LUMINESCENT RECOMBINANT SENSOR BACTERIA FOR THE ANALYSIS OF BIOAVAILABLE HEAVY METALS

PhD thesis

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ABSTRACT

In this work bacterial sensors responding to heavy metals by increase in luminescence were constructed and used for the quantification of bioavailable, i.e., potentially biologically hazardous fraction of metals in (environmental) samples.

Altogether four new bacterial sensor strains: for the dectection of Zn, Cr, Cu and Hg and its organic forms were constructed. The sensors responded to the target metals already at sub-toxic levels enabling the determination of metals starting from 5.4 mg of Zn^{2+} l^{-1}, 0.01 mg of $Cr_2O_7^{2-}$ l^{-1}, 3.2 mg of Cu^{2+} l^{-1}, 0.00005 mg of CH_3Hg^+ l^{-1} and 0.003 mg of Hg^{2+} l^{-1}. With the exception of the Cr sensor that was specific only towards the target metal, the sensors responded also to some non-target metals. Luminescent Hg and As sensor bacteria were alginate-entrapped and incorporated into a fibre-optic sensor system applicable for field analysis of bioavailable heavy metals.

For the quantitative analysis of bioavailable fractions of metals from natural samples, soil or sediment suspensions and their respective particle-free extracts were analysed with sensor bacteria. Reproducibility of the assay even for soil suspensions was acceptable (CV=22%). The novel aspects of this test protocol involve the parallel analysis of the samples using luminescent control bacteria that are not induced by heavy metals enabling thus to take into account the toxic or stimulative effects of samples to bacterial luminescence and quenching of luminescence by turbidity or colour. The developed protocol is applicable to all assays where thes matrix and other conditions may interfere with the test results.

Bacterial sensors were used to analyse bioavailable heavy metals from a large panel of environmental samples of different origin and contamination level (altogether 144 soils and sediments) mostly focusing on soils. All samples were analysed for physico-chemical properties and concentration of total heavy metals. The bioavailable amount of heavy metals to sensor bacteria in soil suspension assay did not correlate with the total concentration of heavy metals in soils. The bioavailable fraction of metals varied from 1 to 46 % of the total metal depending on the metal, soil type and other factors.

The bioavailable fraction of Cu in Cu-polluted soils analysed with bacterial sensors was almost identical with the results obtained with yeast-based sensor cells indicating that the bioavailability of metals in soils for pro-and eukaryotic cells may be similar.

The parallel analysis of soil-water suspensions (contact assay) and respective particle-free extracts showed that the bioavailability of Hg, Cd and Pb was remarkably higher in soil suspensions compared with their water-extracted fractions indicating thus the presence of “particle-bound bioavailable fraction”in soil suspensions.

Bioavailability data from bacterial sensors and the results from chemical surrogate extraction methods (e.g., by using weak acid or salt solutions), often used to predict the mobility and bioavailability of metals showed no significant correlation. Moreover, the bioavailability of Cu, Pb and especially Cd (29-fold difference) was over-estimated by the chemical extraction. Thus, according to the results from this work the bioavailability of

Cd could not be adequately predicted by chemical extraction methods, at least for the bacteria used in the current work.

The fibre-optic biosensors for Hg and As were successfully used for the analysis of bioavailable fractions of these metals from natural samples. The bioavailability of Hg in polluted soils determined with alginate-entrapped sensor bacteria on optical fibres was remarkably lower than that measured with non-immobilised sensor cells. Thus, the alginate-immobilised bacteria may successfully model the reduced bioavailability of metals to e.g., biofilms for studying the efficiency of biocides.

This study showed that the luminescent recombinant bacterial metal sensors are powerful tools for the rapid assessment of already sub-toxic bioavailable concentrations of heavy metals in different environmental matrices. This is extremely important as the effects of sub-toxic metals are not usually detectable by other ecotoxicological test-organisms, however may be transferred via the food-web and cause chronic toxicity effects in biological systems. Thus, the development and use of biosensors in the environmental hazard assessment should be encouraged.

KOKKUVÕTE

Käesolevas töös "Rekombinantsed luminestseeruvad sensorbakterid biosaadavate raskemetallide määramiseks" konstrueeriti ning kasutati luminesteeruvaid spetsiifiliselt raskemetalle "äratundvaid" bakterirakke selleks, et teha kindlaks metallide biosaadavat ehk elusloodusele potentsiaalselt kahjulikult mõjuvat osa. Sensortüved konstrueeriti, viies peremeesbakterisse geneetilisi elemente, mis looduses kodeerivad raskemetallide resistentsus-mehhanismide regulatoorseid valke, mis reeglina koosnevad metalli äratundvast valgust ning promootorist, millele see valk seostub. Et metalli seostumist valguga visualiseerida, fuseeriti need metalli-äratundvad elemendid lutsiferaasi geeniga (*lucFF*). Insenergeneetiliste konstruktsioonide tulemuseks olid sensorbakterid, mille luminestsents indutseeriti kvantitatiivse vastusena rakku sisenenud (s.t. biosaadavatele) metallidele.

Kokku konstrueeriti neli uut sensortüve: Zn, Cr, Cu ja Hg ning selle orgaaniliste vormide (eriti olulised nende kõrge toksilisuse tõttu) määramiseks. Väärib märkimist, et konstrueeritud sensorid olid võimelised kvantifitseerima juba väga väikseid (sub-toksilisi) koguseid raskemetalle (määramispiir mg l^{-1}: 5.4 Zn^{2+}, 0.01 $Cr_2O_7^{2-}$, 3.2 Cu^{2+}, 0.00005 CH_3Hg^+ ja 0.003 Hg^{2+}). Vaid Cr-sensor oli täiesti spetsiifiline sihtmärk-metalli suhtes, kusjuures kõik teised konstrueeritud sensortüved olid indutseeritavad ka mõnede mitte-sihtmärk metallidega. Eelnevalt konstrueeritud Hg ning As sensorbakterid immobiliseeriti alginaadi maatriksisse ning kasutati fiiber-optiliste biosensorsüsteemide väljatöötamisel, mida on lihtne kasutada *in situ* testimisel.

Looduslike proovide relevantseks analüüsiks töötati käesoleva töö käigus välja uudne testprotokoll, mis võimaldab määrata biosaadavat raskemetallihulka mis tahes proovis. Protokolli uudsus seisneb luminestseeruvate ent raskemetallide poolt mitte indutseeritavate nn. kontrollbakterite kasutamises, mis võimaldab võtta arvesse proovi kaasmõju testbakterite luminestsentsile. Testprotokolli kasutati edukalt nii tahkete proovide kui nende veetõmmiste analüüsiks. Meetodi varieeruvus (CV) oli 22 %, mis on igati aktsepteeritav bioloogilise testi puhul läbiviiduna looduslikel proovidel.

Bakteriaalseid metallisensoreid kasutati biosaadavate raskemetallide analüüsiks kokku 144 pinnase-ja setteproovist. Tulemused näitasid, et raskemetallide koguhulk proovides ei korreleerunud nende biosaadav hulgaga, mis moodustas sõltuvalt metallist ning proovist 1 kuni 46 % koguhulgast.

Pärmidel põhinevate biosaadavat vaske ära tundvate sensorrakkude ning analoogiliste bakteriaalsete sensorite tulemuste kokkulangevus näitas, et raskemetallide biosaadavus pro- ja eukarüootsetes mikroorganismides ei pruugi erineda.

Mullasuspensioonide ning tahketest osistest vabade vesiekstraktide paralleelne analüüs sensorbakteritega näitas, et Hg, Cd ja Pb biosaadavus oli tunduvalt kõrgem nn. kontakt-testis (analüüsiti mullasuspensioone), kus bakteritele osutus biosaadavaks ka algselt osakestele-seondunud metalli-fraktsioon. Seetõttu, mõõtmaks relevantseid biosaadavusi muldades, on oluline tagada otsene kontakt mulla ja testorganismi vahel.

Raskemetallidega saastatud muldade keskkonnariski pinnases hinnatakse sageli keemiliste analüüsidega, kasutades biosaadavuse ennustamiseks metallide desorbeerimist nõrkade happe- või soolalahustega. Et hinnata nende meetodite ennustusjõudu, võrreldi pinnaseproovidest keemiliselt ekstraheeritud metallihulki sensorbakterite abil mõõdetud biosaadava fraktsiooniga neis proovides. Selgus, et andmed omavahel ei korreleerunud ning et keemilised meetodid ülehindasid Cu ja Pb poolt põhjustatud riski elusorganismidele 3 korda ning Cd poolt põhjustatud keskkonnariski koguni 29 korda. Seega, vähemalt antud töös kasutatud testbakteritele ei ole keemilised ekstraktsioonimeetodid relevantsed Cd biosaadavuse hindamiseks tahketes proovides.

Hg ning As fiiber-optilist biosensorsüsteemi kasutati edukalt nende metallide biosaadavate hulkade määramiseks looduslikest proovidest *in situ* tingimustes. Selgus, et Hg biosaadavus saastunud pinnaseproovides oli alginaati-immobiliseeritud sensorbakteritele tunduvalt madalam kui mitte-immobiliseeritud sensorbakteritele. Seetõttu võib alginaat-immobiliseeritud baktereid soovitada modelleerimaks ainete biosaadavust biokiledes, nt. biotsiidide efektiivsuse määramiseks.

Kokkuvõtteks võib öelda, et käesolevas töös konstrueeritud ja kasutatud bakteriaalsed sensorid on igati sobivad, määramaks spetsiifiliselt raskemetallide biosaadavust ka väga keerulistes keskkonnamaatriksites nagu mullad ja setted. Kuna sensorbakterite abil on võimalik detekteerida ka sub-toksilisi metallihulki, siis võimaldavad sensorbakterid hinnata metallide liikumist elutust loodusest elusasse (s.t. sisenemist toiduahelasse) juba väga varases staadiumis, kus tänu väga madalatele kontsentratsioonidele veel otsest akuutset toksilisust ei esine, küll aga võivad efektid aja jooksul kumuleeruda ja põhjustada kroonilist toksilisust.

Seega, rekombinantsed sensorbakterid on äärmiselt perspektiivsed biotestid raskemetallide keskkonnaohtlikkuse määramisel.

LIST OF PAPERS

The present dissertation is based on the following papers and some unpublished results. The papers are referred to in the text by their Roman numerals I-VI.

I **Ivask, A., Hakkila, K. , Virta, M**. 2001. Detection of organomercurials with sensor bacteria. Analytical Chemistry 73(21):5168-5171

II **Ivask, A., Virta, M., Kahru, A.** 2002. Construction and use of specific luminescent recombinant bacterial sensors for the assessment of bioavailable fraction of cadmium, zinc, mercury and chromium in the soil. Soil Biology and Bichemistry 34: 1439-1447

III **Ivask, A., François, M., Kahru, A., Dubourguier, H. C., Virta, M., Douay, F.** 2004. Recombinant luminescent bacterial sensors for the measurement of bioavailability of cadmium and lead in soils polluted by metal smelters. Chemosphere 55:147-156

IV **Peltola, P., Ivask, A., Åström, M., Virta, M.** 2005. Lead and Cu in contaminated urban soils: extraction with chemical reagents and bioluminescent bacteria and yeast. Science of the Total Environment 350(1-3): 194-203

V **Kahru, A., Ivask, A., Kasemets, K., Põllumaa, L., Kurvet, I., François, M., Dubourguier, H. C.** 2005. Biotests and biosensors in ecotoxicological risk assessment of field soils polluted with zinc, lead and cadmium. Environmental Toxicology and Chemistry 24(11): 2973-2982

VI **Ivask, A., Green, T., Polyak, B., Mor, A., Kahru, A., Virta, M., Marks , R.** Analysis of bioavailable Hg and As from samples polluted by mining activities by using optimised fibre-optic biosensors and non-immobilised luminescent bacterial sensors. Submitted to Biosensors and Bioelectronics

Copies of the papers I-VI have been included in the dissertation.

AUTHOR´S CONTRIBUTION TO PUBLICATIONS

I Angela Ivask was responsible for the planning of the experiments as well as their practical implementation. Most of the interpretation of the data and writing was also done by Angela Ivask.

II Angela Ivask was responsibe for the planning of the experiments and performed also the practical work. Most of the interpretation of the data and writing was also done by Angela Ivask, who is the corresponding author of this paper.

III Angela Ivask was responsible for the planning and practical realization of the experiments with bacterial sensors. She participated in the interpretation of the bioavailability data and was responsible for most of the writing as she is also the corresponding author.

IV Angela Ivask was responsible for the planning and implementation of the experiments with Cu and Pb sensor cells. She participated in the interpretation of the bioavailability data and writing.

V Angela Ivask was responsible for the planning and performance of the experiments with bacterial sensors. Handling and partial interpretation of the bioavailability data from sensors was also done by Angela Ivask. She also participated in writing.

VI Angela Ivask participated in the construction and optimisation of the biosensor system. She was responsible for the planning and practical realization of the experiments with non-immobilized sensor bacteria. Most of the handling and interpretation of the data was also done by Angela Ivask, who is the corresponding author for this manuscript.

TABLE OF CONTENTS

INTRODUCTION

1.1 HEAVY METALS

1.1.1 Characteristics of heavy metals

Metals with a density above 5 g $cm^{3\ -1}$ have been referred to as heavy metals. Most heavy metals are transition elements with incompletely filled *d* orbitals and thus, high ligand binding ability (Beveridge et al., 1997). Over half (53) of the 90 naturally occurring elements could be thus classified as heavy metals. In the periodical table heavy metals could be found between vanadium (V) and half-metal arsenic (As), zirconium (Zr) and antimony (Sb) and between lanthanum (La) and polonium (Po). Also the lanthanides and actinides can be refered to as heavy metals (Nies 1999). Although arsenic is not a metal but metalloid, it has been often discussed together with heavy metals due to its toxicity and density above 5 g $cm^{3\ -1}$..

Some heavy metals including Zn, Cu, Mn, Co, Ni are essential for living organisms as trace nutrients (thus, heavy metals are called also as trace elements), being however toxic in concentrations above the physiological need. Some heavy metals have no role in living organisms and some, such as lead (Pb), cadmium (Cd) and mercury (Hg) are highly toxic to biota already at very low concentrations.

1.1.2 Heavy metals in the nature

Heavy metals have always been a part of the nature. In the early stages of the Earth during extensive volcanic activity, considerable amounts of heavy metals were released from the Earth´s crust. Nowadays, most of heavy metals are immobilized in basic rocks whereas their concentrations in different rock types may vary considerably. For example the highest concentrations of mercury can be found in shale, limestone and sandstone (up to 0.29 mg kg^{-1}) and the highest concentrations of chromium can be found in ultrabasic rocks such as serpentinite (up to 2980 mg kg^{-1}) whereas the concentrations of cadmium and zinc are relatively similar in different rock types (Alloway 1995).

Heavy metals can be mobilised from their natural sources due to either natural (rock weathering or volcanic activity) or anthropogenic processes (consumption of fossil fuels, mining metals for industrial needs etc.). It has been estimated that the inputs of metals from anthropogenic sources exceed the contributions from natural sources by several fold thus altering the global cycles of trace elements (Adriano 2001). For example, approximately 20 heavy metals including mercury, arsenic, cadmium, lead and nickel are released into the atmosphere due to fossil fuel burning (Morel 1997). In addition to exhaust gases also solid and aqueous wastes originating usually from industries, herbicides or fertilisers may be the possible sources of heavy metal pollution. Once released, heavy metals may be deposited into the water, following accumulation in sediments or into the soil creating thereby polluted habitats and affecting all kinds of living organisms, from bacteria to humans (Academies 2004). The main route for intake of toxic metals by humans is food. As plants grown in

polluted soil may accumulate large amounts of heavy metals, their consumption is one of the most important causes for metal poisoning (Landis et al., 1999). Also the direct ingestion of polluted soil by children (a study in the USA has revealed that children between 1 and 6 years take up in average 117 mg of soil a day (Möller et al., 2005)) may be a route for metal entrance to human body.

In fact, there are only few heavy metals that can be found in environment in the form and concentration, which may cause harmful effects in living organisms. These are cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), tin (Sn), zinc (Zn) and arsenic (As) (Academies 2004) Most of them will be discussed also in following chapters. These elements plus antimony (Sb), beryllium (Be), selenium (Se), silver (Ag) and thallium (Tl) constitute the 'priority pollutant metals' established by the U.S. Environmental Protection Agency (USEPA) based on potential hazard to human health (Academies, 2004).

In order to control the release and hazard of heavy metals in nature, their concentrations are continuously monitored and according to the natural background values, toxicity to living organisms, bioaccumulation and some other factors a `permitted limit value` (PLV) has been set and fixed in environmental legislation. PLV is the highest concentration of a heavy metal in the environment that presumably poses no risk to living organisms. PLV has been set differently for every metal and medium (water and soil in living or industrial areas). Due to the differences in natural background concentrations of metals in different areas the PLV values may vary in different regions, however a recommended value has been set by the EU (directive 86/278/EEC, see Table 1.1).

Table 1.1 *Summary of occurrence and permitted limit values (PLV) of selected heavy metals in soils presented as mg kg^{-1} dwt.*

Heavy metal	Concentration in the Earth's crust	Concentration in soils directly not affected by any pollution source	Concentration in polluted soils	PLV in soils according to EU directive 86/278/EEC	PLV in soils in Estonia [a]	
					Living area	Industrial area
Cd	0.2[b]	0.06 - 1.1[c]	up to 750[c]	1 - 3	5	20
Zn	75[c]	10 - 300[c]	up to 37200[c]	150 - 300	500	1500
Pb	14[c]	<42[d]	up to 7900[d]	50 - 300	300	600
Hg	0.05[c]	0.02 - 0.19[c]	up to 100[c]	1 - 1.5	2	10
Cu	50[c]	1 - 50[d]	up to 10250[e]	50 - 140	150	500
Cr	100[c]	40[f]	[g]	n.d.	300	800
As	1.5[c]	<10[d]	up to 2500[d]	n.d.	30	50

[a] *(KKM, 2004)*
[b] *(Lugon-Moulin et al., 2004)*
[c] *(Alloway 1995)*
[d] *(Adriano 2001)*
[e] *(Peltola et al., 2003)*
[f] *(Losi et al., 1994)*
[g] *the highest reported values have been found in natural soils, 125 000 mg kg^{-1}*
n.d. – not defined

1.1.2.1 Cadmium

As said above, cadmium belongs among the 'priority pollutant metals' of USEPA. The net production of Cd in the world in 1986 was 19 000 tonnes, whereas the biggest producers were Eastern block countries and the the biggest consumer USA (Moore, 1991). Cadmium occurs in the nature mostly as Cd^{2+}. As all heavy metals, it is naturally present at low concentrations in water and soil. The natural background concentrations in waters are low ranging from 0.1 (seawater) to 0.5μg l^{-1} (river water) (Lugon-Moulin et al., 2004) and the average concentrations determined form non-polluted soils over the world is usually less than 1 mg kg^{-1} (ppm) (see Table 1.1) (Alloway 1995; Lugon-Moulin et al., 2004), although this value can differ between different soil types. Naturally occurring Cd ores can usually be found in association with Zn ore sphalerite (the cadmium content is frequently between 0.1 and 5%) and thus, most of the anthropogenic cadmium is released to the environment as byproduct of Zn ore mining (Vig et al., 2003). Cadmium is mainly used for electroplating, batteries (Nies 1992), production of paints and pigments and as anticorrosive agent in alloys like steel and brass (Morel 1997). Relatively high concentrations of cadmium can also be dispersed into soil by waste disposal, burning of fossil fuels and by means of phosphate fertilisers (Alloway 1995; Morel 1997). In Australia, for example, the input of Cd from fertilizer usage has been estimated to be approximately 1.6 g ha^{-1} yr^{-1}(Nursita et al., 2005). Approximately 13 000 tons of Cd is released into the environment from human activities annually (Il'yasova et al., 2005).

