CRANFIELD UNIVERSITY

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Bacteria in Heavy Metal Contaminated Soil: Diversity, Tolerance and Use in Remediation Systems

CRANFIELD HEALTH

PhD Thesis

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Supervisors:

Prof. Naresh Magan and Prof. Paula Castro

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Abstract

The objectives of this project were to determine the bacterial diversity in a heavily contaminated metal region of Portugal. Both traditional and molecular based methods were used to identify tolerant strains and species. The most tolerant species were subsequently identified and utilized for examining the potential for using them to immobilize specific metals from contaminated waste streams by comparing different support materials.

Heterotrophic bacterial populations were isolated and characterized from a contaminated industrial area in Northern Portugal. In a first sampling, 278 strains were isolated in different solid media. To assess the diversity of this ecological site and to select representative strains, the isolates were screened by using Random Amplified Polymorphic DNA (RAPD)-PCR profiles. Phenotypic characterization, phylogenetic analysis by sequencing the 16S rRNA genes and metal tolerance tests with zinc (Zn), cadmium (Cd) and arsenic (As) were performed with the selected strains. Recovered gram-positive isolates were related to class *Actinobacteria* and *Bacilli*. The majority of the isolates were related to genera *Microbacterium* and *Bacillus*. Strains from the genus *Arthrobacter* were also well represented. 16S rRNA gene sequence similarity of the gram-negative isolates showed that they were related to classes γ-*Proteobacteria*, β-*Proteobacteria*, α-*Proteobacteria* and *Flavobacteria*. The most frequently isolated taxa were γ-*Proteobacteria*, related with the genus *Pseudomonas*, where a large number of isolates were clustered. These genera are common in metal contaminated environments. Many of the strains (approx. 17) had a high level of tolerance to the heavy metals tested. A total of 13 isolates were not able to grow when metals were present. In a second sampling the soil rhizosphere was screened for bacterial populations, using metal-based selective media for isolation. About 42 strains were recovered when metal supplemented media was used. The gram-positive population were predominantly *Bacilli* and *Actinobacteria* members. *Bacillus*, *Microbacterium* and *Arthrobacter* were the most common gram-positive genera. Gram-negative genera were from the same classes as in the first sampling however *Sphingobacteria* was present. γ-*Proteobacteria* and *β-Proteobacteria* were the most common taxa. The isolates were shown to be very resistant to Zn and As, with about half of the isolates able to grow with Cd present. Interestingly, no strains

could grow in the presence of metal mixtures. Despite the number of strains recovered in both samplings the majority of the isolates were clustered within a very small number of genera.

During the sampling periods two strains showing low similarity to other bacteria were isolated. These strains were characterized and studied in detail justifying their classification as representing two novel species of the genus *Chryseobacterium*. The names proposed for these organisms are *Chryseobacterium palustre* sp. nov. (type strain 3A10^{T}) and *Chryseobacterium humi* sp. nov. (type strain ECP37^{T}).

Three isolates 1C2, 1ZP4 and EC30 belonging to genera *Cupriavidus*, *Sphingobacterium* and *Alcaligenes* respectively, showing high tolerance to heavy metals, were selected for further study in immobilised systems for Zn and Cd removal. In most cases, matrices (alginate, pectate and a synthetic cross-linked polymer) with immobilised bacteria showed better metal removal. 1C2, a strain belonging to the *Cupriavidus* genera, was able to increased the removal of Zn; EC30, a bacteria related to *Alcaligenes*, was the most promising candidate for Cd removal, especially when combined with the synthetic polymer. Removal of metals as single or in binary mixtures was also assessed. Cd removal was most effective when single metal solutions were tested using immobilised bacteria and examining metal matrixes. Based on the strains used and the matrices tested, best results were obtained for removal of Zn from binary mixtures with Cd. Potential exists for further studies to exploit these bacterial strain to develop effective bioremediation approaches for the removal of heavy metals from waste water streams.

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Bacteria in Heavy Metal Contaminated Soil: Diversity, Tolerance & Use in Remediation Systems

Table of contents

Nomenclature

A = angstrom
API = analytical profile index
As = arsenic
As_2O_3 = arsenic trioxide
As_2O_5 = arsenic pentoxide
ATP = adenosine triphosphate
BLAST = Basic Local Alignment Search Tool
bp = base pairs
ca = circa
Cd = cadmium
$CdCl_2$ = cadmium chloride
CFU = colony forming units
cm = centimetres
DNA = deoxyribonucleic acid
DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen
EU = European Union
g = grams
G+C = guanine cytosine ratio
g cm^{-3} = grams per cubic centimetre
h = hours
ha = hectares
Hg = mercury
H_2O_2 = hydrogen peroxide
HPLC = high performance liquid chromatography
IARC = international Agency for Research on Cancer
Kg = kilograms
KH_2AsO_4 = potassium hydrogen arsenate
LMG = Laboratorium voor Microbiologie
m = meters
M = molar
mg/g = milligrams per gram
mg l^{-1} = milligrams per liter
min = minutes
ml = milliliters
mg/Kg = milligrams per kilogram
mol l^{-1} = mole per liter
mM = milimolar
mm = millimeter
NCBI = National Center for Biotechnology Information
NBRC = NITE Biological Research Center
nm = nanometers
OD = optical density
PAUP = phylogenetic analysis using parsimony
Pb = lead
PCR = polymerase Chain reaction
ppb = parts per billion
ppm = parts per million

PVC = polyvinyl chloride
R&D = research and development
RAPD-PCR = random amplified polymorphic DNA - PCR
RNA = ribonucleic acid
SH = sulfhydryl
t = tones
^{T} = type strain
UV = ultra-violet
vol/vol = volume per volume
w/v = weight per volume
Zn = zinc
$ZnCl_2$ = zinc chloride
% = percent
µm = micrometre
µl = microlitres
µM = micromolar
ºC = degree Celsius

List of Tables

List of Figures

Chapter 1: Literature Review and Objectives

1.1 Heavy metals as soil pollutants

Heavy metals represent a great environmental concern, because of their widespread use and distribution, and particularly their toxicity to human beings and the biosphere. However, they also include some elements that are essential for living organisms at low concentrations (Alloway, 1990). These elements are usually transition metals. They have high densities (>5 g cm^{-3}) when compared with other materials (Baird and Cann, 2005), (Table 1.1). Heavy metals include essential elements such as Iron (Fe) and Zinc (Zn) as well as toxic metals like Cadmium (Cd), and Mercury (Hg).

Table 1.1 Densities of some heavy metals and other substances (adapted from Baird and Cann, 2005).

Substance	**Density (g/cm^3)**
Mercury (Hg)	**13.5**
Lead (Pb)	**11.3**
Zinc (Zn)	**9.16**
Cadmium (Cd)	**8.7**
Arsenic (As)	**5.8**
Aluminium (Al)	**2.7**
Magnesium (Mg)	**1.7**
Water (H_2O)	**1.0**

Heavy metals have an adverse effect on human physiology and other biological systems (Bailey *et al.*, 1999, Kobya *et al.*, 2005). They show a great affinity for other elements such as sulphur disrupting enzyme functions in living cells by forming bonds with this group. Cadmium, lead and mercury ions have the ability to bind to cell membranes, interfering with the cell transport processes (Bailey *et al.*, 1999; Manahan, 2004). Heavy metals may also stimulate the formation of free radicals and reactive oxygen species, which may lead cells into oxidative stress (Dietz *et al*., 1999). They are non-biodegradable and tend to accumulate in the tissues of living organisms, a process called bioconcentration (Bailey *et al.*, 1999; Baird and Cann, 2005; Kobya *et al*., 2005).

Metal speciation influences toxicity of a given metal. This is related to the chemical form or species of the element, i.e. speciation. Speciation depends on physical and chemical characteristics of the soil and waterways (Mulligan *et al*., 2001, Baird and Cann, 2005). Water and soil pH, redox potential, organic, carbonate, and oxide contents influence metal speciation and mobility (Mulligan *et al*., 2001), because interactions such as complexation and adsorption may remove some ions from biological activity (Baird and Cann, 2005). Simple and complex cations are more mobile, while chelated cations are slightly mobile. The metals become mobile after oxidation or weathering. Kabata-Pendias (1992) has shown that the speciation of zinc and cadmium are organically bounded, exchangeable and water soluble.

Soil is a complex mixture of mineral (e.g. clay) and organic (e.g. humic substances), aqueous and gaseous components. It is a dynamic system with variations in moisture content, pH and redox potential conditions. These properties interfere with the form and availability of metals (Alloway, 1990). Soil and heavy metal

interactions can be understood on the basis of ion exchange, surface adsorption and/or chelation reactions. Humic substances have the ability to form complexes with heavy metals due to their functional groups. Heavy metal retention by soil also depends on ionic strength, pH, type of clay minerals present, type of functional groups and competing cations (Alloway, 1990; Evangelou, 1998). Metal absorption increases with increasing pH. Heavy metals particularly are most mobile under acid conditions and increasing the pH by liming usually reduces their bioavailability (Alloway, 1990). The adsorption of ions by soils has been quantitavely described by using Langmuir and Freundlich adsorption isotherms (Alloway, 1990). The basic assumptions of this model are that adsorption takes place at specific homogeneous sites within the sorbent and once a site is occupied by a metal ion no further sorption is possible (Alloway, 1990; Ayyappan *et al*., 2005).

1.1.1 Cadmium

Cadmium (Cd) is a member of group II-B of the periodic table and is a relatively rare metal (Alloway, 1990) which makes it uncommon in most "natural" soils and waters (Evangelou, 1998). The average content of Cd in soil is less than 1 ppm, with the normal range 0.005-0.02 ppm in plants (Mulligan *et al*., 2001). It is very similar to zinc, undergoing joint geochemical processes, and its oxidation state is (like zinc) +2 (Manahan, 2004). Cd is rendered as a by-product of the mining and smelting of lead and zinc (Alloway, 1990; Evangelou, 1998; Baird and Cann, 2005). The production of this metal has increased rapidly in the last few decades from 11000 t in 1960 to 19000 t in 1985 (Alloway, 1990). This heavy metal is used in semiconductors, nickel-cadmium batteries, electroplating, PVC manufacturing, various alloys, pigments and control rods for nuclear reactors (Alloway, 1990;

Evangelou, 1998). Soil and water contamination by Cd originates from the mining and smelting industries, atmospheric pollution, sewage sludge application and burning of fossil fuels (Alloway, 1990; Evangelou, 1998).

Cd has no essential biological function and is thus highly toxic to living organisms. Chronic exposure to cadmium in humans has several toxic effects, such as high blood pressure, kidney, lung, liver and testes damage (Alloway, 1990; Evangelou, 1998; Manahan, 2004; Baird and Cann, 2005). Cd is also associated with a disease called Itai-Itai, meaning "it hurts" in Japanese (Evangelou, 1998; Baird and Cann 2005), and it is characterised by bone pain, pathological fractures and signs of renal impairment (Marazioti, 1998).

1.1.2 Arsenic

Arsenic (As) belongs to group V-A of the periodic table it is a very poisonous metalloid. Chemically As is very similar to phosphorus, and forms colourless crystalline oxides like As_2O_3 and As_2O_5 readily soluble in water (Los Alamos National Labs, 2009). As and As species have a broad spectrum of applications such as insecticides [e.g. lead hydrogen arsenate, Scheele's green and more recently monosodium methyl arsenate (Los Alamos National Labs, 2009). As is also used in pyrotechnics, in bronzing and for hardening other metals. New uses include doping agent in solid-state devices and as a laser material (gallium arsenide) to convert electricity into coherent light. Although highly poisonous and carcinogenic some species of As have been used in the treatment of some diseases namely syphilis and cancer. The main sources of As in the environment include semiconductors industry, fossil fuels, mining and smelting activities. Herbicides, fungicides and fertilizers are

also contributors to As pollution (Alloway, 1990; Evangelou, 1998, Jackson *et al*., 2005).

As is a know poison, it disrupts ATP and it inhibits various enzymes like lipothiamide pyrophospahtase, and succinate dehydrogenase (Manahan, 2004; Baird and Cann, 2005). The metabolic interferences caused by As may lead to death from multi-organ failure (Casarett and Doull's, 2001). The IARC recognizes As and its compounds as group 1 carcinogens, and the European Union (EU) lists arsenic pentoxide, arsenic trioxide and arsenate salts as category 1 carcinogens.

1.1.3 Zinc

Zinc (Zn) is part of group II-B of the periodic table and it is relatively abundant. Zn is a bluish-white lustrous metal, very malleable, and is moderately reactive that it combines with oxygen and other non-metals. The common oxidation state of zinc is Zn^{2+} (Los Alamos National Labs, Zinc, 2009). Zn is widely used in corrosion-resistant steel coatings, brass alloys, paint pigments, wood preservatives, dry-cell batteries, cosmetics and pharmaceuticals (Evangelou, 1998; Manahan, 2004). The industry also uses a large amount of Zn to produce die-castings, which contribute to emissions to the atmosphere, water and soil (Harrison, 2001). The principal sources of Zn contamination in the environment are industrial wastes, metal plating and acid mine drainage (Evangelou, 1998). Sewage and animal wastes constitute a source of zinc in soils. This is not very significant but it is known that soils have the capacity to accumulate high concentrations of this metal (Alloway, 1990).

Zn in contrast to the other two heavy metals discussed previously is an essential trace element for animals, plants and bacteria necessary for life. It is estimated that 3.000 human proteins contain Zn, which plays an important role in the catalytic activity of proteins. Zn shortage signs in animals include hair loss, skin lesions, diarrhoea and growth depression for plants typical signs are interveinal chlorosis, impaired growth, and malformation of stems and leaves (Alloway, 1990). Although Zn is an essential element, excess of this metal causes reduced weight in animals (Marazioti, 1998), reduced iron function, impaired immune system, and nausea. High concentrations of Zn can also interact with thiols and block essential reaction on the cell (Hantke, 2005). Zn is phytotoxic in large quantities to plants (Manahan, 2004).

1.2 Microbial diversity

The knowledge about prokaryotes remains incomplete and controversial despite all the modern technological advances. Due to their abundance and diversity they obviously play a key role in biochemical processes such as primary production, organic matter and nutrient cycling in soil and marine environments, nitrogen fixation, and the microbial interaction with plants (Madigan *et al*, 2004; ; Doney *et al*., 2004). The estimated total number of prokaryotes species on the planet is said to be 10^{30} (Dykhuizen, 1998; Whitman *et al*, 1998). Specifically, average cellular densities of 10^{10} cells per gram of soil and 10^{6} cells per millitre in sea water have been reported. After analysing these numbers the conclusion can be made that the prokaryotic biomass represents more than half of the total biomass on our planet (Whitman *et al*, 1998).

1.2.1 Soil microbial diversity

From phylogenetic diversity in soil it is estimated that a gram of soil contains approximately 6000 species (Torsvik *et al*., 1990; Curtis, *et al*., 2002). Molecular based studies on soil samples have supported this idea (Lunn *et al*, 2004). To add to the high reported values of microbial diversity, the diverse metabolic capabilities of microorganisms enabled them to inhabit all types of habitats. Bacteria can be found in environments which offer good conditions for life such as soils, rivers, estuaries, oceans, and other organisms (symbiosis, parasitism, mutualism), but also in extreme environments like hydrothermal vents, sea ice, acid mine drainage, saline basins and soda lakes and deserts. (Deming, 2002; Horner-Devine *et al*., 2004; Irwin and Baird 2004; Tiago *et al.,* 2004).

Microbial communities are constituted by structural clusters of microbial species, each playing different and complementary roles (Torsvik and Ovreas, 2002). The laboratory characteristics of an organism determined *in vitro* rarely reflect its real properties in the environment. Furthermore, several authors have confirmed that bacterial communities, diversity and structure are influenced by spatial and temporal variables such as temperature (Panswad *et al*., 2003), salinity (Bernhard *et al*., 2005), pH, nutrients present (Mills *et al*., 2003), and contamination with pollutants (Li *et al*., 2006).

When studying the microbial diversity within a microbial community several aspects such as microbial phylogenetic diversity and the microbial functional diversity are key points to properly describe a microbial community (Schloss and Handelsman, 2004). Culture dependent techniques only identify a small proportion of the total

microorganisms sampled, as these grow at best under the enriched condition provided (Chihching *et al*, 2007). Since the advent of modern microbiology microbiologists have based their studies on tools such as microscopy, staining methods and pure cultures (Brehm-Stecher and Johnson, 2004). But once bacteria have similar shapes like rods or spheres, it is almost impossible to differentiate microorganisms based on morphology. Other traditional tools such as biochemical properties and metabolic activities are also highly unreliable, changing according to environment adaptation, not providing a robust frame for classification of microorganisms. For the above reasons, molecular based methods are now considered essential to characterise microorganisms and microbial communities (Ovreas and Torsvik, 1998).

The rapid development of novel molecular techniques has offered new ways of studying microorganisms in diverse environments. The first proposed cultivation independent approaches to study microbial populations were proposed by Pace *et al.* (1985). This consisted of the analysis of the 5S or 16S rRNA gene sequences directly in nucleic acids extracted from environmental samples. The advent of the Polymerase Chain Reaction (PCR) technology facilitated the experimental performance of this analysis. More recently, PCR amplification of the 16S rDNA sequences from environmental bacterial DNA, cloning of amplicons and comparison of the obtained sequences with already know sequences allow their assignment to a given phylogenetic group within a phylogenetic tree. This approach is now commonly utilized to determine the phylogenetic diversity in microbial communities. As a consequence of using these new techniques the number of recognized phyla has greatly increased, from 11 phyla in 1987 (Hugenholtz *et al*., 1998) to 53 phyla, 25 of which do not include culturable members (Handelsman, 2004).

Currently the technique most commonly used in microbial ecology is the analysis of the ribosomal RNA genes (rRNA). Several advantages have contributed for this: rRNA is part of the protein-synthesising cell machinery, thus these molecules occur in all bacterial cells with a high level of functional and structural preservation; their primary structure is divided into several segments that vary from extremely conserved to extremely variable (Van-de-Peer *et al*, 1996), thus conserved and variable regions can be used to study evolutionary relationships between closely related organisms; the highly conserved segments allow rRNA sequences to be aligned and target sequences for the design of PCR primers and/or hybridisation (Giovannoni *et al*., 1988); rRNA sequences are long enough to allow statistically significant phylogenetic analysis.

The reasons stated above make rRNA the molecule of choice for establishing phylogenetic relationships. Especially 16S rRNA has been extensively used due to its size (± 1500 nucleotides) providing enough information to infer about phylogenetic relationships and also because there is a great number of complete 16S rRNA sequences available in databases making comparison a straightforward procedure (Cole *et al*., 2005).

1.2.2 Bacteria in heavy metal contaminated soil and sediments

In a worldwide sense, heavy metal contaminated environments represent a common environmental problem constituting a major hazard for ecosystems and human health with expensive cleanup costs. The input of heavy metals by industry and agriculture has led to the release and improper disposal of enormous amounts of heavy metals (Ansari and Malik, 2007). Heavy metals can be found in soils as free

cations, as complexes (e.g. $CdCl_3^-$, $ZnCl^+$) with organic and inorganic ligands, and associated with soil colloids (Wang *et al.,* 2010), they can accumulate in biological systems finding their way into the food web via different mechanisms (Giller *et al*., 1998).

