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Name DEVELOPMENT OF A WHOLE-CELL BASED BIOSENSOR TECHNIQUE FOR ASSESSMENT OF BIOAVAILABILITY ANDTOXICITY OF HEAVY METALS IN SOIL

DEVELOPMENT OF A WHOLE-CELL BASED BIOSENSOR TECHNIQUE FOR ASSESSMENT OF BIOAVAILABILITY AND TOXICITY OF HEAVY METALS IN SOIL

by

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Development of a Whole-cell Based Biosensor Technique for Assessment of Bioavailability and Toxicity of Heavy Metals in soil

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ABSTRACT

The aim of this study was to develop a suitable monitoring protocol for mediated amperometric whole-cell biosensors for *in situ* assessment of heavy metals in soil. *E. coli 8277*, *Pseudomonas 9773*, *Pseudomonas 9046* and *Pseudomonas 8917* were screened as biosensor catalysts to select the sensitive biosensor configurations to heavy metals. A new protocol was developed for monitoring heavy metals in defined solution, soil pore water, and *in situ* in soil. This study also demonstrated the applications of mediated amperometric bacterial biosensors for *in situ* assessing the bioavailability and toxicity of heavy metals in freshly spiked soils or historically contaminated soils, and mixture toxicities of heavy metals.

It was found that the biosensors incorporating selected bacterial strains were appropriately sensitive to copper, but less sensitive to Zn, Pb, and Hg, compared to Microtox assay. The advantage of the mediated amperometric bacterial biosensor system is its *in situ* application in soils. The present study demonstrated that soil pore water does not accurately reflect conditions of soil ecosystem, and that *in situ* bioassays are more reliable for determining the bioavailability and toxicity of heavy metals. This is the first reported use of disposable whole cell biosensors for *in situ* heavy metal bioavailability and toxicity assessment. The biosensor protocol developed here can be adapted to allow the incorporation of dfferent bacterial biocatalysts for applications in soil quality assessment, screening of sites for contamination 'hot spots', and the evaluation of soil degradation or rehabilitation from metal pollution. Mediated amperometric bacterial biosensors are not analyte specific, their response reflecting the metabolic impact of the combined chemical and physical properties of the environment to which they are exposed. In assessing the toxicity of soil samples from fields using these biosensors, it is vital to get appropriate control soil samples. The conditions of soil samples also need to be well defined.

The sensitivity of the mediated amperometric whole-cell biosensors to heavy metals need to be further improved. Investigations are also required to determine how the natural conditions affect the application of the biosensor system in the field.

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DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Bedfordshire. It has not been submitted before for any degree or examination in any other University.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BOD	Biochemical oxygen demand
CEC	Cation exchange capacity
3,5-DCP	3,5-Dichlorophenol
DOC	Dissolved organic carbon
DTPA	Diethylene-triamine-pentaacetic acid
ECB	European Chemical Bureau
EC*x*	Effective concentrations
EDTA	Ethylenediamine tetraacetic acid
EEC	European Economic Community
EPA	Environmental Protection Agency
ETC	Electron transport chain
FA	Fulvic acid
FeCN	Potassium ferricyanide
FIAM	Free ion activity model
GFP / *gfp*	Green fluorescent protein / gene
HA	Humic acid
ICP-AES	Inductively coupled plasma atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
ISO	International Organisation for Standardisation
LIRANS	Luton Institute for Research in Applied Natural Sciences
LUC / *luc*	Eukaryotic luciferase protein / gene
LUX / *lux*	Prokaryotic luciferase protein / gene
OECD	Organisation for Economic Co-operation and Development
MC	Moisture content

pBQ	*p*-Benzoquinone
SOM	Soil organic matter
TBLM	Terrestrial biotic ligand model
TRAP	Toxicity Relationship Analysis Program
WHC	Water holding capacity

1 Introduction

1.1 Background

Contamination of soil with heavy metals is a worldwide problem that could threaten the sustainability of essential soil functions (Nriagu and Pacyna, 1988; Giller et al., 1998). Assessing the ecological risk of soil contaminated with heavy metals in order to regulate for protection and management of soils is one of the most important tasks for toxicologists, risk assessors and regulatory agencies. Most current regulations governing metal toxicity in soils are based on the total metal concentration which is determined after digestion with strong acids or extraction with organic solvents (Tandy et al., 2005), however, these methods are not able to distinguish between available (potentially hazardous) and non-available (potentially non-hazardous) fractions of metals to biological systems. As a result, the risk of heavy metal contamination in soil to humans, animals and plants has been frequently overestimated. Many approaches have been developed and employed to determine bioavailable heavy metal concentrations in the environment, including chemical analytical methods and bioassays (Kong et al.,1995; Rensing and Maier, 2003). However, due to the combined effect of the soil properties, soil organisms and metal characteristics on bioavailability and toxicity, assessment and measurement methods for bioavailability and toxicity of heavy metals in soil has been a challenge (Rensing and Maier, 2003; Basta et al., 2005). On the other hand, the heavy metal pollution is usually site-specific because of the commonly existing spatial differences of soil properties, testing extensive spatial areas for individual metals can be time consuming and

expensive. A rapid bioassay which is able to be used in soil for *in situ* toxicity and bioavailability assessment is urgently needed.

1.2 Bioavailability and toxicity of heavy metals in soil

Metals can be classified into two groups based on their function in biological systems, i.e., essential metals and non-essential metals. Essential metals such as Na, K, Mg, Ca, V, Se, Cr, Fe, Co, Ni, Cu, Zn and Mo play roles as both bulk and trace essential elements for organisms and enzymes in soil. Na, K, Mg, and Ca are regarded as bulk elements, while Fe, Zn, Cu, Co and other metals are generally required only as trace elements for growth and metabolic functions (Hughes & Poole, 1989). Metals such as Cd, Hg, Sn, Pb, Ag and Al have no biological value or role and are toxic for living organisms at very low concentrations (Gadd, 1992). However, elevated concentrations of both essential and non-essential heavy metals in the soil can lead to symptoms of toxic damage and the inhibition of growth of most organisms.

In soil ecosystems, most heavy metals sorb to the soil matrix, becoming less mobile and thus less toxic or non-toxic to living organisms (Welp and Brűmmer, 1997). Environmental hazard of heavy metals in soils depends to a large extent on their bioavailability. Therefore, it is important for risk assessment to distinguish between bioavailable and non-bioavailable fractions of metals in soil, and to understand the factors that could possibly affect the bioavailability and toxicity of metals.

1.2.1 The definitions of bioavailability and toxicity of metals

The biological availability of heavy metals relates to the quantity that is accessible by the organism (Van der Zee et al., 2004). Rensing and Maier (2003) defined the bioavailable metals as the fractions that can interact with surrounding microbial cells or other biota, they usually exist as the soluble, or easily leachable ionic form of the metal (Schultz et al., 2004). Several studies (Van der Meent et al., 1990; Van Gestel and van Diepen, 1997; Løkke, 1994) suggest that the dissolved fraction in soil pore water is bioavailable. However, some particle-associated metals are also available to biota under certain conditions. For environmental risk assessments involving soil and sediments, this definition implicitly includes the extent to which a substance can desorb, dissolve, or otherwise dissociate from the environmental medium in which it occurs to become available for absorption. Peijnenburg et al. (1997) highlighted that bioavailability should be handled as a dynamic process that comprises two distinct phases: a physicochemically driven desorption process (environmental availability) and a physiologically driven uptake process (environmental bioavailability).

The toxicity of metals depends on both the properties of metals and the sensitivity and resistance of the organisms being investigated. According to Luckey and Venugopal (1977), the constitutional toxicity of a metal reflects its binding capacities to living systems (tissues, cells, organelles, or biological macromolecules) and, therefore, the analysis of the interactions between metals and biological structures, and the physical and chemical properties of metals might explain both the inherent toxicity and the overall toxicity of a metal in a

biological system. Bioavailability is a prerequisite for toxicity, it is the potentially hazardous fraction although it does not necessarily result in toxicity.

Bioavailability is a complex term dealing with species-specific and dynamic processes. It is strongly associated with the species considered and with the type of exposure, including metal speciation. The complexity of parameters and interactions is not always sufficiently considered in toxicity testing.

1.2.2 Factors influencing the bioavailability and toxicity of metals in soil

Metal bioavailability is strongly influenced by soil properties and soil organisms. The main soil properties affecting metal bioavailability include pH, redox potential, organic matter (both particulate and dissolved fractions), clay content (especially 2:1 clay minerals), ionic strength and species (Sposito, 1989; Alloway et al., 1990; Moore, 1994), temperature, and soil moisture content.

1.2.2.1 pH

The pH is one of the most important factors affecting the bioavailability and toxicity of heavy metals in soils both directly and indirectly. The pH affects many aspects of the interactions between microbes and heavy metals, resulting in increasing or decreasing the metal toxicity, some of the data are even contradictory (Babich and Stotzky, 1986).

The mechanisms involved in pH-metal toxicity relations have not been clearly defined, there are several aspects that probably affect the assay system. Firstly, pH affects the chemical speciation of some divalent metal (M^{2+}) (Hahne and Kroontje, 1973). Increasing pH may cause the formation of complex hydroxylated species of the metals. Different hydroxylated metal species exert toxicities that are different from those of the respective divalent cations. pH also influences metal speciation by affecting the solubility of the metal (Kovács et al., 2006). Secondly, pH affects the extent of complexation and binding of metals to organic constituents in the growth medium, including to the cells themselves (Sakaguchi el al., 1979; Wu el al., 2003). In natural environments, pH influences the mobility of metals by influencing their complexation with humic acid, cellulose, clay minerals, hydrous metal oxides, clay mineral-humic acid and clay mineral-cellulose complexes, sediment, and soil. With most studies demonstrating that as the pH is increased, the compexation of heavy metals with these inorganic and organic constituents is also increased, resulting in a lower metal availability because of the competition of metal cations with protons for sorption sites. Thirdly, pH affects the metabolic state of the cell (Johnson el al., 2007), and the specific responses to a metal at different pH levels may reflect the altered physiology of the cells.

1.2.2.2 Redox potential

Redox potential also influences metal speciation and in turn their bioavailability. The redox potential (*Eh*) of an environment is established by oxidation-reduction reactions that tend to be relatively slow, particularly in soil environments. However, microbial activity can dramatically influence the rate and

establishment of redox potential in soil. Reducing conditions (negative *Eh*) found in anaerobic media can result in metal precipitation with media components. For example, in saturated soil systems which is normally under reducing conditions, cationic metals such as Fe^{2+}, Cd^{2+}, and Pb^{2+} combine with sulfides to form nontoxic, insoluble sulfide deposits (Kong et al., 1998). However, under oxidizing conditions (positive *Eh*), metals are more likely to exist in their free ionic form and exhibit increased water solubility. In addition, pH may decrease slightly or even dramatically under oxidizing conditions so as to further increase the solubility of metals (Rensing and Maier, 2003).

1.2.2.3 Organic matter

Soil organic matter (SOM) is thought to be one of the most important factors governing solubility and bioavailability of metals in soil-plant systems. Many studies have shown that heavy metals bound to organics, such as organic acids, humic and fulvic acids (HA and FA), and ethylenediamine tetraacetic acid (EDTA), either soluble (Babich and Stotzky, 1983; Hart, 1981; Wallace, 1982), or particulate (Babich and Stotzky, 1982; Nienke and Lee, 1982; Salim, 1983), are less toxic to the microbiota than are their free forms. The effects of soil dissolved organic matter on behaviors of soil heavy metals were summarized as complexing with heavy metals, competing with metals for adsorption sites, and affecting soil pH value.

Metal ions with different speciation, such as copper and mercury, may be more significantly complexed by dissolved humic substances than by inorganic anions, due to higher stability constants of humic acid-metal complexes (Merian,

1991). Kungolos et al. (2006) reported that the presence of HA decreased the toxicity of Cu, increased the toxicity of Pb, but did not significantly affect the toxicity of Zn. The possible reason was that the Pb complexes with humic acids are bioavailable to a higher extent than the soluble phases of Pb.

1.2.2.4 Clay mineral content

Clay minerals are highly structured hydrous aluminosilicates characterized by high surface activity, and have been shown to decrease metal bioavailability by adsorbing heavy metal cations. The extent of adsorption of heavy metals to clay minerals is directly related to their cation exchange capacity (CEC): the greater the CEC, the greater the amount of heavy metal cations adsorbed (Farrah and Pickering, 1977; Reid and McDuffie, 1981). Babich and Stotzky (1982) pointed out that clays with high CEC, such as montmorillonite, appear to reduce metal bioavailability and toxicity most.

The adsorption of heavy metal cations to clay minerals is affected by other abiotic factors. For example, the adsorption of Cd, Cu, Pb, and Zn to kaolinite, illite, and montmorillonite increased as the pH increased. The background concentration of competing cations also affects the adsorption of heavy metals to clay minerals, as increasing the concentration of Ca^{2+} and Mg^{2+} reduced the adsorption of Ni^{2+} (Mattigod et al, 1979) and Cu^{2+} (Gupta and Harrison, 1981).

1.2.2.5 Water content

The water content of soil influences metal species distribution between solid and solution phases, and also the level of microbial activity. Tom-Petersen et al. (2004) reported that the bioavailable copper to total copper ratio in soil water extracts decreased with decreasing moisture in the soil. Possibly a low DOC turnover in dry soil as a consequence of reduced microbial activity can partly explain a higher proportion of strongly complexed Cu in these soils. Aelion and Davis (2007) also reported an increasing toxicity as soil moisture increased. On the other hand, soil water content also affects the redox potential of soil. For example, the increase of soil water content could result in reducing conditions and a decreased bioavailability of metals (also see Section 1.2.2.2).

1.2.2.6 Temperature

Temperature, although not affecting the chemical speciation of heavy metals, does influence the rate of chemical reaction, the rate of metal uptake by the biota (Prosi, 1989), and the general physiology of the target organisms. The target organism usually appears to be less sensitive to a heavy metal when exposed at a temperature optimal for the process being studied than when exposed at a suboptimal temperature. Yongue et al. (1979) reported that Cr^{6+} was more toxic to *Euglena gracilis* survival at 31.5 °C than at 20°C.

1.2.2.7 Inorganic ionic composition

The soil solution is essentially a weak electrolyte composed of a variety of inorganic, as well as organic, cations and anions of different valencies. The types and concentrations of inorganic cations normally present in soil influence the toxicity of heavy metals to the biota, as competition for sites on cell surfaces between these cations and the cationic speciation forms of the heavy metals affects the extent of uptake by the cells (Babich and Stotzky, 1986). Furthermore, the background cations in soil mediate the mobility of heavy metals by influencing their adsorption to clay minerals and other particulates. As described above, increasing the concentration of Ca or Mg reduced the adsorption of Ni or Cu to kaolinite. Other inorganic anions, such as CO_3^{2-}, S^{2-}, and PO_4^{3-}, also influence heavy metal toxicity. Depending on the pH and/or *Eh*, these anions may interact with heavy metals to form insoluble salts, which are unavailable for uptake by the microbiota.

1.2.2.8 Soil organisms

Soil organisms may influence metal bioavailability and toxicity by two mechanisms: (a) modify the soil environment resulting in influencing physicochemical sorption mechanisms of metals. Marschner and Romheld (1983) reported that the production of protons, exudates, and metabolites released in the rhizosphere by the roots and the microorganisms can modify the pH by as much as one or two units, thus favoring the desorption of metals from soil particles. Gadd's (2004) results also proved that particle-bound metals are released due to the activity of microorganisms and plant roots. (b) species-

specific uptake mechanisms. For example, the selective uptake of plant may change the equilibration process of metal sorption. On the other hand, different organisms possess different sensitivities and tolerances to different metals, therefore, for risk assessment, the target organisms always need to be taken into consideration.

1.3 Bioavailability and toxicity assessment of metals in soil

Bioavailability is a concept that is easily understood but difficult to measure in both laboratory and environmental systems, because the bioavailability is a complex function of many factors, and also the bioavailable metal content depends on the defined assay conditions. There are many approaches developed to measure bioavailability and toxicity of metals, including analytical chemical measurement of metal concentrations combined with metal speciation modeling, and toxicity bioassays.

1.3.1 Analytical chemical methods

Standard analytical chemical techniques are able to precisely determine the total and specific species of metals. Total concentration of metals in soils does not give accurate information about bioavailable fractions to organisms, however, very few tools are available for getting a proper estimation on how much of the total concentration of a certain metal is bioavailable for transportation or uptake into living organisms. For several decades extraction techniques have been applied as a tool to provide information about the operational speciation of heavy metals in soils and sediments. (Peltola et al., 2005). The techniques

employed include both sequential extractions and pore water collection. A sequential extraction procedure developed by Tessier et al. (1979) has been widely used. It uses increasingly strong extractants to release trace metals associate with (a) exchangeable and cation exchange, (b) carbonate, (c) metal oxide or reducible, (d) organic and sulfide, and (e) residual mineral phases. The extractants that have been used are diethylene-triamine-pentaacetic acid (DTPA), ethylene diamine tetraacetic acid (EDTA), acetic acid, and the mineral acids, HNO_3 or HCl (Adriano, 1986). This approaches give a useful approximation of environmental availability of metals. The problem is that the extractant or leaching solution has a strong influence on the results, and are selective to different metal fractions. Impellitteri et al. (2003) showed that 0.01 M $CaCl_2$ extraction was the best predictor of plant (barley, lettuce, and mustard) zinc concentration; whereas 0.01 M HCl extraction was the best predictor of plant copper concentration.

Many studies demonstrated the relationship between metal speciation and their biological effects, and considered the free metal ion as the bioavailable species (Allen and Hansen, 1996). The free metal ion concentration is determined by the chemical equilibrium between three metal-containing phases in the soil: (a) the labile or exchangeable adsorbed phase, (b) the dissolved complexed phase, and (c) the dissolved free metal ion (Fujii *et al*., 1983). Plant availability and uptake of trace metals are generally thought to be related to the free metal ion concentration in soil (Peijnenburg et al., 1997). There are, however, also problems related to (i) the reliability of equilibrium constants, (ii) the description of the complexation behaviour of naturally occurring organic ligands, and (iii) the description of the adsorption behaviour of naturally occurring adsorption phases (Hendrickson and Corey, 1983).

Soil pore water is the dominant exposure medium for most soil-dwelling organisms to water-soluble chemicals (Hund-Rinke and Kördel, 2003), especially relevant for most soft-bodied soil organisms such as earthworms, enchytraeids, nematodes, and protozoans (Vijver et al., 2003). Many studies concluded the metal bioavailability from the bioassays in soil pore water. However, it may be not true for hard-bodied organisms such as springtails, mites, isopods, carabids, spiders, diplopods, millipeds, and harvestmen, because they may be able to absorb metals combined with soil particles through skin or digestive system. Therefore, soil pore water may not overall reflect the bioavailable metals in soils.

In summary, analytical methods are very useful for explanation of bioavailability or toxicity of metals, however, judgements based solely on chemical analyses of total metal concentrations or certain metal species in soils do not give accurate indications of the soil's potential toxic effect (Tiensing et al., 2002; Gu et al., 2004; Ragnvaldsson et al., 2007). These methods need to be related to the exposure route of organisms.

1.3.2 Bioassays for bioavailability and toxicity assessment

Bioassays are procedures that use living material to estimate chemical effects. They have been widely used to predict or assess the adverse effects of heavy metals on populations, communities, and ecosystems. Only bioavailable metal fractions could affect or potentially affect the metabolic activities of organism, therefore, bioassays are able to give an indication of metal bioavailability, or an

explicit interpretation of toxicity. Different contact bioassays (where the organisms are in contact with solid matrix during the exposure) incorporating invertebrates, e.g., worms (Peijnenburg et al., 1999), plants and enzymes, soil microorganisms (Welp and Brummer, 1997) have been used to determine the bioavailable fractions of heavy metals and their transfer to living organisms from soils.

1.3.2.1 Invertebrate tests

As soil dwelling organisms, terrestrial invertebrates are wildly used for ecotoxicological tests. There are a tremendous variety of invertebrates in the soil, which are distributed in the different soil profiles, and play an important role in the terrestrial ecosystem. Among these organisms, earthworms are the most commonly used for toxicity assessment, and earthworm standardized test methods have been well developed for the acute toxicity tests (OECD, 1984a; ISO, 1998). Heavy metals could affect the reproduction of invertebrates (juvenile or cocoon), and even be lethal to them. Therefore, reproduction and mortality are generally used as measuring parameters. A numerous of studies have analysed the relation between earthworm production and the quantities of metals (including total metal and different fractions) in soils, so as to define the bioavailability of metals, or identify the levels of soil ecosystematic risk. Attention needs to be paid to the exposure routes of invertebrates to soil when to predict the available metals. As described above, soft-body worms adsorb metals from soil pore water, thus metal in solid phase of soil is not available; however, some soil-ingesting worms may directly take up metals from the solid phase (Peijnenburg et al., 1997). In comparison with microorganism tests, earthworms

are insensitive to heavy metal pollution (Kahru et al., 2005). Invertebrate tests for acute toxicity take 48 h or longer, and chronic toxicity tests can take weeks. For large-scale soil pollution studies, time and labour consuming could be another disadvantage of the earthworm tests.

1.3.2.2 Plant tests

Plant test systems have also been widely applied for toxicological assay. Plant toxicity tests include germination, plant growth, or growth of roots (ISO, 1993, 1995; OECD, 1984b). The seed germination test is relatively insensitive to heavy metals, the probable reason is that at this stage, the seedlings derive their nutrients mostly from seed reserves rather than from the soil. This test thus provides a poor indication of soil contamination, while the root elongation assay provides a more sensitive measure of toxicity (Kapustka, 1997). The extent of nodulation in the legume root nodule symbiosis appears to be very sensitive to heavy-metal contamination (Neumann et al., 1998). The accumulation of metals in plant tissues is also generally used as an indicator of bioavailable metals (Chaignon and Hinsinger, 2003; Zhang et al., 2004). For risk assessment, testing of a wide variety of plants is requested, including regionally specific wild plants to cover the variability of the flora. Since plants have higher resistance and adaption mechanisms, it may be difficult to directly identify the bioavailable amount of metals through the amount in plant tissues or inhibition effect of metal on plant growth.

Although plant tests are highly ecologically relevant, they have some drawbacks in their application for toxicity and bioavailability assessment. The plant tests

described in the guidelines are usually conducted in small pots with a ratio between soil and roots that differs significantly from field conditions. As a result, the plants may take up larger amount of test substance than they would under the field conditions (Hund-Rinke and Kördel, 2003). It also requires a lot of time and space, thus can be costly to apply to large numbers of soil samples from the site of investigation.

1.3.2.3 Enzyme toxicity tests

Enzymes in soils originating from animal, plant and microbial sources play an important role in nutrient cycles, thus enzyme activity is a good indicator of soil quality. Most enzyme toxicity tests are based on the inhibitory effect of chemicals on enzyme activity. Enzyme biosynthesis inhibition have also been suggested serving as a basis for toxicity assays (Reinhartz et al., 1987). A wide range of enzymes have been explored for use in toxicity bioassays in aquatic environments and soils. These include dehydrogenases, ATPases (Bitton and Koopman, 1992), esterases, phosphatases (Tyler, 1976), urease (Douglas and Bremner, 1971), luciferase (Obst et al. 1988; Xu and Dutka 1987), *β*-galactosidase (Dutton et al. 1988, 1990), protease, amylase, and *β*-glucosidase (Obst et al. 1988).

Dehydrogenases, urease, and phosphatases are the enzymes most utilized in soil toxicity assays. Dehydrogenase enzymes catalyse the oxidation of substrates by transfer of electrons through the electron transport chain (ETC). Urease enzymes and phosphatases play a major role in the nitrogen cycle, in the mineralization processes of organophosphorus substrates. Since these

enzymes are highly relevant to ecological function of soil system, enzymatic assays are representative of the metabolic capacity of the soil. Numerous studies have been carried out to measure the effect of heavy metals on enzyme activity or enzyme biosynthesis and are found to be particularly sensitive to heavy metals. Stuczynski et al (2003) pointed out that Zn had a substantial inhibitory effect on soil dehydrogenase, acid and alkaline phosphatase, urease, and nitrification potential, Pb strongly affected soil urease.

β-galactosidase is an enzyme that converts lactose to galactose and glucose. Biosynthesis of *β*-galactosidase is induced by lactose or lactose analogs. *De novo* biosynthesis of *β*-galactosidase in *Escherichia coli* was found to be sensitive to many toxic chemicals, including pesticides and heavy metals (Dutton et al., 1988; Reinhartz et al., 1987). In contrast, assays based on the inhibition of *β-galactosidase* activity have been found to be senitive only to heavy metals (Bitton and Koopman. 1992; Dutton et al. 1988). Another hydrolase that has been used in toxicity testing is α-glucosidase, which cleaves the 1,4-glucoside linkage of maltose. Dutton et al. (1990) found that inhibition of α-glucosidase biosynthesis in *Bacillus licheniformis* performed well in comparison to Microtox or assays based on biosynthesis of *β-galactosidase.* Campbell et al. (1993) showed that the α-glucosidase biosynthesis assay is particularly sensitive to heavy metals.

For metal toxicity assessment, enzyme toxicity assays may be sensitive, rapid and inexpensive. However, there are some disadvantages for their application: (1) many factors other than metals could affect enzyme activity, such as soil pH, moisture, temperature, redox potential, organic matter, other inhibitors and activators, which make it difficult to find a simple relationship between a single

enzyme activity and the amount and form of heavy metals; (2) enzyme activities could to some extent alter the soil conditions that affect the bioavailability of metals.

1.3.2.4 Microorganisms

Microrganisms are considered to be especially suitable to act as a clear mirror of environmental pollution, or to function as early warning systems due to their ubiquity, size, and versatility and their important role in foodwebs, recycling elements, and maintaining the soil structure (Elsgaard et al., 2001; Filip, 2002). The enormous wealth and diversity of microbial species makes them a potentially valuable source of bioassay materials encompassing a range of metabolic types which could be exploited in monitoring systems. In addition, soil microbes have been proven more sensitive to heavy metal pollution than other members of soil biota (Giller et al., 1998).

Bacterial assays have been widely developed for assessing the toxicity of environmental samples, according to the principle of the tests, these can be classified into Microtox, ATP-based assays, growth inhibition assays, and ecological effect assays.

- Microtox

Luminescent bacteria have been widely used for toxicity testing. The first commercial assay was proposed in 1979 under the name of Microtox using the marine bioluminescent bacterium, *Photobacterium Phosphoreum* (Bulich 1979).

The basic principle is that bioluminescent bacteria produce bioluminescence by a branch of the electron transport system driven by specific enzymes. Toxic chemicals can adversely affect the light output of these bacteria, and the light output can be monitored in the region of 470 to 510 nm (Johnson et al., 1974). This assay has been adopted by many laboratories and used in the assessment of the toxicity of sewage effluents, complex industrial wastes (oil refineries, pulp, and paper), fossil fuel process water, sediment extracts, sanitary landfill, and hazardous waste leachates (Munkittrick et al., 1991). Toxicity assays using Microtox showed a general agreement with fish and invertebrate tests (Curtis et al., 1982; Giesy et al., 1988; Sanchez et al., 1988).

Microtox has been shown to be very sensitive to many heavy metals, such as Cd, Co (V), Cr (VI), Cu, Hg, Pb, and Zn (Bulich et al., 1981; Dutka and Kwan, 1981; Elnabarawy et al., 1988; Qureshi et al., 1984). For contaminated soil testing, it has been used in the soil pore water or soil extracts (Tandy et al., 2005). The specific sensitivity of Microtox® could be due to the sensitivity of the marine bioluminescence bacteria, but this bacteria requires the Microtox procedure to be adjusted at certain pH and salinity values (2% NaCl, pH 6.5), which may influence the bioavailability of toxic compounds to organisms. Therefore, its validity for terrestrial (soil) and freshwater systems has been widely questioned.

- ATP-Based Assays and Growth Inhibition Assays

Adenosine triphosphate (ATP) is a classical indicator of active microbial biomass in environmental samples. It is conveniently and easily measured via determination of the light production resulting from the reaction of ATP with

firefly luciferin and luciferase. As a means to assess toxicity, Brezonik and Patterson (1972) introduced the use of ATP in toxicant screening in activated sludge system. Since then, many studies have confirmed that ATP-based assays were sensitive tests for estimating the toxic effect of envirionmental samples (Kennicutt 1980; Parker and Pribyl 1984). Xu and Dutka (1987) developed a ATP-TOX assay which is base on both the growth inhibition, via ATP measurement, of bacteria such as *E. coli* PQ37, and luciferase activity.

Growth inhibition assays measure the impact of toxicants on growth inhibition of pure or mixed bacterial cultures (Alsop et al. 1980; Trevors 1986). Another approach is to measure bacterial growth rate inhibition as a basis of toxicity. Such tests are of shorter duration (2 h) than biomass based assays (6-18 h). The bacteria *Pseudomonas fluorescense, Spirillum spp.,* and *Aeromonas spp* have been used to determine the toxicity of heavy metals (Dutka et al. 1983; King and Dutka 1986; Perez-Garcia et al. 1993). Compared to bacterial growth inhibition assays, the bioassay based on growth inhibition of the alga *Selenastrum capricornutum* had the lowest EC_{50}s of heavy metals (Hickey et al., 1991; St-Laurent et al., 1992). However, those assays are generally less sensitive than Microtox (Paran et al. 1990).

- Ecological effect assays

The basis of this approach is to measure the inhibition impact of toxic chemicals on the nutrient cycle. The effect of toxic chemicals on the carbon cycle is conveniently determined by measuring microbial respiration. Several approaches are available for measuring respiration with oxygen electrodes, manometers, or electrolytic respirometers (King and Dutka 1986). Nitrification is

generally considered a process sensitive to toxicants. Nitrobacter or nitrifiers naturally present in soil, water, or wastewater have been shown to be sensitive to heavy metals (Chang and Broadbent 1982).

Heavy metal may affect the soil bacterial community resulting in the change of microbial diversity or composition of microbial populations. Many studies have shown that short-term or long-term exposure to toxic metals results in the reduction of microbial diversity and activities in soil (Lasat, 2002; McGrath et al., 2001). Kunito et al. (1999) used the sensitivity-resistance index to estimate the toxicity of copper on bacteria in soils. It was found that the ratio of Cu-resistant bacterial number to total bacteria was significantly correlated with the amount of exchangeable Cu in the soils.

