Environmental Biosensors:
A Microbiological View

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Abstract

In this mini-review, the potential of using microorganisms to design biosensors for detecting environmental pollutants is analyzed and discussed. A distinction is made between a classical biosensor (CB) and a whole-cell biosensor (WCB), emphasizing their structural components and the possibility of using whole microorganisms as their bioreceptor elements. The advantages and disadvantages of using prokaryotic microorganisms as opposed to eukaryotic microorganisms are described. Likewise, the advantages of using protozoa (ciliates) over other eukaryotic microorganisms are also shown. We analyze the current bibliography on biosensors built on microorganisms as bioreceptors of pollutant molecules, such as inorganic (metal (loid)s) or organic (xenobiotics). New trends, such as the prokaryotic riboswitches, microbial two-component systems where the pollutant can be simultaneously detected and bioremediated, along with advances in synthetic biology, are shown as promising tools in the design of environmental biosensors.

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Introduction

The Anthropocene is the geological epoch that has been proposed to succeed the Holocene (the current epoch of the Quaternary Period), due to the tremendous global impact that human activity has on all the ecosystems of our planet. During the last two centuries, the anthropogenic activity has not only caused a significant accumulation of greenhouse gases (such as CO_2) but has also increased levels of both inorganic (metal(loid)s) and organic (xenobiotics) pollutants. This activity has serious impacts on climate (climate change we are already undergoing), biodiversity, environment, and human health.

Certain metal(loid)s, mainly those misnamed "heavy metal(loid)s," are among the most abundant, toxic, and persistent inorganic environmental pollutants (Hill 2004). Although one-third of the elements from the periodic table are essential to life, they can be toxic at high concentrations, and other nonessential elements are very toxic to living beings at very low concentrations. Diverse anthropogenic sources, such as mining and other industrial activities, have substantially increased the metal(loid) content present in the atmosphere and in many terrestrial or aquatic ecosystems (Peñuelas and Fillela 2002). Metal(loid)s can, directly or indirectly, originate reactive oxygen species (ROS) with significant alterations in proteins, nucleic acids, and lipids (Leonard et al. 2004; Valko et al. 2005). A new confirmation of the pollution of our planet is a recent report on Antarctica, in which atmospheric aerosols have been analyzed during the austral summer (2016–2017) (Caceres et al. 2019). The average mass concentration of particulate matter in some places of the coast of the Antarctica region was as high as 28.2 μg/m^3. About 100 times more lead and about 600 times more chromium have been found in the air than in the soil in this region, showing a remote anthropogenic origin (Caceres et al. 2019). Organic compounds that are not part of living beings (xenobiotics) can also be an important source of environmental pollution. However, unlike metal(loid)s, many of them can be completely degraded by microorganisms to their final components (CO_2 + H_2O). But, like metal(loid)s, they can be very toxic too.

Because of the ecological, sanitary, and economic consequences of these two big groups of environmental pollutants, they are considered to be priority in ecotoxicology, with the aim of minimizing the exposure to animals or humans. It is difficult to predict the global effects of increasing the different types of environmental pollutants, so there is an overriding need to develop screening methods for environmental monitoring. This need is both for the detection of pollutant compounds and monitoring of bioremediation processes of ecosystems contaminated by inorganic or organic compounds. These chemical pollutants can be measured using molecular recognition or chemical analysis, such as absorption spectroscopy, mass spectroscopy, gas chromatography, polarography, and others. However, these techniques

require qualified personnel, present a high cost, and it is not possible to carry out in situ analysis. In addition, important ecotoxicological parameters such as bioavailability, toxicity, and genotoxicity can only be assayed using living cells (Gutierrez et al. 2015, 2017). Likewise, the most sensitive screening methods to detect pollutants are those incorporating biological components, which are used as targets for an active substance or pollutant. These detection tools that incorporate biological material or are living cells are known as biosensors or bioreporters. Therefore, we can distinguish two types of biosensors: the classical or conventional biosensors (CBs) and the whole-cell biosensors (WCBs) (Gutierrez et al. 2017).

We can define CBs as integrated bioreceptor-physicochemical transducer devices, which consist of three different parts: a bioreceptor or biological recognition element, which interacts with the pollutant molecules; a physicochemical transducer, which converts the biological response into a measurable physicochemical signal; and a microelectronic processor of this signal, which amplifies and converts it into a numeric record. The biological component can be macromolecules (enzymes, antibodies, nucleic acids, etc.) or whole cells (microorganisms or cells from multicellular organisms). We can consider four main different types of transducers: electrochemical (potentiometric or amperometric), optical (spectrophotometric or fluorometric), piezoelectric, or thermometric. To construct these CBs, we need to have both biological and physicochemical knowledge, which frequently involves an interdisciplinary cooperation among different specialists.

The second type of biosensors, WCBs, was introduced as an alternative to CBs (Belkin 2003; Van der Meer and Belkin 2010). WCBs use prokaryotic or eukaryotic whole cells as single reporters, which incorporate both bioreceptor and transducer elements into the same cell. In general, this involves that organisms used as WCBs are experimentally modified to incorporate transducer capacity or increase their sensitivity against the pollutant. Unlike CBs, these WCBs have the advantageous feature of carrying out both *in situ* and *ex situ* analyses. When using WCBs, two different types of bioassays can be distinguished; *turn off* or *turn on* assays (Belkin 2003). *Turn off* bioassays are similar to standard toxicological bioassays, so the sample toxicity is evaluated from the inhibition degree of a specific cellular activity, such as growth inhibition, respiration rate, motility depletion, etc., or an unspecific cell viability. In these bioassays, the toxic concentration is proportional to the measurement of the cellular function inhibition or the cell mortality percentage. The molecular reporters used in *turn off* bioassays are under a constitutive gene expression (Gutierrez et al. 2017); therefore, by increasing the toxicity of the sample, cell viability is affected, and the expression of the reporter gene decreases. An example of this type of bioassay is the one marketed by the company NCIMB (UK) and called MARA (Jouanneau et al. 2017). This bioassay is based on the growth inhibition of 11 microbial strains (including 10 bacteria and 1 yeast), and cell viability is measured in microtiter plates (96 wells) by assessing of the intensity of a redox red dye which acts as a marker of cellular metabolic activity. Another possibility is to use natural bioluminescent bacteria strains (LumiMARA bioassay), such as *Aliivibrio fischeri* or *Photobacterium phosphoreum* among others, so the pollutant toxic effect is assessed by the bioluminescence inhibition emitted by the cells (Jung et al. 2015).