Soil is a complex mixture of materials of mineral (e.g. clay) and organic (e.g. humic substances) origin, aqueous and gaseous components. It is a dynamic system with variations in moisture content, pH and redox conditions. These properties interfere with the form and availability of metals (Alloway, 1990). Soil and heavy metal interactions can be understood on the basis of ion exchange, surface adsorption and/or chelation reactions.

These contaminated soils and sediments harbour organisms, both prokaryotes and eukaryotes, able to deal with pollution (Zettler *et al*., 2002; Baker and Banfield, 2003). Microorganisms are key elements for recycling nutrients and heavy metals imposes a chronic stress upon the decomposer subsystem, and a variety of experimental systems and regimes have been investigated (Moffet *et al*, 2003). Some of these organisms have the ability to modify the physicochemical conditions of their surrounding environment either by detoxification, metal homeostasis, precipitation or solubilization, redox transformations or by metabolic exploitation (Bruneel *et al*., 2006; Hetzer *et al*., 2006; Guiné *et al*., 2007)

The environmental stress caused by heavy metals, generally decreases the diversity and activity of soil bacterial populations leading to a reduction of the total microbial biomass, decrease in numbers of specific populations such as rizhobia and a shift in microbial community structure (Sandaa *et al*., 1999; Wang *et al*., 2010). Soil

microbial population responses to heavy metal contamination provide a relevant model for ecological studies to assess the influence of environmental characteristics (Guo *et al.*, 2009). Several studies have demonstrated that metals influence microorganisms by affecting their growth, morphology and biochemical activity (Sandaa *et al*., 2001; Tsai *et al*., 2005; Pérez-de-Mora *et al*., 2006) and diversity (Dell'Amico *et al*., 2008). The response of the bacterial populations to heavy metal contamination depends on the concentration and bioavailability of metals itself and is dependent by multiple factors such as the type of metal and microbial species (Hassen *et al*., 1998). High concentrations of metals (both essential and non-essential) harm the cells by displacing the enzyme metal ions, competing with structurally related non-metals in cell reactions and also blocking functional groups in the cell bio-molecules (Hetzer *et al*., 2006). Microbial survival in heavy metal polluted soils depends on intrinsic biochemical properties, physiological and/or genetic adaptation including morphological, as well as environmental modifications of metal speciation (Abou-Shanab *et al*., 2007). Studies on the effects of metals on soil bacteria have been conducted showing that short term contact causes the selection of resistant bacteria within weeks. A more prolonged exposure to metals slowly selects resistant bacteria. On the other hand long term exposure to metals leads to the selection/adaptation of the microbial community which then thrives in polluted soils (Pérez-de-Mora *et al*., 2006; Dell'Amico *et al*., 2007, Chihching *et al*., 2008). The presence of different metals together may also have greater adverse effects on the soil microbial biomass/activity and diversity than those caused by single metals at high concentrations (Renella *et al*., 2005).

Study of the adaptive microbial responses usually focuses on the phenotypic changes observed. Adaptation can also be accessed via the characterisation of the molecular mechanisms of resistance. Different techniques can be employed to investigate these mechanisms such as PCR, DNA hybridisation and subsequent analysis of restriction fragment length polymorphism (RFLP), or amplified ribosomal DNA restriction analysis (ARDRA) (Guo *et al*., 2009). A great advantage these techniques have over the more traditional techniques is that they can be targeted specifically to genes revealing the molecular mechanisms of adaptation (Abou-Shanab *et al*., 2007).

The ability of some microorganisms to tolerate heavy metals and the ability of some to promote transformations that make some metals less toxic, make organisms that live in heavy metal contaminated sites potentially useful in bioremediation. Bioremediation strategies are dependent on the knowledge of the *in situ* microbial diversity targeting the most resistant strains. By characterising the microbial communities, the taxa able to survive and remain active in the extreme environments can be identified and potentially targeted for bioremediation purposes (Akob *et al*., 2007). In order to optimize and develop remediation processes, more studies about the bacterial populations that inhabit these sites are required.

1.2.3 Interactions of microorganisms with heavy metals

Low concentrations of certain metals such as zinc, copper, cobalt and nickel are essential for the metabolic activity of bacterial cells. Other metals like Pb, Cd, Hg and Cr have no known effects on cellular activity and are cytotoxic (Chen *et al*., 2005a; 2005b; Abou-Shanab, 2007). It is known that microbial activity plays an

important role in the metal speciation and transport in the environment (Spain and Alm, 2003). In high concentrations, heavy metal ions become toxic to cells. Due to the fact that some heavy metals are necessary for enzymatic functions (e.g. Zn) and growth, the cell has different mechanisms for metal uptake, this can be accomplished by bioaccumulation or biosorption.

1.2.3.1 Bacteria

Bacterial surface structures are of extreme importance to understand their interactions with the surrounding environment, especially with metals. Bacteria can be Gram-negative or Gram-positive depending on the composition of the cell wall membrane. Gram-negative cell walls are a multilayered structure with an outer membrane containing lipopolysaccharide (e.g. lipopolysaccharide layer [LPS]), phospholipids and a small peptidoglycan layer. On the other hand, Gram-positive cells have as much as 90 % of the cell wall consisting of peptidoglycan in several layers, with small amounts of teichoic acid usually present (Madigan *et al*., 2003; Guiné *et al*., 2007). These structures are negatively charged and can interact with metal ions (Guiné *et al*., 2007).

Bioaccumulation is a substrate specific process, driven by ATP (Spain and Alm, 2003; Errasquin and Vazquez, 2003) and is an active process of heavy metal uptake. Three mechanisms of metal transport into the bacterial cell are known: passive diffusion, facilitated diffusion and active transport. Some of the active transport systems are metal selective. However, there are some exceptions, Cd can be transported by the same transporters as Zn (McEldowney, 1993). A disadvantage of bioaccumulation is the recovery of the accumulated metal which has to be done by

destructive means leading to damage of the biosorbent structural integrity (Ansari and Malik, 2007).

Biosorption refers to other mechanisms that are driven by the chemiosmotic gradient across the cell, not requiring ATP and it is primarily controlled by physico-chemical factors. These include adsorption, ion-exchange and covalent bonding and may occur either in living or dead biomass and is considered as an alternative to conventional methods of metal recovery from solutions (Spain and Alm, 2003; Chen *et al*., 2005a; 2005b), being a passive metal uptake system. Both Gram-negative and Gram-positive bacteria have their cell wall charged with a negative charge. This is due to carboxyl, hydroxyl and phosphyl groups, thus in the presence of positive heavy metal cations these groups are very important in cation sorption (Marazioti, 1998). Biosorption has a possible application as a process for the removal and concentration of heavy metals from wastewater (Errasquin and Vazquez, 2003). However, the cost of the biomass plays an important role in determining the cost of a biosorption process, thus a low-cost biomass is an important factor when considering practical application of biosorption (Chen *et al.,* 2005a; 2005b).

Various microorganisms show a different response to toxic heavy metal ions that confer them with a range of metal tolerance (Valls and de Lorenzo, 2002). Bacteria may achieve this in different ways either through biological, physical or chemical mechanisms that include precipitation, complexation, adsorption, transport, product excretion, pigments, polysaccharides, enzymes, and specific metal binding proteins (Gadd, 1992; Marazioti, 1998; Hetzer *et al*., 2006). From a metabolic point of view a group of metal-chelating proteins called metallothioneins, are very important

in bacterial metal tolerance (Marazioti 1998; Valls and de Lorenzo, 2002). Metallothioneins are small cystein-rich polypeptides that can bind essential metals (e.g. Zn), and non-essential metals (e.g. heavy metals) (Marazioti, 1998).

Other resistance mechanisms include active efflux, complexation, reduction and sequestration of the heavy metal ions to a less toxic state (Nies, 1999). These tolerance mechanisms are generally plasmid driven, which greatly contributes to dispersion from cell to cell (Collard *et al*., 1994, Valls and de Lorenzo, 2002), chromosome resistance was also related in some bacterial species (Spain and Alm, 2003; Abou-Shanab *et al*., 2007).

Environmentally isolated metal resistant bacteria have been studied in more detail in bioremediation processes. *Cupriavidus metallidurans* CH34 has been shown to bioremediate heavy metal polluted soils and water. This strain has the capacity to accumulate Selenium (Se), Gold (Au) and to volatilize Hg through reactive processes (Guiné *et al*., 2003; Sarret *et al*., 2005; Reith *et al*., 2006). *Pseudomonas stutzeri*, isolated from a foundry soil, was shown to be resistant to the toxic effect of chromium up to 1 mM and anaerobically reduce Cr (VI) up to 100 µM (Tsai *et al*., 2005).

The interest in heavy metal uptake by bacteria has increased in recent years, especially because of the biotechnological potential that microorganisms have for the removal and/or recovery of metal contaminants (Valls and Lorenzo, 2002; Errasquin and Vazquez, 2003). Bacteria are good biosorbents and with the proper R&D may be in the near future a good alternative for the removal of metals from the environment (Errasquin and Vazquez, 2003).

The processes by which microorganisms interact with metals are diverse and their locations in the bacterial cell is shown in Figure 1.1

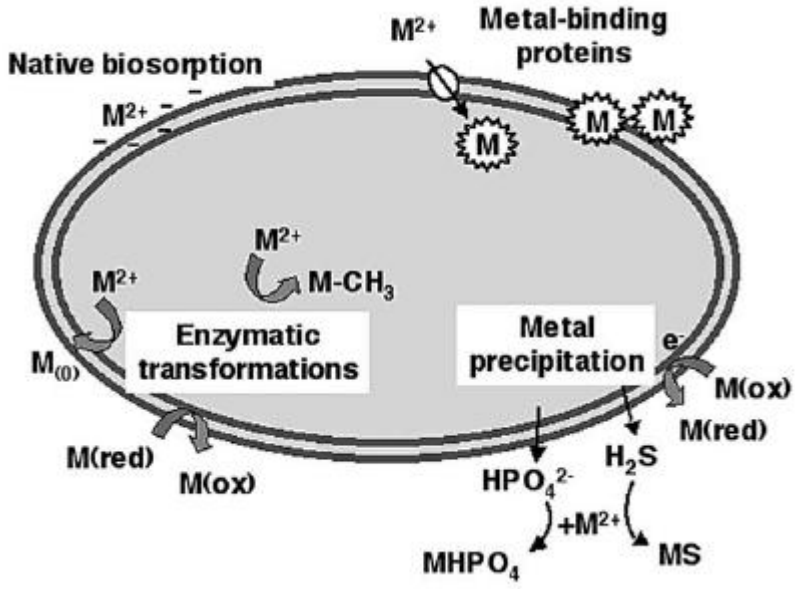


Figure 1.1 Bacterial interaction with heavy metal ions (M^{2+}) in the environment, with reference to the cellular compartment where bacterial response happens (Valls and de Lorenzo, 2002)

(i) Cadmium (Cd)

Gram-negative bacteria show more resistance to Cd than Gram-positive ones. This difference can be attributable to the complex cell wall structure of Gram-negative microorganisms (Jjemba, 2004). Microbial resistance is probably linked to the presence of metallothionein-proteins that bind and detoxify several heavy metals. Cd resistance in microbial cells is mostly attained by active efflux via an energy-dependent mechanism (active transport) to pump out cadmium cations through specific efflux pumps (Jjemba, 2004). The Cd adsorption capacity of *Chlorella*

pyrenoidosa was induced by light exposure (Sirianuntapiboon and Ungkaprasatcha, 2007), and probably the same happens for some groups of bacteria.

(ii) Arsenic (As)

A number of microorganisms have the ability to use either the oxidized or the reduced form of arsenic as electron acceptors in their metabolism. An even greater number of microorganisms can resist its toxicity through the *ars* gene (Oremland and Stolz, 2003). As resistant organisms were isolated as expected from arsenic contaminated environments, but laboratory strains of bacteria such as *Escherichia coli, Pseudomonas aeroginosa* show resistance to high levels of As (Jackson *et al*., 2005). Phylogenetic analysis of the genes involved in arsenic resistance indicate that they might be more abundant in microorganisms than previously thought (Jackson and Dugas, 2003; Jackson *et al*., 2005). This fact implies that microorganisms living in an arsenic free environment may have arsenic resistance. These microorganisms can have a role in transforming arsenic that may become available through pollution and may be important in local clean up strategies and represent a background reservoir of As resistant microorganisms. (Jackson *et al*., 2005). Oxidation of As by bacteria has been observed in Acid Mine Drainage (AMD) and in similar environments (e.g. hot springs) (Battaglia-Brunet *et al*., 2002; Oremland and Stolze, 2003; Bruneel *et al*., 2006).

(iii) Zinc (Zn)

Microorganisms have to maintain the metal concentration in their cells carefully, and Zn is no exception. Bacterial cells maintain a delicate balance between the Zn requirements and their toxicity in several ways: storage mechanisms via

metabolic pathways (e.g. metallothioneins) in which Zn is detoxified and stored in the cell (Blindauer *et al*., 2002), Zn is expelled out of the cell by different low and high affinity exporters (Nies, 2003) and high and low affinity uptake systems that regulate Zn uptake at different levels of transporter synthesis and activity (Hantke, 2005). Zn in synergy with other metals may also enhance toxic effects in anaerobic and aerobic microorganisms. High concentrations of Zn may reduce protein and ATP content, interact with nucleic acids and enzyme active sites, altering the membrane and leading to cell death (Vega-López *et al*., 2007).

1.2.3.2 Fungi

Some heavy metals are essential for the fungal metabolism (e.g. Zn) while others do not have any known role in metabolism (e.g. Pb and Cd). When in excess, essential and non-essential metals become toxic, and this toxicity could be many times greater than required (Baldrian, 2003). Heavy metals present in the environment can interact with extracellular enzymes of fungi, but to cause a physiological response heavy metals have to be taken up by fungi.

The metal uptake systems are usually present in the cell membrane. Heavy metals can be co-transported using the same transporters of essential metals because of their low specificity (Gadd *et al.*, 1990; Baldrian, 2003). The uptake depends on the membrane potential and it is co-transported with calcium (Ca). At a subcellular level, 50% of the metal is bound to the cell wall, 30% stays in the cytoplasm and the remaining 20% is transported to vacuoles. Studies made with *Paxillus involutus*, have shown that Cd uptake involves a rapid binding to the cell wall and a carrier mediated transport more slowly in the cell (Blaudez *et al*., 2000). Fungi can also take a

preventive approach that reduces the uptake of heavy metals. This involves the reduction in the availability of heavy metals via various extracellular precipitation and complexation strategies which could also involve enhanced impermeability to heavy metals (Gadd and Griffiths, 1978).

Fungal cell walls contain basically cellulose, chitin, glucan, and mannan. In addiction to this the cell wall of fungi is composed by proteins, lipids, pigments and polyphosphates (Negishi, 2000). Passive uptake of heavy metals my occur onto the cell wall via ion exchange reactions and complexation with the functional groups of the cell components, without any type of metabolic control (Kuyucak and Volesky, 1988). Similarly to what happens in bacteria it is supposed that functional groups like phosphoryl, carbonyl and hydroxyl (Gadd, 1990) may be involved in this mechanism also called biosorption (Chen *et al*, 2005a; 2005b). Like in bacteria, biosorption using fungal biomass seems to be a very promising technique for the removal of heavy metals. Work conducted by Puranik and Paknikar (1997) have demonstrated that *Streptoverticillium cinnamoneum* biomass was effective as a biosorption substrate for Pb and Zn.

After entering the fungal cell, heavy metals interfere with the individual reactions and metabolic processes. When heavy metals are present at a toxic level the fungal growth rate decreases and this is often accompanied by changes in the mycelium. These changes could be in the fungal colour, e.g. *Trametes versicolor* produces a brown pigment when in the presence of Cd, *Schizophyllum commune* normally has a creamy mycelium but in the presence of Pb gives origin to a black mycelium (Baldrian, 2003).

Figure 1.2 illustrates some of the mechanisms involved in the uptake of heavy metals by fungi.

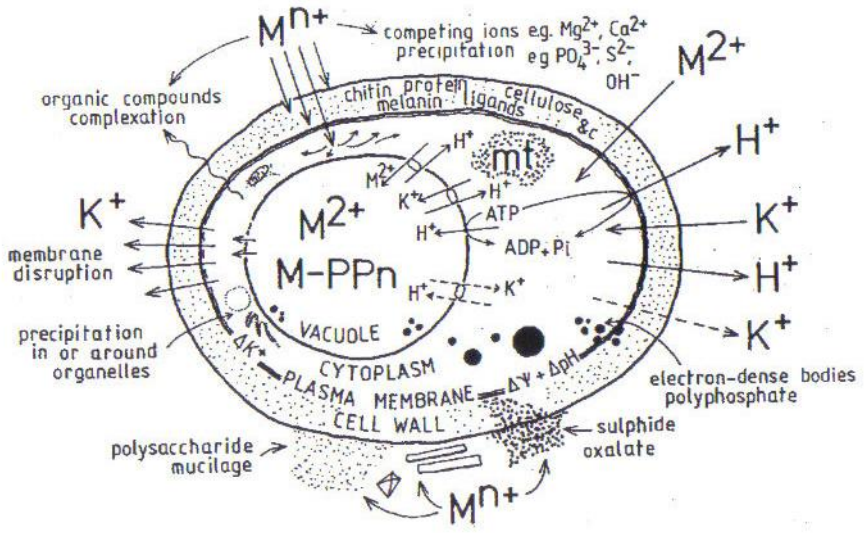


Figure 1.2 Mechanisms involved in heavy metal uptake by fungi (Gadd, 1986)

Heavy metals are toxic to fungi because of their role as enzyme inhibitors and protein denaturers (Gadd and Griffiths, 1978). Metals like Hg bind to SH groups that are responsible for the regulation of enzyme sites causing irreversible inactivation. In contrast Cd has the capacity to bind aromatic amino acid residues in enzymes and cause oxidative damage to proteins (Stohs and Bagchi, 1995). Heavy metals interfere with the reproduction of many fungi, where the spore formation and germination are very sensitive when compared with mycelial growth (Baldrian, 2003; Pawlowska and Charvat, 2004).

Fungi have developed some resistance mechanisms against the toxicity caused by heavy metals. The first line of defence is metal immobilisation by means of extracellular and intracellular chelating compounds. Heavy metals can be chelated by

small peptides like phytochelatins or metallothioneins (Gadd *et al.*, 1990; Negishi, 2000; Baldrian, 2003). One common chelator in fungi is oxalate which provides a way of immobilization of soluble metals in insoluble oxalates, decreasing their availability (Sayer and Gadd, 1997). Another type of metal binding compound is called melanin which is associated with the fungal cell wall. Melanin and related proteins are able to absorb some metals (Gadd and De Rome, 1988; Gadd *et al*., 1990). Work by Rizzo *et al*., 1992, discovered that in *Armillaria* sp. melanin was able to take up Zn, Al, Cu and Fe (Baldrian, 2003).

