Metal Requirements and Tolerance

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INTRODUCTION

Definition of Metals

Metals are defined chemically as all elements on the left side of the periodic table, including the alkali and alkaline earth metals (groups 1A and 2A) and the transition metals (groups 3A to 2B). Also included in the transition metals are the heavier elements of groups 3B to 6B, specifically the elements including and below Al, Ge, Sb, and Po. Elements to the right of and above the aforementioned are defined as nonmetals. This definition includes trace elements often included with metals in the environment; for example, Se and As are often considered along with heavy metals, even though they are not metals. Discussion of these elements will be undertaken here only as it enhances discussion of the metals. When considering metals of biological and environmental importance, the alkali and alkaline earth metals are usually not considered metals. The term "heavy metals" has become popular for those group 3A and heavier metals. These are sometimes required by biological systems but are often toxic if present at concentrations only slightly higher than required. It is the heavy metals that will be the focus of this chapter, primarily because of the toxicity of many of the elements included in this grouping.

It is impossible to offer a comprehensive view of all facets of metal interactions with microorganisms in this chapter, given the diversity of the metals, the breadth of their function, and the number of problems associated with quantitative measures of individual chemical species in microbiological systems, combined with the resultant fact that standard methods for examining metals in these microbiological systems are not well developed. Instead, this presentation will endeavor to provide general information to inform the decision-making process about how to examine the metal-microbe interactions. Approaches will be suggested, but detailed methods cannot be specified. Most importantly, several pitfalls associated with metal-related questions will be identified, with appropriate cautions for the investigator. Perhaps the best advice to one whose curiosity motivates research into metal-microbe interactions in an environmental context is to collaborate with a geochemist who shares curiosity about the behavior of metals in a biogeochemical setting. Such collaboration can yield results that have meaning beyond the confines of the experimental laboratory system and will produce information that can be extrapolated to the environment in a quantitative manner.

Biological Importance of Metals

Metals are critical components of many biochemical systems (12, 15, 18). The lighter metal ions (Na^+, K^+, Ca^{2+}, and Mg^{2+}) are used as enzyme cofactors and are important components of ion pumps that drive oxidative phosphorylation and help to maintain osmotic balance within cells. Mg^{2+} is also involved in the stabilization of ribosomes, nucleic acids, and cell membranes. Several anionic polymers contain K^+, Ca^{2+}, and Mg^{2+}. These ions are abundant in nature, and at most environmentally encountered concentrations they represent little threat to cellular function. Very high concentrations, such as may be present in brines, disrupt the osmotic conditions of cells, causing them to lose water rapidly. Death from osmotic shock usually follows quickly. Additionally, high concentrations of these ions can disrupt the electrical stability of cell membranes, causing them to cease to function and to rupture.

Many of the heavy metals are also important components of biological systems; they are required at low concentrations for growth and metabolic function. For example, microorganisms have evolved mechanisms that vary in specificity to accumulate Cu, Zn, Fe, Ni, Mn, and Co from the surrounding environment (15). The essential metals serve several functions: (i) they represent prosthetic groups in many proteins and dictate the configuration of the active site of enzymes; (ii) they serve as cofactors for some enzymatic reactions; (iii) they serve as multidentate centers for porphyrin molecules; and (iv) they serve as redox centers, transferring electrons in important redox reactions in cells. Note that these functions are not mutually exclusive—the cytochrome system has a series of porphyrin-like components that have iron as their center (Fig. 1). The iron in the cytochromes serves as an electron transfer (redox) site in the electron transport system and changes from Fe(III) to Fe(II) and back as electrons are moved along the cytochrome chain. Examples of the main metal functions in biochemical processes are given in Table 1. Most organisms require iron, manganese, zinc, copper, and cobalt, and several organisms also require molybdenum. As a result, many microbiological media are formulated to contain small amounts of these elements (Table 2). For

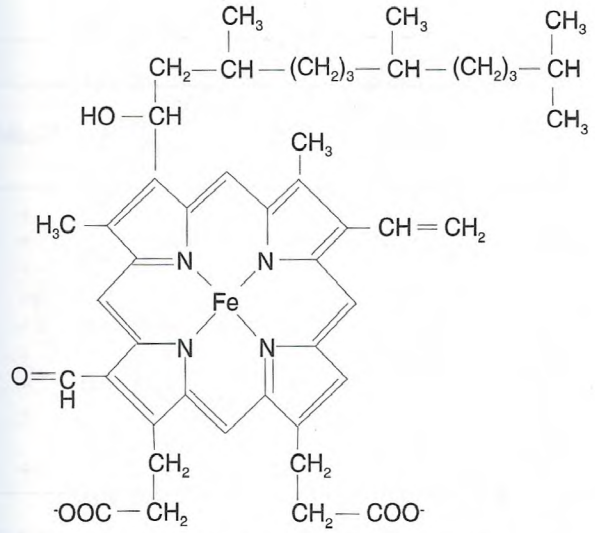

FIGURE 1 Heme A, the prosthetic group of the class A cytochromes. Note the porphyrin structure that contains iron as the redox center.

example, the trace element solution HO-LE, which is used for the enrichment of other media requiring added trace metals, contains (per liter of solution, dilute salts to 1 liter with distilled water) H_3BO_3, 2.85 g; $MnCl_2 \cdot 4H_2O$, 1.8 g; sodium tartrate, 1.77 g; $FeSO_4 \cdot 7H_2O$ 1.36 g; $CoCl_2 \cdot 6H_2O$, 0.04 g; $CuCl_2 \cdot 2H_2O$, 0.027 g; $NaMoO_4 \cdot 2H_2O$, 0.025 g; and $ZnCl_2$, 0.020 g (2). This solution can then be added to a medium to ensure the delivery of required trace elements to the microbes. A typical dilution of HO-LE solution would place 1 ml in 1,000 ml of medium solution (to give final metal concentrations in the range of micrograms per liter) (9).