Compared to invertebrate or plant tests, microorganism toxicity tests are simple, cost effective, and rapid (Balsalobre et al., 1993; Bitton and Dutka, 1986). Microorganism tests are faced with the same problems as enzymes tests. As the microorganism reaction to toxicants are very complex, a single microbioassay may not be able to interpret the adverse effect of a toxicant, or not be sensitive to multi-inhibitors, a battery of tests approach is required.

1.4 Biosensors for toxicity and bioavailability assessment of metals

Established techniques for monitoring environmental pollution or assessing the toxicity of chemicals often involve the use of expensive equipment, centralized laboratory facilities and skilled labour. There is a need for the development of rapid, sensitive and inexpensive techniques to enable the continuous monitoring

and rapid screening for pollution in ecological systems. Biosensors exploit the fact that selected biochemically generated signals can be converted into electrical signals using a transducer and, consist of a biological component such as isolated enzymes, tissues, immunosystems, organelles, or population of whole cells in close proximity to the surface of a transducing element (Lowe 1985). Since they possess the advantages of sensitivity, simple operation, fast response and small size, biosensors, to a great extent, meet the requirements for environmental monitoring and toxicity assessment.

In 1962 Clark and Lyons pioneered the concept of a biosensor. They proposed immobilizing enzymes on electrochemical detectors to form “enzyme electrodes” in order to continuously monitor glucose in cardiovascular surgery. Since then, a variety of biosensors have been developed for different applications. Basically, a biosensor system consists of a biological recognition element and a transducer. Table1.1 shows the components that may be used to construct a biosensor. There are many approaches that researchers employ to select a combination of bioaffinity elements and signal transducers so as to meet the analytical requirements for the proposed application. In this study, enzyme and microbial (whole-cell based bacterial) biosensors are reviewed because they are the most utilized in environmental monitoring and toxicity assessment.

Table 1.1 Components that may be used to construct a biosensor*

Biological recognition elements	Transducers
Organisms	Potentiometric
Organelles	Amperometric
Tissues	Conductimetric
Cells	Impedimetric
Enzymes	Optical
Enzyme components	Calorimetric
Receptors	Acoustic
Antibodies	
Nucleic acids	

* This table is quoted from Mulchandani and Rogers (1998).

1.4.1 Enzyme based biosensors

1.4.1.1 Principles and types of enzyme biosensors

Enzyme based biosensors are analytical devices that combine an enzyme with a transducer to produce a signal proportional to target analyte concentration. This signal could involve a change in proton concentration, release or uptake of gases, such as ammonia or oxygen, light emission, absorption or reflectance, and heat emission. The transducer converts this signal into a measurable response, such as current, potential, temperature change, or absorption of light, through electrochemical, thermal, or optical means (Mulchandani, 1998).

According to the types of transducer used, enzyme biosensors can be classified into 3 groups, electrochemical based enzyme biosensors, thermal enzyme biosensors, and optical enzyme biosensors.

1.4.1.1.1 Electrochemical based enzyme biosensors

Three types of electrochemical transducers are used for construction of enzyme electrodes: potentiometric, amperometric, and conductometric. Conventional potentiometric enzyme biosensors consist of an ion-selective electrode, or a gas-sensing electrode, coated with an immobilized enzyme layer. The enzymatic reaction with the analyte generates a change in potential resulting from ion accumulation and depletion. Potentiometric sensors measure the difference between the transducing electrode and a reference electrode under conditions of zero electron flow. A variety of enzymes have been used in construction of potentiometric biosensors, such as urease, glucose oxidase, glutaminase, β-glucosidase, nitrite reductase, etc (Kaun and Guilbault, 1987). Since potentiometric biosensors show a logarithmic relationship between the electrode potential and the analyte concentration, small errors in the measured potential can lead to large errors in the analyte concentration reported. Therefore, the requirement of a very stable reference electrode may be a limitation of these potentiometric sensors (Luong et al., 1991).

In contrast to potentiometric sensors, amperometric enzyme biosensors operate at a fixed potential with respect to a reference electrode, and measure the current generated by the oxidation or reduction of species at the surface of the working electrode. Amperometric biosensors are based on redox enzymes.

These enzymes use molecular oxygen as an electron acceptor and produce hydrogen peroxide in the reaction with their substrates. Either the oxygen consumption or the hydrogen peroxide production can be determined by measuring the substrate concentration. Amperometric electrodes usually respond faster than ion-selective electrodes since they do not depend on mass transfer interfaces. However, they are less selective since they monitor all electron transfer reactions at the applied potential.

Many enzyme-catalyzed reactions involved a change in ionic species resulting in a change in the conductivity of the reaction solution. The conductometric transducer was developed to measure the change in solution electrical conductivity. The measurement of solution conductance is non-specific, which could limit the application of conductometric sensors where the specificity plays a significant role.

1.4.1.1.2 Thermal enzyme biosensors

Thermal enzyme sensors are based on the principle that the heat evolved in an enzymatic reaction can be utilized to calorimetrically determine the amount of substrate produced (Mattiasson et al., 1981; Mandenius et al., 1984). In the thermal enzyme sensors, the enzyme is attached directly to the temperature transducer, or placed in a temperature-controlled column and the heat of reaction is measured by recording the increase in temperature between the inlet and outlet as the sample flows through the column.

1.4.1.1.3 Optical enzymes sensors

The principle of optical enzymes sensors is to monitor the change in optical properties, such as UV/ vis absorption, bio-and chemiluminescence, reflectance and fluorescence brought by the interaction of the biocatalyst with target analyst. The oxidation and reduction of the NAD(P)H during enzymatic reactions catalysed by dehydrogenase can be monitored by measuring the NAD(P)H fluorescence, and the changes in the fluorescence intensity then related to the substrate concentration. Gautier et al. (1990) investigated the use of luminescent enzyme systems linked to optical transducers for the determination of sorbitol, ethanol, and oxaloacetate at the nanomolar level.

1.4.1.2 Application of enzyme biosensors in toxicity assessment of heavy metals

Mattiasson et al. (1978) developed an enzyme biosensor to detect copper by the activity of a tyrosinase apoenzyme immobilized onto an oxygen electrode. This biosensor was copper specific. The amperometric enzyme biosensors coupled with urease-glutamic dehydrogenase were developed for the rapid screening of toxic metals in polluted samples (Rodriguez et al., 2004). The principle was to monitor NADH consumption using screen-printed three electrode configuration whose oxidation current is correlated to urease activity. The presence of heavy metals in the samples inhibits the urease activity, resulting in a lower NH_4^+ production and therefore a decrease in NADH oxidation.

Biosensors incorporating enzymes as the biocatalyst fulfil the criterion of rapid monitoring, and high specificity. But, the purification procedure is laborious,

expensive, time-consuming, and also their inherent specificity rules out their use as broad-band detectors. Ideally, a multienzymes system *in vitro* could offer the required sensitivity and speed of response, but would be practically difficult.

1.4.2 Whole-cell based biosensor

Divies (1975) first reported a whole cell organism sensor for assaying ethanol using a cellulosic pellicle of *Acetobacter xylinium* at an oxygen electrode. Since then, many types of whole cell biosensor have been developed. Whole cell based biosensors are measurement systems combining analytical devices and whole cells that produce biological signals as the recognition element (Rawson, 1993; Van der Schalie et al., 2001). The use of whole cells as biocatalysts in biosensors has many attractions, such as a high degree of stability, an enormous wealth of known species covering a wide spectrum of metabolic types (allowing the choice of a suitable strain for a given purpose), low cost for growing and harvest (unlike enzymes or antibodies, there is no requirement for costly extraction and purification prior to use as biocatalysts), and the ability to perform complex reaction sequences, etc (Heim et al., 1999; Daunert et al., 2000). Whole-cell based biosensors have some unique features:

- They give functional information about the effect of a stimulus or inhibition on a living system, that result from metabolic stimulation or toxic impact. Those parameters can not be probed with molecular recognition or chemical analysis;
- The systems are easily automated, capable of on-line monitoring, and low cost;
- They posess a high degree of stability and reproducibility.

- They can reflect ecological information of an environment *in situ*, especially in complex environment.
- they can sense only the bioavailable fractions of the detected analyte, thus allowing its differentiation from the non-available fraction. Such information might be highly valuable for risk assessment and for the selection of suitable remediation options.

Compared to enzyme sensors, however, whole-cell based biosensors do have some disadvantages, including slow response, low sensitivity, and less specificity.

1.4.2.1 Broad spectrum (nonspecific) whole-cell biosensors and analyte specific whole-cell biosensors

Whole-cell biosensors can be classified into two types, broad spectrum and analyte specific whole-cell biosensors, according to whether the biosensor response is induced by a specific compound or not. Most whole-cell biosensors which combine naturally isolated bacterial cells are non-specific. They can respond to a range of changes in their environment or conditions that decrease or increase the metabolic activities of bacteria because of the characteristics of bacterial cells being a multi-enzyme system. Conditions such as pH, salinity, temperature, metals, xenobiotics and many other compounds are potential inhibitors or stimulators of bacterial metabolic activities. These broad spectrum biosensors have been widely used to assess the sum negative impact of test samples on living systems, and mainly for early warning system or toxicity screening purposes.

The specific biosensors rely on genetic engineering to generate transcriptional fusions between inducible promoters (including a regulatory gene encoding a repressor or an activator protein) and different reporter genes. The reporter genes are transcribed from promoters that are regulated by a repressor activator protein sensitive only to a specific compound. Since the pioneering work of King et al. (1990) in the construction of a *lux* fusion for the specific detection of naphthalene and salicylate, there has been a steady stream of such constructs responsive to distinct very diverse compounds, ranging from metals to sugars and from alkanes to aromatic hydrocarbons (Daunert et al., 2000; Kohler et al., 2000).

The main advantages offered by the specific whole-cell biosensors are that they can detect certain chemicals in the environment, especially a complex environment, and sense only the bioavailable fraction of the detected chemical. Such information is particularly valuable for assessing the bioavailability of chemicals in the environment. Whilst they are not likely to provide more sensitive, accurate detection and quantification of specific pollutants than standard analytical chemical techniques (Kohler et al., 2000). However, standard analytical chemical techniques are not able to determine the bioavailability of metals.

The broad spectrum nature of whole-cell biosensors can be both an advantage and disadvantage, depending on the purpose of the test. The multi-enzyme systems within cells give the potential for the biosensors to respond to a wide range of chemicals, including some unknown toxicants, or other physical stresses. The biosensors respond to the combined effect of all factors, such as

temperature, pH, toxicants, which are of particular interest in ecological risk assessment. However, the broad spectrum nature make them inappropriate for use where analyte specificity is required.

1.4.2.2 Whole-cell based biosensors for environmental application

Due to the ability to respond to changes in their environment, Whole-cell-based biosensors are mainly applied in the environmental field, especially for use in eco-toxicity tests and environmental monitoring (Gaisford et al., 1991; Rogers and Gerlach, 1999; Rensing and Maier, 2003). These biosensors have been developed for assaying a wide range of substances, such as biochemical oxygen demand (BOD), a variety of organic materials, and heavy metals. The widely used whole-cell biosensors include BOD biosensors based on oxygen electrode, bioluminescent biosensors, and amperometric biosensors.

1.4.2.2.1. Bioluminescent whole-cell biosensors

Bioluminescence have been extensively used as the reporter of whole-cell based biosensors applied in environmental monitoring (Rabbow et al., 2002; Weitz et al., 2002). A variety of bioluminescent bacteria have been employed as biosensor biocatalysts. Luminous bacteria are ubiquitously distributed, but mainly exist as marine forms. Bioluminescence is also characteristic of numerous marine and few land organisms other than bacteria (Girotti et al., 2008). The *lux* operons of bioluminescent bacteria consist of at least five required genes, *luxCDABE*, only *luxA* and *luxB* are required for bioluminescence. The *luxA* and *luxB* proteins form

a dimer structure that catalyses the formation of fatty acids from long-chain aldehydes. During the reactions (1) and (2) a reduced flavin mononucleotide ($FMNH_2$) is oxidized to FMN in the presence of molecular oxygen, with its breakdown a photon of blue light ($\lambda \approx 490$ nm) is emitted. The light production that is correlated with the metabolic state of the organism can be measured by a luminometer, fluorimeter, or scintillation counter. The exposure of luminescent bacteria to noxious substances or dangerous conditions usually results in the change of their light intensity, which is employed to develop the bioluminescence whole-cell biosensors.

$$NAD(P)H + FMN + H^{+} \xrightarrow{NAD(P)H:FMN\ oxidoreductase} NAD(P)^{-} + FMNH_2 \qquad (1)$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{Luciferase} FMN + RCOOH + H_2O + LIGHT \qquad (2)$$

Bioluminescence biosensors are currently used extensively for environmental monitoring. The most suitable species include *Vibrio fischeri (V. fischeri,* classified as *Photobacterium fischeri)*, *V. Harvey*, *P. leiognathi* and *Pseudomonas fluorescens*. *V. fischeri* was the first and most employed luminescent strain, and the most sensitive to a wide range of chemicals. Bioluminescence biosensors based on *Vibrio fischeri* are more sensitive than other bacterial assays such as nitrification inhibition, respirometry, ATP luminescence and enzyme inhibition. Moreover, they show good correlation to toxicity bioassays such as those employing algae, crustacean, fishes, etc (Parvez et al., 2006). The genes required for bioluminescence can be cloned from several bioluminescent bacteria and transferred to other more applicable bacterial species, in particular, *E. coli*, on plasmids (Xi et al., 1991; Marincs et al., 1994;

Voisey et al., 1998), which widened the application of bioluminescent bacterial biosensors for environmental monitoring. Most of such measurements are non-specific, because any condition that decreases the metabolic activity of the biosensor strain will reduce the production of light.

Some specific biosensors have been reported for toxic compounds assessment. A novel approach for the bacterial whole-cell sensor is to use recombinant DNA technology to construct a plasmid or other vector system in which a strictly regulated promoter is connected to a sensitive reporter gene (Rensing and Maier, 2003). The most interesting promoters for environmental analysis are found in bacteria that survive in extreme environments contaminated by, for example, heavy metals or organic compounds. The ability of bacteria to survive in a contaminated environment is usually based on a genetically encoded resistance system, the expression of which is precisely regulated. The best studied example is the mercury resistance (*mer*) operon. It operates the function of the reduction of Hg(II) to Hg^0 (by the *mer*A gene product, the mercuric reductase) and degradation of methylmercury (by the *mer*B gene product, the organomercurial lyase) so as to reduce the toxicity of mercury to the bacterial cell. The *mer* promoter is activated when Hg(II) binds to the regulatory protein *MerR*.

Bioluminescent sensors, based on induction of *mer-lux*, have been developed for the specific detection of bioavailable Hg (II) (Selifonova et al., 1993). Toscione and Belfort (1993) also used a genetically engineered strain of *E. coli* as a bioluminescent sensor for the detection of mercury. Using this principle, the specific bioluminescent bacterial sensors have also been developed to detect copper, thallium, chromium, cadmium, arsenic, lead ions, zinc, or cobalt (Corbisier et al., 1993, 1999; Rouch et al., 1995; Tauriainen et al., 1997, 1998).

1.4.2.2.2 Mediated amperometric whole-cell biosensor

Rawson et al. (1987, 1989), Gaisford et al. (1991) and Richardson et al. (1991) have reported the development of the mediated amperometric whole cell biosensors. This technique has been successfully commercialised as CellSense^{TM}, by a consortium comprising the University of Bedfordshire, WRc plc, AstraZeneca and the Environment Agency of UK. The basic principle is shown in Fig.1.1. The oxidised form of the mediator (such as potassium ferricynide, p-benzoquinone, 2,6-dimethylbenzoquinone) is reduced by the metabolic activities (electron transport chain) of the bacterial cells (or other biocatalyst) and subsequently reoxidised at the working electrode. Electron donation by the mediator from the bacterial cells to the sensor electrode results in a steady current flow. The magnitude of the current is related to the metabolic activities of the biocatalyst. Changes in metabolic activity due to adverse effects on the biocatalyst resulting in a change in current.

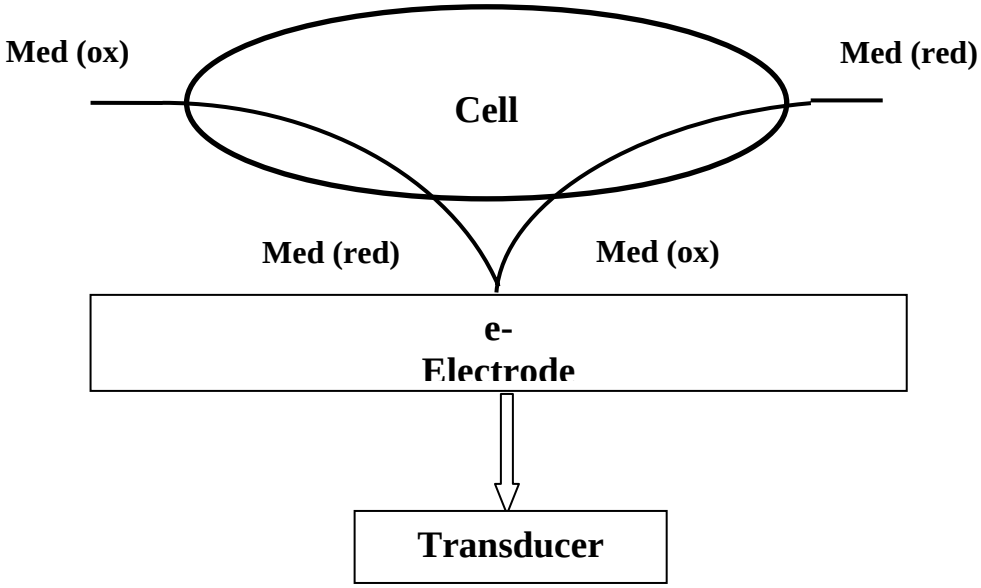


Fig. 1.1 Principle of mediated amperometric whole-cell based biosensor. Med (ox) refers to the oxidised form of the mediator, while Med (red) is the reduced form of the mediator.

The CellSense^{TM} biosensor system consists of CellSense instrument which has 32 reaction vessels allowing individual biosensors to be monitored, CellSense sensor, and computer which show the biosensor signals and conduct the toxicity analysis (shown in Fig1.2 and Fig.1.3).

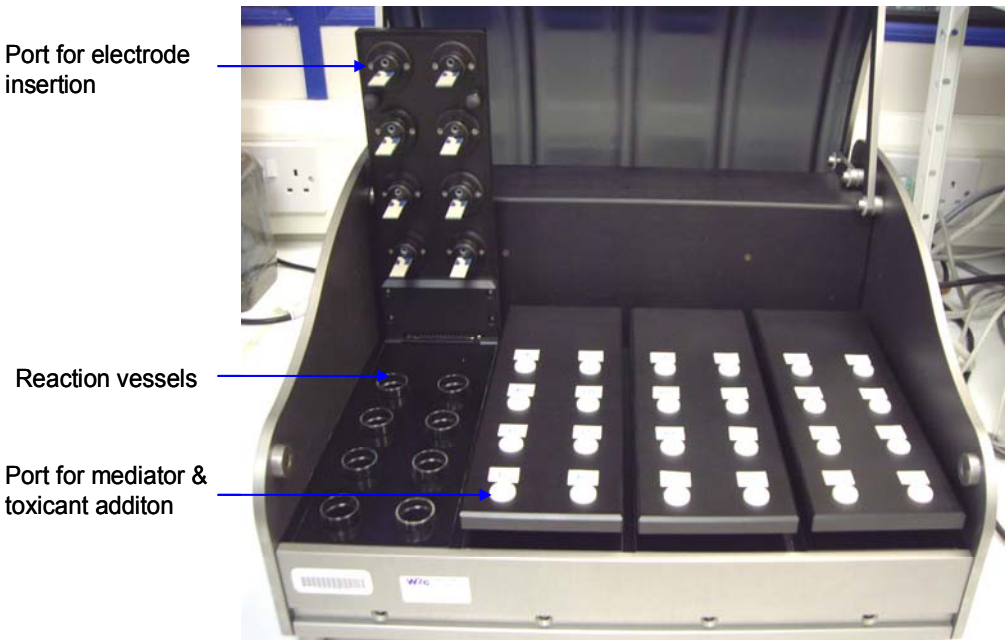


Fig.1.2 CellSense™ instrument

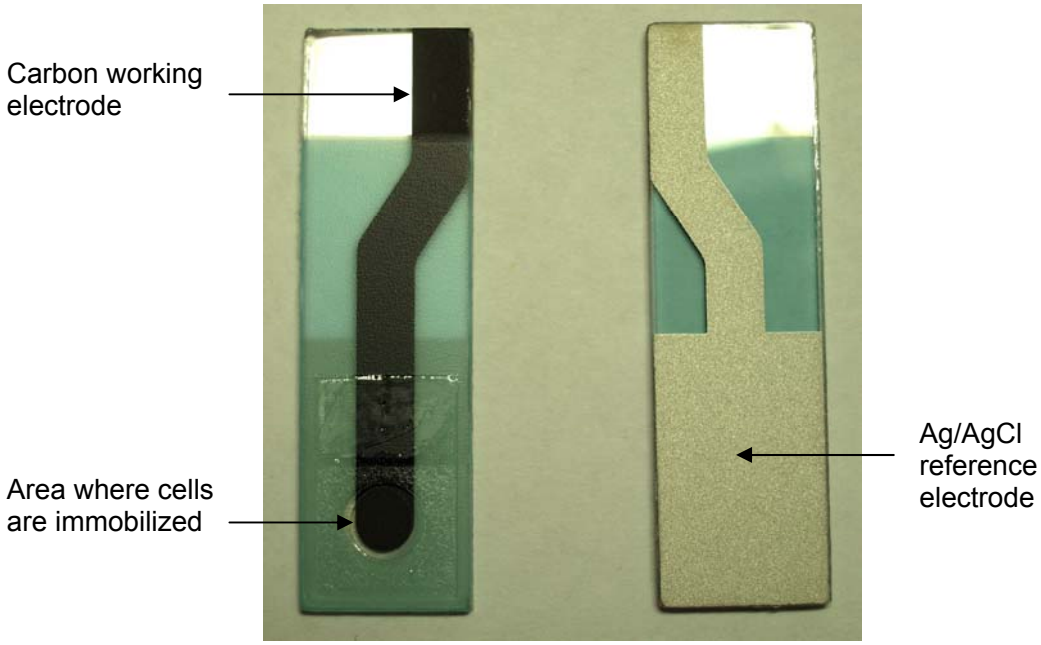


Fig. 1.3 CellSense™ sensors. The figure on the left displays the carbon working electrode where the cells are immobilized. The figure on the right shows the back of the sensor with Ag/AgCl reference electrode.

This CellSense^{TM} biosensor system has been used to detect the adverse effect of various environmental and industrial products including herbicides and other organic pollutants on different types of biological cells. *E. coli*, *Bacillus spp, pseudomonads*, nitrifying bacteria, activated sludge, photosynthetic cyanobacterium, algae, fish cells all have been incorporated into biosensors for the use of toxicity assessment or environment monitoring (Richardson et al., 1991; Pandard and Rawson, 1993; Polak et al., 1996). The CellSense^{TM} biosensors offer several advantages over conventional enzyme sensor or other biosensors:

- Ease of use, high degree of stability and prolonged storage capacity. Biosensors can be freeze dried and stored for up to 6 months, allowing tests to be undertaken at anytime and anywhere, without requirement of cell culture or enzyme extraction and purification steps.

- A wide range of applications. Due to the multi-enzyme systems within the cells, the sensors have the ability to responsing to a variety of physical & chemical stressors or stimulis. In addition, an enormous wealth of known species are able to be used as biocatalyst of the sensors, thus there is a potential for developing biosensors capable of working in different kinds of environment, even in extreme environments.

- The sensors are disposable and low cost.

1.4.2.3 whole-cell based biosensors for bioavailability and toxicity assessment of heavy metals in soil

Total metal concentration in soil does not provide accurate data on bioavailability or toxicity, however, whole-cell biosensors only sense bioavailable compounds in soil. Hence the luminescence-based and fluorescence-based bacterial biosensors that are relevant to soil ecosystems have been widely used to assess the toxicity of contaminated soil by heavy metals (McGrath et al., 1999, 2001; Chaudri et al., 1999, 2000; Vulkan et al., 2000; Tandy et al., 2005).

Giller et al. (1998) identified *Rhizobium leguminosarum* bv. *Trifolii* as a particularly sensitive indicator of soil pollutants. Based on this, Paton et al. (1997) developed an ecologically relevant *lux*-based biosensor using *Rhizobium leguminosarum* bv. *Trifolii* to assess the acute and chronic ecotoxicity of heavy metals contaminated soil. The results demonstrated the sensitivity and rapid response of those biosensors. Applying similar procedures, Palmer et al. (1998), Chaudri et al. (2000) determined the toxicity of Cd and Cu, or Zn and Cu ions in a soil extract by use of a *Rhizobium*-based luminescence biosensor. Turpeinen *et al*. (2003) studied the arsenic bioavailability in soils with an arsenic-specific bioluminescent *E. coli* strain. After random insertion of *P.fluoresens* strain DF57 with a Tn5:*luxAB* promoter probe transposon, a mutant induced by copper was selected and used to assess bioavailable copper in soil solutions (Tom-Petersen et al., 2004). The toxicity of mercury and arsenite in soil extracts was also determined by using *P.fluoresens* OS8 (pTPT11) and *P.fluoresens* OS8(pTPT13), respectively (Petänen and Romantschuk, 2003). Three different soil types (humus, mineral, and clay) were initially spiked with mercury and

arsenite to determine the efficacy of these strains with the different soil characteristics. The results of the tests showed that these mercury and arsenite biosensors were capable of detecting these metals, irrespective of the type of soil. Liao et al. (2006) developed a green fluorescent protein (GFP)-based bacterial biosensor to assess the heavy metal bioavailability in sediments, or in soils using soil-water extracts. This GFP reporter system has some advantages over colorimetric enzyme assay and bioluminescence, such as no requirement of exogenous substrates and pH adjustment.

These biosensor tests were carried out with soil extracts, however, soil extracts do not exactly present the properties of soil. Leitgib et al. (2007) compared the toxicity of metal contaminated whole soil and their water extract using different bioassays, most bioassays showed more harmful effect of the contaminated soil than the tests using soil extracts. Therefore, the direct contact environmental toxicity tests are needed to meet the requirements of environmental toxicology: reliability, sensibility, reproducibility, rapidity and low cost.

In addition, the recombinant luminescent bacterial sensors were used for the specific quantification of the bioavailable concentrations of cadmium (Cd-sensor) and lead and cadmium (Cd-Pb sensor) (Tauriainen et al., 1998) in both aqueous extract and contact exposure. The recombinant luminescent bacterial sensors for heavy metals are unique and powerful tools for sensitive and specific quantification of bioavailable fractions of heavy metals in the case of different exposure types (Ivask et al., 2002).

Ivask et al. (2004) developed two recombinant bacterial sensors, one responding specifically to cadmium and the other to lead and cadmium to

determine the bioavailability of these metals in soil-water suspensions, using the soil-water ratio of 1:9 (w/v). The suspensions were shaken for 24 h before biosensor tests. The results showed that 90-fold more Cd and 21-fold more Pb proved bioavailable if the sensor bacteria were incubated in soil suspensions rather than soil extracts. The authors concluded that there are some particle related bioavailabe Pb and Cd in soils to bioluminescent bacterial biosensors. In this case, contact exposure indicated markedly higher bioavailability and potential hazard.

The physical-chemical characteristics of soil-water suspension is closer to that of the soil than to soil extractions, but soil-water suspension still differ from solid phase of soil, since the bioavailability of heavy metals can be changed by the soil moisture content, especially after 24h shaking. Therefore, it is important to develop a biosensor system which is able to be applied *in situ* for risk assessment of heavy metal contaminated soils.

1.5 Summary

- Measuring the bioavailability and toxicity of metals in soil is challenging because of the complex effect of physical-chemical properties of soil and the diversity of interaction mechanisms between organisms and metals.

- Standard analytical chemical techniques are likely to provide more sensitive, accurate detection and quantification of specific pollutants than the bioassays, but they are not able to distinguish between the bioavailable and non-

bioavailable fractions of heavy metals. Therefore, they do not give accurate indications of toxicity of metal contaminated soils.

- Bioassays are able to interpret the toxicity and bioavailability of metals in soils. Invertebrate, plants, enzymes, soil microorganisms tests have been developed for this purpose, some of them are standard methods of toxicity assessment. For large-scale soil pollution investigation or risk assessment, however, those methods have some disadvantages. Both invertebrate and plants assays are time and labour consuming; enzyme assays are readily influenced by other physical-chemical, or biological factors of soils, so that it is difficult to interpret the results. All these bioassays involve complex laboratory techniques, and thus need to be conducted by professionals in the laboratory.

- A variety of whole-cell biosensors have been developed for environmental applications. They possess a series of remarkable advantages, such as rapid response, sensitivity, stability, low cost, in comparison with conventional methods. A number of recombinant luminescent bacterial sensors have been developed to measure the toxicity and bioavailability of heavy metals. Some are metal-specific biosensors which are very sensitive. Some researcher also applied these luminescent bacterial sensors to detect the bioavailability of heavy metals in soils. However, they are only used in soil extractions, or soil /water suspensions with a soil /water ratio of 1:5 or 1:10 rather than solid phase of soil.

- A mediated amperometric whole cell biosensor was developed for toxicity assessment and environmental monitoring. It has the advantage of other whole-cell based biosensors, and employs disposable electrodes which can incorporate a number of whole cell biocatalysts. Therefore, it has a wide applications in different environments, and a potential application in soil monitoring.

1.6 Aim and main objectives

This study recognises the advantages of mediated amperometric whole cell biosensors, and in particular the potential application of disposable electrodes *in situ* exposed to soils. The aim of this study was to develop a suitable monitoring protocol of mediated amperometric whole cell biosensors for *in situ* assessment of heavy metals in soils. The objectives were:

- Selecting biocatalysts sensitive to selected heavy metals;

- To develop protocols for monitoring heavy metals in defined solution, soil pore water, and *in situ* in soil;

- To evaluate the applications of mediated amperometric whole cell biosensors for assessing the bioavailability and toxicity of heavy metal artificially or historically contaminated soils *in situ,* and mixture toxicity of heavy metals.