The relationship of heavy metals and fungi are not yet fully understood but experiments made with some basiodiomycetes have delivered promising results. They have a cell wall mainly composed of polysaccharides and peptides that have a good capacity for heavy metal binding (Baldrian, 2003). Table 1.2 depicts some values obtained from work using *Phanerochaete chrysosporium.*

Like bacteria some fungi could be a good alternative for removal of toxic heavy metals from the environment, even more when it is suggested that fungi are more tolerant of heavy metals as a group than bacteria (Hiroki, 1992; Rajapaksha *et al*., 2004).

Table 1.2 Maximum sorption capacities of *Phanerochaete chrysosporium* mycelia with different heavy metals, adapted from Baldrian, 2003.

Metal	**Sorption capacity (mg g^{-1} dry weight)**
Cd	**110**
Cu	**60**
Hg	**61**
Pb	**108**

Dead cells accumulate heavy metals to an equal or even greater extent than living cells. This is because cell surfaces are ionic due to the presence of ionised groups in the cell polymers, "attracting" heavy metals. Ion exchange is believed to be the principal mechanism for metal uptake (Hawari and Mulligan, 2006). Waste biomass is readily available from industry (e.g. penicillin production). The use of dead biomass eliminates any nutrient requirements. Work by Niu *et al.*, 1993, has shown an uptake of 116 mg/g of Pb when using *Penicillium chrysogenum* biomass (Bailey *et al*., 1999).

1.3 Remediation techniques for removal of heavy metals from soil

Different techniques are used to contain and/or remove heavy metals from contaminated soil. Although several technologies are available for the treatment of contaminated sites, the selection depends on contaminant and site characteristics, regulatory requirements, costs and time constraints (Riser-Roberts, 1998).

1.3.1 Physico-chemical techniques

(i) Isolation and containment

Heavy metal contaminants can be isolated and contained, to prevent their movement and reduce their permeability. To accomplish this physical barriers made of different materials are used for capping, and vertical and/or horizontal containment. Capping has been used with good results to reduce the water intake. Vertical barriers are used to reduce the movement of groundwater. Solidification/stabilization techniques are very common in the United States, because they contain the contaminants, lowering the labour and energy costs (Mulligan *et al*., 2001).

(ii) Mechanical separation

This technique aims at the removal of larger clean particles from smaller polluted particles. This method has been used in mineral ore processing and now in remediation of heavily contaminated soils (Mulligan *et al*., 2001).

(iii) Chemical treatments

Chemical reactions such as oxidation and reduction can be used to decrease the mobility of heavy metal contaminants. This is commonly used in treatment of contaminated water. This method involves the addition of chemicals such as potassium permanganate, hydrogen peroxide, or chlorine gas. Chemical treatments have the advantage of being performed in situ, but also may add a new source of contamination (Mulligan *et al.*, 2001).

(iv) Electrokinetics

This technique involves passing low intensity electric currents between a cathode and an anode inserted in the contaminated soil. An electric gradient generates movement by electromigration, and electrophoresis. The metals can be removed by electroplating or precipitation or recovering the metals by pumping the waste from which it originated. This technique has been used in Europe (Mulligan *et al*., 2001).

(v) Soil washing

Heavy metals can be removed from soils adding different chemicals to soil. This can be done in reactors. These chemicals can be inorganic or organic acids such as sulphuric acid as acetic acid respectively; chelating agents like ethylenediaminetetraacetic acid (EDTA) can also be used. The cleaned soil is then returned to its former location. The effectiveness of this technique depends on the soil characteristics (Mulligan *et al.,* 2001).

The feasibility of using biodegradable biosurfactants of bacteria and yeasts origin have been tested *in situ* with some promising results for the removal of heavy metals (Mulligan *et al*., 1999).

(vi) Ion exchange

This is one of the more common techniques for heavy metal removal. In this process ions of a given species are displaced from an insoluble exchange material by different ions in solution. The materials used for the exchange include zeolites, chelating resins, microbial and plant biomass. Ion-exchange techniques are highly pH dependent. A drawback to this technique is the high operating costs (Metcalf & Eddy, 2003).

1.3.2 Biological Techniques

Removal of heavy metals using living organisms is still in its infancy. These methods include bioleaching, oxidation/reduction reactions and biosorption. Bioleaching may involve either fungi or bacteria. *Thiobacillus* sp are responsible for the oxidation of inorganic sulphur compounds (Mulligan *et al*., 2004), forming sulphuric acid. This can be utilised for desorbing the metals in the contaminated site by substitution of protons. *Aspergillus niger* offers also a promising alternative due to its citric and gluconic acid production, these particular acids can act as chelating agents for heavy metal removal (Mulligan *et al.*, 2001; 2004).

Biosorption is another promising biochemical process, which can be applied for the removal of low concentrations of heavy metals in water (Mulligan *et al*., 2001). It involves the removal of heavy metals by passive binding to non-living biomass (Chen *et al.,* 2005a; 2005b). This is a passive process and is independent of metabolic control in which heavy metals are deposited in cell walls by means of ion exchange reactions and complexation with determined functional groups of the cell wall components (Kuyucak & Volesky, 1988; Negishi, 2000). Biosorption has some advantages such as, it is independent from metabolism; rapid and independent of temperature; substrates for biosorption are readily available and are easily regenerated (Negishi, 2000).

Biosorption has been found to be very selective depending on the typical binding profile of biosorbents (Ansari and Malik, 2007).

Bioremediation techniques, used as an *in situ* treatment, offer several advantages over the conventional chemical and physical treatment technologies, particularly for diluted and widely spread contaminants (Radhika *et al*., 2006).

1.4 Objectives and thesis outline

As focused in previous sections, the different remediation techniques more commonly used to address heavy metal contaminated environments, can be costly, and may only be used for the upper layers of the soil. The use of bacterial cell biomass to remove or immobilise heavy metal contaminants offers a possible low cost method for the remediation of metal contaminated sites. Research on this subject has resulted in the discovery of several bacterial and fungal species with the capacity to tolerate and immobilise metals in soil and water. However, more information on metal resistant organisms is fundamental and seems to be an important pathway to pursue. The search for bacteria and fungi populations adapted to metal-stressful conditions may be a good approach to find species or strains having the capacity to tolerate or accumulate metals and with potential applications for bioremediation strategies.

The work presented in this thesis investigates the diversity, the metal tolerance and identification of tolerant bacterial species or strains which can be used in heavy metal uptake systems and potential use in remediation systems in metal contaminated sites.

The overall aims of this research were the following:

1. Isolation and characterisation of bacteria from metal contaminated soil;
2. Characterise the bacterial populations;
3. Assess metal tolerance and resistance of the isolated strains which can be used in HM uptake systems;
4. Testing of different support matrices for bacterial immobilization for examining potential applications of the chosen bacterial;
5. To establish an immobilised system for the removal of heavy metals from aqueous streams using HM resistant bacteria with bioremedial applications.

Chapter 2: General methods

2.1 Site and soil sampling

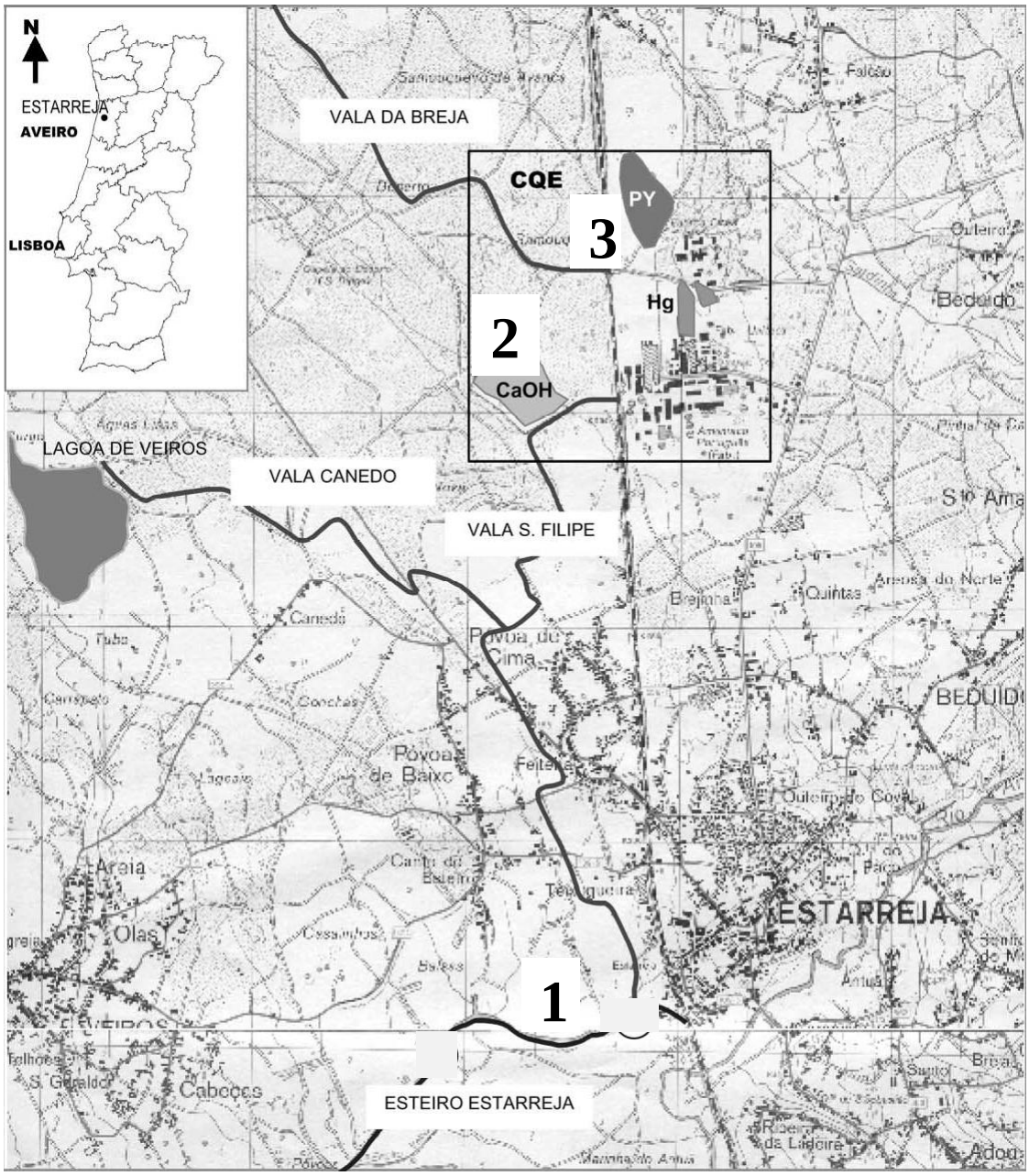
2.1.1 Site description

The study site is a metal polluted site located near an industrialised area of Northern Portugal - Estarreja -, where contamination has been released via wastewater into a nearby stream - "Esteiro de Estarreja" -, heavily contaminating the sediments and the soil of the banks of this water course with metals, especially Zn (Atkins, 1999). Despite this contamination scenario, the vegetation on the banks of the stream remains prolific.

The chemical industry has a five decade history at Estarreja, turning it into one of the most important industrial areas in Portugal. This increased industrial activity over many years has contributed to make Estarreja one of the most contaminated regions in Portugal, due to both the direct discharge of effluents into the nearby water streams and uncontrolled waste disposal in soil (Costa and Jesus-Rydin, 2001). The impact of the recurrent pollution on the environment and health has made this a problem that needs to be addressed. Figure 2.1 shows the area.

The major part of the polluting industry in Estarreja is located inside what is known as the "*Complexo Químico de Estarreja*" (Estarreja Chemical Complex). This industrial area has an area of approximately 2 km^2 and is located 1km from the town of Estarreja. According to estimates (Costa and Jesus-Rydin, 2001) huge deposits of solid waste by-products are stored inside the complex: ±150.000 t of pyrite waste, containing As, Pb, Zn and other contaminants; ± 60.000 t of sludge contaminated with

Hg; 300.000 t of calcium hydroxide sludge. Regarding liquid effluents, until 1975 they were discharged directly into the local water streams, causing the contamination of the agricultural fields downstream. However, some of the industries still discharge their effluents directly. The effluents emitted by the Estarreja industry contain mainly aniline, benzene, monochlorobenzene, As, Hg, Zn, and Pb.



1- Site 1
2- Site 2
3- Site 3

Figure 2.1 Location of the main industrial units and solid waste deposits in the study site (adapted from Costa and Jesus-Rydin, 2001).

Soil samples from three different sub-sites were recovered, on three occasions, March 2006, April 2006 and September 2006.

i) Sub-site 1 (Fig. 2.1 and 2.2)- located on the banks of a stream, mainly contaminated with Zinc (Zn), Arsenic (As) and Lead (Pb) all above the limits established by the European Union (EU) Directive 86/278/EC. The pH of the soil was 7.1-8.1 to a depth of 10 cm. The soil composition in decreasing order was sand (52 %), silt (32 %) and clay (8 %) (Oliveira *et al*., 2001). The lower part of the bank was periodically covered by the stream, whilst the top of the bank was not and hence water contents varied. The bank had abundant vegetation mainly comprising *Phragmites australis* with small reeds and grasses; *P. australis* was the only species found on the lower sections of the bank.

Figure 2.2 Detail of sub-site 1.

ii) Sub-site 2 (Fig. 2.1 and 2.3)- located on a 10-ha dry sediment deposit resulting from the production of polyvinylchloride (PVC). The sediment had a pH of ±12 and was mainly composed of $Ca(OH)_2$ (80 % total) with low levels of organic carbon, N and P. The site had scarce vegetation, mainly small trees of *Pinus* spp. and *Salix atrocinerea* Brot., together with less frequent *Pteridium aquilinum* (L.) Kuhn, *Conyza bonariensis* (L.) Cronq, *P. australis* and *Juncus effusus* L (Balsa et al. 1996).

Figure 2.3 Detail of sub-site 2.

iii) Sub-site 3 (Fig. 2.1 and 2.4)- located upstream site 1, periodically flooded soil with a pH of 4.5/5 to a depth of 10 cm. It shows high levels of contamination by As, Zn and Mercury (Hg). The soil was composed mostly by sand (83 %), silt (8 %) and clay (9 %). Vegetation was abundant, mainly comprising *Pinus* spp, *Acacia melanoxylon* Aiton, *P. australis* and *J. effusus*. (Oliveira *et al*., 2001).

Figure 2.4 Detail of sub-site 3

2.1.2 Soil sampling regime

Soils and sediment cores were sampled on the study sites from the uppermost 10 cm by using a soil borer (6 cm diameter). Soil sampling was made at five different spots in each site the five collection spots in each site were separated by 10 m from each other, in order to cover a high range of soil metal concentrations, profiting from the heterogeneity of the site contamination. At each sampling location, 10 sub-samples were taken and mixed together to account for the spatial variation that may occur within the soil environment. Soil samples for bacterial analysis were then transported to the laboratory at 4 ºC and were assayed within 12 h of sampling.

2.2 Soil analysis

2.2.1 Soil pH

Soil suspensions were prepared with 1 gram of sieved soil in 9 ml of sterile ultra-pure (UP) water and the soil solution pH was measured using a surface-testing Sentix® Sur pH electrode (WTW, Hoskin Scientific). All assays were conducted in triplicate.

2.2.2 Total phosphorous and nitrogen determination

Soil samples were oven dried at 40 ºC for 48 h and passed through a 1 mm sieve. Samples for total phosphorous (P) and nitrogen (N) were digested at high temperatures (up to 330 ºC) with a Se and salicylic and sulphuric acids mixture and determined by colorimetry. For total N colorimetric determination, two reagents were added to the digests: reagent 1, consisting of a mixture of a $5x10^{-2}$ mol l^{-1} dissodiumhydrogenphosphate buffer (pH=12.3) and a 4 % bleach solution and reagent 2 consisting of a mixture of a 1 mol l^{-1} salicylate solution, a $1x10^{-3}$ mol l^{-1} sodium nitroprusside solution and a $3x10^{-3}$ mol l-1 EDTA solution. For total P colorimetric determination, two different reagents were added to the digests: reagent 1, consisting of a $3x10^{-2}$ mol l^{-1} ascorbic acid solution and reagent 2 consisting of a mixture of a $6x10^{-3}$ mol l^{-1} antimonyl tartarate solution, a $5x10^{-3}$ mol l^{-1} ammonium molybdate solution, 0.7 mol l^{-1} H_2SO_4 and an anticoagulation agent (Wetting aerosol 22). The elements concentration on the resulting preparations was determined on an UNICAM HELIOS spectrophotometer (Waltham, USA), at 660 nm for N and 880 nm for P. The intervals of standards used in the determinations of P and N were, respectively, 0 to 5 mg $l^{-1}$and 0 to 30 mg l^{-1}; dilutions of the samples of up to 300 and 20 times,

respectively for P and N, were prepared whenever necessary to fit the calibration curve obtained through the analysis of the standards. All of the above methods were based on Houba *et al*. (1995)

2.2.3 Soil metal analysis

Soil analyses were performed by a certified analytical company (DPM, Lisbon, Portugal). Potassium (K), sodium (Na), magnesium (Mg), calcium (Ca), copper (Cu), iron (Fe), nickel (Ni), chromium (Cr), Zn, lead (Pb) and mercury (Hg) were determined using standard atomic absorption spectrometry techniques described by Pratt. As was measured colorimetrically (Hesse, 1971).

2.3 Microbiology methods

2.3.1 Preparation of soil samples

For the isolation and enumeration of the culturable bacterial population, one gram of soil samples was taken and suspended in a test tube containing 10 ml of sterile 0.85 % (w/v) saline solution supplemented with Tween 20 (Sigma). The soil samples were vortexed for 10 min to extract soil microorganisms from the soil (Standard Laboratory Practice at Escola Superior de Biotecnologia).

2.3.1.1 Protocol for serial dilution (CFU's)

For serial dilutions 1 ml of the suspended soil samples was added to 9 ml of sterile 0.85 % (w/v) saline solution to make a one in 10 dilution (10^{-1}), then one ml of this dilution was added to 9 ml 0.85 % (w/v) saline solution to make a one in 100 dilution (10^{-2}). This procedure was repeated until 10^{-8} dilution was reached. Triplicate

samples of 100 µl of each dilution were spread onto agar plates for microbial counts (Alef and Nanninpiri, 1996). Diluted samples were plated onto different bacterial media Plate Count Agar (PCA), Trypticase Soy Agar (TSA), Alkaline Buffered Medium 2 (ABM2) and Malt Extract Agar (MEA) agar plates for fungi, and incubated at 25 ºC, for up to 10 days. Colonies were monitored after 24 h and then daily for bacterial cultures, and fungal counts were observed between 2 and 10 days with recorded counts every 48 hours.

