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Ecotoxicology and Environmental Safety 56 (2003) 140–147
Ecotoxicology and Environmental Safety
http://www.elsevier.com/locate/ecoenv

# Issues underlying use of biosensors to measure metal bioavailability

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Received 20 March 2003; accepted 20 March 2003

**Abstract**

Heavy metal-mediated toxicity in the environment is dependent on bioavailable metal concentrations both internal and external to microbial cells. Both internal and external metal bioavailability are influenced by multiple factors in the soil environment. External factors include pH, redox potential, ionic strength, organic matter and clay content. The internal bioavailable metal concentration is dependent on both the aforementioned external factors, as well as metal uptake and efflux activities that are specific for each microorganism. The metal-specific biosensors discussed in this article can be used to measure internal metal bioavailability.


*Keywords:* Bioavailability; Transition metals; Heavy metals; Uptake; Efflux; Biosensor

## 1. Metal bioavailability in the environment

Metal speciation and the resulting bioavailability rather than total metal concentration determines the overall physiological and toxic effects of a metal on biological systems (Bernhard et al., 1986; Hughes and Poole, 1989; Morrison et al., 1989; Roane et al., 1996). Total metal refers to all metal present in a given environment. In contrast, one can define external bioavailable metal as the soluble, ionic form of the metal. This is the metal that can interact with surrounding microbial cells or other biota. To understand the difference between total and external bioavailable metal, one must understand metal speciation in the system. The speciation of a metal refers to the various forms of the metal present including the soluble, exchangeable, carbonate-bound, oxide-bound, organic matter-bound, and residual fractions (Tessier et al., 1979; Davis et al., 1993; Brown et al., 1999). At any given time in an environmental system, the external bioavailable metal is considered to be the soluble fraction. However, if equilibrium conditions change, specifically if the soluble metal fraction is removed, some metal forms will quite readily become soluble and bioavailable. Metal forms that are most subject to this type of change are the exchangeable, and the carbonate-, oxide-, and organic matter-bound fractions. In contrast, the residual fraction is composed of stable, low-solubility crystalline metal forms that do not readily become bioavailable. Metal speciation and the resulting formation of the various fractions is dependent on the combined effects of time, pH, redox potential, ionic strength, and in the case of soils, organic matter and clay content (Sposito, 1989; Alloway, 1990; Brierley, 1990; Moore, 1994).

### 1.1. pH and redox potential

At high pH, metals tend to form insoluble metal mineral phosphates and carbonates, whereas at low pH they tend to be found as free ionic species or as soluble organometals. Consider a medium containing phosphate, perhaps the most common buffer constituent used in microbiological media. Even a small change in pH can decrease metal solubility and hence metal bioavailability by several orders of magnitude. For example, according to the MINEQL+ geochemical speciation model, the solubility of cadmium at pH 6 in the presence of 1.3 mM phosphate is 88 mM. Increasing the pH to 7 reduces cadmium solubility to 10 mM.

Redox potential also influences speciation. The redox potential ($E_h$) of an environment is established by oxidation–reduction reactions that tend to be relatively slow, particularly in soil environments. However, microbial activity can dramatically influence the rate

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0147-6513/03/$ - see front matter 
doi:10.1016/S0147-6513(03)00057-5

and establishment of redox potential in soil. Reducing conditions (negative $E_h$) found in anaerobic media can result in metal precipitation with media components. For example, only 1% of the total zinc added to acetate enrichment anaerobic cultures in the work of Majumdar et al. (1999) was in the aqueous phase. A similar situation occurs in saturated soil systems in which carbonates and sulfides are present. Under these conditions, cationic metals such as $Fe^{2+}$, $Cd^{2+}$, and $Pb^{2+}$ combine with sulfides to form nontoxic, insoluble sulfide deposits. The amount of sulfide present in soil systems can often be increased substantially by the activity of sulfate-reducing bacteria such as *Desulfovibrio* species, which reduce sulfate to sulfide. For example, Kong (1998) found that the soluble metal concentration in sediment slurries initially amended with 20 mg/L cadmium, copper, or chromium were below detection limits of 0.03–0.04 mg/L. Furthermore, at 100 mg/L added metal, only 1 mg/L cadmium and $<0.12$ mg/L copper and chromium were found in the aqueous phase.