As the result of large-scale release of Cd into the environment, Cd levels in arable soils have significantly increased during the 20th century, reaching even 750 mg kg^{-1} dwt. (Alloway 1995), which is over 750 times higher than the natural average background values and 250 times higher than the recommended PLV in soil (Table 1.1). If compared with the values reported to affect the soil microflora (50 mg kg^{-1} of Cd has been shown to cause 50% inhibition of soil respiration (van Beelen et al., 1997)).

1.1.2.2 Zinc

Although Zn is an essential microelement for all organisms, it has been considered as one of the priority pollutant metals.

Zn occurs in the nature mostly as Zn^{2+}. Most rocks in the Earth's crust contain zinc in varying amounts (in average 75 mg kg^{-1} (Alloway, 1995)), usually in form of sphalerite (ZnS). The average natural background concentration of Zn in soil is 50 mg kg^{-1}, however it may vary from 10 to 300 mg kg^{-1} in different regions (Alloway 1995).

The main sources of zinc contamination in the environment are industrial plants producing metallic zinc (Diels et al., 1990). Metallic zinc is thereafter used for alloys such as brass, for iron galvanisation, fabrication of batteries, steel protection against corrosion but also in pharmaceuticals, medical equipment, cosmetics, fertilizers and animal feed (Nies 1992; Alloway 1995). Total worldwide anthropogenic emissions of Zn in 1983 were estimated to be about 132 000 tons a year, whereas total emissions from natural sources comprise only about 45 000 tons a year (Wolff et al., 1999). Due to the

extensive release of Zn into the environment soils with Zn concentrations up to 37000 mg kg^{-1} could be found (Alloway 1995). This is about 100 times more than the PLV value (Table 1.1) and may certainly cause adverse effects in soil organisms. For example 50 % mortality or earthworms (14-day test) has been already observed at soil Zn concentrations of 1000 mg kg^{-1} (Spurgeon et al., 1994) and effects on soil respiration has already been observed at 55 mg kg^{-1} (van Beelen et al., 1997).

1.1.2.3 Lead

Lead has been widely used in industrial applications and is thus an abundant, globally well-distributed environmental pollutant. Lead has been used since ancient times: the history of Pb smelting by humans reaches back to 3000 years B.C., when the environmental pollution by Pb began. Jerome Nriagu has been stated in his book "*Lead and Lead Poisoning in Antiquity*" (1983) that lead has contributed to the decline of Roman empire: lead could be the cause of the serious health problems of ancient Romans, mainly madness (for comparison, the consumption of Pb by Romans was about 100 fold compared with the amount consumed by an average American in 1980s) (US EPA). Even though many of the toxic effects of lead were known already among ancient Romans, lead become used as a gasoline additive in the 1920s. From 1960 to 1970 tetraethyl-lead was used to enhance the octane number of gasoline and starting from 1970 less hazardous chemicals were introduced to replace Pb. Nowadays, regulations governing the uses and release of Pb into the environment (including the ban of using Pb in gasoline) have been instituted in most of the countries.

In the nature Pb occurs mainly as galena ore (PbS), which contains a number of associated heavy metals like Ag, Zn, Cu, Cd, Cd, Sb, Tl, Se, Te released into the environment as by-products during Pb smelting (Alloway 1995). It has been reported that bout 87% of the Pb produced in US 1994-1995 was used in storage batteries, 4.4 % was used for ammunition and smaller amounts for other uses like production of paints and dyes, pesticides, analytical reagents, explosives (Johnson 1998). Pb-containing paints (containing up to 100 mg of Pb $cm^{3\ -1}$) have been of concern especially for children accidentially ingesting paint-slices flaking off from walls (Rachel´s Hazardous Waste News, 1994). In addition to the anthropogenic activities lead can also be release from natural sources but this is certainly a minor source for lead contamination.

According to the review by (Johnson 1998) the concentrations of Pb measured in sea surface waters range from 15 to 50 ng l^{-1}, which is about 2 magnitudes higher than the estimated prehistoric concentration (0.6 ng l- l^{-1}), lead concentrations in freshwaters range from 1 to 23 μg l^{-1} and in sediments from 40 (rivers and rural streams) to 350 (urban and industrial areas) mg kg^{-1}. One sources for soil pollution by Pb are shooting ranges, where considerable amounts of Pb have been mobilized from used ammunition. Pb concentrations in soils near shooting ranges may exceed >2000 mg kg^{-1} (Peltola et al., 2003). The concentrations of Pb in soils near lead smelters may reach even 7900 mg kg^{-1} (Adriano 2001), which exceeds the PLV about 26 times (Table 1.1) and

may have toxic effects for soil biota (already 400 mg kg^{-1} of Pb in soil has been shown to cause 50 % inhibition in soil respiration, (van Beelen et al., 1997)).

1.1.2.4 Mercury and its organic forms

Mercury is most toxic metal in the list of priority pollutant metals. The natural background concentrations of mercury are also considerably lower than those for other toxic heavy metals (Table 1.1). In non-polluted freshwaters the concentration of mercury ranges between 1-20 ng l^{-1} (ppt) (Morel et al., 1998), in unaffected sediments the concentrations are in μg kg^{-1} (ppb) range (Baldi 1997) and in non-polluted soils the range is from ten to a few hundred μg kg^{-1} (Summers, 1986; Alloway, 1995).

Merury is usually deposited as sulfide (cinnabar, containing up to 10 000 mg kg^{-1} of Hg), which is found in large quantities in magmatic rocks, from where it could be released naturally by volcanic activity. Sedimentary material rich in organic carbon, such as shales, can also contain relatively high concentration of mercury (Fitzgerald et al., 2004).

Released mercury occurs in the nature as elemental (Hg^0), ionic (Hg^{2+}) or organic mercury, particularly methylmercury or dimethylmercury (Morel et al., 1998; Leermakers et al., 2005). In the atmosphere, 95 % of the total mercury is in the elemental state Hg^0 and is slowly (this process may take about 27 years allowing the efficient transport of Hg around the globe) oxidised to the ionic state Hg^{2+} in the presence of ozone and other oxidants (Morel et al., 1998) (Figure 1.1).

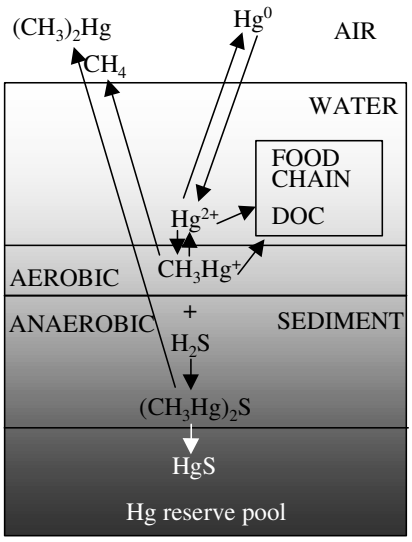


Figure 1.1 *The biogeochemical cycle of mercury after (Baldi 1997). Under aerobic conditions, Hg^{2+} can: a) be reduced by mercury-reducing bacteria to the volatile Hg^0; b) accumulate in the food chain; c) be adsorbed to dissolved organic matter (DOC); d) be transformed to methylmercury. Methylmercury can be: a) accumulated in the food chain faster than Hg^{2+}; b) adsorb to DOC; c) in the presence of H_2S under anaerobic conditions formed to unstable sulphide $(CH_3Hg)_2S$, which will be quickly transformed to the volatile dimethylmercury $(CH_3)_2Hg$ and solid mercuric sulphide (HgS).*

Once oxidised, 60 % of atmospheric mercury is deposited to land and 40 % to water. In water, Hg^{2+} may be reduced by Hg-resistant bacteria or photochemically and return to the atmosphere (Morel et al., 1998). Another part

of Hg^{2+} will be methylated by bacteria to methylmercury (MeHg) (Baldi 1997) (Figure 1.1). Methylmercury is produced by microbes, mostly in aerobic conditions: the biggest Hg methylation activity has been shown to occur in the water-sediment interface and the most upper layer of sediment (Callister et al., 1986). In addition, pH (Winfrey et al., 1990), redox potential (Compeau et al., 1984), temperature (Callister et al., 1986) and nutrients (Furutani et al., 1980) have been shown to affect the mercury methylation. It has been shown that up to 1.3 % from the total mercury in sediment may be methylated (Callister et al., 1986). 90 % of total Hg in muscle tissue of top marine predators has been found to be methylmercury and therefore, MeHg has been considered to be the main biomagnifying species of mercury (Baldi 1997). One of the most famous examples of the biomagnification of methylmercury was in years 1948 to 1960, in Minamata, Japan, where the occurrence of this mercurial in water caused poisoning in thousands of humans who were eating fish contaminated by MeHg (Harada 1995): over 70 humans died and 700 were poisoned (Timbrell 1995). The level methylmercury in fish from Minamata Bay was 5-10 mg kg^{-1} whereas the FDA (USA Food and Drug Administration) has set 1 mg kg^{-1} as maximum permitted concentration in seafood (U.S. FDA, 1982). It has to be mentioned that compared with inorganic mercury the highly lipohilic methylmercury is considerably more toxic to living organisms affecting primarily the nervous system (Elhassani, 1982).

Methylmercury produced by microbes is also degraded by bacteria (demethylation) (see Figure 1.2) and the total concentration of MeHg in the environment is the resultant of these two opposite processes (Baldi 1997). Demethylation occurs mainly in aerobic sediments and is started when the minimal concentration of methylmercury needed for the induction of methylmercury degradative genes is achieved (Baldi 1997). A very minute amount of mercury will be transformed dimethylmercury (DMeHg, present only at concentrations of fM l^{-1}), easily volatile and extremely toxic organic Hg species (Filippelli et al., 1993).

In anaerobic conditions in the presence of H_2S extremely insoluble and very stable mercuric sulphide (HgS) will be formed, which is conserved in the Earth´s natural Hg reserve pool (Figure 1.1).

The main anthropogenic activities leading to the mobilization of mercury are mining (also gold mining, where mercury is used as extractant) (de Kom et al., 1998; Malm 1998), papermaking (Hg has been used as disinfectant), agriculture (the use of organomercurial fungicides, mainly methylmercury for the treatment of seeds), the use of mercurial catalysts in industry and disinfectants in hospitals (Summers 1986; Morel 1997), combustion of fossil fuels and chloralkali plants (Baldi 1997). The biggest mercury mines are located in Spain, Slovenia, China, Algeria and Kyrgyz. The production of Hg in Almadén, Spain started already during the ancient Roman Empire and reached its maximum capacity in 1941. This mine is nowadays one of the main Hg suppliers in the world (annual delivery 1000 tonnes, whereas the total World´s Hg output is ca 9000 tons) (EC, 2005). The recent EU strategy however will ban the trade of Hg from 2011 onwards (EC, 2005). It is estimated that the input of atmospheric mercury has tripled over the past 150 years, which indicates that about two thirds of the mercury now in the environment is of anthropogenic

origin (Morel et al., 1998). The integrated estimate for total loadings from globally dispersed, anthropogenic mercury emissions between 1890 and 1990 is 200,000 tons and about 95 % of this mercury is sequestered in sediments and soils (Fitzgerald et al., 2004). It is estimated that Hg concentration in soil in the most polluted regions can reach 100 mg kg $^{-1}$. (Alloway, 1995), which exceeds the natural background for 1000 times and PLV for 100 times (see Table 1.1). It must be noted that these concentrations are far above the amounts (> 1 mg kg^{-1}) reported to affect the soil microflora (Welp et al., 1997). Similarly to the soils, the polluted sediments may contain several orders of magnitude higher concentrations of Hg than sediments in clean areas (Baldi 1997).

The main exposure pathways of Hg to humans is through the consumption of marine fishery products: fish, especially tuna and swordfish, shellfish, crustaceans. The maximum permitted level of Hg in fish products set by FDA, as mentioned above, is 1 mg kg^{-1} (determined as methylmercury). The Estonian limit for Hg in fish products is 0.5 mg kg^{-1} (RT I 2000) and it should be mentioned that usually the concentrations remain far lower than this set value. According to Estonian Environmental Monitoring Programme the concentrations in fish from coastal areas remain below 0.02 mg kg^{-1} (Seiremonitor). Well-known outbreaks of Hg poisonings are the contamination of Minamata Bay discussed above and the poisoning of bread in Iraq in 1972 caused by inadvertent misuse of grain treated with organomercury fungicides (Leermakers et al., 2005).

1.1.2.5 Copper

Similarly to Zn also copper is a micronutrient for both plants and animals at low concentrations and at the same time belongs among priority pollutant metals. The most important Cu ores are primary sulfate minerals, such as bornite (Cu_5FeS_4) and chalcopyrite ($CuFeS_2$) (Adriano 2001). Cu belongs among the most important metals to man: its application fields include electrical industry (wires, the use of Cu as heat conductor), metallurgy (alloys), agriculture (bactericide and fungicide, algicide), food industry (feed additive, growth promoter, disease control agent in livestock and poultry production) (Adriano 2001). In 1980-s emission of Cu from anthropogenic sources (metallurgy, waste incineration, coal and wood combustion, agriculture) (56300 t year^{-1}) has been considered to be about 3 times higher than the load from natural sources (18500 t year^{-1})(Adriano 2001). Historically the uses of Cu cookware in domestic and small- industrial scale, are probably the cause of Cu pollution near many former workshops. Peltola et al., (2003) measured up to 10 250 mg kg^{-1} of Cu near pitch production facility where Cu tunes had been used for tar cooking. This is 300 times higher than the normal natural background (Adriano 2001). Those high concentrations may have already mortal effects on soil organisms. For example, soils with Cu concentrations >2000 mg kg^{-1} have already reported to cause 100 % mortality for earthworms (Koster et al.,, in press) and soil microflora has been shown to be affected from 55 mg kg^{-1} of Cu in soil (van Beelen et al., 1997).

Naturally occurring concentrations of copper in surface seawaters have been measured from 0.03 to 0.23 μg l^{-1} and from 0.2 to 30 μg l^{-1} in freshwater

systems whereas near pollution sources the Cu concentrations in water may easily reach 100 μg l^{-1}. In waters from mining areas extreme concentrations up to 200 000 μg l^{-1} have been reported.

1.1.2.6 Chromium

Chromium can occur in the environment in two different oxidation states, $^{+3}$ and $^{+6}$ whereas the Cr (III) is more stable (Losi et al.,, 1994; Barnhart 1997). It has been shown that both, the reduction of hexavalent chromium chemically or by microbial action and the oxidation of trivalent chromium by atmospheric O_2 occurs in the nature (Losi et al.,, 1994). Hexavalent Cr is usually present in natural conditions as CrO_4^{2-} and major trivalent Cr forms may include various organic complexes and hydroxides that are usually relatively insoluble in water and precipitates as $Cr(OH)_3$ keeping aqueous concentrations generally low (Losi et al., 1994). On the other hand, Cr (VI) is more toxic (mainly due to its carcinogenic properties, De Flora, 1990) and the majority of Cr (VI) compounds are much more soluble and remain in the solution in aqueous conditions and therefore, can be detected in drinking water and groundwater. However, it has to be mentioned that analytical distinction between different oxidation states of Cr is relatively difficult and does not belong among the standard methods (special pre-treatment is required, Vitale et al.,, 1997). Thus, despite of different toxicity of Cr (III) and (VI) there is only one permitted limit value set for Cr in the environment (see Table 1.1).

The natural background concentrations of Cr in soils depend greatly on the type of basic rocks and may vary even from 10 mg kg^{-1} to 125 g kg^{-1} (Losi et al., 1994). However, 40 mg kg^{-1} could be considered as the average natural background concentration of Cr in soils (Losi et al., 1994). The average natural concentration of Cr in freshwaters is 1 μg l^{-1} (Losi et al., 1994).

The main anthropogenic sources of chromium pollution are metallurgy (production of various alloys used in e.g. stainless steels), pyrotechnics and photography, production of catalysts, pigments, dyes, glues, adhesives, explosives and matches (Losi et al., 1994; Barnhart 1997; Vitale et al., 1997). Considerable amounts of Cr are loaded to terrestrial environment with fertilizers and sewage sludge, where the concentration of chromium may reach even 99 000 mg kg^{-1} (Adriano, 2001).

1.1.2.7 Arsenic

As stated above, arsenic is not a metal but a metalloid (also called as semi-metal) with properties similar to heavy metals. Arsenic, particularly arsenic oxide (As_2O_3) is one of the most famous poisons used since Middle Ages, and probably the most famous victim of this poison is Napoleon Bonaparte (Jones et al., 1982). Even nowadays As is mostly used due to its toxic properties. Its most intensive uses are in agriculture, where As_2O_3 has been used as the raw material for arsenical pesticides and in wood preservatives. As has also been used in ceramic and glass industry and in production of special lead and copper alloys. (Adriano 2001) The primary sources for As production are Cu, Zn, Pb, Hg, Au and other ores, from which smelting process As is recovered as by-product (Nriagu et al., 1988).

Arsenic is present in environment mostly in 2 oxidation states, (V) and (III) whereas the prevailing species in oxic conditions is As (V). It occurs naturally in all environmental matrices: it is a major constituent of more than 245 minerals (Adriano 2001). Thus, the soils originating from As-rich minerals like sulfide ores, contain remarkably higher concentrations (up to 8000 mg kg^{-1} compared with 4-6 mg kg^{-1}, which is the average value in soils (Adriano, 2001)). Leaching of arsenic from minerals may also cause groundwater contamination. Probably the most famous example of this is the water crisis in Bangladesh, where the groundwater coming from recently installed tube wells exceeds the World Health Organization (WHO) guideline for drinking water (10 $\mu g\ l^{-1}$) by 5 times as standard and even by 200 times in some areas (Hossain, 2006). According to (Adriano, 2001) the amount of As released to the environment by natural mobilization is about 7800 t $year^{-1}$ whereas the anthropogenic load from both agricultural and industrial sources exceeds this amount for 3 times.

1.1.3 Analytical measurement of heavy metals

Several chemical methods: mass spectrometry (MS), atomic fluorescence spectrometry (AFS) or atomic absorption or emission spectroscopy (AAS or AES) together with chromatographic techniques or inductively coupled plasma (ICP) are used for the detection of heavy metal contents in both from liquid and solid samples. Different techniques can be used in the sample preparation but the obligatory steps include heating and acid (nitric or sulphuric acid, or their mixture) digestion in order to destroy organic material in the sample and release all bound heavy metals (Beveridge et al., 1997). This step in preparation, attempting to mobilize maximum amount of metals is similar for all sample types. Despite of the method used, the sample preparation for chemical analysis is relatively laborious and time consuming. Talking about environmental samples, the limits of determination of chemical methods are usually enough to measure the concentrations of heavy metals naturally occurring in the environment (Clement et al., 1997; Vazquez et al., 1997; Vitale et al., 1997; Bowles et al., 1998).

Although different sample preparation protocols enable to determine also the metal speciation (i.e. the chemical form in which the metal occurs), these analyses are not carried out routinely because of their relative complexity (Hughes et al., 1991). For example it is possible to differentiate between chromium (III) and chromium (VI) in the sample (Vitale et al., 1997). This kind of speciation is very important because of significantly different toxicity of the two valence states of chromium: Cr(VI) may cause mutagenic effects whereas Cr(III) is known as micro-nutrient (Losi et al., 1994). Also, differentiation between mercury ions and organic compounds of mercury is possible, which is of great importance because of the very high toxicity of organomercury compounds (Bowles et al., 1998; Harrington 2000). However, as pointed out previously, such kind of analyses are not belonging among traditional methods and are usually complicated to perform, if low detection limits are requested.