Colony forming units (CFU) per millilitre (ml) was calculated using the formula:

$$\frac{\text{Mean Count}}{\text{(Dilution used} \times \text{amount plated [ml])}}$$

2.3.2 Media preparation

The media for the isolation were selected for their ability for the growth of a wide variety of the microbial populations and to study their size and composition, in accordance to this the following basic media were selected for isolation:

i) Plate Count Agar (PCA) (Merck), prepared according to the manufacturer recommendations

ii) Malt extract agar (MEA) (Oxoid), prepared according to the manufacturer recommendations

iii) Trypticase soy agar (TSA) (Pronadisa), prepared according to the manufacturer recommendations, but 100 ml of a defined buffer solution at a concentration of 1 M was added to the final volume of 1 liter of medium.

iv) Alkaline buffered medium 2 (ABM2), a media used for alkaline environments and also suited for growth of other heterotrophic bacteria. ABM2 contained the following components per liter of medium: 5 g of yeast extract (Difco),

5 g of tryptone (Oxoid), 1 g of α-ketoglutaric acid monopotassium salt (Sigma), 15 g of agar (Lab M), 100 ml of a macronutrients solution 10x concentrated, 10 ml of a micronutrients solution 100× concentrated; and 100 ml of a specific buffer solution at concentration of 1M. The 10× concentrated macronutrients solution contained per liter: nitriloacetic acid (Sigma) 1 g, $CaSO_4.2H_2O$ (Merck) 0.6 g, $MgSO_4.7H_2O$ (Merck) 1 g, NaCl (Merck) 0.8 g, KNO_3 (Merck) 1.03 g, $NaNO_3$ (Merck) 6.89 g, $NaHPO_4$ (Merck) 1.11 g. The 100x concentrated micronutrients solution contained per liter: $MnSO_4.H_2O$ (Merck) 0.22 g, $ZnSO_4.7H_2O$ (Merck) 0.05 g, H_3BO_3 (BDH Analak) 0.05 g, $CuSO_4.5H_20$ (Merck) 0.0025 g, $Na_2MoO_4.2H_2O$ (BDH Analak) 0.0025 g, $CoCl_2.6H_2O$ (Merck) 0.0046 g.

100 ml of a defined buffer solution at a concentration of 1 M was added to the final volume of 1 liter of medium.

The media used were modified by adjusting the pH to three different pH values (5, 6 and 7), selected for their ability for the growth of a wide variety of the microbial population, using buffer solutions at concentrations of 100 mM. Media with pH 10 was also used for site 2 only, due to the difficulties involved in working at the pH of the site (pH 12). The buffer solutions were autoclaved separated from the other components of the different media and were mixed after cooling to 45 ºC.

2.3.2.1 Buffer solutions

To adjust the media to the different pH values different buffer solutions were used. All buffer solutions were prepared as stock solutions at 1M according to Gomori (1990) and autoclaved separately. For the preparation of each medium, 100 ml of each stock solution were used to reach a final concentration of 100 mM. The following

buffer solutions were used to adjust media to the different pH values: citrate buffer to adjust to pH 5 and 6; phosphate buffer to adjust to pH 7; carbonate-bicarbonate buffer to adjust to pH 10. The pH value of each lot of media was verified prior to use, on the media surface, with a surface testing pH electrode Sentix® Sur (WTW, Hoskin Scientific).

2.3.2.2 Heavy metal supplemented media

Microbial counts and isolation was also made using media supplemented with Cd, Zn and As, supplied as $CdCl_2$, $ZnCl_2$ and KH_2AsO_4 from sterile stock solutions, to final concentrations ranging from 50 to 1000 mg L^{-1}.

The variants of media PCA, MEA, TSA and ABM2 with metals were prepared exactly in the same way. The metal stock solutions contained 10000 mg L^{-1} of $CdCl_2$ (Sigma), $ZnCl_2$ (Sigma) and KH_2AsO_4 (Sigma). Appropriate volumes of the metal solutions were mixed with the media after being autoclaved. All the other steps were as described previously for the media without metals.

2.3.3 Bacterial isolation and storage of the bacterial strains

Bacterial isolates were enumerated daily in the different cultivation media. From the 7^{th} day single colonies were selected based on their different morphological and colours and were streaked using a sterile loop. All strains were then streaked to PCA and TSA. Fungal isolates were streaked onto MEA. All isolated strains were incubated at 25 ºC.

All strains were purified by subculturing and were stored in cryopreservation tubes as described by Feltham (Feltham *et al*., 1978). Each tube contained a media containing the following components per liter of medium: 1 g yeast extract (Difco), 5 g Tryptone

(Difco) and 15 % (vol/vol) of glycerol (Sigma). Pure cultures were taken from the petri plate using 10 µl sterile loops and then suspended on the sterile cryopreservation media. These suspensions were then stored at -80 ºC.

2.3.4 Determination of metal tolerance of the isolated population

The metal tolerance range for growth was determined on TSA solid medium. Cd, As, and Zn were tested using final metal concentrations ranging from 250 to 1000 mg L^{-1}. A mixture of the three metals was also tested using 250 mg L^{-1} of each metal. For determination of growth, pure cultures were spread onto TSA with different metal concentrations and then incubated at 25ºC for 72 h and observed daily. Growth was then evaluated as follows: –, no growth; +, poor growth; ++, good growth; and +++, exuberant growth, by comparison with control – media containing no metal.

2.3.5 Carbon utilisation

2.3.5.1 API 20 E

API® 20 E (bioMérieux, France) were developed as a microbial identification system for *Enterobacteriaceae* and other non-fastidious, Gram-negative organisms which uses 21 miniaturizes biochemical tests. The API 20 E strip consists of 20 microtubes containing different dehydrated substrates. These tests are inoculated with a bacterial suspension to test. During incubation, metabolism produces colour changes that are either spontaneous or revealed with the addition of reagents.

2.3.5.2 API 20 NE

API® 20 NE is a standardize system for the identification of non-fastidious, non-enteric Gram-negative bacteria combining 8 conventional tests, and 12

assimilation tests. It also can be used in environmental isolates to study their metabolic profiles.

The API 20 NE system consists of 20 microtubes containing dehydrated substrates. The conventional tests are inoculated with a bacteria suspended in Ringer solution which reconstitutes the media. During incubation, bacterial metabolism produces colour changes that are either spontaneous or revealed by adding the advised reagents. The assimilation tests are inoculated with a minimal medium (API® AUX) and bacteria will grow if they are capable of using the corresponding substrate.

2.3.5.3 API 50 CH

The API® 50 CH test strip allows the study of carbohydrate metabolism of bacteria. It consists of 50 microtubes containing an anaerobic zone for the study of fermentation and an aerobic portion for the study of substrate oxidation and assimilation. The first microtube is the negative control. The remaining tubes contain a given amount of dehydrated substrate, belonging to the carbohydrate and its derivates (heterosides, polyalcohols, uronic acids).

These substrates may be metabolized by different pathways:

1. assimilation is indicated by growth of the organisms in the cupule of the microtube
2. oxidation is shown by a colour change in the microtube cupule and its due to the aerobic production of acid detected by a pH indicator present
3. fermentation is indicated by a colour change in the tube portion of the microtube and is due to the anaerobic production of acid detected by the presence of a pH indicator

The API 50 CH system may be used to test any of the discussed biochemical pathways. The medium used for inoculation should be chosen according to the pathway to be studied and to the nutritional requirements of the organism to be tested.

2.4 Characterisation of the bacterial isolates recovered

2.4.1 Bacterial colony morphology

Colony morphology of the isolates was determined using a magnifying glass (WILD; Heerbrugg) after a 24 h incubation period at 25 ºC. Colony morphology and colour were registered.

2.4.2 Bacterial cell morphology

Bacterial cell morphology was determined with an optical microscope (Laborlux K, Leitz) of slides containing cultures of the isolated strains after a 24 h incubation period at 25 ºC.

2.4.3 Gram staining

Gram staining of the isolated strains was carried out using the Hucker method as described by Doestch (1981). Pure colonies were fixed to a glass slide. Slides were then covered by crystal violet (Merck) for one minute and gently rinsed with water. Gram iodine (Merck) was then applied for one minute and again rinsed with water. Slides were then washed with ethanol and after rinsed with water. They were then covered by safranin (Merck) for one minute and excess safranin rinsed with water.

2.4.4 Catalase

Catalase was determined as described by Smibert and Krieg (1981). A drop of H_2O_2 3 % (v/v) was placed in a slide bacterial cells were added. Presence of catalase was noticed by the formation of oxygen bubbles.

2.4.5 Cytochrome oxidase

The presence of cytochrome oxidase enzyme was determined as described by Smibert and Krieg (1981). A solution of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), (Sigma), 10 mg L^{-1} in distilled water. A few drops of the TMPD solution were placed in Watmann paper filter and a bacterial colony was added. Presence of the enzyme was noticed by the immediate appearance of a bluish colour

2.5 Molecular Biology

2.5.1 DNA extraction

Total bacterial DNA was extracted from pure cultures using a rapid method of extraction (*"Boiling Method"*); for some strains and a DNA extraction kit was used.

2.5.1.1 Rapid bacterial DNA extraction – *"Boiling Method"*

Bacterial DNA was extracted from pure cultures using an adapted protocol described by Sambrook (1989). One to three colonies of each bacterial isolate were taken with a sterile loop and suspended in 50 µl of ultra-pure water. The suspension was then incubated in a water bath at 100 ºC for 10 min. The bacterial suspension were briefly vortexed and placed in ice for 5 min. Suspensions were then centrifuged at 12000 ×g for 5 min using a bench centrifuge (Eppendorf Centrifuge 5415D). Cell

pellet was then discarded and the supernatant containing the DNA was transferred to a new tube and kept at -20 ºC (Wiedmann-al-Ahmad *et al*., 1994).

2.5.1.2 Bacterial DNA extraction

For some of the bacterial isolates the previous DNA extraction method was not effective. Genomic DNA Purification Kit (MBI Fermentas) is designed to isolate double-stranded DNA from Gram-positive and Gram-negative bacteria and other sources. The procedure requires 20-25 minutes, involves extraction only with chloroform and does not require phenol extraction nor proteinase digestion. DNA isolation is based on lysis of the cells with a subsequent selective DNA precipitation with detergent. The genomic DNA is concentrated and desalted by ethanol precipitation. DNA obtained by this method can be used for all molecular biology procedures.

200 µl of each bacterial sample was suspended in 400 µl of the lysis solution provided. Samples were then incubated for 5 min at 65 ºC. 600 µl of chloroform was then added to each sample and the tubes inverted 3-5 times. The upper aqueous phase containing the DNA was transferred to a new tube and 800 µl of the precipitation solution provided in the kit was added and mixed thoroughly for 2 min, after which a centrifugation step was carried out at maximum speed. The supernatant was then discarded completely. The DNA pellet was dissolved in 100 µl of NaCl and 300 µl of cold ethanol was added. The samples were then kept at -20 ºC for 10 min allowing the DNA to precipitate. Samples were centrifuged for 4 min and supernatant discarded. After the remaining ethanol evaporated 100 µl of sterile ultra-pure water was added to dissolve the DNA that was then kept at -20 ºC (Wiedmann-al-Ahmad *et al*., 1994).

2.5.2 Electrophoresis

2.5.2.1 Agarose gels and DNA visualisation

Electrophoresis through agarose gels is the standard method used to separate, identify and purify DNA fragments. Mobility of DNA fragments is determined by their size, thus larger fragments take more time to move through the gel. Therefore gels with high concentration of agarose were used to visualise small fragments of DNA and also to allow for a better separation of fragments (RAPD-PCR), whereas less concentrated gels were used for larger fragments (16 rRNA). To visualise DNA in agarose gels it is necessary to use fluorescent dyes (Sambrook *et al*., 1989). Ethidium bromide binds to DNA causing intense fluorescence. Ethidium bromide can be used to detect single and double-stranded nucleic acids. Ultraviolet light is absorbed by the DNA and transmited to the dye, resulting in a visible red colour.

DNA fragments were separated by electrophoresis in agarose gels ranging between 1 % and 2 % (depending on fragment sizes) and run in Tris-Acetic-EDTA (TAE) buffer (BioRad). Ethidium bromide was incorporated in the agarose gels used. The samples were mixed with a loading buffer (bromophenol blue). In each gel 3µl of 100 bp PCR Molecular Ruler (BioRad) was also added into the 1st and last wells. Samples were then run for 4 h at 60 V for Random Amplified Polymorphic DNA - PCR (RAPD-PCR) and for 45 min at 70 V for all other PCR's.

2.6 Polymerase Chain Reaction

The polymerase chain reaction (PCR) can multiply DNA molecules by up to a billion fold in the test tube, yielding large amounts of specific genes. PCR makes use

of the enzyme DNA polymerase, which copies DNA molecules. Oligonucleotides, *ie* short nucleic acid polymers typically with fifty or fewer bases, are designed to act as primers and bind to homologous regions of the DNA. A PCR cycle consists of three steps:

1. *Strand separation (denaturing)* – the DNA double strand is separated by heating the solution to 95 ºC;
2. *Hybridization of primers (annealing)* – the solution is cooled to 55 ºC to allow each primer to hybridize to a DNA strand;
3. *DNA systhesis (extension)* – the solution is then heated to 72 ºC, the optimal temperature for the *Taq* DNA polymerase. The polymerase elongates both primers in the direction of the target sequence because DNA synthesis is in the 5' to 3' direction.

These 3 steps constitute one cycle of the PCR amplification and can be carried out several times just by changing the temperature of the reaction mixture. The thermal stability of the polymerase makes it feasible to carry out PCR in a close container (e.g. "*eppendorf*" tube), no reagents are added after the 1st cycle. PCR has several features which makes a crucial technique in the amplification of DNA (Berg et al., 2002):

1. The sequence of the target may not be known, it is only required is the knowledge of the flanking sequences to amplify;
2. The target is usually much larger than the primers. Targets larger than 10 kb can be amplified by PCR;
3. Primers do not need to be perfectly matched to flanking sequences to amplify targets. Using primers derived from a known gene sequence it is possible to search for variations.

4. High specificity of the technique because of the stringency of hybrydisation at high temperature. Stringency relates to the necessary closeness of the match between target and primer.
5. High sensitivity. PCR had the ability to amplify a single DNA molecule.

2.6.1 Random Amplified Polymorphic DNA - PCR (RAPD-PCR)

The RAPD-PCR method was first used by Williams *et al*. (1990) to produce unique genetic fingerprints. This method uses short primers, usually 10 nucleotides long, to amplify multiple genomic loci, that by electrophoresis in agarose gels produce strain specific profiles. RAPD has proven to be a technique very versatile in the identification and also for the generation of fingerprints of nonclinical prokaryotic phylotypes (Ronimus *et al*, 1997).

i) RAPD-PCR protocol

Random amplified polymorphic DNA (RAPD) analysis was used to group bacterial isolates. Crude cell lysates were used as DNA templates. Amplification reactions were performed in a total volume of 25 µL containing: 0.75U *Taq* polymerase (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP) (Bioron), 0.6 µM primer OPA3 (MWG-Biotech) (5'-AGTCAGCCAC-3') and 0.5 µL of crude cell lysates. Samples were subjected to 45 cycles of amplification (Bio-Rad PTC-1148 MJ Mini Thermal Cycler), as follows 1 min at 94 ºC, 1 min at 34 ºC, 2 min at 72 ºC, and a final extension step of 10 min at 72 ºC. The fragments were analysed by electrophoresis in a 2 % agarose gel in Tris-acetate-EDTA buffer.

2.6.2 16S rRNA PCR

Pace *et al.* (1985) first proposed cultivation independent approaches to study microbial populations. Their approach consisted of the analysis of the 5S or 16S rRNA gene sequences. The development of the PCR technology and primer design that can be used to amplify almost entire genes enhanced the performance of this analysis. PCR amplification of environmental 16S RNA sequences, cloning the resultant amplicons and comparison of these sequences with sequences previously known allows their assignment to a given phylogenetic group encompassed in a phylogenetic tree. 16S rRNA PCR methods are simple, rapid and universally applicable.

i) 16S rRNA amplification protocol

The genomic DNAs were extracted for 16S rRNA gene sequence determination, as described in section 2.5.1 of this chapter. PCR amplification of the 16S rRNA gene was done as described by Rainey *et al.* (1996). Primers 27F (MWG) (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (MWG) (5'-TACCTTGTTACGACTT-3') were used to generate partial 16S rRNA gene sequences. Amplification reactions were performed in a total volume of 50 µL containing: 0.5 µL *Taq* polymerase (Promega, 5U), 5 µL mM $MgCl_2$ (1.5 mM), 0.4 mM of each deoxynucleoside triphosphate (dNTP) (Bioron), 0.5 µL of both primer 27F and 1492R, 1.25 µL of Dimethyl Sulfoxide [DMSO] (Sigma) and 2 µL of bacterial cell lysates. Samples were subjected to 30 cycles of amplification (Bio-Rad PTC-1148 MJ Mini Thermal Cycler), as follows 5 min at 95 ºC, 1 min at 94 ºC, 1 min at 55 ºC, 1 min and 30 sec at 72 ºC, and a final extension step of 10 min at 72 ºC. 16S

rRNA fragments were analysed by electrophoresis in a 1 % agarose gel in Tris-acetate-EDTA buffer.

ii) 16S rRNA purification

PCR products to be sequenced had to be subjected to a purification step, for that purpose a commercial kit was employed. Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) can be used to purify DNA from solution, typical recoveries are >80 % from solution. GFX purification of PCR products from solution removes 99.5 % of primers and free nucleotides and can be performed in less than 5 minutes. The purification was carried out following the manufacturer instructions. Sequencing of the purified PCR products was carried out by Macrogen Inc. – Seoul, South Korea.

2.7 Heavy metal analysis in removal assays

2.7.1 Heavy metal solutions

Stock solutions of cadmium, zinc and were prepared using $CdCl_2$ (Sigma) and $ZnCl_2$ (Sigma), with a 10000 mg L^{-1} concentration.

2.7.2 Atomic absorption spectrophotometry

Atomic absorption spectrophotometry was used to quantify heavy metals in solution in removal experiments. Atomic absorption spectrophotometry is a very sensitive technique used to analyse different compounds that are present in trace amounts including heavy metals. Atoms are excited by flame vaporization, and the radiation emitted when the atoms return to a ground state is measured in unexcited non-ionised molecules. The generation of ground state atoms of the test solution using

an air/ethylene flame is a key aspect in this technique. The atoms can be excited when a specific energy generated by a lamp made with the same material that is under analysis tracks them.

The spectrophotometer used for analysing the heavy metals was a Hitachi Z-8100 atomic absorption spectrophotometer with Zeeman correction. For the analysis performed the lamp current was 7.5A and the analysis wavelength for Cd, and Zn were 228.8 nm and 213.9 nm, respectively. Standards for Cd and Zn were prepared within the concentration range of each heavy metal and calibration curves determined. All samples were aspirated for at least 5 seconds before taking a reading. When the measurement showed high coefficient of variation, the system was re-calibrated. After the analysis of each run of samples, the system was checked using the original heavy metal solution corresponding to the heavy metal that was under analysis.

Chapter 3: Bacterial diversity in a heavy metal polluted site located in Northern Portugal

3.1 Introduction

In a worldwide sense pollution of soils, sediments and water with heavy metals has been an unfortunate by-product of industrialization, and consequently has become a global environmental issue. Agricultural and industrial activities have led to the release of high amounts of heavy metals that not only lead to deterioration of the soil for agriculture industry, but also threaten the depending fauna and flora and also human health through the food chain (Ansari and Malik, 2007). The increased deposition of heavy metals of anthropogenic sources in the environment has increased greatly and it is necessary to remediate contaminated soils.