Under oxidizing conditions (positive $E_h$), metals are more likely to exist in their free ionic form and exhibit increased water solubility. In addition, pH may decrease slightly or even dramatically under oxidizing conditions. The classical example in this case is the formation of acid mine drainage, where sulfide and sulfur are oxidized by *Thiobacillus thiooxidans* to sulfuric acid, resulting in pH values ranging as low as $\leqslant 2$. This further adds to increasing the solubility of metals.

### 1.2. Binding components in medium and soil systems

Many laboratory media contain metal-binding (e.g., yeast extract) and metal-precipitating (e.g., phosphate or sulfate salts) constituents that can bind cationic metals and reduce metal bioavailability. The following data illustrate this quite clearly. MINEQL+ was used to model the effect of increasing concentrations of phosphate on the solubility of cadmium. Results showed that cadmium solubility is reduced from 88 to 50, to 17, to 2, to 0.1 mM as the phosphate concentration is increased from 0, 0.13, 1.3, 13, to 130 mM, respectively.

In the soil environment, organic matter and clay mineral content are important factors that can reduce metal bioavailability. Metal complexation with organic matter is demonstrated by a study showing that only 0.01 mg/L cadmium was required to inhibit trichloroaniline dechlorination in a mineral-dominated soil, whereas 0.2 mg/L cadmium was required for inhibition in an organic matter-dominated soil (Pardue et al., 1996). This increase in tolerance to cadmium was correlated to saturation of metal-binding sites on the organic matter. In a later study, only soluble cadmium was reported to inhibit dehalogenation in microcosms containing cadmium-contaminated sediment (Jackson and Pardue, 1998). Clay minerals have also been shown to reduce metal bioavailability. Clays with high cation exchange capacities, such as montmorillonite, appear to reduce metal bioavailability and toxicity most (Babich and Stotzky, 1977). The impact of clays on the bioavailability of toxic metals has even prompted investigations into the use of clays to reduce metal toxicity.

The susceptibility of metals to pH, redox, organic matter, and clay demonstrates how important it is to define clearly the chemical parameters of metal-containing systems. These system parameters will strongly influence the external bioavailability of metals. Despite the known importance of metal bioavailability, it is a difficult parameter to measure in both laboratory and environmental systems. As a result, few studies provide metal speciation information, and an enormous range of metal concentrations have been reported to have toxic effects on microbial activity. In fact, three to six orders of magnitude separate reported inhibitory metal concentrations.

## 2. Measurement of bioavailable metal using whole-cell biosensors

The transport and fate of metals in soil is influenced by both biotic and abiotic factors. Soil microorganisms are responsible for fundamental ecological processes, including biogeochemical cycling of transition (heavy) metals. Improved understanding of microbial metal homeostasis and of metal resistance strategies should lead to new approaches to environmental decontamination. It should also be possible to increase the rates of restoration of contaminant-impacted soils and optimize methods used to maintain or increase soil fertility through knowledge about microbial populations and how microbes cooperate to affect mineral dissolution, degradation of organic compounds, immobilization of ions, precipitation of minerals, and changes in solution chemistry. Interference by heavy-metal contamination in such microbe-mediated processes may greatly affect the quality of the biosphere. For example, a site contaminated with cadmium or lead might inhibit $N_2$ fixation by free-living bacteria or mineralization of organic substances and thus affect soil fertility. However, heavy-metal-mediated toxicity is not correlated with total metal concentration in soils but rather depends on the external and internal bioavailable concentration of the metal contaminants. It is therefore of interest to develop biosensors that can measure only the internal bioavailable portion of a metal. This has been attempted and will be described in a later section. Reporting of bioavailable metal concentrations is a vital step in the process of standardizing experiments to determine the impact of metals on microbial activities in the environment. There are many approaches to

measuring bioavailable metal, including analytical measurement of metal concentrations, metal speciation modeling, and toxicity testing. In addition, there are a number of promising tools in development intended to accurately quantify bioavailable metal concentrations in more complex systems such as microbiological media and soil including immunoassays and biosensors.

