Science Progress (2003), 86 (3), 179–202

Microbial transformation of metals and metalloids

ANDREA RAAB AND JÖRG FELDMANN

Throughout evolution, microbes have developed the ability to live in nearly every environmental condition on earth. They can grow with or without oxygen or light. Microbes can dissolve or precipitate ores and are able to yield energy from the reduction/oxidation of metal ions. Their metabolism depends on the availability of metal ions in essential amounts and protects itself from toxic amounts of metals by detoxification processes. Metals are metabolised to metallorgano-compounds, bound to proteins or used as catalytic centres of enzymes in biological reactions. Microbes, as every other cell, have developed a whole range of mechanisms for the uptake and excretion of metals and their metabolised compounds. The diversity of microbial metabolism can be illustrated by the fact that certain microbes can be found living on arsenate, which is considered a highly toxic metal for most other forms of live.

1. Introduction

All life on earth depends on the availability of metals and metalloids. Metals like copper are defined as elements, which among other characteristics are good heat and electrical conductors, whereas metalloids like selenium are elements, which show only in a certain crystallographic configuration metallic features. In the following, metals include both metals and metalloids. Metals are ubiquitous distributed in low concentration all over the earth, but locally the concentration can increase several-fold. The cycle from weathering of rocks by wind and water to the re-precipitation of metals from wind and water, also taking into account the influence of volcanoes and the like though not that of living organisms, forms the so-called geochemical part of the distribution of metals. The influence of living organisms on the distribution of metals together with the geochemical part forms the biogeochemical cycle.

University of Aberdeen, School of Physical Sciences, Department of Chemistry, Meston Walk, Old Aberdeen, AB24 3UE, Scotland, UK, email: j.feldmann@abdn.ac.uk

Since the first forms of life, metals have played an important role in catalysing energy-yielding and synthetic reactions. The solubility of metals can have a strong influence on whether or not a species can survive. Cells have developed a whole range of mechanisms to deal with a metal deficiency or surplus. So-called primitive forms of life, prokaryotic and eukaryotic bacteria, archaea and fungi (short microbes) have developed an especially rich variability of mechanisms to deal with unfavourable environmental conditions. Microbes have learnt to live under aerobic or anaerobic conditions in extremely harsh conditions, like hot acidic springs (hyperthermophilic) or very salty waters (halophilic), as well as in more moderate environments (mesophiles).

Metals can be subdivided into essential and toxic elements for each organism. Metals that are essential for most organisms from a simple prokaryote to mammals are iron, copper, zinc, selenium, cobalt, manganese, molybdenum, calcium, magnesium, sodium and potassium. Metals that are essential for some organisms and toxic for others are arsenic, lead, boron and tungsten. However, it should not be forgotten that also essential metals can be toxic when their concentration is higher than the metabolic need. In Table 1a–c, a list of biological important elements is given, in which different types of elements are differentiated according to their type of binding.

This paper summarises the up-to-date knowledge of the involvement of microbes in the biogeochemical cycle of metals: mobilisation and precipitation, essentiality, toxicity and utilisation in cells. Examples from the biogeochemical cycle of iron and arsenic, both represent different types of elements, are used to explain the effects of microbes in more detail.

2. Biogeochemical cycles of metals

Weathering of rocks, volcanic activity, evaporation of water and biological activity together with anthropogenic activity are responsible for the emission of metals into the atmosphere and hydrosphere where upon they are redistributed onto the earth's surface by rain and dust and thus closing the cycle. The distribution of metals between solid (rock/sediment), soluble and volatile species was thought for a long time to result purely from geological processes. The knowledge about the influence of microbes on this distribution grew over the last few decades. Microbes can dissolve metal ions from minerals for instance iron from pyrite and convert them into soluble species. Their metabolic processes can also operate in the opposite way and lead to the formation of insoluble species like magnetite. Microbes are able to metabolise metals to organometallic compounds (soluble

Table 1a *Table of biological important elements*

Principal elements	H, C, O, N, S, P
Elements in ionic form	Na, K, Rb*, Sr*, Cl, I, Br*, Ca, Mg
Elements in coordination complexes	Fe, Cu, Zn, Mn, Co, Mo, W*, Cr, V*, Ni
Elements in organometallic compounds	Se, As*, Ge*, Sb*, Pb*, Cd*, Bi*, Te*, Tl*, I, Co, Sn*, Hg*

* essentiality not established

Table 1b *Some coordination complexes and metalloproteins*

Metal name	Function	
Fe	Hemoglobin, cytochrom C, myoglobin	Oxygen transport, oxygen storage
Cu	Amine oxidase, Cu/Zn-SOD	Oxygen reduction, dismutation of superoxide anion radical
Zn	Zinc finger proteins, alcohol dehydrogenase	Structural
Mn	Mn-SOD	Dismutation of superoxide anion radical
Co	Vitamin B12, methylcobalamine	Transfer of methyl-group
Mo	Sulfite oxidase, nitrogenase	Oxidation of sulfite to sulfate, nitrogen fixation
W	Formaldehyde ferredoxin oxidoreductase	Oxidation of aldehydes
Cr		Insulin production ?
V	Haloperoxidase	Oxidation of halides

Table 1c *some organometallic compounds found in the environment and in biota*

Metal	Gas/water/sediment	Biota
As	MMA(V), DMA(V), Me_3As, Me_2AsH	AsB, AsC, arsenosugars
Se	Me_2Se, Me_3Se	Se-cysteine, Se-methionine
Te	Me_2Te	
Sb	Me_3Sb, Me_2SbOH, $MeSb(OH)_2$	Me_3SbO, $MeSbO(OH)$
Ge	$MeGeO(OH)_2$	
Sn	$Me_xSn(OH)_{4-x}$, Me_4Sn	
Hg	$MeHgOH$, Me_2Hg	
Cd	$MeCd^+$	
Tl	Me_2Tl	
Pb	Me_4Pb, $Et_{4-x}PbMe_x$	

or volatile) *e.g*. dimethylarsinic acid or trimethylarsine. The contribution of these biologically created metal-compounds or biologically mobilised or precipitated metals to the distribution of metals in the environment is as important as the pure physicochemical reactions themselves. A schematic overview of the biogeochemical cycle of arsenic is given in Figure 1 as an example for the complexity of these

