

Complexing agents from microorganisms

L. Birch and R. Bachofen

Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich (Switzerland)

Summary. The majority of extracellular complexing ligands produced by microorganisms are summarized as being of low molecular mass (< 10,000 daltons) and are usually released as part of metal detoxification processes. These exudates appear to exhibit strong metal-binding characteristics, often reducing metal toxicity. Under certain conditions microbes produce metal-specific compounds of low molecular mass called siderophores; although these are normally specific for iron they also have relatively high affinities for radionuclides such as Pu and facilitate their uptake into cells. The occurrence of specific actinide complexing agents has been recorded.

The breakdown of lignins and cellulosic material produces large macromolecular compounds called humates. These contain multiligand sites and display a wide range of complexing abilities. They form both soluble and insoluble complexes with toxic elements with various results. Humates also considerably influence adsorption of metals to substrate surfaces and at high pH may compete with OH⁻ ions for metal binding.

As well as with extracellular ligands, metals can interact directly with microorganisms by accumulation in subcellular compartments or by adsorption on bacterial surfaces.

Key words. Complexation; microorganism; exudate; siderophore; metallothionein; humate; heavy metal; actinide.

Introduction

More and more xenobiotic compounds, especially toxic metals, are being deposited as waste in soils. As soil microorganisms perform a seemingly endless range of fundamental chemical reactions, often under extreme environmental conditions^{31, 55, 107}, it is becoming increasingly important to determine the extent to which bacteria can transform metals and furthermore to evaluate the possible environmental effects of these transformations. Many metals of economic and environmental significance are known to be chemically modified by bacteria, which may lead, for example, to heavy metal and radionuclide solubilization^{32, 35, 61} and the volatilization of lead or mercury¹¹⁴. Metal transformations can have various consequences, both beneficial for example, the solubilization of gold from gold-bearing ores and detrimental ones, such as the release of toxic metals from compounds buried in soil. Indeed, mixed soil cultures have been implicated in the mobilization and release of heavy metals (e.g. cadmium)^{19, 20} and of radionuclides⁴² both under laboratory and field conditions. The specific mechanisms involved in remobilization have not been identified and furthermore, relatively little is known about the influence of bacterial by-products, in particular microbial complexing agents, on the transformation of metals.

In a natural system such as soil, metals are present in various oxidation states often with widely differing properties. The chemical significance of complexing agents in biological systems thus stems from the fact that they have the potential to change the physical state of metals in solution. As a result of complexation, the accumulation, toxicity and ability of elements to be transported across membranes, all of which involve the formation of complexes, may be significantly altered. Microorganisms are ubiquitous in soils and it seems likely that the compounds they produce play a major role in the speciation

and circulation of cations in the environment. A comprehensive discussion of complexation processes in aquatic systems can be found in Buffle¹⁵.

Complexation

Complexes are 'species formed by the association of two or more simpler species, each capable of independent existence'⁹². This can be represented simply as follows⁶²:

Donation/sharing of a pair of electrons

Metal + Ligand \rightleftharpoons Metal complex

Me L MeL
ion or containing a
atom functional
 group (such
 as = O, -N =, -S -)

Any compound, which through the above process can form a chemical combination with a cation, is a complexing agent or 'complexant'. It may contain only a single site or several sites where metals can be bound. Each of these complexing sites includes a functional group (examples in table 1⁹). When a compound possesses at least