In addition to their role as described in the preceding paragraph, metals can serve as energy sources and electron acceptors. Some bacteria have developed efficient mechanisms for linking the energy given off by the exergonic oxidation reactions involving metal elements. On the other hand, other bacteria have developed mechanisms for transferring the electrons produced during the oxidation of reduced organics (or possibly even some other metals) to oxidized metal ions. Despite a great deal of work on iron- and sulfur-oxidizing bacteria and the recent findings of abundant enzymatic iron reduction in anaerobic environments, this area of inquiry is still in its infancy. Only a few "standard" techniques exist, and many of these are being reconsidered in light of the newer discoveries.

Environmental Importance of Metals

Metals are a natural part of most environments; however, in some cases certain ions may be present in high concentrations due to an enhanced abundance in a particular mineral body. More often, high concentrations of metals arise from anthropogenic activities, especially those that involve the processing of metallic substances, i.e., mining, smelting, printing, electroplating, and battery manufacture and disposal (Table 3). Metal ions, for example Cu^{2+}, have seen extensive use in herbicides and fungicides, and many antifouling coatings act by leaching metal ions or metal-containing substances from the coating surface. Many of the same elements required by microbes at low concentrations are toxic to the same organisms when present in concentrations found in many contaminated environments (15). Additionally, some elements (e.g., Al, Cd, Hg, Pb, and Sn) seem to have no essential biological function but are also taken up and accumulated by microorganisms. These metals are also often toxic at concentrations found in contaminated environments (15, 27).

The presence of elevated concentrations of heavy metals has been associated with increased numbers of metal-resistant microbes; however, resistant bacteria are also often found in environments with metals present at background concentrations (see, e.g., references 10, 13, and 21). The presence of metal-resistant bacteria, even in large numbers, cannot be used as direct evidence for metal contamination. In areas where the metals themselves may present no great risk to the environment or to plant, animal,

TABLE 1 Some enzymes that require metal ions as cofactors[a]

Metal ion	Enzyme(s)
Zn^{2+}	Alcohol dehydrogenase
	Carbonic anhydrase
	Carboxypeptidase
Mg^{2+}	Phosphohydrolases
	Phosphotransferases
	Hexokinase
Mn^{2+}	Arginase
	Phosphotransferases
Fe^{2+} (Fe^{3+})	Cytochromes
	Peroxidase
	Catalase
	Ferridoxin
Cu^{2+} (Cu^{+})	Tyrosinase
	Cytochrome oxidase
K^+	Pyruvate phosphokinase (also requires Mg^{2+})
Na^+	Membrane-bound ATPase (also requires K^+ and Mg^{2+})
Co^{2+} (Co^{+})	Cobalamin (vitamin B_{12})
Mo^{4+} (Mo^{5+})	Nitrogen fixation
	Nitrate reduction

[a] Based on a table in reference 18.

TABLE 2 Trace elements commonly added to microbiological media[a]

Element	Presence in medium formulation								
	Trace element mixture	HO-LE	SL-6	SL-7	SL-8	SL-10	A-5	Metals "44"	Wolfe's
Al									+
B	+	+	+	+	+	+	+	+	+
Ca[b]	+								+
Co	+	+	+	+	+	+	+	+	+
Cu	+	+	+	+	+	+	+	+	+
Fe	+	+		+	+	+		+	+
Mn	+	+	+	+	+	+	+	+	+
Mo	+	+	+	+	+		+		+
Ni			+	+	+	+		+	
Zn	+	+	+	+	+	+	+	+	+

[a] Data from reference 2. For specific formulary, consult that reference.
[b] Most media are formulated to contain Na, K, Ca, and Mg, although these elements are often specified as cations for addition of major anionic nutrients ($NaNO_3$ or $MgSO_4$, for example).

or human use of the resources in that environment, the metals may play a role in maintaining antibiotic resistance once attained. Many of the antibiotic resistance genes are plasmid borne, and in clinical isolates they tend to be present on the same plasmids as are the resistance factors for metals such as mercury (15). Nonclinical environmental isolates tend not to have linked antibiotic and mercury resistance. If the plasmids from the clinical isolates are transferred in the environment, however, elevated concentrations of metals could, by providing selective pressure for metal resistance, also provide an indirect selective pressure for antibiotic resistance.