On the other hand, in *turn on* bioassays, a quantifiable molecular reporter is fused to an inducible gene promoter to be activated by a specific or group of environmental pollutants. In these WCBs, the sample toxicity or pollutant concentration is proportional to the reporter molecule gene expression. This reporter signal may reach a maximum value (critical pollutant concentration); after reaching this value, the reporter signal decreases due to the greater toxicity of the sample that can lead to cell mortality. This critical pollutant concentration value will depend on the degree of cellular resistance to the pollutant.

In general, *turn off* bioassays are usually quite unspecific because the reporter signal decreases as a result of a broad range of cytotoxic effects originated by very different pollutants, while *turn on* bioassays or CBs (using specific molecules as bioreceptors) are habitually more specific, as induction of the gene reporter or interaction with the molecular bioreceptor only takes place when the specific pollutant is present. Therefore, the WCB specificity will depend on the degree of the gene promoter specificity to be induced by an exclusive pollutant or a chemically related group of pollutants. CB specificity will depend on the specificity degree of the interaction between the bioreceptor and the pollutant. With regard to specificity, both *turn on* WCBs and CBs have been classified into effect- and compound-specific biosensors (Yagi 2007). Effect-specific biosensors respond to physicochemical environmental changes (e.g., pH, temperature, or osmotic changes) or to a chemically diverse group of pollutants that induce a type of cellular stress response (e.g., oxidative stress or protein toxicity). Compound-specific biosensors respond to only one type of pollutant or compounds with similar chemical features (e.g., metal(loid)s). For some other specialists, the specificity-based classification of CBs or WCBs may be divided into three classes: class-I biosensors which only respond to a specific or exclusive pollutant by increasing the reporter signal; class-II that responds to a specific cellular stress, like oxidative stress, by increasing the reporter signal; and class-III responding unspecifically to different pollutants or environmental stressors (Gutierrez et al. 2017).

All these types of biosensors (CBs and WCBs) can be useful to detect the presence of organic or inorganic pollutant molecules in the environment. In addition, they can be also used for biomonitoring or testing the progress of a bioremediation process after detecting the chemical nature of the pollutant.

Environmental Biosensors: Why Use Microorganisms?

Microorganisms are the most abundant living beings on our planet. The estimated number of microbial genomes in the biosphere is around 10^{29} to 10^{30} (Huse et al. 2010; Kallmeyer et al. 2012), which exceeds the estimated number of galaxies (10^{11}) present in our observable universe. It is between 2 and 3 orders of magnitude of the total number of animal and plant cells together on our planet. In the oceans, they represent 90% of the weight of all organisms. Therefore, they are not only quantitatively important but also qualitatively important, since they occupy all known ecosystems. This adaptation to different ecosystems has contributed to the origin of many different

metabolisms and physiologies. Microorganisms participate in many biogeochemical cycles, with some exclusive physiologies, such as the atmospheric nitrogen fixation or the anaerobic ammonium oxidation. Microorganisms interact with both biotic and abiotic components of their ecosystems, and these interactions are essential for ecosystem functions (Shahsavari et al. 2017). They have developed different mechanisms to counteract the toxic action of inorganic and organic contaminants, and they can eat many organic pollutants of anthropogenic origin (biodegradation). This wide adaptation to different habitats makes them very useful tools when we want to design biosensors for the detection of environmental pollutants.

The complexity of the biotic and abiotic elements that make up an ecosystem makes the design of an environmental biosensor more complex than those biosensors designed for industry or clinical use. The possible interactions of the microbial biosensor (CB or WCB) with unknown elements existing in a specific ecosystem, affecting the interpretation of the detection, are the main handicap of this type of biosensors. However, in spite of that, the majority of reported CB or WCB are based on prokaryotic or eukaryotic microorganisms (Verma and Singh 2005; Gutierrez et al. 2017; Metha et al. 2016). The main reason lies in the greater facility to obtain the sufficient quantity of a specific purified macromolecule (enzyme, antibody, etc.) or whole microorganisms for generating sufficiently quantifiable signal to design the biosensor. Therefore, it is easy to address using microorganisms which have, in general, a high growth rate. Microbial cultures are more manageable and cheaper than isolated enzymes, and the same enzyme, used as bioreceptor in a CB, presents more activity in the microbial cell due to the optimal microenvironment supplied by the cell itself (Verma and Singh 2005). Many microorganisms can be easily manipulated and grown on a wide variety of different media or culture types. Likewise, many of them can be genetically modified to facilitate the biosensor design improving the sensibility (Wan et al. 2019a; Wang et al. 2014, 2015) or specificity (Wang and Buck 2014; Wang et al. 2013) to a specific environmental pollutant. In the case of WCBs, this technological capacity is essential due to necessity to incorporate the transduction capacity into the cell. Also, as stated above, microorganisms are distributed all over the planet, occupying all known ecosystems, which constitutes a great advantage if the biosensor designer is looking for a particular microbial capability to detect a specific environmental pollutant. An example of this is offered by *Ralstonia metallidurans*, a bacterium adapted to toxic metals (Mergeay et al. 2003), colonizing industrial sediments, soils, or wastes with a high content of metal(loid)s. From the knowledge on metal-resistant mechanisms and their regulation obtained from this bacterium and other metal-resistant microorganisms, several types of biosensors detecting metals have been designed (Diels et al. 2009; Leth et al. 2002; Tseng et al. 2014). The existence of cellular resistance mechanisms against metals or xenobiotics is really important when we want to design a WCB to detect these pollutants, because the regulatory genes and promoters involved in these mechanisms can be used for the design of the biosensor.