Table 1. Functional groups of known complexing compounds⁹

Basic		Acidic	
-NH ₂	(amino)	-CO ₂ H	(carboxylic)
=NH	(imino)	-SO ₃ H	(sulphonic)
-N=	(tertiary acyclic or heterocyclic nitrogen)	-PO(OH) ₂	(phosphonic)
=CO	(carbonyl)	-OH	(enolic, phenolic)
-O-	(ether)	=N-OH	(oxime)
-OH	(alcohol)	-SH	(thioenolic & thiophenolic)
-S-	(thioether)		
-PR ₂	(substituted phosphine)		
-AsR ₂	(substituted arsine)		

two functional groups, which attach to the metal in such a way that a ring is formed then it is called a chelate (from the Greek 'chele' meaning lobster's claw)³⁰. In most natural systems, which are chemically undefined, individual ligands are virtually impossible to identify so the concept of the overall complexation capacity of a system is used. This value is the total ligand concentration (L) capable of binding metals (note, by definition, this may include binding by other mechanisms). A wide variety of techniques have been used to investigate the ability of natural systems to bind metal ions including anodic stripping voltammetry^{39, 101}, ion specific electrodes¹⁶, solubilization methods (such as Cu(OH)₂ solubilization), MnO₂ precipitation, and dialysis^{13, 51, 81}. They have revealed that complexation capacity is a function of several parameters, including pH, metal and ligand concentrations, ionic strength, and redox conditions. Although basically all environmental parameters can be influenced by microbial action it is the type of ligand present which we are most interested in. If we take soil as an example, then there are two groups of microbial compounds which can be considered as complexing agents:

- 1) *by-products of microbial metabolism and degradation*, e.g. simple organic compounds (low molecular weight organic acids and alcohols) or macromolecular humic and fulvic acids such as those released as a result of the degradation of higher plant material,
- 2) *microbial exudates induced by the presence or absence of specific metal ions*, e.g. iron binding siderophores and toxic-metal binding proteins, and including those many microbial exudates as yet uncharacterized.

By-products of microbial metabolism

Low molecular weight organic acids and alcohols

Under optimal conditions and in the presence of oxygen, complex organic substrates are degraded via different metabolic pathways to the end products, carbon dioxide and water⁹⁴. In waste sites, and most soils, however, suboptimal conditions usually exist, e.g. low water po-

tential, oxygen stress, pH differing from neutrality or nutrient limitations. Under these hostile conditions microorganisms exist but metabolic pathways are incomplete, facilitating the release of a variety of low molecular weight organic acids and alcohols. Francis⁴¹ observed the presence of low molecular weight organic acids and alcohols in the leachates from shallow land burial sites of radionuclides. It was suggested that the compounds originated from the microbial decomposition of complex organic material under anaerobic conditions. The complexing ability of the acids and alcohols was not determined. To a large extent, the organic materials present as substrates dictate the range of organic compounds which are released. Various organic acids released during microbial degradation have been investigated for metal complexation ability, some examples of which are listed in table 2. From the experimental data available, it is virtually impossible to evaluate the complexation capacities of these metabolic products as they are dependent on environmental conditions and are usually uncharacterized. Thus mathematical models and chemically-defined constants have been used to simulate complexation effects. Morel⁷⁴ used mathematical modelling in an attempt to predict the complexing capacity of the total organic component in natural waters. He suggested that for organic complexation to be important, it is necessary that either the ligands exhibit a high affinity for the metal or that they are present in very high concentrations. The calculation of the complexation capacities of various amino acids and small chain molecules at pH 10, i.e. the expected pH when concrete is present, showed that the complexation capacity of most small chain acids (except citrate) was too weak to be detected by conventional methods¹⁸. Based on purely chemical data, the complexing abilities of several organic acids were compared to three synthetic complexes¹. They were observed to decrease in the order below, although this depends on the oxidation state of the metal being complexed:

DTPA > EDTA > NTA > humic acids – tricarboxylic acids > dicarboxylic acids > monocarboxylic acids. It is