DIFFICULTIES IN WORKING WITH METALS

Speciation

Metals tend to exist as polyvalent ions (frequently as more or less oxidized species such as Hg^{2+} [mercuric] and Hg^{+} [mercurous] or Cu^{2+} [cupric] and Cu^{+} [cuprous]), and they also tend to form a variety of aqueous-phase complexes with dissociated water molecules. In the presence of species such as HS^{-} or S^{2-}, metals often form soluble complexes with ions other than OH^{-}. The identity of the dominant species is therefore a function of both pH and the redox potential (E_h, sometimes expressed as the negative logarithm $-p\varepsilon$, which is the hypothetical electron activity at equilibrium). The relationship between acid-base equilibrium and redox equilibrium for solutions of given bulk composition is often expressed by means of E_h-pH diagrams (see, e.g., Fig. 2 for Hg). By examining the diagram, one can determine the dominant species of a given metal in a solution of the strength and composition specified for the calculations used to generate the diagram. For a detailed discussion of this subject, refer to appropriate geochemistry references such as reference 33. The multivalency and the complex formation exhibited by metals makes identifying the species to which microbes are sensitive a more complex task than simply determining the total concentration of the element in the bulk solution.

Microbial processes are sensitive to metals and metal speciation (see reference 13 for an early review). As a single example, Tatara et al. (41) observed that ferric iron (added at 1 to 20 μM) and copper (0.25 mg of $CuSO_4 \cdot 5H_2O$ $liter^{-1}$) inhibited the mineralization of CCl_4 by a *Pseudomonas* strain at neutral pH but that the inhibition was relieved when the pH was increased to 8.2, thus causing the precipitation of the iron (and presumably the cop-

TABLE 3 Comparison of anthropogenic inputs of some heavy metals versus inputs from natural sources[a]

Metal	Input of metal (10^3 metric tons/yr) derived from			Ratio of anthropogenic to weathering input
	Weathering	Mining (anthropogenic)	Industrial emissions (anthropogenic)	
Antimony	15	55	41	6.4
Cadmium	4.5	19	24	9.6
Chromium	810	6,800	1,010	9.6
Copper	375	8,114	1,048	24
Lead	180	3,100	565	20
Mercury	0.9	6.8	11	20
Molybdenum	15	98	98	13
Nickel	255	778	356	4.4
Zinc	540	6,040	1,427	14

[a] Data from reference 27.

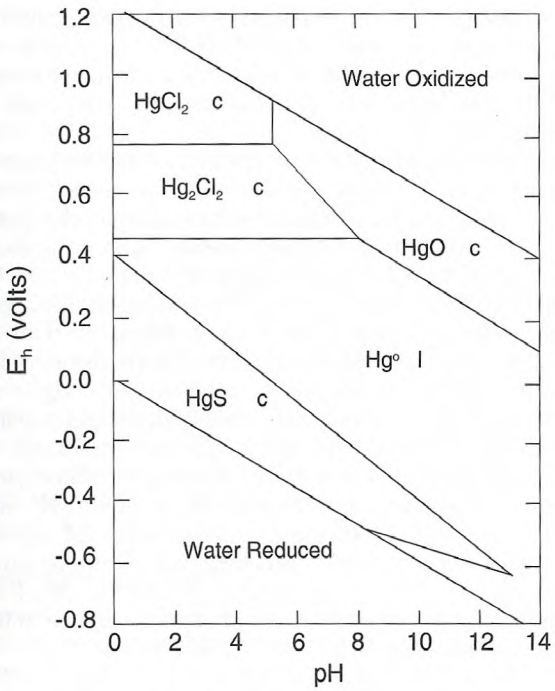

FIGURE 2 An example of an E_h-pH diagram for equilibrium speciation calculations for Hg. The zones represent regions of stability for the phases shown. Boundaries are points at which the dominant species in adjacent regions will be present in equal concentration. Centers of regions represent conditions under which the species indicated in that region will be at the highest concentration and those surrounding it will be at their lowest respective concentrations. Specific conditions for these calculations are 25°C and 1 atm pressure in a system containing 1 mM Cl^- and 1 mM total sulfur as SO_4^{2-}.

per). The effect of the metal on the mineralization process changed with the metal species, even though the total amount of metal added to the system (as $CuSO_4$) did not change. The combined metal concentration-environmental condition spectrum must be accounted for in determining the exact effect of metals on any microorganism or microbial process.

The species of metal present (and therefore the bioavailability of the metal) changes with pH, E_h, and other constituents in a complex manner. The equilibria are not simple, two-component reactions. To assist with determining the speciation of inorganic ions, a number of computer programs have been developed which carry out the large number of calculations quickly and efficiently. Routine use by microbiologists is not easily accomplished, however, and the models suffer from some severe disadvantages. The best of the equilibrium speciation models is the WATEQ family (5). Models such as WATEQ are not designed for use by inexperienced individuals; the entry of data into the model for calculation is not always straightforward. Moses and Herman (24) provided a small program to serve as a data entry module for WATEQ, but changes in the WATEQ code itself are often required to make the program work on a specific computer platform.