Among the microorganisms that can be used to design biosensors are both prokaryotes and eukaryotes. With regard to the microbial type used in WCBs to detect metal(loid)s, about 85% of these are based on genetically modified bacteria

(Magrisso et al. 2008), while a 15% are based on eukaryotes, and the majority of them are yeasts. However, among eukaryotic microorganisms, there is the possibility of using microbes from the three different taxonomic groups: fungi, microalgae, and protozoa (Gutierrez et al. 2017). The "eukaryotic" character is particularly important because, in general, environmental biosensors are geared toward detection of potential toxic pollutants affecting mainly eukaryotic organisms (including humans). The existence of a greater similarity of metabolism, genome, and cell organization of eukaryotic cell-based biosensors with potential organisms (plants and animals) undergoing chemical pollution makes the comparison of detection level and toxicity safer and more effective. A similar situation is found among the number of biosensors for detecting xenobiotics; prokaryotic-based biosensors exceed eukaryotic one in a ratio of approximately 10:1.

Metal(loid) Biosensors

To design metal(loid) CBs or WCBs, we can consider two basic types of microorganisms: the naturally existing or wild type and those genetically modified (anthropogenic origin). The wild-type strains usually present a peculiar feature which can be used to design the metal(loid) biosensor, such as bioluminescence, color, pigmentation, or any other characteristic that can be altered by metal(loid) toxicity. As previously indicated, these are the *turn off* bioassays. On the other hand, genetically modified microorganisms could be used in both *turn off* or *turn on* bioassays to design CBs or WCBs.

After an analysis of the published works, in the last 25 years, on CBs and WCBs to detect metal(loid)s, using complete microorganisms as bioreceptors (CBs) or bioreceptor-transducers (WCBs), we have seen that the number of WCBs is more than twice (78%) as much as CBs (22%) (Fig. 1a). It is probably due to the greater complexity of the multidisciplinary work involved in designing CBs. Regardless of the type of biosensor (CB or WCB), the frequency of using prokaryotes exceeds that of using eukaryotes. In CBs, the prokaryote/eukaryote ratio is 59% : 41%, while in WCBs, this ratio is 79%:21% (Fig. 1b). Although eukaryotes have some advantages as WCBs (Gutierrez et al. 2015), many specialists prefer to continue using bacteria because they are easier to grow and manipulate. Among WCBs, *turn on* bioassays are more abundant (87%) than *turn off* bioassays (13%) (Fig. 1c). This indicates that much more specific biosensors are being searched for the detection of a given compound than those that respond to general stress caused by any environmental pollutant.

Bacteria-Based Metal(loid) Biosensors

In this section, we will review the metal(loid) biosensors designed using bacterial elements or complete bacteria. Electrochemical (amperometric or potentiometric) transducers are the majority (60%) among bacterial CBs, in second place are optical
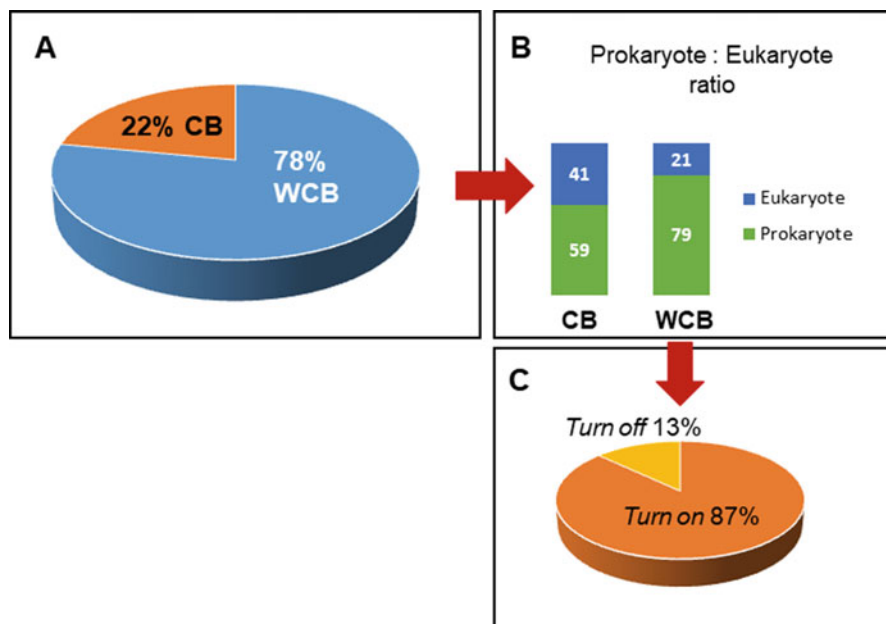
Fig. 1 Statistical analysis of metal(loid) biosensors. *CB* classical or conventional biosensor, *WCB* whole-cell biosensor

transducers (30%), and in the last position are the acoustic transducers (10%). Among bacterial WCBs, bioluminescence-based transducers have been widely used (75%), while fluorescence and colorimetric-based transducers have been less used (about 17% and 8%, respectively).

The following are some examples of CBs designed using bacteria. Immobilized whole bacteria, without genetic modifications, have been used in metal CBs, such as the bioluminescent bacterium *Photobacterium phosphoreum* on a cellulose nitrate membrane to detect chromium (Lee et al. 1992) or the cyanobacterium *Anabaena torulosa* embedded in a cellulose membrane to detect Cu(II), Pb(II), and Cd(II) (Wong et al. 2013). The presence of these toxic cations reduces the photosynthetic (cyanobacterium) or bioluminescent activity by changing the fluorescence or bioluminescence quenching of these cells. The release of photosynthetic oxygen is also inhibited under the metal(loid) presence, and the oxygen reduction can be detected by an oxygen electrode (Shing et al. 2008). Bacterial enzymes have been also used to design metal CBs, under the assumption that toxic metals can inhibit the enzyme activity and show a direct correlation between the enzyme inhibition rate and metal toxicity. Enzymes, such as alkaline phosphatase, glucose oxidase, or urease, among others, have been used to detect Cd(II), Pb(II), Zn(II), Ni(II), or Co(II) (Berezhetskyy et al. 2008), in addition to Hg(II), Ag(I), Cu(II), and Fe(III) (Guascito et al. 2008; Ilangovan et al. 2006; Samphao et al. 2012).

