Chapter 13
Microbial Biosensors for Metal(loid)s

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Abstract In this chapter we carry out an updated review on metal(loid)s biosensors using microorganisms as bioreceptor element of a classic biosensor or as a whole-cell biosensor. We analyze the potential advantages and possible disadvantages to use prokaryotic or eukaryotic microorganisms in metal(loid) biosensors. Likewise, the presence or absence of a cell wall in the microbial system can determine the degree of permeability of the target molecule to be detected. Sensitivity versus specificity of the biosensor is also discussed. We call attention on the necessity to carry out more bioassays using real environmental samples, and not only laboratory prepared once. A greater interest on designing biosensors using protozoa is also reclaimed, because these eukaryotic microorganism are much more sensitive to metal(loid)s than other microorganisms, and they share a higher degree of functional conservation with human genes than do other eukaryotic microbial models. Finally, a collection and analysis of the main metal(loid) microbial biosensors and genetic constructs potentially useful to design metal biosensors is reported.

Abbreviations

CB	Classical or conventional biosensor/s
GFP	Green fluorescent protein
MT	Metallothionein
WCB	Whole-cell biosensor/s

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13.1 Introduction to Metal(loid) Biosensors: Basic Concepts

Certain metal(loid)s (mainly those considered as "heavy metal(loid)s") are among the most abundant, toxic and persistent inorganic environmental pollutants (Hill 2004). Anthropogenic sources, mainly mining and industrial activities, have substantially increased the metal(loid)s content in the atmosphere and in may terrestrial and aquatic ecosystems (Peñuelas and Fillela 2002). This is the main reason to consider to these toxic compounds a priority in ecotoxicology, with the aim of minimizing the exposure of animals or humans. It is difficult to predict the global effects of increasing the different types of environmental pollutants, so there is an pressing need to develop screening methods for environmental monitoring. This necessity is for both, the early detection of environmental pollution by metal(loid)s and/or for testing the bioremediation process of a metal(loid) polluted ecosystem.

Low metal(loid) concentrations can be measured using molecular recognition or chemical analysis, such as absorption spectroscopy, mass spectroscopy, chromatography, polarography, among others. These techniques require qualified personnel and involve a high cost, and in addition, is not possible to carry out in situ analysis by using these techniques. On the other hand, critical ecotoxicological parameters such as bioavailability, toxicity and genotoxicity, can only be assayed using living cells. The most sensitive screening methods for detecting pollutants are those that incorporate biological components that are used as targets for an active substance or pollutant. In general, these screening techniques are known as biosensors or bioreporters. We can distinguish two types of biosensors: the classical or conventional biosensors (CB) and the whole-cell biosensors (WCB).

The CB can be defined as an integrated bioreceptor-physicochemical transducer device. This biosensor consists of three different elements: 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 it and converts it into a numeric record (Fig. 13.1). The biological components can be macromolecules (such as enzymes, antibodies, nucleic acids, etc.) or whole-cells (prokaryotic or eukaryotic microorganisms or cells from multicellular organisms). At least, four main different types of transduction elements or transducers can be considered: electrochemical (potentiometric/amperometric), optical (spectrophotometry/fluorometry), piezoelectric or thermometric. Construction of these biosensors requires biological and physicochemical knowledge, which involves an interdisciplinary cooperation among different specialists, making construction more difficult and expensive. About the second type of biosensor (whole-cell biosensor), several authors have introduced the concept of the WCB as a very useful alternative to CB (Belkin 2003; Van der Meer and Belkin 2010). The main difference between both types of biosensors is that WCB use a whole prokaryotic or eukaryotic cell as a single reporter, incorporating both bioreceptor and transducer elements into the same cell (Fig. 13.1). This means that

organisms used as WCB are, in general, experimentally modified to incorporate transducer capacity or increase their sensitivity. Another advantageous feature of these biosensors is the possibility to carry out both in situ or ex situ analysis.

When using WCB, two types of bioassays can be considered: *turn off* or *turn on* assays (Belkin 2003). *Turn off* assays are quite similar to general toxicological bioassays. In this case, the sample toxicity is estimated from the degree of inhibition of a cellular activity, such as growth inhibition, respiration rate, motility depletion, etc., or a specific reporter constitutive gene expression. In these bioassays, the toxic concentration is proportional to the measurement of any cellular function inhibition (Fig. 13.2). In *turn on* assays, a quantifiable molecular reporter is fused to a specific gene promoter, known to be activated by the chemical or environmental pollutant. Therefore, in this second type of bioassay, the sample toxicity is proportional to the gene expression of the reporter molecule (Fig. 13.2). These screening methods can be applied to detect the presence of both, any environmental pollutant causing general cellular stress or a specific pollutant (like metal(oid)s).

Turn off assays are more unspecific, because the signal decreases as a result of a broad range of cytotoxic effects, while WCB using *turn on* assays (based on an inducible gene expression) or CB (using specific molecules as bioreceptors), are usually more specific, as induction of the gene reporter, or interaction with the molecular bioreceptor, only takes place when the pollutant is present. The WCB specificity will therefore depend on the degree of the gene promoter specificity to be