Included in the model is a large table of thermodynamic data associated with the solubility and appropriate equilibria for a given element. In general, the models work well for speciation calculations in dilute inorganic solutions, and they usually contain options for calculating speciation in more concentrated solutions (seawater and brines). However, to use them properly, the options must be understood by the user, and few microbiologists will be familiar with the specifics of these calculations. The thermodynamic data are not complete, and for all but a few compounds they do not exist at all. The most recent version of WATEQ will allow the entry of humate and fulvate, and reasonable complexation estimates can be obtained from the model if the concentrations of these specific substances are known. Thus, speciation models are not helpful in determining the most likely form of a metal in a complex, organic-rich microbiological medium. Furthermore, the calculations are based on equilibrium assumptions. Microorganisms are well known for their proclivity to move a thermodynamic system away from equilibrium. Additionally, the models assume no kinetic constraints; that is, they assume that the system being modeled is at equilibrium and that any change (requiring a recalculation) to a new equilibrium state occurs instantly. While there are many drawbacks to the use of equilibrium speciation models, microbiologists collaborating with geochemists will find the results of their use helpful in interpreting experimental results in terms of more realistic dissolved-metal concentrations. The models can be especially useful in studies involving organic-poor solutions or natural waters. In such cases, a complete chemical analysis is possible (in fact, the entire solution composition may often be defined by the investigator), so that speciation of the metal can be determined and changes in the concentration of the various species with changing conditions of pH, E_h, temperature, ionic strength, solid mineral phases, etc., can be related to changes in the behavior of the microbe or assemblage being examined.

Analytical Considerations

Not all metal species can be readily analyzed by some techniques. Atomic absorption spectroscopy (AA) measures the total amount of a metal in a digest or in solution, but it cannot differentiate among the dissolved species present. The digests or extracts often include material that was in the solid phase in the original sample. For aqueous samples in which interest is focused on the total metal concentration, AA is an excellent analytical tool. The simplest AA techniques involve aspiration of a small amount of sample into the flame of the instrument. Detection limits for flame AA vary with the metal but are generally in the range of 0.01 to 0.1 mg $liter^{-1}$ (1). Modifications such as the use of a graphite furnace (electrothermal method) instead of the flame burner can lower the detection limit 20- to 1,000-fold. Many elements can be determined at levels as low as 1.0 μg $liter^{-1}$ (1).

Plasma emission spectroscopy is also an excellent method for the quantitative analysis of metals at low concentration (1). The detection limits for the heavy metals compare favorably with those obtained from graphite furnace AA, although the latter is slightly more sensitive in most cases. Choosing between the methods for microbiological applications will depend more on the availability of instrumentation than on other factors.

Specific ion electrodes can detect ionic forms of the metals and are quite specific as to what forms are sensed. A Cu^{2+} electrode, for example, can be used to determine the amount of cupric ion in a given solution. In many applications, however, there are interfering materials that preclude accurate determination of the metal ions, which may be present in extremely low concentration. Ion chro-

matography can sometimes be adapted to differentiate among metal species (20), but this approach is not as sensitive as other techniques, for example graphite furnace AA, in which larger samples may be concentrated prior to the actual analysis.

METAL REQUIREMENTS

The requirement of any given metal ion by a microbial process is most appropriately determined in synthetic media, where the potential for inclusion of minute amounts of material found in environmental systems can be eliminated or controlled. No standard procedures for this type of determination exist, but some general guidelines can be suggested. In most cases, the organisms of interest will be heterotrophs. Thus, organics in the medium are required. The concentrations of organics should be kept as low as possible to avoid rendering the added metals unavailable due to complexation with the medium components. Complexation is likely to be a more serious problem in undefined, complex, organic-rich media (e.g., nutrient broth, peptone, and yeast extract). However, complexation effects on metal requirements in culture are less likely to be problematic than complexation effects on metal toxicity. In the latter case, the investigator often wishes to know how much of a supplement must be added to the medium to produce optimum growth. To understand the form of the metal being used, it would be best to avoid a highly rich, complex medium, and it should be noted that the amounts of metals necessarily added to a complex medium may not reflect the amounts of metals required to support microbial growth and metabolism in water or soils.

All medium components, organic and inorganic, should be examined for trace metal contamination. Microbes can often obtain minute but adequate amounts of trace elements that occur as contaminants in medium ingredients. For this type of assay, only the purest media and salts obtainable should be used. The use of double-distilled water (with the second distillation in scrupulously cleaned glass) is recommended. Deionized water may be extremely low in total ions but may contain just enough of the target ion to support the growth of the test organism. Whenever possible, the tests should be conducted in liquid medium with as low an organic content as practical. If the medium is a simple one (a single carbon source consisting of a carbohydrate, for example), the speciation model approach may be used to estimate the amount of a given metal species present in the medium. Diffusion of metals in agar can be a kinetic limitation on availability when metal concentrations are extremely low, and agar is known to complex with a variety of metal ions (30), lowering the actual concentration of metal delivered to the microbial cell.

RESISTANCE TO METALS

Most metal toxicity arises from the reaction of metal ions with proteins. For example, the toxicity of mercuric ions arises from the aggressive binding of the ions to sulfhydryl groups in organic molecules. This action can inhibit macromolecular synthesis and enzyme activity. Proteins often contain R—SH groups that control the tertiary and quaternary structure of the molecule. Binding by Hg^{2+} can destroy that structure and inactivate the enzyme. Disulfide bonds are also sensitive to Hg^{2+} binding, and transcription and translation are particularly sensitive (14, 34). An increase in mercury resistance is thought to have been mediated by the use of compounds such as phenyl mercury and thimerosal as disinfectants (16). This concept is supported by the observation of a decreasing resistance to mercury coincident with abandonment of mercurials as disinfectants (29).