Genetically engineered bacteria have been mainly used to design metal WCBs. Here we describe some of the most relevant. Some WCBs presumably specific to one or two metals have been reported. To detect As(III), a sensing construct formed by the *arsR* gene promoter (arsenic-sensitive promoter) from *Escherichia coli* fused to *mtrB* gene (involved in metal reduction pathway) from *Shewanella oneidensis*, a bacterium with great capacity to reduce metal ions and living in environments with and without oxygen. Accordingly, the output signal is bioelectrochemical (Webster et al. 2014). Another WCB with a high specificity to detect As(III) is also based on the promoter of the operon *ars* from *E. coli*, with a P_{ars}::*arsR* construct fused with the reporter gene *gfp*, so the signal measurement is fluorescent (Li et al. 2015). To improve the sensitivity of this WCB, the authors carried out directed evolution experiments, where mutants were selected after three generations obtaining strains with a 12-fold increase of their response to As. Likewise, this WCB was tested with environmental samples contaminated with As, which means a positive validation for this environmental biosensor. Other similar constructs based on the *ars* promoter and the *arsR* regulator gene, fused with different fluorescent or luminescent reporter genes, have been designed (Huang et al. 2015; Hu et al. 2010; Jia et al. 2019; Merulla and van der Meer 2016; Preveral et al. 2017; Sharma et al. 2013; Wan et al. 2019b; Wang et al. 2014, 2015).

Several recombinant strains from both Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*E. coli* and *Pseudomonas fluorescens*) bacteria have been constructed to express bioluminescence reporter genes (luciferase system) to be used as metal WCBs (Gutierrez et al. 2017; Ivask et al. 2009). Both *turn off* and *turn on* bioassays have been carried out using different bacterial strains of which five for detecting Cu(II) or Hg(II) were target metal specific, whereas eight other strains showed a lower specificity level, responding to diverse metals (Cd(II), Hg(II), Zn(II), and Pb(II)) (Gutierrez et al. 2017). Other multi-metal(loid) response WCBs using *E. coli* as bacterial recipient are those reported by Branco et al. (2013), Hou et al. (2015), and Kim et al. (2016). Another *E. coli*-based WCB for detecting chromate in environmental samples use the plasmid pCHRGFP1 containing the construct P_{chrB}::*GFP* (fluorescence signal) and presumably do not respond to other metal(loid)s (Branco et al. 2013). The promoter P_{chrB} was isolated from the Gram-negative aerobic bacterium *Ochrobactrum tritici*, which has been also used as WCB by these authors. Other nonclassical bacterial species used as WCB are the Gram-negative *Salmonella enterica*, which, like other *E. coli*-based WCBs, hosting the construct P_{golB}::*GFP*, has been used to detect Au (Cerminati et al. 2011). The soil bacterium *R. metallidurans* has been used as a WCB (*turn on* bioassay) to detect Cr (II) by using the *lux* reporter gene system (Corbisier et al. 1999). Another strain of *R. metallidurans* has been used as WCB to detect Ni(II) and Co(II) in soil samples, after transformation with the megaplasmid pMOL1550 containing the *cnr* operon promoter (Ni(II) and Co(II) resistance system) (Tibazarwa et al. 2000) fused to *lux* reporter gene (Tibazarwa et al. 2001).

A WCB, to detect Cd(II) and Zn(II), has been designed using the *cadA* gene promoter from *S. aureus* (Yoon et al. 1991) fused to the firefly luciferase reporter gene into the plasmid pT0024, using cell chassis of both *S. aureus* and *B. subtilis*

(Tauriainen et al. 1998). In the cyanobacterium *Synechococcus*, the *smt* locus contains *smtB* (a *trans*-acting repressor) and the metal-regulated *smtA* (encoding a metallothionein). Depending on the environmental bioavailability of metal(loid)s, *smtB* gene controls the gene expression of *smtA* (Erbe et al. 1996). Based on this system and using the same cyanobacterium, a multi-metal(loid) response WCB has been designed, harboring the plasmid construct *Smt:luxCDABE*. This WCB presents a linear response to several metals, such as Cd(II), Cu(II), Zn(II), Co(II), Hg(II), and Ag(I), with sensitivity ranking of Hg > Cu > Ag > Co > Zn > Cd (Martin-Betancor et al. 2015).

Other bacterial genetic constructs have improved both specificity and metal detection and quantification (Bernard and Wang 2017; Wan et al. 2019b; Wang et al. 2013). For instance, a specific Cu-WCB based on an *E. coli* strain containing a plasmid with the construct $P_{copA}::GFP$ has been reported (Kang et al. 2018). This bacterial strain has a deletion affecting the *copA* gene, which is involved in cellular copper export, so this mutation makes this strain more sensitive to Cu(II) than the wild type. Under small Cu amounts (μM), this WCB responds specifically to Cu, no responding to other metals.

Dual sensing WCBs are another very useful monitoring possibility as it can detect two different metals with different sensitivity. A dual sensing WCB based on *E. coli* has been designed to detect bioavailable As(III) or As(V) and Cd(II) in polluted soil samples (Yoon et al. 2016a). It carries two different genetic constructs: $P_{ars}::$ *mCherry* (a monomeric red fluorescent protein) and $P_{znt}::eGFP$ (enhanced GFP version) or the cross-combination fusions $P_{ars}::eGFP$ and $P_{znt}::mCherry$. The first construct responds to As(III) mainly, but also to As(V), while the second one responds to Cd(II). This dual WCB was seen to respond mainly and simultaneously As and Cd, but no against other metal(loid)s (Yoon et al. 2016a).