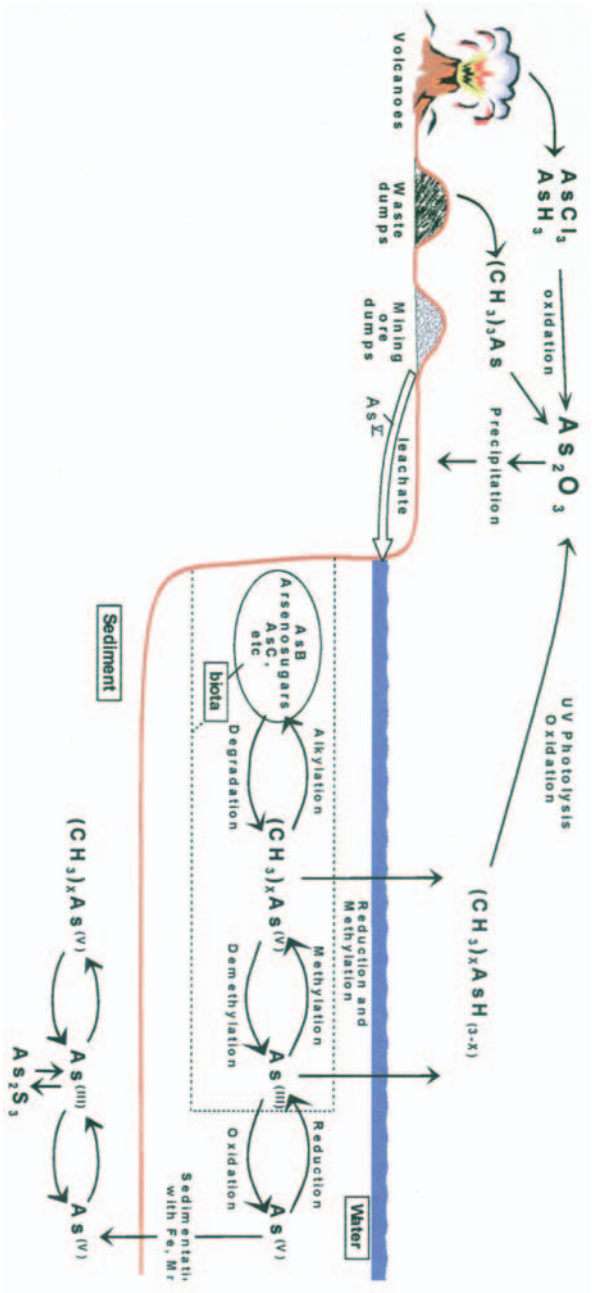

Fig. 1 *Schematic biogeochemical cycle of arsenic showing the transformations of arsenic taking place in sediment and water with and without the influence of microbes, and the chemical changes taking place in the atmosphere*

cycles, which are always interconnected. Most of the transformations are mediated by microbial activity. Methylation and demethylation takes place, in the water phase as well as in the sediment.

2.1 Mobilisation

Microbes, like all other cells, can take up only soluble metals for their metabolism. Microbes have two possible routes to overcome a deficiency of a particular metal with restricted solubility in their environment: they can stop their growth and wait until the environmental conditions improve, or second they can actively increase the solubility of the metal.

2.1.1 Passive mobilisation

The bioavailable metal concentration in the environment is dependent on the solubility of the metal under the conditions in place. The solubility is influenced by physicochemical processes and the presence of living cells, since the uptake of metals by cells shifts the equilibrium so that the dissolution rate of minerals increases.

2.1.2 Active mobilisation

The active mobilization of metals from insoluble material can follow three different pathways: (a) by growing directly on the surface of the insoluble material so that enzymes on the cell wall can interact directly with the solid; (b) by the extrusion of chelating molecules to bind the metal and the uptake of the metal-containing complex; (c) by excreting "electron shuttle" molecules, which transfer the electrons in the respiration chain, from the cellular inside to the insoluble metal outside of the cell and by reducing the metal increase its solubility

In the case of iron, microbes are known to use all three pathways for the mobilisation of essential iron. Iron-respiring bacteria use options one or three, whereas option two is exploited by nearly every microbe living under iron-restricted conditions. Iron-respiring bacteria, growing on or near the surface of iron-containing minerals, take part in the process of iron solubilisation. They use the exogenic reduction of the ferric to the ferrous iron for energy-yielding reactions as part of their respiration metabolism. *Geobacter* species[1] grow directly on the surface of iron-containing ores. They can only utilise iron when growing in direct contact with the mineral. The enzymes for this process are probably membrane-bound in the *Geobacter* species, because direct contact of the enzyme with the insoluble metal is necessary for the electron-transfer. Species of the *Shewanella* and

Geothrix families[2,3] use option three and extrude small organic molecules with a high redox potential to transfer electrons created intracellular to the extracellular iron-containing mineral. They can grow without direct contact to the iron-containing mineral. The electron-transfer necessary for the yielding of energy from the reduction of the extracellular ferric to ferrous iron is conducted by "electron shuttles" which are small quinone-like organic molecules[4] or melanin[5]. Strong ferric chelates (siderophores) are excreted by nearly every microbe to satisfy the metabolic need for iron in case of iron deficient conditions. These molecules are small organic molecules synthesised and excreted specifically for the uptake of iron. Their affinity to ferric iron is very high, so that they shift the solubility balance and therefore increase the solubility of iron. The microbes express transport systems for these iron-transporters, which are often highly specific for the iron-containing complex and vary from species to species.

Another example for the mobilisation of metals is the reduction of hydrological less mobile arsenate to the more mobile arsenite. Microbes reduce arsenate to arsenite for two distinctly different reasons, one is to use the reduction to yield energy and the other is to protect the cell from toxic effects of arsenate (see below). The energy yielding reduction is used by microbes of very different morphological backgrounds. Today microbe strains using this metabolic pathway to yield energy are known to occur in freshwater mesophile[6], halo-alkaliphile[7], sulphate-reducing[8], hyperthermophile[9] and enteric bacteria[10] families. For example, members of the *Sulfolobales* family grow directly on sulphur ores and are obviously able to mobilize heavy metals especially arsenic directly from the ores[11]. The enzymes involved in these processes are not yet characterised. Arsenate-respiring bacteria can use different ways for yielding energy form the arsenate-reduction[12] (Equation (1)). The products of these reactions are either further metabolised or excreted. The influence of arsenate- and/or iron-respiring microbes on the arsenic cycle has become more and more a reason of concern, for instance in Bangladesh, where millions of people suffer from highly contaminated well water. Microbial activity is probably partly responsible for the increasing concentration of arsenic in these well waters.