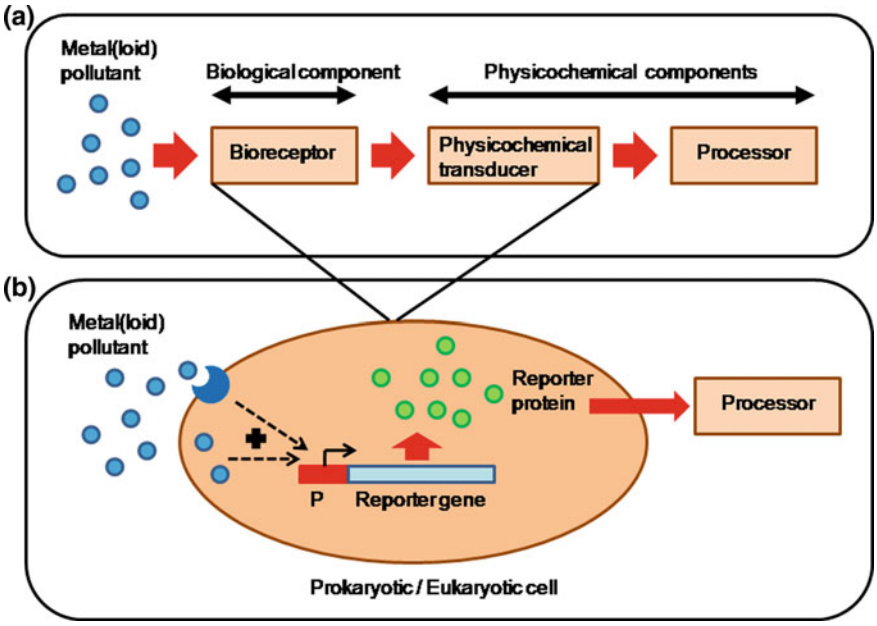

Fig. 13.1 Schematic representation of elements configuring classic and whole-cell biosensors. This figure is based on another previously published (Gutierrez et al. 2015)

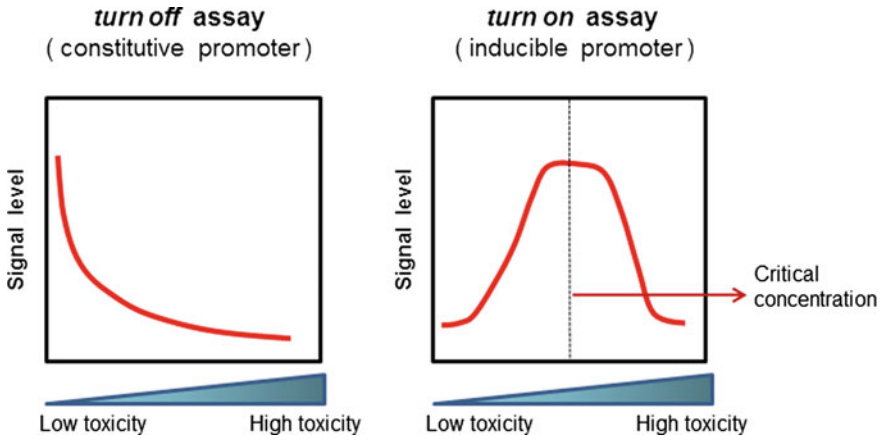

Fig. 13.2 *Turn off* and *turn on* bioassays. *Turn off* bioassays use constitutive promoters; therefore the signal level from the reporter gene decreases proportionally to toxic pollutant concentration. *Turn on* bioassays use inducible promoters; in this case the reporter signal level increases with the pollutant concentration. This reporter signal may reach a maximum value (critical concentration), after which decreases due to the increasing toxic concentration effect on the cell. The value of critical concentration will depend on the degree of cellular resistance to the pollutant. This figure is based on another previously published (Gutierrez et al. 2015)

activated by an exclusive pollutant or a chemically related group of pollutants. On the other hand, the CB specificity will depend on the specificity degree of the interaction between the bioreceptor and the pollutant. With respect to specificity, both *turn on* WCB and CB can be divided into effect- and compound-specific biosensors (Yagi 2007). Effect-specific biosensors respond to physicochemical environmental changes (e.g., pH, temperature or osmotic changes) or chemically diverse pollutants that give rise to a type of toxicity (e.g., oxidative stress or protein damage). On the other hand, compound-specific biosensors respond to only one type of pollutant or compounds with similar chemical features (e.g., metal(loid)s). For some specialists the before specific-based classification of CB or WCB, may be divided in three classes: (1) class-I biosensors that only respond to a specific or exclusive pollutant increasing the signal, (2) class-II biosensors that respond to a specific cellular stress (e.g., oxidative stress) increasing the signal, and (3) class-III biosensors that respond unspecifically to different pollutants or environment stressors.

In the last ten years, the number of publications reporting metal(loid) biosensors has been doubled, and about 85% of these are based on bacteria (Magrisso et al. 2008), while about 15% are based on eukaryotes (being yeasts the majority of them). Several reviews focused on general or specific aspects of biosensors to detect metal(loid)s have appeared in the recent years, such as Walmsley and Keenan (2000), Gu et al. (2004), Belkin (2003), Van der Meer et al. (2004), Kröger and Law (2005), Verma and Singh (2005), Yagi (2007), Magrisso et al. (2008), Van der

Meer and Belkin (2010), Gutierrez et al. (2015), Mehta et al. (2016). In this chapter, only biosensors (CB and WCB) using whole microorganisms or microbial macromolecules for detecting metal(loid)s present in environmental samples are considered.

13.2 Advantages and Disadvantages of Using Whole Microorganisms as Biological Recognition Components in CB and WCB

A majority of reported CB and WCB for metal(loid)s detection are based on prokaryotic or eukaryotic microorganisms (Verma and Singh 2005; Gutiérrez et al. 2015; Mehta et al. 2016). For an experimental point of view, is more easy to get a high microbial biomass than to reach the necessary amount of a specific purified macromolecule (enzyme, antibody, etc.) for getting the sufficiently quantifiable signal in any biosensor. So, this can be resolved using organisms with a high growth speed or short generation time, features that are almost exclusive of microorganisms. Microbial strains are cheaper than isolated enzymes, and the same enzyme used as biological component in a CB present more activity into the microbial cells owing to the optimal micro-environment provided by the cells (Verma and Singh 2005). Another advantages using microorganisms is that most of them can be easily manipulated and grown on a wide variety of different media or culture types. Recent advances in microbial genetic analyses and their genetic modification, and an increasing number of sequencing microbial genomes have facilitated the design and development of microbial biosensors with improved selectivity toward metal(loid)s or any other pollutants. In the case of WCB this technological capacity is essential due to necessity to introduce a transduction capacity into the cell. Furthermore, microorganisms are distributed all over the world, and occupy all known ecosystems, which constitutes a great advantages when the biosensor designer is looking for a particular microbial capacity to detect a specific environmental pollutant. For instance, the β-protobacterium *Ralstonia metallidurans* (formerly known as *Alcaligenes eutrophus*) is specifically adapted to ecosystems with a high content of metals, such as industrial and polluted biotopes or metallurgic wastes (Mergeay et al. 2003). From the knowledge on the mechanisms of metal(loid) resistant and their regulation obtained from this bacterium and other metal-resistant microorganisms, several types of metal biosensors have been designed (Corbisier et al. 1999; Leth et al. 2002) (Table 13.1).