In ecotoxicology, it is important to monitor pollutants both before and during its remediation. A novel strategy to test arsenic bioavailability in soil samples by direct (*in vivo*) or indirect (*in vitro*) measurement using an *E. coli*-based WCB has been reported by Yoon et al. (2016b). As previously reported, the plasmid construct has $P_{ars}::eGFP$, which shows a high specificity to As. This WCB was applied to detect As(III) and As(V) in both laboratory and polluted soil samples. The authors (Yoon et al. 2016b) also described a standardized protocol to measure bioavailable As in soil samples. Hence this WCB was seen to be a useful tool to evaluate the efficiency of soil arsenic remediation processes.

Likewise, metal(loid) environmental pollution monitoring is as important as bioremediation of the pollutant. An ideal situation would be to have a biosensor to detect a specific pollutant but also to bioremediate that same pollutant. These are WCBs based on two-component regulatory systems. The bacterial two-component systems have been engineered as synthetic biotechnological platforms for both monitoring and bioremediation purposes (Ravikumar et al. 2017). These WCBs simultaneously sense and remove (by bioadsorption) metals from the environment. For instance, detection and removal of Cu^{2+} ions in the *E. coli* surface have been achieved using the two-component system CusSR which allows for exogenous copper detection via membrane-associated kinase (Ravikumar et al. 2011). The CusSR two-component is a regulatory system for copper homeostasis in *E. coli*.

The gene *ompC* codes an outer membrane pore protein (porin), which is induced by high osmolarity and temperature. CusSR system activates the expression of *CusCFBA* operon, and *CusC* gene encodes a copper-binding peptide (CBP). Authors (Ravikumar et al. 2011) obtained a bacterial adsorption system constructed by the integration of chimeric *OmpC::cusC* under the P_{cusC} promoter located in the plasmid pCC1056, to respond to and adsorb exogenous copper. Other two-component regulatory system-based microbial biosensors coupled with bioadsorption have been designed for metal sensing and bioremediation or biorefinery of some organic compounds (Ravikumar et al. 2017).

Bacterial-WCBs Based on Riboswitches

Riboswitches are regulatory elements (noncoding sequences) within a mRNA molecule, able to bind different metabolites (purines and derivatives, cofactors, amino acids) and metal ions (Mg(II), Ni(II), Co(II)) as ligands. They regulate mRNA expression by transcription termination/anti-termination or translation inhibition or activation. The mRNA regulatory or switching sequence is most often located in the 5´-UTR, as a stretch of RNA preceding the translation starting site, although in some eukaryotic mRNAs, the riboswitch regulates splicing at the 3´-UTR (Edwards and Batey 2010). Riboswitches have two domains; the aptamer domain (acting as a receptor that specifically binds a ligand) and the expression platform or coding region (ORF: open reading frame). A riboswitch can adopt different secondary structures effecting gene expression depending on whether a target ligand is bound. When ligand is not bound, the expression platform incorporates the switching sequence into an anti-terminator stem-loop, and transcription starts through the mRNA coding region. But, when ligand binds, the switching sequence is incorporated into the aptamer domain, and the expression platform folds into a terminator stem-loop, inducing transcription stop.

In addition, mRNA translation activated by riboswitches (Serganov and Nudler 2013). The expression of ORFs can be repressed by sequestration of the ribosome entry site, blocking the translation initiation. When a ligand is attached to a riboswitch, the formation of an anti-sequester hairpin can be facilitated, opening the entrance to the ribosome and initiating the translation of the mRNA.

Riboswitches are very common in Gram-positive bacteria, where they control the expression of around 4% of the genes (Lünse et al. 2011). Recently (Machtel et al. 2016), riboswitches have been considered as a new tool for WCB design. We can select a riboswitch responding to a specific metabolite or ligand through the in vitro selection process known as SELEX (systematic evolution of ligands by exponential enrichment). This experimental methodology originates the aptamer domain of a riboswitch responding to almost any metabolite (including metals). These riboswitches bind ligands even in very low concentrations in a dose-dependent manner (Fig. 2). Although it is known that riboswitches binding metal cations and anions exist (McCown et al. 2017; Wedekind et al. 2017), a WCB has not yet been built to
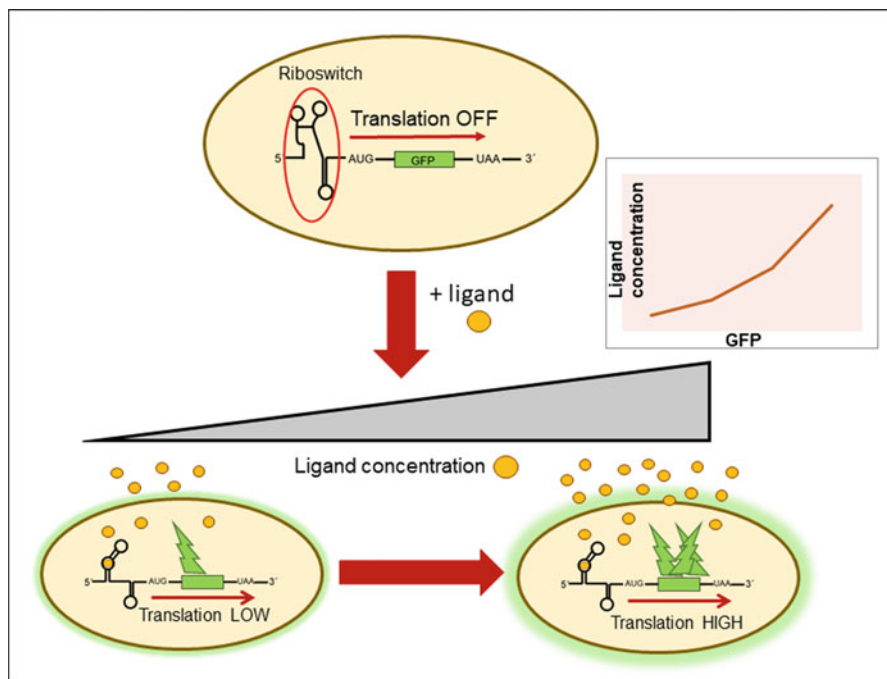
Fig. 2 Schematic of a riboswitch-based cell biosensor and its response to an increasing ligand concentration. *GFP* green fluorescent protein, *AUG* start codon, *UAA* stop codon

detect metal(loid) based on these molecular sensing systems. However, we know the existence of WCBs to detect organic compounds (drugs or xenobiotics), which will be discussed in the corresponding section. Riboswitches as molecular tools to design WCBs for detecting both metal(loid)s or xenobiotic compounds appear as a promising area in biosensorization of environmental pollutants (Findei et al. 2017).