2.2 Immobilisation and sequestering

Microbes in metal rich environments can play a role in the precipitation of metals by forming non-crystalline insoluble metal species *e.g.* from arsenic or biomineralisation of magnetite from iron.

Equation 1: some energy-yielding reaction used by As^{5+} respiring microbes

$Acetate^- + 2\ HAsO_4^{2-} + 2\ H_2AsO_4^- + 5\ H^+ \rightarrow 4\ H_3AsO_3 + 2\ HCO_3^-$

$Lactate^- + 2\ HAsO_4^{2-} + 2\ H^+ \rightarrow 4\ H_2AsO_3^- + acetate^- + HCO_3^-$

$2\ H_3AsO_3 + O_2 \rightarrow HAsO_4^{2-} + H_2AsO_4^- + 3\ H^+$

$1/2\ H_2AsO_4^- + 1/2\ H_2 \rightarrow 1/3\ As^0 + 1/2\ H_2O$

$1/3\ HAsO_4^- + 1/2\ H_2 \rightarrow 1/3\ As^0 + H_2O$

2.2.1 Precipitation

The formation of insoluble but not crystalline metal species is known for several microbes. Biological precipitation of metals is best studied for arsenic and iron, other metals for which biological immobilisation is known are nickel, calcium, selenium and zinc. The ability of microbes to precipitate soluble and therefore highly mobile metals can be used for bioremediation of contaminated sites. Experiments to use this ability are under way, for example by using members of the *Desulfuromonas* and *Geobacter* families to precipitate uranium from contaminated aquifers[13]. These bacteria are able to reduce soluble hexavalent uranium to insoluble tetravalent uranium with acetate as electron donor. The use of these bacterial abilities *in situ* still requires additional research in order to determine the exact physiological conditions necessary for bacterial growth.

The formation of insoluble arsenic species by microbes is another for bioremediation studies interesting example. Recent research showed that the *Desulfotomaculum auripigmentum* (isolated from surface sediment of a fresh water lake) can form arsenic trisulphide (orpiment) by respirative reduction of arsenate and sulphate to arsenite and sulphide and oxidation of lactate to carbondioxide (Equation (2)). The sensitivity of the reaction toward molybdate-inhibition of the involved enzymes points to an iron–sulphur-cluster as active centre of the enzyme. The mineral is formed extra- and intracellularly at concentrations, where abiotic formation is not possible. However, orpiment precipitated by biological activity can form a nucleus for

Equation 2: Reaction of aresenate and sulphate reduction by *D. auripigmentum*

$$CH_3\text{-}CHOH\text{-}COO^- + 2\ HAsO_4^{2-} + 4\ H^+ \rightarrow CH_3\text{-}COO^- + 2\ HAsO_2 + CO_2\ (g) + 3\ H_2O$$

$\Delta G^{0,} = -172$ kJ/mol lactate

$$CH_3\text{-}CHOH\text{-}COO^- + 0.5\ SO_4^{2-} + 0.5\ H^+ \rightarrow CH_3\text{-}COO^- + 0.5\ HS^- + CO_2\ (g) + H_2O$$

$\Delta G^{0,} = 89$ kJ/mol lactate

the mineralisation in the bulk milieu[14]. The transport mechanism of orpiment from the inside of the cell to the outside is not exactly known, but there are experimental results that suggest that membrane-bound orpiment particles can be transported through the cell wall. In the presence of arsenate and sulphate, first the arsenate is reduced, since this reaction yields more energy for the microbe. The reduction of sulphate and the following precipitation of orpiment is a way to keep the intracellular concentration of arsenite low and so the energy-yield of arsenate-reduction high. It is not yet known whether *D. auripigmentum* possess an arsenite excretion pathway like other bacteria. In contrast, arsenite oxidising bacteria like *Agrobacterium albertimagni* and *Thermus* HR13 seem to be unable to precipitate arsenate species.

2.2.2 Biomineralisation

The formation of crystalline, inorganic species under the influence of cells is known as biomineralisation. The formation of bones by higher organisms from calcium phosphate as well as the formation of magnetite particles by magnetotactic bacteria belongs to this category. Iron precipitates are formed either passively by microbes, which serve simply as crystallisation points or actively as in the case of magnetotactic bacteria. Part of the biogeochemical iron cycle is formed by magnetotactic bacteria that synthesise intracellular monocrystalline magnetite (Fe_3O_4) particles approximately 50–100 nm in size. These bacteria have been studied since the 1970s and occur in freshwater and marine environments. These magnetite-forming bacteria have a number of gene encoded specific proteins that play a crucial role in forming magnetite particles[15]. The intracellular formation of magnetite is poorly understood. It is believed to be a four-step process, involving the uptake and reduction of iron, formation of ferric oxides by partial oxidation, formation of ferrihydrite complexes and finally formation of magnetite. These biologically formed magnetite particles are enclosed by a thin organic membrane (magnetosomes) and can therefore be distinguished from physicochemically formed magnetite. The size and number of magnetosoms depends upon the bacterial species and on the environmental concentration of iron, oxygen and several other chemicals. Extracellular formation of magnetite particles by ferric iron-reducing microbes and bacteria combining the oxidation of ferrous to ferric iron with the reduction of nitrate produce smaller, more irregular particles that are not surrounded by an organic membrane.

Iron can also precipitate in other mineralogical forms depending on the availability of other ions like sulphate and carbonate. By

coexistence with anaerobic iron-oxidisers like *Ferroglobus placidus*, an iron cycle, which may have existed already within early life forms on Earth, is formed.

3. Essentiality and toxicity

Metals can be divided in essential and toxic elements for a given organism. Essential metals are all metals, which are necessary for the function of metabolic pathways. Toxic metals inhibit specific or non-specific metabolic pathways and therefore damage or kill the organism.

3.1 Essentiality

The essentiality of metals is influenced by their availability, their reactivity/suitability for a certain reaction and the coexistence of other species. Aluminium for instance is the most abundant metal on earth, but since it has a low solubility, it is not essential for cells. Soluble iron in contrast to aluminium, abundant during the anoxic period of the evolution is today a growth limiting metal. Due to its high reactivity at a wide range of redox-potentials, it is still an essential metal and is not replaced with another metal.