Metal resistance is frequently determined by plasmid-carried genes in bacteria. Mechanisms of resistance to metals generally involve either a transformation mechanism or an efflux mechanism. A transformation-type mechanism is exemplified by the mercury reductase system, i.e., the enzymatic reduction of the Hg^{2+} ion to elemental mercury (Hg^0). The elemental form is much less toxic than the mercurous ion (35, 36), and the physical properties of Hg^0 greatly decrease its availability to the microbes. Hg^0 is virtually insoluble in water, and it has a high vapor pressure, which leads to rapid evaporation. Cadmium resistance, on the other hand, is considered to occur by an efflux system in which it is pumped out of the cell by a Cd^{2+}/H^+ antiport system (14). Other plasmid-associated Cd and Cu resistance mechanisms involve metallothionein-type molecules (19, 28) or siderophore production (8). For a thorough review of mechanisms of metal resistance in bacteria, see reference 32.

General Considerations of Resistance Testing

For many compounds, standard protocols exist for the determination of resistance to the compound. For antibiotics, disk sensitivity assays that represent a comparative standard among investigators are used. Such standards do not exist for metals. Obviously, there are some common areas in practice that allow statements to be made about the presence or absence of metal resistance, and to some extent a quantitative response to metal concentrations may be made. Problems associated with unknown speciation of the metals as inorganic complexes, or especially as organometallic complexes with components of growth media, make difficult an exact determination of how much of what metals the bacteria can actually resist.

Resistance to specific medium components, in this case metals, serves as a method for the selection or isolation of organisms known to be resistant to a certain metal or for the determination of the degree of resistance to that metal. The degree of resistance is usually expressed on the basis of the total amount of metal added to the medium (see, e.g., references 10, 17, 21, and 31). Metal-tolerant organisms can often be enumerated by plating water samples or dilutions of sediment or soil samples on agar-solidified media to which some concentration of metal salt has been added. Mills and Colwell (21) reported very high concentrations of metals (e.g., 250 mg per liter of medium) that had to be added to agar media to achieve reasonable reductions in the number of organisms in estuarine water. Ramamoorthy and Kushner (30) noted that the actual concentration of free metal ion in microbiological media was substantially lower than the concentration added due to complexation of the metal ions with organic components of the media.

The main problem with inclusion of metals in organic-rich media is that the concentration of metal added to the medium, and therefore the concentration to which growing organisms are considered resistant, is generally much higher than the active concentration of metal in, for example, lakewater or seawater. The true MIC, i.e., the level of true resistance, is therefore generally much lower than that demonstrated through a medium-supplement based assay. For example, Sunda et al. (38–40) used specific ion elec-

trodes to demonstrate that the actual toxic copper species was free Cu^{2+}, which was present in only small amounts in comparison with the total copper present; the strength of the relationship for some bacteria was so great that they suggested that the response of the organism might be used as a measure of the cupric ion activity (38). Similarly, free-metal (ionic) cadmium was also shown to be the toxic species in a study of shrimp (37).

The use of molecular probes to detect genes coding for metal resistance in bacteria is certainly feasible and in some cases may be desirable. Probes can be prepared for specific genes after they have been sequenced; for example, the *mer* gene (which confers resistance to mercury) can be probed in isolates and in communities (7, 44), as can several other genes for metal resistance (44). However, there appears to be no general metal resistance gene, and the variety of mechanisms employed by bacteria for resistance to metals (32) makes impractical probing for anything other than a specific gene that regulates a specific resistance.

Inhibition of Growth

Several methods to determine the resistance of bacteria to metals in solution have been suggested. No method has been adopted as a standard procedure, and the methods presented here all have some significant disadvantages. Nevertheless, each could be used to give a relative measure of the resistance of an organism to different levels of metal, of the resistance of an organism to different metals at the same or different relative concentrations, or of the resistance of different organisms to one or more concentrations of one or more metals. Obviously, the more complex the relationships being examined, the more the complexity of the procedure becomes important. As always, it is important to restate that in complex media containing organics and many other ions, the exact concentration of the toxic species will rarely, if ever, be known accurately.

Agar Dilution Method

An agar dilution method similar to that used for antibiotic resistance assays (45) has been used by Nieto et al. (25, 26) to evaluate metal tolerance in halophilic bacteria. This technique simply involves the use of agar-solidified media with a range of metal concentrations included. Nieto et al. (26) placed 21 spots of liquid inocula (with 10^4 to 10^5 cells) on each plate with an automatic pipettor, incubated the plates for 2 days, and looked for visible growth. Resistance was reported as the highest concentration of metal (actually metal added to the medium) that allowed growth of the organisms.

Continuous Culture

Mayfield et al. (20) used a continuous-culture system to examine the differences in growth kinetics of a target organism in a dilute growth medium with or without metals present. Such a system has a distinct advantage in that by careful tuning of the system, organisms can be grown in media that very closely resemble the natural conditions of the organisms (assuming an aquatic lifestyle). The use of dilute organic substrates in chemostats also allows for better control of metal speciation. The effects of different metal concentrations can be determined by adjusting the concentration and examining the response in the steady-state growth parameters. Both lethal and sublethal effects on growth can be observed. There is a distinct disadvantage in terms of the number of strains that can be examined in this fashion and the length of time necessary to obtain results. Continuous-culture systems can also be used to select slower-growing, metal-resistant strains from a general assemblage of nonresistant strains.

