields, ε = molar extinction coefficient, FL=Fluorescence, CL=Chemiluminescence, RI = Radioisotope, BL=Bioluminescence, CM=Chemiluminescence, CR=Colorimetric, EC = Electrochemical.

bioluminescence of the MWCBs was observed.

Cardemil et al. have constructed two *E. coli*-based reporter systems for the measurement of phosphate and ammonia in coastal and suburban watersheds by fusing *E. coli* promoters *phoA* and *glnAp2* in front of the *luxCDABE* operon of *Aliivibrio fischeri* (Cardemil et al., 2010). In a limited or starved condition phosphate regulon transcriptional regulator *phoB* and nitrogen regulon *glnGF* activates *phoA* and *glnAp2* promoter and produces bioluminescence. The detection range was between 1 and 24 ppm of phosphate and 0.1–1.6 ppm of ammonia. Cyanobacteria-based MWCBs for the detection of bioavailable nitrogen in aquatic ecosystems have also been reported (Muñoz-Martín et al., 2014). Briefly, the *glnA*, *nirA* and *gifA* promoters were fused with *luxCDABE* reporter. The MWCBs responded linearly to the addition of combined nitrogen in the forms of NH_4^+ and NO_3^-. Green fluorescent protein (GFP) and β-galactosidase based biosensors have also been developed and deployed to assess nitrogen levels in the form of nitrate

Table 2
Microbial Whole-Cell Biosensors for use in Environmental and Agricultural Monitoring.

Target analyte	Host microorganism(s)	Promoter/reporter construct	Detection limit/Range	References
N (NH_4^+ or NO_3^-)	*Nostoc* sp. PCC 7120	*glnA/nir/gifA-luxCDABE*	50–500 μM	Muñoz-Martín et al. (2014)
	S. elongatus PCC 7942	*glnA/nir/gifA-luxCDABE*	10–500 μM	Muñoz-Martín et al. (2014)
	E. coli	*glnAp2′-luxCDABE*	0.1–1.6 ppm	Cardemil et al. (2010)
P (PO_4^{3-})	*E. coli*	*phoA′-luxCDABE*	1–24 ppm	Cardemil et al. (2010)
Cu^{2+}	*E. coli* XL1-Blue	PcusC–rfp	26 μM	Ravikumar et al. (2012)
	E. coli TOP10	*cusR-PcusC-gfp*	12 μM	Wang et al. (2013)
	C. metallidurans	*copSR-PcopT-rfp*	24.3 μM	Chen et al. (2017b)
	C. metallidurans	*copSR-PcopQ-MjDOD*	87.3 μM	Chen et al. (2017b)
	Wild-type and mutant *E. coli* BL21	*PcopA-EGFP* pCDF-Duet-*CueR*	0.3–5000 μM	Kang et al. (2018)
Zn^{2+}	*E. coli* XL1-Blue	PzraP–gfp	16 μM	Ravikumar et al. (2012)
	B. megaterium strain WH320	*smtB-EGFP*	1×10^{-6} M	Date et al. (2007)
	P. putida X4	*PczcR3-EGFP*	5–55 μmol/L	Liu et al. (2012)
As^{3+}	*E. coli* TOP10	*arsR-ParsR-gfp*	0.1 μM	Wang et al. (2013)
	E. coli	*arsR-O/P-luxCDABE*	0.05–0.8 μM	Sharma et al. (2013)
	E. coli DH5α	*ars-pr-mCherry*	2.79 ng/mL	Yoon et al. (2016)
Cr^{6+}	*A. baylyi*	*PrecA-luxCDABE*	520 mg/kg	Song et al. (2014)
	E. coli	*recA-luxCDABE*	260 mg/kg to 2600 mg/kg	Jiang et al. (2017)
Cd^{2+}	*D. radiodurans*	pRADI-P0659-1-crtI	50 nM-1 mM	Joe et al. (2012)
	P. putida 06909	*gfp-lacI^q-PcadR-Ptac-cadR*	0.01 μM (1.12 ppb)	Wu et al. (2009)
	E. coli DH5α	*znt-pr-EGFP*	3.6 ng/mL	Yoon et al. (2016)
Ni^{2+}	*Synechocystis* sp. PCC 6803	*nrsR-PnrsBACD-luxAB*	0.2 μM	Peca et al. (2008)
Pb^{2+}	*E. coli* DH5α	*ΔpbrA-PpbrRT-pbrR-gfp*	0.001 μM	Jia et al. (2018)
Hg^{2+}	*P. putida*	*merR-egfp*	<40 μg/kg	Wei et al. (2014)
	E. coli	*merR-luxCDABE*	0.24 μg/L	Zhang et al. (2016)
Co^{2+}	*Synechocystis* sp. PCC 6803	*coaR-PcoaT-luxAB*	0.3 μM	Peca et al. (2008)
Sb_2S_3	*B. subtilis* ars-23	*arsR-lacZ*	2.8×10^{-8} M	Date et al. (2007)
Naphthalene	*B. sartisoli* RP007	*phn-luxAB*	0.17 μM	Tecon et al. (2010)
BTEX	*E. coli* DH5α	*LuxAB-TbuT*	0.24 μM (Toluene)	Tecon et al. (2010)
Organophosphorus (Parathion/4-nitrophenol)	*E. coli* MG1655	*DmpR-Pdmp-RFP*	10 μM	Chong and Ching (2016)
parathion and methyl parathion	*E. coli*	*PchpA–atsBA* pBBR–*chpR*	2 μM	Whangsuk et al. (2016)
OH-PCBs	*E. coli*	*hbpR−PhbpC−luxAB*	5×10^{-8} M	Turner et al. (2007)
Phenanthrene	*P. putida*	*phnS-luxCDABE*	<24 mg/kg	Wei et al. (2014)
2,4-DNT	*E. coli* K12 strain MG1655	*yqjF-luxCDABE ybiJ-luxCDABE*	5 mg/L	Yagur-Kroll et al. (2014)
2,4,6-TNT	*E. coli* K12 strain MG1655	*yqjF-luxCDABE ybiJ-luxCDABE*	N/A	Yagur-Kroll et al. (2014)
C_{14}-HSL	*A. tumefaciens*	*traG-lacZ-TraR*	10^{-12} M	DeAngelis et al. (2007)
$3OC_6$-HSL	*E. coli* DH5α	*PnptII-gfp/ahlR/T1−4/PahlI-mcherry/T1−4*	5×10^{-8}–1×10^{-5} mol/L	Deng et al. (2014)
Sodium Dodecyl Sulfate (SDS)	*P. aeruginosa* PAO1	*sdsB1-EGFP*	0.1 ppm	Dey et al. (2020)
2-hydroxylbiphenyl and biphenyl	*E. coli* DH5α	*hbpR-PhbpC-luxAB*	0.30 μM	Tecon et al. (2010)
3-phenoxybenzoic acid (3-PBA)	*E. coli* DH10B	anti-3-PBA-VHH p*AmilCP_*J104	3 ng/mL	Riangrungroj et al. (2019)

ions in rhizosphere and intracellular concentrations (DeAngelis et al., 2005; Koch et al., 2001). A detailed review on MWCBs for the detection of nutrients in the soil can be found elsewhere (Renella and Giagnoni, 2016). A current challenge in the field of nutrient monitoring in soil is how to properly assess large areas of land. MWCBs offer an advantage that they can assay soil samples in common high throughput formats, such as in 96-well plates, allowing for samples to be collected from multiple locations and assayed in parallel at relatively lower costs.

2.1.2. Microbial whole-cell biosensors for monitoring of metallic trace elements

Although MTEs are generally considered to be toxic, some are essential for plant growth, albeit in very low amounts. Soil that contains less than 100 parts per million (ppm) of Cu^{2+}, for example, is healthy soil essential for plant growth (Romić et al., 2014). If MTEs accumulate in larger quantities, however, they become a threat to the environment, food security, and overall health of humanity. The primary source of MTE pollution in the environment stems from the waste disposal activities of industrialization and urbanization (Hu et al., 2013). As such, it is of crucial importance to continuously monitor soils, industrial effluents, and drinking water for the presence of MTEs.

Various inducible reporter systems have been employed in the construction of MWCBs for MTEs, including luciferase, β-galactosidase and green/red fluorescent protein (GFP/RFP), and blue fluorescent protein (Bereza-Malcolm et al., 2015). MWCBs for the detection of Cu^{2+}, Zn^{2+}, Nickel (Ni^{2+}), Cadmium (Cd^{2+}) and other MTEs (Table 2) have been engineered with multiple resistance/regulatory genes from different microorganisms. These include regulatory genes such as copper sensing transcriptional repressor (*csoR*) (Chillappagari et al., 2009), zinc uptake regulation protein (*ZuR*) (Ma et al., 2011), and cadmium resistance transcriptional regulatory protein (*cadC*) (Kumari et al., 2021). When it comes to the development of MWCBs for MTEs, however, specificity and selectivity pose significant challenges. Microbial organisms have evolved to quickly adapt to a changing and hostile microenvironment in which the availability of required and non-essential MTEs can drastically vary. Many of the regulatory proteins for metal resistance not only respond to the binding of their specific metal but also to cognate metallic trace elements (Reyes-Caballero et al., 2011). As such, MWCBs harboring these regulatory proteins will yield significantly stronger signals to their specific MTE but will also generate a weaker signal to other cognate MTEs. Efforts to address these shortcomings in sensitivity and selectivity, such as with directed evolution, will be discussed in the

Current Challenges and Solutions for the Broader Application of Microbial Whole-Cell Biosensors section further into this review. While some investigators have focused on developing MWCBs for MTEs relevant to soil health, others have focused on developing MWCBs for detecting heavy metal ions known to contaminate potable drinking water.

MTE contamination of drinking water poses serious public health risks in regions around the world. Arsenic contamination of underground drinking water has posed an ongoing threat to the health of countless individuals, especially in regions such as South East Asia. While working with a living sensor grants the advantage of detecting bioavailable levels of an analyte, it can be limited in its use if the analyte of interest is toxic to the host microbe, such as with MTEs. This disadvantage can be overcome in some cases by incorporating resistance genes which allow MWCBs to detect such analytes at levels that would normally kill the organism. By inserting the *arsR* regulatory gene upstream of the *lacZ* reporter gene and inserting the *smt* operon upstream of the *egfp* reporter protein, Date et al. developed *Bacillus subtilis* and *B. megaterium* spore-based MWCBs for the detection of arsenate, arsenite, and zinc ions (Date et al., 2007). The detection limits for these spore-based sensors were reported to be 1.6 × 10-7 M, 2.8 × 10-7, 1 × 10-6 M, respectively. A similar, *E. coli*-based MWCB has also been reported (Preveral et al., 2017).