Essential metals form either part of an active enzymatic centre, or they are structure-forming elements. Redox-active metals can play an active role in enzymatic reactions, whereas structural metals are not redox-active. The concentration of essential metals in an organism is carefully regulated, since a lack of metal will switch off essential metabolic pathways and a surplus is likely to inhibit metabolic pathways. The uptake and excretion of metal ions is regulated by the expression of specific and non-specific transporters. The concentration of these transporters in the cellular membrane is often back regulated by the available concentration of the free metal in the cell. Metals essential for all cells are among others iron, zinc, copper, selenium, magnesium, manganese, molybdenum and sodium. Other elements are essential for the survival of specific organisms for example arsenate for arsenate-respiring microbes. Microbial enzymes and proteins often contain unusual metals. Some hyperthermophilic microbes use for instance tungsten instead of molybdenum as catalytic centre in aldehyde dehydrogenases, because of the enhanced solubility of tungsten in contrast to molybdenum at higher temperature.

3.1.1 Catalytic centres

Metal-containing enzymes (metalloenzymes) are part of every metabolic cycle, whether it is the synthesis of energy-rich molecules like ATP, the transcription of DNA, or protein/carbohydrate synthesis.

Examples of metalloenzymes in microbes are the widespread sulfite-reductase-hemoproteins[16]. In sulphate-reducing microbes, they are part of the respiration chain, whereas in all other microbes they are essential for the synthesis of sulphur-containing amino acids. They contain heme-bound iron, plus an iron-sulphur cluster. An iron-protoporphyrin forms the active centre of the enzyme. Some members of this family also contain calcium ions for structural purposes. Photosynthetically active bacteria of the *Rhodospirillaceae* family use an enzyme-complex containing four heme-bound iron ions and a fifth non-heme iron ion in addition to other different cofactors. This enzyme-complex converts solar energy to biochemically available energy in the form of energy-rich ATP. Some of the biological iron-clusters are shown in Figure 3.

3.1.2 Electron-transfer reactions

Electron-transfer reactions are an essential part of the respiration chain in cells, and are independent of the kind of respiration. These reactions are catalysed by a wide variety of enzymes. In prokaryotic organisms, most enzymes of the respiration chain are localised at the cytoplasm membrane. In eukaryotic organisms, they are found mostly at the mitochondrial membrane and in the mitochondria. Protons or small organic molecules like acetate or lactate are oxidized. The reduction partner is depending on the organism, it can be oxygen in aerobic organisms, ferric iron in iron-reducing bacteria or other molecules or ions. The transfer of electrons is mostly handled by iron-containing enzymes of the cytochrome C family. The heme-bound iron in these enzymes forms the active electron-handling interface between the transfer steps. The energy yielded by these transfer reactions is normally stored in form of ATP. Heme-containing proteins are especially useful for these reactions. Their redox potential, depending on the ligands, covers a wide range from –300 to + 400 mV.

3.1.3 Structural functions

Non-redox active metals, like zinc, magnesium or calcium, are often used to stabilise specific protein structures or to enable interactions between enzymes and their cofactors. Magnesium bound to ATP is a good example of the latter. Members of the ubiquitous copper–zinc superoxide dismutase (Cu,Zn-SOD) contain a redox-active copper and a structural zinc ion in each subunit. The zinc ion stabilises the enzyme conformation during the redox-cycle of the copper. Both ions share a common ligand, which helps in the stabilisation of the protein channel leading to the active centre of the enzyme. The amino acid sequences of eukaryotic and prokaryotic Cu,Zn-SODs

are quite similar, showing that the Cu,Zn-SOD enzymes have been around since the early evolution catalysing the dismutation of superoxide anions to oxygen and water. There are probably several hundred proteins and enzymes in organisms, which all use zinc to stabilise the structure. One of the most essential protein families utilising zinc for that purpose are the so-called zinc-finger-proteins, which are essential during the transcription of DNA.

3.2 Toxicity

Metals become toxic for organisms when their concentration is higher than the demand from the metabolism, at this point the metal can act as inhibitor of metabolic pathways by strongly binding to enzymes or by forming unwanted radicals or less stable reaction-products and therefore wasting energy. Cells have developed different mechanisms to avoid the toxicity of metals, a selective uptake regulated by the metabolic need for the metal, an efficient excretion mechanism or specific metabolic pathways by which the toxic form of the metal is transformed into a non- or less toxic form.

3.2.1 Production of radicals

Radicals are atoms or molecules with an unpaired electron. They are generally very reactive and act as starting point of chain reactions. Radicals formed in this way can for instance polymerise essential unsaturated fatty acids in the cellular membrane and therefore change the physicochemical characteristics of the membrane. They can also damage carbohydrates, proteins and DNA.

Iron is one of the metals where the toxicity is based on its ability to act as starting point for radical generation, especially of extremely reactive oxygen or nitrogen radicals. Therefore, the uptake of iron must strictly be regulated. Iron toxicity is based on the ability of ferrous iron to catalyse the formation of the highly reactive hydroxyl radicals from hydrogenperoxide or other radicals from lipidperoxides (Fenton reaction, Equation (3)). In aerobic living microbes' traces of free iron can start a deadly cycle of oxidation/reduction and the simultaneous production of radicals, since aerobic conditions always mean that superoxide anion radicals are formed (Haber-Weiss reaction, Equation (3)).

3.2.2 Inhibition of energy-yielding or enzymatic reactions

A whole group of metals act as toxins by binding either reversible or irreversible to the active centre of enzymes or DNA and therefore

Equation 3: Fenton reaction (1 a/b) and Haber-Weiss reaction (2)

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad (1a)$$

$$Fe^{2+} + LHO_2 \rightarrow Fe^{3+} + OH^- + LH^{\bullet}$$

(L = lipidperoxide or other peroxides) (1b)

$$Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2$$

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^- \text{ (+ traces of } Fe^{3+} \text{ or } Fe^{2+}) \quad (2)$$

effectively blocking essential metabolic reactions. Some metals can, by replacing other elements in their biological position, change the reactivity of the product and exhibit toxic properties by this way. Arsenic for instance can block enzymatic centres in its trivalent form by binding to free sulfhydryl-groups[17]. On the other hand, arsenate is structurally very similar to phosphate, which it can replace in different reactions for example in the synthesis of ATP from ADP. The spontaneous hydrolysis (arsenolysis) of arsenate-ATP does not yield energy. The cellular respiration chain can be inhibited by arsenate and arsenite via the uncoupling of the oxidative phosphorylation of ADP or by the inhibition of the NADH and NADPH reduction through arsenite binding to one of the reductases. The mechanisms by which organoarsenic compounds unfold their toxicity are not known in detail. However, it is known for arsenic as well as for chromium and other metals, that the species in which the metal is bound has great influence on the toxicity of the metal (toxicity of some arsenic compounds in Table 2). For arsenic the toxicity varies several-fold depending on the compound[18,19].