Table 2. Examples of the complexing action of microbial by-products

Products of microbial origin	Action	References
Tricarboxylic acids	Complexation of Mn	99
Citric acid	Formation of complexes with Pu (10 with citric acid, 6 with iso-citric acid)	71
Catechol	- Various actinides - Implicated in the uptake of germanium into microbial cells	1 23, 60
Oxalate, salicylate, acetate, lactate, pyruvate and polypeptides	Solubilized uranium	70
Uncharacterized low molecular weight anions found in leachates from seepage trenches	Solubilized ⁹⁹ Tc, ⁶⁰ Co, ²³³ U	40, 41
Uncharacterized low molecular weight ions produced by <i>Bacillus thuringiensis</i> in Mo-deficient medium	Complexed Mo	57
Uncharacterized organic ligands in saline, alkaline lake water	Maintained elevated concentrations of the normally insoluble Th ⁴⁺ , Pa ⁵⁺ , U ⁶⁺ , and Pu ions	2

interesting to note that in some instances humic acids can form complexes comparable to those of synthetic chelating agents such as NTA.

Large macromolecular ligands

The microbial degradation of complex organics, primarily lignin and cellulose compounds, releases large macromolecular compounds typically representing 40–90 % of the DOC. These macromolecules collectively termed the humates, have such a range of structures that they have not yet been fully characterized, although certain functional groups, such as carboxyl, alcohol, and phenol are common to all humic macromolecules. They are generally differentiated into three fractions: 1) compounds that are soluble in dilute alkali and precipitate at low pH, called humic acids; 2) the acid soluble compounds of low molecular weight termed fulvic acids; and 3) a residual humic fraction that cannot be extracted by either dilute acid or alkali^{95,96}.

Numerous reactions take place between metals, minerals, and humates, for example metals such as Hg can be adsorbed on humate compounds¹⁰⁹. Humic acids alter both the adsorption on particulate matter^{28,102}, and the bioavailability and toxicity of some heavy metals⁴⁶. Humic acids can also reduce radionuclides; for example Np(V) in the presence of humates is reduced to the more easily adsorbed Np(IV)⁷⁷ and Pu(V) to Pu(IV)^{12,93}. Pu(V) can be rapidly reduced to Pu(III) and (IV) in aquatic sediments⁸⁴, which is again thought to be a consequence of the presence of humates. Due to the complicated structure of humates and the presence of multiple binding sites, it is difficult to determine discrete formation constants, even when model compounds are used^{14,45}. Nevertheless, various effects have been observed under a variety of environmental conditions. Complexation by humic acids, isolated from soils, lake sediments and waters, was shown to solubilize both ²⁴¹Am¹⁰² and Th^{72,76} although, in contrast, naturally occurring DOM consisting of humic substances and diatom exudates were observed to have no appreciable effect on the complexation of ²³⁷Pu or ²⁴¹Am³⁷.


The dissociation of protons from amino groups present on the humic acids causes new binding sites to be created and means that complexation of metals such as Zn, Cu and radionuclides to humates is highly pH-dependent^{66,89,116}. At alkaline pHs competition by anions for binding sites also becomes increasingly more important and on theoretical grounds, carbonate and hydroxide chemistries are expected to dominate^{59,105}. However, carbonate and hydroxide precipitation can be prevented by the presence of humates. The presence of large quantities of dissolved cations such as Ca²⁺ and Mg²⁺, as found in 'hard' freshwaters, can hinder complexation by humates presumably as a result of increased competition for available binding sites on the humate ion^{66,102}. It has been suggested^{18,100} that humic acids, rather than complexing metals to form dissolved species, actually main-

tain the presence of colloidal metal species, a result observed with Cu¹⁸. A real complexation constant is therefore difficult to obtain as truly dissolved and colloidal species cannot be easily distinguished experimentally. Thus Morel⁷⁴, in his mathematical modeling of natural aquatic systems, concluded that trace concentrations of weak organic ligands such as those released by microorganisms would not be able to compete with inorganic ligands for metal complexation. Several authors have predicted this effect^{27,59,74}; however, in natural systems there are few quantifiable results demonstrating it. Tetravalent actinides show formation constants of 10⁴⁶ with OH⁻ and thus actinide OH⁻ species would be expected to predominate; however, the above studies indicate that in organic-rich environments, humates may have a greater affinity for metals than some inorganic ligands and even form relatively strong complexes. The influence of the smaller organic acids in heterogeneous systems is not yet clear.