2 Materials and methods

In this study a series of investigations were carried out to develop an appropriate protocol of mediated amperometric whole-cell biosensors for assessing the toxicity of heavy metals, including optimizing conditions for harvesting cells to construct biosensors, selecting sensitive bacterial strains as biocatalysts, and setting up a suitable monitoring regime. Subsequently, this protocol was applied to assess the toxicity and bioavailability of metals in freshly spiked soils and historically contaminated soils. The mixture toxicity of metals was also investigated. Soil sample collection, soil property analysis, and soil preparation for biosensor testing was carried out at Rothamsted Research (Harpenden, UK), all the other work at the Luton Institute for Research in the Applied Natural Sciences at the University of Bedfordshire.

2.1 Chemicals

All substrates, *p*-benzoquinone and toxicants were AnalaR grade and supplied by Sigma-Aldrich (UK). Zinc chloride, mercuric chloride, copper chloride and lead nitrate were used to make standard stock solutions for assessing toxicities. Nutrient agar and nutrient broth (No.2) were supplied by Lab M.

2.2 Biosensor construction

2.2.1 Biocatalysts and which culture maintenance

Escherichia coli 8277 (NCIMB 8277) and *Pseudomonas putida* (NCIMB 9773, 9046 and 8917) were selected as biocatalysts. The reasons for using *E. coli* in this study were that *E. coli* had been reported as sensitive bacteria to a range of toxicants (Reinhartz, et al., 1987; Hansen and Sørensen, 2001), including Cellsense^{TM} biosensor assay (Gaisford et al., 1991; Rawson, 1993), and also being easy to handle in the laboratory. *Ps. putida* strains were chosen as biocatalysts for toxicity testing because they are common soil bacterium. Also, *Ps. Putida* is the bioassay organism in the ISO *Ps. Putida* growth inhibition assay. All the biocatalysts were obtained as freeze dried cultures from The National Collections of Industrial and Marine Bacteria Ltd, Aberdeen. Freeze dried cultures were resuspended in 0.5 ml nutrient broth, and three categories of culture were established on 3 nutrient agar slopes in Universal bottles, reference, closed and working cultures. The reference and closed slopes were used as stocks, held at 4 °C until required. Working cultures were prepared weekly. The closed slope was subcultured every month to give new reference, closed, and working cultures.

2.2.2 Culturing & harvesting cells for biosensor construction

Organisms were subcultured from working slopes held at 4 ºC into flask with 20 ml nutrient broth. These batch cultures were transferred to an orbital incubator

at 150 rpm and either 37 ºC (*E. coli*) or 25 ºC (*Pseudomonas*). Cells were harvested every 2h after inoculation and immobilized onto the biosensor electrodes, to determine the optimal harvest conditions. The optimal harvest time was subsequently used for all biosensor constructs. Aliquots (1 ml) of diluted culture were centrifuged at 10,000 g for 2 min, using an Eppendorf microfuge, and the supernatant discarded. The cell pellet was then resuspended in 1 ml sterile 0.85% saline, recentrifuged at 10,000 g for 2 min and the supernatant again discarded. This washing procedure was repeated twice. Finally, the washed cell pellet was resuspended in sterile 0.85% saline to give a certain range of biomass loading. Uniformity of biomass loading can be obtained by adjusting optical density of the culture sample to a pre-determined value before subsampling and subsequent harvesting and immobiization. The optical density of the culture ($O.D._{430nm}$) was measured on a Philips spectrophotometer.

2.2.3 Electrode component

The disposable sensors consisted of a carbon working electrode, with a working electrode surface area of 5 mm in diameter surrounded by a adhesive region, and a Ag/AgCl reference electrode, screen printed on a polycarbonate substrates (developed with Danielson Ltd., Aylesbury). The working electrode was poised at a potential of 550 mV.

2.2.4 Immobilisation of biocatalyst

3 µl aliquots of cell suspension was loaded onto 0.2 µM polycarbonate membrane (Whatman International, Kent, UK), and once dry, the bacteria loaded side was placed facing towards the electrode. The filter was then secured onto the adhesive region of the working electrodes.

2.3 Cellsense™ assays

2.3.1 Preparation of substrate for Cellsense™ assays

A fresh stock solution of 0.85% saline was used to prepare a substrate cocktail (containing equal concentrations of D-glucose, sodium succinate and sodium lactate) with a final concentration of 5 mM in the assay vial.

2.3.2 Redox mediator

p-Benzoquinone was freshly prepared before biosensor measurement as stock solution in 0.85% saline to give a concentration of 20 mM. *p*BQ was dissolved in ethanol (95%, AR) first, then saline was added to make up to the final concentration. The ethanol concentration was 5% in the stock solution.

2.3.3 Protocol for toxicity assessment using CellTMsense (Protocol A)

The substrate solution of 9.8 ml was dispersed into each magnetically stirred vial placed in the Cellsense^{TM} instrument. Biosensors were put into the connectors in the instrument lid. The biosensors was held in the substrate solution for at least 15 min before measuring their signals. Measurement started by running the Cellsense^{TM} software, for 1 or 2 min, 200 µl pBQ stock solution was then added into every vial so as to give 0.4 mM pBQ, by lifting the instrument lid. The data collection restarted when the lids were closed. Once stable signals had been achieved toxicity tests were started by adding known concentrations of the test toxicant to each test vial. Controls were treated with deionised water at the same volume as other treatments. There were 8 replicates for each treatment. A 0.5 h exposure time was chosen to calculate the effective concentrations (EC_x) (eg, EC_{50} refers to the concentration of a toxicant which cause a biosensor response of 50% inhibition).

The protocol A was used to test the sensitivity of 4 bacterial strains (*E. coli 8277*, *Ps.9773*, *Ps.9046*, and *Ps.8917*) to Pb, Zn, and Hg in solution. A new protocol was developed for copper toxicity measurement with defined solution, soil and soil pore water samples (described in Chapter 3). This protocol was also employed to measure toxicities of Zn and Pb in defined solution, soil and pore water samples so as to allow comparison among solution and soil measurements.

2.4 Electrochemical assay

An electrochemical assay was employed to determine whether there was any interaction between metal ions and the pBQ redox couple under conditions likely to be encountered in biosensor assay.

Measurements were made using an electrochemical workstation PGSTAT10 (Eco Chemie BV) and a laboratory computer running GPES 4.9 software. Voltammograms were recorded in cyclic voltammetry (staircase) normal mode with pre-treament at +200 mV for 2 s and then a scan from +200 mV to the first vertex at -400 mV to the second vertex at +800 mV. The step size was 1 mV and the scan rate was 50 mV s^{-1}. Two scans were set and the second scan was saved to disc. Test solutions (10 µl) which were prepared immediately or 1h before test were pipetted on to the electrode area of the sensor and covered with a microscope slide cover slip so as to produce a thin layer cell. A single screen-printed electrode was used for all the measurements of each metal, and it was rinsed and dried each time after use. The screen-printed electrodes used here were printed in the lab of LIRANS, University of Bedfordshire (Luton, UK).

2.5 Soil property measurement and soil sample preparation

2.5.1 Soil samples

A loamy sand soil (J) used in copper toxicity assessment was obtained from Woburn, UK. Soil samples were collected from the upper 20 cm of a field, which had not been cultivated for several years and had no evidence of

contamination. The air dried soil sample was sieved through a 2-mm mesh and stored at room temperature. Another 9 soils were collected and stored by Rothamsted Research (Harpenden, UK). The locations and land use of these soils are shown in Table.2.1.

Table 2.1 General information of the sampling sites

Location	Code	Land use	Soil texture class	Applications in this study
Arthur Richwood, UK	A	Agricultural	Loamy Peat	Chapter 4, 6
Dorset, UK	B	Agricultural	Clay loam	Chapter 6
Newcastle, UK	C	Agricultural	Clay loam	Chapter4, 6
Rothamsted,UK	D	Agricultural	Silty clay loam	Chapter 6
Wood Walton, UK	E	Agricultural	Loamy Peat	Chapter 6
Northern France	F	Zn smelter	Loam	Chapter 6
Avonmouth, UK	G	Zn smelter	Silty clay	Chapter 6
Devon, UK	H	As mine	Sandy loam	Chapter 6
Budel, Belgium	I	Zn smelter	Loamy peat	Chapter 6
Woburn, UK	J	Agricultural	Loamy sand	Chapter 4,5,6

2.5.2 Basic soil properties

2.5.2.1 Soil pH

The pH values of soil were measured with a soil : water ratio of 1:2.5. 10g soil was weight out into a vial, and 25 ml deioned water was added. After shaking, the vial was left for 30 min, shaken again and left to stand for a further 30 min, then shaken once more and pH measured immediately. There were 3 replicates for each soil sample, including a standard sample whose pH value is known.

2.5.2.2 Soil moisture content and soil water holding capacity

Soil moisture content (MC) was determined as all data about soil is reported on an oven dried soil basis. It was measured using the "105 °C oven dry" method. The formula of calculation is:

$$\% \text{ MC} = \frac{(Wfs - Wds) * 100}{Wds}$$

Wfs refers to fresh soil weight, and *Wds* refers to oven dry soil weight.

Soil water holding capacity (WHC) represents the amount of water held by the soil after water has ceased to drained away under the influence of gravity. At this point the soil is said to be at field capacity and represents 100% WHC. This determination is necessary for spiking soils, so that the amount of water

contained in the soil samples can be standardised to a stipulated fixed amount of water per soil sample. It was measured following this procedure:

50 g soil in triplicate was weighed onto 3 separately weighed Whatman No.42 filter paper (15 cm diameter), then filter paper with soil sample was placed into a funnel with a collecting container underneath. Deionised distilled water was added to thoroughly soak the soil until there is approximately 5 mm depth of water on the surface of the soil. The soil should be fully saturated with water, then left to drain overnight or long enough until the water ceased to drain away. The funnels were covered with cling film to prevent evaporation from the soil surface. The wet soil and filter paper was weighed, and placed in an oven at 105 °C overnight to dry. The dry filter paper and soil were weighed again. The weight of water held by filter paper was determined by following the same steps. The percentage WHC was calculated by using these formulas:

*% WHC of soil = Wt of water retained by soil * 100 / Wt of oven dry soil*

Wt of water retained by soil = (Wt of wet soil + FP) – (Wt of oven dry soil + FP) – (Wt of water held by FP)

Wt of oven dry soil = (Wt of oven dry soil + FP) – (Wt of dry FP)

Wt of water held by filter paper = Wt of wet FP – Wt of dry FP

2.5.2.3 Soil major and trace elements

Total C and N were determined using a LECO combustion analyzer (LECO CNS 2000, St. Joseph, MI). Total concentrations of metals, other major and trace elements were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, Fison ARL Accuris, Ecublens, Switzerland). Prior to analysis, soil samples were digested and prepared as follows:

About 0.250 g dry and finely ground soil in triplicates were accurately weighed into a Pyrex tube, including a couple of Standards and Blanks in the run. Samples were added 5 ml Aqua regia (4 ml HCl, then 1 ml HNO_3), then left to predigest overnight. The pre-digested samples were transfered to the cool Carbolite heating block to digest overnight. After cooling, 5 ml 20% HNO_3 was added, and whirlmixed well. The samples were reheated to 80 °C for 30 min. Ultra pure Reverse Osmosis water was added to make up to exact 20 ml for ICP-AES analysis.

2.5.3 Preparation of soil samples

Air dried soil samples were amended with $CuCl_2.2H_2O$ at a moisture content of 75% WHC. 10000 mg kg^{-1} copper stock solution was spiked to soils to obtain three Cu concentrations, which were 200, 500, and 1000 mg kg^{-1} copper, the control samples were made by adding the same amount of deionised water to ensure the same moisture content. Copper stock solution was made up in deioned water. The samples were equilibrated for 3 d at constant moisture content, 20 ± 2˚C in darkness, prior to biosensor measurement. Soils were also

amended with zinc, lead, or metal mixtures (see Section 2.7), under the same conditions.

2.5.4 Soil pore water extraction

Soil pore water was extracted from soil samples equilibrated for 3 d after metal or deioned water amendment. Solutions were removed by centrifugation at 3,000 g for 15 min (sample F, H, J) or 23,000 g for 15 min (sample A, B, C, D, E, G, I) using a double chamber method. Immediately after extraction, soil pore water samples were filtered using 0.2 μm cellulose acetate membrane filters, and the pH value, dissolved organic carbon (DOC), and metal and other trace elements measured. Each soil was extracted and analyzed in triplicate. The total concentrations of metals and trace elements in soil pore water samples were determined by the methods shown above. Toxicity tests on soil pore water were undertaken at the same time as soil samples in parallel.

DOC of soil pore water was measured using a DOC analyzer (Thermalox; Analytical Sciences, Cambridge, UK).

2.6 The toxicity and bioavailability assessment of copper in soils

2.6.1 Copper toxicity and bioavailability assessment

A loamy sand soil sample (J), a clay soil sample (C), and a soil with relatively higher organic matter sample (A) were spiked with copper in the laboratory for

toxicity and bioavailability assessment. Both *E. coli 8277* and *Ps.9773* biosensors were employed for solid phase of soil and soil pore water measurement. The percentage inhibition of biosensors by soils or soil pore water was calculated to determine the toxicity of copper. The calculation method was described in Section 2.9.1.

2.6.2 Effect of moisture content on toxicity of copper in soil

Loamy sand soil sample was spiked with copper at the moisture content of 50%, or 100% WHC to detect the effect of the soil moisture content on the toxicity of copper. The samples were kept under the same condition as described in section 2.5.3. *E. coli 8277* biosensors were used for soil samples and soil pore water samples measurement. Percentage inhibitions of biosensor signals by soil at different moisture content or corresponding soil pore water are compared. The properties of soil pore water extracted from different treatments were also analysed to study the effects of soil moisture content on the bioavailability of copper.

2.6.3 Effect of pH on copper toxicity to biosensors

1 M HCl or 1M NaOH solution were used to adjust pH of 0.85% saline, copper solution, or soil pore water.

2.6.3.1 Effect of pH on biosensor signals

The pH of 0.85% saline solution was measured first (6.8). HCl solution was gradually added into saline solution to adjust their pH into 5.5, 4.5, and 3.5, respectively. Both *E. coli 8277* and *Ps.9773* biosensors were exposed to saline solution with different pH for 3h to determine the effect of pH on biosensor responses. Unmodified saline solution was used as a control to calculate the percentage inhibition of biosensors by saline solution with lower pH.

2.6.3.2 Effect of pH on the toxicity of soil pore water

Loamy sand soil was spiked with Cu and the soil pore water extracted using the method described in section 2.5.3 and 2.5.4. It was prepared independently from other tests. The pH of soil pore water was measured immediately after extraction. According to the results, two values (the highest and lowest) were chosen to adjust the pH of soil pore water. In this case, pH values of soil pore water extracted from control, 200 mg kg^{-1} copper, 500 mg kg^{-1} copper, and 1000 mg kg^{-1} copper soil treatments were 6.8, 5.4, 4.9, and 4.5, respectively. 6.8 and 4.5 were chosen to modify the soil pore water in order to compare the toxicity of copper in soil pore water with the same pH as unadjusted control sample or the sample with the lowest pH. The pore water samples extracted from different treatments were adjusted to pH = 6.8 by adding 1M NaOH solution, or pH = 4.5 by adding 1 M HCl solution, respectively. Then, both *E. coli 8277* and *Ps.9773* biosensors were exposed to soil pore water samples

with the same pH. For calculating the percentage inhibition of biosensors by copper contaminated soil pore water, each control soil pore water with the same pH treatment was used as the corresponding control. The possible effects of pH adjustment on copper concentrations or other characteristics of pore water were ignored, as the amount of HCl or NaOH solution added was considerably small in comparison with those of soil pore water samples.

2.7 Toxicity assessment of heavy metal mixtures

Mixture toxicities of copper + lead, or copper + zinc in solution, solid phase of soils, and soil pore water to biosensors were investigated.

2.7.1 Toxicity assessment of heavy metal mixture in solution

A range of concentrations of two metal mixture solutions — copper + lead, and copper + zinc were made up with 0.85% saline solution (shown in Table 2.2). Saline solution was used as the control in biosensor tests. Preliminary range finding experiments were carried out to enable selection of the concentrations of two metal mixture. These metal concentrations listed in Table 2.2 were selected, according to their EC_{50} values, in order to detect the single and combined toxicity of two metals. 250 mg l^{-1} copper stock solution, 4000 mg l^{-1} lead stock solution, and 5000 mg l^{-1} zinc stock solution were used to make the different concentrations of metals.

Both *E. coli 8277* and *Ps.9773* biosensors were exposed to the single metal solution or metal mixture solution at the defined concentrations for 3hr. The

percentage inhibition of biosensors by any single metal or metal mixtures in solution was calculated for studying the toxicity of metal mixture.

Table 2.2 Metal concentrations of solution treatments for mixture toxicity ssessment.

copper and lead (mg l^{-1})		copper and zinc (mg l^{-1})	
copper	lead	copper	zinc
0	0	0	0
0	50	0	100
0	100	0	200
0	200	0	500
0	400	2	0
2	0	2	100
2	50	2	200
2	100	2	500
2	200		
2	400		

2.7.2 Toxicity assessment of heavy metal mixture in soil or soil pore water

The loamy sand soil (J) was spiked with Cu, Pb, Zn, Cu + Pb, or Cu + Zn at the moisture content of 75% WHC, to obtain a range of Cu and Pb, or Cu and Zn concentrations (shown in Table 2.3). Those concentrations were selected based on the range finding experiments in order to determine the possible toxic interactions of two metals. 10000 mg l^{-1} copper, 5000 mg l^{-1} lead, or 5000 mg l^{-1}

zinc stock solution were used to spike the soils to give the different concentrations of metals in soil. Soil samples were incubated under the same conditions described in Section 2.5.3. Soil pore water was extracted after 3 d incubation, and the properties of soil pore water samples measured.

Both *E. coli 8277* and *Ps.9773* biosensors were exposed to the treated soil or soil pore water samples for 3hr, respectively. The percentage inhibition of biosensors by any single metal or metal mixtures in soils or soil pore water was calculated to determine the toxicity of metal mixture.

Table 2.3 Metal addition to soil treatments for mixture toxicity assessment.

copper and lead (mg kg^{-1})		copper and zinc (mg kg^{-1})	
copper	lead	copper	zinc
0	0	0	0
0	500	0	500
0	1000	0	1000
0	2000	0	2000
500	0	500	0
500	500	500	500
500	1000	500	1000
500	2000	500	2000

2.8 Toxicity assessment of historically contaminated soils

Six uncontaminated soil samples (A, B, C, D, E, J) which possess different characteristics were used to determine their effects on *E. coli 8277* and *Ps.9773* biosensor responses. Four contaminated soil samples (F, G, H, I) were used for toxicity testing. Uncontaminated soils A and D were used as control for calculating the inhibition percentage of biosensors by contaminated soils. Certain amount of deioned water was added into all soil samples so as to give a moisture content of 75% WHC, then soil samples were kept 3 d for the biosensor measurement, under the same condition as described above.

The levels of 0.05M ethylene diamine tetraacetic acid (EDTA) (MAFF, 1986) and 0.01M $CaCl_2$ (Houba, et al., 1996) extractable metals were measured as indicators of bioavailable metals. For EDTA extractable measurement, 10 g air dried soil sample was added with 50 ml 0.05M Na_2EDTA solution, then shaked for 1 hr at 20 °C. After shaking, soil suspension was settled for 5 min, then filtered through No. 40 filter paper to get clear solution. The analysis of the extract was carried out by ICP. For 0.01M $CaCl_2$ extractable metals measurement, 5 g air dried soil sample was added with 50 ml 0.01M $CaCl_2$ solution, then shaked for 2 hr at 20 °C. After shaking, soil suspension was centrifuged at 3000 RPM for 15 min at 20 °C, then the supernatant was filtered through a 0.45 micron membrane filter using a syringe. Aristar conc. HCl was added into soil extract to give a final volume of 5% HCl, then the soil extract samples were analysed by ICP/ ICP MS.

2.9 Data analysis

2.9.1 Calculation of inhibition and ECx

In all the Cellsense^{TM} assays described above the effect of the toxicants was presented as the percentage inhibition of biosensors. The percentage inhibition calculated by comparing the biosensor current before & after exposure. Biosensor signals before & after exposure were normalized with regard to the corresponding control. The ECx represents the concentration of a compound where x % of its maximal effect is observed. The observed ECx value of Hg, Zn, and Pb in solution (Chapter 3), following Protocol A, were estimated by toxicity analysis with Cellsense^{TM} instrument based on the least squares regression method.

The observed EC_{50} and EC_{10} copper concentrations for soil or soil pore water, following Protocol B, were estimated by fitting the log-logistic model using the Toxicity Relationship Analysis Program (TRAP), version 1.0 (U.S. EPA, 2002).

2.9.2 Comparison of biosensor response to different uncontaminated soil samples

Comparison of biosensor responses to different uncontaminated soil samples was presented as normalized post-exposure responses divided by normalized pre-exposure responses, thus eliminating the effect of intra-batch difference among biosensors.

2.9.3 Toxicity assessment of historically contaminated soil samples

Toxicity levels of contaminated samples are expressed as percentage inhibition of the biosensor responses normalized with respect to the selected uncontaminated control soil responses. The percentage inhibition was calculated as described above.

2.9.4 Statistical analysis

Each experiment was repeated at least 3 times for the statistic analysis. Microsoft Excel™ was used for standard error calculation. One-way ANOVA (Post Hoc Multiple Comparisons) was used for the difference analysis by SPSS 12.0.1 for windows. The significant level was $p<0.05$.

3 Optimization of mediated amperometric whole-cell biosensor protocol for assessing the toxicity of heavy metals

3.1 Introduction

A mediated bacterial biosensor was successfully developed to assess the toxicity of some organic toxicants such as 3,5-DCP, Chlorophenols, Synprolan, Polyhexanide, Proxel paste, Atrazine, and industrial effluents (Rawson and Willmer, 1989; Gaisford et al., 1991; Rogerson, 1997), which offered the advantages of rapid testing, low cost, sensitivity, ease of use, and scope for online application. The preliminary work also showed that it has potiential applications in monitoring metals and assessing their toxicity. The current study sought to develop suitable biosensor configurations which could be used to assess the toxicity of heavy metals. An efficient system should include: (i) bacterial strains sensitive to heavy metals incorporated into the disposable biosensor electrode; (ii) optimized biosensor construction so as to give a strong and stable signal of biosensors; and (iii) a suitable monitoring regime.

There are numbers of bacterial strains that may be suitable for configuration of biosensors, depending on the application. The factors considered in this study were that the biocatalysts should be sensitive to test toxicants, stable under standard operating conditions (temperature, pH, *etc.*), ease of culture, maintenance and immobilisation of the cells in the biosensor. *Escherichia coli* (NCIMB 8277) and three *Pseudomonas putida* strains were selected for incorporation into the biosensors. *E. coli* has been known to be sensitive to

many toxicants, and *Ps. putida* strains were selected as they are typical soil microorganisms. Both *E. coli* and *Ps. putida* have previously been used as biocatalysts for whole-cell biosensors (Rogerson, 1997; Bhatia, 2005).

Both *p*-benzoquinone and potassium ferricyanide had been used as mediators for amperometric whole cell biosensors (Richardson et al., 1991; Patchett et al, 1989). Ferricyanide was not recommended as the mediator for monitoring heavy metals, due to its reaction with metal cations during exposure of the biosensors (Weast, 1988). Therefore pBQ was assessed as mediator for monitoring the effect of heavy metals on bacterial metabolic activities. It was necessary to determine whether pBQ possibly interacts with heavy metals.

3.2 Optimal conditions for harvesting cells

The culture age of cells at harvesting for incorporation into the biosensor should be such as to provide cells with a high metabolic activity capable of generating a stable biosensor signal. The chosen harvest time also needs to be convinent for carring out the Cellsense^{TM} assay post-incorporation into the biosensor configuration.

Growth measurements of 4 species of bacteria were taken by measuring the $O.D._{430}$ of cell culture at different incubation times. Fig.3.1 showed that all four bacterial strains followed a logarithmic growth curve. Following a lag period of between 4–8 hrs, the cell culture grew slowly. The stationary phase was reached between 10 and 12 hrs with *E. coli*, but later than 18 hrs for *Ps. Spp*. Rawson (1993) reported that mid-to-late exponential phase was optimal for

biosensor construction. Cells were harvested after 12 h, 14 h and 16 h incubation, and immobilized onto the biosensor electrodes, then their amperometric responses were compared. Monitoring of the biosensor signals of the different strains showed that they were all able to give a stable biosensor signal. For convenience the bacterial cells were harvested after 16 h, allowing overnight incubation, and the construction of biosensors and biosensor testing to be completed the following day.

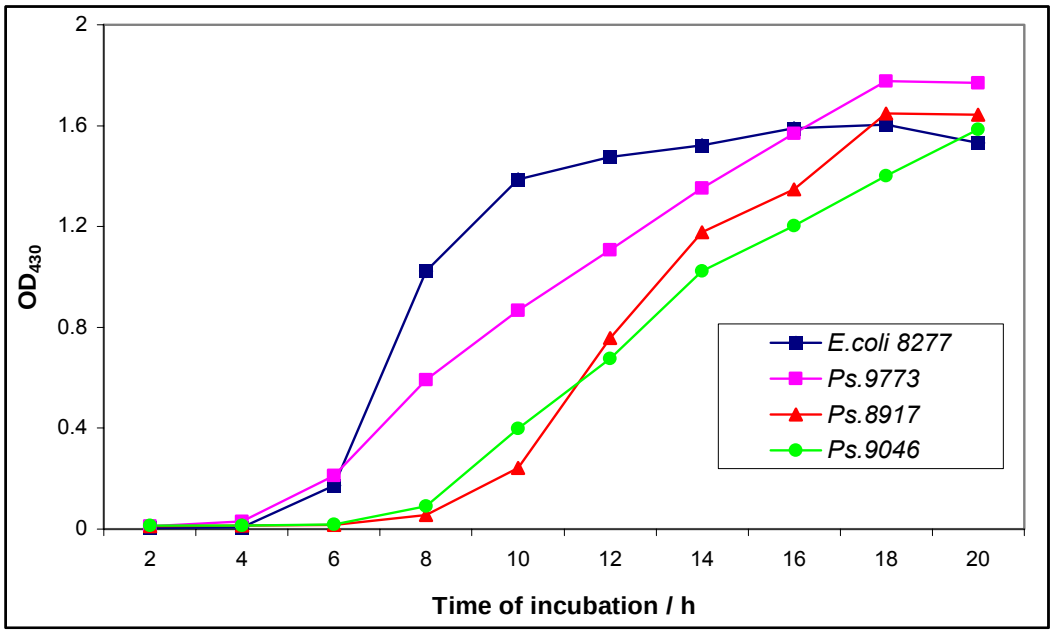


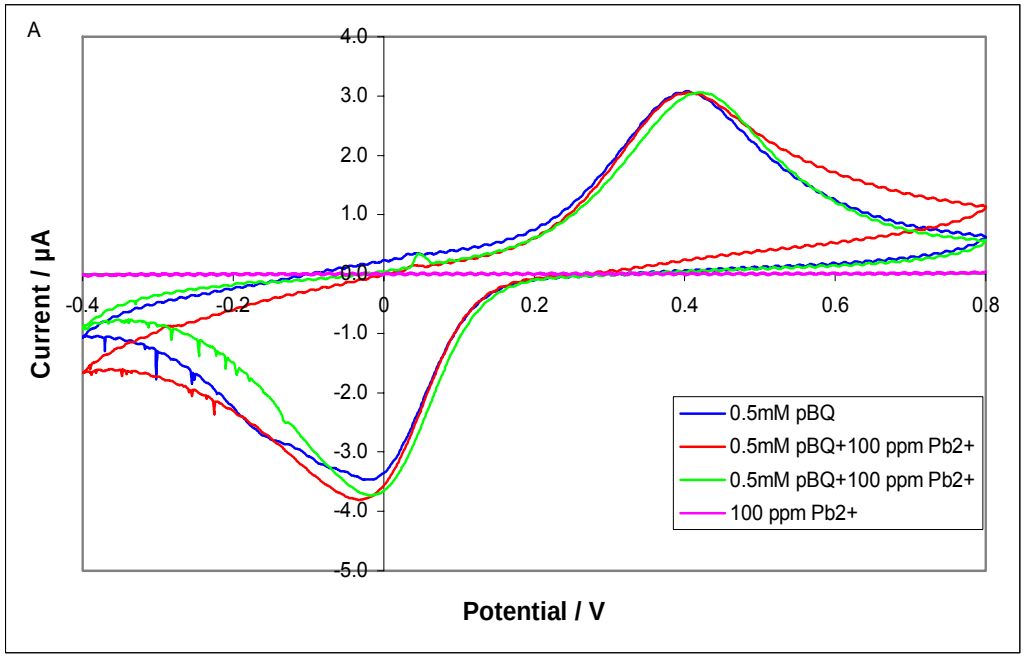
Fig.3.1 The optical density of cell cultures of 4 bacterial strains, after different incubation times at 37 ºC (*E. coli.8277* cuture), or 25 ºC (three *Pseudomonas* cuture). Culture OD was measured by mixing 200 µl batch culture with 800 µl nutrient broth in 1 ml plastic semi-cuvets.