4. Uptake and excretion

Cellular membranes are formed by hydrophobic lipid bilayers, which are nearly impenetrable for charged compounds. In the course of evolution, membrane-spanning transporters were developed for the exchange of ions between the surrounding environment and the intracellular space. These transporters either form simple pores, by which energy-independent diffusion is possible or they transport their freight through membranes by consuming energy.

4.1 Passive cellular uptake and excretion by simple diffusion

Simple diffusion of metals through cell membranes is not a biologically relevant process, simply because of the hydrophobic nature of cell membranes and the hydrophilic nature of the ions. Diffusion through cell membranes is used by gases like oxygen, nitrogen or methane. Diffusion is energy independent since it is driven by a con-

Table 2 *Arsenic species found in environment and biota and their determined acute toxicity for male mice in mg/kg body weight* [33-30]

Nr. of R	Trivalent Species	LD_{50}	Pentavalent Species	LD_{50}
0	$As(OH)_3$	4.5 (rat)	H_3AsO_4	14–18 (rat)
	AsH_3	3		
1	MMA(III)		MMA(V)	1,800
1	$MeAsH_2$			
2	DMA(III)		DMA(V)	1,200
2	Me_2AsH			
3	Me_3As (TMA(III))	8,000	TMAO	>10,000
3			DMAE	?
3			DMAA	
3			Me_2As-sugar-OH,-SO_4, etc.	>12,000[34]
3			Arsenolipids	?
4			TMA^+	900
4			Me_3As-sugars	
4			AsC	6,540
4			AsB	>10,000
4			AsB-2	?

centration gradient. It is likely that volatile metal compounds for example Me_3As, Me_2Hg or AsH_3, synthesised by the metabolism in the cell, are also excreted by passive diffusion.

4.2 Active cellular uptake

Molecules spanning cellular membranes for transporting other molecules are often protein-complexes with hydrophobic and hydrophilic domains. They can form pores suitable for passive transport through the membrane, like members of the permease family. These proteins span the membrane, but they are not able to transport against a concentration gradient. They allow energy independent unhindered diffusion of hydrophilic compounds, like water, through hydrophobic membranes "downhill". Active transport of molecules, often against a concentration gradient, is an energy demanding process, which is also facilitated by *trans*-membrane transporters with binding sites for the transported molecule and often ATP as energy deliverer. All of these transporters can either be selective for a specific molecule or molecule-group or non-specific transporters. As an example, the up-to-date knowledge about the uptake of arsenate and arsenite is briefly discussed in the following paragraph. A schematic overview of the cellular arsenite and arsenate uptake is given in Figure 2.

Arsenate uptake takes place via phosphate-transporters, because of the structural similarity of phosphate and arsenate ions. So far,

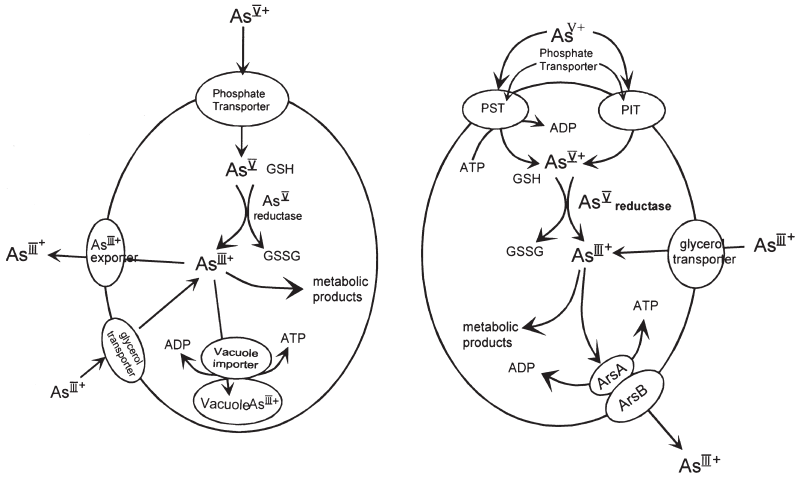

Fig. 2 *schematic uptake and excretion of As(V) and As(III) by prokaryotic and eukaryotic microbes, the scheme of the prokaryotic cell (right) shows that these cells have a more complex way to handle inorganic arsenic, whereas in eukaryotic cells (left) less proteins and enzymes are directly involved in the handling of inorganic arsenic*

examination of prokaryotic microbes showed they possess two different phosphate transporters, one with a high affinity for phosphate and one with a lower affinity. Both transporters consist of a quite complex protein structure with different subunits. Eukaryotic microbes express a phosphate transporter similar to the low affinity transporter of the prokaryotes. Their expression is regulated via a feedback mechanism. Members of the low-affinity Pit-family are expressed predominantly when the phosphate concentration in the environment is high. These transporters belong to the permease transporter channels. Members of the Pst-family are ATP-dependent phosphate transporters. The affinity of the Pit-transporter for arsenate is higher than that of the Pst-transporter[20].

The uptake of arsenite ($As(OH)_3$) or the structurally similar antimonite ($Sb(OH)_3$) is facilitated by transporters, which also transport glycerol, water and other polyols. Transporters of this kind are involved in the osmoregulation of every cell. The ability of glycerol-transporters to transport arsenite is probably a result of the similarity of arsenite to other polyols[21].