As an example, the industrial activity of the region of Estarreja, Northern Portugal, over the years has contributed to make it one of the most contaminated regions in the country, due to both direct discharge of effluents in the nearby water streams and uncontrolled waste disposal over the soil (Costa and Jesus-Rydin, 2001). This polluted area presents high levels of metals, mainly zinc (Zn), but also lead (Pb) and arsenic (As) that are above the limits established by EC Directive 86/278/EC (Atkins, 1999; Oliveira *et al*., 2001). The effluents emitted by the Estarreja industry contained mainly aniline, benzene, monochlorobenzene and heavy metals. Solid waste by-products are stored inside the complex and consist of ±150.000 t of pyrite waste, containing mainly As, Pb, and Zn; ± 60.000 t of sludge contaminated with Hg; and 300.000 t of calcium hydroxide sludge (Costa and Jesus-Rydin, 2001).

Heavy metal contamination can have significant effects on the indigenous microbial populations. Heavy metal presence in the environments generally causes a decrease in the diversity and activity of soil microbial populations, and affects the ecological balance of population interactions within the community. Heavy metals may reduce species composition and limit microbial proliferation. Heavy metals also have been shown to affect microbial activity, such as nitrogen fixation in rhizobia (Wei *et al*., 2008). The toxicity of heavy metals results from the interaction of metals with enzymes and inhibition of bacterial metabolism (Gasic *et al*., 2006). Metal effects on natural soil communities are complex and difficult to characterise because of many complex factors involved. The existence of highly heterogeneous bacterial populations in soils means that the methodology to assess its activity has to be non-specific in order to include the various contributions from all the diverse group of organisms (Barros, *et al*., 2003). On account of the difficulties involved in cultivating heterotrophic bacteria, bacterial population composition have been lately characterized using culture-independent methods, such as 16S rRNA gene cloning, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment analysis (T-RFLP) (Eiler and Bertilsson, 2004; Kolmonen *et al*., 2004; Giovannoni and Stingl, 2005). However, these methods do not allow the characterisation of heterotrophic bacteria in the environment.

Heavy metal contaminated environments harbour organisms, both prokaryotes and eukaryotes, able to deal with pollution (Zettler *et al*., 2002; Baker and Banfield, 2003). The ability of some microorganisms to tolerate heavy metals and the ability of some to promote transformations that render them less toxic, make organisms that live in heavy metal contaminated sites potentially useful in bioremediation.

Bioremediation strategies are dependent on the knowledge of the *in-situ* microbial diversity targeting the most resistant strains. By characterising the microbial communities, the taxa able to survive and remain active in extreme environments can be identified and potentially targeted for bioremediation purposes (Akob *et al*., 2007). In order to optimize and develop remediation processes, more studies about the bacterial populations that inhabit these sites are required.

The purpose of this study was to isolate and phylogenetically characterize the heterotrophic aerobic cultivable populations present in the contaminated study area. Moreover heavy metal tolerance of the isolated populations was also tested.

3.2 Materials and methods

3.2.1 Samples and sampling site

Soil samples were recovered at Estarreja, Portugal. A large industrial complex surrounds the area considered in the present study. Soil was collected near a stream "Esteiro de Estarreja" in three different locations.

i) Site 1- located on the banks of a stream, mainly contaminated with Zinc (Zn), average 898.9 mg/kg, Arsenic (As), average 1495.9 mg/kg, and Lead (Pb), average 835.4 mg/kg (Oliveira *et al*., 2001).

ii) Site 2- located on a 10-ha dry sediment deposit resulting from the production of polyvinylchloride (PVC).

iii) Site 3- located upstream from site 1, periodically flooded soil with a pH of 4.5/5 to a depth of 10 cm. It shows high levels of contamination by As, average 237,4

mg/kg, Zn, average 39.2 mg/kg and lead (Pb), average 18.2 mg/kg (Oliveira *et al*., 2001).

Chemical characterisation performed by a recognized state laboratory (DPM, Lisbon, Portugal) is presented in table 3.1 with permission. Potassium (K), sodium (Na), magnesium (Mg), calcium (Ca), copper (Cu), iron (Fe), nickel (Ni), chromium (Cr), Zn, lead (Pb) and mercury (Hg) were determined using standard atomic absorption spectrometry techniques described by Pratt. As was measured colorimetrically (Hesse, 1971). The method to determine Nitrogen (N) and Phosphorous (P) were based on Houba *et al*. (1995). The pH was determined in water and the electrical conductivity in ultra-pure water.

Table 3.1 Chemical properties of the soil from the three assessed sites.

Chemical properties	***Site 1***	***Site 2***	Site 3
Conductivity (µS/cm)	278.0 b	5,500.0 b	258.0 b
pH (H_2O)	7.1–8.1 b	12.6 b	4.1 b
Total organic carbon (g/kg)	46.5 a	5.1 c	40.0 d
N (g/kg)	2.9 b	0.3 c	0.6 b
P (mg/kg)	4.6 b	36.0 c	12.5 b
K (mg/kg)	151.0 b	12.0 b	3.8 d
Na (mg/kg)	2,500 b	51,000 c	3.1 d
Mg (mg/kg)	0.4 b	0.7 c	29.6 d
Ca (mg/kg)	0.1 b	48,100 c	28.2 d
Total metals (mg/kg)			
Zn	898.9 (125–3,620) a	8.7 c	39.2 d
Pb	835.4 (16–3,740) a	71.3 c	18.2 d
Hg	66.6 (0.30–275) a	0.1 c	3.5 (0.73–8.90) a
As	1495.9 (45–5,620) a	<0.9 b	237.4 (35–955) a
Cr	26.0 b	17.3 c	7.0 b
Ni	37.3 b	4.3 c	3.5 d
Cu	0.0 b	23.0 c	10.0 d
Fe (g/kg)	16.8 b	1.3 c	9.2 d

a From Atkins (1999) (average and range); **b** Determined during this study; **c** From Balsa *et al*. (1996); **d** From Leitão *et al*. (1994)

Soil samples for bacterial analysis were recovered directly from the different sub-sites on two occasions, March 2006 and April 2006. Soil samples were then transported to the laboratory at 4 ºC and were assayed within 12 h of sampling.

3.2.2 Enumeration and isolation of the heterotrophic populations

For the isolation and enumeration of the bacterial populations, samples were serially diluted and plated onto different isolation media, and the plates were then incubated at 25 ºC for up to 10 days. The cultures were observed daily for enumeration, and all different morphological colony types were isolated. Isolates were purified by sub-culturing on the corresponding isolation media with 15 % (vol/vol) glycerol and were stored at –80 ºC.

3.2.3 Isolation media

The following media was used: plate count agar (PCA) (Pronadisa), trypticase soy agar (TSA) (Pronadisa), alkaline buffered medium 2 (ABM2), and malt extract agar for fungi isolation (MEA) (Oxoid). The media was modified by adjusting the pH of each of the basic compositions to four different pH values 5, 6, 7 and 10 (for site 2 only), by using buffer solutions at a final concentration of 100 mM.

3.2.3.1 Media composition and preparation

PCA, MEA and TSA were prepared according to the manufacturer's instructions, but 100 ml of a defined buffer solution at a concentration of 1 M was added in a final volume of 1 liter of media. ABM2 was prepared as described in chapter 2, section 2.4.1.

The buffer solutions were autoclaved separately from the other components of the different media and were mixed after cooling to 45 ºC.

3.2.3.2 Buffer solutions

Different buffer solutions were used to adjust the media to different pH values in order to isolate a wide variety of organisms with different environmental requirements. All solutions were prepared according to Gomori (1990) and were autoclaved separately. The following buffer solutions were used to adjust the media to the different pH values: citrate buffer to adjust the different media to pH 5 and 6, phosphate buffer to adjust the different media to pH 7, and carbonate-bicarbonate buffer to adjust the different media to pH 10.

3.2.4 RAPD analysis of isolates

Random amplified polymorphic DNA (RAPD) analysis was used as a primary method to group the isolates. Crude cell lysates were used as DNA templates, as described by Widmann-al-Ahmad *et al*. (1994). The RAPD protocol is described in chapter 2, section 2.6.1.

3.2.5 Morphological and biochemical characteristics of representative strains

Cell morphology was examined by phase-contrast microscopy after cultivation on PCA and TSA. Gram staining was determined as described by Doestch (1981), cytochrome-oxidase and catalase activities were determined as described by Smibert and Krieg (1981).

3.2.6 Phylogenetic analysis of representative bacterial strains

The extraction of genomic DNA for 16S rRNA gene sequence determination, and PCR amplification of the 16S rRNA gene was carried out as described by Rainey

et al. (1996), as described in section 2.7.2.. Sequencing of the purified PCR products was performed by Macrogen Inc. (Seoul, Republic of Korea).

For phylogenetic analyses, the sequences were aligned by using the Clustal-X program (Thompson *et al*., 1997). Trees were constructed with PAUP* version 4.0b10 (Swofford, 2003), using the neighbour-joining method (Kimura two-parameter distance optimized criteria). For each isolate, sequences from the closest isolated and identified strains based on a BLAST search (Altschul *et al*., 1997) of GenBank were selected as reference sequences. The robustness of the phylogenetic tree was confirmed by using bootstrap analysis based on 1000 resamplings of the sequences.

3.2.7 Determination of heavy metal tolerance of the representative strains of the isolated population

As and Zn were the heavy metals more prevalent on site and they were selected for the heavy metal tolerance tests. Although not analysed Cd was also selected for the metal tolerance tests due to the fact of being a metal closely associated to Zn in cases of metal contamination and also due its toxicity even at low levels.

The heavy metal range for growth was determined on solid media. For comparison purposes, a media on which all of the representative strains could grow was chosen. For that reason TSA buffered to pH 7 was used. Media preparation was carried out as described previously.

For determination of growth, 24 h cultures were ressuspended in saline solution (0.85 %, w/v, NaCl) to a turbidity equivalent to a McFarland no. 2 standard (Smibert and Krieg, 1981). 0.1 ml were spread onto TSA agar adjusted to pH 7 and then incubated

at 25 ºC for 72 h. Growth was then quantitatively described as follows: -, no growth; +, poor growth; ++, good growth; and +++, exuberant growth.

3.3 Results

3.3.1 Enumeration of the microbial populations

The soil on the study sites had a chemical composition dominated by heavy metal ions mainly in sub-site 1 and 3. Total organic carbon was very low on site 2. Sub-site 1 presented a pH of around 7, and sub-site 3 a pH of 4. On the other hand site 2 was highly alkaline (pH 12.6). Chemical characterization of the soil is presented in detail in Table 3.1.

The number of heterotrophic bacterial populations recovered was dependent on the media composition. The Colony Forming Units (CFU) varied between 6.58 CFU Log_{10}/g (for MEA medium buffered at pH 7 and incubated at 25 ºC) and 7.69 CFU Log_{10}/g (for TSA buffered at pH 7 and incubated at 25 ºC) for sub-site 1. CFU's at sub-site 3 varied from 5.52 CFU Log_{10}/g (for MEA medium buffered at pH 6 and incubated at 25 ºC) and 7.51 CFU Log_{10}/g (for TSA buffered at pH 6 and incubated at 25 ºC). These values were obtained for the first isolation period. During the second isolation period a small variation in CFU's was observed (Table 3.2). The highest levels of recovery, reaching 7.69 and 7.16 CFU Log_{10}/g, were verified in TSA and ABM2 both with pH7, respectively. No heterotrophic bacteria were recovered for site 2 in the media used. Lower counts were registered for fungi/yeasts on MEA (Table 3.2), and again no colonies were recovered in plates inoculated with samples derived from site 2.

Table 3.2 Microbial CFU's per weight

Media	Sub-Site 1 CFU_{10}/g		Sub-Site 2 CFU_{10}/g		Sub-Site 3 CFU_{10}/g	
	Samplings					
	1st	2nd	1st	2nd	1st	2nd
PCA	6.90	5.96	NG	NG	7.06	7.04
TSA	7.69	6.98	NG	NG	7.51	6.89
ABM2	7.16	6.06	NG	NG	7.12	6.12
MEA	6.58	5.47	NG	NG	5.52	5.69

NG, no growth detected

3.3.2 Bacterial isolation

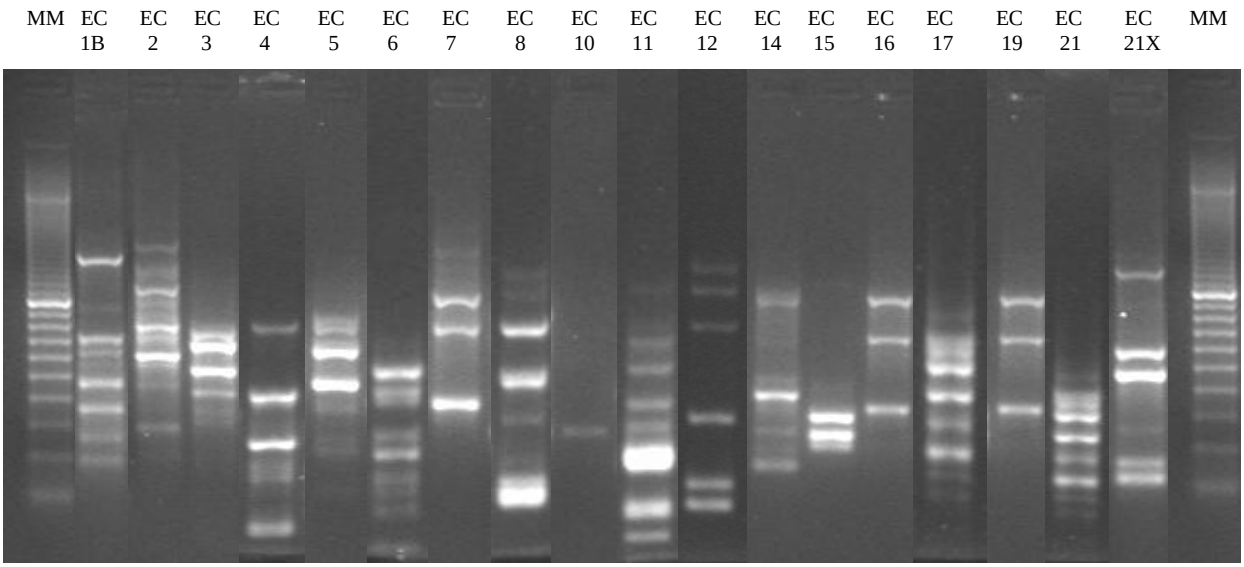
Throughout the sampling periods, a total of 278 bacterial strains were recovered from site 1 and 3. 85 strains were isolated from site 1 (labelled EA*, EB†, and EL* – *E=Estarreja, A & L=site 1, TSA, ABM2; † E=Estarreja, B=site 1 PCA;, numbers represent the isolate number).

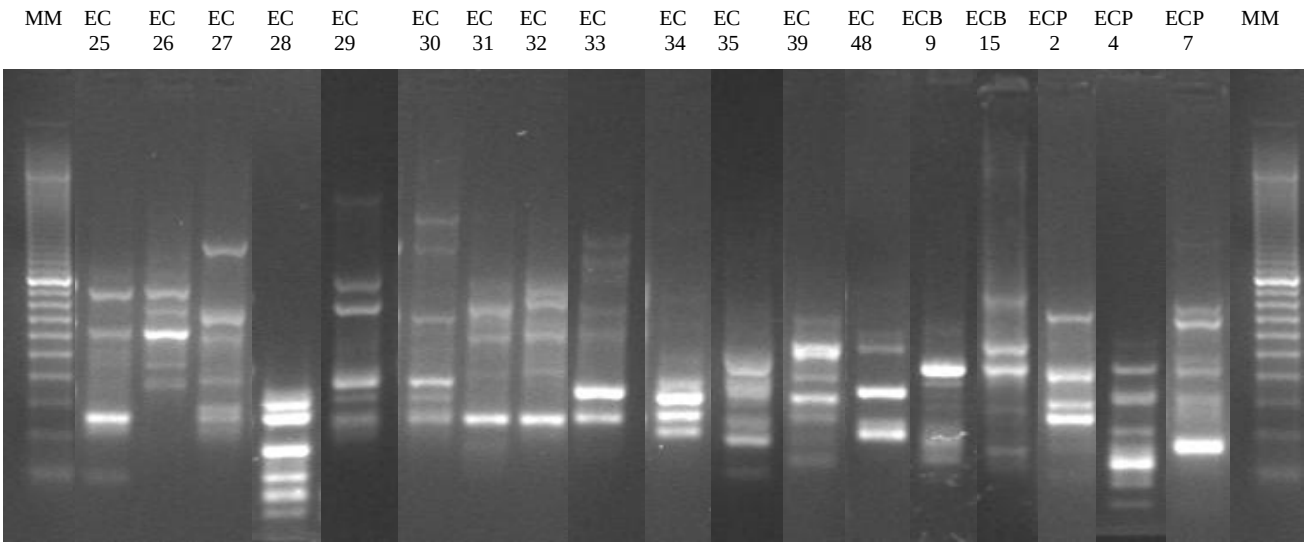
193 strains were isolated from site 3 (labelled EC‡, ECP♯, ECX‡, S3ᵊ, ED‡ and EDP♯ – ‡E=Estarreja, C-D & X= site 3, TSA, ABM2; ᵊS3=Site 3, ABM2; ♯E=Estarreja, C & D=site 3, P=PCA, numbers represent the isolate number). No viable isolates were recovered from sub-site 2 in any of the sampling stages. The strains were subcultured in media – PCA, TSA and ABM2 – different from that in which they were isolated initially. Interestingly, the only media capable of supporting the growth of the totality of the isolates was TSA, so for comparative purposes this media was used to grow the isolates for further characterisation.

3.3.3 Grouping the bacterial isolates

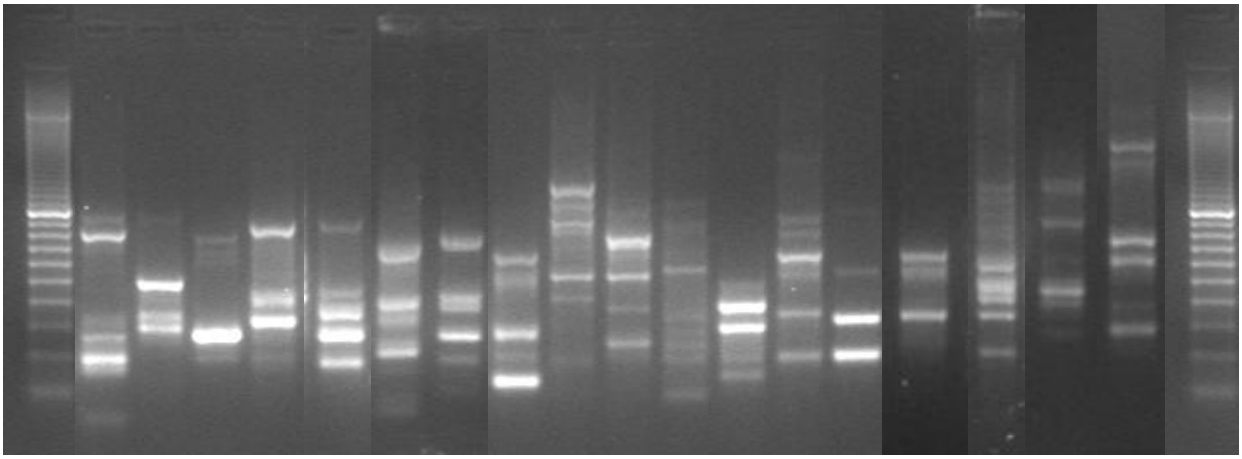
Primary grouping of the 278 isolates was performed by RAPD analysis in an attempt to find similar strains that may have been isolated several times. Using this approach 103 different RAPD profiles were recognized (Fig 3.1). This procedure together with

analysing the morphological and biochemical characteristics of the isolates, made it possible to identify strains with the same RAPD patterns. On the other hand, some strains had similar morphological and biochemical characteristics, but had distinct RAPD patterns. Several bacterial strains (18) had RAPD profiles that showed no bands or had very light ones In order to choose representative strains of the isolates recovered and to ensure the preservation of the diversity, further characterization of all of the types with different RAPD profiles was made.