Among eukaryotic microorganisms, there is the possibility to use microbial cells from three different taxonomic groups; fungi, microalgae and protozoa. The "eukaryotic" feature is particularly important because, in general, biosensors aimed at the detection of potential environmental toxic substances affecting other eukaryotic organism (including humans). The existence of a more similar metabolism, genome, and cellular organization in microbial eukaryotic biosensors with

Table 13.1 Microbial biosensors for metal(loid)s or microbial genetic constructs useful to design metal biosensors

Metal(loid)s[a]	Microorganism		Biosensor[b]		Reference
	Prokaryotic	Eukaryotic	CB	WCB	
Cd(II) > Ni(II) = Zn(II) > Cu(II)	*R. leguminosarum*			Bioluminescence (*turn off*)	Paton et al. (1997)
Cr(II)	*R. metallidurans*			Bioluminescence (*turn on*)	Corbisier et al. (1999)
As(III)	*S. aureus*			Bioluminescence (*turn off*)	Corbisier et al. (1993)
Cu(II), Pb(II), Cd(II)	*A. torulosa*		Optical (fluorescence)		Wong et al. (2013)
Cd(II)	*E. coli*		Electrochemical		Souiri et al. (2012)
Cd(II), Hg(II)	*E. coli*		Acoustic		Gammoudi et al. (2010)
Pb(II) > As(V) > Cd(II) > Cu(II) > Zn(II) > Hg (II)		*T. thermophila*		Bioluminescence (*turn on*)	Amaro et al. (2011)
Cd(II) > Hg(II) > Zn(II) > Cu(II) > Pb(II) > As(V)		*T. thermophila*		Bioluminescence (*turn on*)	Amaro et al. (2011)
Cd(II)		*T. thermophila*		Bioluminescence (*turn on*)	Amaro et al. (2014)
Cu(II)		*S. cerevisiae*		Electrochemical (*turn on*)	Tag et al. (2007)
Cu(II)	*R. metallidurans*		Optical (bioluminescence)		Leth et al. (2002)
Cu(II)	*T. chuii*[c]		Electrochemical (potentiometric)		Alpat et al. (2008)
Cr(VI)	*Thiobacillus* sp.[d]		Electrochemical		Oh et al. (2011)
Cd(II), Cu(II)	*A. torulosa*		Electrochemical (amperometric)		Chay et al. (2005)

(continued)

Table 13.1 (continued)

Metal(loid)s[a]	Microorganism		Biosensor[b]		Reference
	Prokaryotic	Eukaryotic	CB	WCB	
Cr(VI)	*A. ferrooxidans*		Electrochemical (amperometric)		Zlatev et al. (2006)
Cd(II)	*C. vulgaris*			Fluorescence (*turn off*)	Nguyen-Ngoc et al. (2009)
Cu(II)		*Circinella* sp.	Electrochemical		Alpat et al. (2008)
Hg(II)		*Chlorella* sp.	Electrochemical (amperometric)		Singh and Mittal (2012)
Cd(II)		*C. vulgaris*	Electrochemical (conductometric)		Chouteau et al. (2004)
Cu(II)	*D. chlorelloides*		Optical		Peña-Vázquez et al. (2010)
Pb(II)		*Phormidium* sp.	Electrochemical		Yüce et al. (2010a)
Cu(II)		*R. mucilaginosa*	Electrochemical		Yüce et al. (2010b)
Ni(II)	*B. sphaericus*		Electrochemical (amperometric)		Verma and Singh (2006)
Cu(II)		*S. cerevisiae*	Electrochemical (amperometric)		Lehmann et al. (2000)
Cu(II), Ag(I)		*S. cerevisiae*		Fluorescence (*turn on*)	Shetty et al. (2004)
Cu(II)		*S. cerevisiae*		Bioluminescence (*turn on*)	Roda et al. (2011)

(continued)

Table 13.1 (continued)

Metal(loid)s[a]	Microorganism		Biosensor[b]		Reference
	Prokaryotic	Eukaryotic	CB	WCB	
Cu(II), Ni(II)		*C. reinhardtii*	Electrochemical (amperometric)		Shitanda et al. (2005)
Hg(II)	*E. coli*			Bioluminescence (*turn on*)	Selifonova et al. (1993)
Cd(II), Pb(II), Sb(III)	*S. aureus*			Bioluminescence (*turn on*)	Tauriainen et al. (1998)
Cd(II), Sb(III), Zn(II), Sn(II)	*B. subtilis*			Bioluminescence (*turn on*)	Tauriainen et al. (1998)
As(V), Sb(III)	*E. coli*			Fluorescence (*turn on*)	Liao and Ou (2005)
As(V), Sb(III), As(III), Bi(III)	*S. aureus*			Colorimetric (*turn on*)	Ji and Silver (1992)
As(V), Cd(II), Sb(III)	*E. coli*			Bioluminescence (*turn on*)	Tauriainen et al. (1999)
Zn(II), Cd(II), Hg(II)	*Synechocystis* sp.			Bioluminescence (*turn on*)	Erbe et al. (1996)
Zn(II), Co(II)	*Synechocystis* sp.			Bioluminescence (*turn on*)	Peca et al. (2008)
Zn(II), Cd(II), Cr(VI), Hg(II), Pb(II)	*E. coli*			Bioluminescence (*turn on*)	Riether et al. (2001)
Cd(II), Hg(II), Zn(II)	*E. coli*			Bioluminescence (*turn on*)	Ivask et al. (2002)
Cd(II), Pb(II)	*E. coli*			Fluorescence (*turn on*)	Shetty et al. (2003)