4.3 Active cellular excretion

Excretion of metals from cells is normally an energy demanding process involving selective or non-selective transport molecule com-

plexes. Some metal ions like iron are normally not excreted in free ionic form. Others like arsenic are excreted as free ions as well as in metabolised form. These exporters are, as the importers, transmembrane protein complexes. Most contain binding domains for ATP or other energy-rich molecules, necessary for delivering the energy for the transport, in addition to the substrate-binding site. Not all metals imported into the cell can be excreted as well. For example iron exporters are, in contrast to importers, virtually unknown. Partial responsibility may lie with iron being a limiting factor for microbial growth in most environments, because of its low solubility. The iron export in iron-respiring microbes has not yet been studied. It may be similar to the only known iron export system in *Escherichia coli*, where iron is exported by a member of the ABC-transporter family bound to a siderophore[22].

Arsenite is the exported form of arsenic, independent of whether arsenate or arsenite was imported into the cell. For all organisms, it is essential to maintain a low cellular level of arsenite, since it is a very effective cellular toxin. Arsenite is able to inhibit every enzymatic activity where sulfhydryl groups are participating. Cells with a defective arsenite export-system are extremely sensitive to the lowest environmental arsenic concentrations. The enzyme systems involved in the excretion of arsenite are known in detail in prokaryotic and eukaryotic microbes. These transporters are either plasmid or chromosomal encoded. In all studied species so far these transporters belong all to the ATP-binding-cassette-protein superfamily, despite their variability. The arsenite-transporter of gram-negative microbes is a complex of two different proteins ArsA and ArsB, where ArsA is the arsenite and Mg^{2+}-ATP binding part and ArsB is the transmembrane protein. Gram-positive microbes do not express ArsA, but a protein similar to ArsB, which fulfils the functions of both ArsA and ArsB.

5. Intracellular metabolism of metals and metalloids

Metals taken up by microbes are metabolised into very different compounds depending on the metal, organism and the metabolic needs of the organism. Metals form coordination complexes with, among others, proteins and lipids or they form compounds containing metal-carbon bonds. Compounds containing metal-carbon bonds play an important role in the environmental transformation and distribution of metals. Coordination complexes, in which the metal is bound by nitrogen, oxygen or sulphur ligands of an organic molecule, are less important for the biogeochemical cycle of metals than species containing metal–carbon bonds, except for dissolution and precipitation.

In contrast to the amino acid composition of metal-containing enzymes, the bond metal is not encoded by the DNA sequence.

The only metal where integration into proteins is determined by the DNA is selenium in the form of Se–cysteine. Se–cysteine often forms the active centre of peroxidases, where its higher reactivity compared to the normal sulphur containing cysteine is advantageous.

5.1 Coordination complexes

Organisms use most metals in form of coordination complexes, where the metal is coordinated through physical interactions with amino acids. Depending on the metal, the preferred coordination partners are nitrogen, sulphur or oxygen atoms from amino acids like histidine, cysteine, glutamate or aspartate. The metal complexation results from free electron pairs on these atoms. Figure 3 shows some of the possibilities for iron. There are enzymes where the active centre is formed by iron or iron-sulphur cluster. Others use additional metals like molybdenum or tungsten in the active centres. Examples of these enzymes can be found in the different oxidoreductase, nitrogenase or dissimilatory nitrate reductase families. The replacement of molydenum by tungsten is currently only known for some hyperthermophilic archaea like *Pyrococcus furiosus*[23].

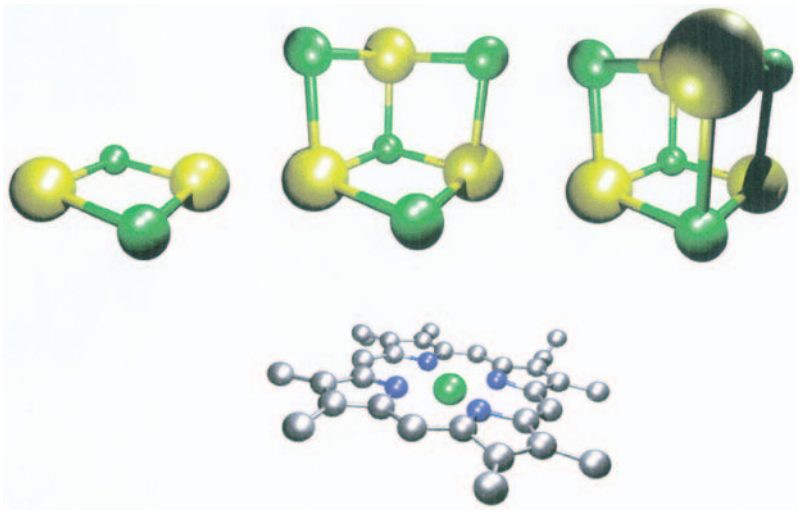

Fig. 3 *Molecular structures of some iron coordination complexes:* Fe_2S_2, Fe_4S_3, Fe_4S_4 *and Fe-heme complex (iron: green, sulphur: yellow, carbon: grey, nitrogen: blue); the coordination to the protein takes place via interaction of the iron atom(s) with sulphur, oxygen or nitrogen atoms of the protein,* e.g. *in haemoglobin*

5.2 Organometallic species

5.2.1 Synthesis

Biologically synthesised metal-carbon species are known for a number of elements, most often one or more methyl-groups are transferred to the metal. Methylated or more complex organic species are either excreted as part of a detoxification process or formed as intermediates in the metabolism. Methylated species are known for arsenic, selenium, tin, germanium, tellurium, antimony, bismuth, mercury, thallium, lead and cadmium[24].

Arsenic and selenium have the most variable and best-known biological metal–carbon chemistry. In Figure 5 the molecular structures of some of these arsenic compounds are given.

Most metals are only methylated by microbes, but arsenic and selenium are also metabolised to carbon-containing species in mammals. Therefore, the methylation process of arsenic is intensely studied, since the toxicity of arsenic is strongly dependent on its species (compare Table 2) and the health of millions of humans is at risk. Challenger was the first to publish a probable metabolic pathway for the methylation of arsenic and selenium and maybe antimony and tellurium[25]. Figure 4 shows the pathway of arsenic reduction and oxidative methylation by cells in some detail. Arsenate reductase (ArsC) is currently the best known enzyme in this pathway. Cofactors for the reduction are often thioredoxin, glutathione or similar molecules. The active centre of the enzyme is formed by the sulfhydryl groups of three cysteines from the amino acid chain. It belongs to another enzymatic family than the arsenate reductase of arsenate-reducing microbes and is not to be confused with this enzyme in the respiration chain. Of the other enzymes involved in the methylation little is known with the exception of the mammalian liver arsenite methyltransferase, MMA(V)-reductase[26] and MMA(III) methyltransferase[27]. The methylation agent in mammalian cells is S-adenosylmethionine (SAM). Other methylating compounds like methylcobalamin may play a role in biological transfer of methyl-groups in microbes as well.