### 2.1. General principles

A number of microbially based bioreporter–biosensor systems have been developed to detect metal contaminants in environmental samples. These systems are becoming increasingly more available as alternatives to traditional analytical methods. Bioreporters provide a unique analytical capability because contaminants are quantified relative to the concentrations experienced by the bioreporter organism as opposed to being relative to the extraction technique that is used for traditional analysis. Thus, these bioreporters can complement other analytical methods by distinguishing the bioavailable fraction from the total amount of contaminant present. For example, Van Dyk et al. (1994) designed two bacterial bioreporters that were identical except that one contained a mutation in a gene known to affect the permeability of the outer membrane to hydrophobic compounds. The response to pentachlorophenol was observed at significantly lower concentrations in the mutant strain with the more permeable outer membrane. It is important also to note that in the same way the efficiency of traditional chemical analyses is limited by the extraction technique, bioreporters are living organisms with associated advantages and limitations. These associated limitations must be fully understood to select the optimal bioreporter for the specific application and to interpret the results accurately.

Numerous bioreporter systems have been constructed. These systems can be broadly categorized according to two sets of criteria. The first set of criteria is based on the type of reporter gene used and how the gene is detected. The types of reporter genes used fall into two categories: those that require substrate addition for assay (e.g., the *lacZ* system) and those that do not (e.g., the green fluorescent protein (*gfp*) system). For systems that require substrate addition, the reporter cells are extracted and a biochemical assay is done wherein specific chromogenic or fluorogenic substrates are added to quantify the enzyme produced by the reporter gene. Reporter genes that do not require substrate addition include those that produce luminescence or fluorescent proteins such as the green fluorescent protein (Burlage and Kuo, 1994). Most of the reporter genes can be fused to any gene of interest in either procaryotic or eucaryotic cells; thus the selection of the optimal reporter system is based on the specific application. Several criteria are considered when selecting a reporter gene. First, the enzyme activity encoded by the reporter gene must be readily distinguishable from similar or competing activity in the cell. Second, the assay for the reporter gene product should be rapid, sensitive, reproducible, and appropriate to the specific application. Finally, a reporter system should be selected with minimal background interference from the samples analyzed (Sambrook et al., 1989). Luciferase genes are widely used reporter genes in procaryotic as well as eucaryotic systems because they provide simple and sensitive detection of gene expression and regulation (Wood and Gruber, 1996). The quantification of light emission (bioluminescence) is one of the most sensitive means of detection, and it can be measured with a liquid scintillation counter or a luminometer or even with X-ray film. This makes it very suitable for environmental monitoring. The *lacZ* gene encoding $\beta$-galactosidase is another widely used reporter gene used in metal or metalloid detection. For example, Ramanathan et al. (1998) developed a highly specific antimonite and arsenite biosensor with a fusion between the *lacZ* and the *arsD* genes. Antimonite was detected at concentration as low as $10^{-15}$ M. The chemiluminescent substrate, Galacton-plus, was also used allowing the $\beta$-galactosidase to be detected by chemoluminescence. The new chemoluminescent sensing system not only simplifies the assay, but improves the detection limits. Unfortunately, the substrate could not penetrate the *Escherichia coli* cell wall, so the reporter cells still had to be removed and treated with polymyxin B sulfate to disrupt the cell membranes to permit a sensitive assay of the $\beta$-galactosidase gene product. This assay prevents the use of *lacZ* biosensors for real-time, nondestructive, in situ quantitation of the gene product. Davies and Geesey (1995) reported the use of the fluorogenic substrate, methylumbelliferyl-galactosidase, for the *lacZ* system. The cell membrane of the bioreporter strain, *Pseudomonas aeruginosa*, was permeable to this substrate, thus the real-time activity of the bacteria could be observed in a continuous culture flow cell by epifluorescent microscopy.