Agar Diffusion Method

In determining the resistance of chemoorganotrophic bacterial isolates to antibiotic chemicals, a rapid assay has been developed that utilizes the diffusion of the antibiotics from paper disks into the agar-solidified medium to achieve a radial concentration gradient from the disk outward into the medium (6). This method was adapted for metal elements by Smith et al. (32a), and further modified by Thompson and Watling (42, 43). The ensuing description follows closely the method of Thompson and Watling (43), but because there is no standard, modifications could be made to improve the technique or to make it more suitable for answering a specific question.

Preparation

1. Reconstitute and sterilize nutrient agar in 100-ml volumes (small volumes make pouring uniform plates easier—if an automatic filler is used, a larger batch of medium may be prepared). Nutrient agar was suggested for use by Thompson and Watling, who indicated that metals diffused uniformly through the emulsion. A less rich medium (e.g., one-half-strength nutrient agar, R2A agar [1], or dilute peptone-tryptone-yeast extract-glucose agar [PTYG]) may cause less metal complex formation with medium components, thus delivering more metal to the organisms.
2. Prepare tubes containing 5 ml of a suitable liquid medium for growing log-phase cultures of the organisms being tested. Thompson and Watling (43) used a tryptone medium in their examination of metal resistance in *Escherichia coli*.
3. Sterilize glass petri dishes containing Whatman AA disks (13 mm in diameter) or equivalent disks in a 160°C oven for at least 1 h.
4. Prepare stock solutions (1 mg of metal ion ml^{-1}) of metal salts. (Minor contamination with other metals can produce artificial results. AA standards are commercially available at this concentration, and they provide uniformly prepared solutions with no, or minimal but known, contamination.)

Procedure

1. Grow cultures of the isolates to be tested on suitable agar plates (presumably the same medium as will be used for the test).
2. Inoculate a few representative colonies into a tube of the liquid medium and incubate for 18 h (or a suitable length of time for the appearance of turbidity—the object is to obtain cells in the logarithmic phase of growth).
3. Make a standard inoculum by transferring 0.1 ml of the 18-h culture to a fresh tube containing 5 ml of liquid medium (which serves as a transfer diluent) immediately prior to the test.
4. Melt the agar, cool it to 50°C, and pour plates containing 10 ml of the medium. Uniform plates are important, since thicker plates will tend to reduce the amount of diffusion of the metal away from the disk. When the agar has solidified, spread 0.1 ml of the inoculum from step 3 evenly over the surface of the plate.
5. Make the metal-impregnated disks by dropping 0.1 ml of the stock metal solution onto each disk (100 μg of metal per disk). Use of an automatic pipettor will facilitate

the requisite uniformity in the disks. Place the disk on the inoculated plate and incubate the plate, at a relevant temperature for a suitable period for the organisms being examined.

6. After incubation, measure the distance from the edge of the disk to the edge of the clearing zone (in millimeters).

7. To determine an apparent MIC, compare the distance from the edge of the clearing zone to the edge of the disk with the distance produced by a concentration determined by the diffusion gradient for that metal in that medium. Figures 3 and 4 show examples from the Thompson and Watling publications. The concentrations needed to determine a diffusion gradient may be measured as follows. Carefully cut strips of agar 15 mm long by 2 mm thick (to the bottom of the agar layer) from the edge of the disk to the edge of the plate. Weigh each strip, digest it with 2 ml of concentrated HNO_3, and evaporate the material to dryness. Add 2 ml of 10% (vol/vol) HNO_3 to dissolve the sample residue. The metal concentration in these samples can then be determined by AA, and the concentration of metal (in micrograms per gram) can be plotted against distance from the disk to obtain the diffusion gradient plots. From these plots, the total metal concentration at the point of inhibition can be determined and reported as the MIC. Keep in mind that this is an apparent value, in that the concentration of the true toxic species of the metal is not determined by this assay.

This procedure provides a fundamental approach to metal resistance testing similar to that commonly used for antibiotic resistance testing. Modifications can easily be made: the medium can be changed, or the metal salts used in the filters can be altered to determine the effect of different forms, e.g., the effect of the anion component or of metal contaminants. Metals could also be mixed in the solutions in different proportions in experiments to look at synergistic interactions in metal resistance. Despite the several advantages that are offered by an approach such as the diffusion assay, all the problems associated with cultivation of microbes are manifested; not least is the limitation on the type of organism (chemoorganotrophs) to which the technique can be applied easily.

Inhibition of Growth Determined by Uptake of [^{3}H]Thymidine

A method for determining the effect of dissolved metals on the growth of bacterial communities in soil has been proposed by Bååth and coworkers (3, 4, 11). The method is based on the assumption that bacterial growth is proportional to the amount of thymidine incorporated into macromolecules in the bacterial cell. For use of this method for bacteria in soils, the bacterial cells are extracted from the soil and the metal of interest is added to the cell suspension. Tritiated thymidine is added, and the suspension is incubated. At the end of the incubation period, the macromolecules (the fraction that is soluble in cold trichloroacetic acid) are extracted from the suspension and the amount of ^{3}H incorporated is measured. For studies of the effect of the metal on the entire community, this method does not select for organisms that can grow on the isolation or enumeration medium. The lack of necessity of supplementing the suspension with large amounts of complex organic mixtures (as are found in many culture media) makes the determination of a reasonable metal concentration and speciation much more straightforward, yielding results that are more environmentally relevant. Although the published procedure is for microbes extracted from soil, the same approach could easily be adapted to microbes in water samples, which would not require extraction prior to testing.