1.2 BIOAVAILABILITY

The term bioavailability has been traditionally used in pharmacology and toxicology to express the systemic availability of a xenobiotic after intravenous or oral dosing. Nowadays, the term bioavailability is also widely used in environmental sciences and has become a key issue in the environmental hazard assessment. There is no unique definition for bioavailability in environmental sciences but a range of different definitions has been used: in practice more or less every author has come out with his own formulation. They range from relatively simple (`the ability of a substance to interact with the ecosystem or an organism`) to much more complex definitions (`the amount/percentage of a compound that is actually taken up by an organism as the outcome of a dynamic equilibrium of organism-bound uptake processes, all in relation to a dynamic set of environmental conditions`). Also, the nature of bioavailability has been differently understood by different authors: some consider bioavailability to represent the accessibility of a soil-bound chemical for possible toxicity and others the fraction, which has entered the cell and become available at a site of biological activity (Semple et al., 2004; Academies, 2004). According to the classification by (Semple et al., 2004) the bioaccessibility (available to cross a cellular membrane from the environment if the organism has access to the chemical) and bioavailability (freely available to cross a cellular membrane, i.e., the transfer between the organism and medium is not taken into account) could be defined as `bioavailability processes`.

The bioavailability issues have been discussed in environmental sciences for about 30 years since it became evident that some contaminants in soils and sediments appear to be less available to cause harm to humans and ecological receptors than is suggested by their total concentration indicating that total levels of contaminants expressed as bulk concentrations may not correlate with their actual biological hazard. Mining sites were some of the first to receive attention as sites where the total amount of contaminant present is not the best indicator of the actual risk for human health. Currently the absolute bioavailability of Pb in soil to humans (e.g., due to the soil ingestion by children) is considered to be about 30 percent of the total amount of metal (default value of U.S. Environmental Protection Agency). Most of the discussion about pollutant bioavailability has referred to soils and sediments. This is due to their great sorption capability for different kind of contaminants, both organic and inorganic (e.g., heavy metals) (Academies, 2004). In following chapters the bioavailability of heavy metals in soils will be discussed in detail.

1.2.1 Bioavailability and toxicity

In general, bioavailability is a perquisite for toxicity as usually only the fraction of chemical entered a living cell may cause adverse effects.

1.2.1.1 Toxicity of heavy metals

As discussed in previous chapters, almost all heavy metals may cause toxic effects in certain concentrations. In order to enter the living cells, the heavy metals have first to be solubilized and thus, the toxic effects may usually only be caused by metal ions. The toxicity of ions of 2-valent metals consists

usually on their ability to bind to sulphydryl groups of proteins, replace the essential metals and thus, inhibit the normal activity of these proteins (Nies, 1999). Compared with other 2-valent metals, mercuric cations have a high affinity for sulphydryl (–SH) and consequently they can disturb almost any reaction where critical proteins are involved (Patra et al., 2005). The high toxicity of mercury to different organisms compared with other metals is also shown in Table 1.2. Due to the great similarities of Cd and Zn ions, Cd may substitute for zinc in Zn-containing enzymes participating in biological reactions like synthesis of DNA, RNA, and protein (Il'yasova et al., 2005). Moreover, in humans, Cd has been shown to sobstitute for Ca^{2+}, being thus a risk factor for osteoporosis (Brzóska et al., 2005). Replacement of Ca^{2+} ions by Pb^{2+} has shown to be the cause for neurological disorders (Goyer, 1997; Belitz et al., 1999). Some metals, like arsenic and chromium (VI) have been shown to cause mutagenicity in living cells (Dong et al., 1994; Losi, 1994). It has been suspected that the mutagenic effects of arsenic are due to the disruption of thiol proteins by trivalent arsenic and the cosequent production of toxic oxygen radicals by these damaged proteins (Gonsebatt et al., 1994). According to Losi (1994) the mutagenic effect of hexavalent cromium is due to its strong oxidizing capabilities.

As seen from Table 1.2 the maximum concentrations of heavy metals laid down in the legislation, are in good agreement with the toxicity data. For groundwater, the lowest permitted limit value (PLV) is for Hg, which is also the most toxic element. The PLV-s for other metals increase in order Cd<As<Pb=Cr<Zn. Althoug the limit value set for Zn exceeds the E(I)C50 for algae and crustaceans, this value is obviously so high due to the beneficial effects of Zn to other organisms.

Table 1.2 *Toxicity (expressed as LD50: dose lethal to 50% of organisms; LC50: concentration lethal to 50 % of organisms; E(I)C50: concentration causing 50% decrease of test parameter) of selected heavy metals to different organisms*

Organism (route of exposure)	**Effect/ Exposure time**	**Pb^{2+}**	**Cd^{2+}**	**Hg^{2+}**	**Zn^{2+}**	**Cr^{6+}**	**As^{5+}**
				mg kg^{-1} body weight			
Rat (oral)	LD50 / acute toxicity	3200 [a]	54 [b]	155 [c]	525 [d]	4 [e]	8 [f]
				mg l^{-1}			
Fish *Oncorhynchus* sp.	LC50 / 96 h	0.3 [g]	0.02 [h]	0.66 (24h) [i]	0.64 [J]	12 [k]	66.5 [l]
Waterflea *Daphnia magna*	EC50 / 48 h	1.8 [g]	0.55 [m]	0.0074 [m]	0.05 [n]	0.7 [k]	1.12 [g]
Algae *Selenastrum capricornutum*	IC50 / 96h	0.07 [o]	0.04 [o]	0.009 [p]	0.1 [o]	0.02 [k]	0.7 [q]

Table 1.2 Continued

Organism (route of exposure)	**Effect/ Exposure time**	**Pb $^{2+}$**	**Cd $^{2+}$**	**Hg $^{2+}$**	**Zn $^{2+}$**	**Cr $^{6+}$**	**As $^{5+}$**
		mg l^{-1}					
Bacteria (luminescent *Vibrio fisheri*, Microtox test)	EC50 / 15 minutes	0.46 [r]	1.7 [r]	0.046 [r]	3.4 [r]	27 [r]	1.07 [r]
PLV in groudwater in Estonia, **mg l^{-1}** [s]		0.2	0.01	0.002	5	0.2	0.1
PLV in soils in Estonia, living zone, **mg kg^{-1}** [s]		300	5	2	500	300	30

[a] *Material Safety Data Sheet, http://www.jtbaker.com/msds/englishhtml/l2434.htm*
[b] *Material Safety Data Sheet, http://www.jtbaker.com/msds/englishhtml/c0105.htm*
[c] *Material Safety Data Sheet, http://www.jtbaker.com/msds/englishhtml/m1573.htm*
[d] *(Domingo et al, 1998)*
[e] *Material Safety Data Sheet, http://www.catalogue.fisher.co.uk/scripts/search.dll?ViewMSDS&SheetNumber=19370*
[f] *http://yosemite.epa.gov/oswer/ceppoehs.nsf/Profiles/7778-44-1?OpenDocument*
[g] *PAN Pesticides database, www.pesticideinfo.org/*
[h] *(Davies et al., 1993)*
[i] *http://avogadro.chem.iastate.edu/MSDS/HgCl2.htm*
[j] *http://www.eprf.ca/ebi/contaminants/zinc.html*
[k] *http://www.omegachemistries.com/pdf%20files/Potassium%20Dichromate.pdf*
[l] *(Hamilton et al., 1990)*
[m] *(Janssen et al., 1993)*
[n] *http://ecb.jrc.it/esis/*
[o] *(Aruoja et al., 2004)*
[p] *http://www.eurochlor.org/upload/documents/document88.pdf*
[q] *http://www.usbr.gov/niwqp/guidelines/Arsenic.pdf*
[r] *(Kaiser et al., 1994)*
[s] *Permitted limit values, set by KKM (2004)*

In general, the PLV-s for soil increase in the same order as the values set for groundwater (Table 1.2). However, compared with the water PLV-s those of soil are 100 to1000-fold higher showing a considerably lower environmental hazard of heavy metals in soils than in water. This lower risk is due to the low bioavailability of heavy metals in soil discussed in next chapter.

1.2.2 Bioavailability and toxicity of heavy metals in soils

As soil is the key component of terrestrial ecosystems being essential for the growth of plants and offering a habitat for many other organisms, the

occurrence of heavy metals in soil is of great importance. However, soil is a very complex system comprising mineral and organic solids, aqueous and gaseous components and also living organisms that have an integrated effect on the behaviour of heavy metals (Alloway, 1995). Soil is formed from combined effects of climate, vegetation, soil organisms and time on rocks and parent materials. The main mineral components in soil are products of rock weathering or oxides of Fe, Al and Mn and the soil organic matter consists of living organisms, dead plant material and colloidal humus. The solid components in soil are usually clustered to aggregates between that there is a system of pores filled either with air or water. The behaviour of heavy metals in soil is mainly affected by the solid soil components because of their ability to sorb ions, which can occur:

i) non-specifically (formation of weak bonds between positively charged heavy metal ions and negative charges of carboxyl and phenolic groups on the surface of soil particles due to cation exchange) or

ii)specifically (formation of covalent bonds between the metal cations and surface ligands such as Al, Fe, Mn oxides),

iii) by co-precipitation (together with clay minerals, Ca carbonates, Fe, Mn oxides, formation of insoluble metal sulphides) or

iv)complexation (e.g., with organic material) (Losi et al., 1994; Alloway, 1995). However, the sorption of different heavy metals occurs in different extent, for example Pb has shown to sorb to soil more than Cu which in turn sorbs to soil more than Cd and Ni (Biddappa et al., 1981). Generally, the sorption of heavy metals in soils is influenced by many factors:

i) pH (heavy metal cations are usually most mobile under acid conditions),

ii) redox potential (decreased redox potential leads to reduced mobility),

iii) organic matter content (generally higher organic matter content decreases metal mobility),

iv) cation exchange capacity, i.e. `the total charge excess of cations over anions for a soil` (Academies, 2004) (the higher the capacity to exchange ions, the higher the adsorption of metals),

v) particle size (the higher the surface area the higher sorption) (Alloway, 1995; Academies, 2004). In addition to the listed soil properties also the `age `of pollution has been shown to play an essential role in metal sorption. A number of studies have shown that heavy metals, which have been stayed in soils for longer periods (so called "aged") are more tightly sorbed than metals, which have been in the soil for shorter time (Alexander, 2000). (Vig et al., 2003) found that the concentration of desorbed Cd in freshly contaminated soil (3 mg Cd kg^{-1} soil) decreased exponentially in time from 0.012mg l^{-1} to <0.0006 mg l^{-1} within 50 days of ageing and explained it by the formation of low-solubility metal precipitates on mineral surfaces.

Mobility and sorption of metals in soil is schematically presented in Figure 1.2 A. It can be seen that a considerable fraction of metals is firmly bound and cannot be mobilized under normal conditions. Thus, this fraction do not usually pose any risk to soil biota as it is not bioavailable. Relatively small fraction of metals in soil is in soluble form and present in soil solution. This is the fraction, which has most often been considered to be bioavailable (Vig et al.,

2003). However, this kind of parallel is true only for some organisms. For example, lately it has been shown that in the case of earthworms the main exposure to heavy metals occurs via dermal contact with pore water (Vijver et al., 2003). For some organisms the bioavailability of metals in soil is not limited to soluble fraction of metals. For example, plants (most used for bioavailability studies due to the importance of plants as human and animal food) may easily take up also weakly soil-adsorbed heavy metals (Figure 1.2 B). This could be due to excretion of different chelating and other metal-mobilizing substances by the roots promoting the assimilation of mobilized metals by plants and also other soil organisms (Morel 1997). Thus, the bioavailable fraction of metals to plants is far from being equal with the water-soluble fraction. Mobilization and enhanced uptake of metals from soils could also be due to activity of other living cells producing extracellular metal-complexing polymers. Bacterial cells can dissolve minerals by direct (enzymatic) or indirect action. For example, an extracellular Cu-reducing protein, has been considered to promote the uptake of Cu by *Enterococcus hirae* cells (Solioz et al., 2003). The indirect dissolution of minerals can be the result of microbial activity connected with production of organic and inorganic acids, and oxidizing agents that can influence soil conditions including changes in pH and redox potential (Chenu et al., 2002; Rensing et al., 2003). Metals can also be mobilized from geological sources by complexation with end products of microbial metabolism or with metal-sequestering agents (siderophores) produced by cells to acquire microelements, necessary for the synthesis of enzymes, vitamins and cofactors (Chenu et al., 2002).

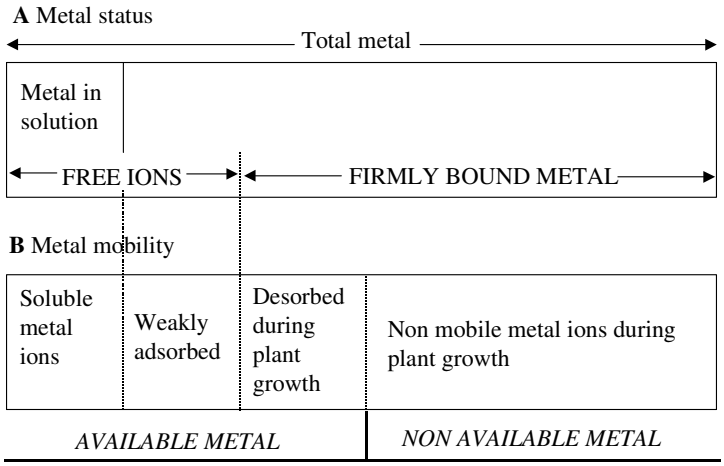


Figure 1.2 *Model of phytoavailable heavy metals in soil by (Morel, 1997).* ***(A)*** *indicates the metal status and* ***(B)*** *shows the mobility and phytoavailability.*

There are a number of processes determining heavy metal bioavailability in soils. They include contaminant interactions with solid/liquid phase (association, dissociation processes, influenced also by the activities of soil organisms as discussed above), transport of contaminants to organisms (either via liquid phase or direct interaction between organisms and solid

phase), the entry of contaminants into living cells (passage through a biological membrane), contaminant accumulation within organisms and possible toxic effects (Figure 1.3).

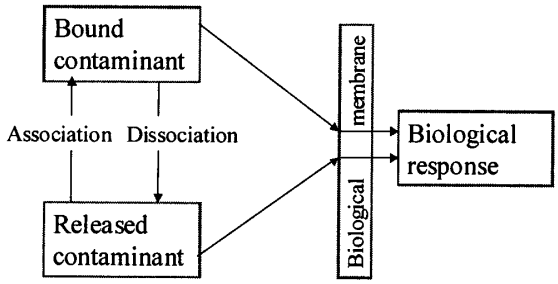


Figure 1.3 *Bioavailability processes in soils and sediments. After (Ehlers et al., 2003)*

Herewith it is appropriate to introduce the term bioaccessibility, which has been defined as the fraction of pollutant, which is available to cross an organism´s cellular membrane (Semple et al., 2004), and thus could be regarded as potentially bioavailable fraction. The most important factor affecting the bioaccessibility of metals in soils is their interaction with solid phase (sorption), which depends greatly on the soil properties as discussed above and thus, varies among different samples (Academies, 2004). The subsequent entrance of bioaccessible metals through membrane and biological response may differ among different organisms (Semple et al., 2004) complicating the uniform understanding of heavy metal bioavailability. Issues connected with determination of heavy metal bioavailability are discussed in next section.

1.2.2.1 Assessment of heavy metal bioavailability in soils

Due to a number of factors influencing the bioavailability of contaminants in soils, which were discussed above, the determination of bioavailability is very complicated and no universal (chemical or biological) test has been developed. However, as stated above, there is a great need for the assessment of real biological effects of metals in soils as in most cases they are not correlated with the total heavy metal content.

There are two possible approaches, which have been used to obtain information on bioavailability.

First – chemical - is the prediction of potentially mobile (possibly bioaccessible) metals by different extraction schemes. Water extraction (weak extraction) or single and sequential extraction with certain extractants (stronger extraction) have been used for that. Weak acetic acid (0.11 M), sodium acetate (1 M), $K/MgNO_3$ (1 M), $MgCl_2$ (1 M) and $Ca(NO_3)_2$ (0.5 M) have been used to predict the mobility of metals in soils (Ure et al., 1995; Basta et al., 2000) and thus, bioaccessibility to soil organisms. Extraction schemes have been developed to predict the bioavailability of metals to higher organisms: a digestive tract model for humans consisting of different extraction steps (simulating the release of pollutants from soil due to gastric juice and intestinal

conditions) (Hack et al., 1996; Ruby et al., 1996). It should be mentioned that predictions based on water-extractability of pollutants often underestimate their bioavailability (see Figure 1.2), whereas the correlations between extraction with different salts, weak acids and bioavailability have in many cases still to be studied: as stated by (Basta et al., 2000) neither the exchangeable fraction of Cd, Pb nor Zn extracted with 0.5 M $Ca(NO_3)_2$ did predict their plant-available fraction. Also, according to the most recent literature, acetic acid extracted fraction has been used for the estimation of metal bioavailability to earthworms in soils under the influence of metal industries, where the acetic acid extractable metal concentrations did not explain earthworm concentrations better than total soil concentrations did (Hobbelen et al., 2004). In fact, due to the lack of information on the prediction capability of the extraction procedures their use in bioavailability assessment has been fairly criticised (Chaignon et al., 2003).

The second approach –biological - to obtain information on heavy metal bioavailability is the use of living organisms. It must be noted that these `bioassays` differently from standard analytical methods determining directly the concentration of metals, determine their effects (i.e., the fraction of metals already entered the cell, bound to the target(s) and having an impact). The effect measured is usually toxicity expessed as mortality, inactivation or decrease in a specific vital parameter (e.g., enzyme activity). The Organisation for Economic Co-operation and Development (OECD) toxicity tests for soils include assays with earthworms (OECD 207, mortality is measured), bacteria (OECD 216 and 217, carbon and nitrogen mineralization by soil microbes is measured) and plants (OECD 208 seed germination is measured). Currently, these tests are mainly applied for testing the environmental hazard of new chemicals. Also, the toxicity test with luminescent bacteria (luminescence inhibition as toxicity measure) initially applied for aquatic toxicity measurements, have been used for the study of soil leachates (Microtox test) as well as soil suspensions (Solid-Phase Flash assay) (Põllumaa et al., 2000 and 2004). The use of micro-organisms in soil analysis has several advantages. First, there is considerable reduction testing volumes compared with plants or animals. Also, the cost and time of analysis is reduced. Second, the use of micro-organisms in soil analysis is relevant as microbes play an unique role in the soil ecosystem (e.g., degradation of organic molecules, mobilization of metals). Moreover, compared with earthworms and nematodes the soil micro-organisms have been reported to be more sensitive towards heavy metal pollution (Vig et al., 2003). However, it should be mentioned that most soil toxicity tests respond to heavy metals in extremely high concentrations. The 14-day *Eisenia fetida* LC50 values for Pb is 4480 mg kg^{-1} of soil, for Zn 1010 mg kg^{-1} and for Cd >300 mg kg^{-1} (Spurgeon et al., 1994). No toxicity for soil microflora (number of heterotrophic bacteria and microbial biomass) was observed in soil containing 1000 mg kg^{-1} of Cd (Fritze et al., 2000). However, if more sensitive parameters (e.g., soil ATP and enzyme content) have been measured, considerably lower concentrations have been shown to affect soil micro-organisms. Welp (1999) reported that the toxicity for soil microbial activity, measured as 50% inhibition in dehydrogenase assay was 652 mg of Pb kg^{-1} of soil, 115 mg of Zn kg^{-1} and 90 mg of Cd kg^{-1} and Dar (1996) showed a decrease of dehydrogenase activity in soil containing 50 mg Cd kg^{-1}. The relatively low sensitivity of soil organisms towards metal pollution

indicates that the bioaccessibility and bioavailability of heavy metals in soils is apparently rather low. The latter is reflected also by relatively high permitted limit values for heavy metals in agricultural soils (see Table 1.1).

One of the issues in using living organism-based tests is the transfer of results from one species to another. As discussed above, the entrance of metal ions to different organisms (e.g., ingestion of contaminated particles by sediment-ingesting organisms, diffusion of dissolved contaminants via the skin of earthworms or uptake of contaminants by bacterial cells) may be different (Semple et al., 2004). In addition, the effects measured by different organisms (toxicity, amount of specific proteins) may be induced by different metal concentrations and thus, when presenting the data or predictions on contaminant bioavailability, the target organism, measured effect and test conditions should always be mentioned.