The second set of criteria is based on the regulation of host genes to which the reporter is fused. Host gene expression can be inducible or constitutive. Inducible bioreporters contain reporter genes that are fused to a gene in an operon or pathway regulated by the concentration of the contaminant of interest. These bioreporters permit a quantitative analysis of contaminant concentration (Stewart and Williams, 1992) and are described later. Constitutive bioreporter systems contain reporter genes that are fused to genes in the microorganism that are expressed continuously as long as the organism is alive and metabolically active. This type of bioreporter is not used to detect a specific contaminant but can be used to evaluate the toxicity of a contaminant. In this case, when a toxic substance is

present, reporter activity will be inhibited. The application of constitutive bioreporters was demonstrated by Paton et al. (1995). Two different constitutive *lux*-marked *P. fluorescens* bioreporters were constructed to determine the effective concentration ($EC_{50}$) of a range of heavy metals. Both biosensors produced luminescence when grown on rich media in the presence of the appropriate substrate, but the bioluminescence decreased with increasing metal concentration. Thus, metal toxicity could be evaluated in terms of the ratio of light produced to that produced in the absence of the contaminant. Consistent graduated results were obtained for copper, zinc, cadmium, chromium, and nickel.

### 2.2. Metal-specific biosensors

Numerous nonspecific microbial whole-cell sensors have been developed that react to nearly any kind of toxic substance (Karube and Nakanishi, 1994). A novel approach for a microbial whole-cell sensor is to use recombinant DNA technology to construct a plasmid or other vector system in which a strictly regulated promoter is connected to a sensitive reporter gene (Fig. 1). A recent extensive review by Daunert et al. (2000) gives an excellent overview. The most interesting promoters for environmental analysis are found in bacteria that survive in extreme environments contaminated by, for example, heavy metals or organic compounds. The ability of bacteria to survive in a contaminated environment is usually based on a genetically encoded resistance system, the expression of which is precisely regulated. The best studied example is the mercury resistance (*mer*) operon whose functions,

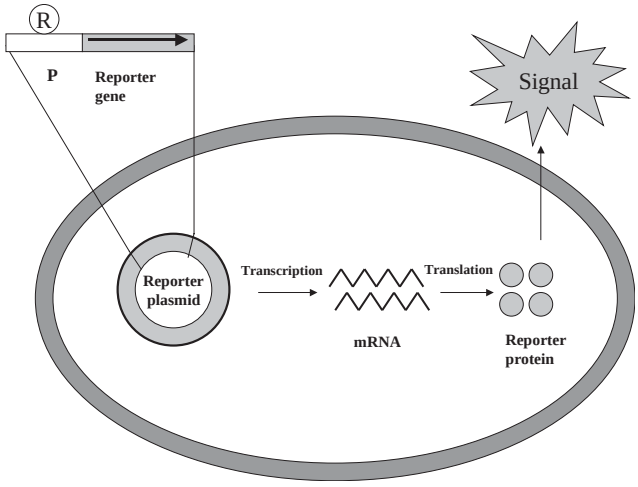


Fig. 1. Regulation of a reporter gene by a regulatory protein. Binding of the regulatory protein R to the promoter P controls transcription, followed by translation of the mRNA to produce the protein. Both of these steps produce multiple copies of the reporter protein, leading to an increased protein concentration.

the reduction of Hg(II) to $Hg^0$ (by the *merA* gene product, the mercuric reductase) and degradation of methylmercury (by the *merB* gene product, the organomercurial lyase), are beneficial because they reduce the toxicity of mercury to the bacterial cell (Silver and Phung, 1996). The *mer* promoter is activated when Hg(II) binds to the regulatory protein *MerR*. Indicator bacteria that contain gene fusions between the promoter of the *mer* operon and a reporter gene are able to detect Hg(II). For example, luminescent biosensors using this principle have been developed for the detection of mercury. Selifnova et al. (1993) constructed three biosensors for Hg(II). Mercury sensitivity was determined in minimal media with resulting values of 0.5, 1, and 25 nM Hg(II) for each of the three strains, respectively. Freshwater, rain, and estuarine (1:3 dilution) water samples were also supplemented with Hg(II) to determine the effectiveness of the bioreporter in environmental samples, and the sensitivity of detection was found to be similar to that observed in minimal media. Other applications include fusions with the copper-resistant promoters *pcoE*. This copper salt biosensor was suitable for analytical determinations (Da et al., 1995) Sensor bacteria in which this promoter–reporter gene concept is operable have been developed to detect mercury, arsenic, cadmium, zinc, and lead ions, and also xenobiotic compounds (Virta et al., 1995; Selifnova et al., 1993; King et al., 1990; Scott et al., 1997; Rensing et al., 1998a; Tauriainen et al., 1997, 1998).