(continued)

Table 13.1 (continued)

Metal(loid)s[a]	Microorganism		Biosensor[b]		Reference
	Prokaryotic	Eukaryotic	CB	WCB	
Hg(II), Cd(II)	*E. coli*			Bioluminescence (*turn on*)	Virta et al. (1995)
Hg(II)	*Vibrio anguillarum*			Bioluminescence (*turn on*)	Golding et al. (2002)
Hg(II), Cd(II)	*Pseudomonas fluorescens*			Bioluminescence (*turn on*)	Petänen et al. (2001)
Hg(II)	*E. coli*			Bioluminescence (*turn on*)	Ivask et al. (2001)
Hg(II), Cd(II), Zn(II)	*E. coli*			Bioluminescence (*turn on*)	Ivask et al. (2002)
Pb(II), Cd(II)	*E. coli*			Colorimetric (*turn on*)	Shetty et al. (2003)
Pb(II), Sb(III)	*E. coli*			Fluorescence (*turn on*)	Liao et al. (2006)
Cu(II), Ag(I)	*E. coli*			Bioluminescence (*turn on*)	Hakkila et al. (2004)
Cu(II)	*P. fluorescens*			Bioluminescence	Tom-Petersen et al. (2001)

[a]The order of sensitivity for tested metal(loid)s is reported, when it is available. [b]The transducer element of biosensors and the type of bioassay for WCB are indicated. [c]In this CB the no-living biomass of the alga *Tetraselmis chui* (biosortion-based CB) is used. [d]In this CB several sulfur-oxidant bacteria (including *Thiobacillus* sp.) are used

those organisms (plants and animals) undergoing a chemical pollution, makes the extrapolation and comparison of results more accurate and reliable.

13.2.1 Bacteria-Based Biosensors

To design CB or WCB, we can consider two basic types of bacteria; the naturally existing or wild-type and those genetically modified (anthropogenic origin). The first one, usually presents a peculiar natural characteristic which can be used for designing a metal(loid) biosensor, such as bioluminescence, a color or pigmentation, or any other feature that can be modified or altered after metal(loid) exposure. These biosensors are based on the inhibition or blocking of a natural bacterial feature, existing a proportional ratio between the metal toxicity and the bacterial signal decreasing (which should be measurable). These biosensor can be considered as *turn off* bioassays.

An example of whole bacteria-based CB is the one using the bioluminescent bacterium *Photobacterium phosphoreum* (Lee et al. 1992), immobilized on a cellulose nitrate membrane and used to detect chromium. Also *Anabaena torulosa* cyanobacterium cells embedded in a cellulose membrane have been used to detect Cu(II), Pb(II) and Cd(II) (Wong et al. 2013) (Table 13.1). The presence of these toxic ions reduce the photosynthetic activity changing the fluorescence quenching of these cells, and the release of photosynthetic oxygen is also inhibited under the metal presence and this oxygen emission reduction is detected by an oxygen electrode (Shing et al. 2008).

Likewise, in metal(loid) CB using enzymes isolated from microorganisms, toxic metals might inhibit the normal enzyme activity, exhibiting a direct correlation between the enzyme inhibition rate and metal toxicity. Consequently a reduction in enzyme activity can be read as a signal, which can be amplified to get the desired sensitivity level (Vel Krawczyk et al. 2000). Several 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), Cr(III), Hg(II), Ag(I), Cu(II), Cd(II), Pb(II), Fe(III), Co(II), Ni(II) or Zn(II) (Guascito et al. 2008; Samphao et al. 2012) and Cd(II) or Pb(II) (Ilangovan et al. 2006).

Genetically modified bacteria could be used in both *turn off* and *turn on* bioassays of CB or WCB. Several recombinant strains from both Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Pseudomonas fluorescens*) bacteria were constructed to express a bioluminescence reporter gene (*lux* genes) to be used as metal(loid) WCB (Ivask et al. 2009). Both, *turn off* and *turn on* bioassays were carried out, and five strains to detect Cu(II) and Hg(II) were target metal specific, whereas eight other strains were induced by Cd(II), Hg(II), Zn(II) and Pb(II), so showing a lower metal specificity. The soil bacterium *R. metallidurans* has been also used as a WCB (*turn on*) to detect Cr(II) using *lux* reporter gen system (Corbisier et al. 1999) (Table 13.1). Another strain of *R. metallidurans* has been used as WCB for detection of Ni(II)

and Co(II) in soil samples, after transformation with the megaplasmid pMOL1550 which contains the promoter of the *cnr* operon (resistance system to Ni(II) and Co (II) present in this bacterium) (Tibazarwa et al. 2000) fused to *lux* reporter gene system (Tibazarwa et al. 2001). The promoter from the *cadA* resistance determinant system for Cd(II) and Zn(II) of *Staphylococcus aureus* (Yoon et al. 1991) fused to the firefly luciferase reporter gene into the plasmid pT0024 has been used to design WCB in *S. aureus* and *B. subtilis* (Tauriainen et al. 1998) (Table 13.1). A WCB to detect Hg(II) has been also genetically constructed by using fusions of the Tn21 mercury resistance operon (*mer*) with *lux* reporter gene system from *Vibrio fischeri*, and the bacterium *E. coli* was used to design the WCB (Table 13.1). The *turn on* bioassays using this Hg-WCB was able to detect bioavailable mercury in water samples at a nM to μM concentration range (Selifonova et al. 1993). Although, they are not commented here, others different bacteria genetic constructs used to design CB or WCB are reported in Table 13.1.

13.2.2 Eukaryotic Microorganisms-Based Biosensors

In general, both CB or CWB using eukaryotic microorganisms are more scarce than those using prokaryotic one (Table 13.1). Probably, it is due to the greater cellular complexity of the eukaryotic cells and relative difficulty to work with them, but however they present several advantages in comparison with prokaryotic cells (see Sect. 13.2). Like in prokaryotic-based biosensors, we can distinguish between two basic types of eukaryotic microorganisms to be used to design both CB or WCB; wild-type and genetically modified microorganisms.