Some models have been developed to predict the heavy metal mobility and bioavailability in soils. However, they are all still in test stage. In mobility models mostly the soil parameters have been taken into account to predict the metal partition between the solid and liquid phase. The number of parameters included varies from three (Sauve et al., 2000) to nine (Francois et al., 2004) whereas the more parameters included the higher the prediction capability. Models for bioavailability should in addition to chemical data (metal partitioning between different phases of the sample, possible bonds between ligands in the sample and metal, form of metals in the conditions present in the sample etc.) take into account the organisms properties and their influence on metal behaviour in soil, which is already more complicated. There is only one widely used model to predict the metal toxicity to living organisms, the biotic ligand model (BLM) (Niyogi et al., 2004), which however has only be applied for aquatic environment. The BLM model takes into account both the chemical speciation of metals (mainly the amount of free ions) and properties of the target organisms (binding of the metals to biological ligands). Historically the first (developed in the beginning of 1990s) and the most well-studied BLM model was the fish gill model, in which the proteins on fish gills were considered as biological ligands (Niyogi et al., 2004). Later also reports of models for other organisms like daphnids (toxicity of metals instead of ligand binding value) (De Schamphelaere et al., 2002) and phytoplankton (ligands on cell surfaces) (Campbell et al., 2002) have been published. No models for bacterial cells have been published so far. Unfortunately, the BLM in the current stage cannot be successfully applied for soils. This is because by this model only the bioavailability of water-soluble heavy metals could be predicted, without taking into account the effect of direct contact between the organism and the soil solid phase and its effects on net bioavailability.

1.3 BACTERIAL RESISTANCE MECHANISMS TOWARDS HEAVY METALS

As discussed above, heavy metals are important elements in the environment being present in the nature already for millions of years. Although many metals are essential for living organisms, all metal ions are toxic at some level. As it was stated already by Paracelsus: poison or remedy – it is dependent on concentration. Fe, Mn, Zn, Mo, Co, Ni, Cu, V, W belong among the group of

essential heavy metals and become toxic mostly at millimolar concentrations. Metals without any known biological importance like Hg, Cd, Pb, Ag may induce toxic effects already at several magnitudes lower concentrations. Most heavy metal cations tend to bind strongly to sulphide groups in proteins by inhibiting their further functionality.

In order to cope with these toxic effects, many organisms living in metal-rich environments have developed mechanisms for heavy metal "immunity". In literature there are two terms to describe this characteristic: metal tolerance and metal resistance.

Metal tolerance can be described as an indirect effect of an organism resisting the toxic effects of metals due to for example possession of an impermeable cell wall, production of extracellular polysaccharide or lack of specific metal transport systems (Beveridge et al., 1997). Metal tolerance can be due to methallothioneins (cysteine-rich metal binding proteins) that are usually acting as intracellular metal depositories, which bind the excess of metal ions but in the case of need deliver metal ions to the respective metalloproteins. Metallothioneins have been found in most animals, higher plants and lower eukaryotes such as yeast but also in prokaryotes such as cyanobacteria from genus *Synechococcus* (Silver et al., 1996) and bacteria from *Pseudomonas* family (Blindauer et al., 2002). Expression of methallothioneins may be regulated and controlled by the heavy metals towards which the tolerance is achieved. For example the expression of a cyanobacterial methallothionein conferring tolerance towards zinc and cadmium is also controlled by these metals (Turner et al., 1995).

Metal resistance is achieved predominantly due to genetically encoded resistance genes which expression is usually very precisely regulated (Silver et al., 1996). The resistance mechanisms towards heavy metals arose obviously shortly after prokaryote life started, in an already metal-polluted world and have been so far found in all known bacterial groups, both eukaryotes and archaea (Ji et al., 1995; Silver 1996). The metal ion resistance systems can be localized in different genetic elements - either in plasmids, transposons or chromosomes whereas frequently, the genes initially found in plasmids have been subsequently found in the chromosomes of other bacteria (Silver, 1996). There are five mechanisms generally proposed for heavy metal resistance in bacteria and other microorganisms:

i) exclusion of the metal by a permeability barrier;

ii) exclusion by active export of the metal from the cell;

iii) intracellular physical sequestration of metal by binding proteins or other ligands to prevent it from damaging the metal-sensitive cellular targets;

iv) extracellular sequestration; and

v) transformation and detoxification (Choudhury et al., 2001). Attention must be drawn also to the fact that the most studied metal-resistant bacteria carry resistance determinants towards several heavy metals as in the polluted areas usually more than one metal is present.

A very uselful model for research on bacterial resistance has been a multi-resistant *Ralstonia metallidurans*, which was found to be resistant to Zn, Cd, Co, Ni, Cu, Cr, Hg and Pb (Mergeay et al., 2003). This strain has been found in highly polluted environments e.g., near metal smelters and the

resistance in this strain is achieved due to 2 megaplasmids, which can be transferred further in population. Until now, in no other bacterial strain the resistance towards so many different heavy metals has been identified. However, the new metal resistance genes are continuously discovered (for example, recently resistance systems towards Cd in *Caulobacter crescentus* (Braz et al., 2005), Cu in *Enterococcus sp*. (Hasman 2005), and an archaeon *Ferroplasma acidarmanus* (Baker-Austin et al., 2005), Ni and Co resistance in *E.coli* (Rodrigue et al., 2005) have been identified). Thus, obviously the current knowledge about both heavy metal homeostasis and resistance systems in bacteria is relatively limited.

1.3.1 Transport of metals into cells

To induce a biological effect, metals must be first transported into the living cells. Non-polar hydrophobic compounds of metals (for example methylated metals such as methylmercury) can diffuse readily through the cell membrane. However, charged ions need a transport system to get into the cells. At first glance, divalent metal cations are structurally very similar (for example the diameter of Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} Cu^{2+} and Zn^{2+} varies only 14%) and metal oxyanions resemble the structures of acid anions (e.g., chromate is similar to sulphate and arsenate to phosphate and thus, are transported into the cells via sulphate and phosphate uptakes systems, respectively) (Nies 1999). Thus, the differentiation between different metal forms is a difficult task for the cell. In most bacterial cells two types of metal uptake systems are present: one is fast, unspecific, constitutively expressed and driven by the chemiosmotic gradient across the cytoplasmic membrane, the other has high substrate specificity, is slower and uses ATP energy (Nies 1999). The well-known fast and unspecific cation transport system in bacteria belongs to MIT Mg^{2+} uptake system family, which facilitates the transports of most cations (also toxic ones, like Cd^{2+}, Pb^{2+}). For oxyanions fast phosphate (used to transport also arsenate) and sulfate (used for chromate transport) uptake mechanisms are known (Nies 1999). The most well-known specific metal transporters belong to ATP-binding cassette (ABC) transporters and are P-type ATP-ases, which exist only for essential metals (Mg^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}). Also the specific uptake systems may "make a mistake" and facilitate the transport of toxic metals. For example, it has been assumed that Cd^{2+} enters the bacterial cells through Mn^{2+} -specific transport systems (Tynecka et al., 1981; Laddaga et al., 1985). Mercury is the only non-essential heavy metal having its own import mechanism (Osborn et al., 1997). This transport system is a part of the resistance mechanism and is discussed in detail in the next chapter.

Once entered the cells the concentrations of the free metal ions are expected to be very low. Most of divalent metals, e.g., Pb(II), Zn(II), Cd(II), and Hg(II), are likely to be present inside cells as complexes with glutathione, tightly bound to specific metal-binding proteins or as yet unidentified "chaperone" proteins.

1.3.2 Mercury resistance

The mercury resistance in bacteria can be achieved due to transformation of Hg ions to less toxic compounds like elementary mercury (will be afterwards volatilised) or in some bacterial species also into methylated Hg forms (Osborn et al., 1997). Although the methylated mercuric compounds are generally considered to be more toxic than Hg^{2+}, for some bacteria these compounds may be of lower apparent toxicity due to the subsequent formation of dimethylmercury, which volatilises even more efficiently from the cells than Hg^0 (Baldi 1997). In this section, only the resistance mechanism leading to the formation of elementary mercury will be discussed.

Mercuric ion is one exception among toxic heavy metals, which has its own uptake system as a part of the resistance mechanism allowing the efficient detoxicification of Hg(II) ions.

Hg resistance operon mer is probably one of the most extensively studied metal resistance mechanisms in bacteria. The organization of *mer* cluster has been found to be similar for all Gram-negative bacteria (Figure 1.4) whereas the structure of *mer* operon in Gram-positive bacteria is somewhat different (Osborn et al., 1997).

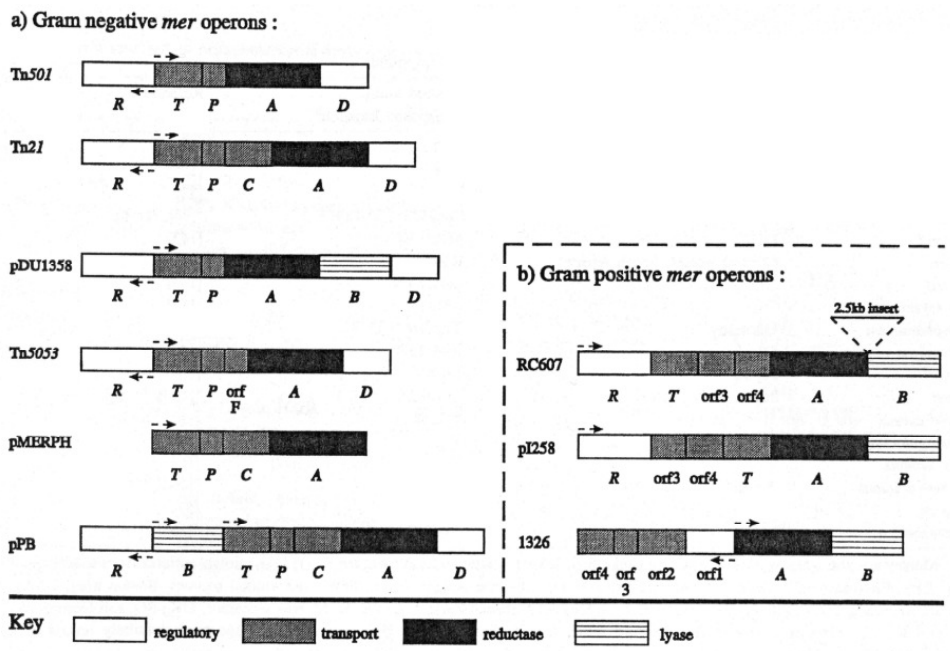


Figure 1.4 *Organisation of genes of the mercury resistance operons (mer operons) from Gram-negative Pseudomonas aeruginosa (transposon Tn501), Shigella flexneri (transposon Tn21), Serratia marcescens (pDU1358, Xanthomonas sp. (transposon Tn5053) Pseudomonas putrefaciens (plasmid pMERPH) and Pseudomonas stutzeri (plasmid pPB) and Gram-positive Bacillus sp. (plasmid RC607), Staphylococcus aureus (plasmid pI258) and Thiobacillus ferrooxidans 1326 chromosome, modified from (Osborn et al., 1997). Direction of transcription is shown with arrows.*

In this review, only the mer operon of Gram-negative bacteria will be discussed. As it can be seen from Figure 1.4, all mer operons from Gram-negative bacteria consist of at least five genes: *merR*, *merT*, *merP*, *merA* and *merD*. In some clusters, e.g., mer operon from transposon Tn21 there is an additional gene *merB*.

Expression of all the *mer* genes in Gram-negative operons is controlled by the operator-promoter region, which consists of two promoters: promoter from which the *merR* is transcribed in counter-clockwise direction (divergently from other *mer* genes) and promoter from which the other *mer* genes are transcribed in clockwise direction (Figure 1.4). This operator-promoter region is controlled by the product of *merR* gene, the regulatory protein of the *mer* operon. This protein belongs to the MerR family of regulators (Brown et al., 2003) with characteristic extended spacer region between –35 and –10 consensus sequences (Stoyanov et al., 2001). The regulation mechanism of the MerR is following: once transcribed from the promoter of *merR*, the MerR protein dimerises, binds to the promoter-operator region of structural *mer* genes between hexamers –10 and –35 and attracts the RNA polymerase establishing a stable non-transcribing pre-initiation complex (allowing the immediate start of transcription when Hg ions will be present). When Hg^{2+} is bound to MerR, an allosteric change in the protein will be induced, which leads to the underwinding of the operator DNA and consequent improved access of RNA polymerase to the transcriptional start site. (Barkay et al., 2003). One MerR has been shown to bind one Hg ion by tri-coordinate Hg-SR_3 (from three cysteine residues) complex (Barkay et al., 2003). MerR has very high affinity to mercury ions inducing the transcription of the *merTP(C)A(B)D* genes already in the presence of femtomoles (10^{-15} M) of mercury (Bontidean et al., 1998). It has been found that there is also an antagonist for MerR – MerD, which binding to the operator region (occurs in much lower affinity than in the case of MerR) induces the constant expression of structural *mer* genes (Barkay et al., 2003). However, the function of this kind of regulation has not been ruled out yet. It should be mentioned that MerR is not completely specific to Hg^{2+} ions but also Cd^{2+} and Zn^{2+} are capable of binding to this protein, however with 100 and 1000 times lower affinity (Caguiat et al., 1999).

The mercury resistance mechanism induced by MerR and Hg ions is schematically presented in Figure 1.5. The resistance mechanism starts with the influx of Hg^{2+} ions into the cell mediated by the products of *merP* (periplasmid Hg binding protein) and *merT* (transmembrane protein transferring the Hg ion to cytosol) (Osborn et al., 1997). In case of some *mer* operons (e.g., in *Thiobacillus*) also a transmembrane protein MerC has been found to participate in the uptake of Hg^{2+}, probably by duplicating the functions of MerT (Misra 1992; Barkay et al., 2003). Once entered the cell, mercury ions bind to the cysteine pair of the mercuric reductase, dimeric MerA either directly or by the medium of cytosolic thiol redox buffers such as glutathione. Here, the Hg^{2+} ions are reduced to Hg^0 in a NADPH-dependent reaction. The non-toxic Hg^0 is thereafter released into the cytoplasm and volatilised from the cell. (Barkay et al., 2003). The mechanism in which only the mercury ions are detoxified is referred to as narrow- spectrum mercury resistance system. The resistance mechanism in which also organic compounds of mercury are detoxified is

called for broad-spectrum resistance system. The resistance towards organomercurials in the broad-spectrum resistance determinants such as in plasmid pDU1358 (Figure 1.4) is achieved due to organomercurial lyase, product of *merB* gene. The MerB enzyme catalyses the cleavage of mercury-carbon bond (Begley et al., 1986), releases appropriate organic groups and Hg^{2+} ions, which will be further detoxified by MerA, analogously to the narrow-spectrum resistance system (Figure 1.5).

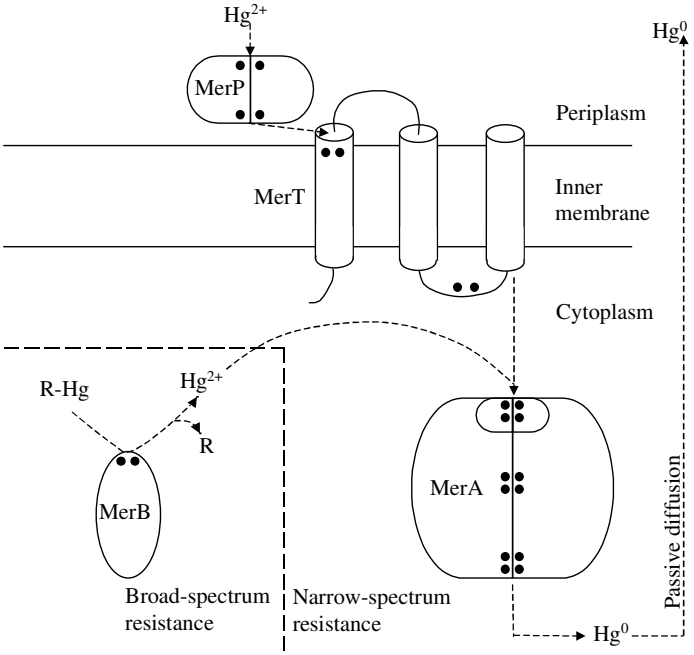


Figure 1.5 *Proposed mechanism of narrow- and broad-spectrum mercury resistance by (Osborn et al., 1997). The cystein residues in proteins essential for the resistance mechanism are marked with dots. See Text for the description of the resistance mechanism.*

1.3.3 Zinc resistance

As zinc belongs to the group of essential heavy metals, its intracellular concentration has to be precisely regulated. Zinc plays a critical role in the catalytic activity and/or structural stability of many enzymes, however in excess it is a potent inhibitor of the respiratory electron transport systems in bacteria.

Usually the bacterial cells cope with the Zn excess by transporting the ions out from the cells. The zinc efflux pumps have been found among P-type ATP-ases (ZntA in *E.coli*, (Rensing et al., 1997 and 1999)), Cation Diffusion Facilitator (CDF) family of transporters (ZitB in *E.coli*, (Grass et al., 2001)) or Resistance-Nodulation-Division (RND) transporter family (*czc* system in *Ralstonia metallidurans* (Nies 1999)). Probably the most studied Zn resistance system is the efflux by ZntA transporter, which will be discussed here in detail.

The *znt* zinc efflux system in *E. coli* consists of two genes, *zntA* and *zntR* that are located separately in the chromosomal DNA of *E. coli*. The product of *zntA* is an ATP-ase involved in the efflux of the Zn^{2+} ions from the cell and the product of *zntR* is a MerR family regulatory protein that controls the expression of *zntA*. (Brocklehurst et al., 1999). If the concentration of Zn^{2+} exceeds the essential concentration for the cell, the excess of Zn ions will bind to ZntR. Upon Zn^{2+} binding, the Zn-ZntR complex will induce some structural rearrangements in the operator region and formation of open transcriptional complex at promoter of *zntA* (Figure 1.6) (Outten et al., 1999).

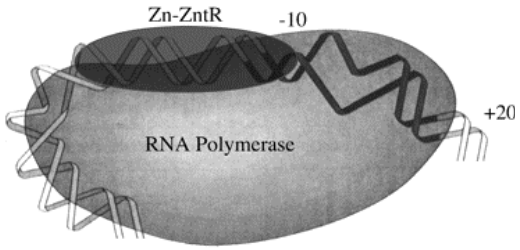


Figure 1.6 *Model for the open RNAP/P_{zntA}/Zn-ZntR complex by (Outten et al., 1999). The formation of open transcriptional complex includes the spooling of DNA upstream the promoter onto the RNA polymerase and distortion of operator/promoter DNA by Zn^{2+}-ZntR complex (bending of promoter to proper direction and facilitating the wrapping characteristic of a productive open complex)*

It has been shown that the induction of *zntA* promoter by ZntR begins in the zinc concentration that is at least an order of magnitude above that which is sufficient for rapid growth (Brocklehurst et al., 1999), obviously because the export system should prevent the loss of the essential metal. However, the ZntA has been shown to mediate also the efflux of other two-valent cations such as Cd^{2+}, Pb^{2+}, Hg^{2+}, Co^{2+} and Ni^{2+} (Beard et al., 1997; Gatti et al., 2000). Therefore, in addition of zinc also the other cations being transported by the ZntA could be the possible inductors of the *zntA*. Accordingly, the inducibility of *zntA* by Cd^{2+}, Pb^{2+} and Hg^{2+} has been reported (Binet et al., 2000).

1.3.4 Copper resistance

Similarly to Zn, copper is an essential trace element for living organisms and toxic when in excess. Thus, the copper homeostasis in organisms should be carefully regulated to avoid on the one hand the deficiency but on the other hand, the accumulation of this metal in the cell. The mechanisms involved in copper homeostasis in bacteria are, however only partially understood. For example, no specific Cu-importing protein has been identified so far (Rensing et al., 2003).