A serious drawback of most of the described systems is that they use plasmids in which a regulated promoter is connected to a reporter gene. This approach may not be suitable for long-term studies of the environment because of the need to take into account such variables as copy number and loss of plasmid. A second concern that is specific to metal biosensors developed thus far is that they may give variable results depending on the ability of the host organism to take up and subsequently pump toxic metals out of the cell. In microorganisms, three biotic variables influence metal homeostasis: uptake, efflux, and complexation. These variables greatly influence results obtained by whole-cell metal biosensors. If microorganisms do not take up metals, there would be no response from an intracellular biosensor. Furthermore, not much is known about specific transporters responsible for transition-metal uptake in microorganisms under conditions of excess metal at the molecular level. Thus, the presence of other metals might greatly influence rates of metal uptake of the metal to be measured. Finally, bacteria are able to pump metals out of the cytoplasm and this would also influence data obtained by whole-cell biosensors. Therefore, one has to examine both bacterial uptake and efflux systems to be able to design reliable biosensors.

## 3. Interaction of metals with microorganisms that influence biosensor measurement

### 3.1. Uptake of transition metals by microorganisms

Recent findings indicate that there is (almost) no free transition metal in living cells (e.g., copper and zinc in *E. coli* and *Saccharomyces cerevisiae;* Rae et al., 1999; Outten and O'Halloran, 2001). New findings from two of the best characterized model organisms, *S. cerevislae* and *E. coli*, and data from genome sequencing projects of numerous microorganisms indicate that metal transporters and metal homeostatic mechanisms are widespread. Metals are usually taken up by specific transporters and not by passive diffusion. This is important for interpretation of toxicity and biosensor measurements. The best characterized homeostatic mechanism for a transition metal in bacteria is that for zinc in *E. coli* (Fig. 2). Therefore, we describe zinc homeostasis in *E. coli* to be able to illustrate that the genetic background and the transporters themselves can have a profound impact on measurement of toxicity and detection.

In *E. coli*, zinc deficiency induces expression of a specific zinc uptake system, ZnuABC, which is an ABC transporter for zinc uptake (Patzer and Hantke, 1998). ZnuA is a periplasmic binding protein, ZnuB is the membrane sector of the pump, and ZnuC is the ATPase catalytic subunit. Genes encoding ZnuABC homologs have also been described in *Haemophilus influenzae* and *Bacillus subtilis* (Lu et al., 1997; Gaballa and Helmann, 1998) but are present in the genome of many bacteria. Under conditions of zinc sufficiency, expression of the pump is repressed by the Fur homolog Zur, which presumably binds to the bidirectional promoter region of *znuA* and *znuBC*. In addition to ZnuABC, zinc is also taken up by ZupT, a member of the ZIP family of proteins (Grass et al., 2002). Members of the ZIP family that are involved in iron and zinc transport were first identified in the plant *Arabidopsis thaliana* and yeast but have subsequently shown to be ubiquitous (Guerinot, 2000). In addition to these well-characterized metal transporters, zinc might also be able to enter via the Pit phosphate uptake system as a metal phosphate (Beard et al., 2000), but this seems to be adventitious. Because zinc is such an important nutrient, it is not surprising to observe redundancy in transport systems. However, the number and families of putative zinc transporters is not conserved in different bacteria and might reflect different physiological needs. Finally, zinc might also be taken up by transporters that also translocate cadmium and lead under toxic conditions. Cd(II) and Pb(II) compete for transport with other physiologically important cations. Transport inhibitor studies suggest that in Gram-positive bacteria such as *Staphylococcus aureus*, *B. subtilis* and *Lactobacillus plantarum* ATCC14917, Cd(II) is taken up by a manganese transport system (Archibald and Duong, 1984; Hao et al., 1999). Recent results in *E. coli* and *Salmonella typhimurium* suggest Cd(II) might also be taken up by MntH, a NRAMP ortholog (Makui et al., 2000; Kehres et al., 2000). The physiological function of MntH seems to be uptake of Mn(II) possibly as protection from hydrogen peroxide. Genomic sequence data can also be used to assign probable Mn(II) transport activity to the NRAMP homologs of *S. aureus*, *B. subtilis,* and *L. plantarum* (Fisher et al., 1973; Perry and Silver, 1982, Hao et al., 1999). Whether bacterial NRAMP orthologs transport zinc is unknown. CorA is a low-affinity magnesium uptake system that can also take up metals such as nickel or cobalt and possibly also zinc (Smith and Maguire, 1998). Because there is a vast array of transporters that are able to transport transition metals such as zinc, it is difficult to predict how external conditions can influence zinc uptake.