13.2.2.1 Microalgae-Based Biosensors

Microalgae are important in biosensor construction for aquatic (freshwater or marine) ecosystems applications (Kröger and Law 2005). Immobilized whole-cells of the microalga *Chlorella vulgaris* has been used to design a conductometric CB based on the inhibition of alkaline phosphatase activity in presence of Cd(II) ions (Chouteau et al. 2004) (Table 13.1). This same microalga was used as a WCB to detect Cd(II), in water suspension or immobilized in a translucent silica matrix. The Cd(II) toxicity affected the algal photosynthetic activity (*turn off* bioassay) resulting in a quenching of cellular fluorescence (Nguyen-Ngoc et al. 2009) (Table 13.1). For monitoring Cu(II) in water supplies the chlorophita *Dictyosphaerium chlorelloides* was used with an optic fiber coupled to the cellular flow or a microwell-plate reader (Peña-Vázquez et al. 2010) (Table 13.1). On the basis of flagellar motility of the microalga *Chlamydomonas reinhardtii* electrochemical biosensing systems for detecting Cu(II) or Ni(II) have been developed (Shitanda et al. 2005) (Table 13.1).

In many of these microorganisms, it is likely that the lack of usable genetic tools for bioengineering considerably limit the construction of improved WCB; in fact,

they are used as wild type strains in both, as bioreceptor elements in CB or cells in WCB (*turn off* bioassays) (Table 13.1). Although, there is an exception related to the microalgal model *C. reinhardtii*, this suitable microalga model has not yet been genetically modified to design biosensors for environmental metal(loid) monitoring, excepting for sensing triazine and urea types herbicides (Lambreva et al. 2013), even though diverse studies have already been done on metal toxicity in this microorganism (Aksmann et al. 2014; De Schamphelaere et al. 2014). Particularly, De Schamphelaere et al. (2014) demonstrated inter-specific differences in Pb(II) sensitivity among three microalgae species (*Pseudokirchneriella subcapitata*, *Chlorella kesslerii* and *C. reinhardtii*), which should be taken in account when designing WCB for detecting this metal. Likewise, Aksmann et al. (2014) report a gene expression analysis of several antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase) under oxidative stress induced by Cd(II), together with an analysis of photosynthetic activity on this alga, which could be useful to select molecular elements to design biosensors for detecting Cd(II).

Taking in account that microalgae present enough qualities to be considered as good potential metal(loid) biosensors, we conclude that this biotechnological aspect has not been yet sufficiently exploited.

13.2.2.2 Filamentous Fungi and Yeasts-Based Biosensors

Both, filamentous fungi and yeasts are well-known eukaryotic microbial models that are widely used in toxicology, biotechnological and basic biological studies. Among them, the yeast *Saccharomyces cerevisiae* is the most widely used eukaryotic microorganism in very diverse biological areas, especially in genetic and bioengineering, and for this reason some authors (Walmsley and Keenan 2000) consider that it has certain advantages as a biosensor to be used with natural polluted environment samples. It is a robust eukaryotic microorganism with a considerably physicochemical tolerance to very diverse chemicals, and good genetic tools that make possible the construction of genetically modified yeasts showing optimized features to design better biosensors.

However, like bacteria, fungi and yeasts have a cell wall that protects the cell and acts as a selective barrier for very different molecules (including substrates used by the biosensor transducer system in WCB), which makes transducer signal emission more difficult. Therefore, in some occasions it is necessary to increase cell wall permeability before using these microorganisms as WCB or bioreceptor in CB, which constitutes an additional difficulty. Mutans with enhanced cell permeability can be used for this purpose (Terziyska et al. 2000; Walmsley and Keenan 2000).

As it also occurs with bacteria and microalgae, yeasts have been used almost exclusively as bioreceptor elements in CB (Table 13.1) (Baronian 2004). The pigmented yeast *Rhodotorula mucilaginosa* has been used to construct a microbial biosensor based on carbon paste for determination of Cu(II) (Yüce et al. 2010b) (Table 13.1). A similar construction was carried out on the filamentous fungus *Circinella* sp., consisting of concentrated whole cells on the carbon paste electrode

surface for Cu(II) detection. This CB is based on the biosorption capacity of the fungus cell wall to Cu(II) ions (Alpat et al. 2008) (Table 13.1). A recombinant *S. cerevisiae* strain has been used to construct an amperometric CB (Lehmann et al. 2000) to detect Cu(II) (Table 13.1). A plasmid containing the copper inducible *cup1* gene promoter and the *E. coli lacZ* gene as a reporter gene was constructed, then this plasmid construct was introduced into *S. cerevisiae* and recombinant strain was immobilized with polyvinyl alcohol on a capillary membrane. If Cu(II) is present in the sample, this recombinant strain is able to utilize lactose as a carbon source, which leads to alterations in the oxygen consumption of the cell. So, changes in the oxygen concentration were quantified by an oxygen electrode (similar to Clark's oxygen electrode for glucose quantification) (Clark et al. 1953). Another amperometric CB using other different recombinant *S. cerevisiae* strain to detect Cu(II) was constructed also using *lacZ* reporter gene (Tag et al. 2007) (Table 13.1).

Few WCB have been designed using yeasts, some of them are: *S. cerevisiae* cells and GFP (green fluorescent protein) as a reporter protein was developed to detect Cu(II) ions (Shetty et al. 2004) (Table 13.1). The transcriptional activator protein AceI present in this yeast was used to control expression of the reporter gene *gfp* (encoding GFP). When Cu(II) ions are present, the AceI protein actives the *cup1* gene promoter located upstream from the *gfp* gene (*Pcup1::gfp*) into a plasmid, there by inducing GFP production. This system is selective for Cu(II) over other metals, except for Ag(II) (Shetty et al. 2004). Another similar *S. cerevisiae* WCB, also for Cu(II) detection, has been constructed using the same promoter (*cup1*) but a different reporter gene (luciferase), and showing a similar detection level for this metal (Roda et al. 2011) (Table 13.1).