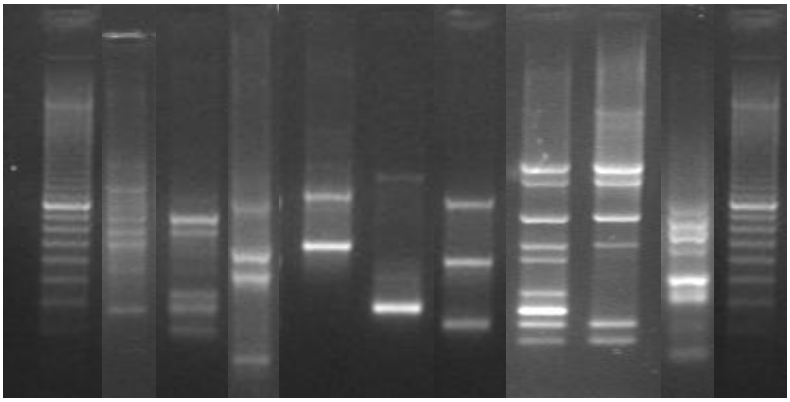




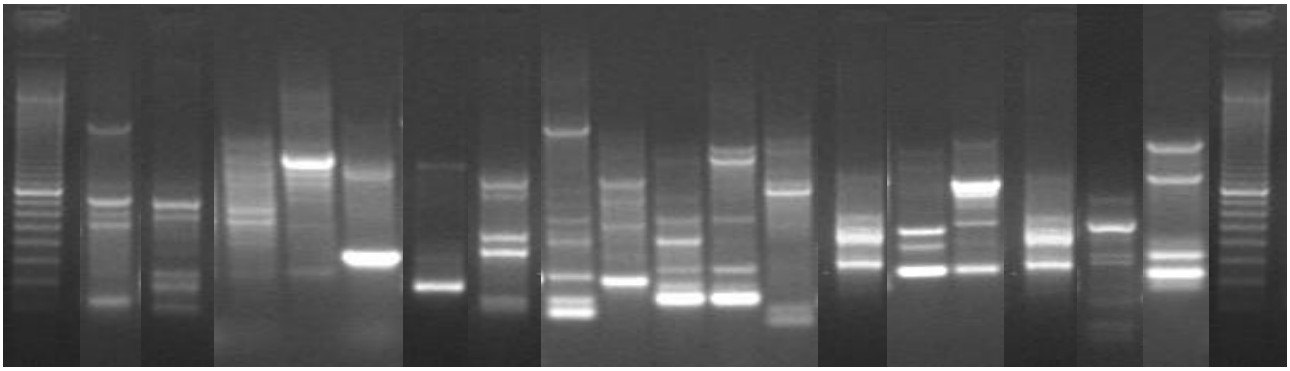
MM ECP 12 ECP 15 ECP 17 ECP 18 ECP 23 ECP 27 ECP 28 ECP 29 ECP 31 ECP 36 ECP 37 ECP 38 ECP 39 ECP 40 ECP X8 ECX 2 ECX 16 ECX 24 MM



MM ED37 ED50 EDP 4 EDP 10 EDP 24 EDP 28 EDP 31 EDP 33 S3X MM



MM EA2 EAL EAMR EAO EAP 2 EAP 3 EAP A1 EAP AA EAP C2 EAP C5 EAP C6 EAP C8 EAP D EAP E EAP M EAP N EAR EAX Y4 MM



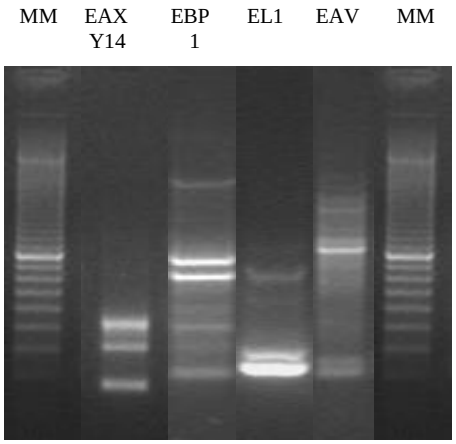


Figure 3.1 RAPD profiles of representative strains of the isolated populations (not all RAPD profiles are shown). – MM: molecular ruler marker. Site 1: EA; EB; EL. Site 3: EC, ECP, ECX, S3, ED and EDP.

3.3.4 Characterisation of the recovered populations

Characterisation of the 103 isolates with different RAPD profiles is shown in Table 3.4. The diversity found in cell and colony morphology and in colony pigmentation was not very significant. From the representative strains of the isolated population 55 phylotypes are Gram-negative and 48 phylotypes are Gram-positive. Interestingly, a large part of the representative strains (45 phylotypes) were isolated at pH 5-6 (sub-site 3) and not surprisingly the rest of the strains were isolated at neutral pH.

Table 3.3 List of the representative isolates and their closest relatives identified by 16S rRNA

Isolate[1]	Phylogenetic group	Closest relatives		
		Identification (NCBI/BLAST assession number)	Source	Similarity (%)
EC1B	*α-Proteobacteria*	*Agrobacterium tumefaciens* strain ORS 3405 (EF054875)	Soil	100
EC2	*γ-Proteobacteria*	*Shewanella baltica* OS223 (CP001252)	Unavailable	99
EC3	*γ-Proteobacteria*	*Shewanella* sp. MK03 (AY690713)	Rhizosphere soil	99
EC4	*β-Proteobacteria*	*Alcaligenes* sp. S-SL-5 (FJ529025)	Contaminated soil of oilfield	99
EC5	*Actinobacteria*	*Arthrobacter* sp. strain CMPC5 (AM936058)	Hydrocarbon-contaminated soil	99
EC6	*β-Proteobacteria*	*Alcaligenes* sp. L-1B (EF059713)	Soil	100
EC7	*γ-Proteobacteria*	*Pseudomonas stutzeri* strain 13636M (EU741093)	Beach sand	100
EC8	*γ-Proteobacteria*	*Pseudomonas* sp. strain AKB-2008-HE5 (AM989275)	Sea water	98
EC10	*Actinobacteria*	*Arthrobacter* sp. strain IK1_95 (AB461069)	Soybean stem	100
EC11	*γ-Proteobacteria*	*Pseudomonas* sp. ARCTIC-P37 (AY573031)	Arctic ice	100
EC12	*γ-Proteobacteria*	*Pseudomonas guineae* LMG 24017.(AM491811)	Antarctic soil	96
EC14	*γ-Proteobacteria*	*Rhodanobacter spathiphylli* strain B39 (AM087226)	Rhizosphere	99
EC15	*Bacilli*	*Bacillus* sp. K22-25 (EU333888)	Soil from cold desert	99
EC16	*Actinobacteria*	*Microbacterium oxydans* strain Hg3 (FJ228159)	Iron mining site sediment	100
EC17	*β-Proteobacteria*	*Achromobacter* sp. B02-07F (FJ542961)	High ergovaline treatment gut	99
EC19	*Bacilli*	*Bacillus weihenstephanensis* strain P2-14 (AY277557)	Hypogean environment	99
EC21	*β-Proteobacteria*	*Alcaligenes* sp., isolate 159 (AJ002804)	Gut of the soil microarthropod	99
EC21X	*α-Proteobacteria*	*Brevundimonas diminuta* strain ATCC 19146 (EU497053)	Unavailable	100
EC25	*Actinobacteria*	*Curtobacterium* sp. S22 (GU120656)	Soil from K-feldspar mine	99
EC26	*Bacilli*	*Bacillus* sp. PCWCS37 (GQ284389)	Sediment of natural spring	100
EC27	*Actinobacteria*	*Leifsonia* sp. WPCB149 (FJ006907)	Freshwater of Woopo wetland	99
EC28	*Actinobacteria*	*Arthrobacter* sp., isolate 21S1 (AM237085)	Rhizosphere soil	99
EC29	*Actinobacteria*	*Microbacterium oxydans* strain L2 (GQ923776)	Soil	98
EC30	*β-Proteobacteria*	*Achromobacter* sp. ZH24 (HM103344)	Agricultural wastes	100
EC31	*Actinobacteria*	*Arthrobacter* sp. 95-0188 (EU086814)	Human clinical specimens	99
EC32	*Actinobacteria*	*Arthrobacter rhombi* strain 11-2 (FJ795684)	Air adjacent to aerial slurry spraying	99
EC33	*Actinobacteria*	*Arthrobacter* sp. sptzw42 (GU377126)	Soil	100
EC34	*Actinobacteria*	*Rhodococcus erythropolis* isolate M14-4 (EF690428)	Rhizosphere	99
EC35	*Actinobacteria*	*Rhodococcus* sp. S43 (GU731245)	Soil enrichment culture with As	99
EC39	*Bacilli*	*Bacillus* sp. strain 01105 (EU520307)	Unavailable	99

EC48	*Actinobacteria*	*Microbacterium* sp. JJD-1 (FJ765512)	Slaughterhouse waste soil	100
ECB9	*γ-Proteobacteria*	*Dyella* sp. 528F-2 (EU872214)	Petroleum polluted soil	100
ECB15	*γ-Proteobacteria*	*Dyella* sp. ATSB10 (EF397574)	Plants	97
ECP2	*γ-Proteobacteria*	*Acinetobacter lwoffii* strain ES-306b (FN393792)	Spacecraft	100
ECP4	*Actinobacteria*	*Arthrobacter aurescens* strain 93-0734 (EU086812)	Human clinical specimens	100
ECP7	*Actinobacteria*	*Arthrobacter* sp. Cr6-08 (GU784867)	Soil	98
ECP12	*Actinobacteria*	*Curtobacterium* sp. 2340 (AY688358)	Human clinical specimens	99
ECP15	*Bacilli*	*Bacillus* sp. MK13 (EF173317)	Hydrocarbon-contaminated soil	100
ECP17	*β-Proteobacteria*	*Pusillimonas* sp. DCY25T (EF672088)	Unavailable	97
ECP18	*Actinobacteria*	*Arthrobacter* sp., strain B2-6.(AJ785759)	Waste water	99
ECP23	*Actinobacteria*	*Arthrobacter* sp. AG3-7 (GU332615)	Longshan potassium mine	100
ECP27	*Actinobacteria*	*Microbacterium* sp. H102 (EF204421)	Raw milk	100
ECP28	*Actinobacteria*	*Curtobacterium pusillum* strain I3 (DQ086779)	Potato tubers	97
ECP29	*α-Proteobacteria*	*Sphingomonas* sp. KH3-2 (AF282616)	Unavailable	99
ECP31	*α-Proteobacteria*	*Sphingomonas* sp. BP-7 (AB276370)	Seawater	99
ECP36	*γ-Proteobacteria*	*Ewingella americana* GTC1277 (AB273745)	Unavailable	99
ECP37	*Flavobacteria*	*Chryseobacterium marinum* strain IMCC3228	Antarctic seawater	96
ECP38	*Bacilli*	*Paenibacillus* sp. R1-12-4 (GU457786)	Soil	96
ECP39	*γ-Proteobacteria*	*Frateuria* sp. Bio32 (AB467342)	Acid sulfate soil	99
ECP40	*Actinobacteria*	*Janibacter* sp. HG106 (FM878648)	Intestinal content from seahorses	99
ECPX8	*Flavobacteria*	*Chryseobacterium taeanense* strain PHA3-4 (AY883416)	Rhizosphere	97
ECX12	*Bacilli*	*Bacillus* sp. CH3 (AM711563)	Andean wetlands	100
ECX16	*Actinobacteria*	*Arthrobacter* sp. 210_5 (FJ938116)	Soil	99
ECX24	*Actinobacteria*	*Arthrobacter* sp. GG7a (GQ332346)	Soil	99
ED37	*γ-Proteobacteria*	*Pseudomonas* sp. LD118 (AM913943)	Brown alga	100
ED50	*γ-Proteobacteria*	*Dyella ginsengisoli* strain LA-4 (EF191354)	Unavailable	99
EDP4	*γ-Proteobacteria*	*Pseudomonas koreensis* strain MS200 (HQ58933)	Unavailable	99
EDP10	*Flavobacteria*	*Chryseobacterium vrystaatense*, strain R-23533 (AJ871398)	Chicken meat	98
EDP24	*γ-Proteobacteria*	*Rhodanobacter* sp. GR18-2 (FJ821730)	Ginseng soils	99
EDP28	*γ-Proteobacteria*	*Pseudomonas* reactans NO8 (FJ972537)	Soil	99
EDP31	*Actinobacteria*	*Rhodococcus erythropolis* strain MJ2 (GU991529)	Soil	99
EDP33	*Actinobacteria*	*Rhodococcus* sp. P15D9 (AM942744)	Rhizosphere of wheat	100
S3X	*γ-Proteobacteria*	*Pseudomonas fluorescens* strain LMG 14677 (GU198127)	River water	100
EA2	*Bacilli*	*Bacillus pumilus* strain MLA14 (EF462914)	Unavailable	100
EAL	*Bacilli*	*Bacillus* sp. SYW2 (FJ601632)	Soil	99

EAMR	*Bacilli*	*Bacillus subtilis* strain B-A (GU980963)	Bagasse	97
EAO	*Bacilli*	*Bacillus* sp. HXG-C6 (GU257957)	Petroleum-contaminated soil	99
EAP2	*Bacilli*	*Bacillus* sp. 'kanghwensis' (AY172987)	Unavailable	99
EAP3	*Bacilli*	*Bacillus* sp. PAMU-1.13 (AB118223)	Unavailable	100
EAPA1	*γ-Proteobacteria*	*Pseudomonas* sp. HMPB1(AM745260)	Unavailable	100
EAPAA	*Actinobacteria*	*Arthrobacter nicotinovorans* THWCSN6 (GQ284335)	Sediment sample of natural spring	100
EAPC2	*γ-Proteobacteria*	*Acinetobacter* sp. KSL5401-052 (GQ497287)	Sewage sludge	99
EAPC5	*Bacilli*	*Bacillus* sp. SYW2 (FJ601632)	Soil	100
EAPC6	*γ -Proteobacteria*	*Acinetobacter* sp. H1(AY663435)	Soil	97
EAPC8	*γ-Proteobacteria*	*Pseudomonas putida* ZFJ-9 (EU931564)	Sugarcane roots	99
EAPD	*Bacilli*	*Bacillus* sp. SGE72 (HM566773)	Soil	99
EAPE	*γ-Proteobacteria*	*Pseudomonas* sp. HMPB1(AM745260)	Unavailable	100
EAPM	*Actinobacteria*	*Arthrobacter* sp. 16.43 (DQ157988)	Xylene and chromate enriched soil	99
EAPN	*γ-Proteobacteria*	*Pseudomonas* sp. IBUN S1901 (DQ813328)	Cultivated soil with sugarcane	99
EAR	*Bacilli*	*Lysinibacillus fusiformis* xfru5 (GQ480497)	Fermentative pit - liquor	99
EAXY4	*γ-Proteobacteria*	*Acinetobacter* sp. strain bk_13 (HQ538659)	Sludge	99
EAXY14	*γ-Proteobacteria*	*Acinetobacter* sp. XMZ-26 (GQ227698)	Glacier soil	98
EBP1	*Bacilli*	*Bacillus cereus* clone B11 (GU003828)	Unavailable	100
EL1	*γ-Proteobacteria*	*Pseudomonas* sp. JLF-a390 (FM201274)	Host plant	99
EAV	*γ-Proteobacteria*	*Pseudomonas* sp. BIM B-86 (GU784939)	Unavailable	97

[1] Strains from **site 1** (EA[*]; EB's; EL) [*]E=Estarreja, A & L=site 1, TSA, ABM2; [†] E=Estarreja, B=site 1 PCA;, numbers represent the isolate number**.**
Strains from **site 3** (EC[‡], ECP[♯], ECX[‡], S3[♲], ED[‡] and EDP[♯] - [‡]E=Estarreja, C-D & X= site 3, TSA, ABM2; [♲]S3=Site 3, ABM2; [♯]E=Estarreja, C & D=site 3, P=PCA, numbers represent the isolate number

3.3.5 Phylogenetic analysis of the recovered populations

The majority of the isolates recovered on sub-site 1 were related to the class *Bacilli* and γ-*Proteobacteria* class (Fig. 3.2-a). The majority of the isolates were related to *Bacillaceae* and to *Pseudomonadaceae* family members. These strains were associated with the genera *Bacillus*, *Lysinibacillus* (Fig. 3.2-b) and to *Pseudomonas* and *Acinetobacter* (Fig. 3.2-c), respectively. Other isolates belonged to the *Actinobacteria* and β-*Proteobacteria* classes presenting a relationship with species of the genera *Arthrobacter* and *Alcaligenes* respectively (Fig. 3.2).

The values of 16S rRNA gene sequence similarity between the isolated populations from sub-site 1 and the described taxa were generally high (98-100 %). 3 strains – EAL; EAPC6 and EAV– showed a calculated similarity of the 16S rRNA gene of only ~97 % with the described taxa (Table 3.3). The phylogenetic affiliation of the recovered isolates is shown in Table 3.3 and Figure 3.6 and 3.7.