Eukaryotic Microorganism-Based Metal(loid) Biosensors

As previously indicated, metal WCBs based on eukaryotic microorganisms are more scarce than those using prokaryotic ones. The main eukaryotic microorganisms used to design both CBs or WCBs to detect metal(loid)s in polluted environments have been selected among microalgae, fungi (including yeasts), and ciliated protozoa (Gutierrez et al. 2017). To detect metal(loid)s, all reported CBs using eukaryotic microorganisms are electrochemical, while an 83% of WCBs are based on bioluminescence and the rest (17%) use fluorescence. The great majority of eukaryotic microbial biosensors use yeasts as the cellular component (yeast-based WCBs are reviewed by Adeniran et al. 2015). Second are microalgae (mainly genera *Chlorella*

and *Chlamydomonas*), followed by protozoa which is represented only by the ciliate *Tetrahymena thermophila* (Amaro et al. 2011, 2014).

The yeast-model *Saccharomyces cerevisiae* is the most widely used eukaryotic microorganism in diverse biological areas, especially in genetic and bioengineering, which leads certain researchers (Walmsley and Keenan 2000) to consider that they can be good candidates to design biosensors for natural polluted environmental samples. However, like bacteria, yeasts have a cell wall protecting the cell and acting as a selective barrier for very different molecules (including substrates used by the biosensor transducer system in WCBs), which makes the transducer signal difficult to be efficiently emitted. For this reason, in some occasions, it is necessary to increase the cell permeability before using them as WCBs or bioreceptor in CBs, which constitutes an additional disadvantage. Mutants with enhanced cell permeability could be used for this purpose (Terziyska et al. 2000; Walmsley and Keenan 2000).

The majority of yeast-based biosensors use the *CUP1* promoter of the *cup1* gene, encoding a copper-binding metallothionein (CUP1), which are used as biosensors for detecting copper. Some examples are given below. A recombinant *S. cerevisiae* strain has been used to design an amperometric CB (Lehmann et al. 2000) to detect Cu(II). Another amperometric CB using other different recombinant *S. cerevisiae* strain to detect Cu(II) has been constructed using the *lacZ* reporter gene (Tag et al. 2007). A biosensor to detect Cu(II) ions in water samples using genetically modified *S. cerevisiae* within immobilized alginate beads and based on the *CUP1* promoter has been elaborated by Vopálenská et al. (2015). To validate this WCB, authors compare the Cu(II) measurements by the biosensor from urban water samples with those results obtained by standard laboratory assays using the same water samples. The transcriptional activator protein AceI present in *S. cerevisiae* has been used to control the expression of the reporter gene *gfp* (Shetty et al. 2004). When Cu(II) ions are present, the AceI protein activates the *cup1* gene promoter fused with the *gfp* gene. This WCB is not selective for Cu(II) because it also responds to Ag(II) (Shetty et al. 2004). Another similar WCB, also for Cu(II) detection, has been constructed using the same *CUP1* promoter but with the luciferase reporter gene, showing a similar detection level for this metal (Roda et al. 2011).

This yeast has been also used to design biosensor for detecting other metals. For instance, the Cd-inducible *SEO1* promoter from the yeast *Hansenula polymorpha* was fused to *gfp* gene, which was introduced in *S. cerevisiae* cells to detect Cd(II) ions (Park et al. 2007). This reporter construct is not specific to Cd(II) because it is also inducible by As(III). Likewise, the *SEO1* promoter from *S. cerevisiae* revealed that it is inducible by multiple metal ions with sensitivity rank of As(III) > Cd(II) > Hg(II), hence also unspecific for Cd(II). A WCB based on an engineered *S. cerevisiae* strain responds to several metal(loid)s (Radhika et al. 2005). This yeast strain harbors a plasmid with the human *CREBBP* gene (encoding a binding protein to *CREB* gene, a cAMP response element binding that acts as a transcription factor) and the construct of P_{creb}::*gfp*. When the yeast sensor is under the toxic metal exposure, a stress pathway is activated and cAMP levels increase. cAMP activates the *CREBBP* gene expression, and, in turn, the CREB protein activates the *CREB* promoter to express the GFP reporter gene to generate the output signal.

Microalgae are also significant microorganisms to design biosensors for aquatic ecosystem (Kröger and Law 2005). *Chlorella vulgaris* immobilized cells have been used to design a conductive CB based on the alkaline phosphatase activity inhibition to detect Cd(II) ions (Chouteau et al. 2004). This same microalga was used as a WCB for detecting Cd(II) in a water suspension or immobilized cells in a translucent silica matrix. The Cd(II) toxicity affected the photosynthetic activity (*turn off* bioassay) resulting a quenching of cell fluorescence (Nguyen-Ngoc et al. 2009). For monitoring Cu(II) in water supplies, the Chlorophyta *Dictyosphaerium chlorelloides* has been used with an optical fiber coupled to the cellular flow or a microtiter plate reader (Peña-Vazquez et al. 2010). The flagellar motility of *Chlamydomonas reinhardtii* has been used to design an electrochemical biosensor to detect Cu(II) or Ni(II) (Shitanda et al. 2005).

Unlike yeasts, the lack of usable genetic tools for bioengineering many microalgae has likely limited the construction of WCBs, perhaps the main reason to use wild-type strains as the bioreceptor elements in both CBs and WCBs. Although *C. reinhardtii* is an exception, this microalga has not yet been genetically modified to design biosensors for environmental metal(loid) monitoring, even though diverse studies have already been carried out to assess metal toxicity with this microorganism (Aksmann et al. 2014; De Schamphelaere et al. 2014). Considering microalgae present sufficient qualities to be selected as good potential metal(loid) biosensors, we can conclude that this micro-algal biotechnological aspect has not yet been adequately exploited.