From a microarray gene expression analysis carry out in the methylotrophic yeast *Hansenula polymorpha*, under Cd(II) treatment, several over-expressed genes were selected (Park et al. 2007). This analysis revealed that the promoter from the *seo1* gene (with an unknown cellular function), fused with the GFP gene, was the reporter construct with the highest GFP expression level with regard to other promoters tested. This reporter construct is not specific for Cd(II) because it is also inducible by As(III). Likewise, the *seo1* promoter from *S. cerevisiae* revealed that this is inducible by As(III) > Cd(II) > Hg(II), being also unspecific for Cd(II). These constructs could be useful to design metal WCB with these eukaryotic microorganisms.

13.2.2.3 Protozoa-Based Biosensors

Among protozoa, ciliates have been extensively used in ecotoxicological analysis (Gutierrez et al. 2008). Ciliates have, at least, two additional advantages with regard to other microorganisms. In first place, unlike bacteria, yeasts or microalgae, ciliates have not a cell wall in their vegetative stage. As it has been previously mentioned, a major limitation in using microorganisms with cell walls as WCB or bioreceptor in CB, is the diffusion of substrates or molecules through the cell wall, resulting in a

lower signal emission or less effective cell response. To prevent this, cells have to be permeabilized by physicochemical or enzymatic methods. Furthermore, the presence of a cell wall could involves a not controlled unspecific metal(loid) biosortion process which would affect the real cellular response to the external metal concentration, when it is not used as a metal biosortion-based biosensor. Using ciliates might therefore avoid or diminish this serious problem, so the absence of a wall in these eukaryotic microorganisms results in greater sensitivity to environmental pollutants and a faster cell response (Martin-Gonzalez et al. 1999; Gutierrez et al. 2003). Secondly, ciliates are eukaryotic cells with a series of metabolic traits that are more similar to those of human cells than bacteria, microalgae, or even yeasts. After completing genome sequencing projects of two ciliate models such as *Tetrahymena thermophila* and *Paramecium tetraurelia* (Aury et al. 2006; Eisen et al. 2006), results shown that they share a higher degree of functional conservation with human genes than do other eukaryotic microbial models. 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 the *Paramecium* proteins are most similar to human proteins (Sperling et al. 2002). Therefore, this similarity with human biology makes it more reasonable to use these eukaryotic microorganisms in ecotoxicological studies (Gutierrez et al. 2008, 2011) or as biosensors to detect metal(loid) or organic pollutants. In addition, ciliates are cosmopolitan microorganisms living in aquatic or terrestrial ecosystems, and can be used as biosensors for monitoring pollutants in both habitats.

T. thermophila has five metallothionein gene isoforms. Two of these (*MTT1* and *MTT5*) are preferably over-expressed under Cd(II) or Pb(II), respectively, though they are also induced by other metals (Diaz et al. 2007; Gutierrez et al. 2011). Both genes, but mainly MTT5, respond quickly and strongly to metal stress, and their promoters have been used to design metal(loid) WCB. The first two WCB using ciliates, to detect metal pollution in soil and aquatic samples, were reported in 2011 (Amaro et al. 2011). These WCB (*turn on* bioassays) were designed using *MTT1* or *MTT5* gene promoters from *T. thermophila* and the firefly luciferase as reporter gene (Table 13.1), then these lineal constructions were introduced into nuclear genome by biolistic transformation. Validation of these WCB was carried out using artificial and natural (soil and aquatic) samples, including methods to detect false positives and negatives. A second type of *T. thermophila* WCB has been constructed with *MTT1* gene promoter and the GFP as a reporter molecule fused to *MTT1* or *MTT5* complete open reading frames into a plasmid (Amaro et al. 2014) (Table 13.1). A comparative analysis of both WCB revealed that: (1) in those using luciferase the minimal exposure time to obtain a detectable signal is ≈1 h, however for GFP-WCB an exposure of ≈2 h is necessary to have a stable signal, indicating a faster response in those with luciferase as reporter gene; (2) for the same MT promoter gene (*MTT1*), the minimum detectable Cd(II) concentration is lower in luciferase-WCB than GFP-WCB, so being luciferase-WCB more sensitive than

GFP-WCB; and (3) the bioluminescence emission from luciferase-WCB viable cells is up to 5 μM Cd(II), while cells with fluorescence emission (GFP-WCB) are viable up to 15 μM Cd(II). GFP-WCB are more resistant to Cd(II) than luciferase-WCB strains, because they have a higher copy number (plasmid constructs) of MTT1 or MTT5 genes (Amaro et al. 2014). Therefore, to detect low Cd (II) concentrations in polluted samples is better to use the luciferase-WCB strain, while for higher Cd(II) concentrations is more reasonable to use GFP-WCB strains.

At present, the only protozoa-based biosensors to detect metal(loid)s are those using the ciliate *T. thermophila* (Amaro et al. 2011, 2014), and although it has been only used as a metal(loid) WCB, in a next future these microorganism might be also used for monitoring other pollutants.

13.3 A Comparative Analysis Among Microbial Metal (Loid) Biosensors

Although the four types of microorganisms (bacteria, yeasts, microalgae or protozoa) can be used to design both CB and WCB for metal(loid) environmental monitoring, they present their advantages and disadvantages, from which the most significant will be discussed in the next sections.