Cu resistance determinants have been identified in many bacterial species, e.g. in *E. coli*, a number of *Pseudomonas* species, *Klebsiella aerogenes,*

Mycobacterium scrofulaceum, Thiobacillus spp., *Enyterococcus hirae*, *Helicobacter pylori*, *Staphylococcus aureus*, *Ralstonia metallidurans* (Brown et al., 1992; Rosen, 2002). The biological responses of a bacterial cell to Cu can be considered in 2 separate parts: one concerns the mechanisms for Cu transformations (speciation) in normal cells, which are usually chromosomally encoded (Cu tolerance) and another covers plasmid-encoded mechanisms by which the high concentrations of Cu are handled in Cu-resistant cells (Brown et al., 1992).

In *E.coli* both types of resistance have been described. The cells can be protected by extreme periplasmid Cu stress by plasmid-encoded *pco* system consisting of 4 genes, which products are working as energy-dependent Cu ion efflux pumps. It has been assumed that before export the Cu ions will be modified to a form, which is unavailable to any Cu uptake system in the bacterial cell envelope (Brown et al., 1992).

Efflux of Cu ions during normal cellular homeostasis occurs mainly via chromosomally encoded CopA, a P-typa ATPase and *cus*-system, a CBA-type proton-cation antiporter system both of which are up-regulated by Cu ions (Figure 1.7) (Rensing et al., 2003).

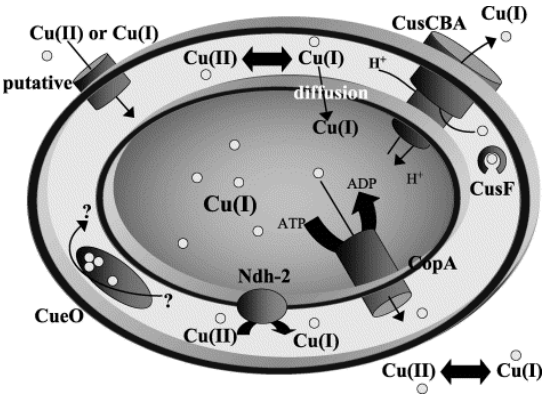


Figure 1.7 *Copper homeostasis mechanisms in E. coli (after Rensing et al., 2003). Shown are the most relevant homeostatic systems. CopA is a Cu(I)-translocating P-type ATPase, Ndh-2 a cupric reductase, CueO a multi-copper oxidase and CusCFBA a four-component copper efflux pump. Copper probably traverses the outer membrane through porins (possibly OmpC and OmpF).*

The substrate for both of these transporters is Cu^+ as most of the intracellular Cu has been assumed to be present in reduced form (Outten et al., 2000). The best studied of these two is CopA protein, which has been assumed to be the central component for cytoplasmic copper homeostasis. Regulation of this protein occurs by a MerR-like regulator (see above). CueR as a response to the intracellular free Cu^+. Also, the up-regulation of P_{copA} has been shown to occur as a response to extracellular Ag^+ , whereas in remarkably lower concentrations (obviously due to the higher toxicity of Ag ions) than in the case of Cu^+ (Stoyanov et al., 2001).

1.3.5 Chromate resistance

Chromate (Cr^{6+}) resistance has been found to be based on either chromate reduction (to Cr^{3+}) (*Pseudomonas fluorescens* LB300) or efflux (*Ralstonia metallidurans*) (Nies 1999). In this review, only the chromate resistance system of *R. metallidurans* will be discussed in detail.

As discussed above *R. metallidurans* is a chemolitotrophic soil bacterium which strain CH34 has been found to carry several heavy metal resistance mechanisms located in two megaplasmids pMOL28 and pMOL30 (Taghavi et al., 1997). The resistance determinants towards chromate in *R. metallidurans* have been found both from chromosome and the megaplasmid pMOL28 (Juhnke et al., 2002). So far six genes have been identified to be responsible for resistance in plasmid pMOL28 and three genes responsible for the resistance, which are located in the chromosome. The 3 genes found in the chromosomal DNA show considerable similarity with the respective genes in plasmid indicating that they have possibly been evolved by duplication during speciation of the genus *Ralstonia* perhaps indicating an adaptation of this bacterium to environments with elevated Cr concentrations. As shown by (Juhnke et al., 2002) due to the sequence similarities the chromosomal and plasmid-encoded systems are connected being able to regulate each others expression. The main protein responsible for the resistance towards Cr is membrane transport protein ChrA, which most probably mediates the efflux of chromate ions from the cell (Cervantes et al., 1992; Taghavi et al., 1997; Peitzsch et al., 1998). The genes for ChrA have been found both in plasmid and chromosome (Juhnke et al., 2002). The recent data have demonstrated that also a plasmid-encoded ChrC protein may be involved in chromate detoxification (Juhnke et al., 2002). The up-regulation of chromate detoxification system occurs via ChrB (Cervantes et al., 1992), which is again duplicated in plasmid and chromosome (Juhnke et al., 2002). The role of ChrB in regulation is also proved by the fact that this gene is missing in non-inducible chromate resistance determinant in *Pseudomonas aeruginosa* (Taghavi et al., 1997). Recent data from (Juhnke et al., 2002) show that the *chr* system may also be repressed by ChrF, however the exact regulation of chromate resistance system in *R. metallidurans* has still not been described in detail.

It is interesting to note that the *chr* system increases the resistance against chromate just 5-fold compared with the cells lacking those systems (Juhnke et al., 2002). This increase is very small compared with other resistance determinants, which may increase the minimal inhibitory concentration (MIC) up to 100 times (e.g., *czc* system conferring resistance towards Cd, Co and Ni, (Rensing et al., 1997)). As stated by (Juhnke et al., 2002) this could be due to rapid interaction of chromate oxyanions with cellular components and subsequent toxic effects that are not compensated by the efflux via ChrA.

1.4 WHOLE-CELL SENSORS

During last fifteen years there has been a great interest in developing whole-cell sensors for the detection of certain, both organic and inorganic substances. A number of publications have been published on bacterial whole-cell sensors for inorganic substances, mainly heavy metals like Al, As, Sb, Cd, Cr, Cu, Hg, Zn

but also for organic compounds like benzene and its derivatives, phenol and its derivatives, naphthalene, polychlorinated biphenyls (PCBs) and others (Köhler et al., 2000). In addition to bacteria also other microbes can be used as sensors. For example yeast cells have been modified to be used as Cu-sensors (Leskinen et al., 2003). In order for a cell to function as a sensor-cell, it has to contain two linked genetic elements: a sensing element and a reporting element: the former senses the target molecule(s) and turns on the latter. Usually the sensing element consists of a regulator gene and its responsive promoter, which originally regulates the expression of a gene or a group of genes necessary for the detoxification or biodegradation of the target analyte. In the case of bacterial heavy metal sensors the sensor elements originate from natural metal resistance systems, where the metal-regulator protein complex induces the expression of metal resistance gene(s) (see above).

ln the case of bacterial sensors for organic compounds the sensitive elements are usually from natural biodegradation mechanisms (Köhler et al., 2000). In most cases in the sensor constructs the natural biodegradative or resistance genes have been replaced by a reporter gene or reporter genes, which activation by the regulator protein in the presence of target analyte could be easily detected (Figure 1.8).

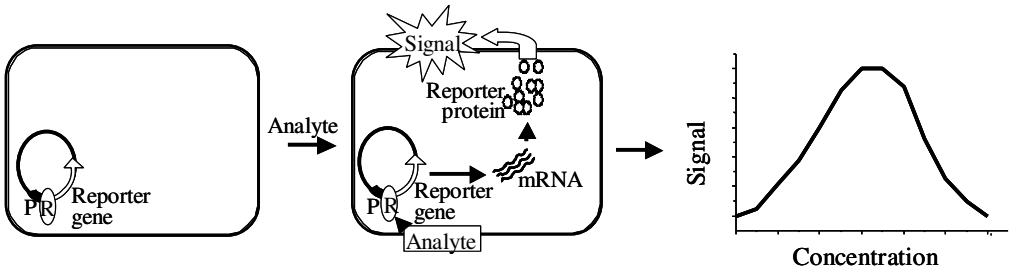


Figure 1.8 *Working mechanism of a bacterial whole-cell sensor, modified from (Virta et al., 2001). Binding of the regulatory protein R to the promoter P controls the transcription of reporter gene(s). Upon binding of the target analyte, transcription from the promoter will be induced leading to an increased protein concentration and thus, signal. The response curve of these kind of sensors is U-shaped due to toxicity of analyte in higher concentrations.*

The most frequently used reporter genes include *lucFF* gene, which encodes firefly (*Photinus pyralis*) luciferase, *lux CDABE* 5-gene cassette (bacterial luciferase system), and the variants of *gfp* gene, which encodes the green fluorescent protein (GFP) (Purohit 2003)).

In addition to sensors reacting to single inorganic or organic compounds, a number of publications can be found on systems sensing a broader class of substances, e.g., DNA disrupting compounds (based on SOS response mechanism in bacteria), compounds causing oxidative stress or protein damage (Belkin et al., 1997). There is a number of publications on yeasts used for identifying of possible endocrine-disrupting compounds, a class of pollutants which are very hazardous especially in the case of chronic exposure,

but on the other hand very difficult to predict by chemical structure (Baker 2001). Analogously to specific sensors also these, so called semi-specific constructs contain a sensing system, consisting on a regulatory (also named as sensory or receptor) protein (in this case reacting to a number of different compounds) and a promoter, connected with a reporter.

1.4.1 Bioluminescence

Bioluminescent reporter proteins, luciferases, belong to the most widely used reporters in sensor cells.

The ease of detection (possible to measure even with portable instruments) and lack of endogenous background activity in most of the cells make them attractive candidates for reporter applications (Lewis et al., 1998).

Luciferases are enzymes with the ability to produce light or luminescence as a by-product of their catalysed reactions (Wilson et al., 1998). Luminenscence has been described in a number of organisms including bacteria, algae (Dinoflagellates), insects (the most well-known are American firefly *Photinus pyralis* and click-beetle *Pyrophorys plagiopthalamus*), fungi (e.g., *Armillaria mellea*) and coelenterates (e.g., sea pansy *Renilla reniformis*)(Campbell 1988). The major function of light emission in many species is the communication (insects use luminescence flashes as signals during courtship whereas the light production occurs in species-specific manner) or need for illumination during hunt for food (luminescent marine bacteria from *Vibrio* family living in symbiosis with deep-sea fish enable their hosts to illuminate their pray) (Campbell, 1988). Historically the luminescence phenomenon has been often connected with supernatural forces. For example, the luminescence from fungi has been considered to indicate the location of a treasure (money-lights), which was seen in the forest at nights (Parmasto 2005).

Although carrying a common name (due to the ability to produce light), the luciferases in different organisms are chemically very different. According to their main origin, they can be divided into bacterial and eukaryotic luciferases (Wilson et al., 1998).

The bacterial bioluminescence system consists of an operon of 5 structural (*lux*) genes (Meighen 1988). The products of *luxA* and *luxB* genes encode for the synthesis of the α and β subunits of the enzyme luciferase, a flavin-dependent monooxygenase catalysing the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aldehyde (RCHO – luciferin of this reaction) to FMN and the corresponding long-chain carboxylic acid with light emission at 490 nm:

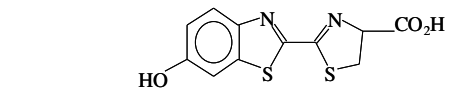
$FMNH_2 + O_2 + RCHO \rightarrow FMN + ROOH + H_2O$ + light (490 nm) (Wilson et al., 1998).

The genes *luxC, lucD* and *luxE* encode for proteins required for the synthesis of reaction substrate, RCHO (Wilson et al., 1998). There are 2 additional genes required for the induction of the bacterial bioluminescence system in natural conditions. The product of *luxR* is a repressor of the *lux* genes and the product of *luxI* catalyses the production of an autoinducer, homoserine lactone, which diffuses freely through the cell membranes, and binds to repressor LuxR. In the wild type system the lux genes will be activated only when enough autoinducer

is present in the environment (at certain cell density), i.e. the luminescence is induced in quorum-dependent manner (Meighen 1988).

For reporter purposes, maximum 5-gene cassette (*lux CDABE)* is used, but it is also possible to use the *lux AB* encoding just for luciferase and add the reaction substrate (luciferin) externally (Lewis et al., 1998). It should be mentioned that the expression of 5 additional genes (*lux CDABE*) is relatively burdensome for a bacterial cell. Moreover, the continuous bioluminescence production requires a considerable amount of the cellular energy (it has been shown that during luminescence the ATP levels in bacterial cells may drop about 10 times, (DeLuca 1978)). The most widely used *lux*-cassettes are from marine bacteria *Vibrio fisheri* or *Vibrio harveyi* (Köhler et al., 2000). However, due to the limited temperature range of these systems (<30°C and <37°C, respectively) in many applications the lux system from terrestrial bacterium *Photorhabdus luminescens* with higher temperature maximum has been used (Lampinen et al., 1992).

For reporter purposes the most widely used eukaryotic luciferase is from firefly *P. pyralis* (*lucFF* gene) (Lewis et al., 1998). The firefly luciferase reaction is very different from that of bacterial luciferase: the only common feature of these reactions is the production of light and need for molecular oxygen and energy (either $FMNH_2$ or adenosin triphosphate, ATP) and therefore, the metabolic activity of the cell. The substrate for the *P. pyralis* luciferase is D-luciferin, which will be oxidised in the presence of ATP to oxyluciferin with light emission at 560 nm (Wilson et al., 1998):



$$ATP + O_2 + \text{D-luciferin} \rightarrow AMP + PPi + CO_2 + \text{oxyluciferin} + \text{light.}\ (560\ nm)$$

The firefly luciferase gene has been successfully expressed both in eukaryotic and prokaryotic cells (Cheng Vollmer et al., 2004). Compared with the prokaryotic luminescence system the energy requirements of firefly luminescence are much smaller and the produced quantum yield is highest among known luciferase systems (0.9 from each oxidized substrate molecule, (Lewis et al., 1998)). Moreover, the linear range of the firefly bioluminescence reaction is up to eight orders of magnitude, compared to the three orders of magnitude for the bacterial system (Naylor 1999). However, the disadvantage of the firefly system is the absolute need for the external addition of reaction substrate, D-luciferin as the genes involved in its synthesis are still not known. In addition, in order to have the luminescent signal, the pH of the surrounding environment (i.e. test medium) should be set acidic in order the D-luciferin to pass the cell membrane: pH 5 has been found to be optimal for *E.coli* (Jawhara et al., 2004) and *Pseudomonas fluorescens* cells (Petänen et al., 2001) and pH 6 for *Streptococcus* (Loimaranta et al., 1998). Thus, the continuous monitoring of reporter gene activity in the case of firefly luciferase is almost impossible and when the pH of surrounding environment (e.g., in the case of analysing

promoter activity in natural environments) is crucial, the pH adjustment may substantially affect the results.

A more novel relatively widely used eukaryotic luciferase is *Rluc* from sea pansy *Renilla reniformis*. Although it has been expressed in *E.coli* (Lorenz et al., 1991), its applications in bacteria have not been developed and improvements, such as those in codon usage, may be necessary (Cheng Vollmer et al., 2004). One advantage in using luciferase from *Renilla* as reporter gene could be the fact that this system does not require ATP and Mg^{2+} (Inouye et al., 1997). However, compared with the firefly system, the quantum yield of this luciferase is rather low (0.055).

1.4.2 The use of whole-cell sensors in environmental analysis

The whole cell sensors represent a significant breakthrough for the monitoring of pollutants in contaminated matrices since they have the unique ability to measure the interaction of specific compounds with biological systems through highly sensitive and specific biorecognition processes. The attractiveness of the use of whole-cell sensors in determination of different bioavailable substances has been emphasized in many papers (Kong et al., 1995; Daunert et al., 2000; Köhler et al., 2000;, Belkin, 2003). The main advantages of this technique include the ease of the procedure (no sample pre-treatment and fast signal detection), rapidity (hundreds of samples in couple of hours), low requirements on the apparatus (if compared with the sophisticated instruments used for chemical analysis) and the relatively low price of the procedure. On the other hand also some disadvantages should be pointed out. One weakness for some types of whole-cell sensors (especially these constructed for organic compounds) is the low specificity (Daunert et al., 2000). The lack of specificity is ususally due to the fact that the resistance and biodegradation mechanisms, which have been used in the construction of the sensors themselves are not specific, i.e., the sensor responds usually to several compounds (due to the simultaneous presence of several metals in nature, the resistance systems often detoxify all of them, or the biodegradative pathways may use many structurally related compounds as substrates) and the specificity is very difficult to achieve. Another weakness is inherent property of all living cells: relatively small detection range (maximum 3 of orders of magnitude) of target compounds, which is determined by the difference in concentrations initiating the detoxifying/biodegradation mechanisms and the ones causing already toxic effects (Köhler et al., 2000).

Due to the well established physico-chemical detection methods, the main application field for the whole-cell sensors in environmental analysis could base on their ability of detecting the bioavailability of contaminants, which is especially valuable as it provides a unique tool to evaluate the hazard of a polluted environment to biota. As discussed above, the fact that the total concentrations of pollutants, e.g., heavy metals in soils or sediments do not provide any information on their bioavailability is well-known (Academies, 2004). Several papers have described that only a relatively small fraction of heavy metals in soils (e.g., 0.12% of the total Hg (Petänen et al., 2003) and 0.4

– 18% of the total As (Petänen et al., 2003)) is available to bacterial cells. Thus, the bioavailability analysis of natural samples in parallel with standard analytical methods would be of great interest. Still, in spite of the great number of bacterial sensors constructed (Köhler et al., 2000) surprisingly few have been applied for environmental analysis. Sensors for Cu (Corbisier et al., 1996), Hg (Rasmussen et al., 2000; Petänen et al., 2001 and 2003), Pb and Cd (Turpeinen et al., 2000; Fritze et al., 2001), Co and Ni (Tibazarwa et al., 2001) and As (Petänen et al., 2001 and 2003) have been applied for analysis of heavy metals from soil samples. It could be said that these papers have been just first attempts to introduce the whole bacterial cells in environmental analysis and their promising results have certainly paved the way for comprehensive future use in environmental hazard assessment.

1.4.3 The use of whole-cell sensors in biosensor systems

A biosensor has been defined as a device for the rapid, accurate, low-level detection of an analyte in body fluids, water and air that combines a biological component with a physico-chemical detector component (www.wikipedia.org; On-line medical dictionary). Most commonly the biological element has been defined as an immobilized macromolecule such as enzyme or antibody and another, less common approach uses the term `biosensor´ more widely and adds also living micro-organisms or sections of organs or tissues as the biological element (Simpson et al., 1998). Herein the use of whole cell bacterial sensors as biological sensing elements is discussed.

It has to be mentioned that the term ´biosensor´ has been used relatively freely by microbiologists: in many papers only the biological elements, living microbial cells with certain sensing capabilities have been defined as `biosensors` (Belkin 2003). Although an isolated microbial strain might serve as excellent sensor in the laboratory, the same microbes taken outside and used as biosensors need to be incorporated into a system that will allow the effective storage and maintenance of the sensing potential (e.g., viability) of cells, access of the analyte to be sensed to these microbes as well as technical interface for on-line capturing of the signal specifically caused by the analyte to be sensed.

As luminescence offers easy detection possibilities with low signal-to-noise ratio, the sensor cells expressing luminescence as reporter have often been used in biosensor systems. Various methods exist for the entrapment of microbial cells into biosensor systems (in the case of luminescent sensors at or near the light-transducing system connected with the physico-chemical detector). These include immobilization in PVA (polyvinyl acetate) matrix (Horsburgh et al., 2002), latex film (Lyngberg et al., 1999), alginate immobilization onto optical fibre tips (Polyak et al., 2000; Hakkila et al., 2004), agar immobilization at the bottom of microtiter plate wells (Mbeunkui et al., 2002) and encapsulation in sol-gel matrices (Rajan Premkumar et al., 2002). A recent advance is the development of Bioluminescent Bioreporter Integrates Circuit (BBIC), in which the optical signal from immobilized sensor cells is automatically detected, processed and the results are communicated (Simpson et al., 1998). The polymeric matrices used for the cell entrapment provide a

hydrated environment containing the nutrients and cofactors needed for cellular activity and growth (Simpson et al., 1998).