2.1.3. Microbial whole-cell biosensors for monitoring of organic compounds

Organic pollutants such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and benzene, toluene and xylenes (BTEX) in soil and wastewater pose significant risks to human health and ecosystems (Robertson and Hansen; Shi et al., 2013). While it is true that these compounds are hydrophobic in nature and therefore considered to be unavailable in soils, they can be a serious threat to ecosystems if allowed to accumulate over time (Kelsey et al., 1996). As such, several MWCBs for the detection of aromatic compounds have been developed over the last 3 decades. Some of these MWCBs include the use of bacterial strains such as *E. coli* for its ease of use, *Nitrosomonas europaea* for its autotrophic features and *Pseudomonas putida* for its natural metabolism of harmful organic solvents into nontoxic composites. These strains have been genetically engineered to harbor genes associated with catabolic pathways for alkanes, alkylsulphonates, and BTEX, in combination with *luxCDABE* as a reporter gene (Brandt et al., 2002; Tecon et al., 2010). Green fluorescent protein (GFP) based biosensors for toluene and benzene have also been reported (Casavant et al., 2003; Stiner and Halverson, 2002). Biosensors for the chlorinated pollutants based on the lux reporter have been constructed in a pUCD607 plasmid system in *P. fluorescens* (Palmer et al., 1998), and in chromosomal integration in *P. putida* (Weitz et al., 2001). By fusing promoter/regulator genes (*bphA1/BphS*) with *luxCDABE* reporter gene, biosensors for polychlorinated biphenyls (PCBs) have also been developed (Hay et al., 2000; Layton et al., 1998). The detection limits for the toluene, 4-chlorobiphenyl, and Aroclor 142, MWCBs were reported at 30 μg/L, 0.15 mg/L, and 1.5 mg/L, respectively (Applegate et al., 1998; Layton et al., 1998).

To measure the bioavailable fractions of petroleum hydrocarbons, which are considered as toxic for aquatic and marine life, biosensors based on luxAB luciferase reporter for onsite bioassays have been developed (Tecon et al., 2010). These biosensors readily detected BTEX such as benzene, toluene, ethylbenzene, and xylenes. For the detection of the trace explosive 2,4,6-trinitrotoluene (2, 4, 6-TNT) and its synthetic by-product 2,4-dinitrotoluene (2,4 DNT) in soil, an *E. coli*-based biosensor was developed by fusing two gene promoters, *yqjF* and *ybiJ,* to either the green fluorescent protein gene *GFPmut2* or to *Photorhabdus luminescens* bioluminescence *luxCDABE* genes (Fig. 3) (Yagur-Kroll et al., 2014). A list of MWCBs for organic compounds such as naphthalene, phenanthrene, BTEX, PCBs, and PAHs has been provided in Table 2.

One of the most widely used standard indicators for the quality assessment of wastewater is the measurement of biochemical oxygen

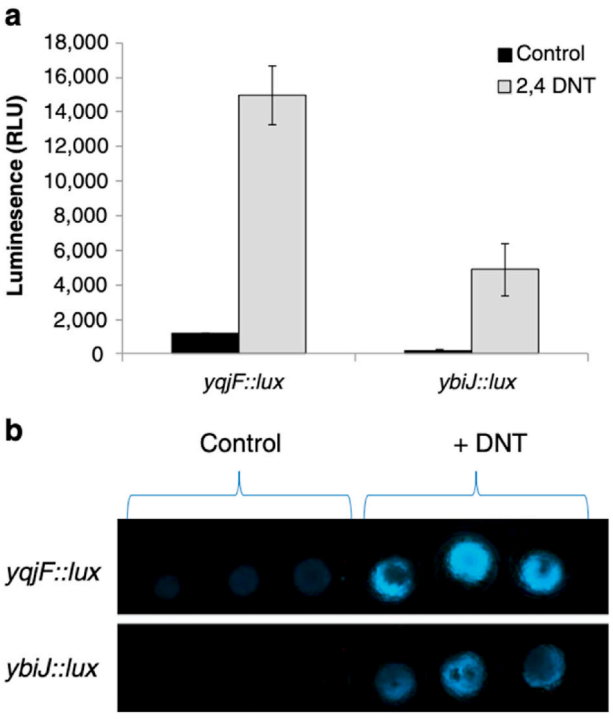

Fig. 3. Detection of 2,4-DNT vapors by agar-immobilized cells carrying either a yqjF:luxCDABE or a ybiJ:luxCDABE fusion in a closed chamber **(a)** or in soil **(b)** following a 3.5-h exposure. Adapted from Yagur-Kroll et al., (2014).

demand (BOD). BOD assessment is an empirical test that reveals the quality of wastewater by determining the levels of dissolved oxygen necessary to oxidize biodegradable organic pollutants within it (Arlyapov et al., 2012). The conventional method which has been approved and is used in international industrial settings to assess BOD is denoted as BOD_5 (Sakaguchi et al., 2007). BOD_5 biosensors rely on oxygen electrodes to measure oxygen consumption by aerobic microorganisms incubated with wastewater samples (Rodriguez-Mozaz et al., 2006). As the name suggests, this assay takes 5 days to produce a BOD measurement.

The extended incubation time of BOD_5 leaves much to be desired, and as such MWCBs for BOD assessment have been developed. Sakaguchi et al. report the successful determination of BOD using MWCBs based on luminous marine bacterium *Photobacterium phosphoreum* and recombinant bioluminescent *E. coli* (Sakaguchi et al. 2003, 2007). The BOD levels of wastewater samples correlated from the bioluminescent signal generated from these MWCBs were comparable to those measured with the standard BOD_5 assay. Additionally, BOD assessment was achieved with significantly lower incubation times (2 h with *P. phosphoreum* and 20 min with *E. coli*) using MWCBs as compared to the 5-day incubation time using the conventional BOD_5 biosensor.

2.1.4. Microbial whole-cell biosensors for detection of quorum sensing molecules

Soils are home to vast and diverse communities of microbes which can, depending on their makeup, be beneficial or harmful to the plants, crops, and ecosystems that rely upon them. These soil-borne microbial communities are one of the most important components of soil ecosystems due to their role in nitrogen fixation (Burns and Hardy, 2012; Mylona et al., 1995), plant remnant decomposition (Naeem et al., 2000), and disease suppression (Mendes et al., 2011). Understanding the inter- and intra-species interactions that occur within these communities and how they, in turn, affect the overall health of the soil is crucial to maximizing soil health. One way to monitor inter- and intra-species communications, as well as establish the microbial fingerprint of soils,

is through the measurement of quorum sensing molecules (QSMs).

Bacterial Quorum Sensing (QS) is a cell-cell communication system based on small molecules known as QSMs or autoinducers (AIs) which controls processes reliant on the synchronized behavior of large communities. These include virulence factor production (Bronesky et al., 2016; De Kievit and Iglewski, 2000), bioluminescence (Bassler et al., 1994; Engebrecht et al., 1983), secondary metabolite production (Barnard et al., 2007), competence (Kleerebezem et al., 1997; Okada et al., 2005), and biofilm formation (Hammer and Bassler, 2003; Miller and Bassler, 2001; Mukherjee and Bassler, 2019). QS hinges on the timely production, release, and detection of QSMs or AIs. As microbial density increases so too do the concentrations of QSMs; once a threshold of QSMs is surpassed molecular cascades are triggered, resulting in population-wide gene regulation (Fig. 4). Microbes employ a large variety of QSMs for inter-species and inter-kingdom communication and have evolved complex QS circuitries to cooperate and/or compete with each other. QS mechanisms typically employ a regulatory protein that, once bound to a QSM, controls transcription of target genes.

Several MWCBs have been developed to monitor a variety of QSMs such as acylated homoserine lactones (gram-negative bacteria), autoinducing peptides (gram-positive bacteria), and autoinducer-2 (both gram negative and positive bacteria), whose structures can be seen in Fig. 5(Burmølle et al., 2005; DeAngelis et al., 2007; Gantner et al., 2006; Scott et al., 2006; Steidle et al., 2001). These studies have revealed that in soil, bacteria release acylated homoserine lactones (AHL) to communicate. Rhizosphere soil is a narrow zone of soil surrounding the root vasculature of plants, characterized by intense biological activity (Kennedy and de Luna, 2005). To investigate the QS dynamics in rhizosphere soil, an MWCB based on *Agrobacterium tumefaciens* was constructed, with a minimum detection limit of 10–12 M for N-acyl homoserine lactones (AHLs), with the exception of C4 homoserine lactone (DeAngelis et al., 2007). The presence of AHLs in soil have also been detected by using *E. coli* MC4100 harboring pAHL-GFP plasmid (Burmølle et al., 2003). By continuing these studies and examining additional QSMs in soil, it may be possible to create a molecular "fingerprint" of healthy and unhealthy soil. While it may not provide information as to the kinds of microorganisms in the soil, unhealthy soil might display a markedly less diverse and diluted range of QSMs than healthy soil does. This "fingerprint" could then be used as an alternative to the more costly and time-consuming sequencing of microbial genomes and transcriptomes for soil health assessment. Detection of AHLs via MWCBs has also been explored as an indicator of food spoilage caused by bacteria found in soil or food processing complexes (Wynn et al., 2018).

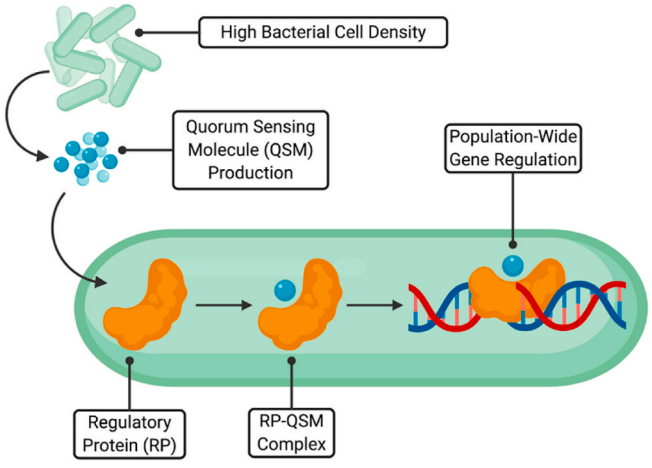

Fig. 4. Bacterial Quorum Sensing. At high bacterial cell density, quorum sensing molecules bind to regulatory proteins and trigger population wide gene regulation.