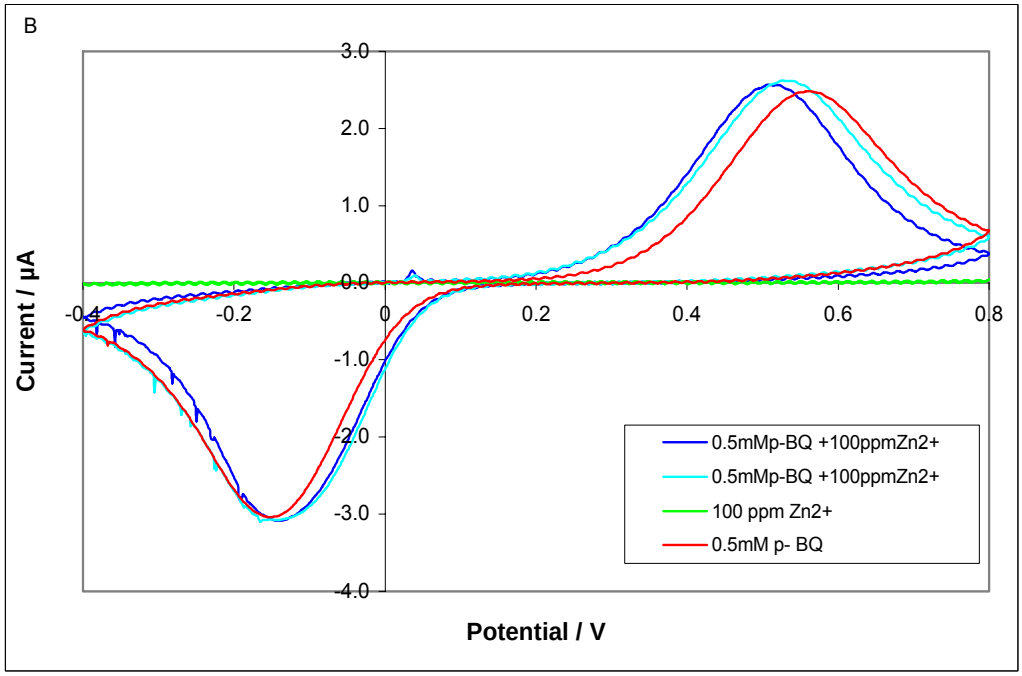
3.3 Investigation of possible interaction between metal cations and mediator

As described above, mediated amperometric bacterial biosensors monitor redox events related to metabolic activity of the bacterial biocatalyst. Interference of metabolism by toxicants would affect redox events of biosensor response (Ramsey and Turner, 1988; Gaisford et al., 1991). However, if the addition of metal species has a direct effect on the mediator, the biosensor signals would not reflect the toxic impact of the metal on bacterial metabolism. It was therefore necessary to investigate whether there was any interaction between metal cation and *p*BQ redox couples. Electrochemical measurements were carried out for investigating the possible interactions between *p*BQ and metal cations (Pb^{2+}, Zn^{2+}, or Cu^{2+}) redox couples. It had been reported that there is no interaction between Hg^{2+} and *p*BQ (Weast, 1988).

Fig.3.2 shows the cyclic voltammograms of *p*BQ, metal cation alone, or mixture of the twos. Metal ions alone (Pb^{2+}, Zn^{2+}, or Cu^{2+}) did not exhibit any electrochemical activity under the given conditions. The oxidation peaks of mixtures of *p*BQ (0.5 mM) and Pb^{2+} (100 ppm) or pBQ and Zn^{2+} (100 ppm) did not make a obvious change from the peak of *p*BQ alone, which indicated that there were no obvious interactions between lead (II) cation and *p*BQ redox couple or between zinc (II) cation and *p*BQ redox couple. However, there was interaction between copper (II) cation and *p*BQ redox couple (as shown in Fig.3.2). The oxidation peak of *p*BQ appeared at +550 mV, but when copper ions were mixed with *p*BQ, then there was an oxidation peak at +350 mV and a shoulder at about +550 mV. When the solution of mixture was left to stand

for an hour, then the peak at +350 mV was a little larger and the shoulder at +550 mV had all but disappeared, indicating an interaction between copper (II) cation and the *p*BQ redox couple. The copper – *p*BQ reaction compromises accurate assessment of the impact of the cation on bacterial biocatalyst.





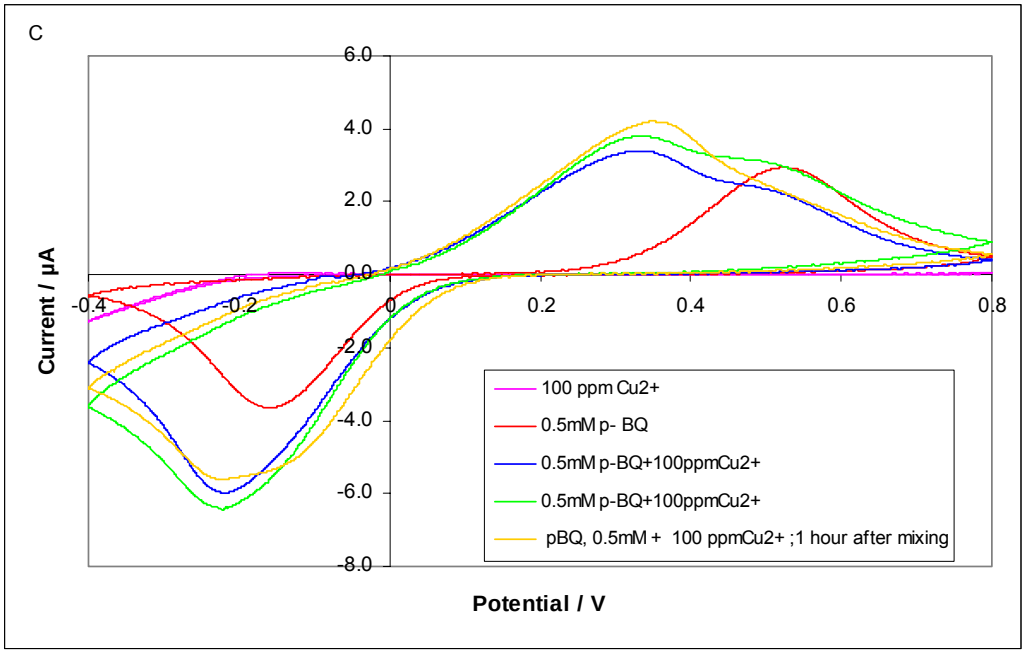


Fig.3.2 Cyclic voltammograms of pBQ, lead nitrate (A), or zinc chloride (B), or copper sulphate (C) and mixtures of each cation and pBQ. Voltammograms were recorded in cyclic voltammetry (staircase) normal mode with pre-treatment at +200 mV for 2s, then a scan from +200 mV to the first vertex at -400 mV and to the second vertex at +800 mV. The step size was 1 mV and the scan rate was 50 mV s^{-1}. Screen-printed electrodes were used for all measurements. Test solutions (10 µl) were pipetted on to the working electrode and covered with a microscope slide cover slip so as to produce a thin layer cell.

Biosensor measurements by protocol A (see section 2.3) would not be affected by cation – *p*BQ interaction in the cases of lead and zinc determination. There are two possible options to overcoming this problem for copper studies: (i) use of a alternative mediator; (ii) employing a method that separates the exposure phase of biosensor to copper, from the electrochemical interrogation of the biosensor post-exposure.

The second option had the advantage that it would make *in situ* application in soils possible. If the process of exposure to metals could be separated from the biosensor monitoring, the biosensor can be exposed directly in soils or other environments *in situ*. This approach was adopted.

3.4 Sensitivity of biosensors with different bacterial strains to metals in solution

The toxicity of metal ions in solution mainly depends on the reactivity of the metal compounds with bacterial cells, the tolerance and resistance of organism, and the physiochemical characters of solution, including pH and temperature. In this study, toxicity tests of lead, zinc, mercury and copper to different bacterial strains were carried out to determine the sensitivity of the different biosensor configurations, with a view to selecting sensitive bacterial strains for incorporation into biosensor monitoring system for assessing the toxicity of heavy metal contaminated soils.

3.4.1 Toxicity of lead to different biosensor configurations

Biosensors immobilized with *E. coli 8277*, *Ps.9046*, *Ps.9773*, and *Ps.8917* were exposed to Pb solution in the concentration range of 20–100 mg l^{-1} for 30min, respectively. *Ps.9046* biosensor was found to be the most sensitive to lead in solution (Table 3.1), with an EC_{50} of 81.26 ± 2.36 mg l^{-1}, however, its lower limit of detection was not obtained. *Ps.9773* biosensor was more sensitive than *E. coli 8277* biosensor. No obvious toxicity of lead to *Ps.8917*

biosensor was detected in the range of concentrations tested, and *Ps.8917* biosensor was rejected for the measurement of lead toxicity. Compared with Microtox–EC_{50} 0.31 mg l^{-1} after 0.5 h exposure (Dutka and Kwan, 1981; Qureshi et al, 1984), all the biosensor configurations had a lower sensitivity.

Table 3.1 Toxicity of lead to different biosensor configurations. Data represent average value in mg l^{-1} ± standard error (n = 3). Exposure time was 30 min.

Biosensors	EC_{10}*	EC_{20}*	EC_{30}*	EC_{50}*
E. coli 8277	15.69± 0.4	36.1 ± 1.5	ND	ND
Ps. 9773	9.63 ± 0.8	25.78 ± 1.3	45.67 ± 3.0	ND
Ps. 9046	ND	ND	20.33 ± 1.3	81.26 ± 2.4

* The observed ECx values were estimated using the software of toxicity analysis of Cellsense™ instrument (also in Tables 3.2 and 3.3).

ND: not detectable.

3.4.2 Toxicity of zinc to different biosensor configurations

Zinc plays an important role as an essential trace element in development, growth and differentiation of all living systems from bacteria to human. However, zinc is also known to be a potent inhibitor of the respiratory electron transport systems of bacteria above a certain concentration (Beard et al., 1995). The toxicity of zinc to 4 bacterial biosensors is shown in Table 3.2. *E. coli* biosensor was the most sensitive to zinc, EC_{10} value was identified as 13.0 mg l^{-1}. The sensitivity of *Ps. putida* to zinc was lower than *E. coli*. EC_{50} was not found with any of the 4 bacteria in the tested range of 20–100 mg l^{-1}. In comparison to Microtox whose EC_{50} was 2.35-3.4 mg l^{-1} (Dutka and Kwan,

1981; Qureshi et al., 1984; Elnabarawy et al, 1988; Paton, 1997), the biosensors were less sensitive.

Table 3.2 Toxicity of zinc to different biosensor configurations. Data represent average value in mg l^{-1} ± standard error (n = 3). Exposure time was 30 min.

Biosensors	EC_{10}	EC_{20}	EC_{30}
E.coli 8277	13.0 ± 1.3	31.6 ± 2.4	52.9 ± 3.3
Ps.9773	93.6 ± 15.7	ND	ND
Ps.9046	93.7 ± 11.9	ND	ND
Ps.8917	20.4 ± 2.8	ND	ND

ND: not detectable.

3.4.3 Toxicity of mercury to different biosensor configurations

The bacterial biosensor were more sensitive to mercury than lead or zinc. EC_{50} values of *Ps.9773* and *E. coli 8277* biosensors were 0.28 and 0.52 mg l^{-1} (Table 3.3), respectively. *Ps.9773* biosensor was the most sensitive to Hg among the three biosensor configurations. However they still showed a lower sensitivity than Microtox, EC_{50} 0.01-0.046 mg l^{-1} (Qureshi et al., 1984; Ribo et al., 1989; Elnabarawy et al., 1988).

Table 3.3 Toxicity of mercury to *E. coli* biosensor and *Ps.*biosensors. Data represent average value in mg l^{-1} ± standard error (n = 3). Exposure time was 30 min.

Biosessors	EC_{10}	EC_{30}	EC_{50}	EC_{70}
E. coli 8277	0.08 ± 0.0	0.26 ± 0.0	0.52 ± 0.0	0.96 ± 0.1
Ps.9773	ND	0.11 ± 0.0	0.28 ± 0.1	0.77 ± 0.1
Ps.9046	0.53 ± 0.1	1.71 ± 0.1	ND	ND
Ps.8917	ND	6.48 ± 1.5	ND	ND

ND: not detectable.

3.5 Development of a new protocol for copper toxicity assessment

As the results showed in section 3.3, *p*BQ interacts with copper in solution, requiring the separation of biosensor exposure to copper from the electrochemical monitoring of biosensors. Therefore, a new protocol was developed for copper toxicity assessment.

3.5.1 Protocol B

The protocol has three steps as shown in Fig.3.3. In step I, the stable current value for each biosensor prior to exposure is determined. Cellsense™ assays start with the same procedure as described in Section 2.3.3, and stop when the biosensor signals have been stable for about 15 min. In step II the biosensors are removed from the Cellsense™ instrument and placed into cuvettes with different concentrations of copper (or other toxicants). A 1 ml

test solution was used in each of the cuvettes and at least 4 replicates for each concentration. An exposure time of 3 hr was used based on the preliminary range-finding experiments. After exposure (step III), the biosensors are returned to their previous position in the Cellsense^{TM} instrument and biosensor monitor begins. Inhibition percentages were calculated by dividing the normalized values of biosensor current immediately after exposure by the normalized values of biosensor current before exposure. Biosensor values were normalized using corresponding controls.

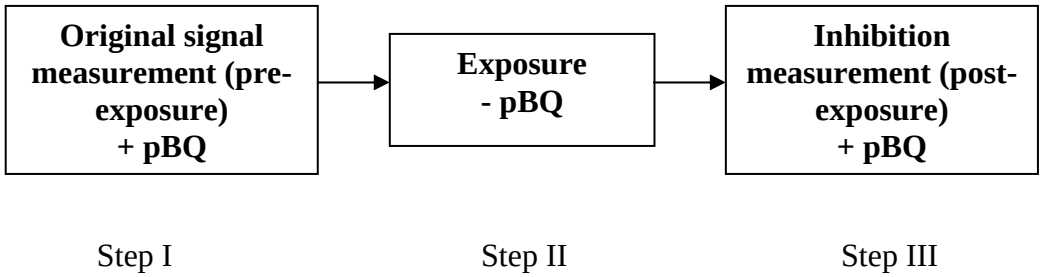


Fig.3.3 Diagram for toxicity measurement of copper solution using biosensor

3.5.2 Validation of the new protocol for toxicity assessment

An example of toxicity measurement of copper using the new protocol is shown in Fig.3.4. Before exposure to toxicant, the *p*BQ mediated biosensor signals fell from initial peak to a lower - stable signal. The initial peak appeared because the metabolic activities of cells were at relatively high levels shortly after being harvested from nutrient broth, then they gradually declined in the bathing medium comprising of substrate and mediator until a lower-stable signal was reached. The average values of biosensor signals in

the 20s periods immediately before exposure to test samples were used as pre-exposure data to calculate the inhibition percentage (shown in Fig.3.4a). After a period of exposure (3h in this case), biosensors were moved back to the bathing medium, and the Cellsense^{TM} monitoring started again. The biosensor signals were initially high, unless the bacterial cells were seriously damaged by the test samples, and then declined gradually to a stable level (shown in Fig.3.4b). The average value of biosensor signals in the 20s periods immediately after exposure were taken as post-exposure data, since it should be precluded that bacterial cells recover during post-exposure in the absence of toxicant. Fig.3.4b also shows that the difference of biosensor signals between different treatments (exposure to different concentrations of copper) reduced with increasing recovery time. As a result, biosensors need to be moved back to Cellsense^{TM} assay as soon as possible after 3h exposure to reduce the possible effect from the recovery of bacterial cells.

A statistic analysis was carried out to determine whether the new protocol was appropriate for toxicity assessment (Fig.3.5). Before exposure to copper, there was no significant difference between the biosensor signals, all biosensors giving similar response, although it is impossible to ensure all are identical. Minor difference is the result of biosensor construction. After exposed to different concentrations of copper solution, the biosensor signals were significantly decreased with higher concentrations showing greatest inhibition. All biosensors were exposed under the same conditions with the exception of the concentrations of copper during exposure period (step II). The significant inhibition of biosensor responses after exposed to metal solution is inferred as being a toxicity effect.

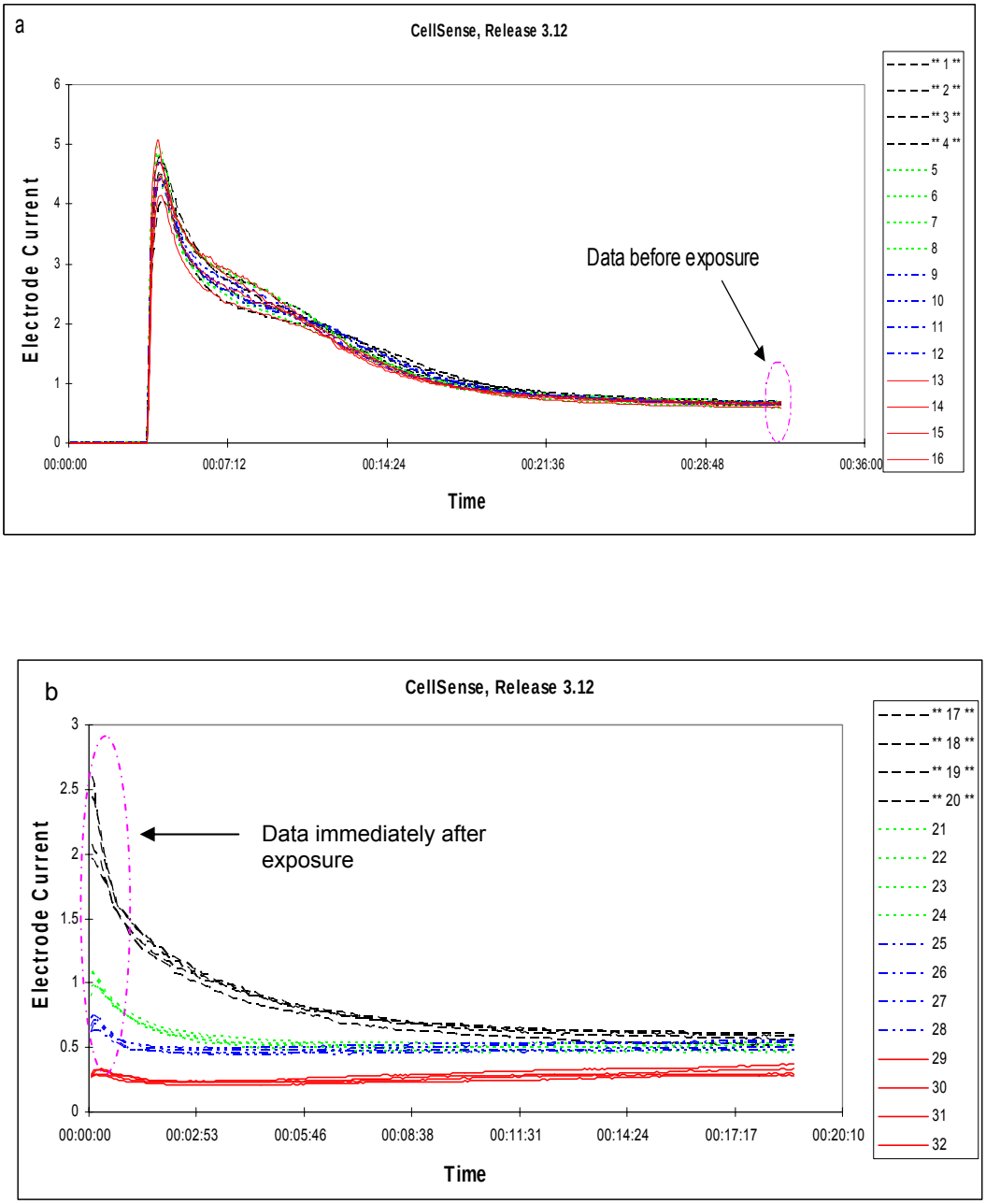


Fig.3.4 *E. coli 8277* biosensor signals before (a) and after (b) exposure to different concentrations of copper solution. The concentrations of copper are as same as shown in Fig.3.5.

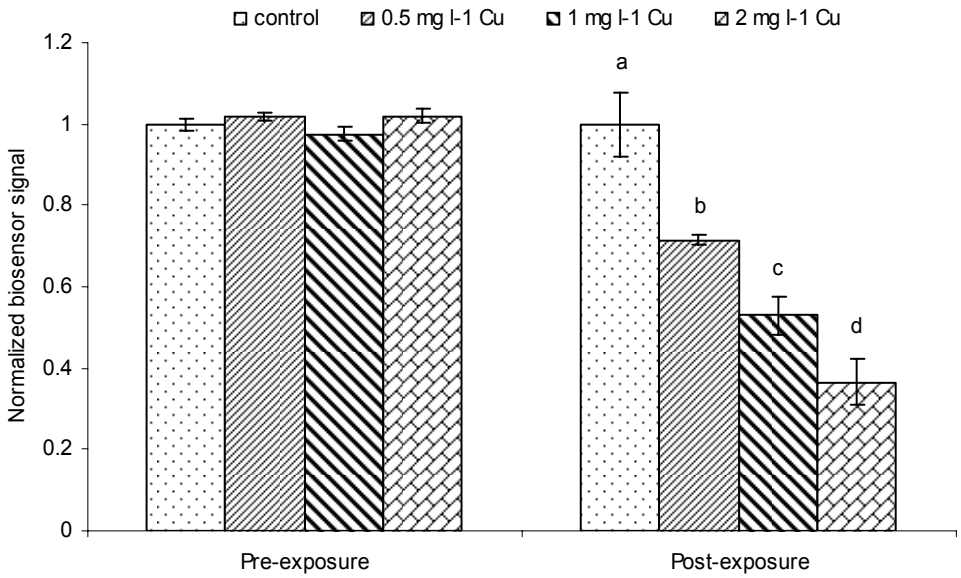


Fig.3.5 Biosensor signal values immediately before and after exposure to different concentrations of copper solution. The biosensor signals are shown as normalized average values in the 20s periods immediately before and after exposure (average value ± standard error, n=3), respectively. Four *E. coli 8277* biosensors were used for each treatment. Exposure time was 3 hours. $CuCl_2.2H_2O$ was used in the test solutions. Different letters above the bars indicate statistical difference at $p < 0.05$.

3.5.3 Toxicity of copper in solution to biosensor configurations

The toxicity of copper in solution to *E. coli 8277* and *Ps. 9773* biosensors was measured following the protocol described above. *Ps. 9046* and *Ps. 8917* biosensors were not used for Cu measurement regarding the stability of biosensors and convenience of bacterial culture. Fig.3.6 shows that the inhibition of both *E. coli 8277* and *Ps.9773* biosensors by copper increased with increasing concentrations. *E. coli 8277* was more sensitive than *Ps.9773* at concentrations of copper lower than 4 mg l^{-1}, and *Ps.9773* gave higher

inhibition than *E. coli 8277* at concentrations of copper above 4 mg l^{-1}. EC_{50} and EC_{10} values were estimated to be 1.44 and 0.05 mg l^{-1} for *E. coli 8277* and 1.73 and 0.11 mg l^{-1} for *Ps.9773* biosensors (shown in Table 3.4). In comparison, the Microtox assays EC_{50} for copper are in the range of 0.13-3.8 mg l^{-1} (Dutka et al., 1981; Qureshi et al., 1984; Elnabarawy, 1988 and Paton et al., 1997). Both biosensor configurations were considered to have appropriate sensitivity to copper for toxicity assessment.

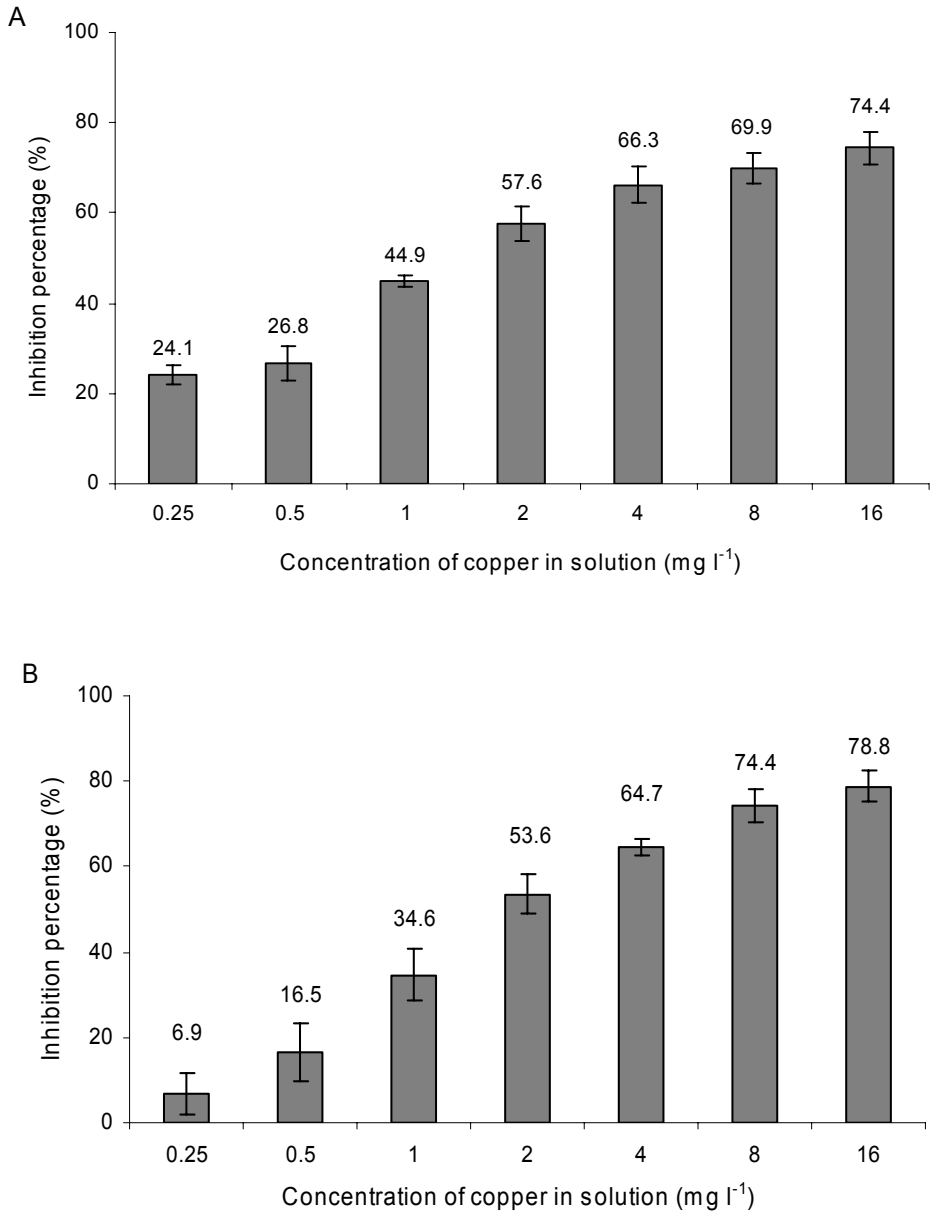


Figure 3.6 The inhibition of biosensors by copper in solution (average value ± standard error, n=3). (A) *E. coli 8277* biosensors; (B) *Ps. 9773* biosensors. Copper solutions ($CuCl_2.2H_2O$) were made up in 0.85% saline solution. 0.85% saline solution was used as a control.

Table 3.4. Estimated EC_{10} and EC_{50} solution copper concentrations (mg l^{-1}) to biosensors

Biosensor	ECx	Estimated value	95% LCL*	95% UCL*
E. coli 8277	EC_{50}	1.44	0.74	2.79
	EC_{10}	0.05	0.01	0.23
Ps.9773	EC_{50}	1.73	1.09	2.74
	EC_{10}	0.11	0.04	0.34

* LCL and UCL refer to the lower confidence level and upper confidence level.

3.6 Discussion on the sensitivity of amperometric whole-cell biosensors to heavy metals

As reviewed earlier, the toxicity of heavy metals depends on both the chemical characteristics of metals and biological resistance mechanism of organisms. In the present study, *E. coli 8277* was found to be the most sensitive to Zn and Cu, but *Ps.9773* was the most sensitive to Pb and Hg, which demonstrate that toxicities of metals are species dependent. Comparing the toxicities of the 4 metals to all 4 bacterial biosensors, Zn and Pb were found to be much less toxic than Cu and Hg. This is consistent with the findings of Duxbury (1981) that toxicity of zinc is quite low compared to the metals Hg, Cd, Cu, Ni, and Co. This might be explained by the cellular homeostatic mechanisms of bacteria. Bacteria are able to pump metals out of the cytoplasm to protect against the metal toxicity. Researchers have reported Zn homeostatic mechanisms (Beard et al., 1997; Rensing et al., 1997). In *E. coli*, zinc efflux is accomplished by both ZntA,

a P-type ATPase, and the CDF protein ZitB. This P-type ATPase is a specific Zn(II), Cd(II), Pb(II) pump, which means that ZntA only transport zinc, cadmium, and lead (Rensing et al., 1998; Sharma et al., 2000). In this case, the lower sensitivity of the biosensors to zinc and lead might result from the decreased internal bioavailability of zinc and lead, since some of the excess zinc was rapidly transported out of the cytoplasm by the homeostatic mechanisms of bacteria. Rensing et al. (1998) also found that a biosensor with a ZntA disrupted strain was able to detect zinc at a low concentration. Therefore, the presence of cellular homeostatic mechanisms greatly influences the toxicity of externally bioavailable metals. Selecting the sensitive bacteria strains which possess a weak mechanism of metal resistance should be a efficient method to improve the sensitivity of biosensors.

This study also made a comparison of sensitivity between amperometric whole-cell biosensors and the Microtox assay. With the exception of copper, the biosensors with all four bacteria were less sensitive to zinc, lead, or mercury than the Microtox assay. The possible explanation is that the bacteria strains employed in this biosensor may be less sensitive to metals than those in Microtox assay. Although the Microtox assay is well known to be sensitive to many heavy metals, its applicability to monitor soils is questionable. Since the bioluminescent bacteria employed in the Mixrotox test is isolated from marine environment, it lacks ecological relevance when it is used to monitor or assess the soil pollution. This study used natural bacteria strains which widely exist in soil system, therefore, the biosensors may be able to assess the ecological risk of polluted soils.

3.7 Summary

- *E. coli 8277* and three *Pseudomonas putida* strains were used to construct biosensors. Bacterial cells harvested and immobilized after 16h (overnight) incubation were found to give strong and stable responses.

- The chosen mediator (pBQ) was found to react with copper, and protocol (A) was not suitable for monitoring copper, therefore, a new protocol (protocol B) was developed. The result demonstrated that protocol B was suitable for copper, and could be generally used for any other metals. The new protocol makes the toxicity measurement of heavy metals in soil *in situ* possible, because the exposure step is separated from Cellsense^{TM} instrument, biosensors may be able to be placed into solid phase of test samples directly. This is a significant advantage of such biosensors.

- In comparison with the Microtox assay, the mediated amperometric whole cell biosensors had a low sensitivity to Zn, Pb, or Hg, but were appropriately sensitive to Cu. The sensitivity of biosensors may be improved by selecting more sensitive bacteria strains.