The sensor cells expressing "self luminescent" luciferin-luciferase system from prokaryotes (*lux* genes) enabling the continuous monitoring of luminescence, have most often been used in biosensor systems. This kind of biosensor systems have been constructed for the detection of genotoxicants (Polyak et al., 2000), heavy metals (Corbisier et al., 1999), organic pollutants like naphthalene and salicylate, (Heitzer et al., 1994), toluene and trichloroethylene, (Applegate et al., 1997). The use of eukaryotic luciferase (*luc* gene) in biosensor applications is not as widespread as this system emits light only upon external addition of reaction substrate, D-luciferin (a unique benzothiazole). Thus, the diffusion of luciferin trough immobilization matrix and the requirement for pH 5 in order to pass the bacterial membranes and enter the living cells (difficult to achieve inside the immobilization matrix) may greatly interfere with the test results. Moreover, D-luciferin is relatively costly if used in high quantities, as required for biosensor applications. These disadvantages have been pointed out also by other authors in conncetion with the biosensor for benzene derivatives carrying *luc* reporter gene (Ikariyama et al., 1997). Interestingly, the authors noted that the induction of luminescence by the analyte was remarkably slower in immobilised cells compared with similar, non-immobilised bacteria, probably due to the interference of immobilisation matrix lowering the bioavailability of the analyte. Thus, the matrix used in cell entrapment should be carefully chosen in order not to interfere with the results especially when measuring low concentration-environmental samples.

Compared with the use of free sensor cells (most often used experimental setup), the biosensors (usually immobilised cells) have a number of advantages including their miniaturized size and portability, which is especially suitable for on-site environmental monitoring (Daunert et al., 2000). Currently there are still some weak points in the routine production of whole-cell biosensor systems (based on matrix-entrapped bacteria) like unstable viability and sensitivity of the cells, however further advanced in biotechnology and transduction mechanisms will ultimately provide the means for rapid and sensitive real-time analyte detection at ultralow levels (Daunert et al., 2000).

AIMS OF THE STUDY

The main aim of this study was to construct and/or use a panel of bacterial and eukaryotic metal sensor including fibre-optic biosensor systems to determine the factors influencing bioavailability of different heavy metals in various environmental matrices.

The sub-tasks of this study were:

1. Construct bacterial sensor strains for the detection of ubiquitous heavy metals Zn, Cu and Cr as well as for highly toxic organic compounds of Hg.

2. Develop a protocol for quantification of bioavailable heavy metals in natural soils and sediments in contact assay. Due to the interference of solid samples with test results, the development of appropriate protocol for these samples is extremely important.

3. Evaluate the test protocol for bacterial and eukaryotic sensors on natural samples and compare the data with surrogate chemical methods for estimation of bioavailable heavy metals.

4. Determine and analyse the factors influencing bioavailability of heavy metals in soils and sediments

5. Develop a fibre-optic biosensor system applicable for field analysis of bioavailable heavy metals

EXPERIMENTAL PART

MATERIALS AND METHODS

2.1 Metal sensor and control strains

Bacterial strains constructed during this work and earlier constructed bacterial and yeast strains used in this study are presented in Table 2.1.

Table 2.1 *Heavy metal sensor and control strains constructed and/or used in this study.*

Strain	Sensor elements: Constructed for	Sensor elements: Regulatory protein/promoter (origin)	Sensor elements: Additional genes	Reporter	Reference
Sensor strains					
Escherichia coli MC1061(pmerR_{BS}B luc[a])	Organic and inorganic Hg	*merR*/P*merA* (*Serratia marcescens* plasmid pDU1358)	*merB*	*lucFF* (firefly *Photinus pyralis*)	(Paper **I**)
E.coli MC1061(pzntluc[a])	Zn	*zntR*/P*zntA* (*Escherichia .coli* chromosomal DNA)		*lucFF*	(Paper **II**)
E.coli MC1061(pSLcueR[a]/ pDNPcopAluc[b])	Cu	*cueR*/P*copA* (*E.coli* chromosomal DNA)		*lucFF*	(Hakkila et al., 2004; partial construction of sensor by A.Ivask)
Ralstonia eutropha AE104 (pchrBluc$_c$)	Cr	*chrB*/P*chrA* (*Ralstonia metallidurans* CH34 megaplasmid pMOL28)		*lucFF*	(Paper **II**)
E. coli MC1061(pmerlux CDABE[a])	Inorganic Hg	*merR*/P*merA* (*Serratia marcescens* plasmid pDU1358)		*luxCDABE* (*Photorhab-dus luminescens*)	(Hakkila et al., 2002)
E. coli MC1061(parslux CDABE)	As	*arsR*/P*ars* (*E.coli* plasmid R773)		*luxCDABE*	(Hakkila et al., 2002)
Bacillus subtilis BR151(pTOO24)	Cd	*cadC*/P*cad* (*Staphylococcus aureus* plasmid pI258)		*lucFF*	(Tauriainen et al., 1998)

Table 2.1 *Continued*

Strain	Sensor elements: Constructed for	Sensor elements: Regulatory protein/promoter (origin)	Sensor elements: Additional genes	Reporter	Reference
Sensor strains					
Staphylococcus aureus RN4220(pTOO24)	Cd, Pb	*cadC*/P*cad* (*S. aureus* plasmid pI258)		*lucFF*	(Tauriainen et al., 1998)
Saccharomyces cerevisiae BMA64-1A(pSALluc-skl)	Cu	promoter CUP1		*lucFF*	(Leskinen et al., 2003)
Control strains					
E.coli MC1061(pTOO02 [d])	Control bacterium (not induced by heavy metals)			*lucFF* (firefly *Photinus pyralis*)	(Paper **III**)
E.coli MC1061(pDNlux)	Control bacterium			*luxCDABE* (*Photorhab-dus luminescens*)	(Leedjärv et al., in press)
S. aureus RN4220(pTOO02 [d])	Control bacterium			*lucFF*	(Paper **III**)
B. subtilis BR151(pCSS962/ pBL1)	Control bacterium			*lucGR* (click-beetle *Pyrophorus plagiopthala mus)*	(Lampinen et al., 1992)
S. cerevisiae BMA64-1A (pRS316luc)	Control yeast			*lucFF*	(Leskinen et al., 2005)

[a] *high copy number plasmid for E.coli, based on pSL1190 (Amersham Pharmacia Biotech)*
[b] *low copy number broad host range plasmid based on pDN18N (Nunn et al., 1990)*
[c] *low copy number broad host range plasmid based on pRK415 (Keen et al., 1988)*
[d] *p602/22 (LeGrice et al., 1987) based shuttle vector for E.coli and B.subtilis*

Heavy metal sensors constructed and/or used in this study contain a plasmid with sensor and reporter elements. The "metal-sensing elements" consist of a regulatory protein specifically recognizing the target metal(s) and a promoter, from which the transcription is regulated by that protein. The promoter is fused with (a) reporter gene(s): *lucFF* , luciferase gene from *Photinus pyralis* or luciferin-luciferase system (*luxCDABE*) from *Photorhabdus luminescens* which expression in controlled by target heavy metal(s). The general structure of a heavy metal sensor cell is presented in Figure 2.1.

In the control cells (Table 2.1) the gene(s) encoding for sensor element(s) are absent and the luminescence in these strains is not induced by heavy metals but expressed from promoter of *lac* operon (P_{lac}).

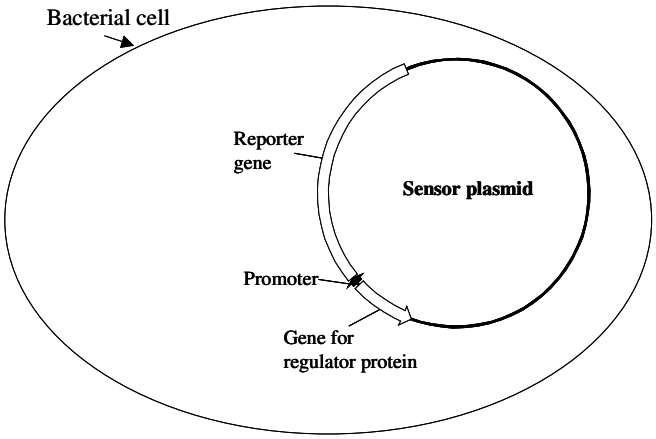


Figure 2.1 *Schematic structure of a heavy metal sensor cell. The "sensing" properties are encoded by a sensor plasmid. In the presence of intracellular metal the regulatory protein switches on the promoter in sensor plasmid and thus, induces the reporter gene.*

2.1.1 Construction of heavy metal specific sensors

The sensor plasmids constructed in this work (Table 2.1) were done by PCR-amplifying the appropriate sensor elements: genes for regulatory proteins and promoters regulated by these proteins from their natural origins. The sensor elements were inserted to plasmids with suitable copy number and host range (see Table 2.1) as fusions with *lucFF* gene (GeneBank accession number M15077). The detailed PCR conditions and plasmid modifications are described in papers **I** and **II**. For the construction of sensor bacteria for organic mercury, an additional gene, *merB* was also PCR-amplified and added to the sensor plasmid pmerR_{BS}Bluc (paper **I**). MerB encodes for organomercurial lyase breaking down the carbon-mercury bond. The sensor plasmids were further transformed to selected bacterial strains, usually to those from which the sensory elements naturally originated (Table 2.1).

2.1.2 Construction of control strains

Control plasmid pTOO02, in which the *lucFF* gene is under the control of *lac* promoter, was transformed to *E.coli* and *S.aureus* (paper **III**) to obtain luminescent control strains *E.coli* MC1061(pTOO02) and *S.aureus* RN4220(pTOO02) not inducible with heavy metals (see Table 2). The strains were used to take into account the influence of natural samples on bacterial bioluminescence.

2.2 Preparation of fibre-optic biosensors

The suspensions of Hg sensor *E.coli* MC1061(pmerluxCDABE) or As sensor *E. coli* MC1061(parsluxCDABE) were mixed with 2 % Ca-alginate (1:1 ratio) and

attached onto optical fibre tips in several layers. The detailed protocol of immobilization is presented in paper **VI**. The optical fibres with hardened tips were then connected with a photomultiplier tube (Polyak et al., 2000) to produce a heavy metal biosensor system.

2.3 Characterization of samples

In this paper data on bioavailable Cd, Pb, Cu, Zn, Hg, Cr and As in 139 natural soils and 1 natural sediment, 1 spiked soil and 3 spiked soil components determined by heavy metal sensor cells are presented. In addition, 200 natural sediments from the Baltic Sea were analysed for bioavailable Cd, Pb and Hg during this work, the data for which are not presented here due to their very low content of bioavailable metals.

2.3.1 Spiked samples

One soil (sandy-loam from Estonia, physico-chemical properties are described in paper **II**) and three standard soil components: montmorrilonite, kaolinite and humic acid (commercially available) were used for spiking. Spiking of the samples was generally performed by mixing of the soil/soil components with heavy metal solutions. For preparation of spiked soil samples 5 ml of $HgCl_2$, $CdCl_2$, $ZnCl_2$ or $K_2Cr_2O_7$ solution was added to 10 g of dry soil and air-dried (paper **II**). For preparation of spiked model soil components 25 ml of 0.8 and 5 $\mu g\ l^{-1}$ aqueous solutions of CH_3HgCl was added to 2 g of montmorrilonite, kaolinite and humic acid and pH was adjusted to 7. The suspensions were equilibrated for 24 h, filtered and air-dried. (Bernaus et al., 2005). For both, spiked soil and model soil components, the concentration of added metals was considered as total.

2.3.2 Natural samples

In all 140 natural samples used in this study the total amount (after solubilization with HF or *aqua regia*) of selected metals were determined by standard analytical methods (ICP-AES, inductively coupled plasma-atom emission spectrometry, ICP-MS, inductively coupled plasma-mass spectrometry or ICP-OES, inductively-coupled optical emission spectrometry) in either ISA (Lille, France), Universitat Autònoma de Barcelona (Spain) or Kalmar University (Sweden). In addition, from 130 samples selected (key) soil physico-chemical parameters were measured (see Table 2.2).

From the analysed samples 110 topsoils originated from the collection of ISA (Lille, France) and were collected from the surroundings of two metal smelters in Northern France (papers **III** and **V**). These were agricultural soils with greatly varying physico-chemical parameters and heavy metal content (see Table 2.2) and were used to determine bioavailable Cd and Pb. The principal component analysis was performed to correlate bioavailability with key soil components (pH, content od $CaCO_3$, silt, clay and organic matter).

Table 2.2 *Characteristics of natural heavy metal polluted samples analysed in this study.*

	Physico-chemical properties					**Content of selected heavy metals (min-max), mg kg^{-1}**					
Samples	**pH**	**Organic matter, g kg^{-1}**	**Clay, g kg^{-1}**	**Silt, g kg^{-1}**	**Sand g kg^{-1}**	**Zn**	**Cd**	**Pb**	**Cu**	**Hg**	**As**
110 soils (papers **III** and **V**)	5.5-8.4	15-114	85-681	94-777	63-756	100 - 1390	1-20	50-1050			
Model soil (code T_{REF})	*7.9*	*32.4*	*209*	*555*	*236*	*1390*	*20*	*1050*			
20 soils (paper **IV**)	5.2-8.5	10-220	n.d.	n.d.	n.d.			14-5323	8 - 12 987		
9 soils and 1 sediment (paper **VI**)	n.d.	n.d.	n.d.	n.d.	n.d.					<8 – 12.5	8 - 701

n.d. – not determined during present study

One model soil (code T_{REF}) (Table 2.2) was selected from the collection of 110 soils from ISA and tested thoroughly for bioavailability and water-extractability of Zn, Cd and Pb (see next chapter).

20 soil samples originated from urban area near Jakobstad, a small town in Finland (paper **IV**) (Table 2.2). The samples varying from sand to organic-rich topsoils were collected from very different locations, contained greatly different amounts of heavy metals (paper **IV**) and were analysed for bioavailable Cu and Pb.

9 soils and 1 sediment analysed in paper **VI** were collected from Aznalcollar mining area in Southern Spain that is one of the main polymetallic deposits in Spain and analysed for bioavailable Hg and As in parallel with fibre-optic biosensor system and conventional non-immobilised sensor bacteria (paper **VIII**).

2.4 Test protocol

2.4.1 Preparation of samples

Natural soil and sediment samples were air-dried and sieved (mesh size 2 mm) before analysis. The samples were mixed with water in ratio 1:12.5 (paper **II**), 1:10 (paper **V**), 1:9 (papers **III**, **IV**, **VI**) or 1:5 (paper **VI**). The influence of liquid-to-solid ratio on water-extracted amount of Cd, Zn and Pb was tested on model soil T_{REF} (Table 2.2) by analysing soil:water ratios from 1:3 to 1:100.

The sample-water suspensions were either mixed by rotation at room temperature during 24 hours (papers **II - V**) or used after short (1-minute) mixing (paper **VI**). The suspensions were either analysed for bioavailable metals directly (contact assay) (papers **II - VI**) or centrifuged prior analysis (usually at 10 - 16 000 g) to determine the water-extracted bioavailable fraction of metals (papers **II**, **III**, **V**). Centifugation forces from 600 to 10 000 g were applied to the suspension of model soil T_{REF} to study the efficiacy of centrifugation in settling of particles containing bioavailable Cd.

Except in paper **II** a set of dilutions (usually applying dilution factor 3) in water were done from each sample (suspension or extract) in order to quantify the bioavailable amount of metals.

2.4.2 Incubation and measurements

Strains used for testing were either cultivated freshly (yeast cells in paper **IV** and bacterial cells to be immobilized in alginate, paper **VI**), or freeze-dried before experiments (**II - VI**). M9 mineral medium (Sambrook et al., 1989) supplemented with 0.5 % of cas-aminoacids was used for pre-cultivation and/or rehydration after freeze-drying of most *E.coli*-based sensor strains (papers **I**, **II**, **IV**, **VI**). LB medium (Sambrook et al., 1989) was used for pre-cultivation and /or rehydration of As sensor *E.coli* MC1061(parsluxCDABE) (paper **VI**) and Pb sensor *S.aureus* RN4220(pTOO24) (papers **III**, **IV**, **V**). Modified SMM medium (Tauriainen et al., 1998) supplemented with 0.5 % of cas-aminoacids was used for rehydration of Cd sensor *B.subtilis* BR151(pTOO24) (papers **III**, **V**). Modified ATCC Cultire medium 425 (paper **II**) was used for cultivation and/or rehydration of *R.eutropha* AE104(pchrluc) and SC medium (Burke et al., 2000) supplemented with appropriate amino-acids was used for cultivation of yeast (paper **IV**).

Heavy metal sensor and control strains at suitable growth phase (usually optical density at 600 nm around 0.6 in the case of bacteria and 1 in the case of yeast) suspended in growth media were mixed with water (control) heavy metal standard in water or sample (dilutions) in ratio 1:1 (papers **I- VI**). In the case of fibre-optic biosensors the fibre tips with alginate-immobilised sensor bacteria were dipped directly into the water, metal dilution or suspension of soil or sediment (**VI**). Luminescence measurements were done after 2-hour or 3-hour (optical-fibre biosensors) incubation at 37 ºC (*E.coli*-based sensors, papers **I**, **II**), 30ºC (sensors based on *R.eutropha*, *S.aureus*, *B.subtilis*, or *S.cerevisiae*, papers **III - V**) or room temperature (in field testing, paper **VI**). This incubation is needed for the synthesis of luciferase from reporter gene(s) induced by the target analyte. In the case of bacterial and yeast cells expressing *lucFF* or *lucGR* as reporters the equal amount of D-luciferin (substrate of the eukaryotic luciferase) was added prior measurement (papers **I**-**V**).

2.4.3 Limit of quantification

Limit of quantification of the sensors was determined according to the variation of background luminescence by formula:

$$LOD = 2\frac{\overline{X}_B + 3SD}{\overline{X}_B} \qquad (1),$$

where LOD is the lowest induction of luminescence statistically above the background, $\overline{X}_B$ is the mean background luminescence value of the sensor (6-8 blanks included in each assay) and SD is the standard deviation. The limit of quantification for the sensor was determined as the concentration corresponding to LOD in standard concentration-effect curve (see example in Figure 2.2. A).

2.4.4 Quantification of bioavailable metals

Induction of bioluminescence in sensors as response to standard solutions of metals or environmental samples was calculated:

$$Induction = \frac{L_S}{L_B} \qquad (2),$$

where L_s is the luminescence of sensors incubated with and L_B of those incubated in water (control). In order to take into account the non-specific effects of environmental samples to bacterial luminescence these samples were analysed with respective control strains (Table 2.3.), the results of which were used for correction of data from sensor strains.

Table 2.3 *The use of controls in correction of luminescent responses of respective metal sensors*

Control	Sensor	Paper
Escherichia.coli MC1061(pTOO02)	*E.coli* MC1061(pSLcueR*/pDNPcopAluc)	**IV**
	E.coli MC1061(pmerR_{BS}Bluc)	(in: Bernaus et al., 2005)
E. coli MC1061(pDNlux)	*E.coli* MC1061(pmerlux)	**VI**
	E.coli MC1061(parsluxCDABE)	**VI**
Staphylococcus aureus RN4220 (pTOO02)	*S. aureus* RN4220(pTOO24)	**III**, **IV**, **V**
Bacillus subtilis BR151(pCSS962/pBL1)	*B. subtilis* BR151(pTOO24)	**III**, **V**
Saccharomyces cerevisiae BMA64-1A (pRS316luc)	*S. cerevisiae* BMA64-1A(pSALluc-skl)	**IV**

According to the results from control cells correction factor (CF) was calculated:

$$CF = \frac{L_B}{L_S} \qquad (3),$$

where L_B is the luminescence of control bacteria in water (control) and L_S luminescence of control bacteria in sample. The results from sensors (equation 2) were corrected by CF to obtain NL, normalised luminescence as follows:

$$NL = CF * induction \qquad (4).$$

The bioavailable amount of metals in a sample was calculated by comparing the induction of the sensor by heavy metal standards (concentration-effect curve, see an example in Figure 2.2. A) and by a sample (see an example in Figure 2.2. B).