2.2. Microbial whole-cell biosensors for biomedical applications

Multicellular organisms are host to vast communities of microorganisms commonly referred to as the microbiome. The symbiotic relationship between an organism and its microbiome involves a seemingly infinite complex web of interactions. It has been recently determined that a reference man of 70 kg can have 0.2 kg bacterial cells by mass and the number of bacterial cells to human cells is roughly 3.8×10^{13} and 3.0×10^{13}, respectively, (roughly 1:1), disproving the popular belief of a 10:1 ratio (Sender et al., 2016). The growing interest in elucidating just how influential our microbial counterparts are to our overall health has resulted in the development of numerous fields of investigation on the human microbiome. MWCBs are increasingly becoming popular methods for use in disease diagnostics and monitoring of pathological conditions via the detection of microbial metabolites in real time because of their ease-of-use, inexpensive production cost, and reproducibility.

MWCBs have been developed for use in monitoring several diseases linked to the microbiome such as urinary tract infection (UTI) (Lubkowicz et al., 2018; O'Connor et al., 2015; Reyes et al., 2020) and inflammatory bowel disease (IBD) (Raut et al. 2012, 2013; Woo et al., 2020). Standard diagnosis methods for UTI's involve culturing methods that can take at least 48h and result in improper diagnosis. To address these issues, a constitutive MWCB bioluminescence assay using intact, viable cells of *Photobacterium mandapamensis* or previously lyophilized cells of *Photobacterium leiognathi* was developed to detect UTI in a rapid and cost-effective way (Reyes et al., 2020). Briefly, in the presence of gram-negative bacteria in urine, the bioluminescence of the MWCBs decreases due to the competition for available dissolved oxygen between the infectious agent and the bioluminescent agent, thus indicating a possible UTI.

Bacteria are known to play a crucial pathogenic role in many infectious and chronic inflammatory diseases including inflammatory bowel disease (IBD), a chronic inflammation of the gastrointestinal (GI) tract. A MWCB system for the detection of AI-2, a universal signaling molecule for both gram-positive and gram-negative bacteria, was developed and optimized (Raut et al., 2013). Their approach was noninvasive and used stool, saliva, and intestinal samples from IBD patients. Other studies have shown that N-acyl homoserine lactones (AHLs) could serve as potential biomarkers for bacterial disease conditions, such as in oral cavity infections (Muras et al., 2020) and cystic fibrosis (Singh et al., 2000). As such, several biosensors targeting N-acyl homoserine lactones (AHLs) have been developed (Kumari et al., 2006; Pearson et al., 1995; Winson et al., 1998). Kumari et al. developed protocols for the rapid, sensitive, and quantitative detection of AHLs in biological and clinical samples using MWCBs (Kumari et al. 2006, 2008). The protocols were developed to address sample matrix effects often found in clinical samples and validated by detecting AHLs in saliva from Crohn's Disease (CD) and healthy volunteers, as well as in stool samples from infants in the newborn intensive care unit (NICU) (Fig. 6). Notably, MWCBs for detection of other clinically relevant small molecules like riboflavin (Si et al., 2016), glucose (Svitel et al., 1998), serotonin (Knecht et al., 2016), and calmodulin antagonists (Dikici et al., 2008) have also been reported.

The development of MWCBs for QSM detection has enabled novel insights into the connection between the gut microbiome and the central nervous system, otherwise known as the gut-brain axis. Knecht et al. recently reported on the possible role of the neurotransmitter serotonin as an interkingdom signaling molecule, seeing as how it was readily detected by MWCBs for AHL detection (Knecht et al., 2016). They went on to show how serotonin can activate the QS circuitry of *Pseudomonas aeruginosa* for virulence factor production and biofilm formation. Medina-Rodriguez et al. deployed MWCBs for QSM detection in a study on microbiome mediated depressive-like behaviors (Medina-Rodriguez et al., 2020). The MWCBs allowed investigators to show how AI-2 correlates with multiple variables of the disease state in both a murine model and human patients, indicating that AI-2 could be a potential

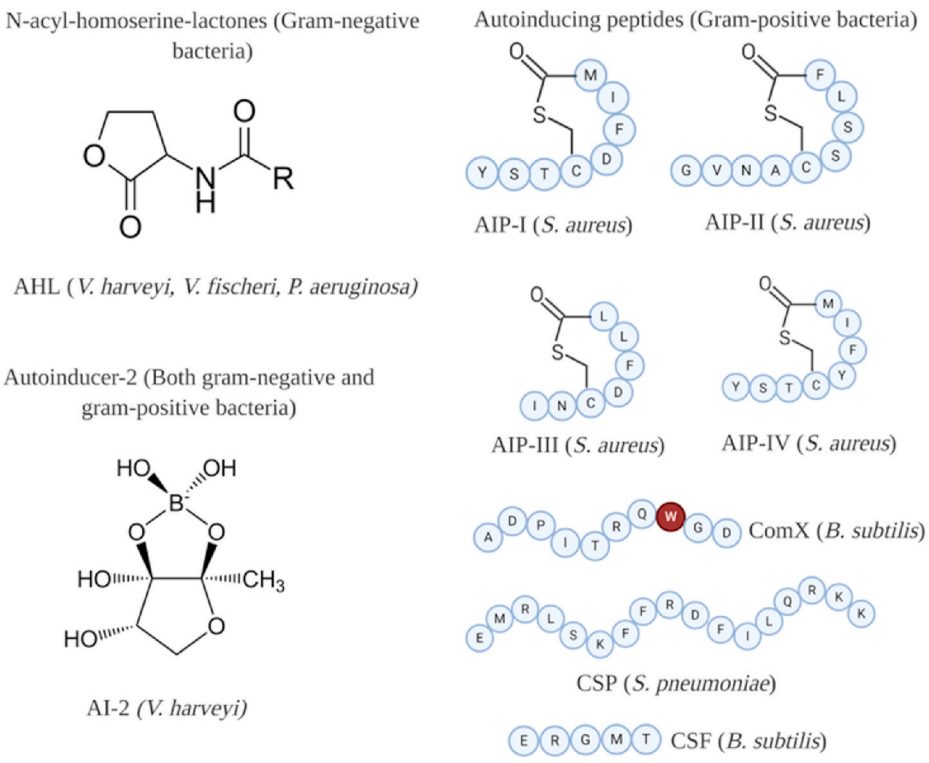

Fig. 5. Bacterial species can employ a large variety of chemical signals called quorum sensing molecules for inter-species and inter-kingdom communication. Gram-negative bacteria mostly rely on a variety short- and long- and short-chain acylated homoserine lactones (AHLs), while Gram-positive bacteria largely employ secreted peptides.

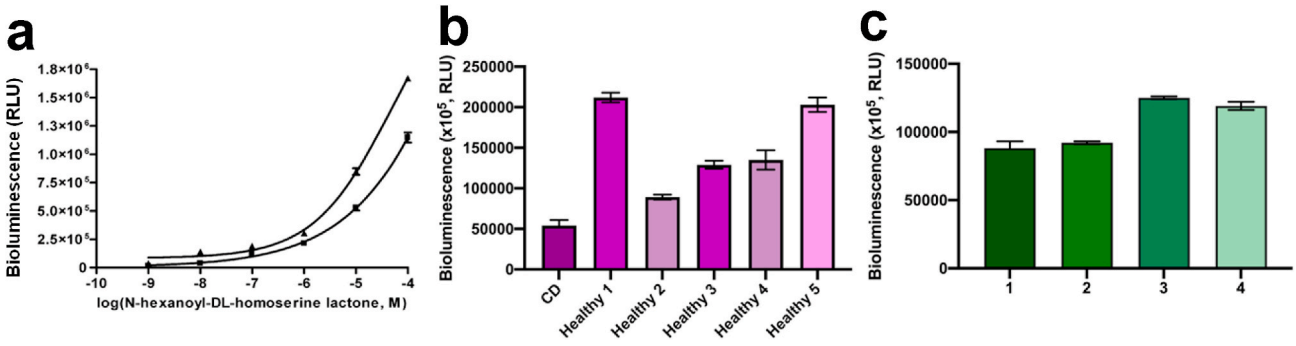

Fig. 6. Kumari et al. employed MWCBs for the detection of AHLs in clinical samples. **(a)** Evaluation of sample matrix effect for biosensing in spiked saliva (▲) and a reference calibration curve (■). **(b)** Detection of AHLs in saliva samples from a Crohn's Disease (CD) patient and five healthy volunteers. **(c)** Detection of AHLs in stool samples from four infants in the newborn intensive care unit (NICU). Adapted from Kumari et al. (2006).

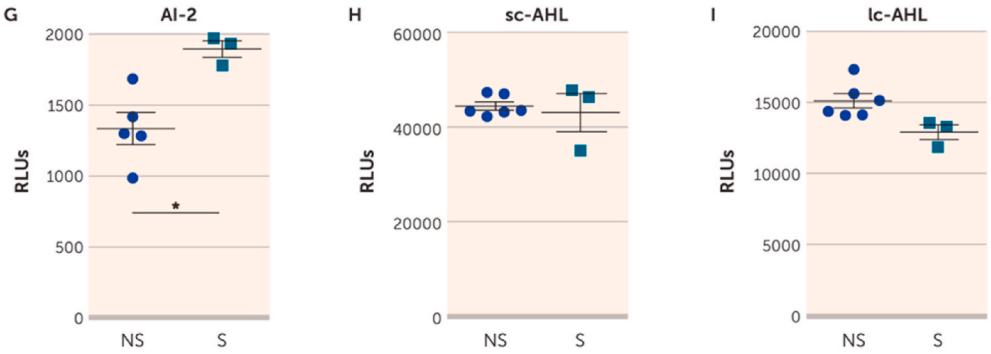

Fig. 7. Levels of the QSMs **(G)** AI-2, **(H)** short-chain AHL (sc-AHL), and **(I)** long-chain AHL (lc-AHL) were measured in the stool of mice subjected to a model of depressive-like behavior (S) or in control (NS) mice using MWCBs. The significantly elevated levels of AI-2 in depressed mice prompted further investigation of its role in depression. Adapted from Medina-Rodriguez et al., (2020).

biomarker of depression (Fig. 7). Similarly, O'Connor et al. employed the MWCBs in a study looking into microbiome alterations in a rat model of spinal cord injury (SCI) and found significantly elevated levels of AI-2 in the intestinal contents of the rats 8 weeks post injury (O'Connor et al., 2018). It is not well understood why patients with SCI often suffer from debilitating bowel disfunction, but their results highlight the need for further investigation of the possible role of QS in this disease state.