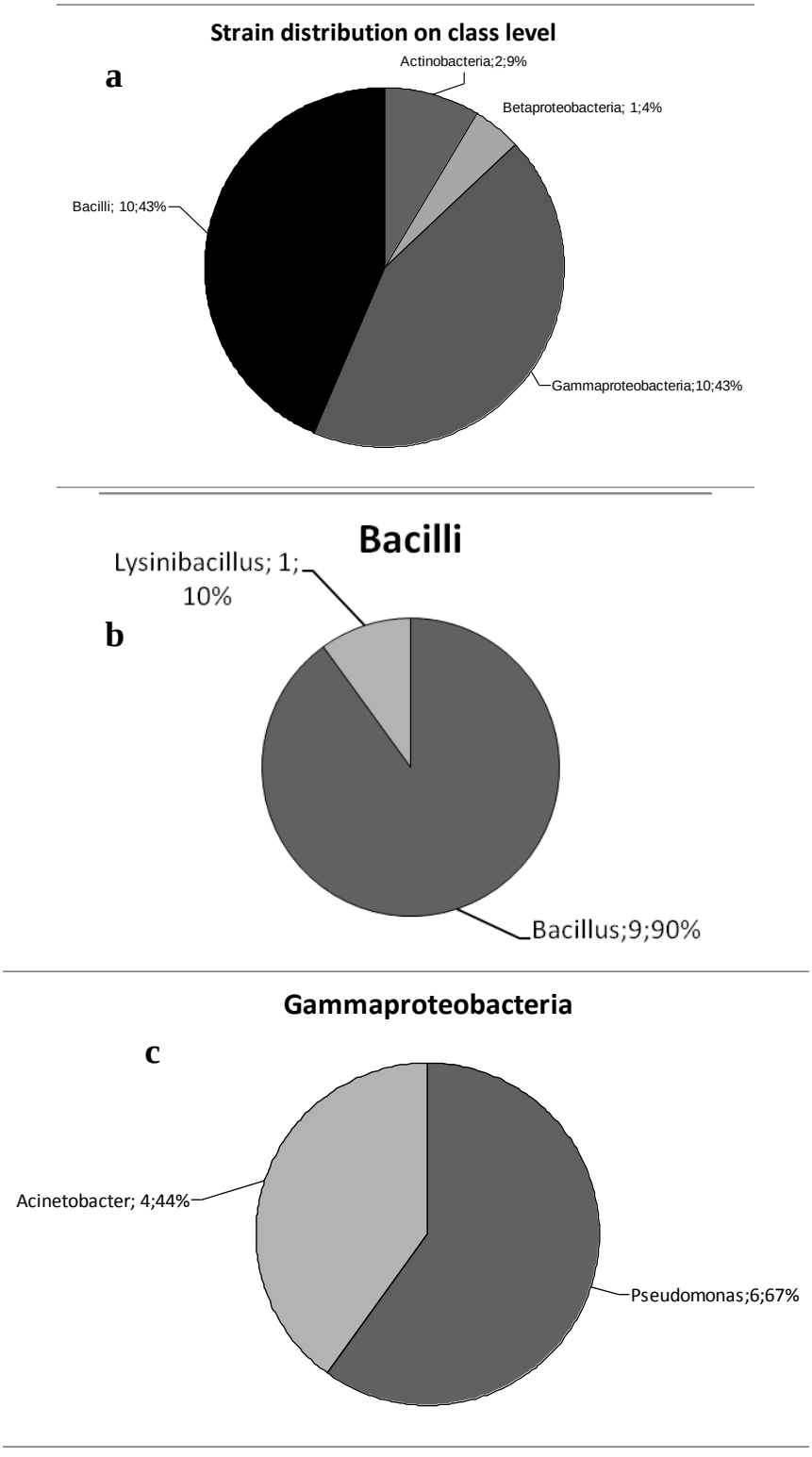


Figure 3.2 Proportions of taxonomic groups represented by the bacterial strains isolated from sub-site 1. The sequences of the isolated bacteria were assigned to class (a) and genus level (b-c). Class Actinobacteria and β-*Proteobacteria* included only one genus and is not shown as a separate pie. The number of isolated strains and their percentage per taxa are given together with the class or genus.

On sub-site 3 the gram-positive isolates were related to the class *Actinobacteria* and class *Bacilli* (Fig. 3.3). The majority of the isolates were related to families *Microbacteriaceae* and *Bacillaceae*. These strains were associated with the genera *Arthrobacter*, *Microbacterium*, *Leifsonia*, *Curtobacterium*, and *Bacillus* (Fig. 3.4). Other isolates, although belonging to *Actinobacteria* and *Bacilli*, presented a relationship with some representatives of the genera *Rhodococcus*, *Janibacter*, *Oerskovia*, and *Paenibacillus* (Fig. 3.4). Values of 16S rRNA gene sequence similarity between isolate ECP38 and genera from the *Bacilli* class was only 96 % when compared with the high sequence similarities presented by the majority of the isolates (Table 3.3). 16S rRNA gene sequence similarity of the gram-negative isolates on sub-site 3 has shown that they were related to classes *γ-Proteobacteria, β-Proteobacteria, α-Proteobacteria, and Flavobacteria* (Fig. 3.3). The most frequent class was *γ-Proteobacteria*, related with some genera within the families *Shewanellaceae, Pseudomonadaceae, Xantomonadaceae, and Enterobacteriaceae* with a relationship with species of genera *Pseudomonas, Rhodanobacter, Shewanella, Dyella, Ewingella*, and *Frateuria* (Fig. 3.5). Values of 16S rRNA gene sequence similarity between isolates EC12 and ECB15, and the described taxa were about 96 and 97 %, respectively (Table 3.3). *β-Proteobacteria* were also well represented, with isolates related to the families *Alcaligenaceae*, *Comamonadaceae* and *Bulkholderiaceae* and closely related to the lineage containing *Alcaligenes, Pusillimonas* and *Achromobacter*. Values of 16S rRNA gene sequence similarity between isolate ECP17 and genera from the *Alcaligenaceae* class was only ~97 % (Table 3.3). *α-Proteobacteria* had representatives of families *Rhizobiaceae, Sphingomonadaceae,* and *Caulobacterales,* related to some taxa within the genera *Brevundimonas, Sphingomonas,* and *Agrobacterium* (Fig. 3.5). *Flavobacteria* was the

least represented class in the population with families *Flavobacteriaceae*, represented by isolates affiliated to genera *Chryseobacterium*.

Several isolates (ECP37 and ECPX8) were related to family *Flavobacteriaceae* of the class *Flavobacteria* (Table. 3.3). Values of 16S rRNA gene sequence revealed similarity between isolate ECP37 with other *Flavobacteria* from the genus Chryseobacterium was 97 %.

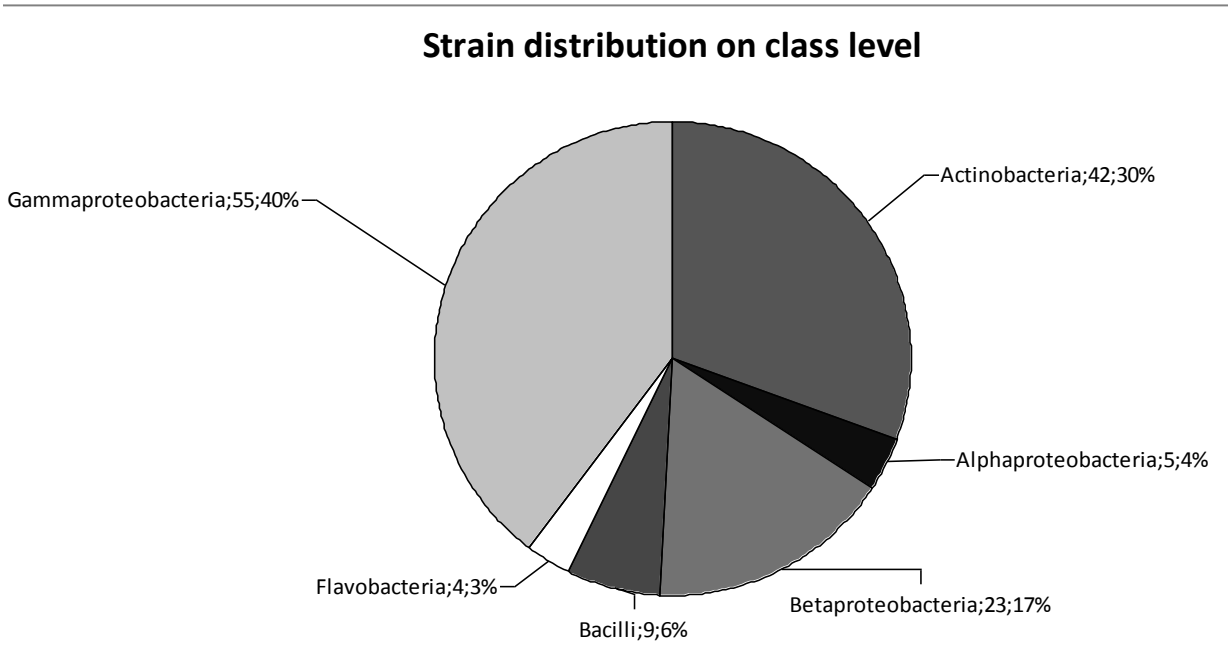
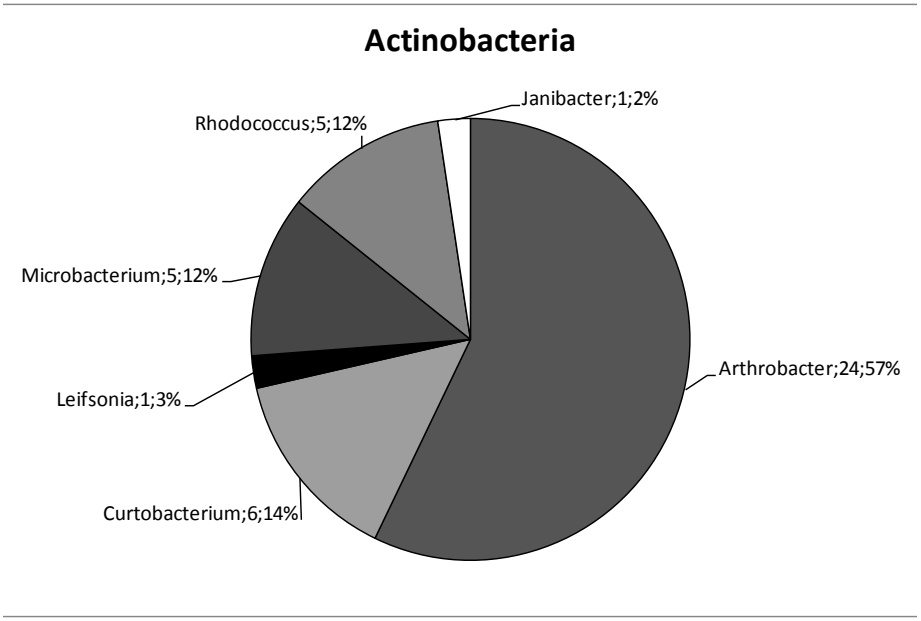


Figure 3.3 Proportion on a class level of the bacterial strains isolated from sub-site 3. The number of isolated strains and their percentage per taxa are given together with the class.



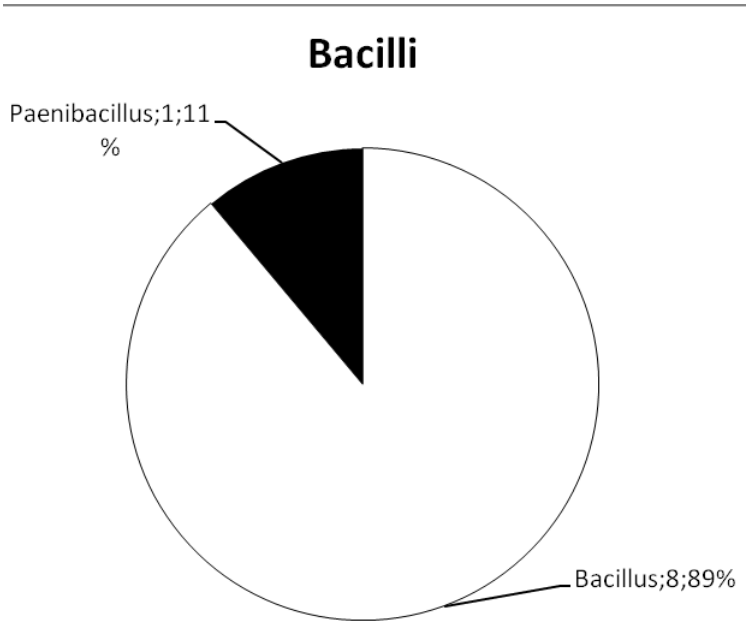


Figure 3.4 Proportions of taxonomic groups represented by the Gram-positive bacterial strains isolated from sub-site 3. The number of isolated strains and their percentage per taxa are given together with the genus.

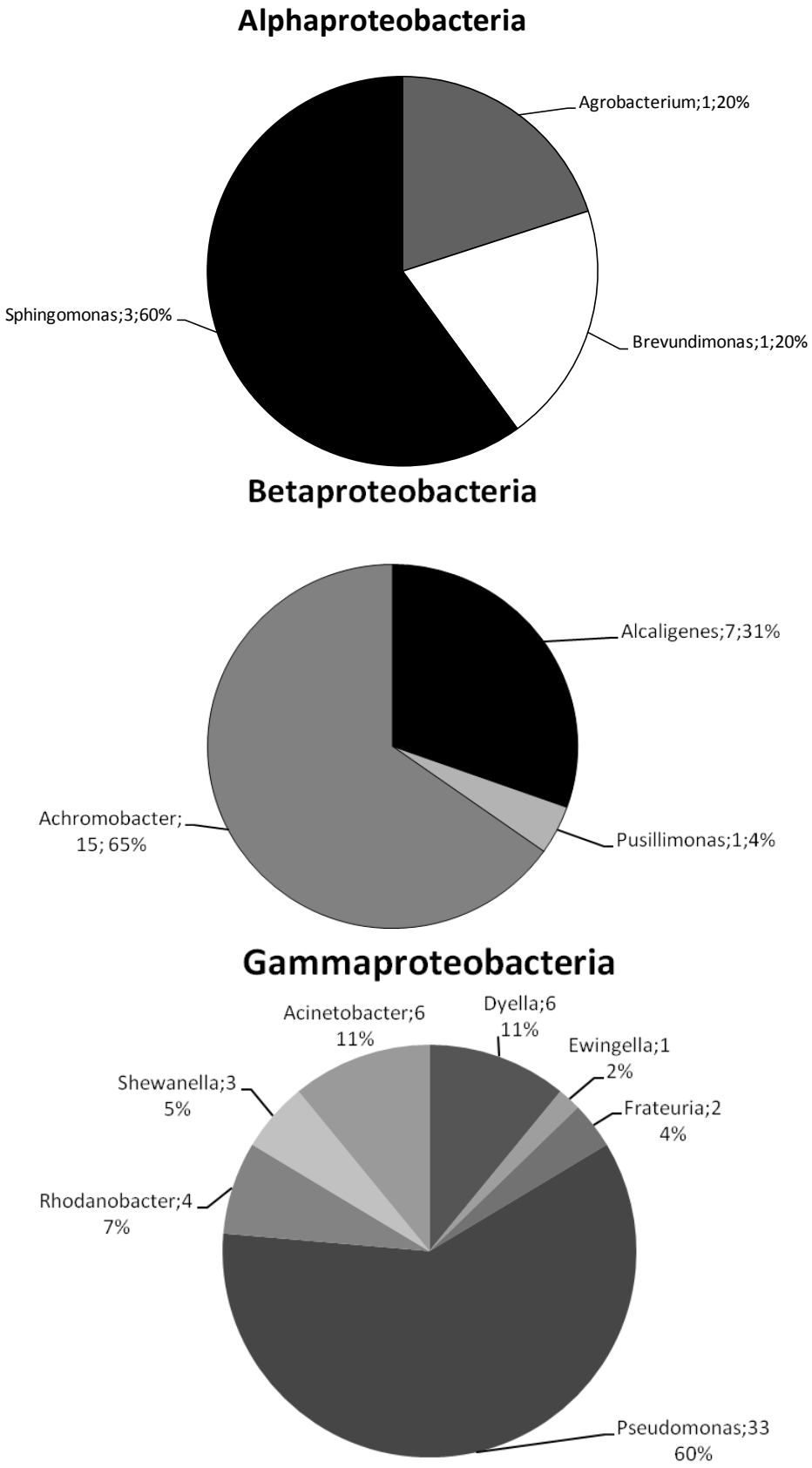


Figure 3.5 Proportions of taxonomic groups represented by the Gram-negative bacterial strains isolated from sub-site 3. Class *Flavobacteria* included only one genus and is not shown as a separate pie. The number of isolated strains and their percentage per taxa are given together with the genus.

In the neighbour-joining phylogenetic tree, the total Gram-positive isolates formed monophyletic groups which were generally supported by high bootstrap values (Fig. 3.4). Eight different bacterial branches can be recognised on the phylogram (Fig. 3.4). The major Gram-positive populations at the study site were composed by strains related to genera *Arthrobacter* and *Bacillus*. These populations had the highest proportion of representative strains (Fig. 3.2) and clones (data not shown) and surely represent an important fraction of the populations present at the study site. Smaller populations of other microorganisms included strains related to *Curtobacterium*, *Microbacterium* and *Rhodococcus*. Other strains were represented only by few strains and clones and constituted very small populations within the total Gram-positive strains (Fig. 3.4).

The Gram-negative population recovered from the study site revealed to be more diverse in terms of the number of genera present. In neighbour-joining phylogenetic tree, the total Gram-negative strains formed monophyletic groups which were generally supported by high bootstrap values (62-100) (Fig. 3.5). In total 7 populations can be recognized and several sub-populations can also be pointed (Fig 3.5). The Gram-negative population with the highest number of members were clearly related to genera *Pseudomonas*. This population can be comparable in size and importance to the two Gram-positive populations mentioned before (*Bacillus* and *Arthrobacter*) and clearly represent an important part of the cultivable heterotrophic populations present at Estarreja. Two of populations with members from the genera *Acinetobacter*, *Shewanella* and *Ewingella* are as *Pseudomonas* members of class γ-*Proteobacteria* (Fig. 3.5), which clearly makes this group the most represented and stressing its importance in the heterotrophic microbial communities inhabiting the study site. Other populations with sizable numbers were clustered within genera

Achromobacter, *Rhodanobacter* and *Dyella* and some members of α-*Proteobacteria*. The bacterial population with more dissimilarity was clustered within class *Flavobacteria* with members of the genera *Chryseobacterium* (Fig. 3.5).