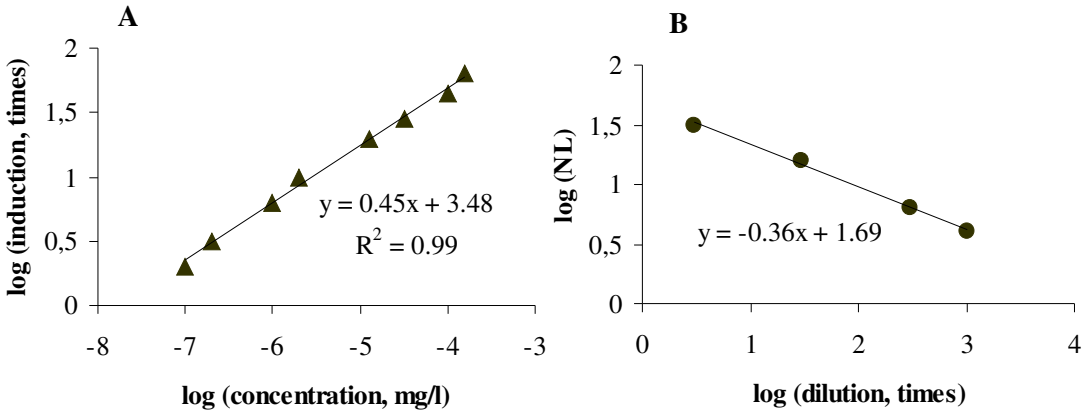


Figure 2.2 *Induction of luminescence in a hypothetical heavy metal sensor by a set of metal sandards (concentration-effect curve) (A) or an unknown sample (induction expressed as normalised luminescence, NL taking into account the non-specific effect of the sample to bioluminescence) (B). The equation of linear regression from standard curve (A) and induction of the sensor by the non-diluted sample (dilution=1; 1.69 in the present case) are to be used to calculate the bioavailable concentration of metals in natural samples.*

The y value in regression equation of concentration-effect curve was substituted with induction (NL value) for non-diluted sample (dilution =1, log(dilution)=0) and the calculated x value was considered as the bioavailable concentration of respective metal in the sample.

RESULTS AND DISCUSSION

3.1 Construction and calibration of heavy metal sensor bacteria

During this work four new sensor bacteria were constructed:

i) *E.coli* MC1061(pmerR_{BS}Bluc) for the detection of mercury and its organic forms (paper **I**),

ii) *E.coli* MC1061(pzntRluc) for the detection of Zn (paper **II**),

iii) *Ralstonia eutropha* AE104(pchrBluc) for the detection of Cr (paper **II**) and

iv) *E.coli* MC1061(pSLcueR/pDNpcopAluc) (Hakkila et al., 2004) for the detection of Cu. It should be mentioned that no sensor for organomercury compounds was constructed before.

These bacterial strains contained sensor elements from the respective heavy metal resistance systems fused with luciferase (*lucFF)* as reporter gene. The structure of sensor plasmid for inorganic and organic mercury, pmerR_{BS}Bluc as an example is shown in Figure 3.1.

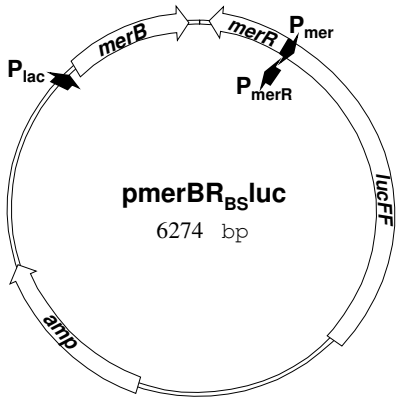


Figure 3.1 *Structure of sensor plasmid for inorganic and organic mercury compounds. Sensor elements: P_{mer} promoter of a natural Hg detoxification system and merR, a regulatory protein interacting with Hg^{2+} and controlling the transcription from P_{mer} are fused with reporter gene lucFF. merB is an organomercury lyase breaking down organic Hg compounds to produce Hg^{2+}. Reproduced from paper **I** by permission from American Chemical Society*

The sensor elements in all constructed plasmids contain a gene encoding for a metalloregulatory protein (*merR*, *zntR*, *chr*B and *cueR* in Hg, Zn, Cr and Cu sensor plasmids, respectively) which upon binding a target metal ion binds to DNA and acts as activator in a promoter controlling the expression of metal resistance genes. This promoter (P_{mer}, P_{zntA}, P_{chrA} *and* P_{copA} in Hg, Zn, Cr and Cu sensor plasmids, respectively) fused with *lucFF* is a second obligatory sensor element. In the case of mercury sensor *E.coli* MC1061(pmerR_{BS}Bluc) an additional gene, *merB*, breaking down the bond between carbon and mercury and releasing Hg^{2+} was used. As expected, the induction of bioluminescence in bacteria containing sensor plasmids was induced by target heavy metals. The induction of the luminescence in a concentration-response manner allowed the quantification of inducing heavy metals from the linear range of the curve. The detection range of constructed sensors for target and some non-target metals are presented in Table 3.1 (from papers **I**, **II** and Hakkila et al., 2004). Only Cr sensor *R.eutropha* AE104(pchrBluc) was target analyte-specific but responded to Cr in its both oxidation states, III ($CrCl_3$) and VI ($K_2Cr_2O_7$). No response towards other tested metals and metalloids, $NaAsO_2$, Na_2HAsO_4 or Na_2MoO_4 was detected. Cu sensor *E.coli* MC1061(pSLcueR/ pDNpcopAluc) was responding to 2 metals, Cu and Ag whereas no induction was observed when $HgCl_2$, $ZnCl_2$, $AlCl_3$ or $Pb(CH_3COO)_2$ were tested. Hg and Zn sensors MC1061(pmerR_{BS}Bluc) and MC1061(pzntRluc) were responding to three 2-valent metals: Zn, Cd and Hg. No induction in these sensors was detected upon incubation with Pb^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+} or Ni^{2+}. Induction of heavy metal resistance mechanisms (used for the construction of the sensor bacteria) by different metals is not surprising as these mechanisms, including regulatory proteins have developed in the nature, where a number of metals are present simultaneously (see also discussion in chapter 1.3).

As seen from Table 3.1, the detection limits of bacterial sensors for different metals differed considerably increasing in order Hg<Cd<Cr<Ag<Cu<Zn. Thus, lowest detection limits were obtained for highly toxic Hg and Cd, which have no known biological function whereas the detection limits for essential micro-elements Cu and Zn were much higher. This sequence is also in correlation with the toxicity of these metals: for example the 24-hour LC50 for *Daphnia magna* to Hg is 0.01 mg kg^{-1} , to Cd 0.64 mg kg^{-1} and for Zn 7.6 mg kg^{-1} (Crisinel et al., 1994; Castillo et al., 2000).

Table 3.1 *Response of sensor bacteria towards different heavy metals*

Sensor and reporter elements	Inducing metals	Detection range, mg l^{-1}
Escherichia coli MC1061(pmerR_{BS}Bluc)	**$(CH_3)_2Hg$**	2.3 – >231000 [a]
	CH_3Hg^+ *(as CH_3HgCl)*	0.00005 – 0.5
	Hg^{2+} *(as $HgCl_2$)*	0.003 – 0.6
	Cd^{2+} *(as $CdCl_2$)*	0.07 – 15
	Zn^{2+} *(as $ZnCl_2$)*	400[b]
E. coli MC1061(pzntRluc)	**Zn^{2+}** *(as $ZnCl_2$)*	5.4 – 1360
	Cd^{2+} *(as $CdCl_2$)*	0.009 – 7.3
	Hg^{2+} *(as $HgCl_2$)*	0.005 – 0.5
Ralstonia eutropha AE104(pchrluc)	**$Cr_2O_7^{2-}$** *(as $K_2Cr_2O_7$)*	0.01 – 6
	Cr^{3-} *(as $CrCl_3$)*	0.2 – 317
E. coli MC1061(pSLcueR/ pDNPcopAluc)	**Cu^{2+}** *(as $CuSO_4$)*	3.2 – 640
	Ag^+ *(as $AgNO_3$)*	0.7 – 9

[a] *Higher concentrations not tested*
[b] *Response detected only in one concentration*

Due to the presence of MerB, organomercury lyase, the MC1061(pmerR_{BS}Bluc) was in addition to inorganic Hg induced by organomercury compounds methyl- and dimethylmercury whereas the induction with methylmercury occurred in about 100-fold lower concentrations than in the case of $HgCl_2$. One explanation for this difference (the breakdown of one molecule of methylmercury should result in one Hg ion, which should lead to similar response towards $HgCl_2$ and CH_3HgCl) could be the remarkably higher lipophilicity of organomercurial compounds and thus their better ability to enter the cell. Surprisingly, the limit of quantification of the sensor for highly toxic and lipophilic dimethylmercury was extremely high, 2.3 mg l^{-1} (10 μM) and the toxicity was not observed even after incubation with 231 g l^{-1}. One possible explanation for this could be the evaporation from the reaction mixture as dimethylmercury is very volatile (Toribara et al., 1997).

3. 2 Applicability of the bacterial sensors for the analysis of environmental samples

3.2.1 Comparison of the luminescence induction in sensor bacteria by standard metal and environmental samples

Before the use of bacterial sensors in the large scale analysis of environmental samples their applicability to environmental analysis was tested on selected samples. Response of a Cd sensor *Bacillus subtilis* BR151(pTOO24) (constructed by Tauriainen et al., 1998) towards Cd standard solution and Cd in water extract of a model soil T_{REF} is presented in Figure 3.2.

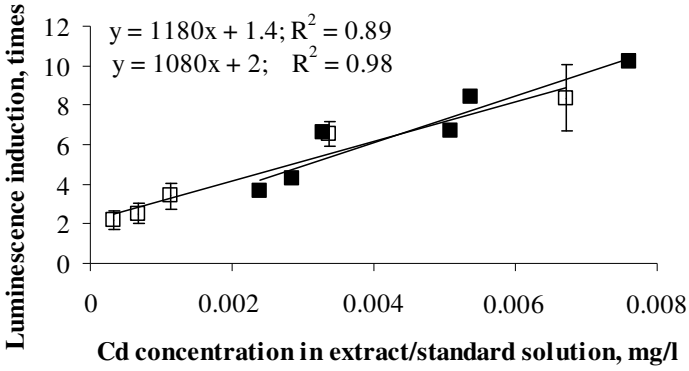


Figure 3.2 *Response of the Cd sensor bacteria to Cd in standard solution (open squares) or soil-water (1:10) extracts (model agricultural soil T_{REF}; total content of Cd 20 mg kg $^{-1}$) (closed squares). For standard curves mean values of 3 independent measurements are presented.*

As the response of the sensor towards Cd standard solution and soil-water extract were almost identical, all the chemically determined Cd in soil-water extract could also be detected by sensor bacteria. Thus, the sensors proved to be suitable for environmental applications and for this particular sample could even substitute the standard analytical methods. However, this is not usually the case for soil suspensions as often the total amount and bioavailable amount of metals, determined by bacterial cells, are different (discussed in more detail in next chapters).

3.2.2 Development of test protocol for complex samples

For more complex environmental samples like soils, sediments or solid waste and even toxic wastewaters the results from sensor cells could be interfered with the toxicity, colour or turbidity (strongly affecting the measurement of luminescence) of the sample. Turbidity of the sample is usually of concern in the case of soil suspensions, whereas color and toxicity may also occur when aqueous samples are studied. Thus, during this work `control bacteria`, which are analogous to sensors but non-inducible with heavy metals were constructed

and a protocol for the analysis of environmental samples incorporating these control cells to correct results from sensor bacteria was developed (paper **III**). *E.coli* MC1061(pTOO02) (paper **III**) was constructed to be used as control for all *E.coli*-based sensors, *Staphylococcus aureus* RN4220(pTOO02) (paper **III**) was constructed as a control for Cd-Pb sensor *S. aureus* RN4220(pTOO24) and earlier constructed *Bacillus subtilis* BR151(pCSS962/pBL1) (Lampinen et al., 1992) was used as a control for Cd sensor *B.subtilis* BR151(pTOO24). The environmental samples were tested with sensor and control bacteria in parallel and the correction factor calculated according to the results from controls (see Materials and Methods) was used to take into account the influence of the sample to bacterial physiology (resulting in changes in production of luminescence, which is tightly connected with energetics) as well as optical quenching of luminescence by solid particles and colour.

As a result of this work a test protocol allowing the calculation of bioavailable heavy metals in any unknown sample by comparing the calibration curves with standard metal and with a row of sample dilutions was developed (papers **II** and **III**) (see Materials and Methods).

3.2.3 Reproducibility of the sensor assay for environmental samples

Reliability of the developed protocol was evaluated by testing the reproducibility of the results with Cd sensor *B.subtilis* BR151(pTOO24) (paper **III**). Variation in bioavailability in repeated experiments ranged from 12 to 22 % being higher for more complex solid samples (soil-water suspensions containing solid particles) and lower for aqueous samples (soil-water extracts). This variation is certainly acceptable for a biological assay performed on natural samples.

3. 3 Bioavailability of heavy metals in environmental samples

In this work different environmental, mostly solid samples: natural soils and sediments, metal-spiked soil and Hg-spiked model soil components were analysed for their content of bioavailable heavy metals. In order to determine the most appropriate conditions for this analysis two approaches were tested: first, as general practice in exotoxicity testing, soil aqueous extracts (leachates) were tested and as a second approach contact tests with soil-water suspensions were performed. 1 to 10 liquid to solid ratio was chosen for the preparation of soil-water suspensions and respective particle-free extracts as it was found to be lowest optimal ratio to solubilize maximal percentage of the metals from soil (data from co-operative experiments with ISA. Lille, France) (Not shown.).

Bioavailability of Hg, Cd, Pb, Zn and Cr in soils determined during this work by bacterial metals sensor cells from either soil-water suspensions or respective centrifuged extracts are presented in Table 3.2. As seen, the bioavailability of Hg, Cd and Pb in soil was not equal to their water-extractability as it has often been presumed but in the case of direct bacteria-soil contact in soil-water suspensions exceeded it up to 115 times. This additional, `particle-bound bioavailable` fraction of metals, which becomes bioavailable when test bacteria are incubated in soil-water suspension is desorbed most probably from solid particles and colloids.

Table 3.2 *Bioavailability of heavy metals in soil-water suspensions and respective particle-free extracts as analysed by bacterial metal sensors*

Metal	Bioavailable (% of the total metal) Soil-water suspension	Soil-water extract	Bioavailability in soil suspension/ in water extract	Paper
Hg^{2+}	40[a]	1.3[a]	30.8	(**II**)
Cd^{2+}	12[a]	0.6[a]	20	(**II**)
	11.5[b]	0.1[b]	115	(**III**)
	1.02[c]	0[c]		(**VI**)
Pb^{2+}	2.8[b]	0.07[b]	40	(**III**)
	0.42[c]	0.048[c]	8.8	(**VI**)
Zn^{2+}	2.6[a]	2.6[a]	1	(**II**)
Cr^{6+}	46[a]	46[a]	1	(**II**)

[a] *metal-spiked soil*
[b] *median value for 50 natural soils*
[c] *median value for 60 natural soils*

Desorption of additional Cd from particles and/or colloids due to activities of bacterial cells was proved for Cd: bioavailability determined by Cd sensors decreased by increasing centrifugation force used to clarify the water suspension of the model soil (T_{REF}) (Figure 3.3). Most of the particles and colloids (84 %) carrying bioavailable Cd were removed from soil-water suspension already by centrifugation at 600 g. Additional 15 % of colloids with bioavailable Cd were removed when the centrifugation was increased up to 10 000g. No bigger colloids carrying bioavailable Cd than those spinned down at 10 000 g were present in this soil-water extract.

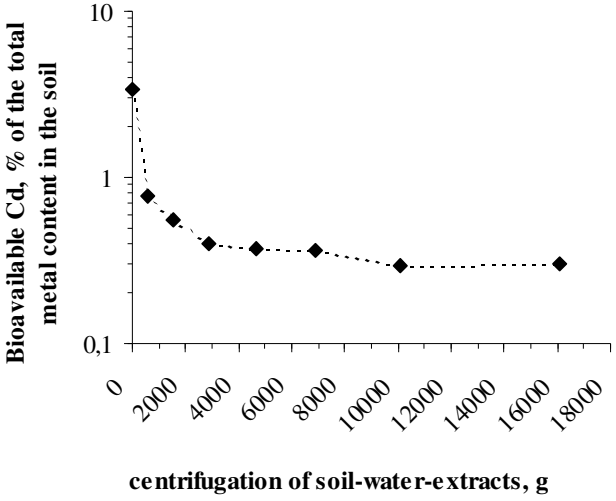


Figure 3.3 *Bioavailable Cd in soil-water suspension (non-centrifuged suspension) and extracts of a model agricultural soil T_{REF}; (total Cd content 20 mg kg^{-1}) clarified by different centrifugation speeds as determined by bacterial Cd sensor Bacillus subtilis BR151(pTOO24)*

Due to the great impact of particle-bound heavy metals on their bioavailability in solid samples, contact tests (by analysing e.g., soil-water suspension) should be used if reliable data on bioavailability of metals in these samples are requested. It should be mentioned that the most relevant data on bioavailability in solid environments could be obviously obtained by *in situ* analysis, however the analysis of water suspensions is the most appropriate if laboratory tests with bacterial sensors will be performed.

3. 4 Bioavailability of heavy metals in natural solid samples

Bacterial heavy metal sensors were used to determine bioavailability of Hg, Zn, Cd, Cr, Pb and Cu in 140 natural soil and sediment samples (120 sampled near heavy metal smelters and 20 originating from urban areas) (papers **III - VI**), an artificially polluted (spiked) soil (spiked with Hg, Cd, Zn and Cr) (paper **II**) and three pure model soil components: kaolinite, montmorillonite and humic acid (spiked with methylmercury) (in: Bernaus et al., 2005). Due to the issues discussed in previous chapter (importance of `particle-bound bioavailable` heavy metals) bioavailability of these metals was determined in contact assays with soil-water suspensions.

3.4.1 Factors influencing heavy metal bioavailability in soil

Bioavailability of Cr, Hg, Cd and Zn in spiked soil was determined in paper **II**. The values obtained in soil-water suspension were relatively high and decreased in order Cr (46% of total)> Hg (40 % of total)> Cd (12 % of total) > Zn (2.6 % of total). High bioavailability of metals in spiked soil has been also shown by other authors: For example, Bontidean et al., (2004) reported on 63% bioavailability of Hg in spiked soil if analysed in contact assay. However, it should be mentioned that the use of spiked soil is appropriate only for modelling and does not take into account different biotic and abiotic factors playing an essential role in sorption of metals to the soil solid matrix in the nature. Thus, the high bioavailability in spiked samples may not reflect the real behaviour of metals in field conditions. Indeed, when suspensions of natural soil samples were tested, generally the bioavailability of metals was lower compared with the spiked soils. Average bioavailability of Cd in 110 soils was around 1 % and the average bioavailable fraction of Pb only 0.42 % (see Table 3.2). However, the bioavailability was varying remarkably in different soils. From 0.1 to 56 % of the total Cd was in bioavailable form in different soils (data for 110 soils). Analogously high variation was shown for Pb and Cu: bioavailability of Pb ranged from 0.1 to 67 % (data for 130 samples) and that of Cu from 2.3 to 47 % (data for 20 samples). Thus, most likely the bioavailability of metals in soils was greatly dependent on soil properties, most important of which are organic matter, texture, pH and cation exchange capacity (Morel et al., 1997). A principal component analysis on the correlation of bioavailable Cd and key soil parameters of 110 agricultural soils from the collection of ISA was done in co-

operation with Institut Supérieur d'Agriculture (Lille, France) and is presented in Figure 3.4.