4 Toxicity and bioavailability of copper in soil *in situ* using mediated amperometric bacterial biosensors

4.1 Introduction

In soil ecosystems, most heavy metals sorb to the soil matrix, becoming less mobile and thus less toxic or non-toxic to living organisms (Welp and Brűmmer, 1997; Bispo et al., 1999). Therefore, total metal concentration does not give accurate information about the risk or potential toxicity of contaminated soils. As reviewed earlier, whole-cell based biosensors are one of the most promising techniques for the detection of the bioavailable metals. Bioluminescence-based bacterial biosensors have been reported for this purpose, and were originally applied for aqueous phase samples or extracts (Parvez el al., 2006). Some researchers (Ivask et al., 2004; Paton, et al., 1997; McGrath et al., 1999; Chaudri et al., 2000) have used these bioluminescent bacterial sensors to measure the bioavailability and toxicity of heavy metals in soils, but they were only used in soil solutions or soil /water suspensions rather than the solid phase of soil. It is important to recognise that bioavailability involves a dynamic process that comprises two distinct phases: a physicochemically driven desorption process and a physiologically driven uptake process (Peijnenburg et al., 1997), furthermore, these processes influence each other. As a result, extracting heavy metals from a soil matrix affects their bioavailablity. A biosensor system is needed that can be used *in situ* to measure bioavailability and toxicity of heavy metal in soil.

A new mediated amperometric bacterial biosensor protocol was validated for copper toxicity assessment, and both *E. coli 8277* and *Ps. 9773* biosensors were found to be appropriately sensitive. In the new monitoring regime, the exposure step is separated from the electrochemical interrogation by the Cellsense^{TM} instrument, the biosensor can be directly placed into the solid phase of soil. This chapter reports on the investigation of the applications of this biosensor system for *in situ* assessing the bioavailability and toxicity of copper in soil.

The soils were freshly spiked to make a series of standard samples with known concentrations of copper. The bioavailability of metals in freshly spiked soils is higher than historically contaminated soils (Oorts et al., 2006), however, the bioavailability of metals decreases quickly after amendment, followed by further decreases at slower rates (Hogg et al.,1993; Ma, 2006). Harter and Lehmann (1983) reported that 95% of the Cu amended to soil slurries was adsorbed to the soil solid phase within the first 15 min after amendment. Therefore, the biosensor tests were carried out with solid phase of soil or soil pore water 3 days after spiked so as to reduce the difference between freshly spiked soils and historically contaminated soils.

Since the bioavailability of metals in soils is very much dependent on the soil properties, including organic matter, clay content, pH, and soil moisture content, this study also investigated the effects of these soil properties on copper bioavailability and toxicity to biosensors in order to define the application conditions of the biosensors.

4.2 Toxicity and bioavailability assessment on copper spiked soil

4.2.1 Basic soil properties

Three types of soils were used, the basic properties are shown in Table 4.1. Soil J is a loamy sand soil with a low level of organic matter. Soil C is a clay loam soil, and its organic matter is also relatively low. The texture of soil A is loamy peat, its organic matter level is 15.05%, which is very high. Soil A was chosen to represent organic soil. The pH of three soils is similar, and the total metal concentrations were all lower than the limit values for concentrations of heavy metals in soil (European Council, 1986).

Table 4.1 Selected soil properties.

Code	Soil Texture	pH	Total C / %	Total Metals or metalloid mg kg^{-1}				
				Cd	Cu	Pb	Zn	As
J	Loamy sand	6.15	1.34	<DL	25	43	123	<DL
C	Clay loam	5.87	1.65	<DL	17	50	72	3
A	Loamy Peat	6.51	15.03	<DL	31	25	95	4

<DL: less than Detection Limit.

4.2.2 Toxicity and bioavailability of copper spiked loamy sand soil

E. coli 8277 biosensors and *Ps.9773* biosensors were exposed directly to soil J samples or soil pore waters for 3 hr. Toxic impacts, as shown by the level of inhibition of biosensor response, are shown in Fig.4.1 and Fig.4.2. The inhibition

of *E. coli 8277* biosensors by copper in soil was 22.9%, 61.4% and 83.3% in soil supplemented with 200, 500 and 1000 mg kg^{-1}, respectively. In comparison, the inhibition of *Ps.9773* biosensors by copper in the soil treatments was lower, with biosensor stimulation at copper concentration of 200 mg kg^{-1}. In copper treated soils, estimated EC_{50} and EC_{10} values were at concentrations of 481 and 141 mg kg^{-1} for *E.coli 8277* and 890 and 490 mg kg^{-1} for *Ps.9773* biosensors.

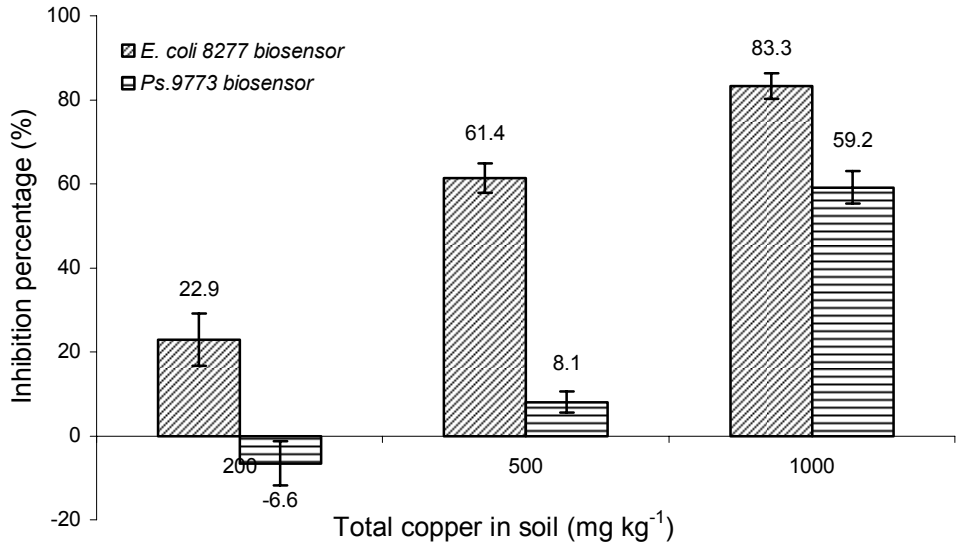


Figure 4.1 Inhibition of biosensors by copper in sandy loam soil J (average value ± standard error). Biosensors were exposed directly in soil samples for 3 h. The test was run in three replicates.

Soil pore water measurements showed a similar trend to the pure copper solution measurements, with the inhibition of *E. coli 8277* higher than *Ps.9773* at lower concentrations of copper contamination, but *Ps.9773* biosensors giving higher levels of inhibition at higher concentrations of copper contamination.

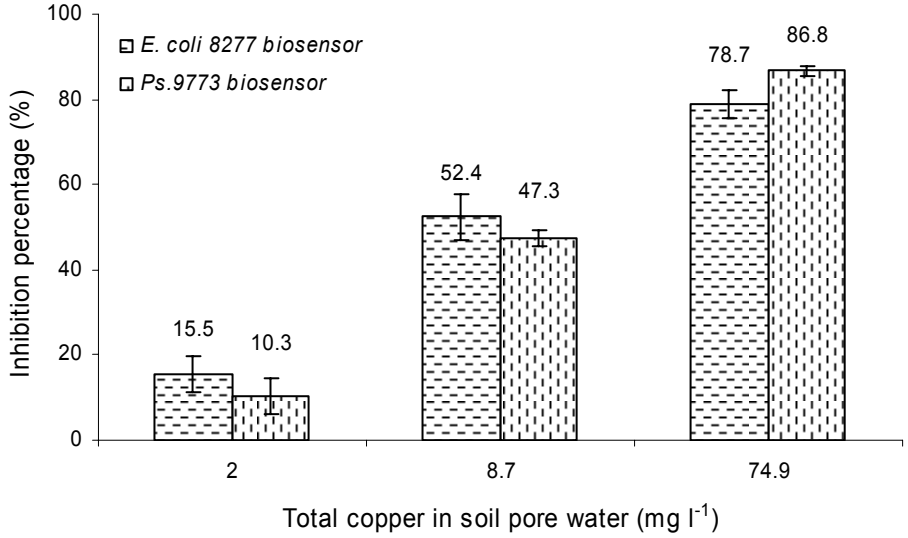


Figure 4.2 Inhibition of biosensors by copper in soil J pore water (average value ± standard error). Soil pore water was extracted from corresponding soil treatments showed in Figure 4.1(see also Table 4.2). The test was run in three replicates.

Table 4.2 shows the selected properties of soil pore water extracted from the control and three copper supplemented samples of soil J. The concentrations of copper in soil pore water were 0.164, 2.015, 8.655 and 74.905 mg l^{-1} from control, 200, 500, and 1000 mg kg^{-1} copper treatment soils, respectively. Based on copper concentrations in pore water, the EC_{50} and EC_{10} values were 8.38 and 0.38 mg l^{-1} for *E.coli 8277* and 9.92 and 1.23 mg l^{-1} for *Ps.9773*. These values are much higher than those in pure copper solution. It can also be seen that the pH values decreased, and the release of Ca, Mg and Zn from soil to pore water increased, with the increase of copper treatment concentration.

Comparing Fig.4.1 and Fig.4.2, both biosensors showed different inhibitions when they were directly exposed to soils and to respective pore water. *E. coli*

8277 biosensors showed less inhibition when exposed to soil pore water than to soil, *Ps.9773* biosensors gave the opposite result.

Table 4.2 Selected properties of soil pore water extracted from sandy loam soil samples amended with different dose of copper.

Treatments (copper addition in soil, mg kg^{-1})	pH	DOC	Ca	Mg	Cu	Zn	Pb
		mg l^{-1}					
0 (control)	7.15	508	96.056	17.596	0.164	0.512	<DL
200	5.98	583	344.88	63.738	2.015	2.721	0.015
500	5.12	563	755.25	122.8	8.655	11.118	<DL
1000	4.54	568	1417.8	196.92	74.905	38.775	0.022

<DL: less than Detection Limit.

4.2.3 Toxicity and bioavailability of copper spiked clay loam soil (C)

Fig.4.3a shows the inhibition of both *E. coli 8277* and *Ps.9773* biosensors by copper in clay loam soil C. Both biosensors were stimulated at copper concentration of 200 mg kg^{-1}. When exposed to soil with 500 mg kg^{-1} copper, *E. coli 8277* showed a very low level of inhibition (3.6%); whereas *Ps.9773* still showed some stimulation. In the case of soil with a copper concentration of 1000 mg kg^{-1}, both *E. coli 8277* and *Ps.9773* biosensors showed inhibition effects of 58.0% and 11.1%, respectively. *E. coli 8277* biosensors were more sensitive than *Ps.9773* to copper in the clay loam soil.

When exposed to the corresponding soil pore water, both biosensors showed inhibition effects with pore water from all three copper spiked soils (shown in Fig.4.3b). The inhibition of *E. coli 8277* biosensors by copper was 21.2%, 31.6% and 50.6% in pore water with copper concentration of 0.67, 0.87 and 10.11 mg l^{-1}, respectively. *Ps. 9773* gave 12.8%, 22.5%, and 52.9% inhibition to the same soil pore water samples.

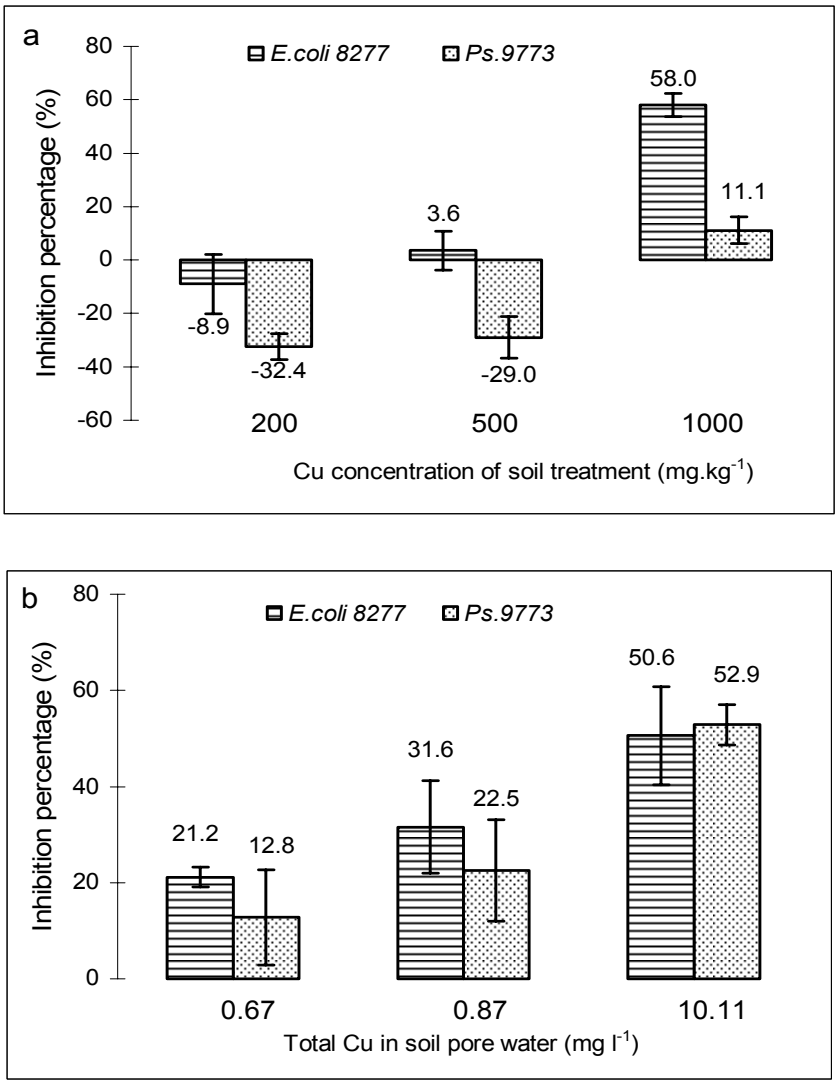


Figure 4.3 Inhibition of biosensors by copper in clay soil C (a) or soil pore water (b). Data is presented as average value ± standard error (n=3). Soil pore water was extracted from corresponding soil treatments (see also Table 4.3).

The properties of soil pore water extracted from the corresponding clay loam soil samples is shown in Table 4.3. The concentrations of copper in soil pore water were 0.02, 0.67, 0.87 and 10.11 mg l^{-1} from control, 200, 500, and 1000 mg kg^{-1} copper treatment soils, respectively. As with sandy loamy soil J, pH values of pore water decreased, and the release of Ca, Mg and Zn from soil to pore water increased, with the increase of copper treatment concentration.

In the case of both solid phase samples and corresponding soil pore water, biosensors gave higher inhibition in pore water than in soil, except when *E. coli 8277* was exposed to soil with copper concentration of 1000 mg kg^{-1}.

Table 4.3 Selected properties of soil pore water extracted from clay soil C samples amended with different dose of copper.

Treatments (copper addition in soil, mg kg^{-1})	pH	DOC	Ca	Mg	Cu	Zn	Pb
		mg l^{-1}					
0 (control)	6.49	655	109.19	35.39	0.02	0.01	0.02
200	5.54	535	179.91	59.58	0.67	0.03	0.02
500	5.45	515	201.74	66.79	0.87	0.03	0.01
1000	4.72	510	615.48	193.77	10.11	0.28	0.01

4.2.3 Toxicity and bioavailability of copper spiked organic soil (A)

Both biosensors were employed to assess the toxicity of copper spiked organic soil A and corresponding pore water. None of biosensors showed inhibition effects either in soil or pore water with all three concentrations of copper, and a trend of increasing stimulation with the increase of copper concentration was observed.

Ps.9773 gave higher stimulation than *E. coli 8277* when it was exposed in soil directly, *E. coli 8277* gave higher stimulation than *Ps.9773* in soil pore water. Comparison of the results from the soil and pore water measurement showed that both biosensors were more stimulated in soil than pore water, except *E. coli 8277* in pore water extracted from 1000 mg kg^{-1} Cu treated soil.

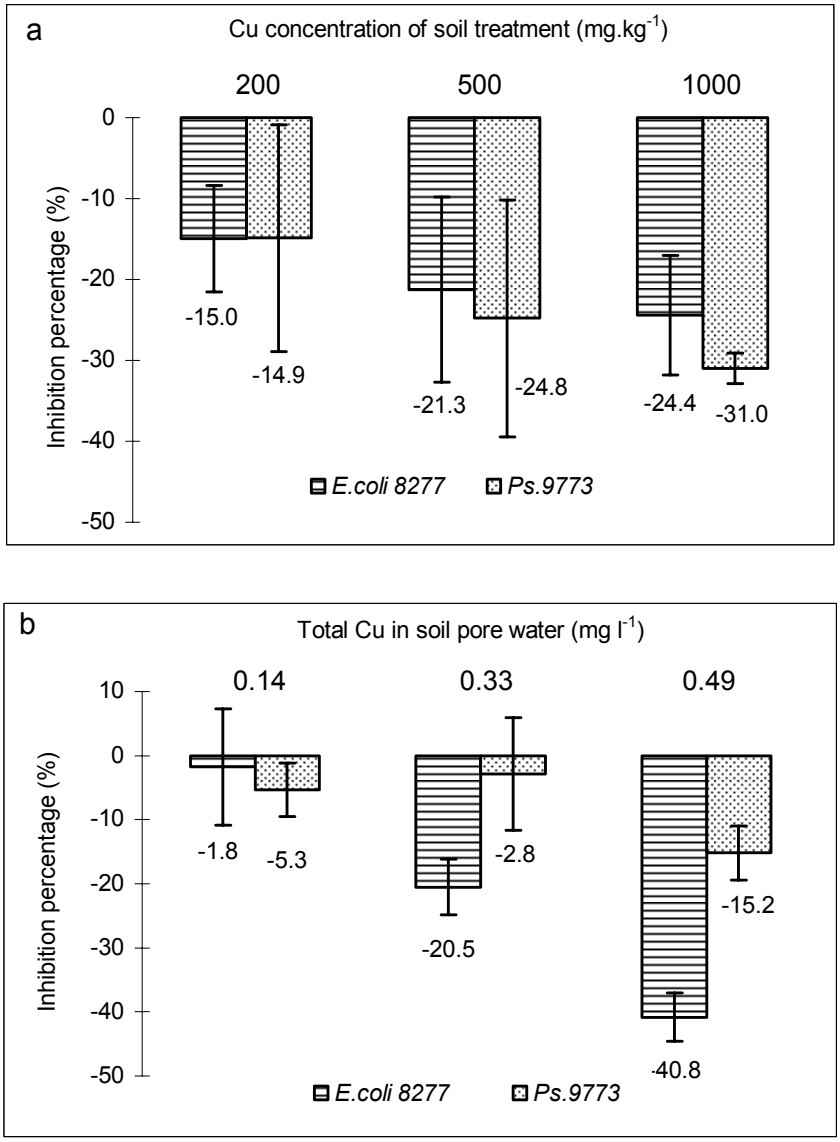


Figure 4.4 Inhibition of biosensors by copper in organic soil A (a) or soil pore water (b). Data is presented as average ± standard error (n=3). Soil pore water was extracted from corresponding soil treatments (see also Table 4.4).

Table 4.4 shows the properties of soil pore water extracted from the treated organic soil (A) samples. The concentrations of copper in soil pore water were 0.02, 0.14, 0.33 and 0.49 mg l^{-1} from control, 200, 500, and 1000 mg kg^{-1} copper treatment soils, respectively, which are much lower than corresponding pore water of clay loam soil C or loamy sand soil J. pH values of pore water were not significantly decreased by the addition of copper. The release of Ca, Mg from soil to pore water increased with the increase of copper treatment concentration, but Zn in pore water slightly decreased.

Table 4.4 Selected properties of soil pore water extracted from organic soil (A) samples amended with different dose of copper.

Treatments (copper addition in soil, mg kg^{-1})	pH	DOC	Ca	Mg	Cu	Zn	Pb
		mg l^{-1}					
0 (control)	6.72	450	336.4	19.31	0.02	0.27	0.02
200	6.66	373	370.03	21.41	0.14	0.08	0.02
500	6.62	343	463.56	27.05	0.33	0.03	< DL
1000	6.55	293	636.07	37.39	0.49	0.02	0.02

<DL: less than Detection Limit.

4.2.4 Discussion on toxicity and bioavailability of copper in different types of soil

- Soil properties greatly influence the toxicity and bioavailability of copper.

The results of the toxicity of copper in loamy sand soil J obtained with the bacterial biosensors are comparable to those of Thakali et al. (2006), who also used a

Woburn soil and obtained EC_{50} values for Cu of 872.8 mg kg^{-1} (tomato shoot yield), 1217.8 mg kg^{-1} (*F. candida* juvenile production), 350.1 mg kg^{-1} (*E. fetida* cocoon production), 1561.4 mg kg^{-1} (glucose induced respiration), and 836.9 mg kg^{-1} (potential nitrification rate). The soils were similar but not identical to those used in this present study, the SOM was higher (4%) but the pH was the same. From this comparison there results indicate that the biosensors are appropriately sensitive to Cu in soil.

This study also demonstrated that soil properties, such as SOM and clay content strongly decrease the bioavailability and toxicity of copper. When biosensors were exposed to different types of soils and corresponding soil pore water at the same concentrations of Cu, the toxicity of loamy sand soil was much higher than clay loam soil, and the organic soil did not show any toxic effect. Comparing the water extractable Cu in different soils, 1.0%, 0.34%, and 0.07% Cu were extracted from loamy sand, clay loam, and organic soils spiked with 200 mg kg^{-1} Cu, respectively. For 500 mg kg^{-1} Cu spiked soils, 1.73%, 0.17%, and 0.07% Cu were soluble in three soils, respectively. For 1000 ppm Cu spiked soils, 7.5%, 1.0%, and 0.5% Cu were extracted. The results indicated that clay content and SOM greatly decreased the soluble copper in soil matrix, which could explain the toxicity results.

Copper was found to have a high affinity for binding to SOM and in soil solution, dissolved organic carbon (DOC) plays a crucial role in the speciation of Cu (Kungolos et al., 2006; Zhao et al., 2006), which can reduce the bioavailability of copper. In the present study, EC50 values for copper in soil pore water from loamy sand and clay loam soils are much higher than for pure copper solution's, whilst the pore water of organic soil gave stimulating effect, even when Cu concentration was 0.49 mg l^{-1}. This indicates that not all soluble copper is bioavailable to bacteria

biosensors. Some free copper ions might be combined with DOC and the bioavailability decreased. The free ion activity model (FIAM) was used to assess the metal toxicity to aquatic organisms (Morel, 1983). However, free ion alone is insufficient to predict the biological response, because H^+ and the hardness cations (e.g., Ca^{2+} and Mg^{2+}) may compete with the metal ions for the binding sites on the cell surfaces, thus modifying the toxic response of the organism to the metal ions (Zhao et al., 2006). Nybroe et al. (2008) concluded that free Cu^{2+} activity is a poor predictor of Cu bioavailability to *Pseudomonas spp.* in samples containing organic ligands, such as EDTA.

Table 4.2 and 4.3 show that increasing the addition of Cu decreased the pH, but increased the release of cations (e.g., Ca^{2+}, Mg^{2+} and Zn^{2+}). Therefore, the toxicity of the soils to biosensors may be not only influenced by DOC, but also by other cations. In fact, the behavior of metals in soil is more complicated than in soil pore water or soil solution. A terrestrial biotic ligand model (TBLM) was developed to assess the toxicity of metals in soil (Thakali et al., 2006), which considered the competition and interactions related to the desorption of metals from the soil solid phase to the solution phase or the transport of metal ions to organism cell surfaces. To apply this model, soil properties need to be well known, however, they are time consuming, and not always readily available. In this study, direct exposure of biosensors to soils made it possible to give a realistic assessment of the bioavailability of metals in the soil system instantly.

- The toxicity results from direct soil contact tests and aqueous tests are different

Bioavailable metals can be defined as those fractions that can interact with surrounding microbial cells or other biota (Rensing and Maier, 2003), they usually

exist as the easily leachable ionic form of the metal (Schultz et al., 2004). Several studies (Van de Meent et al., 1990; Van Gestel, 1997; Løkke, 1994) suggested that the dissolved fraction in pore water is bioavailable. However, some particle-associated metals have been found to be available to biota under certain conditions. Ivask et al. (2002) reported that up to 115-fold more Cd and 40-fold more Pb proved to be bioavailable to sensor bacteria incubated in soil suspensions (the ratio of soil/water was 1:10 w/v) rather than in corresponding soil-water extracts. Brandt (2006) reported that less than 0.16% of soil particle-associated Cu is directly available to the applied *Ps. fluorescens* biosensor strains in soil-water mixtures having a soil to water ratio of 1:10. Petänen and Romantschuk (2003) suggested that the *Pseudomonas bacteria* can actually release mercury from solids through biomobilizing particle-bound heavy metal. Those results indicate that it is important to assess the bioavailability of metals in soil using direct soil contact methods rather than soil extraction methods. Although soil suspensions reflect more real soil conditions than soil extracts, natural soil conditions could be changed by a high ratio of liquid to soil and the bioavailability of metals may also be changed. In this present study, the biosensors were exposed directly in soil at a moisture content of 75% WHC, enabling the toxicity and bioavailability assessment of copper *in situ*.

Kahru et al. (2005) pointed out that the higher toxicity of soil-water suspensions, compared to the toxicity of respective extracts, can be considered to indicate the presence of particle-bound bioavailable toxicants in the sample. Our results with loamy sand soil showed that *E. coli 8277* biosensors experienced a higher level of inhibition in soil than in the corresponding pore water, which may indicate that there is some particle-bound bioavailable copper in soil samples. However, with *Ps.9773* biosensors, the inhibition by soil pore water at different concentrations of copper was always higher than with soil samples, and a stimulatory effect was seen with a

copper concentration of 200 mg kg^{-1}. With clay loam soil, the toxicity of soils was lower than corresponding pore water to both biosensors, even *E. coli 8277* was stimulated in soil at 200 mg kg^{-1} Cu concentration. One possible explanation is the presence of soil compounds that compensate for the toxic effects of copper. Aruoja et al. (2004) reported that algal growth was stimulated by different substances present in the soil, resulting in compensation for the toxic effects caused by heavy metals, and the higher stimulatory effects of soil-water suspensions compared to the extracts could be explained by the additional leaching of nutrients during algal growth. Another possible reason why clay loam soil samples were less toxic than corresponding pore water is that clay minerals might protect bacteria from exposure to soil solution. Ladd et al. (1995) reported that bacteria in soil may not always be exposed to the equilibrium solution activity of heavy metals because they are protected by clays.

The different inhibitory effects of Cu in soil and soil pore water on *E. coli 8277* and *Ps.9773* biosensors in this study is difficult to interpret, but they emphasize that soil pore water does not give an accurate reflection of soil conditions. The *in situ* exposure of organisms to the solid phase of soil is a more reliable approach to toxicity and bioavailability assessment of heavy metals.

4.3 Effect of soil moisture content on the toxicity and bioavailability of copper

Biosensors had been exposed in copper spiked soils at moisture content of 75% WHC and corresponding pore water samples to assess the toxicity of copper. Since soil moisture content may influence the toxicity and bioavailability of heavy metals in

soil (as reviewed in section1.2.2.5), there is a need to investigate whether soil moisture content would affect the toxicity and bioavailability of copper in soil to biosensors. *E. coli 8277* biosensors were employed for this purpose. Figure 4.5 a & b show that the toxicities of soil at moisture content of 100% WHC were higher than that at 50% WHC, especially when copper concentrations in soil were 200 and 1000 mg kg^{-1}. At all soil moisture contents, soils were more toxic than the corresponding pore water, which is consistent with the results with the same soils at a moisture content of 75% WHC (see section 4.2.2).

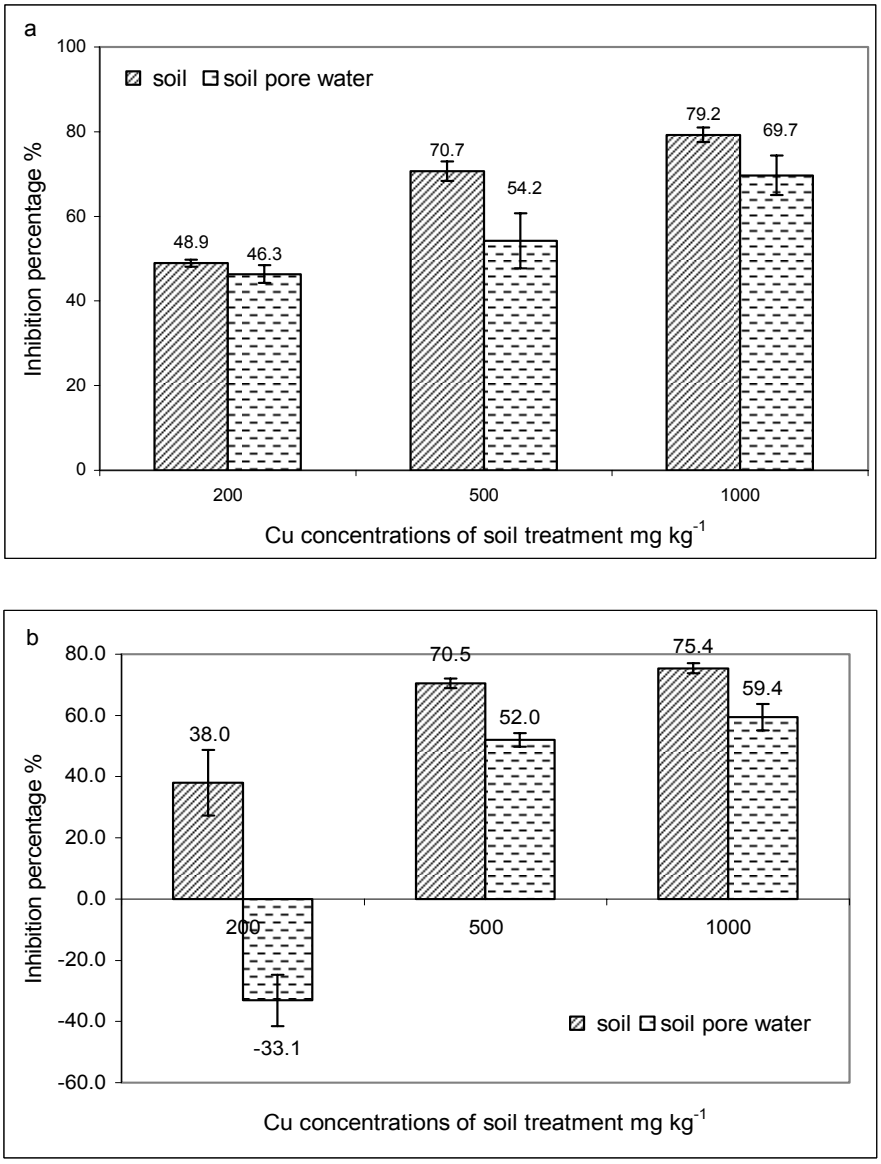


Figure 4.5 Inhibition of *E. coli 8277* biosensor by copper in loamy sand soil (J) or corresponding soil pore water at a soil moisture concent of 100% WHC (a) or 50% WHC (b). Data is presented as average ± standard error.