Ciliated protozoa have been extensively used in ecotoxicological studies (Gutierrez et al. 2008). With respect to the cell sensitivity level and biosensing toxicity for humans, ciliates have, at least, two additional advantages with regard to other microorganisms. In first place, unlike bacteria, yeasts, or microalgae, ciliates do not have a cell wall in their growth vegetative stage. As it has been indicated in previous reviews (Gutierrez et al. 2015, 2017), microorganisms with a cell wall to be used as WCB or bioreceptor elements of CBs have an important limitation since the selective diffusion of molecules through their cell walls results in a lower emission or less effective cell response to pollutants. In several cases, to address this barrier, cells have had to be permeabilized by physicochemical or enzymatic methods. In addition to this difficulty, the presence of a cell wall may involve a non-specific, uncontrolled metal(loid) biosorption process, which may affect the real cellular response to the external metal concentration, when cells are not used as a biosorption-based biosensor. This problem might be solved using ciliates in the biosensor designing, because the absence of cell walls leads to a high sensitivity and rapid response to a variety of environmental pollutants (Martin-Gonzalez et al. 1999; Gutierrez et al. 2003).

In second place, ciliates are eukaryotic cells with an animal biology, which present a series of metabolic traits more similar to those of human cells than bacteria, microalgae, or even yeasts. The genome sequencing projects of two ciliate models, *Tetrahymena thermophila* and *Paramecium tetraurelia* (Aury et al. 2006; Eisen et al. 2006), have shown that they share a higher degree of functional conservation with human genes than do other eukaryotic microbial models. For instance, humans and *T. thermophila* share more ortholog genes with each other (about 2280) than are

shared between humans and the yeast *S. cerevisiae* (Eisen et al. 2006). Likewise, the scores of *P. tetraurelia* proteins against human proteins are the highest with regard to the scores of yeast proteins to human proteins, suggesting that *Paramecium* proteins are most similar to human proteins (Sperling et al. 2002). These similarities with human biology makes it more reasonable to use these eukaryotic microorganisms in ecotoxicological studies (Gutierrez et al. 2008, 2011) or to design biosensors for monitoring toxicity of metal(loid)s or xenobiotic compounds for humans, in aquatic or terrestrial ecosystems.

Unfortunately, there are few currently published ciliate-based biosensors. Only the ciliate *T. thermophila* has been used to design WCBs for metal(loid)s, which have been validated using natural (soil and aquatic) metal polluted environmental samples (Amaro et al. 2011). These *turn on* WCBs have been designed using promoters of *MTT1* or *MTT5* metallothionein genes from this ciliate and the firefly luciferase as the reporter gene. These lineal constructs were then introduced into macronuclear genome by biolistic transformation (Amaro et al. 2011). A second type of *T. thermophila* WCB has been designed using *MTT1* gene promoter and the *gfp* reporter gene fused to the *MTT1* or *MTT5* complete ORF into a plasmid (Amaro et al. 2014). A comparative analysis of both metal WCBs is reported in Gutierrez et al. (2017).

Although the three types of eukaryotic microorganisms (yeasts, microalgae, or protozoa) can be used to design both CBs or WCBs for metal(loid) environmental monitoring, they all have certain advantages and disadvantages, which have been previously discussed (Gutierrez et al. 2017).

Xenobiotic Biosensors

Like metal(loid) biosensors, xenobiotic biosensors can be designed using both wild-type and genetically modified microorganisms. The great diversity of microbial capabilities to biodegrade or biotransform xenobiotics is the basis to design biosensors to detect these toxic compounds. In the design of these biosensors, prokaryotes predominate over eukaryotes probably due to the greater capacity of bacteria to degrade very different organic compounds compared to eukaryotes. Unlike what happens with metal biosensors, CBs to detect xenobiotics are the majority (88%) against WCBs (12%). The common physicochemical transducer used in these xenobiotic CBs is electrochemical. Due to the scarcity of xenobiotic biosensors based on eukaryotic microorganisms, in this section, we will explore exclusively those biosensors based on prokaryotes.

Bacteria-Based Xenobiotic Biosensors

Like metal(loid) biosensors, bacterial two-component systems have been used to design xenobiotic biosensors. Aromatic compounds are very abundant organic environmental pollutants. Many bacteria can feed on these organic compounds as a carbon source. Several two-component regulatory systems are involved in the

catabolism of aromatic compounds, by inducing and activating the corresponding aromatic metabolizing pathway. *Pseudomonas putida* degrades toluene and other benzene derivatives; the first four steps of the catabolic pathway involve the sequential action of seven *tod* genes, which form part of the *tod* operon (Zylstra et al. 1988). This operon can be induced by different aromatic compounds (toluene, xylene, benzene, ethylbenzene) and is modulated by a two-component regulatory system. Engineered microbial biosensors based on bacterial two-component systems, like *P. putida* among others, can be very useful to construct synthetic biotechnology platforms for monitoring, bioremediation, and biorefinery (Ravikumar et al. 2017).

Recently, microbial fuel cell-based biosensors have attracted great interest due to their sustainability, low cost, and applications including anaerobic digestion process monitoring or water quality detection (Zhou et al. 2017). The voltage originated by microbial fuel cells is correlated with the amount of a specific substrate, presenting a linear correlation. Several studies have reported that these biosensors can detect organic compounds, such as p-nitrophenol, formaldehyde, or the antibiotic levofloxacin. An important limitation of these biosensors is their low sensitivity, as the detection range is above the currently allowed contaminant level. Therefore, an improvement in sensitivity will be necessary for their widespread application.