3. Challenges and current solutions for the Broader Application of Microbial Whole-Cell biosensors

As discussed above, the ever-expanding field of MWCBs can be applied to a variety of needs across environmental, agricultural, and medical applications. On the sensor side, work continues to generate new and advanced MWCBs, both by learning from the capabilities of nature and through novel genetic constructs. Additionally, there is much that can be learned by taking sensors developed for one field and employing them to investigate a whole new sample set, which can be achieved with a significantly lower cost considering the use of MWCBs. There are, however, certain challenges in the way of broadening the application of MWCBs. Herein, we will describe these challenges and ongoing efforts to address them.

3.1. Safe deployment

Due to the living nature of MWCBs, when it comes to the design of analytical platforms based on MWCBs, all challenges revolve around this very fact. In a recent publication, Lobsiger et al. provide an excellent framework of criteria for the development of MWCB analytical devices based on successfully implemented point-of-care biosensors such as the home pregnancy test (Lobsiger and Stark, 2019). They argue that MWCB devices need to be engineered such that the viability of the cells is maintained from production through its final readout, given that accuracy and sensitivity is reliant on cell viability. Furthermore, devices need to adhere to strict biosafety standards that allow for the inactivation and safe disposal of the MWCBs, ensuring the safety of both the user and the environment.

Ultimately, these challenges come down to how the biosensor cells will be immobilized for use in an analytical device. Immobilization of the living cells is crucial, seeing as how it would be difficult to ensure safe handling and distribution when liquid cultures are involved. The chosen immobilization approach needs to take into consideration cell viability, long-term storage, safe handling, and proper disposal. Furthermore, it should provide enough mechanical stability such that it prevents cell leakage without sacrificing the efficient access of target analyte molecules via diffusion (Shing et al., 2013; Yoetz-Kopelman et al., 2016). This is a particularly important detail, seeing as how upon immobilization additional matrix effects are introduced that might affect the MWCBs ability to detect the analyte of interest. It is therefore of utmost importance that diffusion through the matrix of immobilization be taken into consideration, in addition to those found in the sample to be analyzed, when designing a MWCB. The literature offers some promising advances in techniques for the immobilization of biosensor cells, specifically with the aim of assisting in transitioning MWCBs from the lab into the field.

One of the more popular immobilization techniques that has seen significant advancements in recent years is the use of hydrogels. Hydrogels consist of cross-linked hydrophilic polymers that can retain large amounts of water in their 3D networks, a feature that is highly beneficial for cell growth (Ahmed, 2015). Of the various kinds of hydrogels available, the more commonly used are agarose and alginate gels. Ma et al. recently published work on agarose-immobilized *E. coli* MWCBs for the screening of lactam species, a highly valued class of cyclic amides for the production of nylon, via fluorescent *mCherry* expression (Ma et al., 2019). They describe a microfabrication process in which engineered *E. coli* are co-encapsulated with melted agarose, forming microgels (~30 μm) containing embedded cells (Fig. 8). They report a dose-dependence behavior of 0–100 mM for the major lactam species butyrolactam, valerolactam, and caprolactam after 16 h of incubation, measured on a high-throughput droplet fluorescence-activated cell sorting (FACS) transducer device. It's important to note the authors suggest immediate use of the MWCB microgels, indicating that they are not particularly well suited for use after long term storage. While this work shows significant promise for the use of agarose-based immobilization, the lengthy response time and limited storage capabilities of these MWCB microgels highlight the difficulty of engineering a MWCB device that meets the criteria of being rapid and amenable to long-term storage.

Similar to agarose, alginate has also been increasingly utilized as a means to immobilize MWCBs. Alginate is an anionic polysaccharide, which forms an open lattice structure after cross-linking via interactions with divalent ions such as Ca^{2+} in a mild and biocompatible process (Lee and Mooney, 2012). The open lattice structure provides a hydrated environment with high porosity levels for cell growth and analyte diffusion (Lobsiger and Stark, 2019). The high porosity of alginate, however, is known for its propensity to leak cells from within its matrix. As such, in an effort to reduce cell leakage, various investigators have experimented with the use of photo-cross linkable polymers to reinforce the alginate gels. Li et al. describe the successful immobilization of *E. coli*-based MWCBs in an alginate-methacrylate based matrix for the detection of quorum sensing molecules via fluorescence of the GFP reporter protein (Li et al., 2017). The photo-cross-linking of glycidil methacrylate in combination with alginate reinforced the microbeads and significantly reduced cell-leakage without sacrificing analyte permeability as compared to plain alginate microbeads. The 100–300 μm microbeads were obtained via an electrostatic extrusion method and displayed a significant response to the analyte after 3 h of incubation, however cell viability was reduced to almost half after 5–6 days of storage at 4 °C.

Another promising form of immobilization is through lyophilization, otherwise referred to as freeze-drying. Lyophilization is a low temperature dehydration process, in which water from a frozen sample is sublimed under vacuum (Lobsiger and Stark, 2019). Sensor cells are typically resuspended in a cryoprotectant solution and spotted onto a platform of choice prior to lyophilization. Stocker et al. report the development of a semi-quantitative paper-based assay for the detection

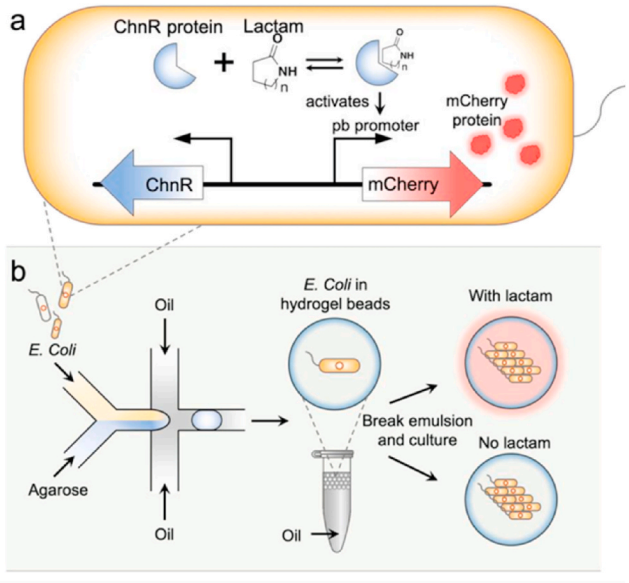

Fig. 8. **(a)** Ma et al. engineered MWCBs for detection of lactam molecules using the fluorescent mCherry reporter and **(b)** immobilized them in agarose microgels. Adapted from Ma et al., (2019).

of arsenite in aqueous samples (Stocker et al., 2003). Briefly, engineered *E. coli* cells with the colorimetric reporter β-galactosidase were lyophilized onto a paper matrix with drying protectant solution. Upon incubation with a sample and subsequent addition of the X-gal substrate the development of blue spots was observed, indicating the presence of arsenite in both standard arsenite solutions contaminated groundwater samples from Bangladesh (Fig. 9). Storage of the lyophilized cells on the paper matrix for 2 months at −20, 4, and 30 °C demonstrated no noticeable loss of induction of the assay. The long-term storage capabilities enabled by the use of lyophilization as an immobilization strategy is one of its most attractive features, with various groups reporting maintenance of cell viability and sensitivity after months and even years of storage (Martin-Betancor et al., 2017; Preveral et al., 2017; Siegfried et al., 2012).

Immobilization techniques have come a long way since the development of the first biosensor, but there are still challenges in the way of developing an affordable, rapid, easy to use, and safely deployable device based on MWCBs. Hydrogels offer excellent matrices for the proliferation of MWCBs and diffusion of the target analyte, but they fall short in their long-term storage capabilities. Lyophilization techniques show great potential for long-term storage, however more work needs to be done to avoid the desorption, shearing, and subsequent contamination of samples with the engineered cells upon re-hydration.

3.2. Analytical performance

Clearly, the most enticing feature of MWCBs is the ability to engineer them for affordable and easy to use detection of a wide range of analytes of interest in a variety of scientific fields. However, assessing the accuracy of a MWCB still relies on its comparison with the results of well-established and highly accurate physio-chemical analytical methods

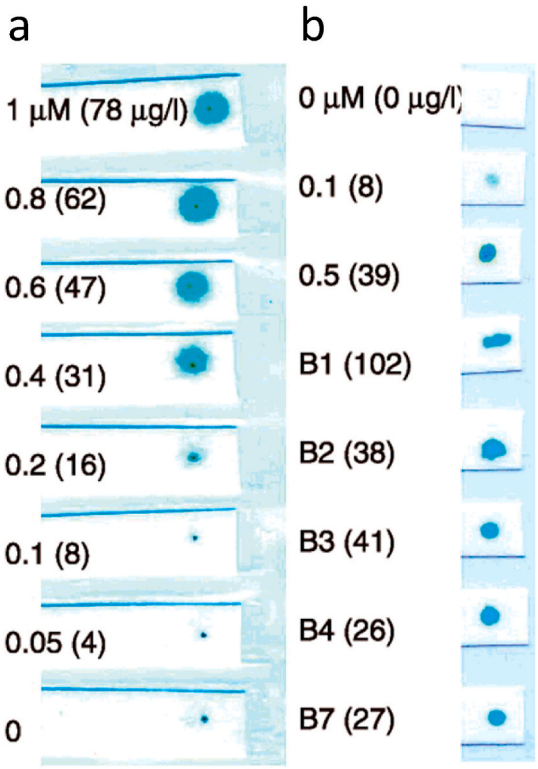

Fig. 9. **(a)** Lyophilized MWCBs on a paper matrix for the colorimetric detection of arsenite at varying concentrations. **(b)** Employment of the MWCB paper strips tests with arsenite standards and five samples from Bangladesh groundwater. Total arsenic concentration (μg of As/L) of the Bangladesh samples measured by atomic fluorescence spectroscopy is indicated in parenthesis. Adapted from Stocker et al., (2003).

such as liquid chromatography mass spectrometry (LC-MS), gas chromatography mass spectrometry (GC-MS), and inductively coupled plasma mass spectrometry (ICP-MS). As accurate as these powerful instruments may be, these analytical platforms are costly and require high technical expertise to utilize. As such, when it comes to choosing an analytical tool for employment in a particular study, investigators may find themselves juggling between selecting an ultra-sensitive physio-chemical analytical tool or a decidedly cheaper, yet comparably less accurate, MWCB. Recent advancements in biotechnology and synthetic biology, however, have made it possible to significantly improve the analytical performance of MWCBs. While MWCBs will certainly not come to entirely replace physio-chemical instrumentation, such advancements are helping make the choice between them less difficult.