An increasing soil moisture might increase the release of metals from soil particles to soil solution, on the other hand, it also might decrease the concentration of metals in soil solution. Table 4.5 and Table 4.6 show the properties of pore water extracted from soil samples at a moisture content of 100% WHC or 50% WHC. The

concentrations of Cu^{2+}, DOC, Ca^{2+}, Mg^{2+}, Zn^{2+} in pore water from control soil, 200 mg kg^{-1} and 500 mg kg^{-1} Cu amended soils were decreased as soil moisture increased. Although soils at moisture content of 50% WHC gave a higher concentration of Cu in pore water than soils at 100% WHC, their toxicities were lower, which indicate that bioavailable Cu in pore water decreased with decreasing soil moisture. Higher DOC in pore water may explain the decreased bioavailability of Cu in pore water extracted from the soils with lower moisture content. Tom-Petersen et al. (2004) reported that the bioavailable copper to total copper ratio in soil water extracts decreased with decreasing moisture in the soil. An increasing toxicity as soil moisture increased was also reported by Aelion and Davis (2007). Those results are consistent with the present study.

Soil is a complex system comprising of solid, liquid, and soil phases. The decrease of the soil moisture content usually results in the increase of soil gas phase, which may decrease the exposure of soil microorganisms to solid or liquid phase of soil. This might be another reason for the decreased toxicity as soil moisture content decreased. In this present study, low soil moisture content may obstruct the close contact of biosensors and soil. Therefore, comparative toxicities of different soil samples would require a uniform WHC to be used with all samples.

Table 4.5 Selected properties of soil pore water extracted from soil samples at a moisture concent of 100% WHC.

Treatments (copper addition in soil, mg kg^{-1})	pH	DOC	Ca	Mg	Cu	Zn
		mg l^{-1}				
0 (control)	7.15	399	66.857	12.044	0.114	0.372
200	5.79	342	242.85	44.295	1.483	1.961
500	5.18	277	535.98	88.335	6.48	8.01
1000	5.12	217	1052.2	143.51	54.521	29.244

Table 4.6 Selected properties of soil pore water extracted from soil samples at a moisture content of 50% WHC.

Treatments (copper addition in soil, mg kg^{-1})	pH	DOC	Ca	Mg	Cu	Zn
		mg l^{-1}				
0 (control)	6.54	665	73.658	13.948	0.226	0.487
200	5.39	510	410.35	81.338	2.121	3.463
500	4.89	442.5	947.25	160.69	10.061	15.066
1000	4.77	370	1126.2	182.67	15.379	21.553

4.4 Effect of pH on toxicity of copper to biosensors

Soil pH is generally accepted as an important factor influencing metal availability and toxicity. As reviewed in Chapter 1, pH affects many aspects of the interactions between microbes and heavy metals, with metal toxicity either increasing or decreasing with changes in pH. The pH could directly affect the metabolic activities of organism resulting in the change of resistance of the organism to heavy metals. Under highly alkaline or acidic conditions, some macromolecules in the organisms can be damaged (Foster, 1999), and some cellular components can also be hydrolysed and enzymes denatured (Atlas and Bartha, 1993), which results in reduction of cell viability. On the other hand, pH could affect metal availability. These two aspects interact, which makes the mechanisms involved in pH-metal toxicity relations very complex. The present study investigated the pH effect on biosensor signals and copper toxicity measurement.

4.4.1 Effect of pH on biosensor signals

Biosensors were exposed to 0.85% saline solutions at a range of pH for 3 hr. Figure 4.6 shows a trend of increasing toxicity with both *E. coli 8277* and *Ps.9773* biosensors as pH decreased. At pH value of 5.5, *E. coli 8277* and *Ps.9773* biosensors gave percentage inhibition of 18.2% and 12.4%, and 39.3% and 41.2% when pH was 4.5. Two biosensor configurations did not show significantly different effect by pH. At a pH of 3.5, however, *Ps.9773* gave a percentage inhibition of 83.7%, which is much higher than *E. coli 8277* (55.2%). The results indicate that

both biosensors were inhibited under the acidic conditions, *E. coli 8277* has a better homeostatic mechanism than *Ps.9773* under the extremely acidic conditions.

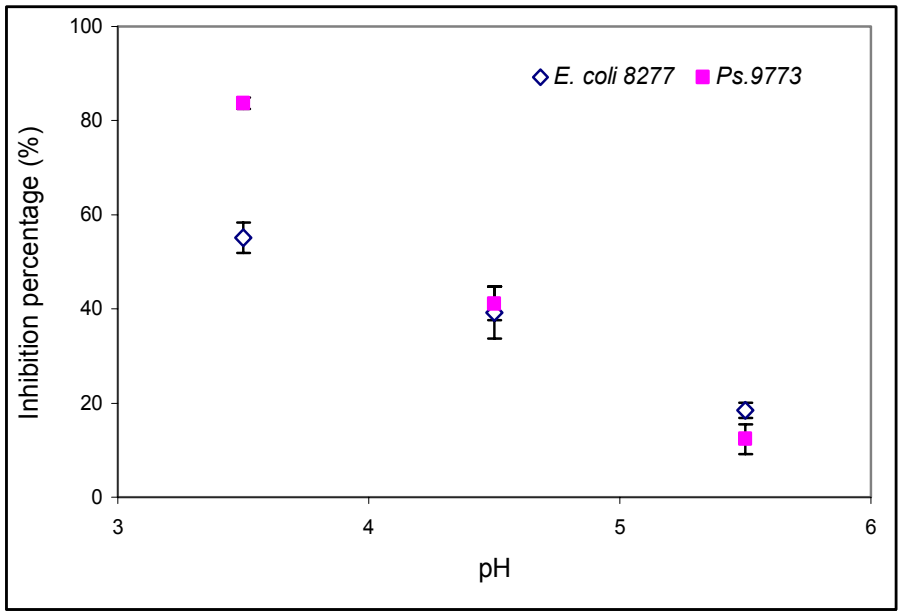


Figure 4.6 Effect of pH on biosensor responses. Biosensors were exposed to 0.85% saline solution at different pH for 3 hr. Unmodified saline solution (pH = 6.8) was used as a control. 1 M HCl or 1M NaOH solution were used to adjust pH of 0.85% saline.

4.4.2 Effect of pH on toxicity of copper in soil pore water to biosensor

The addition of Cu to soils decreases the pH of soil pore water. In this case, the pH values of soil pore water from control, 200, 500, and 1000 mg kg^{-1} copper soil treatments were 6.8, 5.4, 4.9, and 4.5, respectively. Without pH modification, the inhibition of biosensor by copper contaminated soil pore water actually results from the combined effects of copper and pH. For comparison of single and combined effects of copper and pH on biosensors, soil pore water samples extracted from different Cu treated soils were adjusted to the same pH, 6.8 or 4.5 to measure their

toxicities to biosensors. A series of pore water samples without pH modification were also used for comparison. When the pH of pore water samples was modified to 4.5, both biosensors showed lower inhibition than to the pore water without pH adjustment (shown in Fig. 4.7). At the pH of 6.8, both biosensors gave higher inhibition to 1000 mg kg^{-1} copper treated soil pore water, but lower inhibition to 200 and 500 mg kg^{-1} copper treated pore water samples than to the pore water without pH adjustment.

As described in Section 2.9.1, the inhibition percentage of biosensor depends on biosensor signals after exposure to control and copper treated soil pore water. The decrease of biosensor signals to control sample or increase of biosensor signals to samples with toxicants could possibly result in a lower inhibition. In this case, biosensor response to the control pore water was decrease by decreasing the pH, however their responses to 200 and 500 mg kg^{-1} Cu treated pore water at pH of 4.5 were increased (shown in Fig.4.8), which could explain why biosensors gave lower inhibition when soil pore water was modified to a pH of 4.5. At the pH of 6.8, biosensor responses to pore water extracted from 1000 mg kg^{-1} Cu treated soil samples were decreased, however those to 200 and 500 mg kg^{-1} copper treated pore water samples were increased, resulting in the lower inhibition of biosensors to 200 and 500 mg kg^{-1} Cu treated pore water, but higher inhibition to 1000 mg kg^{-1} Cu treated pore water.

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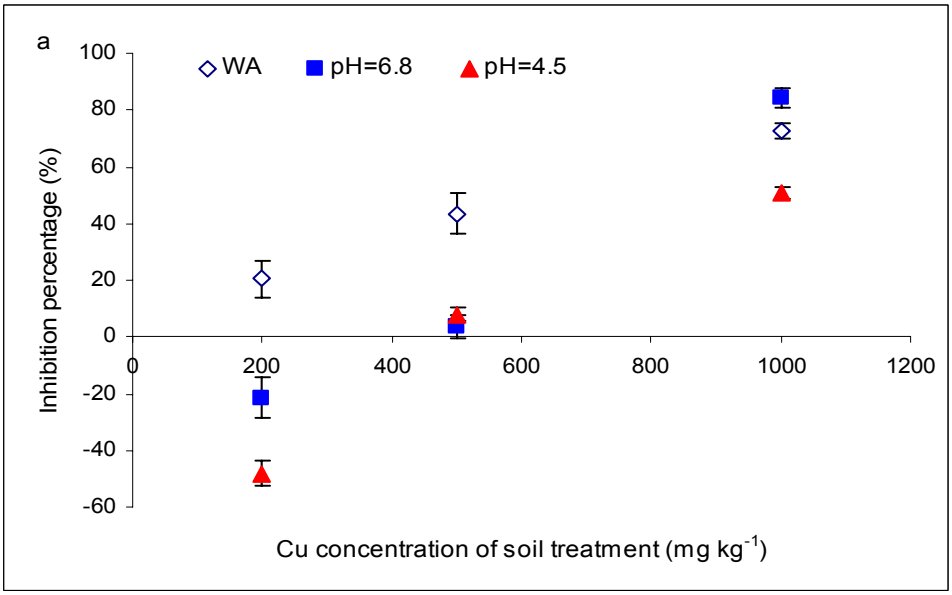
The pH could produce two opposite effects on biosensor response at the same time. An increase of pH might reduce the competition of protons with Cu^{2+} for sorption sites on bacterial cells and increasing the bioavailability of copper, resulting in the increase of Cu toxicity; whilst, increasing the pH could also increase the biosensor signals as shown in Fig.4.6. The biosensor testing showed the combined effects of

Cu and pH. These results demonstrate that pH not only affects the bioavailability, but also the toxicity of Cu to bacteria. In addition, it may be concluded that pure copper toxicity to biosensors is lower than the combined effect of Cu and pH. The assays which only consider the effect of pH on bioavailabilities of heavy metals alone rather than the combined effect, or any bioassays requiring the pH adjustment of test samples would not be able to precisely assess the risk of heavy metal contaminated soils. The biosensor assay for using *in situ* in soil would be a reliable method for rapid risk assessment. pH needs to be well defined when biosensors are applied for assessing the toxicity or bioavailability of metals in soils.



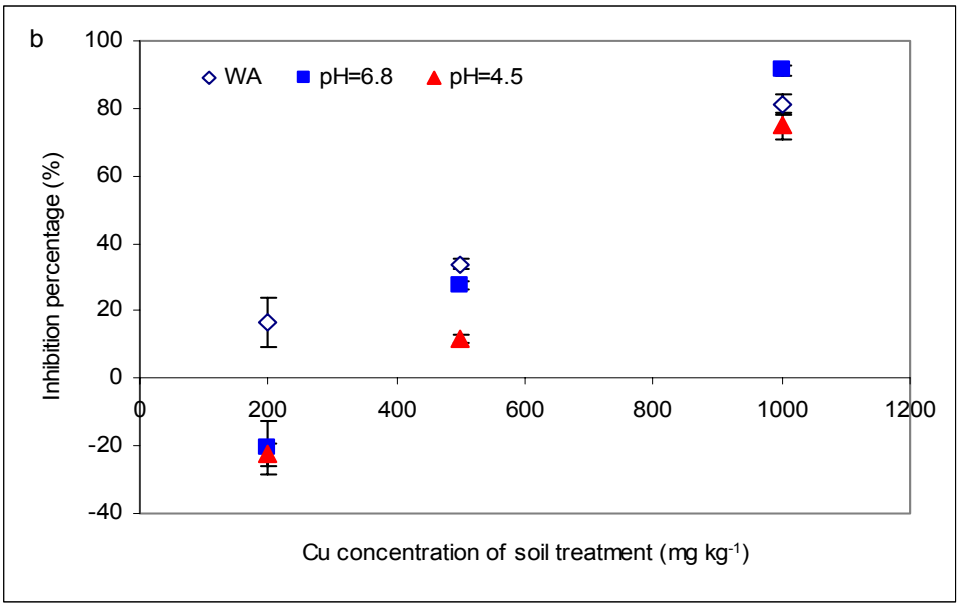
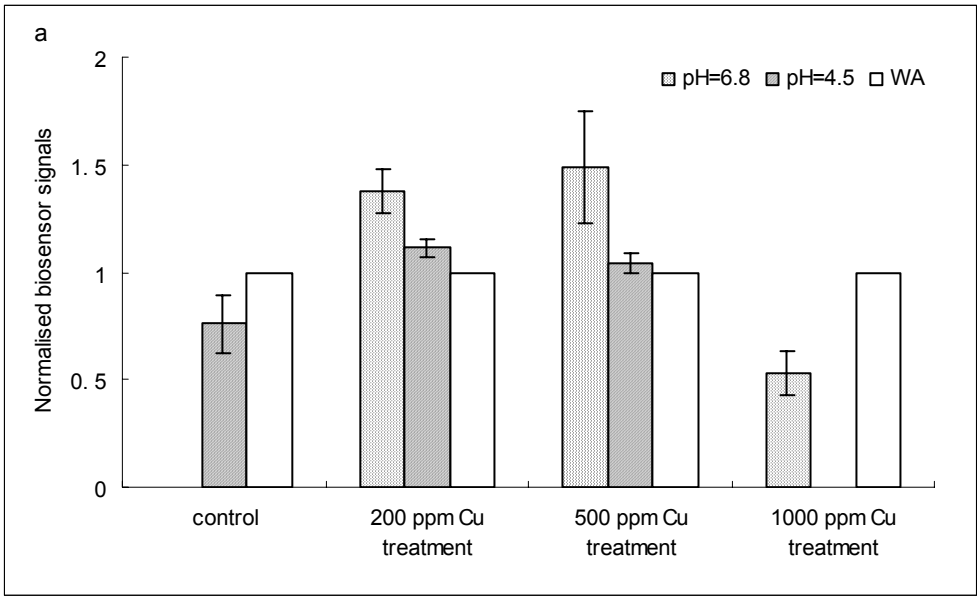


Figure 4.7 Inhibition of biosensors by soil pore water at different pH. a) *E.coli 8277* biosensor; b) *Ps.9773* biosensor. Pore water was extracted from copper treated loamy sand soil (J). The exposure time was 3 hr. The percentage inhibition of biosensors was calculated by taking each control soil pore water with the same pH treatment as corresponding control. WA means without adjustment of pH.



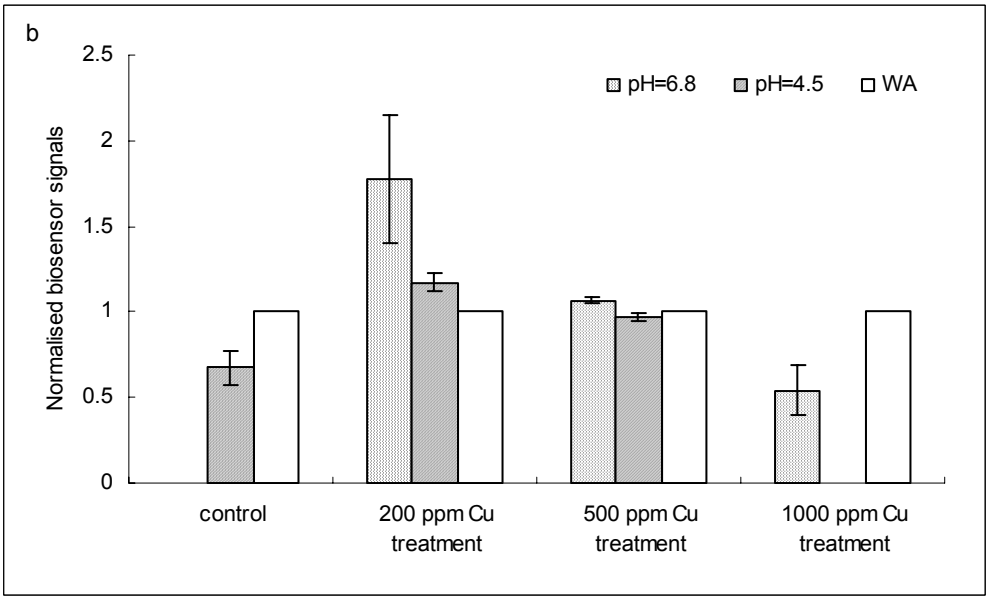


Fig.4.8 Normalized biosensor responses to Cu treated soil pore water at different pH. a) *E. coli 8277* biosensor; b) *Ps.9773* biosensor. The biosensor responses to pore water samples at modified pH were normalized with respect to the corresponding pore water samples without pH modification. WA means without adjustment of pH.

4.5 Summary

- The new protocol allows the mediated amperometric bacterial biosensor system to be applied for *in-situ* assessment of bioavailability and toxicity of copper in soil. Both biosensors showed different inhibition effects when exposed to solid phase or soil pore water. However only solid phase measurements reflect whole soil exposure pathways. Therefore, it is important to develop a bioassay for *in situ* assessment of contaminated soils. The biosensor system is sensitive to copper compared to other bioassays, and is relatively rapid taking less than 4 hrs. In contrast plant and invertebrate tests can take several days or even months, and soil suspension assays with the Alga *Selenastrum capricornutum* take 48h to 72h (Aruoja et al., 2004). The freshly made biosensors are able to maintain a stable signal for 4-6 h. Bacterial biosensors also can be freeze-dried, and resuscitated as and when required, allowing assays to be under-taken at any time.

- The toxicity and bioavailability of copper to biosensors depends on the soil properties. High SOM and clay content strongly decrease the bioavailability and toxicity of copper. Therefore, biosensors may be not able to detect the toxic effects of relatively high concentrations of copper in soils with high SOM or clay content. The pH should be always considered as an important factor for toxicity and bioavailability assessment of heavy metals. In the case of biosensors for toxicity assessment, pH not only changes the bioavailability of copper, but also affects biosensor directly. The inhibition of biosensors by copper contaminated soil or pore water reflects the combined effect of pH and bioavailable copper. Soil moisture content also influenced the toxicity and bioavailability of copper to

biosensors. Increasing soil moisture content from 50% WHC to 100% WHC increased the toxicity of copper. In summary, the soil conditions need to be well defined when biosensors are used for assessing the toxicity and bioavailability of heavy metals in soils.

- Mediated amperometric bacterial biosensors are not analyte specific, their response reflects the metabolic impact of the combined chemical and physical properties of the environment to which they are exposed. The bacterial biosensors used in this study, whilst showing sensitivity to copper, will be affected by other soil components. Nutrients in soil could compensate for toxic effects of heavy metal, resulting in a decreased sensitivity of biosensors. However, soils are rarely polluted with a single contaminant but usually with a mixture of pollutants and their subsequent metabolites (McGrath el al., 1995). The broad spectrum nature of the bacterial biosensors response is an advantage in acute toxicity assessment of copper contaminated soils, and complex polluted fields as well.

- *E. coli 8277* and *Ps.9773* biosensors responded differently to solid phase of soils and soil pore water. Since the bioavailability of metals is usually organism-specific, it is always better to use more than one species for ecotoxicity assessment. Generally speaking, *E. coli 8277* biosensors were more sensitive to copper than *Ps.9773* biosensors. The mediated amperometric bacterial biosensor protocol described here can be adapted to allow the incorporation of different bacterial biocatalysts for other applications in soil quality assessment.

5 Toxicity assessment of metal mixtures (copper with zinc or lead) using biosensors

5.1 Introduction

Risk assessment of anthropogenic toxicants is primarily derived from experiments conducted with single substances (ECB, 2003), however, contaminated soils usually contain mixtures of chemicals rather than a pure substance. Both the physico- and biochemical behaviour of chemicals are known to change in the presence of other compounds, which leads to effects that cannot be estimated properly from a single-substance point of view (Pokarzhevskii & Van Straalen, 1996). Therefore mixture toxicity (combined toxic effects of metal mixtures) has been a subject of ecotoxicological interest for several decades (Hermens et al., 1984; Altenburger et al., 1996; Silva et al., 2002; Backhaus et al., 2003). Evaluating the mixture toxicity of heavy metals and studying the toxicity interaction of metals are important for risk assessment of metal contaminated soils. Some results show that additive, synergistic and antagonistic effects of heavy metal mixtures have all been found (Khalil et al., 1996; Van Gestel and Hensbergen, 1997; Sharma et al., 1999). The term additive effect is used to describe a “no interaction action” or a zero interaction between components. A toxicity greater than additive is described as a synergistic effect, while the term antagonistic effect is commonly used to describe a toxicity lower than the additive effect (Könemann and Pieters, 1996). Various models have been used to evaluate the effects of metal mixtures on organisms and analyse the toxicity interactions of metals (Loewe and Muischnek, 1926; Bliss, 1939; Hewlett and

Plackett, 1959). These models allow evaluation only if equitoxic mixtures are antagonistic, additive, or synergistic and it is not possible to predict the effects of randomly chosen metal mixtures. The only reliable way to determine toxicity of mixtures is to test it using bioassays although understanding of the mechanisms of action of each component may help to make a priori predictions. Recently, Invertebrate and plant tests have been used to assess the mixture toxicity of metals in soils. Khalil et al. (1996) assessed cocoon production and growth of the earthworm *Aporrectodea caliginosa* after exposure to mixtures of cadmium, copper, and zinc. Posthuma et al. (1997) studied the single and joint effects of copper and zinc to pot worm *Enchytraeus crypticus*. Luo and Rimmer (1995) reported the mixture effect of copper and zinc on plant uptake of these metals. These approaches allow the evaluation of mixture toxicity, however they are time and space consuaming (Aruoja et al., 2004). There is need for a rapid, low cost bio-assay that can be applied to assess the acute toxicity of metal mixtures in soils *in situ*.

The present study has demonstrated the use of mediated amperometric bacterial biosensors for *in situ* assessment of toxicity of copper spiked soil. Whole-cell biosensors are not specific to any metals, and have potential applications in mixture toxicity assessment. Both *E. coli 8277* and *Ps.9773* biosensors were employed to investigate the mixture toxicity of copper and zinc, and copper and lead.

In the soil ecosystem the joint effects of a metal mixture could be influenced by many potential interactions. Three interaction levels are usually addressed: 1) interactions in soil, 2) interactions during uptake, and 3) interactions in the body. Different exposure routes of biosensors enable the toxicity

measurement and the investigation of the interaction mechanisms of metals in soil ecosystem to be carried out. In this present study, the biosensors were exposed to defined metal solutions, soils spiked with single or mixed metals, and corresponding soil pore water samples, respectively. Certain concentrations of Cu and Pb mixture, or Cu and Zn mixture were chosen to determine the mixture toxicities in solutions or soils following the range finding experiments described in Section 2.7.

5.2 Single and mixture toxicity of Cu and Pb

5.2.1 Toxicity of Cu and Pb mixture in solution

E. coli 8277 and *Ps. 9773* biosensors were exposed to lead solutions or concentrations of lead plus 2 ppm copper solutions for 3 hr. The percentage inhibition was shown in Figure 5.1a and b. The inhibition of *E. coli 8277* biosensors by lead in solution was 17.5%, 18.7%, 15.4%, and 21.1% at lead concentrations of 50, 100, 200, and 400 mg l^{-1}. The increase of Pb concentration from 50 to 400 mg l^{-1} did not significantly increase the toxicity to *E. coli 8277* biosensor. 2 mg l^{-1} Cu caused 53.3% inhibition of *E. coli 8277* biosensors, however the mixture toxicity of 2 mg l^{-1} Cu plus Pb in the range of 50 to 400 mg l^{-1} was not significantly higher than the toxicity of copper alone. The inhibition of *Ps. 9773* biosensors by lead in solution was 54.4%, 59.9%, 71.8%, and 83.6% at lead concentrations of 50, 100, 200, and 400 mg l^{-1}. The mixture toxicity of lead and copper to *Ps. 9773* biosensors was higher than any single metal toxicity, but the difference between mixture toxicity and single lead toxicity tended to decrease with increasing lead concentration.

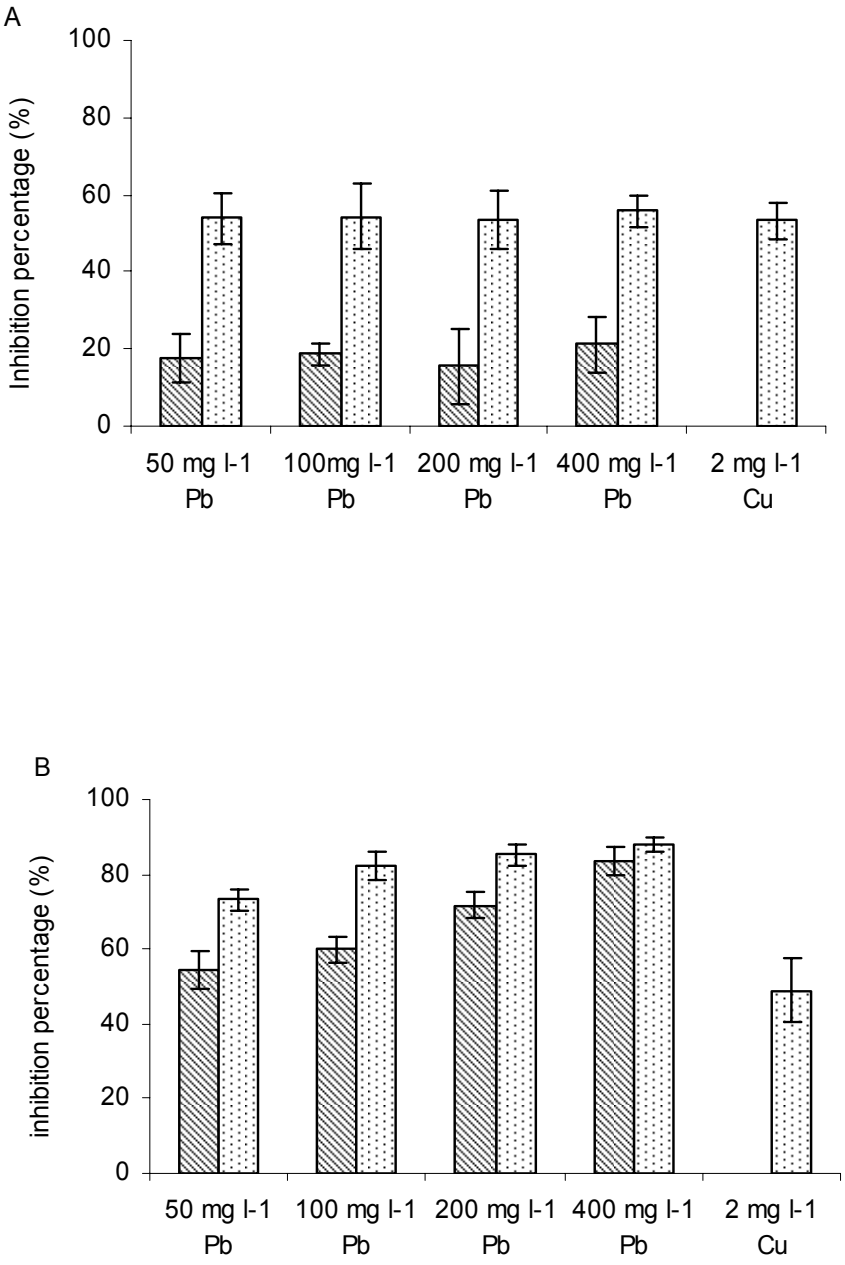


Figure 5.1 The inhibition of biosensors by copper, lead or lead plus copper in solution (mean value ± standard error). (A) *E. coli 8277* biosensors; (B) *Ps. 9773* biosensors. ▨: Pb; ⬚: Pb + 2 mg l^{-1} Cu. Exposure time was 3 hr.

5.2.2 Single and mixture toxicity of Cu and Pb in soil

Soil samples were spiked with Pb or mixtures of Cu and Pb. Both *E. coli 8277* and *Ps. 9773* biosensors were used to study the toxicity of Cu and Pb mixture in soil through different exposure pathways (solid phase of soils and soil pore water). The results are shown in Figure 5.2. The percentage inhibition of *E. coli 8277* biosensor increased with increasing concentration of lead when exposed *in situ* in soil. The mixture toxicity of copper and lead to *E. coli 8277* and *Ps. 9773* biosensors was higher than the toxicity of copper or lead alone at any spiked concentrations in soil. When exposed to corresponding soil pore water, the inhibition of both *E. coli 8277* and *Ps. 9773* biosensors by mixture of copper and lead was higher than either single toxicity when lead addition to soils were 1000 mg kg^{-1} or 2000 mg kg^{-1}, but it also showed lower toxicity of Cu and Pb mixture when lead addition was 500 mg kg^{-1}.