The method, as presented here, outlines the report of Bååth (4). For details that would be useful in adapting the method to water or to a specific soil situation, the reader is referred to the original articles. The extraction efficiency

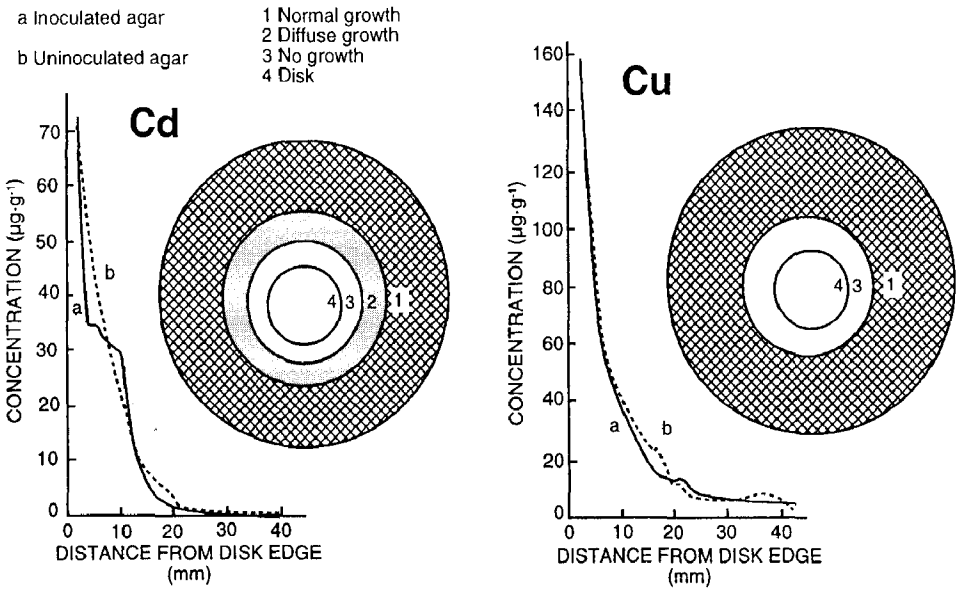

FIGURE 3 Diffusion gradients associated with Cd- and Cu-impregnated disks in the agar diffusion assay with nutrient agar as the medium. The figure also illustrates the appearance of the clearing zones surrounding the disks. Note the alteration of the gradient in the inoculated Cd-containing plates, which arises due to Cd uptake by the bacterial cells at the edge of the clearing zone. Reprinted from reference 43 with permission.