Experimental results show that the methylation process in microbes is similar to that in mammalian cells[28]. The difference between methylation in microbes and higher organisms is the number of methyl-groups attached to the arsenic. Microbes are known to produce mono-, di- and trimethylated arsenic species. Mammals can normally only methylate arsenic to mono- and dimethylated species. Tetraalkylated arsenic compounds such as arsenobetaine and tetramethylarsonium ion have been found in fish and shellfish. Marine algae contain even more complex organoarsenicals like the so-called

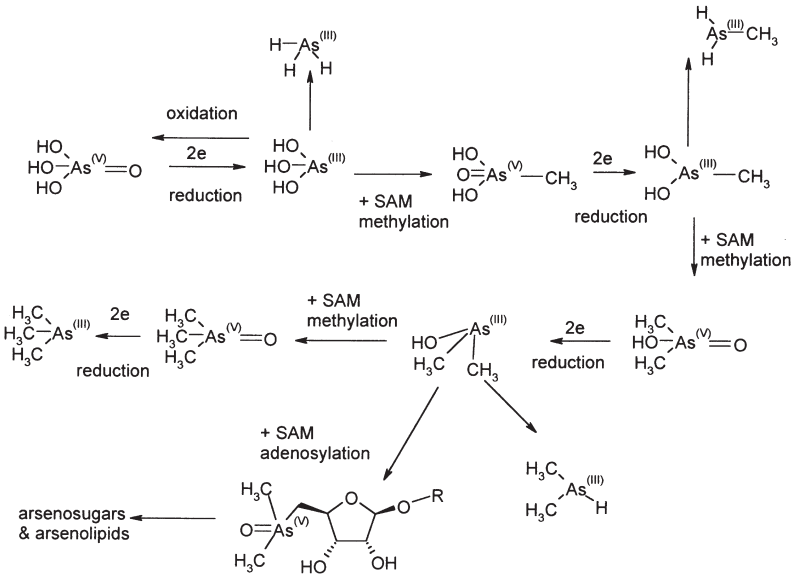

Fig. 4 *Simplified overview of the biological transformation of inorganic arsenic to the different organoarsenicals involving two electron reduction and oxidative methylation or adenosylation; microbes are not known to do adenosylation but they transfer up to four methyl groups to the arsenic and excrete arsenic either as trivalent hydrides (volatile) or as pentavalent methylated species (soluble in water)*

arsenosugars, where instead of a methyl group an adenosyl-group is transferred from SAM to DMA(III) (see Figure 5 for some structures of arsenic species). The function of these organoarsenicals is entirely unknown.

5.2.2 Degradation

Microbes can break down compounds with metal–carbon bond to the free metal ion. The enzymes involved in these reactions are not yet known. It is known that organoarsenic compounds are broken down by the stepwise removal of carbon, so to say a reversal of the methylation process. Arsenosugars, for example, are broken down in soil by first removing the side-chain of the sugars, and then the ribose moiety before the dimethyl-group is broken down[29].

5.3 Volatile metal compounds

Microbes are also known to form hydrides from arsenic, selenium and some other elements. This process is often, but not exclusively observed in methane-generating microbes, for instance at landfill

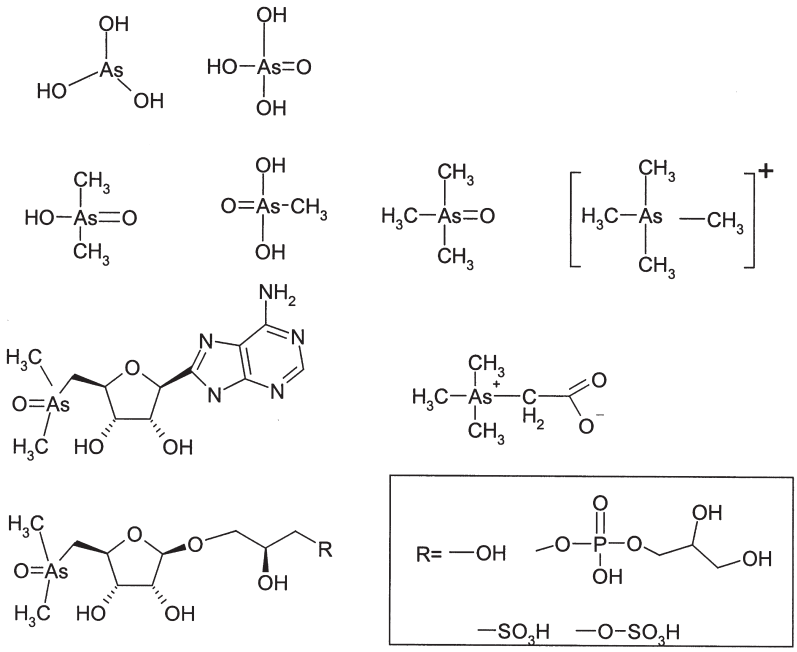

Fig. 5 *Structures of some biological important arsenic compounds (with the exception of the two inorganic species of arsenic, the element is always bound to at least one carbon atom).*

sites[30]. The hydride formation in these microbes is probably via metabolic pathways of the methane formation. The hydrides contain either only hydrogen or a mixture of methyl-groups and hydrogen. The enzymatic mechanisms for the hydride formation are not known, but there are reasons to assume that some of the microbes use metals as a proton sink in the respiration chain. Another possible reason for the synthesis of the highly toxic hydrides is the necessity for fast excretion of large amounts of metal by simple diffusion through the cellular membrane.