As previously described, the molecular recognition events between the regulatory protein, analyte, and promoter heavily influence the analytical performance of a given MWCB. These molecular recognition events are of utmost importance due to their direct impact on analytical parameters such as signal-to-noise ratio, dynamic range, selectivity, sensitivity, response time and limit of detection of a given MWCB. As such, methods aimed at manipulating genetic circuitries to improve the activity of biomolecules have given investigators the opportunity to enhance the analytical performance of their engineered MWCBs. Directed evolution, a cyclic process that alternates between gene diversification and screening for or selection of functional gene variants, is one such technique (Packer and Liu, 2015).

Perhaps the most well-known example of employing directed evolution to change and/or enhance the fundamental properties of biomolecules is the work done by the Tsien lab at the University of California. Throughout the years they have developed a vast range of fluorescent proteins whose wild-type genetic makeup has been altered to increase their brightness and even modify their excitation and emission wavelengths (Rodriguez et al., 2017). The use of directed evolution is not limited to reporters, as it can also be employed to produce variants of promoter regions with improved regulatory properties. A great example of such work was demonstrated by Yagur-Kroll et al. They implemented directed evolution to vastly improve the analytical performance of their MWCB for the detection of 2,4,6-trinitrotoulene (2,4-TNT) and 2,4-dinitrotoluene (2,4-DNT) (Yagur-Kroll et al., 2015). The investigators subjected the *yqjF* promoter employed by their MWCB to four sequential random mutagenesis rounds and screened the promoter variants for enhanced analytical performance. The investigators report an up to 4450-fold increase in the intensity of the luminosity response, an approximately 42-fold enhancement in signal-to-noise ratio, a 5-fold lowering of the detection limit (from 24.2 mg/l to 4.8 mg/l), and a significant reduction in the response time (from 150 to 70 min).

Directed evolution is clearly a powerful approach to systematically improve the analytical performance of MWCBs. However, investigators wanting to utilize directed evolution to evaluate the best possible combination of genetic components are presented with the daunting task of screening countless possible iterations of their circuit. Berepiki et al. set out to address this issue and demonstrated how statistical modeling can vastly improve the optimization of genetic systems of MWCBs (Berepiki et al., 2020). The investigators employed definitive screening design (DSD), a statistical modeling approach, to efficiently optimize the performance of a protocatechuic acid (PCA) MWCB. Without implementing DSD, a total of 10,648 combinations would have been needed to fully explore the gene expression space. With it, DSD reduced the total experimental configurations down to 13. Overall, the investigators developed a PCA MWCB with an over 30-fold improvement in output signal, and a 25% increase in dynamic range when compared to the original PAC MWCB. Notably, this was all done without understanding the binding affinities of the DNA and ligand binding domains or relying on detailed mechanistic and/or kinetic knowledge. This work demonstrates the potential for efficient optimization of MWCB genetic systems by tuning component expression levels in a systematic and highly efficient way based on statistical modeling.

While some investigators have taken to directly modifying the genetic makeup of the regulatory protein and promoter, others have looked to employing cascaded amplifying circuitries to engineer ultra-sensitive MWCBs. Recent work done by Wan et al. demonstrates the vast potential of this approach, seeing as how they were able to drastically improve the performance of two arsenic and mercury MWCBs (Wan et al., 2019). To do so, the investigators integrated three multilayered coupled signal amplifiers into the genetic circuitry of arsenic and mercury MWCBs (Fig. 10).

Briefly, in both the original arsenic and mercury MWCBs, the constitutively expressed regulatory proteins de-repress their cognate promoters and trigger expression of the *gfp* reporter upon binding to the MTEs. The investigators replaced the reporter from the original MWCB with a transcriptional amplifier (Amp 30^E). A second transcriptional amplifier (Amp 2) was added such that it was under transcriptional control of Amp 30^E, followed by a third transcriptional amplifier (Amp 3), under transcriptional control of Amp 2. The arsenic and mercury regulatory circuits, Amp 30^E, Amp 2, and first half of Amp 3 (regulatory proteins), were engineered into a low-copy number plasmid. The *gfp* reporter was placed under transcriptional control of the cognate promoter of Amp 3 in a high-copy number plasmid, thus establishing a signaling cascade. Using this methodology, the investigators substantially increased the sensitivity of the arsenic and mercury sensors from detection limits of >10 and > 50 parts per billion (ppb) to ≤ 0.1 and ≤0.01 parts per billion (ppb), respectively. Furthermore, this approach improved output readout 750- and 200-fold when detecting arsenic or mercury, respectively. The use of cascading amplifying circuitries shows significant potential as a tool for effectively fine-tuning the analytical performance of MWCBs, helping pave the way for their real-world applications.

Due to the living nature of MWCBs, small differences in cell viability between analytical runs can result in signal fluctuations, thereby also affecting read-out reproducibility and detection limits. To address this, investigators have largely relied on the use of optical density measurements and cell counting techniques to determine the quantity of cells in an analytical run. However, these techniques provide no information as to the quantity of viable cells producing a signal and therefore are not the best methods to ensure reproducibility of the measurements. In that regard, other approaches have been explored where duplex reporter

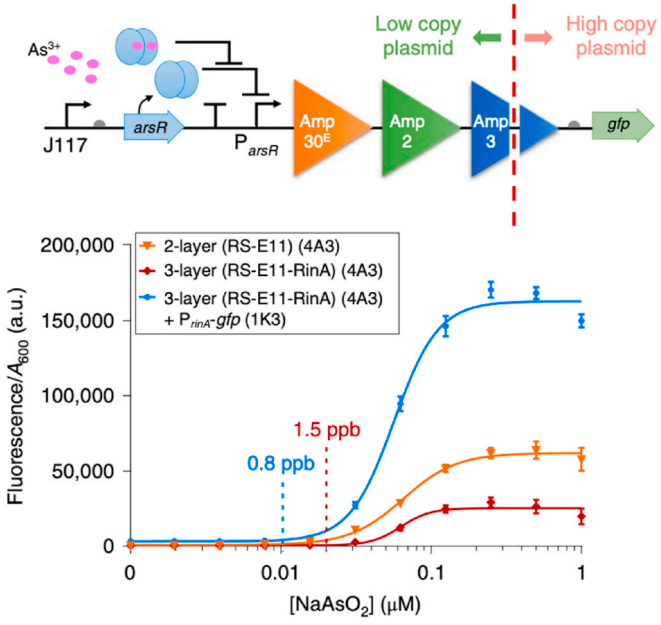

Fig. 10. Employment of cascaded amplifying circuits implemented on two plasmids vastly improved the output of an arsenic MWCB. Adapted from Wan et al., (2019).

methodologies have been employed to improve the analytical reproducibility of MWCBs. Mirasoli et al. report incorporation of a second constitutive reporter into the plasmid of a MWCB for L-arabinose detection (Mirasoli et al., 2002). The signal generated by the second reporter protein functioned as an internal response correction system for nonspecific stimuli and cell viability. In the context of adverse experimental conditions, the underestimation of L-arabinose concentration was revealed and corrected by taking into account the expression of the second reporter gene, providing an internal baseline signal of cell viability and metabolic activity. A similar cadmium MWCB has been developed by cloning both green and red fluorescent proteins onto a single vector (Bereza-Malcolm et al., 2017). Expression of the green fluorescent protein was regulated by cadmium while the signal generated by the constitutively expressed red fluorescent protein was indicative of metabolic health and cell viability. While more work needs to be done to further address this issue, the use of duplex reporter methodologies could help provide more reliable and consistent MWCB outputs by taking into account secondary signals indicative of microbial viability.

A novel approach based on cellular aggregation to strengthen the signal generation and sensitivity of MWCBs has recently been reported. Chen et al. report the development of a MWCB which couples bioluminescence and cell aggregation to provide lower detection limits (Chen et al., 2020). A previously developed mercury MWCB, based on the MerR regulatory protein and *luxCDABE* reporter, was modified to carry genes for either nMagHigh or pMagHigh. nMagHigh and pMagHigh are photoswitchable proteins which are expressed on the surface of *E. coli*. Cocultures of bacteria displaying nMagHigh and pMagHigh aggregate into larger clusters under blue light due to the blue light-dependent heterodimerization of these two proteins. The investigators found that the biological light generated by *luxCDABE* was capable of inducing dimerization of the light-switchable proteins, resulting in cellular aggregation. Furthermore, when incorporated with the mercury MWCBs, the synergistic effect between bioluminescence production and photo-switchable cellular aggregation resulted in a 10-fold signal enhancement in low concentrations of mercury. While MWCB sensitivity remains a significant challenge, highly promising work such as the ones previously described are helping pave the way towards achieving the engineering of highly sensitive MWCBs.

There is a growing interest in engineering MWCBs for use as multiplex assays in which various analytes can be detected from a single analytical run. This could be achieved by engineering MWCBs with multiple differentiable reporters, each under the control of distinct promoters specific to each analyte of interest. Yoon et al., for example, report on a multiplex MWCB for the simultaneous detection of cadmium and arsenic in contaminated soils (Yoon et al., 2016). The investigators transformed *E. coli* cells with two sets of plasmids, one for the detection of arsenic via expression of a red fluorescent protein and another for arsenic detection via expression of green fluorescent protein. The MWCBs displayed strong signal induction in the presence of arsenic and cadmium, however weaker signals were generated in the presence of other MTEs such as Zinc. While MWCBs are highly amenable for multiplex assay development, the reliability of these assays is hindered by the level of specificity and selectivity of the regulatory proteins and promoters employed to construct them. Notwithstanding, the various efforts previously described to tighten the regulatory properties of these molecular recognition events are also enabling the development of highly specific multiplex MWCBs.

Finally, MWCBs can vary greatly in their response times. In inducible MWCBs, microbial cells must first detect a bioavailable analyte and subsequently produce a quantifiable signal, all while maintaining cellular viability. Many factors can play a role that will affect the response time of an inducible MWCB, such as the permeability of the microbes to a given analyte, the diffusion rate of an analyte through the sample matrix, the complexity of genetic circuitries employed, the strength of the underlying molecular recognition events, the metabolic

rate of the microbial cell, and the type of reporter molecule employed. Furthermore, efforts aimed at improving the analytical parameters of a MWCB, such as incorporating additional regulatory components, could result in the lengthening of the response time due to increased metabolic load.