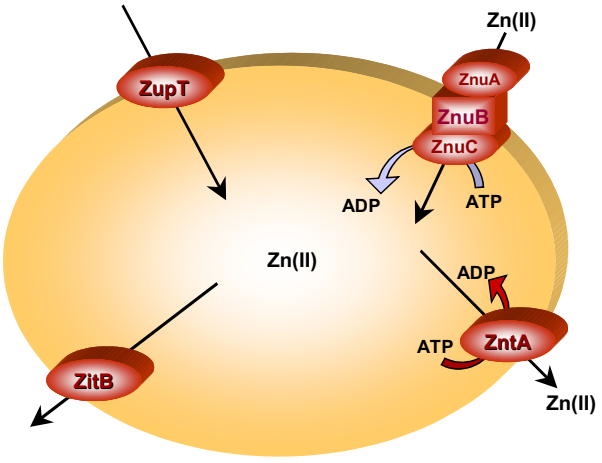


Fig. 2. Zinc transport systems in *E. coli*. In this model the known zinc transport system are shown. Under conditions of zinc deficiency zinc is taken up by ZupD and ZnuABC (Grass et al., 2002; Patzer and Hantke, 1998). ZupT belongs to the ZIP family of metal transporters (Guerinot, 2000) and ZnuABC is an ABC transport system. Zinc-translocating efflux pumps are the P-type ATPase ZntA and the CDF protein ZitB (Rensing et al., 1997; Grass et al., 2001b). Interestingly, *E. coli* seems to have at least two transporters responsible for uptake and for efflux. ZnuABC and ZntA are both ATPases and are probably more powerful transporters than ZitB or ZupT who might be responsible for zinc homeostasis under physiological conditions.

### 3.2. Metal efflux systems in bacteria

Four ubiquitous families of related proteins have been shown to transport metals out of cells in bacteria. These are the cation diffusion facilitator (CDF) family (Paulsen and Saier, 1997), the P1-ATPases (also called Cpx- or soft-metal P-type ATPases) (Axelsen and Palmgren, 1998), the major facilitator superfamily (Saier et al., 1999; Grass et al., 2001a), and the resistance–nodulation–cell division (RND) family (Nies, 1999). Of

these families, only the CDF family is exclusively involved in the transport of metals. The RND family is restricted to Gram-negative bacteria, because RND proteins usually form a multimeric complex with proteins of the membrane fusion protein familiy and the outer membrane factor family of proteins. These complexes transport the substrate across the cytoplasmic and outer membrane into the extracellular medium (Nies, 1999).