Microbial exudates

The ligands described previously exhibit a non-specific binding of a wide range of metals. Microbial exudates differ from these ligands in that they exhibit exceptionally high metal affinities even though they may be present in trace quantities. These compounds are produced for the efficient uptake of essential metal ions or form part of a complex mechanism to protect cells from toxic elements. The production and release of these strong chelating agents for the complexation of essential metal ions has been widely observed in various microorganisms, for plants in terrestrial systems^{48,82} and phytoplankton in marine systems⁷.

Siderophores

In microorganisms probably only one system, that of the solubilization and transport of Fe(III) molecules has been studied in detail^{78,79}. Iron is an essential element for the metabolism of all living organisms. It is often present, however, in a form unobtainable to many cells. In order to overcome this problem, aerobic and facultative anaerobic microorganisms have developed a method for the solubilization and transport of Fe(III). Microbes produce relatively low molecular weight, Fe(III)-scavenging ligands termed siderophores^{78,79}. These ligands are essentially specific for Fe(III) and enhance the availability of Fe by complexing with the insoluble Fe(III) ion to form a soluble complex which can be transported into the cell via a specific transport mechanism¹⁰³. Siderophores are compounds that possess catecholate R = H, OH; X = O, N/phenolate [ - C - R] or hydroxamate -CO-N(O-)- as their binding groups^{73,78}. Enterobactin and ferrichrome (fig. 1) are considered to be the two prototype structures of the catecholate and hydroxamate groups, respectively⁷³, and

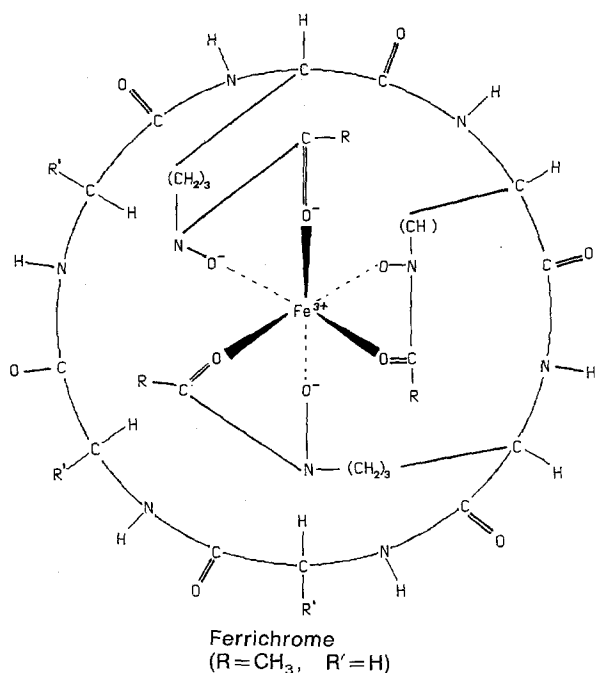
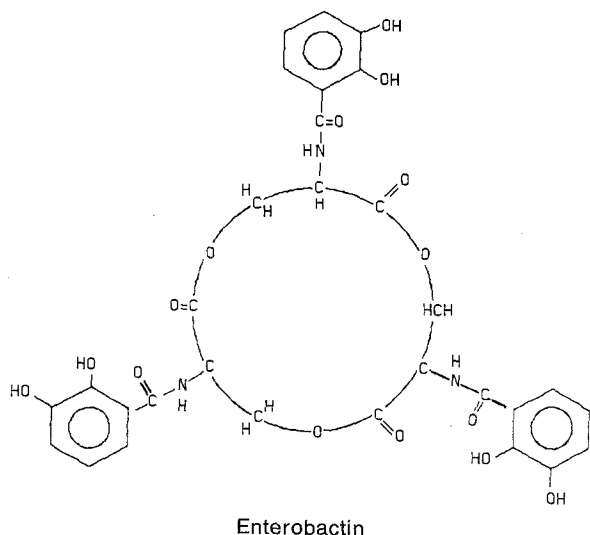