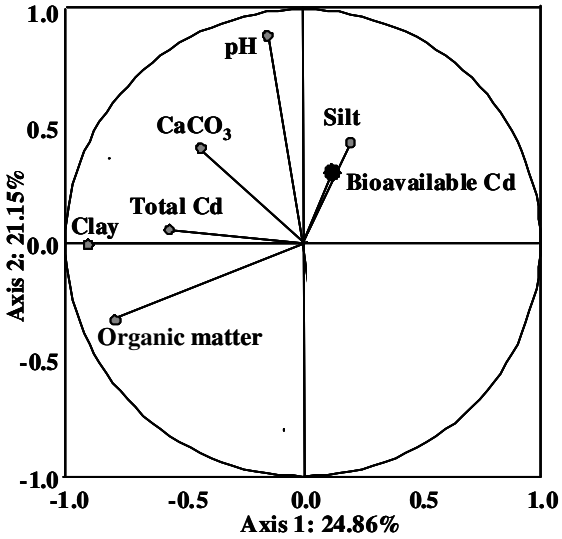


Figure 3.4 *Principal component analysis of key soil parameters and bioavailable fraction of Cd in 110 agricultural soils from the collection of ISA (see the content of heavy metals and physico-chemical properties from Table 2.2). Analysis was done in co-operation with ISA, Lille, France.*

The bioavailable Cd proved to be strongly negatively correlated with the soil organic matter. Weak positive correlation was found with silt, $CaCO_3$ content and pH. No significant correlation was detected between the bioavailable and total concentration of Cd in soil.

In addition to soil physico-chemical properties also interactions of metal ions with soil minerals may have an effect to bioavailability. During this work it was demonstrated that bioavailability of metals in soils may depend on the type of bondage. The analysis of bioavailable methylmercury with organomercury sensor *E.coli* MC1061(pmerR_{BS}Bluc) from spiked soil model components (montmorillonite and humic acids) showed that bioavailability of methylmercury was higher (56 % of total) in the case of montmorrilonite - common soil clay-mineral forming more ionic type bonds with methylmercury, compared with the bioavailability of methylmercury sorbed on humic acid (13%) - organic component of soil forming more covalent type bonds with methylmercury (Bernaus et al., 2005).

3.4.2 Correlations between bioavailability data from different organisms

*3.4.2.1 Comparison of data from bacterial and yeast cells (paper **IV**)*

As seen from Figure 3.5, the bioavailability of Cu determined with prokaryotic (bacterial) and eukaryotic (yeast) Cu sensor cells was almost identical. Moreover, the correlation between the data from these sensors was high (Figure 3.5).

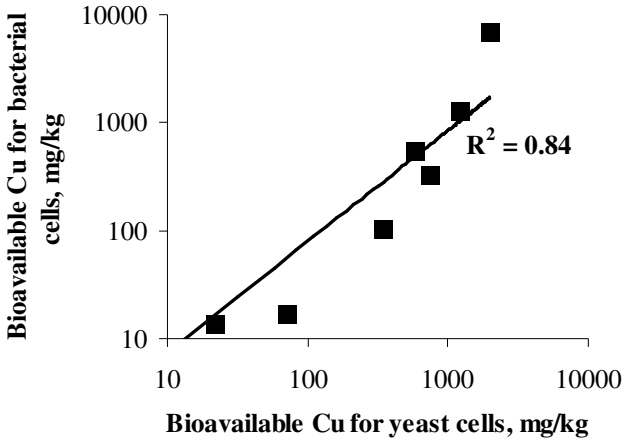


Figure 3.5
Bioavailable Cu in suspensions of 7 soils determined by bacterial and yeast Cu-sensor cells. Data from paper ***VI.***

Thus, according to the results from paper **VI** the bioavailability of Cu did not depend on differences in membrane/envelope of different type pf cells (even pro-and eukaryotic) but similar amounts of metal was desorbed from soil solid matrix and transported into yeast or bacterial cells enabling the transfer of data on bioavailability between these organisms.

3.4.2.2 Comparison of data on bioavailability of heavy metals: recombinant sensor bacteria vs ecotoxicological tests (paper ***V****)*

The combined chemical, ecotoxicological and biosensor-based hazard evaluation study was conducted on 60 smelter-influenced soils containing (mg kg^{-1}) Cd 1-13, Pb 50-653 and Zn 100-1198. Although heavy metal concentrations in the studied soils were high, the toxic effects of water extracts were observed only in few samples and in few biotests (e.g., algae *Selenastrum capricornutum*). For most of the aquatic test organisms the bioavailable concentrations of metals in soil-water extracts were either subtoxic (e.g., crustaceans) or the adverse effects were compensated by soil nutrients etc. (e.g., natural photobacteria, algae). Analogous effects by soil nutrients masking the toxicity of heavy metals for algae were observed by Aruoja et al., (2004).

Thus, only few aqueous extracts of these 60 soils showed toxic effect in conventional ecotoxicity tests (toxicity was registered as inhibition of the test organisms by more than 20% compared with the control). In this respect Cd-sensor bacteria were much more sensitive compared with the above-mentioned toxicity tests: as much as 10 soil-water extracts induced the luminescence of these sensors. The sensors proved even much more sensitive when soil suspensions were studied: 40 soil-water suspensions out of 60 induced the luminescence of Cd-sensor bacteria, indicating the desorption of Cd induced by direct contact of bacteria with soil particles. Thus, biosensors can be considered as "early-warning" biotests on hazard of heavy metals that already at very low

concentrations of bioavailable heavy metals give a warning signal on entrance of heavy metals into living cells.

Although the mechanisms of enhanced bioavailability of particle-bound pollutants in soil suspension assay are not yet known, the induction of the sensor bacteria clearly shows that even particle-bound heavy metals can be released and transferred into bacterial cells. Thus, one may expect a transfer through membranes of any biological system and in addition further transfer via the food-web. Even if no acute toxic effect is observed, this transfer may be significant for a long-term accumulation and chronic toxicity effects in biological systems. Thus, development and use of biosensors - excellent tools for mechanistic studies and signalling hazard already at subtoxic level - should be encouraged.

3.4.3 Correlations between chemical mobility and bioavailability

Bioavailability of metals in solid samples like soils and sediments has often been attempted to assess by their chemical mobility determined after treatment with "mild" chemical extractants releasing the most easily exchangeable heavy metals. During this work correlations between bioavailability (as determined by metal sensor bacteria) and chemical mobility of Cd, Pb (mobility determined after extraction with 0.11 M acetic acid: first step in BCR sequential extraction procedure - a three-step sequential extraction procedure for sediment analysis proposed by The Standards, Measurements and Testing Programme of the European Commission) and Cu (mobility determined after extraction with 1 M Na-acetate) were studied (papers **IV**, **V**) (Figure 3.6). Correlation between chemical mobility and bioavailability was very good in the case of Cu (Figure 3.6. C) whereas the correlations for Cd and Pb were remarkably weaker (Figure 3.6., A,B). As a rule, the chemical mobility was higher than bioavailability and exceeded the latter for 29 times as average in the case of Cd (data from 157 soils) and 3 times as average in the case of Cu (data from 14 soils) and Pb (data from 42 soils). However, as seen from Figure 3.6. these values vary greatly in different soils depending most probably primarily on the soil physico-chemical properties.

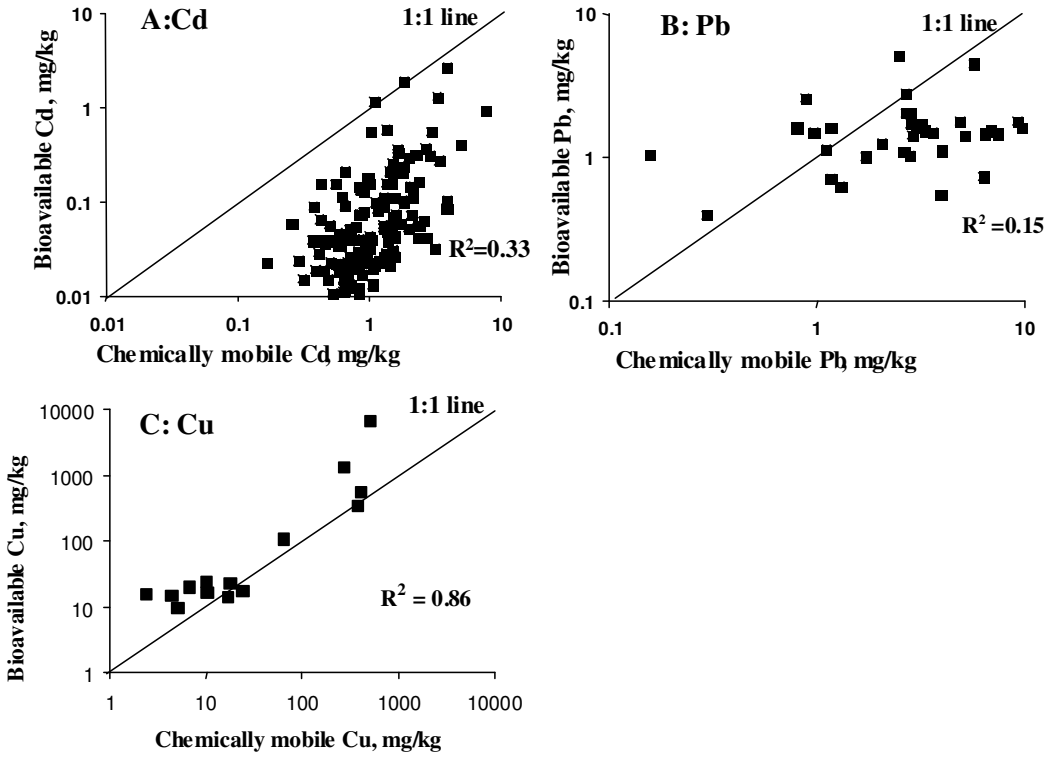


Figure 3.6 *Correlation between bioavailability (as determined by respective bacterial sensors) and chemical mobility (as determined by "mild" extractants 0.11 M acetic acid in the case of Cd and Pb and 1 M Na-acetate in the case of Cu) of Cd (data from 157 soils) (**A**), Pb (data from 42 soils) (**B**) and Cu (data from 14 soils) (**C**). 1:1 lines and R^2 values are indicated. Combined data from papers **IV** and **V**.*

3.5 Construction of fibre-optic heavy metal biosensors and their application on environmental samples

During this work the bacterial Hg and As sensors, *E.coli* MC1061(pmerluxCDABE) and *E.coli* MC1061(parsluxCDABE) constructed previously by Hakkila et al., (2002) (Table 2.1.) were immobilised in alginate matrix and integrated to an optical-fibre biosensor system developed in the National Institute for Biotechnology in the Negev (Beer-Sheva, Israel) (paper **VI**). Differently from bacterial sensors described earlier in this work, these sensor cells expressed bacterial luciferase system (*luxCDABE* genes containing all components for bioluminescence production) as reporter. The limit of quantification for the constructed biosensors and for the respective non-immobilised sensor cells is presented in Table 3.3.

Table 3.3 *Limits of quantification for fibre-optic Hg and As biosensors and of respective non-immobilized sensor cells. From paper* ***VI****. Reproduced by the permission from Elsevier.*

Sensor for (strain):	**Format of the assay**	**Limit of quantification, µg l^{-1}**
Hg (*Escherichia coli* (pmerluxCDABE)	Immobilized on optical fibres	2.6 ± 0.9
	Non-immobilized (free) sensor bacteria	0.03 ± 0.001
As (*Escherichia coli* (parsluxCDABE)	Immobilized on optical fibres	141 ± 32 [As (V)] 18 ± 4.1 [As (III)]
	Non-immobilized (free) sensor bacteria	80 ± 1.6 [As (V)] 8 ± 0.2 [As (III)]

The limits of quantification for both Hg and As biosensors were lower compared with non-immobilised sensor cells. For As the difference was relatively small: 1.8-fold in the case of As(V) and 2.2-fold in the case of As (III). However, for Hg the difference was about 100-fold, most probably due to the complexation of Hg^{2+} with alginate matrix resulting in lower apparent bioavailability of Hg. Thus, when environmental samples are analysed, the results obtained by alginate-immobilised sensor bacteria may rather model the reduced bioavailability of metals to, e.g., biofilm-entrapped micoorganisms: one targets for development of biocides for different (e.g., pulp and paper) industries. Despite of the higher limit of quantification (i.e., poorer sensitivity) of the fibre-optic biosensors compared with non-immobilised bacteria the former still work in the environmentally relevant ranges. Moreover, especially if further optimisation of the system (e.g., selection of immobilisation matrix) would be carried out, the fibre-optic biosensors have several advantages over non-immobilised bacteria, of which the main ones are ease of use of the system and possibility of continuous *in situ* tracking of a site.

As mentioned above, one advantage of the use of immobilised sensor cells instead of bacterial suspension would be their easy use *in situ* conditions. Thus, applicability of the constructed biosensors was tested on natural soil and sediment samples (paper **VI**). The biosensors proved useful and allowed the quantification of bioavailable Hg and As from natural samples. However, the bioavailable fraction detected with biosensors remained lower (20 times in the case of Hg and 3.4 times in the case of As) than what was seen with conventional non-immobilised sensor bacteria. This difference was most likely due to the `particle-bound bioavailable` fraction of metals in soils and sediments (see section 3.3), that was bioavailable to free sensor bacteria in contact assays. However, in case of alginate-entrapped sensor cells the direct contact was possible only for bacteria on the biosensor surface areas whereas the cells near to optical fibre contributing most to the light output of the sensor were masked by several layers of alginate matrix.

CONCLUSIONS

- During this work four new bacterial sensor strains - gene-modified bacteria - that produce luminescence as a response to bioavailable heavy metals were constructed:
  1. Zn-sensor *Escherichia coli* MC1061(pzntluc) with limit of quantification 5.4 mg of Zn^{2+} l^{-1}
  2. Cr-sensor *Ralstonia eutropha* AE104(pchrluc) with limit of quantification 0.01 mg of $Cr_2O_7^{2-}$ l^{-1}
  3. Cu-sensor *Escherichia coli* MC1061(pSlcueR/pDNPcopAluc) with limit of quantification 3.2 mg of Cu^{2+} l^{-1}
  4. Hg- and organomercury-sensor *Escherichia coli* MC1061(pmerR_{BS}Bluc) with limit of quantification 0.05 μg of CH_3Hg^+ l^{-1} and 0.003 mg of Hg^{2+} l^{-1}.

- Hg-sensor bacteria *E. coli* MC1061(pmerluxCDABE) and As-sensor bacteria *E. coli* MC1061(parsluxCDABE) constructed by Hakkila et al. (2002) were alginate-entrapped and incorporated into a fibre-optic sensor system applicable for field analysis of bioavailable heavy metals. The limit of determination for fibre-optic biosensors was 0.0026 mg of Hg^{2+} l^{-1}, 0.018 mg of As^{3+} l^{-1} and 0.14 mg of As^{5+} l^{-1}. The fibre-optic sensors were constructed in co-operation with Ben-Gurion University of the Negev

- All constructed sensors responded to target metals in environmentally relevant concentration range. Cr-sensor was induced only by Cr. Cu sensor was co-induced by Ag and Hg. Zn sensor was co-induced by Hg, Cd and Zn and Hg sensor with Cd.

- A protocol for quantification of bioavailable heavy metals in natural soils and sediments using recombinant bioluminescent sensor bacteria was developed. Reproducibility of the assay even for soil suspensions was acceptable (CV= 22 %). The novel aspects of the protocol involve the parallel measurements of the samples using control bacteria that are not induced by heavy metals but otherwise similar to the sensor bacteria used for metal analysis. Thus, the analysis made by control bacteria takes into account the toxic or stimulatory effects of the samples to bacterial luminescence but also the quenching of luminescence light by turbidity/colour of the environmental sample (such as soil suspensions etc). This protocol is applicable to all assays where test matrix and/or conditions may interfere with the test results.

- The constructed sensors were applied to large panel of environmental samples (140 natural soils and sediments, 1 spiked soil and 3 spiked soil components analysed as water suspensions and extracts) for the analysis of bioavailable heavy metals. The data obtained with recombinant microbial sensors were combined with data obtained by surrogate

chemical methods currently used in risk assessment studies as well as with toxicity data for a battery of non-specific ecotoxicological tests

a) The bioavailable amount of heavy metals to sensor bacteria in soil suspension assays did not correlate with their total concentrations in the soils. The bioavailable fraction of metals varied from 1 to 46 % of the total metal in the soil depending on the metal, soil type and other factors.

b) The bioavailable fraction of Cu in copper-polluted soils analysed with bacterial sensors was almost identical with the results obtained with yeast-based sensor cells indicating that the bioavailability of metals in soils for prokaryotic and eukaryotic cells may be similar

c) The parallel analysis of soil-water suspensions and (centrifuged) particle-free extracts showed that the bioavailability of Hg, Cd and Pb in soil suspensions was remarkably higher (up to 115-fold for Cd and 40-fold for Pb) than their water-extracted fraction.

d) Bioavailability data from bacterial sensors and the results from chemical surrogate extraction methods (often used to predict the mobility and bioavailability of metals for risk assessment purposes) were not significantly correlated. As a rule, the chemical extraction methods over-estimated the bioavailability of metals if compared with bacterial sensors. In the case of Cu and Pb, the average difference was 3 times but in case of Cd the bacterial sensors showed 29 times lower bioavailability. Thus, according to the data from this work, prediction of bioavailable Cd with chemical extraction methods is not relevant at least for the bacteria used in the current work.

e) The fibre-optic biosensors for Hg and As were successfully used for analysis of bioavailable fractions of these metals in soils. The bioavailable fraction of Hg in polluted soils analysed with alginate-entrapped sensor bacteria (a biosensing component of the sensor system) was remarkably lower than for the non-immobilised bacteria. Thus, the alginate-entrapped bacteria could be suggested for modeling of the reduced bioavailability of metals and other toxicants in biofilms, e.g., for studying the efficiency of biocides.

SUMMARY AND OUTLOOK

This study showed that the luminescent recombinant bacterial metal sensors are powerful tools for the rapid assessment of bioavailable concentrations of heavy metals in different environmental matrices already at sub-toxic level. This is extremely important as the effects of sub-toxic metals are not detectable by other test-organisms, however bioavailable metals may be transferred via the food-web and cause chronic toxic effects in biological systems. Thus, development and use of biosensors - excellent tools for mechanistic studies and signalling hazard already at subtoxic level of pollutants - should be encouraged.

In the future we are planning to continue our work in following directions:

1) to construct more sensitive sensor bacteria by e.g., by knocking out the heavy metal transport proteins and thus disrupting the metal homeostasis in the host bacterial cell
2) to construct more specific sensors (or modify the test format) ensuring the selective response only to a target analyte.
3) to elucidate the mechanisms for elevated uptake of particle-bound metals in the case of direct contact of test bacteria with soil particles
4) to elaborate models for prediction of bioavailability of heavy metals in different (environmental) samples applicable for risk assessment purposes

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LIST OF PUBLICATIONS

Publications in CC journals

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3. Ivask, A., Francois, M., Kahru, A., Dubourguier, H. C., Virta, M., Douay, F. 2004. Recombinant luminescent bacterial sensors for the measurement of bioavailability of cadmium and lead in soils polluted by metal smelters. Chemosphere, 55, p. 147-156

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6. Peltola, P., Ivask, A., Åström, M. and Virta, M., 2005. Lead and Cu in contaminated urban soils: Extraction with chemical reagents and bioluminescent bacteria and yeast. Science of the Total Environment, 350 (1-3), 194-203

7. Kahru, A., Ivask, A., Kasemets, K., Põllumaa, L., Kurvet, I., Francois, M. and Dubourguier, H.C. 2005. Biotests and Biosensors in ecotoxicological risk assessment of field soils polluted with zinc, lead and cadmium. Environmental Toxicology and Chemistry, 24(11), 2973-2982

8. Leedjärv, A., Ivask, A., Virta, M., Kahru, A. Analysis of bioavailable phenols from natural samples by recombinant luminescent bacterial sensors. Chemosphere, in press (accepted for publication, 2006)

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