In certain cases, response times can be quite rapid. For example, certain nonspecific and specific MWCBs can produce a result within 1–15 min (Bulich and Isenberg, 1981; Reyes et al., 2020; Rothert et al., 2005). However, response times and detection limits are intimately related. In many cases the response time needs to be lengthened to allow for improved detection limits via increased production of the reporter protein, yielding higher signal-to-noise ratios. Thus, the tradeoff between response times and detection limits allows for tailoring of these parameters to the needs of a given MWCB application.

3.3. Shelf-life

Another challenge in the way of achieving widespread use of MWCBs is their modest shelf life. While lyophilization techniques might address some of the long-term storage issues, the very nature of physiologically active microorganisms presents a significant bottleneck in the efficiency of such immobilization techniques. This challenge can be overcome by developing MWCBs based on physiologically inactive, e.g. spore forming, microorganisms. Certain microorganisms, such as *Bacillus*, *Clostridium*, and *Saccharomyces* can form spores when exposed to nutrient deficient or harsh environments that are unfavorable for their growth (Knecht et al., 2011; Wynn et al., 2017). Sporulation is a process that results in the protection of DNA in a hard, dry coating. Microorganisms in this dormant state can survive many years, with some reports of spore dormancy spanning more than 25 million years (Desnous et al., 2010). In the presence of favorable nutrient-dense environments, sporulated microorganisms can return to their physiologically active state in a process called germination. Furthermore, spores have the added benefit of being highly resilient towards environmental insults such as pH, temperature, radiation, and chemical changes.

Work done by Date et al. demonstrated the feasibility of engineering spore-based MWCBs (Date et al., 2007). Briefly, the investigators employed *Bacillus subtilis* and *Bacillus megaterium* strains for the development of sensing systems for arsenic and zinc via the *lacZ* and *gfp* reporters, respectively. They were able to demonstrate how the analytical performance of their *Bacillus*-based sensing system was consistent with that of other sensing systems developed for arsenic using nonsporulating bacteria, such as *E. coli*. Notably, they found that the analytical performance of their spore-based sensors did not change significantly after submitting them to several sporulation-germination cycles (Fig. 11).

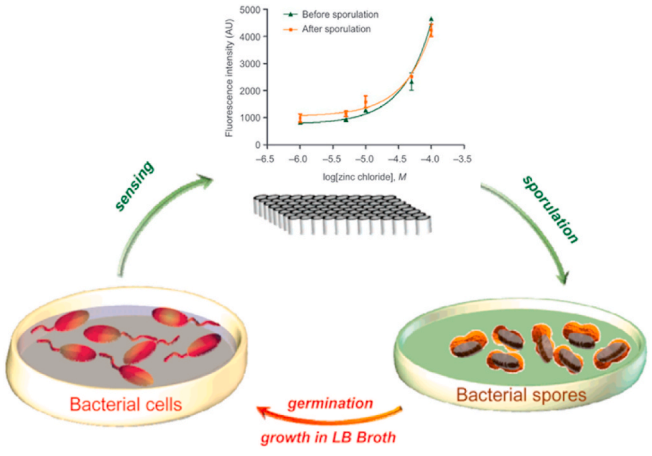

Fig. 11. The analytical performance of spore-based MWCBs is not affected by sporulation and subsequent germination cycles. Adapted from Wynn et al., (2017) and Date et al., (2007).

Furthermore, both of the spore-based sensing systems retained their analytical performance after being stored at room temperature for at least 6 and 8 months, respectively, highlighting the superior storage capabilities of spore-based MWCBs when compared to non-sporulating MWCBs.

Aside from long-term storage capabilities, the use of spores presents an opportunity to deploy MWCBs in harsh environments. A study published on astrobiology, for example, showed that *Bacillus subtilis* strain WN511 spores on artificial meteorites survived hypervelocity (1.2 km/s) and high temperature (145 °C) (Fajardo-Cavazos et al., 2005). As seen in Fig. 12, Sangal et al. demonstrated the stability of their Bacillus-based arsenite MWCB after exposure to four lab-simulated weather conditions, dry heat (37 °C), wet heat (27 °C, 90% humidity), low temperature (−20 °C), and extreme dryness (room temperature in a desiccator), for a period of 12 months (Sangal et al., 2011). Each month, spore aliquots from each condition were transferred to fresh growth media and allowed to germinate. Then, the stability and analytical performance of the germinated bacteria were monitored through bioanalytical assays. The investigators found that the viability and sensing abilities of the germinated cells were conserved upon storage, regardless of the different storage conditions, for the entire duration of the 12-month study.

The use of spore-forming microorganisms as vehicles for MWCB development which are highly amenable to long-term storage opens the door to their implementation into other analytical platforms, such as microfluidics. Date et al. demonstrated this possibility by incorporating their arsenic and zinc *Bacillus*-based MWCBs into centrifugal compact disk (CD) microfluidic platforms, resulting in a portable sensing system for fast on-field analysis (Date et al., 2010). Their work illustrated that spore-based sensing can effectively be performed on a microfluidic platform at various levels of complexity of the assay, regardless of the requirement of adding substrate for generation of analytical signal.

The choice of immobilization technique, genetic circuitry, and microorganism all factor greatly into the design of an analytical device based on MWCBs. Immobilization techniques each offer a unique way to safely deploy MWCBs, however they each come with their own pitfalls. The use of physiologically active microorganisms significantly lowers the incubation time of the MWCBs but are less amenable to long-term storage. Physiologically inactive microorganisms such as sporulated *Bacillus* species addresses many of the shelf-life and storage concerns for non-spore based MWCBs, however these added benefits come with extended incubation and response times due to the additional germination step. Some investigators are looking into curtailing this extended

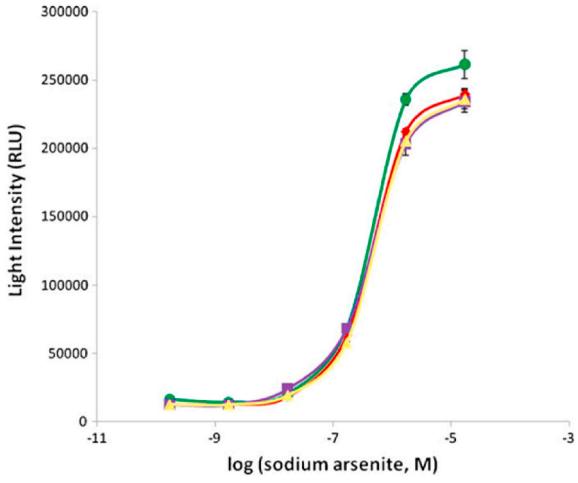

Fig. 12. Spore-based MWCBs are highly resilient to environmental challenges. The viability and sensing ability of a spore-based MWCB was maintained after 12 months of storage in cold (□), dry heat (●), desiccation (■), and wet heat (▲). Adapted from Sangal et al., (2011).

incubation time by exploring the possibility of expressing the necessary MWCB proteins on the outer surface of the spore. Work done by Chen et al., for example, has demonstrated the feasibility of immobilizing multiple enzymes on the surface of *B. subtillis* endospores in a stoichiometrically controllable manner (Chen et al., 2017a). Clearly, the field of MWCBs will progressively evolve to address each of these concerns, as knowledge on biological systems continues to grow, and emerging biotechnologies continue to develop.

4. The future of microbial whole-cell biosensors

While we have described MWCBs as best suited for use in affordable, easy to use, point of care devices, as well as some of the challenges in the way of their broader in-field implementation, there is some truly exciting work in the literature that is expanding beyond what was thought to be possible with MWCBs. From ingestible devices to their incorporation with drone technologies, the future of MWCBs looks incredibly promising. In the following section, we will highlight some of the work that is paving the way for the future of MWCB technologies and offer our thoughts on other possibilities for the future of MWCBs.

Liu et al. describe the development of a set of living materials and devices based on stretchable, robust, and biocompatible hydrogel-elastomer hybrids that host various types of MWCBs employing GFP as the reporter protein (Liu et al., 2017). The investigators combined the hydrophilic and stretchable qualities of polyacrylamide (PAAm)-alginate hydrogels with the air permeability and mechanical robustness of polydimethylsiloxane silicone elastomer to develop a hydrogel-elastomer hybrid material that maintained cell viability and displayed high mechanical robustness. They envisioned the development of a living patch that can be fixed onto human skin for the chemical detection of components relevant to human sweat or blood, as well as a glove with MWCBs integrated at the fingertips for potential environmental monitoring. As a proof-of-concept, a living patch harboring MWCBs for the detection of rhamnose and a glove harboring MWCBs for the detection of rhamnose (Rham), β-D-1-thiogalactopyranoside (IPTG), and AHLs, on three fingertips (Fig. 13). The living patch visibly fluoresced within 4 h of adhesion to skin swabbed with Rham, and the glove fluoresced in response to gripping cotton balls soaked in Rham and IPTG at the two corresponding fingertips also within 4 h. The living patch and biosensing glove show the potential of wearable living materials and devices for healthcare and environmental monitoring.

In another study, Wang et al. envision the use of genetically tractable microbial cells to create multifunctional, moisture-responsive interfaces (Wang et al., 2017). Inspired by the shape-transforming nature of living cells in response to moisture gradients, they speculated that a bilayer-structured biohybrid film could change shape and functions simultaneously with humidity change. Briefly, constitutive *E. coli*-based MWCBs expressing GFP were microprinted in parallel lines on a latex surface to produce a multifunctional biohybrid film. When introduced to dry conditions, the contractile forces generated by cell dehydration bent the film while GFP fluorescence intensity correlated directly with humidity. To push their concept further, they designed ventilating bio-flaps composed of the biohybrid material which opened up in response to sweat production during exercise and implemented them onto prototypes of a running suit and shoe (Fig. 14). The flaps on the running suit opened up after 5 min of exercise, removing sweat from the body and helping cool the wearer, while the running shoe exhibited both shape and fluorescence intensity change under different humidity conditions. This innovative and futuristic take on the incorporation of MWCBs as functional features of clothing presents a scalable and adaptable technology that has pushed the boundaries of what was thought to be possible with MWCBs.