Figure 1. Structure of enterobactin and ferrichrome

many of the structures of the siderophores can be extrapolated from these two basic structures. For further information, readers are referred to the reviews of Neilands^{79,80}, where detailed structures (when known) are presented for all siderophore groups. Over the past few years many siderophore or siderophore-like compounds have been identified from various biological systems widespread in the prokaryotic and eukaryotic world^{21,26,33,34,50,86} and are still being discovered^{50,69}.

Although siderophores are primarily specific for Fe(III) (specific formation constant 10^{30}) they can complex other metals, e.g., gallium(III), chromium(III), aluminium(III) (where only an unstable complex is formed)¹⁷,

scandium (Sc), indium (In)⁹¹ as well as several essential metal ions, e.g., Mg, Mn, Ca⁸². The bioaccumulation of Ga from highly alkaline wastewaters is thought to be mediated by siderophores (Gascoyne, pers. comm.). Radionuclides such as Pu(IV) can also be complexed and it has been suggested that iron sequestering agents could play an important role in the complexation of actinides especially as Pu(IV) and Fe(III) and Pu(VI) and Th(IV) exhibit similarities in their biochemical and chemical characteristics¹⁷, ²⁴¹Am and ⁵⁹Fe also show similar behaviour in adsorption onto biological surfaces and in organic complex formation¹¹¹.

The transport of Pu, Th(IV), Np(V), Am, and Cm by eukaryotic Fe transport proteins has already been demonstrated¹¹³, so it would be interesting to study the extent to which actinides can be substituted for Fe(III) molecules and transported across microbial membranes in a manner similar to that of the siderophore transport mechanism, especially since the substitution of essential manganese by the toxic metal cadmium has already been observed in microorganisms^{85,110}.

Toxic metal binding compounds

In addition to the ligands released for the acquisition of essential elements, low molecular mass compounds exist which are produced in response to the presence of toxic elements. One such group of metal binding proteins called 'metallothioneins' was first recorded in terrestrial mammals. These low molecular mass (6–10 kd) proteins are characterized by a high cysteine content (ca 30%) and a high binding capacity for group B metals. They are synthesized when cells are exposed to heavy metals, such as Cd, Cu, Zn, and Hg^{22,98}. In plants and algae, similar metallothionein-type polypeptides termed 'phytochelatins' are released in response to a variety of heavy metals (Cd²⁺, Cu²⁺, Hg²⁺, Pb²⁺, Zn²⁺)^{47,63}. In eukaryotic organisms, these metallothioneins and phytochelatins have an important function in metal detoxification. A parallel mechanism is now thought to exist in prokaryotic cells where the production of exudates in cultures of both phytoplankton and bacteria in response to metal exposure is well documented^{38,58}. The exudates have often been shown to protect the organism from the toxic effects of metals such as Cu⁹⁷ and Cd⁵³. The production of metal-binding and -precipitating proteins is observed when *Sarcina urea* is grown in the presence of 1 mM concentrations of Sr²⁺, In²⁺, Ce⁴⁺ or Pd²⁺ ions¹⁰. Exudates released by *Pseudomonas* sp. in the presence of cadmium were characterized and found to resemble metallothioneins but with a smaller cysteine residue content^{52,90}.