In *E. coli*, zinc efflux is accomplished by both ZntA, a P1-ATPase, and the CDF protein ZitB (Beard et al., 1997; Rensing et al., 1997; Grass et al., 2001b). ZntA is activated by ZntR, a MerR homolog that is an activator of *zntA* (Brocklehurst et al., 1999; Outten et al., 1999). Disruption of *zntA* results in sensitivity to Zn(II), Cd(II), and Pb(II). The strain did not exhibit increased sensitivity to any other metal ions, including copper and silver, suggesting that this P-type ATPase is a specific Zn(II)/Cd(II)/Pb(II) pump. ZntA has been shown to catalyze ATP-coupled accumulation of $^{65}$Zn(II) and $^{109}$Cd(II) in everted (inside-out) vesicles of *E. coli*, where accumulation in everted membrane vesicles is equivalent to efflux from intact cells (Rensing et al., 1997, 1998a; Beard et al., 1997). ATPase activity of purified ZntA was only stimulated by Zn(II), Cd(II), Pb(II), and Hg(II) (Sharma et al., 2000) and not any other metal ions, again showing that ZntA is specific for these metals. ZntA homologs are widespread in Gram-negative bacteria, including *S. typhimurium*, *Vibrio cholerae*, *Yersinia pestis*, *Deinococcus radiodurans*, and *Proteus mirabilis* (Rensing et al., 1998b, 1999). The CDF family has been found in all three domains of life: bacteria, eucaryotes, and archeae. Representative examples include CzcD from *Ralstonia metallidurans*, ZitB (formerly YbgR) from *E. coli*, ZRC1 and COT1 from yeast, and ZnT-1 and ZnT-2 from mice (van der Lelie et al., 1997; Kamizono et al., 1989; Conklin et al., 1992; Palmiter et al., 1996; Palmiter and Findley, 1995; Grass et al., 2001b). The CDF proteins encompass six predicted transmembrane domains and variable potential metal binding sites. All CDF proteins transport zinc, while some members of this family are able to transport additional cations such as cobalt and cadmium (Anton et al., 1999).

### 3.3. Transporters can influence biosensor measurements

In addition to zinc, ZntA also transports cadmium and lead (Rensing et al., 1998a; Sharma et al., 2000). This knowledge was helpful in constructing a lead biosensor (Rensing et al., 1998a). However, these studies also described how the genetic background of different *E. coli* strains can have a profound influence on the response of an intracellular biosensor. In this case, the presence of *zntA* prevented a zinc response from the biosensor, because excess zinc was rapidly transported out of the cytoplasm. However, a biosensor in a *zntA*-disrupted strain was able to detect extracellular zinc (Rensing et al., 1998a). Without going into further detail, this example is used to illustrate a point: the response of an intracellular biosensor to extracellular concentrations of metal is dependent on transporters mediating uptake and efflux. This is true for all organisms but can be more readily observed in organisms that are further genetically characterized, such as *E. coli* or *S. cerevisiae*. Furthermore, uptake of certain metals is also dependent on concentrations of other metals. For example, the magnesium transporter CorA can also take up cobalt and nickel, which can be toxic to cells at high concentrations (Smith and Maguire, 1998). Thus, magnesium can act as a competitive inhibitor of CorA-mediated cobalt and nickel uptake. Another example is phosphate uptake. The Pit system is responsible for low-affinity phosphate uptake but can also transport metal phosphate into the cells and is expressed constitutively (Harris et al., 2001). Phosphate deficiency would lead to induction of the high-affinity Pst-system, possibly leading to higher specificity and decreased uptake of metal phosphates. Another interesting observation is the uptake of antimony and arsenic through the glycerol facilitator family in *E. coli*, *S. cerevisiae*, and probably humans (Sanders et al., 1997; Tamas and Wysocki, 2001). This suggests that both As(III) and Sb(III) are unexpectedly recognized as polyols. Recent results also suggest that the toxic effect of copper in *E. coli* might be interference of copper with iron uptake. The impact of excess copper, again, is dependent on the availability of alternative routes of iron uptake (unpublished observations). These points clearly illustrate the difference between external bioavailable metal, which is the soluble fraction, and internal bioavailable metal which is dependent on host factors such as metal transporters.

## 4. Conclusions

Metal specificity and affinity of transport proteins determines rates of metal translocation and distribution. Therefore, the presence of cellular homeostatic mechanisms greatly influences the toxicity of the external bioavailable metal concentration.

## Acknowledgments

This work was supported in part by Grant 2 P42 ESO4940-11 from the National Institute of Environmental Health Sciences, NIEHS, (to R.M.), in part by Grant CHE 0133237 from the National Science Foundation (to R.M.) and by Grant EEC9908280 from NSF (to C.R.).

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