While some investigators have focused on developing wearable analytical and functional devices, other investigators have turned to exploring the possible use of MWCBs in ingestible devices. Mimee et al. describe the development of an ingestible micro-bio-electronic device

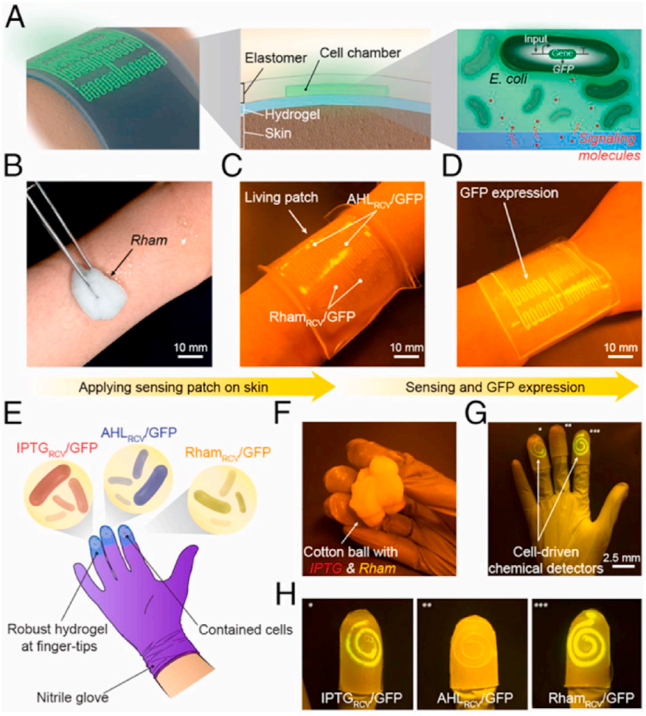

Fig. 13. (A) Liu et al. engineered MWCBs using the GFP reporter and immobilized them in a hydrogel-elastomer hybrid biomaterial. **(B–D)** Proof-of-concept of a living patch for the chemical detection of components relevant to human sweat and/or blood. **(E–H)** Proof-of-concept of a wearable glove with immobilized WCBs on each fingertip for multiplex sensing in environmental monitoring. Adapted from Liu et al., (2017).

(IMBED) that combines engineered MWCBs together with ultra-low-power microelectronics to enable in situ detection of gastrointestinal biomolecules associated with health or disease (Fig. 15) (Mimee et al., 2018). Engineered *E. coli* for the detection of heme from lysed red blood cells via the luminescent *luxCDABE* reporter were coupled with photodetectors embedded into an electronic device which process the signal and transmit it wirelessly to an external radio or cellular phone for convenient readout. The MWCBs lie in individual wells separated from the outside environment by a semipermeable membrane that confines the cells and allows for the diffusion of small molecules. The investigators were able to show their IMBED could detect the presence of heme in a porcine model of gastrointestinal bleeding. They also highlight the flexibility of their device by demonstrating detection of thiosulfate and AHL in a fluidic environment, by simply swapping out the MWCBs for detection of said analytes. Their integration of MWCBs with semiconductor electronics offers opportunities to transform diagnosis, management, and monitoring of health and disease.

Other investigators have taken an alternative approach to ingestible MWCBs by exploring the possibility of utilizing MWCBs as probiotics for detection and management of disease. Mao et al. recently developed a MWCB for the detection and suppression of *Vibrio cholerae* (Mao et al., 2018). They took advantage of the knowledge that *V. cholerae* is sensitive to acidic environments, and so chose to experiment with the common gut colonizer *Lactococcus lactis*. *L. lactis* displays strong acidification capabilities, so much so that the investigators were able to demonstrate that administration of *L. lactis* in an infant mouse model for cholera significantly reduced aggressive advancement of cholera. They next explored the possibility of engineering an *L. lactis*-based MWCB for the detection of CAI-1, a *V. cholerae* quorum sensing molecule, via production of the colorimetric β-lactamase reporter. The investigators were able to demonstrate successful detection of *V. cholerae* infection via colorimetric analysis of fecal samples in an infant mouse model for

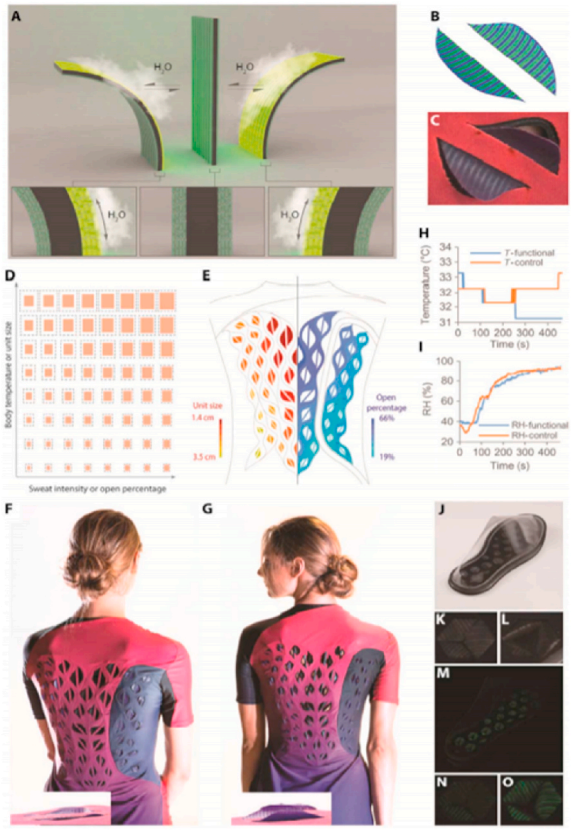

Fig. 14. (A-O) Wang et al. developed proof-of-concept moisture-responsive clothing and shoes based on their MWCB biomaterial. Adapted from Wang et al., (2017).

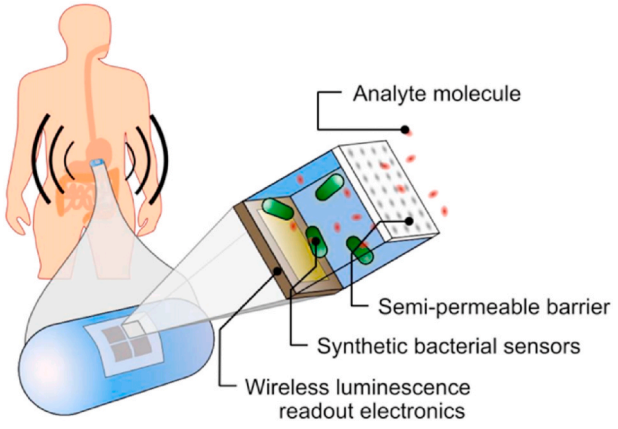

Fig. 15. Mimee et al. developed an ingestible micro-bio-electronic (IMBED) device based on MWCBs for in vivo detection and wireless readout of gastrointestinal biomolecules associated with health or disease. Adapted from Mimee et al., (2018).

cholera. Furthermore, the combination of natural and engineered *L. lactis* was able to detect cholera and protect the mice from *V. cholerae* propagation (Fig. 16). Their work sets the stage for dietary intervention and disease screening based on probiotic MWCBs as a strategy for early disease diagnosis as well as combat pathogenic diseases.

Another interesting development in the MWCB field is its implementation with emerging drone technologies. Lu et al. describe the development of a portable incubation system that allows for growth of MWCBs during continuous environmental monitoring in remote locations (Lu et al., 2015). The design of the system consists of a microfluidic channel for microorganismal inoculation, a temperature regulating system, and a mechanical enclosure to house all components. The microfluidic channel was fabricated using polydimethylsiloxane (PDMS), a transparent, gas permeable, and chemically inert material. The system itself weighed about 200 g, was able to be lifted by an unmanned aerial vehicle (UAV), and also capable of regulating the temperature such that it remained at 37 °C precisely and effectively in different environmental conditions (Fig. 17). The authors describe potential applications of their portable incubator system for air pollution sensing in remote and unreachable areas, given the PDMS microfluidic channel allows for effective gas diffusion. MWCBs for the detection of known air pollutants, such as CO2, O3, and NO2, could be paired with the portable incubation system and UAV's for real-time, cost-effective, monitoring of environmental and air pollution.

Other investigators are exploring the possibility of incorporating MWCBs into autonomous robots for environmental monitoring of remote ocean areas. Bayat et al. report the development of the Envirobot, a bio-inspired environmental monitoring platform (Fig. 18) (Bayat et al., 2016). This autonomous marine vehicle is based on existing segmented anguilliform swimming robot, with long range communication capabilities and versatile flexible environmental sensor integration. Given the amenability of MWCBs for miniaturization, their integration into autonomous vehicles such as the Envirobot presents an exciting venue for MWCB deployment for environmental monitoring (van der Meer, 2016).

The rapidly advancing capabilities of cellular phone devices have made them an enticing transducer for the development of MWCBs. Some of the studies previously described in this review, such as Mimee et al.'sIMBED device and Reyes et al.'s MWCB for UTI detection, explored this possibility (Mimee et al., 2018; Reyes et al., 2020). The IMBED device was capable of sending real time heme sensing data to an external cellular device, while the Reyes team developed specialized cellphone-based UTI bioluminescence extinction technology (CUBET). The CUBET system is a 3D printed instrument designed to fit onto the camera of a cellular phone, retrofitted with chambers for lyophilized bacteria. Once the sample is added into the chambers, all that is needed is a cellular phone to capture images of the well to determine if the samples are positive or negative for UTI. Other examples of cellphone-based MWCB systems include Kim et al.'s bioluminescent-based analyte quantitation by smartphone (BAQS) technology, and Lu et al.'s LumiCellSense (LCS) system (Kim et al., 2017; Lu et al., 2019). These technologies show great potential for the eventual development of an at-home or in field diagnostic device, in which users can monitor their own health with nothing more than a MWCB and their cellular phone.

MWCBs have also been shown to have great potential for use in counterterrorism initiatives. We previously described Yagur-Kroll et al.'s work with directed evolution to develop MWCBs for the sensitive detection of 2,4,6-TNT and 2,4-DNT (Yagur-Kroll et al., 2015). 2,4, 6-TNT is frequently used as the main explosive component in munitions such as landmines. 2,4-DNT is a synthetic byproduct of 2, 4, 6-TNT and due to its relative volatility, permeates through plastic landmine components and is often considered a suitable marker for the presence of buried explosives. Work by this group has demonstrated the feasibility of employing these MWCBs for the remote detection of buried landmines (Belkin et al., 2017). By developing a laser-based optoelectronic system for the remote detection and quantification of bacterial fluorescence, and deploying it in tandem with MWCBs for 2,4-DNT detection immobilized in 3-mm-diameter alginate beads, the investigators were able to identify and map 13 buried landmines from a distance of 20 m in a small field test. This work is highly promising, and further optimization of the MWCBs and remote detection system could one day result in a reliable system for the detection of buried improvised explosive devices in war ravaged countries that could save countless lives.