There are distinct modes of action of complexing agents in biological systems; they may complex metals in solution, in extracellular polymers on the cell surface⁵⁶ or in subcellular compartments in the cell¹¹⁵ (summarized in fig. 2). Any one of these processes can result in the reduction of toxicity. Complexes are formed which by virtue of

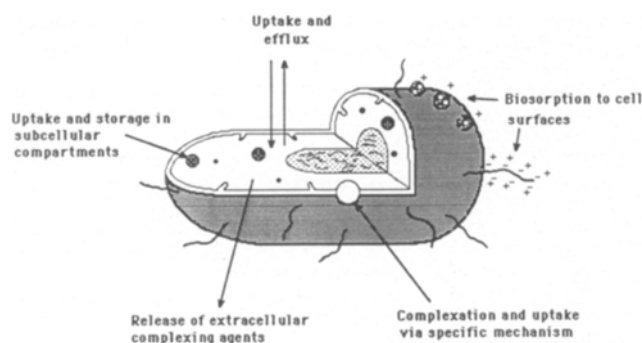


Figure 2. The various mechanisms by which metals can interact with microbial cells

their size or charge cannot pass through the cellular membrane, or are insoluble and precipitate. Alternatively, low molecular weight complexes can be formed which enter the cell by diffusion but can be then either expelled back into the media¹¹⁰ or stored in subcellular compartments^{65,83}. The different mechanisms are thought to provide one explanation for the differences in toxicity observed for certain metals, although environmental factors may also have a significant role⁵. Metals complexed with organics show, in general, toxicities different from those of the free forms of the same metals^{6,105}. There are considerable differences in the production of complexing compounds, depending on the growth phase of the organism; cells in the log phase produce different complexing material from those in the stationary phase³⁶. In *Pseudomonas* sp. polyphosphates are believed to be produced in the early phase of growth whilst in the later stages of growth, binding proteins are believed to be released⁹⁰. Similarly, nitrogen-deficient diatoms (*Thalassiosira* sp.) produce a smaller ligand-to-cell ratio than control algae⁵⁴.

Microbial chelating agents need not necessarily be released into the medium but may remain in the subcellular compartments⁶⁵ or on the exterior surfaces on the cell, e.g. in the form of polysaccharides or polymer matrices^{11,56}. They form part of the cell wall or capsule, and act as efficient metal biosorbents⁷⁵. In some cases, adsorption is followed by internalization either via an active process, as is the case for essential metals and a few toxic metal ions, e.g., cadmium⁶⁵, or by a passive diffusion process which is thought to be the main uptake route for most toxic metals⁵⁴. An extended review on the processes of accumulation and precipitation of heavy metals has been published by Gadd^{43,44}.

The search for other metal-complexing mechanisms similar to that of the siderophore transport system, or of a metallothionein type, has revealed the existence of other metal-binding agents, but none with the specificity shown by the iron system. *Pseudomonas aeruginosa* for example, produces uranium and thorium complexing ligands⁸⁷ in response to the presence of U and Th but it is not known whether these complexing agents are truly specific for U or Th. The siderophores are the only com-

plexing agents where a true 'cause and effect' has been established in microbiology, although Cd and Co chelates have been identified in other biological systems. Under laboratory conditions exudates, such as metal binding proteins, do modify the transport properties of trace metals in the immediate vicinity of cellular membranes, but their effect in a natural large-scale system remains unknown. Furthermore, despite evidence for the existence of such exudates, qualitative and quantitative information on the nature of their associations is lacking. This is primarily a result of the biological diversity present and the analytical problems encountered in the analysis and isolation of such complexes.