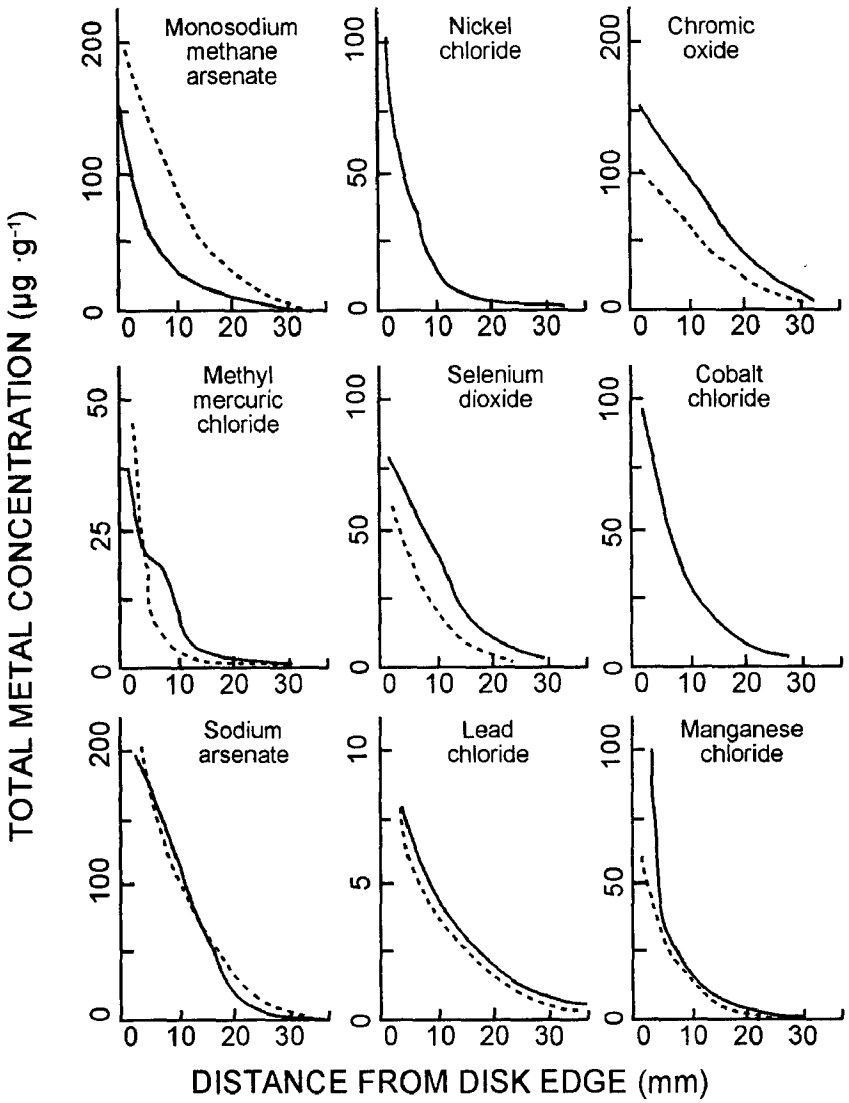

FIGURE 4 Diffusion gradients associated with several trace elements used in the agar diffusion assay reported by Thompson and Watling (42). Solid lines represent gradients in inoculated agar, and dotted lines represent gradients in uninoculated agar.

for bacterial cells reported by Bååth (4) was 10 to 30% and depended on the soil type.

Procedure

1. Homogenize 10 g of soil (the amount necessary may vary depending on the numbers of organisms and their activity) with 200 ml of distilled water in a high-speed blender or homogenizer for 10 min.
2. Sediment the solids in a centrifuge at 750 × g for 10 min.
3. Decant the supernatant through glass wool, and place 1- to 4-ml portions into plastic vials.
4. Add solutions containing the necessary concentration of the metal to be tested. For this assay, a convenient final volume is 5 ml. Be sure to add distilled water or an appropriate salt solution to controls that contain no added metal.
5. Hold the vials at room temperature for 10 min before the addition of [*methyl*-^{3}H]thymidine.
6. Add [^{3}H]thymidine and incubate for 1 to 2 h. (Note: The amount of thymidine added and the incubation time used will vary from study to study. All considerations appropriate for use of the [^{3}H]thymidine method for assaying bacterial productivity apply here. For a specific and detailed discussion, see chapter 31. It is also recognized that various individuals conduct the thymidine uptake procedure with different protocols. The Bååth (4) procedure is presented as an approach that can be modified as necessary to suit the needs and practices of individual laboratories.
7. At the end of the incubation period, terminate all reactions by adding 1 ml of 5% formaldehyde solution.
8. Filter the suspension through a 0.45-μm-pore-size membrane filter prewashed three times with 1% sodium-hexametaphosphate.
9. Wash the filter with cells in place with three 5-ml portions of 80% ethanol and then with three 5-ml portions of ice-cold 5% trichloroacetic acid.
10. Trim away the edges of the filters, and place each filter into a liquid scintillation vial.

11. Add 1 ml of 0.1 M NaOH, and heat to 90°C for 1 to 2 h to solubilize macromolecules.
12. Allow the vials to cool, add liquid scintillation cocktail, and count the radioactivity with a liquid scintillation counter.
13. Compare the thymidine incorporation with the concentrations of metals added (including the controls).

For water samples, the cells may be collected directly on the filter without any extraction.

Inhibition of Specific Metabolic Processes

A number of studies have examined the effect of added metals on specific enzymatic activities or on microbial processes. In many cases, the assay is often quite simple and involves the addition of a concentration of metal ion to a solution used to test enzymatic activity. The assays can suffer from the same speciation-complexation problems that plague other approaches, although again, the use of dilute solutions can minimize the problem. Speciation modeling can often be accomplished for the assay solutions. The use of whole cells for the test may avoid complexation of the metal with the purified or semipurified enzyme (protein) if enzyme complexation is not the mechanism of toxicity in the environment.

Examples of the enzyme activity assays for testing metal toxicity include that of Montuelle et al. (22). They examined dehydrogenase activity and β-glucosidase activity in bacteria isolated from the sediments of polluted and unpolluted rivers and placed in solutions in the presence or absence of added metals. As mentioned above, Tatara et al. (41) examined the mineralization of CCl_4 as affected by the presence of iron and copper and the effect of environmental conditions on the degree of toxicity. Other methods of measuring microbial growth and activity may be readily adapted to examine the effect of metals on the organisms simply by including different concentrations of metal ions in the test solutions. In all cases, care must be taken to use only the purest of chemicals and, whenever reasonable, to determine the dominant metal species in the test solution.

The activity assays also have the ability to provide direct evidence of synergistic relationships (also possible at a more crude level when classical culture methods are used) by allowing the mixing of various combinations of metals at different concentrations. Additionally, sublethal effects may be readily observed since they appear as an inhibition in an activity. Such an observation may lead to the documentation of chronic inhibition in the environment.

The determination of metal requirement and resistance is not always straightforward. Metal loadings for culture media can easily be determined, but these results are operational definitions for the culture conditions and may have no relationship to the actual toxic levels in an environmental situation. Thus, caution should always be used in the quantitative extrapolation to the environment of laboratory results involving metals. Laboratory studies will continue to be of great value in the interpretation of field-derived observations, and experiments that examine the molecular basis of metal requirement and resistance should continue apace. Qualitative or mechanistic conclusions may follow directly from laboratory studies, but quantitative relationships, and especially regulatory guidelines, based on such procedures should be viewed with some skepticism.

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