Recently, NASA launched the BioSentinel mission as the sole biological experiment to fly on the Space Launch System's first Exploration

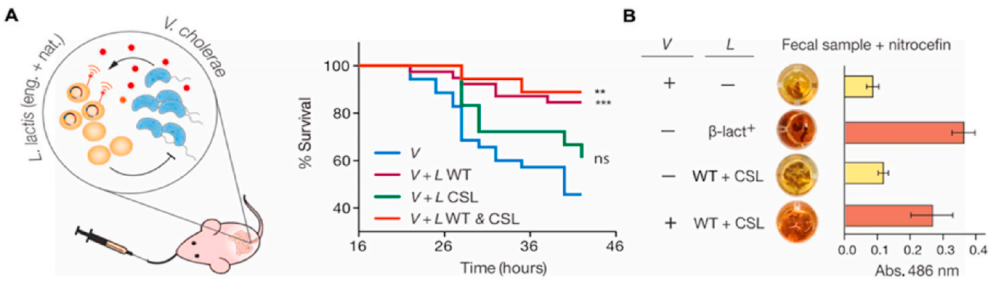

Fig. 16. Mao et al. demonstrate successful **(A)** amelioration and **(B)** detection of cholera infection via probiotic administration of MWCBs in an infant mouse model of cholera infection. Adapted from Mao et al., (2018).

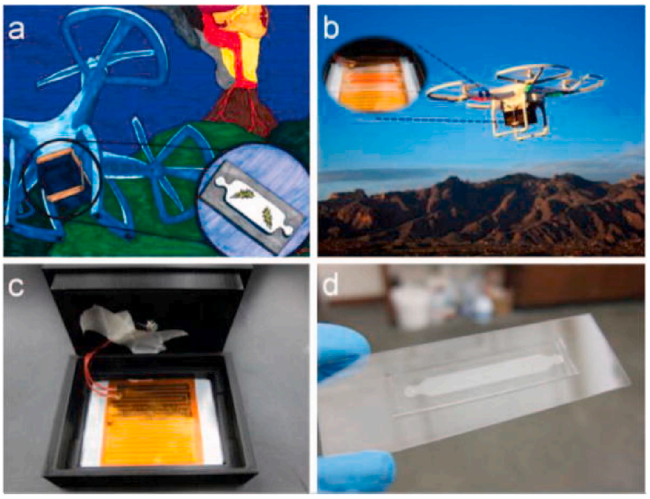

Fig. 17. (a–d) Lu et al. developed a light-weight, portable incubator system that can be attached to unmanned aerial vehicles to house MWCBs for the detection of environmental pollutants in remote and unreachable areas. Adapted from Lu et al., (2015).

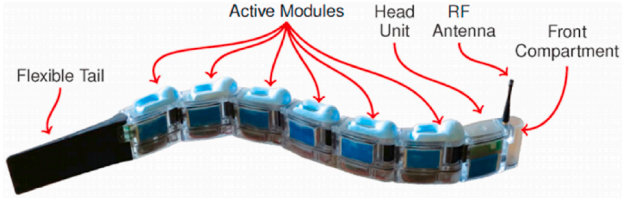

Fig. 18. Envirobot with six active modules, flexible tail, head unit, RF antenna, and front compartment. Adapted from Bayat et al., (2016).

Mission (EM-1). The primary objective of BioSentinel is to develop a biosensor to detect and measure the impact of space radiation on living organisms over long durations beyond Low Earth Orbit (LEO). While the biosensor employed in BioSentinel is not a MWCB, it is based on the microorganism *S. cerevisiae*. Work such as this opens the door for the incorporation of spore-based MWCBs in future space missions. Based on the results of the BioSentinel mission, future spore-based MWCBs could be designed to help monitor molecules within spacecraft relevant to the health of the astronauts inhabiting them.

The field of nanotechnology is providing new technological capabilities to investigators in a variety of fields, including those in MWCB development. In a recently published opinion, Guo et al. discuss the advent of a third generation of whole-cell sensing systems heralded in by the fusion of synthetic biology with nanotechnology (Guo et al., 2020). They speculate on the vast capabilities that could be enabled by coating whole-cell sensing systems with biomimetic nanomaterials. These include the ability to magnetically orient sensing cells to specific locations for onsite detection or for safe removal from the environment via their coating with magnetic nanoparticles, and even eliminating the concern of potential cell leakage by coating the cells with restrictive nanocages which minimize the risk of horizontal gene transfer. While more work is needed at the frontiers of synthetic biology and nanotechnology to fully realize these visions, MWCBs will undoubtedly see a radical transformation in the years to come.

Finally, we envision a future in which the potential of MWCBs is fully realized by their complete integration for the development of smart cities (Fig. 19). Miniaturized, autonomous devices mounted on UAV's and marine vehicles carrying MWCBs for the detection of a variety of analytes could continuously monitor water and air pollution in industrial sites, urban and rural counties, as well as in agricultural and environmental land. The massive amount of data generated could be remotely sent to central computing hubs where programs dictated by artificial intelligence process and interpret the data, yielding continuous real-time information on air, water, and soil quality.

5. Conclusions

MWCBs are a highly versatile platform for the development of affordable and accessible analytical devices, offering an enticing alternative to their more cumbersome and expensive analytical counterparts. The two classes of MWCB, constitutive and inducible, can be used to determine the toxicity of a sample or quantify bioavailable levels of a target analyte, respectively. Constitutive MWCBs largely rely on observing the decrease in a constitutively generated signal in response to the toxicity of a given sample, while inducible MWCBs employ genetic circuitries to generate a quantifiable signal in response to a target analyte in a dose-dependent manner. Given the wide array of genetic circuitries and reporters that can be employed in their development, MWCBs are highly adaptable for use with samples from numerous scientific fields.

MWCBs have been developed for the detection of analytes relevant to environmental and agricultural monitoring. These include analytes of interest typically found in soil and water samples such as micronutrients, MTEs, organic compounds, and quorum sensing molecules. Additionally, MWCBs have also been developed for use in biomedical applications. MWCBs have been used to develop diagnostic devices for the detection of diseases such as UTI's as well as to further the understanding of the microbiome in other conditions such as Crohn's disease. While they have seen success in the laboratory, there are still various challenges facing the field MWCB development.

Given the living nature of MWCBs, many of the difficulties facing their development revolve around this fact. However, there has been significant progress in addressing these concerns. Regarding safe handling and disposal, there are several encouraging technological developments in immobilization techniques such as in lyophilization and hydrogel entrapment. As recombinant and statistical modeling techniques have progressed, so too have the efforts in improving sensitivity concerns. Furthermore, the use of spore-forming microorganisms shows

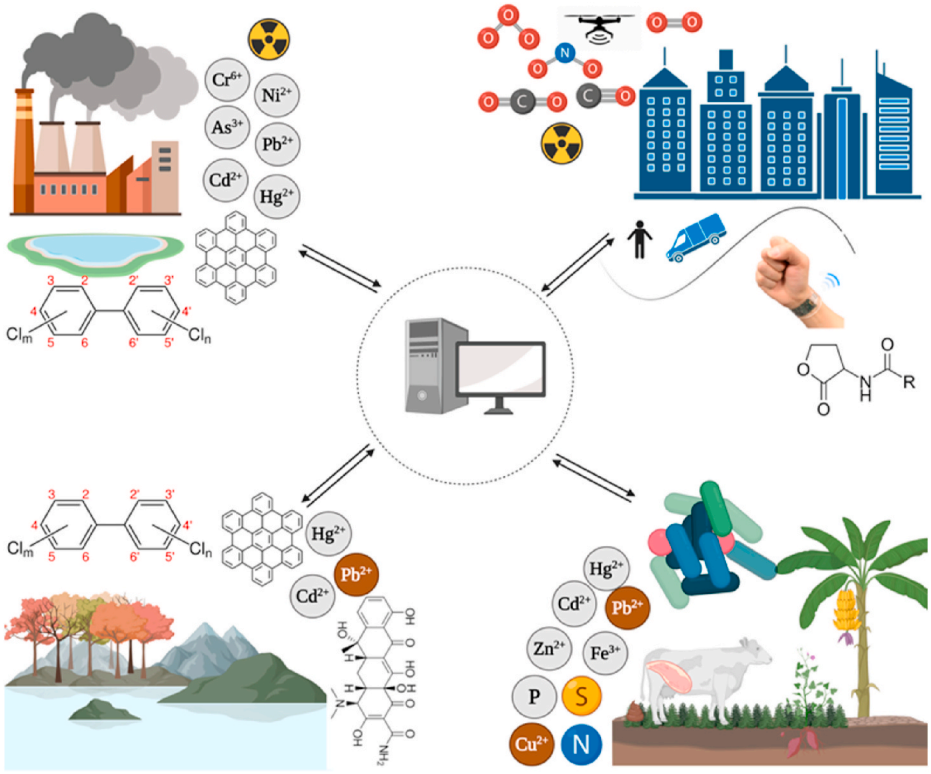

Fig. 19. The future of smart urbanization where continuous real-time monitoring of air, water, and soil is enabled by MWCBs.

great promise in significantly extending the shelf-life and handling of MWCBs.

Finally, the high versatility and adaptability of MWCBs has shown great promise for their potential use in novel and exhilarating ways. From their incorporation into ingestible devices for real-time analysis of gastrointestinal metabolites, to wearable clothing and living patches, and assimilation with autonomous robots and drones for the monitoring of otherwise inaccessible environments, MWCBs are greatly amenable for their use in new frontiers. As work continues to be done in their development and optimization, MWCBs will become an increasingly useful and enticing analytical tool.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

SD and SKD thank the following grants from the NIH R01GM127706 and 1R01MH110415-01A1; NSF-CBET-1841419 and CBET-2041413; Ministry of Defense Israel-4441018452; DoD-W81XWH2010697; and Firefighters Cancer Initiative State of Florida appropriation #2382A. SD is also grateful to the NIH for grants R01MH104656 and UL1TR002736, and to the Miller School of Medicine of the University of Miami for the Lucille P. Markey Chair of Biochemistry and Molecular Biology. Figs. 2, 4, 5 and 19 were created with BioRender.com.

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