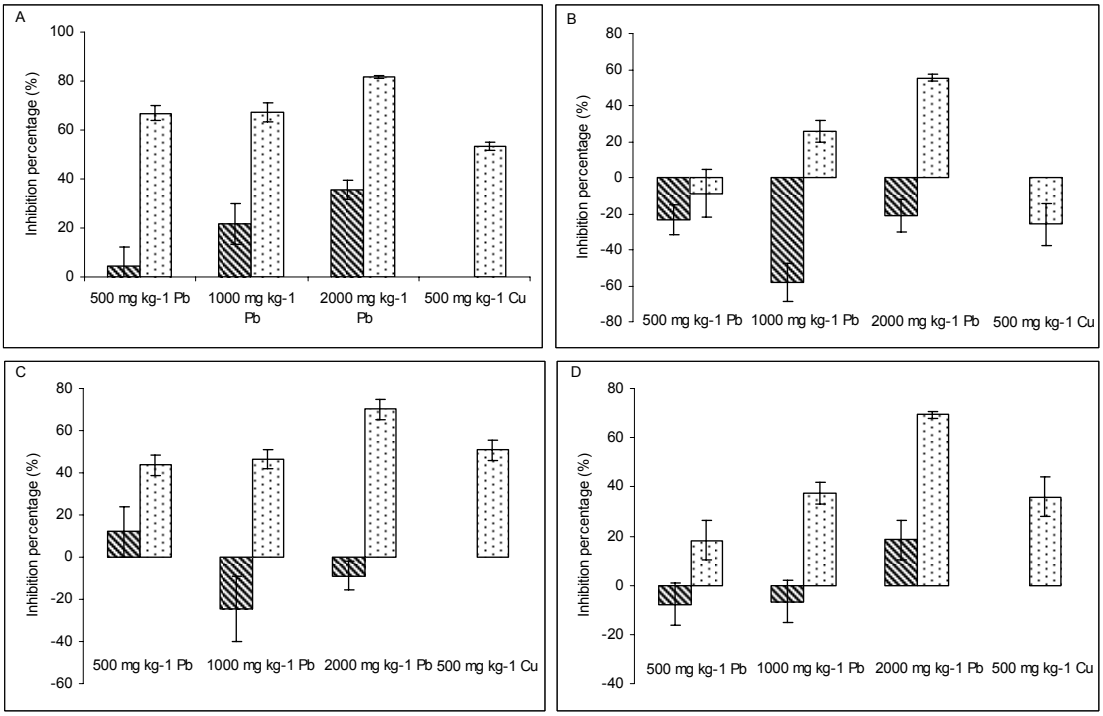


Fig.5.2 The inhibition of biosensors by copper, lead or lead plus copper in soil or soil pore water (mean value ± standard error). (A) *E. coli 8277* biosensor response to soil; (B) *Ps. 9773* biosensor response to soil; (C) *E. coli 8277* biosensor response to soil pore water; (D) *Ps. 9773* biosensor response to soil pore water. ▨ : Pb; ⬚ : Pb + 500 mg kg^{-1} Cu. Biosensors were exposed to soil or pore water samples for 3 h.

Comparing the single or mixture toxicities of Cu and Pb *in situ* in soils and pore water, *E. coli 8277* showed a higher inhibition when exposed *in situ* in soils than in pore water, however, *Ps. 9773* gave opposite results.

The properties of soil pore water extracted from the spiked soil samples were determined to investigate the effects of metal amendment on soil solution and how the metals influence their desorption from soil particles to solution (shown

in Table 5.1). Lead appeared to be adsorbed to a large extent. Only 0.05%, 0.03%, and 0.22% Pb were water extractable from 500, 1000, and 2000 mg kg^{-1} Pb spiked soils. The addition of copper to soil increased the levels of soluble lead in pore water, 0.08%, 0.20%, and 0.66% Pb were extracted from 500, 1000, and 2000 mg kg^{-1} Pb spiked soils when 500 mg kg^{-1} Cu was added. The increase of lead addition also increased the desorption of copper from soil to pore water. There was also a trend shown by other cations such as Ca^{2+}, Mg^{2+} increased with increasing lead or copper addition. The addition of Pb did not change pH of pore water as much as Cu, pH was slightly increased by the addition of 500 or 1000 mg kg^{-1} Pb. As a result, the addition of Pb to soil did not clearly change pH of pore water extracted from Cu spiked soils.

Table.5.1 The properties of soil pore water extracted from soil samples spiked with different doses of copper, lead, or copper plus lead.

Soil treatment	pH	DOC	Ca	Mg	Cu	Pb
		mg kg^{-1}				
control	6.41	495	107.63	20.251	0.142	0.003
500 mg kg^{-1}Pb	7.01	438	455.49	82.567	0.156	0.252
1000 mg kg^{-1}Pb	6.51	465	82.55	15.313	0.099	0.34
2000 mg kg^{-1}Pb	5.31	375	230.72	42.668	0.071	4.512
500 mg kg^{-1}Cu	4.89	348	806.18	126.59	3.282	0.002
500 mg kg^{-1}Cu + 500 mg kg^{-1}Pb	5.16	378	649.08	109.86	3.575	0.385
500 mg kg^{-1}Cu + 1000 mg kg^{-1}Pb	4.94	405	844.07	134.5	4.284	2.023
500 mg kg^{-1}Cu + 2000 mg kg^{-1}Pb	4.68	285	1211.2	173.75	7.24	13.289

5.2.3 Discussion

As described above, the toxicity interactions of heavy metal mixtures are usually studied at three levels. In this study, metal concentrations in pore water to some extent reflected the interactions in soil, since it represent the bioavailable fractions of metals in soil. This data could be used to evaluate how the desorption of metals from soil particles to solution is affected by other metals. Mixture toxicity of metals to biosensors in solution and pore water reflects both levels of interactions during uptake by bacteria and in the cell. During uptake, metal cations compete for sorption sites on the surface of cells and target sites inside of cells. The differences between solution and pore water are that they may contain different metal species, and their pH are different. Metals in pore water may exist in the form of free ions and complexes with inorganic or organic ligands, which affect their bioavailability. Mixture toxicity to biosensors in solid phase of soil also reflects the two levels of interactions during uptake and in the cell, whereas the uptake route includes all the pathways including through pore water and soil particles. Therefore, toxicity assessment based on direct contact approaches are more realiable for risk assessment. Exposure of biosensors to different medium is helpful to interpret the mixture toxicity and the interactions of metals in soil ecosystem.

The addition of Pb slightly increased Cu in pore water, and Cu addition increased Pb in pore water. Heavy metals compete with each other for sorption sites, resulting in one metal displacing the weaker competing metal from soil particles into the soil solution (Atanassova, 1999; Van Gestel &

Hensbergen, 1997). The outcome of the competition mainly depends on the concentration ratio of the competing metals and their respective sorption characteristics. The increased Pb and Cu concentrations in pore water partly explained the results that the mixture toxicity of Cu and Pb to both biosensors in solid phase of soil or pore water was higher than either single metal. An antagonistic effect of Cu and Pb in defined solution or soil pore water on the response of both biosensors can be estimated from the biosensor tests. The pattern of response to Cu and Pb mixture in the solid soil samples seemed to be additive. This results are consistent with Laskowski & Hopkin (1996) that the toxicity of Cu / Pb to juvenile and adult garden snail consumption showed concentration-additive and antagonistic, respectively. However, the biosensor tests were designed to detect the total toxicity of metal mixtures instead of evaluating the pattern of toxicity interaction of metals. A more suitable model is needed to give an accurate interaction pattern of metals.

The toxicity of Cu and Pb mixture to *E. coli 8277* biosensor in soil *in situ* was higher than in pore water, however *Ps.9773* biosensor gave opposite results. This indicates that two bacteria species probably have different uptake and resistance mechanisms to metals. However soil pore water does not give an accurate reflection of soil conditions, measurement in solid phase of soil would be more ecologically relevant for risk assessment. Therefore, *E. coli 8277* biosensor is more sensitive for *in situ* screening and assessing soils contaminated by Cu and Pb.

Comparing the results between solution and soil measurement, lead doesn't increase the mixture toxicity of copper and lead in solution to biosensors, however it does in soil *in situ* or pore water measurement. The possible

explanation is that the soluble Pb may not be the main species of uptake, and formation of organic Pb complex species may result in the increased toxicity as increasing the concentration of Pb in soil. Slaveykova (2007) reported that the bioavailable Pb to algae in the presence of fulvic acid was greater that expected for the same free lead ion concentrations in the absence of fulvic acid. Fulvic acid adsorbed to algae may give rise to additional binding sites for Pb (II). It has also been reported that the presence of humic acid increased the toxicity of Pb (Kungolos et al., 2006; Tsiridis et al., 2006). In this study, *E. coli 8277* biosensors showed low sensitivities to Pb in solution, 17.5% inhibition by 50 mg l^{-1} Pb. However, 21.5% and 35.4% inhibition were abstained with 1000 and 2000 mg kg^{-1} Pb spiked soil respectively, whilst the concentrations of Pb in corresponding pore water were just 0.34 and 4.512 mg l^{-1}.

E. coli 8277 showed a trend of increasing inhibition as the increase of Pb addition to soils, however, *Ps. 9773* didn't show any inhibition effect in the Pb concentration range of 500 – 2000 mg kg^{-1}, although it was more sensitive than *E. coli 8277* to Pb in defined solution. This indicates that the mechanisms of Pb uptake from soil is species-specific. *E. coli 8277* biosensors are more suitable for *in situ* assessment of toxicity of Cu and Pb mixture.

5.3 Toxicity of Cu and Zn mixture

5.3.1 Toxicity of Cu and Zn mixture in solution

Both *E. coli 8277* and *Ps. 9773* biosensors were exposed to zinc solutions or concentrations of zinc plus 2 mg l^{-1} copper solutions for 3 hr. The percentage inhibition of biosensors is shown in Fig. 5.3. *E. coli 8277* biosensors showed inhibition responses to individual metals of 49.5% to 2 mg l^{-1} Cu, and 38.2%, 45.7%, and 44.0% at Zn concentrations of 100, 200, 500 mg l^{-1} respectively. Whilst the toxicity of Cu and Zn mixtures to *E. coli 8277* biosensors at the three mixture concentrations was greater than for each individual metal. Unlike *E. coli 8277* biosensors, the mixture toxicity of Zn and Cu to *Ps.9773* was even lower than either single metal solution. In comparison of two biosensor responses to Zn, *E. coli 8277* was more sensitive at a lower concentration of Zn, whereas *Ps.9773* gave higher inhibition at higher Zn concentrations.

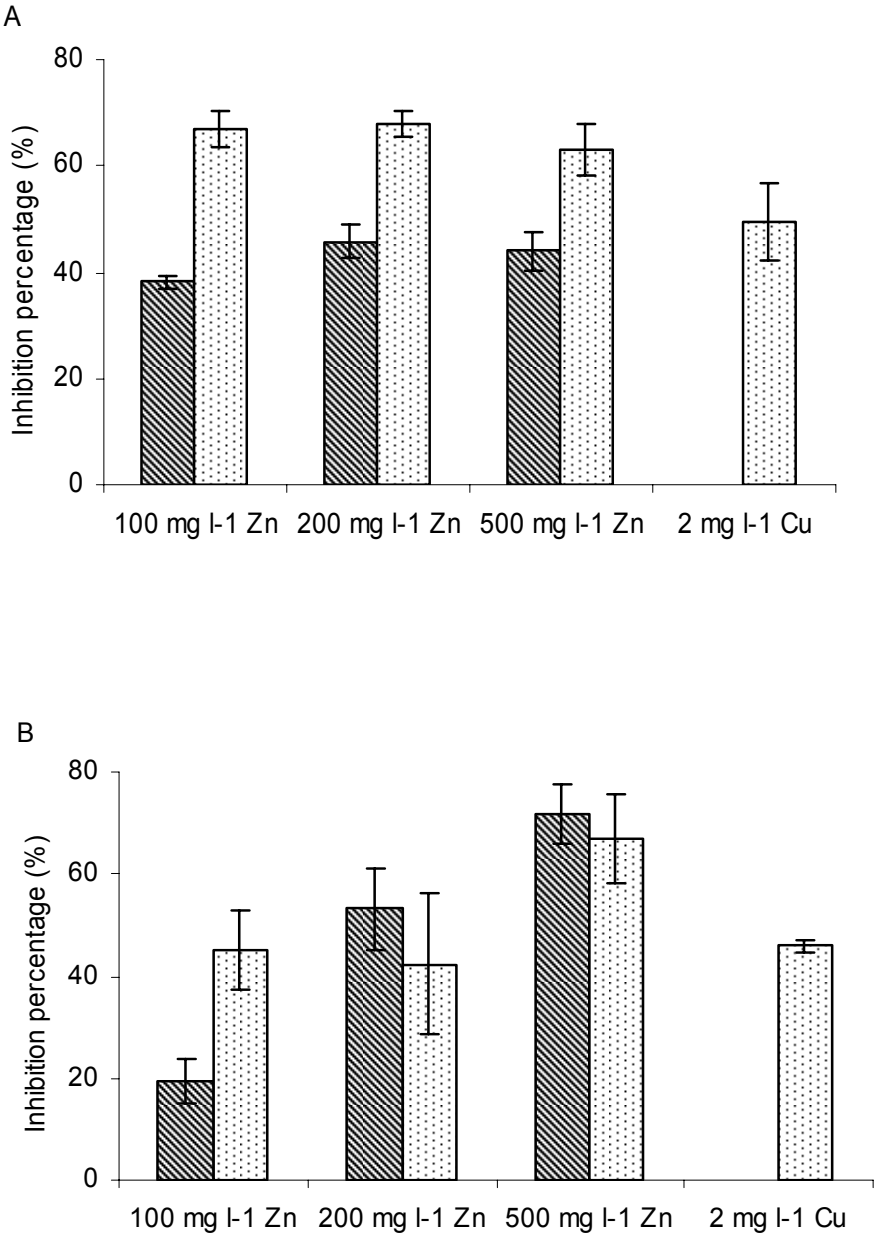


Figure 5.3 The inhibition of biosensors by copper, zinc or zinc plus copper in solution

(mean value ± standard error). (A) *E. coli 8277* biosensors; (B) *Ps. 9773* biosensors. ▨ : Zn; ⬚ : Zn + 2 mg l^{-1} Cu.

5.3.2 Single and mixture toxicities of Cu and Zn in soil

Single and mixture toxicities of Cu and Zn in soil to *E. coli 8277* and *Ps. 9773* biosensors were shown in Figure 5.4. The inhibition of *E. coli 8277* biosensor by Zn spiked soils was 19.7%, 30.3%, and 66.8% when soil was spiked with 500, 1000, and 2000 mg kg^{-1} Zn, respectively. The toxicity of Cu and Zn mixture to *E. coli 8277* in solid phase of soil was higher than either single metal. Increasing the addition of Zn increased the mixture toxicity. *Ps.9773* biosensor only showed inhibition effect when the soil was spiked with 2000 mg kg^{-1} Zn. Whereas in 500 and 1000 mg kg^{-1} Zn spiked soil, the stimulation effect was shown. The toxicity of Zn and Cu mixture to *Ps.9773* in solid phase of soil was also higher than either single metal, and it increased as increasing Zn addition to soil.

When exposed to soil pore water, both biosensors showed higher inhibition by Cu and Zn mixture than any single metal, and the total toxicity of Cu and Zn mixture was increased as increasing Zn. Unlike in solid phase of soil *Ps.9773* biosensor showed inhibition effect in soil pore water at any Zn treatments.

Comparing the exposure to soil and pore water, *E. coli 8277* biosensor gave the similar inhibition by Cu and Zn mixture, however, *Ps.9773* biosensor clearly showed higher inhibition by soil pore water than soil.

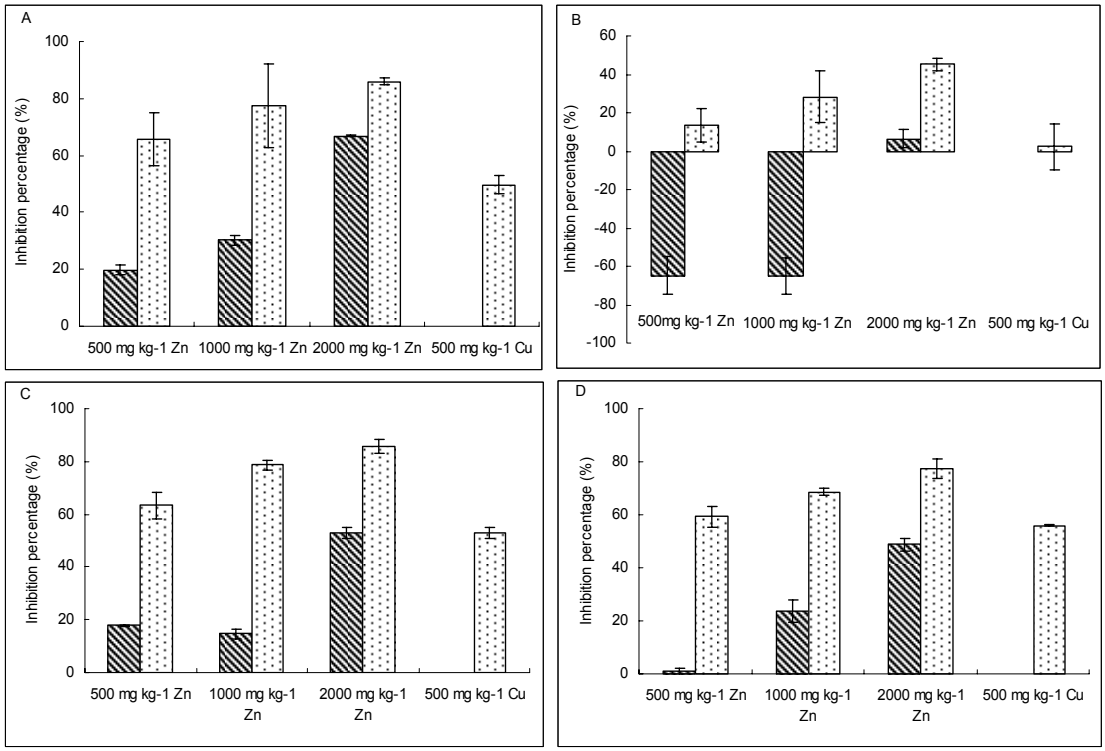


Fig.5.4 The inhibition of biosensors by copper, zinc or zinc plus copper in soil or soil pore water (mean value ± standard error). (A) *E. coli 8277* biosensor response to soil; (B) *Ps. 9773* biosensor response to soil; (C) *E. coli 8277* biosensor response to soil pore water; (D) *Ps. 9773* biosensor response to soil pore water. ▨ : Zn; ⬚ : Zn + 500 mg kg^{-1} Cu. Biosensors were exposed to soil or pore water samples for 3 h.

The properties of soil pore water extracted from corresponding soils were shown in Table 5.2. Zn concentrations of soil pore water extracted from 500, 1000, and 2000 mg kg^{-1} Zn spiked soils were 99.29, 491.29, and 1657.4 mgl^{-1}, respectively. It indicates that soluble Zn was dominating species in soils. Soluble Cu was increased as increasing Zn addition. On the other hand, the addition of 500 mg kg^{-1} Cu also greatly increase Zn concentration in soil pore

water. pH of soil pore water appeared to be decreased by addition Cu or Zn to each other. The increase of Zn addition to soils also increased the desorption of other cations, such as Ca^{2+} and Mg^{2+}.

Table.5.2 The properties of soil pore water extracted from soil samples spiked with different doses of copper, zinc, or copper plus zinc.

Soil treatments	pH	DOC	Ca	Mg	Cu	Zn
				mg kg^{-1}		
Control	6.88	358	51.03	13.14	0.12	0.302
500 mg kg^{-1} Zn	5.17	398	590.32	137.99	0.06	99.29
1000 mg kg^{-1} Zn	5.15	483	1141.9	219.03	0.06	491.29
2000 mg kg^{-1} Zn	4.83	470	1658.2	296.79	0.09	1657.4
500 mg kg^{-1} Cu	5.04	412	620.75	143.1	5.27	8.01
500 mg kg^{-1} Zn + 500 mg kg^{-1} Cu	4.84	386	1117.4	227.31	12.23	295.36
1000 mg kg^{-1} Zn + 500 mg kg^{-1} Cu	4.71	440	1468.9	273.38	21.07	996.07
2000 mg kg^{-1} Zn + 500 mg kg^{-1} Cu	4.52	455	1894.8	327.29	38.41	1834.8

5.3.3 Discussion

Zn demonstrates less affinity to SOM (Stevenson, 1982), therefore, it is much more extractable than copper. In this case, 19.9%, 49.1%, and 82.9% of total Zn were extracted from 500, 1000, 2000 mg kg^{-1} Zn spiked soils. Based on concentrations of metals in pore water, copper increased the extractability of

zinc, and zinc addition increased the extractability of copper as well. Luo and Rimmer (1995) found the increase of zinc by copper, whereas zinc addition hardly affected copper extractability. Posthuma et al. (1997) also reported that copper reduced the sorption of zinc to soil, but copper sorption was inert for zinc addition. The different conclusions might result from different soils, the interactions of metals in soil are to some extent dependent on soil properties, such as SOM, pH, etc. besides, the incubation time of the metal spiked soils could change the extractability of metals (Tye et al., 2004).

The joint effects of Cu and Zn have been investigated by some groups. Luo and Rimmer (1995) reported that copper increased zinc concentrations in the plant *Hordeum vulgare*. Posthuma et al. (1997) also found that zinc uptake of earthworm from soil solution was stimulated by copper, but copper uptake was not stimulated by zinc. Based on those findings, the higher mixture toxicity of Zn and Cu to biosensors in soils maybe mainly contributed by an increase of zinc uptake. Different interaction patterns of metal mixtures were also reported by using different organisms and in different environment. Antagonistic effects of Cu and Zn were found by Kraak et al. (1994) using freshwater mussel tests and Negilski et al. (1981) using marine shrimp tests. Korthals (1997) found Cu/Zn effects were slightly antagonistic for soil nematodes judged by total metal concentrations, but concentration-additive when exposure was expressed by 0.01 M $CaCl_2$-extractable concentrations. In the present study, the interaction of Cu and Zn seemed to be antagonistic in defined solution and soil pore water, and more like concentration-additive in solid phase of soils. Although the conclusion of interaction pattern of metal mixtures can not be drawn directly from the biosensor tests, the mixture toxicities to biosensors can be explained by those interaction patterns.

It is well known that the ecotoxicity of metals can vary over several orders of magnitude depending on the soil characteristics (Lock et al., 2000). Chaperon and Sauvé (2007) reported that the enzyme responses in relation to the total soil metal combinations were synergistic for the agricultural soil and antagonistic for the forest soil. The present study used only one type of soil, different effects might be found for mixture toxicity of metals in different soils.

5.4 Summary

- Mediated amperometric bacterial biosensors were used to assess the single and mixture toxicity of Cu and Pb or Zn in soil *in situ*. The mixture toxicity of copper and lead in soil was higher than either single concentrations of copper or lead to both *E. coli 8277* and *Ps. 9773* biosensors. *E. coli 8277* biosensor showed higher inhibition by Cu and Pb mixture in solid phase of soil than in soil pore water, however, the results were opposite with *Ps. 9773* biosensor. The mixture toxicity of Cu and Zn to both biosensors were higher than either single metal in both solid phase soil and pore water measurement.

- The important advantage of using this biosensor system is that the biosensors are able to rapidly give the information of overall joint effect of metal mixtures in soil, which is also of most ecotoxicological interest. The biosensor results from this study are insufficient to identify the pattern of toxicological joint effects, such as antagonistic, additive, or synergistic. However, it is helpful to understand the mixture toxicities of metals to

biosensors by using those interaction pattern. As many reseachers found, the joint effects of metal mixture are greatly influenced by soil charateristics, it is not always realistic to predict the ecotoxicological risk by using the joint toxic effect models. The biosensor systems are able to be applied to analyse the interactions of metal mixture at various levels (soil, uptake, or inside of organisms), such as between solid phase and liquid phase of soil, desorption from soil and uptake by organisms, since it can be used in solution, and directly in soil as well.

- Mixture toxicity of metals is organism specific, because organisms have special uptake mechanism in soil ecosystem. It is necessary for ecotoxicity assessment to employ multi-species, and selecting sensitive organisms is also important for rapid screening the contaminated soils by metal mixture. In this case, *E. coli 8277* biosensor was more sensitive than *Ps.9773* to both Cu and Zn mixture and Cu and Pb mixture. Mixture toxicity of metals and their interactions at different levels might be different in different type of soil. It would be interesting to study the effect of soil characteristics on the mixture toxicity of metals.

6 Toxicity measurement of historically contaminated soils *in situ* using biosensors

6.1 Introduction

Bioassay determination of metal-contaminated sites is complicated by the effect of a wide range of physico-chemical factors in soils. Broad spectrum toxicity assays provide an indication of the impact of the combined environment on the test organism, that may well be influenced by many chemical agents in the complex soil medium. This, in many applications, has also the advantage of bioassay assessment as it provides information on toxicity and not simply analyte concentration. Alternative approaches include soil extraction linked to chemical analysis, which has been used successfully to determine metal contamination levels. However, this approach is of limited value in determining the bio-availability and potential toxicity of contaminants and the methods used are also complex and costly, and require a high level of operator skills (Conder and Lanno, 2000; Peijnenburg et al., 1999). It is difficult to estimate toxicity purely from concentration levels, especially as many sites are contaminated with more than one metal (Tandy et al., 2005). Although analytical methods could give some bioavailability information by extracting metals with different extractants, it is still very difficult to estimate the levels of toxicity. Metal cations would compete for the uptake sites of target organisms, thus changing the metal bioavailability. *In situ* toxicity testing using invertebrate and plant species are able to provide an assessment of the toxicity of a soil sample, but they are time and space consuming (Aruoja et al., 2004). There is a need for a rapid, low cost bioassay that can be applied to *in situ* screening of contaminated soils.

This study has proved that such mediated cell-based biosensors are suitable for *in situ* monitoring of heavy metals in artificially contaminated soils. They are sensitive to copper, and also gave significant inhibition to soils contaminated by Zn or Pb. The question addressed in this part of the studies was can these biosensors be used to determine toxicity in historically contaminated soils. It has been reported that the bioavailibility of metals in freshly spiked soils significantly decreased with increasing incubation time (Oorts et al., 2006). It was important to know if the biosensors were still sensitive to historically contaminated soils. In order to assess the toxicity of a sample, an appropriate control soil is required, because biosensors response to the test samples has to be compared with those of a control sample, allowing the level of inhibition to be determined. However, it is not always possible to find a representative uncontaminated soil sample from a contaminated area, and the selection of appropriate control soils is an important step in applying biosensor technology to soil testing. The aim of this study was to demonstrate the feasibility of using the mediated amperometric whole cell biosensors, for in situ acute toxicity assessment and screening of metal contamination in soils. Six uncontaminated soil samples and four metal contaminated soil samples were chosen to test the biosensor responses. The effect of uncontaminated soils on biosensor response and the toxicity of contaminated soils to biosensors are discussed in this chapter.

6.2 The characteristics of contaminated and uncontaminated soil samples

The characteristics of 6 uncontaminated (A, B, C, D, E, J) and 4 contaminated (F, G, H, I) soil samples are shown in table 1. All six uncontaminated soil samples were

collected from agricultural fields. They possess different soil texture, pH and organic matter content, which could possibly affect biosensor signals. The total metal concentrations were all lower than the limit values for concentrations of heavy metals in soil (European Council, 1986), although they vary from sample to sample. Three samples (F, G, and I) were collected from soils in the vicinity of Zn smelters, the Zn concentrations were 3064, 3504, and 897 $mg.kg^{-1}$, respectively. They also had high levels of other metals, including Pb, Cu, or Cd. Sample H was a soil from a site close to an arsenic mine, the concentration of As reached 3700 $mg.kg^{-1}$, and Cu contamination level was also very high (958 $mg.kg^{-1}$). The pH of all contaminated soils were lower than uncontaminated soils except for sample C.

Table 6.1. Selected characteristics of uncontaminated and contaminated soil samples

Code	Land use	pH	Total C / %	Total Metals or metalloid mg kg^{-1}				
				Cd	Cu	Pb	Zn	As
A	Agricultural	6.51	15.03	<DL	31	25	95	4
B	Agricultural	8.15	6.66	0.6	11	20	56	<DL
C	Agricultural	5.87	1.65	<DL	17	50	72	3
D	Agricultural	7.1	2.38	<DL	8	32	47	<DL
E	Agricultural	7.55	18.95	<DL	33	25	90	4
F	Zn smelter	5.91	3.79	16.2	79	955	3064	9
G	Zn smelter	4.63	3.58	53.8	285	4358	3504	58
H	Arsenic mine	5.3	4.24	<DL	958	91	120	3700
I	Zn smelter	5.27	2.76	7.4	23	136	897	8
J	Agricultural	6.15	1.34	<DL	25	43	123	<DL

<DL: less than Detection Limit.

6.3 Effect of different uncontaminated soil samples on biosensor signals

To get an appropriate control is often a problem for ecological risk assessment, especially in field-oriented research. Nonaffected areas will always differ from the influenced area. In experimental plots it is extremely difficult to match treated and control plots in all relevant aspects (Eijsackers and Løkke, 1992). The question addressed here was does the use of different uncontaminated soils as controls significantly affect biosensors toxicity assessment?

Both *E. coli 8277* and *Ps.9773* biosensors were employed to test their responses to 6 uncontaminated soils or soil pore water. The results showed that the *E. coli 8277* and *Ps.9773* biosensor responses, both for soil pore water and *in situ*, with soil samples from uncontaminated sites varied from sample to sample (Fig.6.1). The results indicated that a range of naturally occurring physicochemical conditions other than heavy metals can affect biosensor response and possibly the response of biosensor to toxicants. This is consistent with the results of Bhatia et al. (2003).

For an accurate toxicity assessment, the control soil sample used should be as similar as possible to the soil characteristics of the contaminated test sample. Jensen et al. (2006) pointed out that the reference soil has to resemble the contaminated soil in essential parameters such as pH, moisture, nutrient content, and organic matter quantity and quality, and preferably reference sites have to be similar to the contaminated site when it comes to slope, illumination, and land management. Further consideration should be given to the matched sites, pre-

treatment data, regional trends, or (even) toxic-standard treatments as control samples.