13.3.1 To Have or not to Have Cell Wall: Advantage or Handicap?

For many prokaryotic and eukaryotic microorganisms to have a rigid wall is essential for survival, and, in general, if this structure is removed the cell dies. To use microorganisms with a cell wall to design metal biosensors has advantages and disadvantages. Microorganisms with a cell wall are more resistant to physicochemical disturbances than those without cell wall, so to immobilize cells to design CB it is more easy using microorganisms with cell wall (bacteria, yeasts or microalgae). This is a good reason because these microorganisms are mostly used to design CB, where cells are in solution or immobilized into an inert matrix. But, another important point to be considered to design any biosensor is the cellular permeability capacity to target molecules (to be detected by the biosensor). Unlike ciliate protozoa, the presence of a cell wall in the rest of microorganisms (bacteria, yeasts or microalgae) may require a preliminary permeabilization process to facilitate the transit of the pollutant through the cell wall. This treatment might disturb the response of the cell to the pollutant modifying the level of the biological signal to be translated by the external transducer system (CB) or the cellular transducer (WCB). Likewise, to obtain the reporter signaling in substrate-dependent reporters, the substrate must cross the cell wall and reach the cytoplasm, where the

enzymatic reaction takes place, or be added to the cells previously lysated. The substrate for eukaryotic luciferase, D-luciferin, is membrane-permeant only in its protonated form (pH 5); at neutral pH, it crosses the plasma membrane very slowly. For this reason, most luciferase-based bioassays are performed using cell extracts (Van der Meer et al. 2004) or permeabilized cells (Lagido et al. 2001). An additional problem to design metal(loid) biosensors using microorganisms with a cell wall, is the possible biosorption process carry out by the wall polymers. It could alter the response of the biosensor to the metal(loid) target to be detected and/or quantified, because a important part of metallic ions would be trapped by the microbial cell wall (biosorption).

As ciliated protozoa do not present a cell wall in their vegetative phase, they have a great advantage over other potential metal microbial biosensors, because there is no need for any preliminary permeabilization treatment or cellular lysis. For instance, in *T. thermophila* permeabilization pre-treatment or cellular lysis is not necessary when used as a WCB with luciferase as a reporter gene, because the luciferin crosses through the cell membrane. In this ciliate, luciferase activity can be measured as efficiently in intact viable cells as in permeabilized cells, and similar induction in vivo and in vitro was observed (Amaro et al. 2011).

13.3.2 Specificity Versus Sensitivity

The majority of microbial biosensors (CB and WCB) respond to two or more metal (loid)s, although some of them show greater specificity (Corbisier et al. 1999; Tom-Petersen et al. 2001; Ivask et al. 2009). It is not easy to find a gene promoter responding exclusively to one metal(loid), in fact metallothioneins (the main proteins binding metals) respond to several different metal(loid)s (de Francisco et al. 2016; Gutierrez et al. 2011). Likewise, the same bacterial operon responding to a specific metal stress has been used to design biosensors for other different metals (Tauriainen et al. 1998). In general, cells are ready to respond to diverse metal(loid) stresses using the same molecular protection system. Probably, the main reason of this is that natural or anthropogenic metal polluted ecosystems present very frequently a mixture of metal(loid)s rather than a single one (Fairbrother et al. 2007; Preston et al. 2000). Therefore, one valuable aim of environmental monitoring may be to determine the overall toxicity of a sample rather than the presence of a specific metal. Metal(loid) specificity is therefore not so important when designing a biosensor to be used for monitoring environmental metal pollution. However, several authors (Elad et al. 2008; Jouanneau et al. 2011) have tried to resolve this problem using a panel of luminescent bacteria as WCB with different stress-responsive gene promoters. These bacteria were treated with different toxic compounds (including heavy metals), and each toxic treatment activated different promoters. From this experimental approach these authors (Elad et al. 2008) were able to identify the toxic elements into the experimental sample within 30 min and with an error rate estimate that did not exceed 3% at a 95% confidence level. Later,

Jouanneau et al. (2011) based on a similar experimental approach, have elaborated predictive decision tree models and by using a specific software they can choose the best decision tree to identify the toxic metal(loid) from a four metal(loid) mixture. This method showed a high correlation (≈98%) for the metal(loid) identification. Although, these two experimental approaches represent good contributions to identify metallic elements from a polluted sample, the problem of the biosensor specificity is not still resolved.

On the other hand, the sensitivity level of the biosensor is really important when trying to detect metals present in very low concentrations, mainly those that are lower than the maximum allowable metal concentrations established by international commissions. A comparative analysis of the ranking of sensitivity values to different metal(loid)s among reported biosensors is summarized in Table 13.2, and described in the following points: (1) with regard to As(V), the *T. thermophila* MTT5Luc strain (WCB with the reporter construct *MTT5::LucFF*) is the biosensor with the highest sensitivity (25 nM) (Amaro et al. 2011) in comparison with other eukaryotic or prokaryotic-based biosensors; (2) for Zn(II) the ciliate *T. thermophila* MTT1Luc strain (with *MTT1::LucFF* construct) (Amaro et al. 2011) together the cyanobacterium *Synechococcus* sp. (with *smtB::luxCBDAE* construct) (Erbe et al. 1996) are the WCB with the highest sensitivity (0.5 μM), while among CB the synthetic phytochelatin-based capacitive biosensor (Bontidean et al. 2003) is the one showing the highest sensitivity (0.1 pM); (3) the *Escherichia coli*-based biosensor (with *cadC::gfp* construct) is the prokaryotic WCB with the highest

Table 13.2 Comparative analysis of the ranking of sensitivity values to different metal(loid)s among reported biosensors

Metal(loid)	Type	Bioreceptor[a]	Sensitivity[b]	Reference
As(V)	WCB	*T. thermophila*	25 nM	Amaro et al. (2011)
Zn(II)	WCB	*T. thermophila*	0.5 μM	Amaro et al. (2011)
	WCB	*Synechococcus* sp.	0.5 μM	Erbe et al. (1996)
	CB	Phytochelatin	0.1 pM	Bontidean et al. (2003)
Cd(II)	WCB	*E. coli*	0.1 nM	Liao and Ou (2005)
	WCB	*T. thermophila*	0.5 nM	Amaro et al. (2011)
	CB	Phytochelatin	0.1 pM	Bontidean et al. (2003)
Hg(II)	WCB	*E. coli*	1 fM	Virta et al. (1995)
	WCB	*T. thermophila*	0.25 nM	Amaro et al. (2011)
	CB	*E. coli*	1 pM	Gammoudi et al. (2010)
Pb(II)	WCB	*E. coli*	0.1 nM	Shetty et al. (2003)
	WCB	*T. thermophila*	50 nM	Amaro et al. (2011)
	CB	Phytochelatin	0.1 pM	Bontidean et al. (2003)
Cu(II)	WCB	*E. coli*	0.3 μM	Hakkila et al. (2004)
	WCB	*S. cerevisiae*	0.5 μM	Shetty et al. (2003)
	CB	Phytochelatin	0.1 pM	Bontidean et al. (2003)