Riboswitch-based WCB for bisphenol A (BPA), a known endocrine disruptor and potential carcinogen present in plastics, food packaging, and drinking water supplies, has been tried in *E. coli* (Zorawski et al. 2016). TetA is a transporter protein that pumps tetracycline out of the cell, making the cell resistant to this antibiotic. In addition, this protein allows Ni(II) to enter the cell, inhibiting growth. *tetA* gene expression is regulated by a riboswitch. The construct $P_{TetA}::tetA::gfp$ was introduced into a plasmid, and then the aptamer domain of the riboswitch was replaced, by PCR, with 40 random bases to generate plasmid libraries. *E. coli* was transformed using these plasmids such that each bacterium harbored only a copy of the plasmid library. A selection was carried out to identify riboswitches expressing TetA-GFP only in response to BPA. Unfortunately, the authors failed to produce a significant increase in output fluorescence under the presence of BPA compared to control (without ligand) (Zorawski et al. 2016). However, this pioneering work could be a first step in the future construction of WCBs using riboswitches to detect xenobiotic molecules.

A selection of research studies reporting xenobiotic biosensors is summarized as follows. CBs using different bacterial species (*Pseudomonas*, *Sphingomonas*, *Ralstonia,* and *Rhodococcus*) immobilized onto a Clark-type oxygen electrode were tested for monitoring the cellular degradation capacity and detection of several xenobiotic compounds especially their chlorinated derivatives (chlorophenols, chlorobenzoates, PCBs, among others) (Beyersdorf-Radeck et al. 1998). This study showed that each bacterium prefers selectively a type of substrate, suggesting for each xenobiotic biosensor a specific type of bacteria should be selected in advance.

A bioluminescent *E. coli* strain carrying the *lux* operon and the promoter region of the *pgi* gene (encoding the glucose-6-phosphate isomerase), which responds to oxidative stress, was used for the construction of a bioluminescent WCB (Niazi et al. 2008). This biosensor is designed on the basis that xenobiotics can induce cell

damage by oxidative stress, such as the herbicide paraquat (Diaz et al. 2016), causing oxygen free radicals.

The *linA* gene encoding the enzyme γ-hexaclorocyclohexane dehydrochlorinase, involved in biodegradation of the pesticide lindane, was cloned and overexpressed in *E. coli*. Subsequently, cells from this lindane biodegradating strain were immobilized on polyaniline film. The hydrochloric acid generation from the lindane biodegradation by the recombinant *E. coli* cells led to a change in the conductivity of the polyaniline film, which was monitored amperometrically (Prathap et al. 2012). This CB detects lindane at a concentration of one part per trillion, and was selective to all lindane isomers, but did not respond to other aliphatic, aromatic chlorides or the end product of lindane degradation.

Another possible technical approach for designing WCBs to detect xenobiotics is the use of immobilized cells in microplates. Whole cells of the bacterium *Sphingomonas* sp., which hydrolyzes the methyl parathion (an organophosphate pesticide and insecticide) into p-nitrophenol (yellow-colored product), were immobilized onto the surface of the polystyrene microplate wells. Para-nitrophenol was detected by a colorimetric method (microplate reader) (Kumar and D'souza 2010). Microplate-based biosensors constitute a convenient system to detect multiple numbers of samples in a single platform.

Organophosphate pesticides are very serious environmental pollutants. Amperometric, potentiometric, and optical CBs based on engineered microorganisms expressing organophosphate-hydrolyzing enzymes, either located intracellularly or anchored to the cell surface, have been designed to detect these dangerous pesticides (Lei et al. 2007). The advantages of using microorganisms over purified enzymes are lower cost and simpler construction.

There are many other examples of potential biosensors for detecting xenobiotics, although most are based on the ability of bacteria to degrade or biotransform these molecules.

Conclusions and Future Perspectives

1. In general, microorganisms used as whole cells or cell factories to design biosensors (CBs or WCBs) for detecting environmental pollutants present greater advantages than using molecules (such as enzymes). In addition, eukaryotic microorganisms have certain advantages over prokaryotic microorganisms, because the comparative analysis with multicellular organisms (including humans) is more reliable than using bacteria.

2. Likewise, protozoa, and mainly ciliates, present a great potential to design both WCBs and CBs, mainly due to its greater sensitivity to environmental pollutants without a cell wall and also due to their greater resemblance to human cells.

3. In almost all prokaryotic and many eukaryotic microorganisms, the presence of a cell wall is a hurdle or inconvenience in the design of a biosensor, because it could hinder permeability or retain (biosorption) pollutant molecules. In addition, using substrate-dependent reporters, substrates must cross the cell wall to reach the

cytoplasm, where the enzymatic reaction takes place. Therefore, it is sometimes necessary to pre-treat the cell to make it permeable to the molecule.

4. With respect to metal(loid) contaminants, the sensitivity of the biosensor is more important than its level of specificity to the target metal. This is because, in the real world, anthropogenic contamination by metal(loid)s is complex with the presence of several metal(loid)s.

5. In general, many CBs or WCBs are exclusively tested in the laboratory, under strictly controlled conditions, but very few of those published are validated using natural samples from metal(loid) or xenobiotic polluted environments. Due to this lack of essential experimentation, to evaluate a biosensor's usefulness for detecting an environmental pollutant, many of the biosensors considered as specific for detecting a certain pollutant molecule are not really so. It is due to the presence of other unknown organic or inorganic molecules, from the soil or aquatic ecosystem, which can interact with the bioreceptor element and interfere with the response. This point is really important for designing functional biosensors to be used in polluted environments in the real world.

6. The future development of biosensors (CBs or WCBs) for the monitoring of environmental pollutants could be developed on the basis of three, still little explored, experimental routes:

   (a) A greater and more intense exploration of prokaryotic riboswitches, to experimentally select those that interact with metals or xenobiotics. This exploration should also extend to eukaryotic microorganisms.

   (b) Enabling the same biosensor with both capabilities: the monitoring of the environmental pollutant and its bioremediation. For this purpose, two-component systems could be essential parts in the design of these bifunctional systems (biosensor-bioremediator).

   (c) A synthetic biology approach. This could facilitate the design of environmental biosensor (mainly WCBs) with multi-input systems based on two or more regulatory gene promoters in the same genetic construct, thereby increasing the capacity of the biosensor to detect simultaneously several different organic or inorganic pollutants in the same environmental sample (Wang et al. 2013, 2014).

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