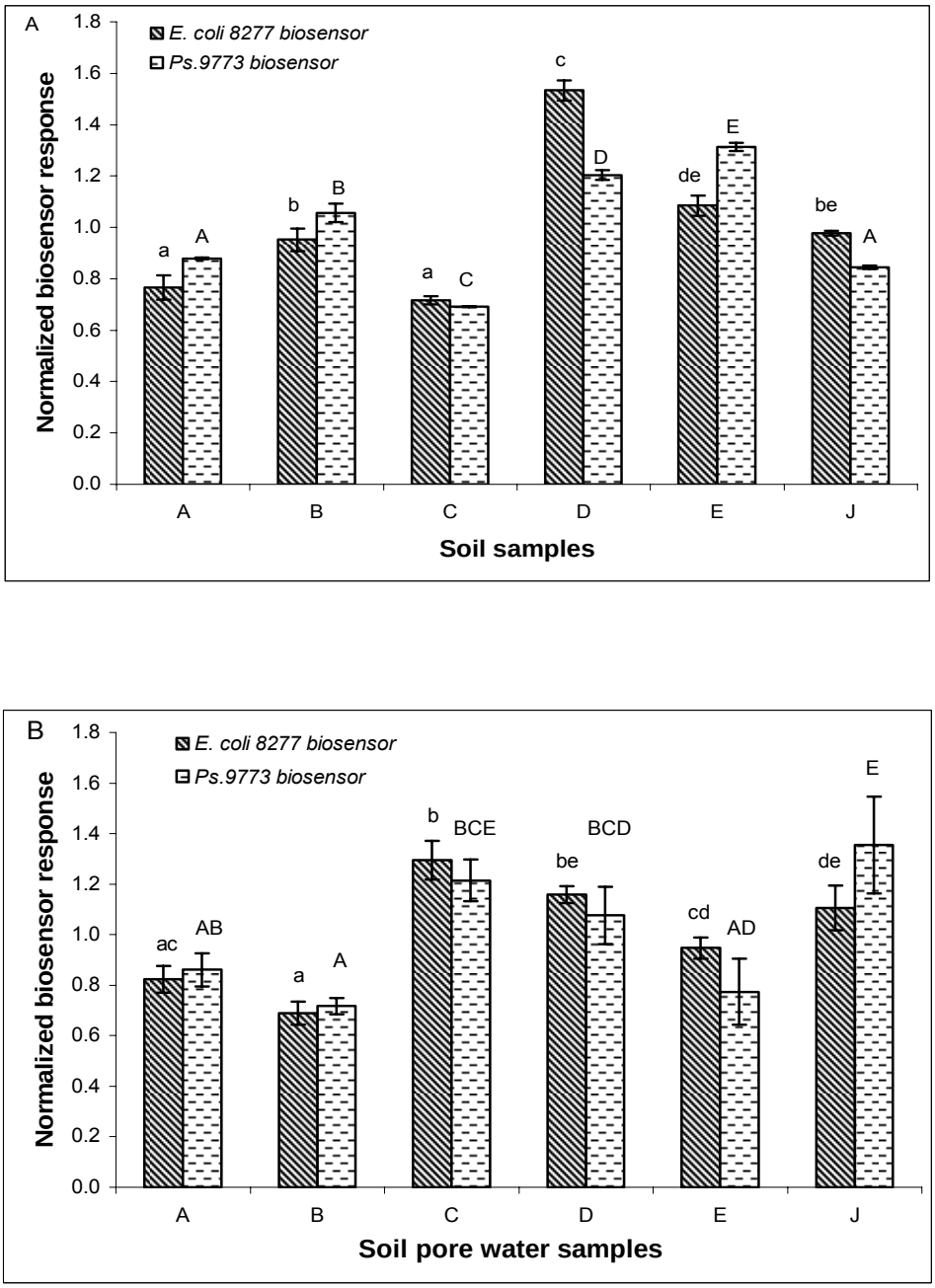


Figure 6.1 Biosensor response to different uncontaminated soils (A) and corresponding soil pore water (B) (average value ± standard error). Biosensor responses were presented as normalized post-exposure responses divided by normalized pre-exposure responses. Different letters above the bars indicate statistical difference at $p < 0.05$, capital letters relate to *Ps.9773* biosensors, lower case to *E. coli 8277* biosensors.

6.4 Toxicity assessment of contaminated soil samples

In this present study, two uncontaminated soils (sample A and D) which had different biosensor responses were chosen as controls, to test whether the inhibition effects of contaminated soils are significant.

E. coli 8277 and *Ps.9773* biosensors were exposed to two uncontaminated (A and D) and four contaminated (F, G, H and I) soil samples or corresponding soil pore water. The results are shown in Fig.6.2. The *E. coli 8277* biosensors showed an inhibition with all four of the contaminated soils and corresponding pore water, irrespective of which of the two uncontaminated soils was used as the control. However, *Ps.9773* biosensors only showed the inhibition with all four contaminated soils when the uncontaminated soil D was used as the control. Stimulation effects were seen with soils F and I when the control was the uncontaminated soil A. It indicated that other soil characteristics could cause a lower biosensor response than toxic metals when the bioavailable metal quantity is relatively low. But, the effect of other soil factors could be minimized by selecting a more appropriate control.

Although the degree of biosensor inhibition varied from sample to sample when different control samples were chosen, the trend of biosensor inhibition with the four contaminated soils was consistent, with the sequence of toxicity G > H > F > I.

The levels of 0.05M EDTA and 0.01 M $CaCl_2$ extractable metals, and metals in soil pore water all reflect bioavailable metal fractions to some extent. Heavy metal bioavailability results from the three extraction methods used in this study are given

in Tables 6.2 and 6.3. Sample G gave high levels of 0.05M EDTA extractable Cu, Zn, Pb, Cd, and also 0.01 M $CaCl_2$ extractable Zn, Cd. Sample H gave high levels of 0.05M EDTA extractable Cu and 0.01 M $CaCl_2$ extractable Zn. In soil pore water, sample G had high concentrations of Cu, Zn, Pb and Cd, and sample H gave the highest concentration of Cu, in comparison with sample F and I. These extractable metal concentrations are helpful in explaining why the toxicities of sample G and H were higher than sample F and I, however, metal concentrations based on extraction methods do not explain biological effects. Besides, the results also showed that the concentration values of heavy metals vary widely depending on the extraction method used.

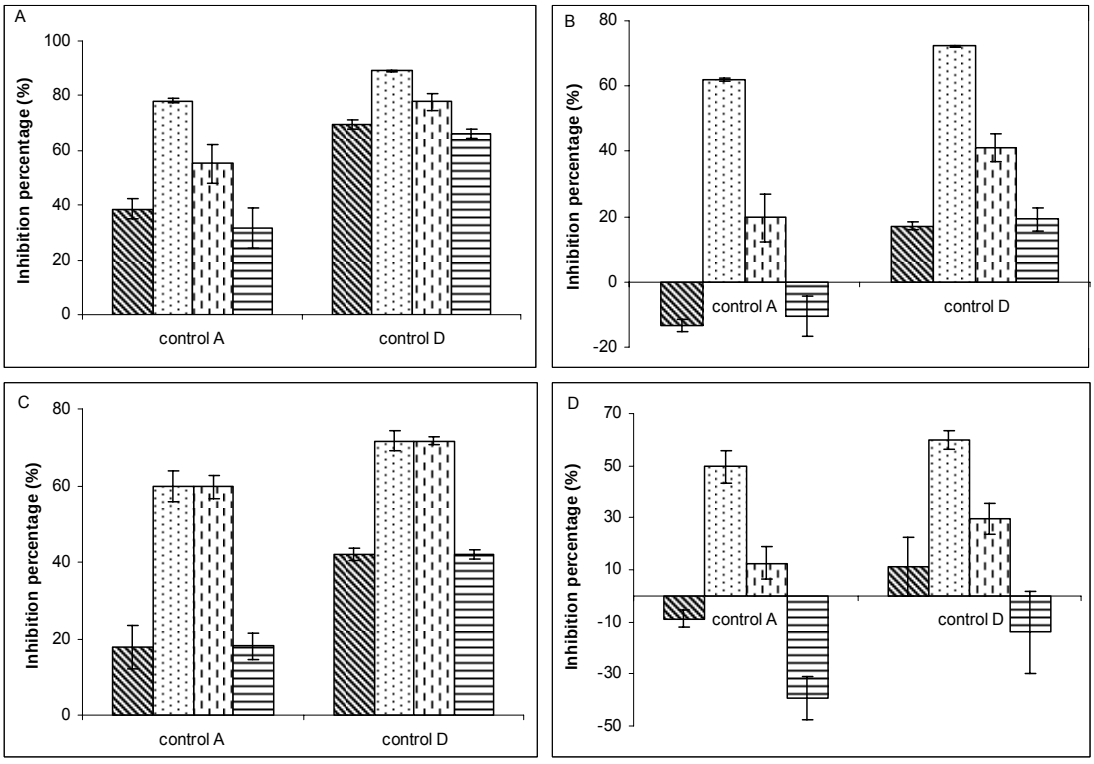


Figure 6.2. Inhibition of biosensors by contaminated soil samples or soil pore water (average value ± standard error). A: *E. coli 8277* biosensor response to soil samples; B: *Ps.9773* biosensor response to soil samples; C: *E. coli 8277* biosensor response to soil pore water; D: *Ps.9773* biosensor response to soil pore water. ▨ : sample F; ⬚ : sample G; ⊞ : sample H; ▤ : sample I. Control A and control D refer to soil sample A and D used as control.

Metal bioavailability and toxicity is not only strongly influenced by soil properties, but also by the complex interactions of metals in the soil ecosystem. Calamari & Alabaster (1980) described three levels of metal interactions in soil: (1) the substrate level, (2) the uptake level and (3) the target level. The first level refers to competitive sorption interactions, resulting in a specific distribution of the interacting metals over the solid and liquid phases of the soil. This process alters the bioavailable fraction of

metals. The second and third level describe interactions of metals during uptake and interactive effects at target sites within an organism, respectively. In this present study, both biosensor configurations gave higher inhibition when exposed to the solid phase of soil than to the soil pore water, which indicates that soil pore water is not able to completely reflect the real interactions of metals in the soil ecosystem. A terrestrial biotic ligand model (TBLM) was developed to assess the toxicity of metals in soil (Thakali et al., 2006), which considered the competition and interactions related to the desorption of metals from the soil solid phase to the solution phase or the transport of metal ions to organism cell surfaces. To apply this model, soil properties need to be well known, any soil extraction linked to chemical analysis alone is insufficient to predict the biological response (Zhao et al., 2006). Assessment of the bioavailability of metals requires the exposure of test organisms to the environment under study.

Different organisms possess different sensitivities and tolerances to metals, which makes the toxicity assessment more complicated when soils are contaminated with more than one metal. In this study, soil samples F and I have different levels of metal concentrations with different metals. Sample I gave slightly greater toxicity than sample F to *Ps.9773* biosensor, at the same time, the converse result was shown with *E. coli 8277* biosensor. The probable explanation is the two bacteria strains have different sensitivities to different metals and different uptake mechanisms. As shown in Chapter 3, 4, and 5, two biosensors have different sensitivities to Cu, Zn, and Pb, and give different inhibition by Cu and Zn or Cu and Pb mixture. It also demonstrated that toxicity of contaminated soils is species-specific. Therefore, ecologically relevant species is suggested for the ecotoxicity risk assessment.

These results also showed that the *E. coli 8277* biosensor configuration was more sensitive than the *Ps.9773* biosensor configuration when exposed in soils directly or in soil pore water. Therefore, *E. coli 8277* biosensor is a better option for screening metal contaminated soils.

Table 6.2. Extractable metal concentrations (mg l^{-1}) of soil samples

Sample code	Cu		Zn		Pb		Cd	
	0.01M $CaCl_2$	0.05M EDTA	0.01M $CaCl_2$	0.05M EDTA	0.01M $CaCl_2$	0.05M EDTA	0.01M $CaCl_2$	0.05M EDTA
A	0.03	0.25	0.92	0.35	0.02	0.17	0.002	0.01
B	0.02	0.06	0.05	0.10	0.13	0.07	0.002	0.01
C	0.01	0.10	0.07	0.06	0.02	0.32	0.003	0.01
D	0.03	0.27	0.14	0.33	<DL	0.80	0.004	0.02
E	0.01	0.21	0.02	0.41	<DL	0.18	0.002	0.01
F	0.01	1.93	0.06	63.32	0.01	27.93	0.493	0.57
G	0.03	6.58	51.05	57.04	0.19	142.06	1.89	1.36
H	0.87	15.76	71.97	0.62	7.32	0.50	0.01	0.003
I	1.37	0.41	0.92	27.45	<DL	3.16	0.13	0.25
J	0.01	0.63	21.57	2.35	0.05	0.86	0.02	0.05

<DL: less than Detection Limit.

Table 6.3. Selected characteristics of soil pore water

Sample code	pH	DOC	Ca	Mg	Cu	Zn	Pb	Cd
		mg l^{-1}						
A	7.17	430	365.34	20.81	0.02	0.09	<DL	<DL
B	7.84	472.5	285.71	4.27	0.03	0.01	<DL	<DL
C	6.5	607.5	120.38	39.20	0.02	0.03	0.01	<DL
D	7.58	337.5	216.63	16.36	0.03	0.01	<DL	<DL
E	7.79	625	449.43	18.41	0.02	0.01	<DL	<DL
F	6.14	29	91.70	12.30	0.06	47.34	0.1	0.26
G	4.39	246.5	123.37	24.70	1.37	111.41	2.26	2.12
H	4.99	542.5	75.01	14.65	3.63	0.6	0.01	ND
I	5.25	255	181.81	93.53	0.07	45.82	0.03	0.14
J	6.45	515	102.23	19.29	0.15	0.54	<DL	<DL

<DL: less than Detection Limit.

6.5 Conclusions

- Mediated amperometric bacterial biosensors were effective in assessing the bioavailability and toxicity of heavy metals in contaminated soil samples. Especially when soils were contaminated with mixed metals, this biosensor test is able to give a clear answer about the total toxicity level.

- For accurate toxicity assessment, an appropriate reference soil sample is required as control. Although it is often difficult to find a reference soil which has exactly same characteristics as contaminated soils have, the variation caused by the difference of soil characteristics between control and test soils

could be minimized by the knowledge of individuals who carry out the soil survey.

- The biosensor tests can be performed *in situ* on soil samples and soil pore water, enabling the screening of sites for contamination ‘hot spots’, and the evaluation of soil degradation or remediation from metal pollution.

7 Conclusions

7.1 Reiteration of aims

Assessing and monitoring the toxicities of heavy metal contaminated soils is one of the key steps in preventing ecological risks, rationally utilizing and efficiently restoring such soils. Standard analytical chemical techniques are able to precisely determine the total and specific species of metals, however, they can not give accurate indications of toxicities of heavy metal contaminated soils due to the `combined effects of the soil properties, organisms and metal characteristics on bioavailability and toxicity of metals. Bioassays are able to measure and interpret the toxicity and bioavailability of metals in soils. A varieties of bioassays, including invertebrate, plant, enzyme, soil microorganism tests have been developed for this purpose. For large-scale soil pollution investigation or risk assessment, however, those methods are usually time consuming and expensive. A number of whole-cell based biosensors have been developed for detecting the toxicity and bioavailability of heavy metals in soils, which are reported as rapid and sensitive. However, they can only be used in soil extractions, or soil suspensions rather than the solid phase of soils. It is known that the toxicity and bioavailability of heavy metals in soils can be changed by extractants or extract methods. Therefore, there is a need to develop rapid bioassays for *in situ* monitoring and assessing the toxicities of soils contaminated by heavy metals.

The mediated amperometric whole-cell biosensor has the advantages of ease of use, low cost, rapidity, and potential applications in soil monitoring. However, the existing protocol of Cellsense ^{TM} was not suitable for direct application in soils.

The main aim of the project was to develop a suitable monitoring protocol of mediated amperometric whole-cell biosensors for *in situ* assessment of heavy metal toxicities in soils. *E. coli 8277* and three *Pseudomonas* strains were screened as possible biosensor in biosensor configurations. A new protocol was developed for monitoring heavy metals in defined solution, soil pore water, and *in situ* in soil. The present study also validated the applications of mediated amperometric bacterial biosensors for *in situ* assessing the bioavailability and toxicity of heavy metals in freshly spiked soils or historically contaminated soils, and mixture toxicity of heavy metals.

7.2 Review of the main findings

7.2.1 Optimization of mediated amperometric whole-cell biosensor protocol for assessing the toxicity of heavy metals

The investigation was carried out to find optimal conditions for harvesting cells to construct biosensors. Bacterial cells harvested and immobilized after 16h incubation were found to give strong and stable responses.

Mediated amperometric whole-cell biosensor measurement of metal toxicities required the protocol to ensure the mediator redox couples would not be affected by metal cations. An electrochemical assay was employed to detect the possible interaction between metal cations and the chosen mediator pBQ. It was found that the pBQ redox couples were affected by Cu, but not by Zn or Pb. Therefore, protocol A which exposes biosensors to toxicant and bathing medium together was

not suitable for monitoring copper. A new protocol (protocol B) was developed and validated for copper toxicity measurement, and could be generally used for any other metals. The new protocol makes the toxicity measurement of heavy metals in soil *in situ* possible, due to the exposure of biosensors to test samples being separated from monitoring by the Cellsense^{TM} instrument. The disposable biosensors can be conveniently placed into solid phase of test samples directly.

This study also compared the sensitivities of mediated amperometric biosensors with 4 different bacterial strains to Cu, Zn, Pb, and Hg in defined solutions, respectively. *Ps.9046* was found to be the most sensitive to lead, *Ps.9773* the most sensitive to Hg, and *E. coli 8277* was found to be the most sensitive to Zn and Cu. In comparison with the Microtox assay, the mediated amperometric bacterial biosensors had a low sensitivity to Zn, Pb, or Hg, but were appropriately sensitive to Cu. EC_{50} values of Cu were estimated to be 1.44 ppm for *E. coli 8277* and 1.73 ppm for *Ps.9773* biosensors.

7.2.2 Toxicity and bioavailability assessment of copper in soil in situ using mediated amperometric bacterial biosensor

The new protocol allows the mediated amperometric bacterial biosensor system to be applied to *in situ* assessment of bioavailability and toxicity of copper in soil. The biosensors are able to be exposed directly to solid phase of soil and soil pore water. Both *E. coli 8277* and *Ps.9773* biosensors showed different inhibition effects to solid phase or soil pore water. The significance of using biosensors for *in situ* toxicity assessment was addressed. In copper spiked loamy sand soil, EC_{50} values were estimated to occur at copper concentrations of 481 ppm with *E.coli 8277* and 890

ppm with *Ps.9773*; in corresponding pore water, EC_{50} values were estimated to be 8.38 ppm for *E.coli 8277* and 9.92 ppm for *Ps.9773* biosensor. This is considered to be appropriately sensitive to copper in soil compared to other bioassays (Thakali et al., 2006), and is relatively rapid taking less than 4 hrs.

Soil properties greatly influence the toxicity and bioavailability of copper to biosensors. High SOM and clay content strongly decreased the bioavailability and toxicity of copper in soils. When biosensors were exposed to different types of soils and corresponding pore water at the same concentrations of total Cu, the toxicity of loamy sand soil was much higher than clay loam soil, and the organic soil did not show any toxic effect in the range of 200 to 1000 ppm Cu.

pH and soil moisture content were also found to affect the toxicity and bioavailability of copper to biosensors. Both *E. coli 8277* and *Ps.9773* biosensors showed increased inhibition effect with decreasing pH of saline solution. The amendment of soil with Cu decreased pH of soil pore water, therefore the biosensor response to pore water resulted from combined effect of Cu toxicity and pH. When soil pore water samples were adjusted to the same pH values (6.8 or 4.5), their toxicity to biosensors were lower than the corresponding pore water without pH adjustment. This indicates that pure copper toxicity to biosensors is lower than the combined effect of Cu and pH. The effect of soil moisture content on toxicity and bioavailability of copper to biosensors was studied. It was found that increasing soil moisture content from 50% WHC to 100% WHC increased the toxicity of copper.to *E. coli 8277* biosensors. Based on those investigation, soil conditions need to be well defined for the application of mediated amperometric bacterial biosensors in toxicity assessment of heavy metals in soils.

Mediated amperometric bacterial biosensors are not analyte specific, their response is affected by other soil components. The broad spectrum nature of these biosensors can be an advantage in assessing the overall toxicity of copper contaminated soils, or complex polluted fields. This study also showed that the bioavailability and toxicity of metals is organism-specific. *E. coli 8277* and *Ps.9773* biosensors responsed differently to solid phase of soils and pore water. In loamy sand soil, *E. coli 8277* biosensors showed higher inhibition effect in solid phase of soils than pore water, however, *Ps.9773* biosensors gave opposite results.

7.2.3 Toxicity assessment of metal mixtures using biosensors

Contaminated soils contain mixtures of chemicals. These chemicals interact with each other in soil ecosystem in different physical-chemical and biological processes, including competing for sorption sites on soil particles, surface of organism cells and inside of cells. The mixture toxicity reflects the overall effect of the interactions of metals on organisms. The mediated amperometric bacterial biosensor assay is able to determine and interpret the acute mixture toxicity. *E. coli 8277* and *Ps.9773* biosensors were used to assess the toxicities of single and mixed Cu with Pb or Zn spiked soils and solution.

The toxicity of Cu and Pb mixture in defined solution appeared to be an antagonistic effect in the response of both biosensors. In soil systems, heavy metals compete with each other for sorption sites, resulting in the redistribution of metal fractions between soil particles and solution. The addition of Pb increased Cu in pore water, and Cu addition increased Pb in pore water. The same phenomenon was observed in soils spiked with Cu and Zn. The mixture toxicity of Cu and Pb to both biosensors

in solid phase of soil or pore water was higher than either single metal due to the external bioavailability of both metals being increased. The patterns of biosensor response to Cu and Pb mixture appeared to be additive in solid phase of soil samples, but antagonistic in soil pore water. Pb did not increase the mixture toxicity of Cu and Pb in defined solution, however it did *in situ* in soil or pore water, which may indicate that Pb does not affect the uptake of Cu by bacteria, but it does affect the desorption of Cu from soil particles to solution.

In defined solution with Cu and Zn, the mixture toxicity was found to be higher than either single metal to *E. coli 8277* biosensors, but lower than single metal to *Ps.9773* biosensors. *Ps.9773* biosensor test showed a marked antagonistic effect with Cu and Zn. Both biosensors showed higher inhibition by Cu and Zn mixture than either metal exposed to either solid phase of soil or pore water. The interactrion of Cu and Zn seemed to be antagonistic in defined solution and soil pore water, but more like concentration-additive in solid phase of soils. The possible explanation is that Zn uptake by bacteria was actually increased because Cu increased the bioavailability of Zn in soils.

The biosensor results from this study are insufficient to precisely identity the pattern of joint toxic effects of metals. The remarkable advantage of using this biosensor system is that the biosensors are able to rapidly detect the overall toxicity of metal mixtures in soil, which is also of most ecotoxicological interest. The joint effects of metal mixtures are greatly influenced by soil characteristics, it is always difficult to predict the ecotoxicological risk of soils contaminated with mixed metals even if there were appropriate joint toxic effect models.

Mixture toxicity of metals is organism specific, because organisms have their special uptake mechanisms for different metals. *E. coli 8277* biosensors were found to be more sensitive than *Ps.9773* biosensors to both Cu and Zn mixtures or Cu and Pb mixtures.

7.2.4 *In situ* toxicity measurement of historically contaminated soils using biosensors

Unlike freshly spiked soil samples in the laboratory, the characteristics of historically contaminated soil samples could vary even if they were collected from very close sites. In addition, they are likely to contain metal mixtures and other unknown chemicals. When using biosensors to measure toxicities of contaminated soils, an appropriate control soil sample is needed. This study tested the effect of six uncontaminated soils on biosensor responses. It was found that the *E. coli 8277* and *Ps.9773* biosensor responses to soil pore water or solid phase of soils varied from sample to sample. The natural physicochemical conditions other than heavy metals could affect biosensor response and possibly the response of biosensor to toxicants. For an accurate toxicity assessment, the control soil sample used should be as similar as possible to the soil characteristics of the test contaminated sample.

These biosensors could be used for comparing the toxicity of contaminated soils under certain circumstances. Two uncontaminated soils (A and D) were chosen as controls to assess the relative toxicity of 4 metal contaminated soils. *E. coli 8277* biosensors showed an inhibition effect with all four of the contaminated soils and corresponding pore water, irrespective of which of the uncontaminated soils used as control. *Ps.9773* biosensors also showed inhibition effect with all four contaminated

soils when uncontaminated soil D was used as control. Stimulation effects were observed with *Ps.9773* biosensors with soils F and I when soil A was the control. Other soil properties could possibly cause a lower biosensor response than toxic metals. The relative toxicity of contaminated soils was consistent when different uncontaminated soils were used as control, with the sequence of toxicity G>H>F>I.

The results of biosensor toxicity tests can be explained by measuring extractable metals since extractable metals reflects bioavailable metal fractions to some extent. The levels of 0.05M EDTA and 0.01 M $CaCl_2$ extractable metals, and metals in soil pore water were determined. Soil G gave high levels of extractable Cu, Zn, Pb, and Cd, and soil H gave the highest concentration of extractable Cu, which may explain the toxicity sequence of 4 contaminated soils. The drawbacks of using analytical chemical technique addressed here are that extractable metal concentrations do not directly give toxicity information, and the results from different extraction methods varied largely from sample to sample. The biosensor test is able to give a clear answer about how toxic the soil samples are to the test organism, and which samples pose the higher risk.

It was also found that both *E. coli 8277* and *Ps.9773* biosensors gave higher inhibition when exposed *in situ* in soils than in soil pore water. *E. coli 8277* was found to be more sensitive to metal contaminated soils than *Ps.9773*.

7.3 Conclusions

This study developed a new protocol of mediated amperometric bacterial biosensors which is suitable for monitoring heavy metals in defined solution, soil pore water,

and *in situ* in soil. The biosensors incorporating selected bacterial strains were appropriately sensitive to copper, but less sensitive to other metals, compared to Microtox. The mediated amperometric bacterial biosensor system may have not the degree of sensitivity to detect a toxic substance at its maximum permissible concentration for protection of the environment, but it can produce rapid assessments, and is a convenient, easy bioassay to use.

The remarkable advantage of the mediated amperometric bacterial biosensor system is its *in situ* application in soils. It has been recognized that soil pore water or other soil suspension can not accurately reflect soil conditions due to their separation from the entire steady-state process of soil ecosystem or changing soil structure, *in situ* bioassays are more reliable for determining the bioavailability and toxicity of heavy metals. In assessing the environmental risk caused by heavy metals, it is important to use the relevant organisms in the toxicity tests. Soil bacteria are a good choice in measuring the effects of toxicants in soils and sediments. *Pseudomonas spp* is an important group of soil microorganisms which play an critical role in soil ecosystem. In this present study, *Pseudomonas* biosensors have been used *in situ* toxicity measurement in soils. Although they were not remarkably sensitive, there is the potential to develop soil relevant biosensors with higher sensitivity. The mediated amperometric bacterial biosensor protocol described here can be adapted to allow the incorporation of different bacterial biocatalysts for other applications in soil quality assessment, screening of sites for contamination 'hot spots', and the evaluation of soil degradation or rehabilitation from metal pollution.

For assessing the toxicity of soil samples from fields, it is vital to get an appropriate control or reference soil sample. Although it is often difficult to find a reference soil which has exactly same characteristics as contaminated soils have, the variation

caused by the difference of soil characteristics between control and test soils could be minimized by the knowledge of individuals who carry out the soil survey.

The conditions of soil samples need to be well defined for using this biosensor system. For example, soil moisture content can change the bioavailability and toxicity of heavy metals. 75% WHC is recommended in this present study since it maintains the natural soil processes.

7.4 Future work

This study raises a range of areas for further reseach.

The sensitivity of mediated amperometric biosensors needs to be improved before their widespread use. One approach would be by selecting more sensitive microorganism species as biocatalysts. Algae have been reported to be the most sensitive towards heavy metals (Vig et al., 2003), and they have been incorporated into this mediated amperometric biosensors for the use of environment monitoring (Pandard and Rawson, 1993). It would be interesting to investigate the sensitivity of algae biosensors to heavy metals. Considering the time limit, only 4 bacterial strains were selected as biosensor biocatalysts in this present study. It would be certainly helpful to improve the sensitivity of this biosensor system if more bacterial strains were tested. Genetically recombinant organisms could largely improve the sensitivity and specificity of biosensors. Microorganisms generally have their specific resistance mechanisms against toxic metals, such as hampering uptake, increasing efflux, or forming complexation in cells, which influence biosensor detection.

Organisms whose resistance mechanisms has been disrupted would be more sensitive. ZntA is one of the powerful transporters which transports Zn out of the cell membrane. A biosensor in a ZntA disrupted strain was found to be more sensitive (Rensing et al., 1998). Genetically constructing sensitive strains for biosensor biocatalytes would be a promising approach to improve the sensitivity of biosensors, which requires an understanding the genetic background of the selected microorganisms.

This project investigated the biosensor response to copper, zinc, lead, and mercury. Further testing is required to determine how biosensors respond to other metals or metalloids, such as cadmium, arsenic. Since polluted soils usually contain several metals, knowing biosensor response to individual metal will help in assessing and interpreting the toxicity and the resulting risk of complex contamination. The mediated amperometric biosensors has been reported to be appropriately sensitive to some organic toxicants such as 3,5-DCP, Chlorophenols, Synprolan, Polyhexanide, Proxel paste, Atrazine, and industrial effluents (Rawson and Willmer, 1989; Gaisford et al., 1991; Rogerson, 1997), therefore, it would be interesting to test the combined effects of heavy metals and organic toxicants on microorganisms by using these biosensors. For field application, investigations are also needed to determine how the natural conditions affect the use of the biosensor system. Unlike in the laboratory with controlled conditions, biosensors would be exposed to widely varying conditions.

8. References

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Appendix: Publications

The following co-authored publications were produced during the course of the research project.

1. Ding.Y., Zhang, T., Rawson, D., McGrath, S. P., 2007. Mediated amperometric whole-cell biosensors for assessment of bioavailability and toxicity of copper in soil. Book of abstracts II International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2007). Seville, Spain.

2. Ding, Y., Zhang, T., Rawson, D., 2008. Mediated amperometric whole-cell biosensors for rapid in situ screening of heavy metal contaminated soils. In: Luo, Y.M., Japenga, J., McGrath, S.P., Zhao, F.J., Newman, L., Vaněk, T., Wong, M.H., Doelman, P., Mench, M., Takagi, K. (eds) Proceedings of SoilRem 2008, Nanjing, China.