[a]Microorganism or biomolecule. [b]Lowest detectable metal concentration

sensitivity to Cd(II) (0.1 nM) (Liao and Ou 2005), while, among eukaryotic WCB, the ciliate *T. thermophila* is that with the highest sensitivity (5 nM) for this metal (Amaro et al. 2011). And, among CB, the synthetic phytochelatin-based capacitive biosensor (Bontidean et al. 2003) shows the highest sensitivity value (0.1 pM) for Cd(II); (4) the *E. coli*-based WCB (with *merR::LucFF* construct) is that reporting the highest sensitivity (1 fM) for Hg(II) (Virta et al. 1995), while, among eukaryotic biosensors, *T. thermophila*-based WCB (Amaro et al. 2011) is the one showing the highest sensitivity (0.25 nM). Immobilized *E. coli* cells used to design an acoustic wave-based biosensor, seen to be the most sensitive for this metal (1 pM) (Gammoudi et al. 2010); (5) the WCB using the bacterium *E. coli* containing the *zntA::lacZ* construct (Shetty et al. 2003) presents the lowest detectable concentration for Pb(II) (0.1 nM), while *T. thermophila*, among eukaryotic WCB, presents the highest sensitivity (50 nM) (Amaro et al. 2011), likewise, the synthetic phytochelatin-based capacitive CB (Bontidean et al. 2003) is that reporting the highest sensitivity (0.1 pM) for Pb(II); and (6) with regard to Cu(II) ions, the *E. coli* strain with *copA::lucFF* construct (Hakkila et al. 2004) presents the highest sensitivity (0.3 μM), and among eukaryotic-based biosensors, a strain of the yeast *S. cerevisiae* with the *cup1::gfp* construct (Shetty et al. 2004), used as a WCB, has the highest sensitivity (0.5 μM) for Cu(II). Again, the synthetic phytochelatin-based capacitive CB (Bontidean et al. 2003) is that showing the most high sensitivity (0.1 pM) to this essential metal. Although these last authors indicate that this phytochelatin-based biosensor is able to detect metal ions in concentration range of 0.1 pM–10 mM, reporting an order of sensitivity ($Zn > Cu > Hg \gg Cd \approx Pb$), they do not indicate the concentration values for each metal. So, we cannot assure the real sensitivity values for each metal detected by this CB.

A summary of several features among different microorganisms which could affect (positively or negatively) the design of a metal(loid) biosensor is showed in Table 13.3.

Table 13.3 Comparative analysis of several features among different types of microorganisms which could affect (positively or negatively) the design of metal(loid) biosensors

Feature	Microorganism			
	Bacteria	Microalgae	Fungi/yeasts	Ciliates
Rapid growth	+++	+++	+++	+++
Easy manipulation	+++	+++	+++	+++
Genetic modification	+++	+	+++	+++
Metal(loid) sensitivity	++	++	++	+++
Cellular immobilization	+++	+++	+++	?
Used as WCB	+++	+	+++	+++
Presence of cell wall	+++	+++	+++	−

(+++): high; (++): low; (+): very low; (−): absent; (?): unknown. See the text for a more extensive explanation

13.4 Concluding Remarks

From this review on metal(loid) microbial biosensors the following general conclusions can be drawn:

(1) In general, microorganisms present more advantages than disadvantages to be used as CB or WCB to detect metal(loid)s from polluted ecosystems, in regard with other organisms. This is due to their easy cultivation and maintenance, their higher growth rate and genetic manipulation facilities. Likewise, eukaryotic microorganisms used as WCB have certain advantages over prokaryotic ones. Among them, the comparative analysis with multicellular organisms (including humans) is more reliable than using bacteria.
(2) The biotechnology for using microalgae as WCB is still underdeveloped, although these photosynthetic microorganisms have a great potential as CB or WCB based on genetic constructs involving photosynthesis genes. Likewise, ciliated protozoa also present a great potential to design both WCB or CB (using isolated molecular metal bioreceptors like metallothioneins).
(3) Microorganisms with cell wall (bacteria, fungi or microalgae) present a considerable disadvantage with regard to protozoa, because the presence of the cell wall could hinder the permeability of the pollutant or hold it by extracellular biosorption. Furthermore, using substrate-dependent reporters, the substrate must cross the cell wall to reach the cytoplasm, where the enzymatic reaction takes place. Therefore, sometimes a pre-treatment to increase cell permeabilization is necessary.
(4) The capacity for sensitivity of a metal biosensor is more important than its level of specificity to a metal. Because, in the real world the anthropogenic environmental metal pollution is generally by several metal(loid)s.
(5) At present, many CB and WCB using microorganisms have been designed to detect metal(loid)s in laboratory experiments, but, in general, very few of them have been validated using bioassays with real environmental samples. After rigorous bioassays using real metal polluted aquatic or terrestrial environmental samples, a lot of the biosensors reported to be specific to only one metal(loid) could be finally considered as non-specific. This is due to the presence of other unknown inorganic or organic components that can interact with the bioreceptor of the biosensor disturbing the response. This point is really important to build useful biosensors to detect metals in environmental samples.
(6) The future development of microbial WCB for environmental metal pollution monitoring could be considerably furthered by applying a synthetic biology approach. This would facilitate the design of WCB with multi-input systems based on two or more regulatory gene promoters in the same genetic construct, thereby increasing the capacity of the biosensor for detecting simultaneously several different metal(loid)s in the same polluted sample.

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