6. Experimental methods used to measure microbial transformation of metals

For the determination of metal species transformed by microbes a whole range of analytical techniques are used. First, the metal has to be determined quantitatively, spectroscopic methods like atomic absorption or inductively coupled plasma with mass spectrometry are used most often. Second, these methods are also useful in the determination of the metal species when combined with a separation

method like gas or liquid chromatography. For the species determination, solid samples have to be transferred into the liquid phase by either extraction or dissolution without changing the metal species present in the solid state. This sample preparation is one of the most crucial steps in the whole process of species identification, since any species-transformation at this stage is very difficult to trace and has a large impact on the results especially if complex questions are to be answered, like the metabolic pathway of a certain metal. The species identification after separation can be done by matching the chromatographic retention times of known standard compounds and spiking of the sample. When an elemental detector, like inductively coupled plasma mass spectrometer, is used as the sole detector after the separation this is the only possibility for identification. It is often difficult to find matching standards for biologically transformed metals, therefore the use of only elemental detection for identification of the species is difficult. The use of organic mass spectrometers, like electrospray mass spectrometer, to determine not the element but the molecular mass of the species in combination with an elemental detector is increasing the probability of the correct identification of a biologically transformed metal[31]. Organic mass spectrometry alone can also not solve the problem of identification, since biological samples contain hundreds of compounds, which are impossible to separate completely within one chromatographic separation, therefore finding the molecular mass belonging to a certain metal species without the knowledge where this species is eluting is impossible even with high-resolution mass spectrometers.

Traditional biochemical methods, like gel electrophoresis and measurement of enzymatic activities, are often used for the determination of the biochemical processes involved in metal transformation. Nowadays genetic or proteomic approaches are often used to identify the enzymes and proteins involved. An overview of the most common methods is given in Table 3.

7. Outlook

Metal speciation in biological and environmental samples is anything but routine analysis. This is indicated in the small number of certified standard reference materials available with certified concentration of metal species. For example, any attempt so far to produce a standard reference material for arsenosugars failed and these are compounds, which are considered as relatively stable during extraction and separation procedures. The real challenge today is to identify unknown metal-containing compounds detected by elemental detectors. For this

Table 3 *A list of analytical methods used for the determination of biological metal transformation and the kind of information gained from the analysis*

AAS / AFS ICP-OES / ICP-MS	Organic MS	MS/MS	Genetic methods (PCR)	Proteomics	(Enzymatic assays)
Elemental composition	Yes	No	No	No	No
Molecular information of purified compound	No	Yes	Yes	Sometimes, if encoded in gene	No
Coupling to GC for on-line purification of complex mixture	Yes	Yes	Yes	No	No
Coupling to HPLC for on-line purification of complex mixture	Yes	Yes	Yes	No	No
Structural information for purified compound	No	Sometimes	Yes	Sometimes, if encoded in gene	No
Information about pathways	No	No	No	Sometimes	Yes

AAS: atomic absorption spectrometer, AFS: atomic fluorescence spectrometer.
ICP-OES: inductively coupled plasma with atomic emission spectrometer.
ICP-MS: inductively coupled plasma with mass spectrometer.
organic MS: mass spectrometer using e.g. electrospray ionisation (ESI) for liquid samples (HPLC) to measure the mass of whole molecules or electron impact ionisation (EI) for gaseous samples (GC) or matrix-assisted laser desorption ionisation (MALDI) for solid samples.
MS/MS: combination of different mass spectrometers, so that fragments created in one are measured in the next MS.

purpose, other techniques such as organic mass spectrometry are used to identify the molecular mass. High-resolution mass spectrometer may bring a new dimension in the field of metal speciation, due to their accurate mass measurements. It is expected that traditional biochemical methods such as gel electrophoresis will find more use in the field of metal speciation, especially for the determination of metal-protein complexes. In addition to this new fleet of conventional techniques, X-ray absorption methods will be utilized for the detection of very labile metal species directly in the unchanged organism[32]. It is for example just emerging that besides the wealth of organoarsenic species, many labile coordination species may occur, which may play a key role in the complex biochemistry of arsenic.

8. Acknowledgment

We thank Dr Bruce F. Milne for the design of the molecular structures.

9. Glossary

ferric ion: iron in the oxidation state III
ferrous ion: iron in the oxidation state II
arsenate: arsenic in the oxidation state V
arsenite: arsenic in the oxidation state III
uranyl: uranium in the oxidation state VI
uranium in the oxidation state II
$As(OH)_3$: arsenite
AsH_3: arsine
MMA(III): methylarsonous acid ($MeAs(OH)_2$)
$MeAsH_2$: methylarsine
DMA(III): dimethylarsinous acid (Me_2AsO)
Me_2AsH: dimethylarsine
Me_3As (TMA(III)): trimethylarsine
H_3AsO_4: arsenate
MMA(V): methylarsonic acid
DMA(V): dimethylarsinic acid
TMAO: trimethyl arsine oxide
DMAE: dimethylarsinoylethanol
DMAA: dimethylarsinoyl acetate
TMA^+: tetramethyl arsonium ion
AsC: arsenocholine
AsB, AsB-2: two forms of arsenobetaine
Me_3As trimethylarsine
Me_2Hg methylmercury
Me: methyl-
metal compounds
orpiment: As_2S_3
magnetite: Fe_3O_4
Ferredoxins: Fe_2S_2, Fe_4S_4, Fe_3S_4
Microbes
mesophile microbes: microbes living in moderate environment (like freshwater)
haloalkaliphile microbes: microbes living in extreme salt concentrations
hyperthermophile microbes: microbes living in extremely hot environments (geyser)
enteric bacteria: microbes living in the intestine
Enzymes, proteins and other biological active molecules
ATP: adenosine triphosphate, source of energy
ADP: adenosine diphosphate, source of energy
NADH: β-Nicotinamid adenine dinucleotide, proton transfer
NADPH: β-Nicotinamid adenine dinucleotide proton transfer
SAM: S-adenosylmethionine, donor of methyl groups
siderophore: small iron binding molecules
Cu,Zn-SOD: copper-zinc superoxide dismutase
cytochrome C: protein family containing the heme-molecule
Pit-transporter: family of transmembrane phosphate transporters
Pst-transporter: family of transmembrane phosphate transporters
ABC-transporter family: group of anion transporters
ATP-binding-cassette-protein superfamily: group of transmembrane transporters using ATP as energy source
ArsA and ArsB: microbial arsenite exporters
Mg^{2+}-ATP: magnesium adenosine triphosphate complex
ArsC: arsenate reductase
arsenite methyltransferase: enzyme which transfers a methyl group from SAM to arsenite
MMA(V)-reductase: enzyme which reduces methylarsonic acid to methylarsonous acid
MMA(III) methyltransferase: enzyme which

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