Discussion

Transformations of metals by bacterial cells is not only dependent on the speciation of the metal but also on a variety of physical, chemical and biological properties of the cells as well as various environmental factors. In natural systems, non-specific binding to particles^{4,77}, presence of competing ions^{18,66}, pH⁵, charge on the metal ion¹⁰⁸ and solubilities all play a role in complex reactions. Additionally a vast array of ligands and complexes, many of which have formation constants that are unknown, exists in natural environments. Despite the complexity of the system and the difficulties involved in measurement there are several cases where complexation effects are believed to have been responsible for the solubilization and release of several toxic elements from waste sites. Strongly complexed radionuclides were observed to migrate in groundwater at a velocity close to that of water itself⁶⁷. Enhanced migration as a result of the formation of organic complexes was observed from the shallow subsurface burial of medium level radioactive waste at Oak Ridge and Maxey Flats burial sites^{24,68} where migration of Pu, Co, U, Th and Ra occurred although the major part of the radionuclides was thought to be complexed with organic ligands originating from the processing of commercial waste, and not of microbial origin. Increased migration of complexed Pu and Am has also been reported in other soil systems²⁵, where it was suggested that the formation of stable protective complexes reduced sorption to geological media and increased the solubility of the radionuclides. Few studies have been conducted that directly examined the mobilization of heavy metals by microbial exudates such as siderophores or metallothioneins. In a natural system where both humic and low molecular weight acids are present the majority of complexed metal ions would be expected to be bound with humates and not with low molecular weight compounds. However, this is not always the case; for example, Cu ions released into lake systems are mainly bound by low molecular mass compounds (mol mass less than 1000 daltons)^{18,104}. In contrast, in a marine system, 90 % of added Hg (as HgCl₂) was associated with particulate, colloidal, and high

molecular mass dissolved organics, and the fraction > 10,000 daltons bound the greatest proportion of metals¹¹².

These observations highlight the problems of measuring a chemical parameter in a biological system. The actual complexing ability observed in situ or in the laboratory may differ strongly from the expected ability calculated on purely chemical principles, and because of the selective mechanisms of binding⁶⁴ its correlation with biological mechanisms is not always satisfactory. These parameters cannot simply be investigated using chemical extraction data because different patterns of uptake and adsorption exist in microbial populations. Cells differ in the complexing agents present and in the types of complexes they form with metals, as well as the location of binding²⁹. Different strains of the same species may also exhibit different properties, e.g. in *Citrobacter* sp., metal uptake mechanisms may either be metal-specific or metal-nonspecific⁶⁵ and in yeast, wild type cells of *Saccharomyces cerevisiae* took up four times as much Cd as a resistant strain of the same species⁸. The proportion of cells reacting with metals may also differ, e.g. less than 50 % of yeast cells took up radionuclides such as uranium, but did so in suicidal proportions, whilst others remained empty of accumulated metal¹⁰⁶. The reason why some cells took up U while others in the same population did not remains to be elucidated. Interpretation of laboratory and field data is often complicated by the fact that laboratory media rich in undefined organic compounds often complex huge quantities of heavy metals, reducing the amount of free metal ions present⁸⁸ and/or reducing toxicity. To try to counteract this effect, minimal media are being developed where complexation effects are either negligible or can be easily monitored³.

Future considerations

It should be evident from this chapter that although detailed information exists on the many individual metal/microorganism interactions, e.g. the production of siderophores, polypeptides etc., information from mixed communities in heterogeneous systems such as soil is sparse. With the increasing need for safe storage of toxic elements and the continued public awareness of environmental problems, the interactions of microbial products with toxic elements may play an important part in the future of waste storage. Complexation not only solubilizes elements but also may precipitate them, both processes which may be involved in recycling, detoxifying or immobilizing toxic metals. The behaviour of such a heterogeneous system as a soil/waste site is as yet too complex to allow the prediction of mobilization effects but certainly merits further research.

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Heavy metal accumulation by bacteria and other microorganisms

G. M. Gadd

Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK

Summary. Bacteria, and other microorganisms, exhibit a number of metabolism-dependent and -independent processes for the uptake and accumulation of heavy metals and radionuclides. The removal of such harmful substances from effluents and waste waters by microbe-based technologies may provide an alternative or additional means of metal/radionuclide recovery for economic reasons and/or environmental protection. Both living and dead cells as well as products derived from or produced by microorganisms can be effective metal accumulators and there is evidence that some biomass-based clean-up processes are economically viable. However, many aspects of metal-microbe interactions remain unexploited in biotechnology and further development and application is necessary, particularly to the problem of radionuclide release into the environment.

Key words. Heavy metals; radionuclides; microorganisms; bacteria; algae; fungi; yeasts; uptake; accumulation; biosorption.