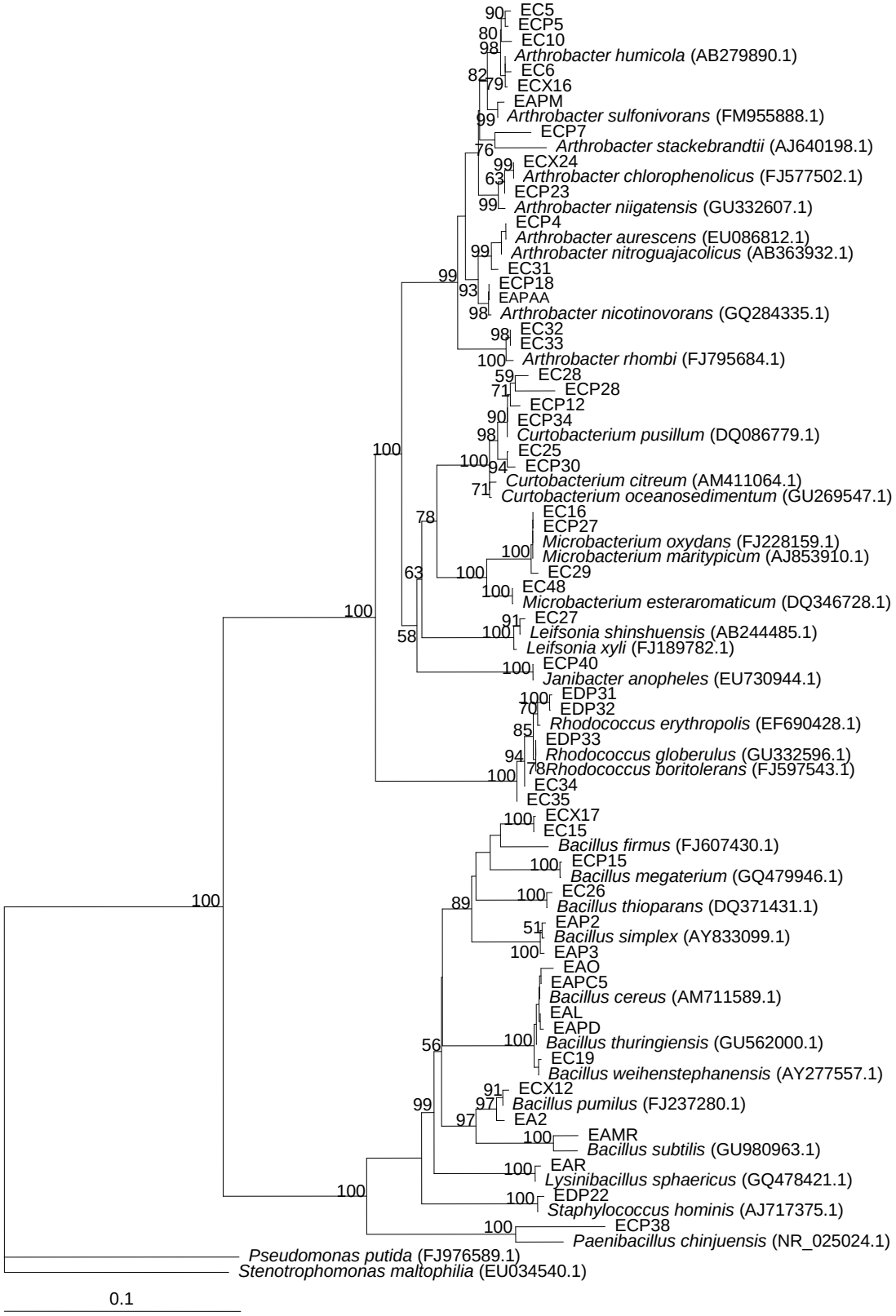


Figure 3.6 Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between sequences of the Gram-positive representatives isolated and some of their closest phylogenetic relatives. The tree was created by the neighbour-joining method. Bootstrap values are shown at nodes. *Pseudomonas putida* (EF204247) and *Stenotrophomonas maltophila* (EU034540) were used as outgroup. Bar, 0.1 substitutions per nucleotide position.

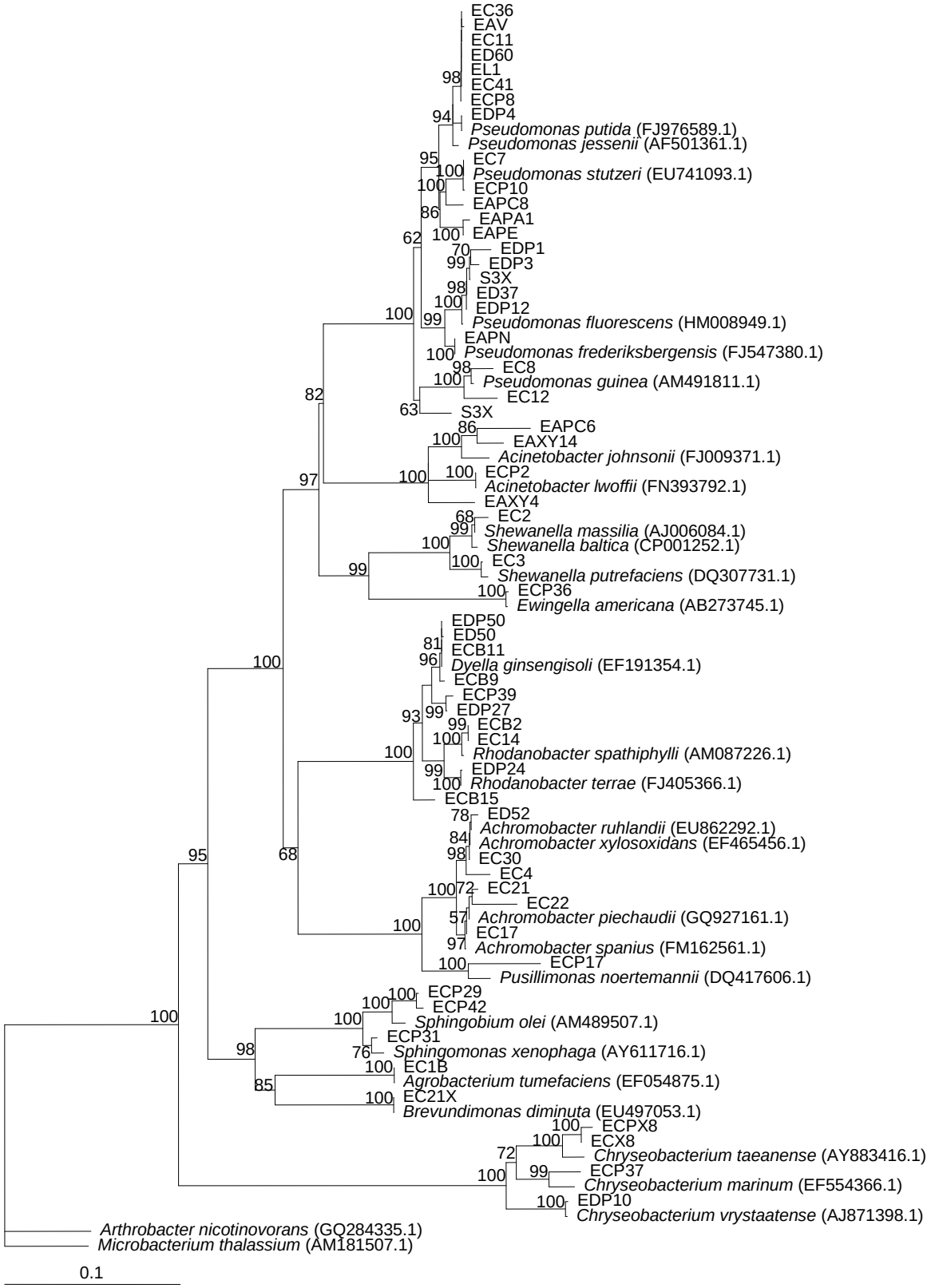


Figure 3.7 Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between sequences of the Gram-negative representatives isolated and some of their closest phylogenetic relatives. The tree was created by the neighbour-joining method. Bootstrap values are shown at nodes. *Arthrobacter nicotinovorans* (GQ284335) and *Microbacterium thalassium* (AM181507) were used as outgroup. Bar, 0.1 substitutions per nucleotide position.

3.3.6 Metal tolerance for growth

Despite the high levels of metal contamination present in this environment, the majority of isolates were sensitive to moderate to high concentrations of metals, and few isolates were able to grow especially at the highest concentration (Table 3.4). The majority of the isolates grew when Zn was present. Most of the isolates had no growth or small growth when Cd was present (Table 3.4). About half the isolates grew when As was present. The representative populations isolated in sub-site 3 were shown to be more tolerant to the heavy metals at the concentrations tested. 12 isolates grew when a metal mix was tested; in contrast, 13 isolates had no apparent metal tolerance and did not grow in the presence of any of the metals tested either alone or as mixtures (Table 3.4). Overall, seventeen strains were capable of growing at high concentration of As, Zn and Cd. Of these seventeen resistant strains thirteen of them were gram-negative and only four were gram-positive. The most resistant bacterial isolates are highlighted in Table 3.4.

Table 3.4 Characteristics of representative strains of the isolated population (most tolerant strains shown in bold).

Strain[2]	Colony pigmentation	Cell morphology	Gram	Metal tolerance (growth at ppm)[1]									
				Cd			Zn			As			
				250	500	1000	250	500	1000	250	500	1000	Mix
EC1B	**White**	**Rod**	-	++	+	-	++	+++	++	++	++	-	-
EC2	**Yellow**	**Short rod**	-	++	+	+	+++	+++	+++	++	++	++	-
EC3	**Yellow**	**Short rod**	-	++	+	-	+++	+++	+++	+	+	-	-
EC4	**Yellow**	**Rod**	-	++	+	-	++	++	+	++	+	-	-
EC5	White	Round rod	+	+	-	-	+++	+++	+++	++	-	-	-
EC6	Yellow	Rod	-	++	+	-	++	++	+	++	+	-	-
EC7	Yellow	Curved rod	-	-	-	-	++	++	++	++	++	+	-
EC8	Beige	Short rods	-	-	-	-	++	++	++	++	++	+	-
EC10	**Yellow**	**Short rod**	+	++	++	+	+++	++	-	++	-	-	-
EC11	Yellow	Short rods	-	-	-	-	++	++	++	++	++	+	-
EC12	Pink	Short rods	-	+	+	-	+	+	+	+	+	-	-
EC14	Yellow	Rod	-	+	-	-	+++	-	-	+	-	-	-
EC15	Red	Rod	+	-	-	-	++	-	-	-	-	-	-
EC16	Yellow	Short rod	+	-	-	-	++	++	++	+	-	-	-
EC17	White	Rod	-	+	+	-	+	+	+	+	+	-	-
EC19	Smooth white	Rod	+	-	-	-	++	++	+	+	-	-	-
EC21	Red	Rod	-	+	+	-	+	+	+	+	+	+	-
EC21X	Milky white	Rod	-	+	-	-	++	++	-	-	-	-	-
EC25	Yellow	Rod	+	-	-	+	-	-	+	-	-	+	-
EC26	White	Large rod	+	-	-	-	-	-	-	-	-	+	-
EC27	Yellow	Short rods	+	-	-	-	-	-	-	-	-	-	-
EC28	Yellow	Rods	+	-	-	-	+	+	+	+	+	+	+
EC29	**Yellow**	**Short rods**	+	+	+	-	+	+	+	+	+	+	+
EC30	**Beige**	**Short rod**	-	++	+	-	++	++	++	++	++	+	+
EC31	**Yellow**	**Short rod**	+	++	++	-	++	++	++	-	-	-	-
EC32	Yellow	Rod	+	-	-	-	-	-	-	-	-	-	-
EC33	White	Rod	+	+	+	-	++	+	+	+	+	+	-
EC34	White	Rod	+	-	-	-	-	++	++	++	+	+	-
EC35	**White**	**Coccus**	+	+	+	+	+	+	+	+	+	+	+
EC39	White	Rod	+	+	-	-	+	+++	-	+	-	-	-
EC48	Yellow	Short rod	+	-	-	-	+	-	-	-	-	-	-
ECB9	Yellow	Rod	-	+	+	-	+	+	+	+	+	-	-
ECB15	Beige	Rod	-	-	-	-	-	-	-	-	-	-	-
ECP2	**White**	**Short rod**	-	++	++	-	+++	+++	++	+	-	-	-

ECP4	Yellow	Rod	+	-	-	-	+	-	-	-	-	-	-
ECP7	White	Short rod	+	-	-	-	+++	-	-	-	-	-	-
ECP12	Yellow	Short rod	+	-	-	-	+++	++	-	++	-	-	-
ECP15	Yellow	Large rods	+	-	-	-	+	-	-	-	-	-	-
ECP17	Yellow	Short rod	-	++	-	-	++	+	-	-	-	-	-
ECP18	Beige	Rod	+	-	-	-	+++	-	-	-	-	-	-
ECP23	White	Short rod	+	-	-	-	++	-	-	-	-	-	-
ECP27	Yellow	Short rod	-	-	-	-	+	+	-	-	-	-	-
ECP28	Yellow	Rod	+	-	-	-	+	+	+	+	-	-	-
ECP29	Yellow	Short rod	-	-	-	-	-	-	-	-	-	-	-
ECP31	Yellow	Rod	-	-	-	-	-	-	-	-	-	-	-
ECP36	White	Short rod	-	-	++	-	++	+	-	+++	+	-	-
ECP37	**Yellow**	**Long rod**	-	++	-	-	+++	+++	+++	+++	+++	+++	+
ECP38	Orange	Large rod	+	-	-	-	-	-	-	-	-	-	-
ECP39	Yellow	Rod	-	-	-	-	-	-	-	-	-	-	-
ECP40	Yellow	Coccus	+	-	-	-	+	-	-	-	-	-	-
ECX8	Orange	Rod	-	+	-	-	++	++	-	-	-	-	-
ECX12	White	Rod	+	-	-	-	+	+	+	+	+	+	+
ECX16	White	Rod	+	-	-	-	-	-	-	-	-	-	-
ECX24	White	Rod	+	+	-	-	+	+	+	+	+	-	-
ED37	Brown	Rod	-	-	-	-	+++	++	+	+++	+	+	-
ED50	**Beige**	**Rod**	-	-	+++	++	+	+++	+++	-	++	++	+
EDP4	Pink	Rod	-	+	-	-	++	+	-	++	+	-	-
EDP10	Orange	Rod	-	+	-	-	++	+	-	-	-	-	-
EDP24	Pink	Rod	-	-	-	-	-	-	-	-	-	-	-
EDP28	**Milky white**	**Rod**	-	++	++	+	++	++	++	++	++	++	+
EDP31	Pink	Coccus	+	-	-	-	-	-	-	+	-	-	-
EDP33	Pink	Coccus	+	-	-	-	-	-	-	++	++	+	-
S3X	**Milky white**	**Rod**	-	+	+	+	+	+	+	+	+	+	+
EA2	White	Rod	+	-	-	-	-	+	-	-	+	+	-
EAL	White	Rod	+	-	-	-	++	++	++	++	++	++	+
EAMR	Milky white	Rod	+	-	-	-	-	+	+	-	+	-	-
EAO	White	Rod	+	-	-	-	-	-	-	-	-	-	-
EAP2	White	Long rods	+	-	-	-	+	+	+	+	+	-	-
EAP3	Milky	Long rod	+	-	-	-	-	++	+	-	++	+	-
EAPA1	Brown	Rod	-	-	-	-	++	++	++	++	+	-	-

EAPAA	Yellow	Rod	-	-	-	-	++	++	++	++	+	+	+
EAPC2	Yellow	Rod	-	-	-	-	-	+	+	+	+	+	-
EAPC5	Pearly white	Rod	+	-	-	-	++	+	-	-	-	-	-
EAPC6	Yellow	Curved rods	-	-	-	-	-	-	-	+	-	-	-
EAPC8	Beige	Rod	-	-	-	-	-	-	-	-	-	-	-
EAPD	Milky	Long rod	+	-	-	-	-	-	-	-	-	-	-
EAPE	Beige	Rod	-	+	-	-	+	-	-	++	+	-	-
EAPM	White	Rod	+	-	-	-	+++	++	+	-	-	-	-
EAPN	Yellow	Short rod	-	-	-	-	-	-	+	-	+	+	-
EAR	**Yellow**	**Rod**	-	++	+	+	-	++	+	-	++	+	+
EAXY4	**White**	**Short rod**	-	++	+	-	+++	+++	+	+	-	-	-
EAXY14	Yellow	Rod	-	+	-	-	++	+	-	-	-	-	-
EBP1	White	Rod	+	-	-	-	++	+	+	-	-	-	-
EL1	Purple	Cocci	-	+	-	-	++	++	+	+	+	-	-
EAV	Grey	Rod	-	-	-	-	++	++	+	+	-	-	-

[1] **-,** no growth; +, poor growth; ++, good growth; +++ exuberant growth.

[2] Strains from **site 1** (EA[*]; EB's; EL) [*]E=Estarreja, A & L=site 1, TSA, ABM2; [†] E=Estarreja, B=site 1 PCA;, numbers represent the isolate number**.**
Strains from **site 3** (EC[‡], ECP[♯], ECX[‡], S3[ᵭ], ED[‡] and EDP[♯] - [‡]E=Estarreja, C-D & X= site 3, TSA, ABM2; [ᵭ]S3=Site 3, ABM2; [♯]E=Estarreja, C & D=site 3, P=PCA, numbers represent the isolate number

3.4 Discussion

This study presents a characterization of soil bacterial composition in the industrial area of Estarreja, Portugal. This has shown that the diversity is narrow in terms of species suggesting that toxicity in this industrial soil has influenced the bacterial community. On sub-site 2 no bacteria were recovered, thus a different isolation approach is necessary to recover members of the cultivable fraction of the bacterial populations. In populations under stress, previously dominant groups may lose their advantage compared to the majority of individuals, when compared to pristine soils, resulting in an apparent increase in *richness* arising from increase in *evenness*; conversely, if many groups became uncompetitive, these diversity measures may decrease (Giller *et al*., 1998).

In order to develop effective strategies for the bioremediation of heavy metal contaminants, a better understanding of the composition and metabolic potential of microbial communities in highly contaminated soils is required. There are many culture dependent or independent methodologies available to investigate the microbial population structure of environmental samples. Denaturing gradient gel electrophoresis (DGGE) could be an adequate option to obtain knowledge on the composition of the microbial community structure. 16S clone libraries are prone to bias when examining microbial diversity in a specific ecological system (Xia *et al*., 2005; Li *et al*., 2006). Restriction fragment length polymorphism (RFLP) on the other hand may overestimate or underestimate the total population size. One microorganism may be identified by multiple RFLP types because a single bacterial genome may have numerous copies of the operon used and a single RFLP type may represent more

than one organism, respectively (Fisher and Triplett, 1999). 16S rRNA is a useful tool for providing qualitative descriptions of the microbial diversity and analysing the phylogenetic evolutionary relationship of microorganisms (Madigan, 2003). The Basic Local Alignment Search Tool (BLAST) is not a program for the determination of phylogenetic relationship, but it can provide the most probable affiliation sequence, which can help building a phylogenetic tree (Guo, *et al*., 2007). In this study, BLAST was used as an assistance tool to analyse the microbial diversity in the soils examined.

One of the primary goals of this work was to determine the bacterial diversity in the industrialised region of Estarreja, to measure the culturable aerobic populations. While the influence of the metals on the microbial communities is not precisely known to date, previous studies have demonstrated that heavy metals can significantly alter the microbial populations and diversity (Tsai *et al*., 2005). Using different medium variants, a total of 278 bacterial strains were recovered in two occasions from this xenobiotically contaminated environment. Despite 103 representative strains being characterized, potentially corresponding to the same number of species, the majority of the isolates were clustered within a small number of genera when compared to pristine soils where the bacterial diversity is normally higher. Therefore, the comparison of the soil bacterial isolates showed that many shared a similar 16S rRNA genes indicating a degree of genetic relatedness. The dominant gram positive populations belonged to the family *Micrococcaceae* and *Bacillaceae* which accounted for more than 50 % of the total bacterial populations on sub-site 1 and 3. The most represented genera were *Bacillus, Microbacterium, Curtobacterium, Rhodococcus* and *Arthrobacter*. The dominant gram negative bacteria belonged to the class *γ-Proteobacteria* with *Pseudomonas* as the most common genera. *β-Proteobacteria* was

also well represented with the most common genera *Alcaligenes* and other members of the family *Alcaligenaceae. α-Proteobacteria* was represented only on sub-site 3 with the genera *Brevundimonas*, *Agrobacterium* and *Sphingomonas*.

Not surprisingly many of the isolated strains had closest phylogenetic relatives isolated from soil and from other contaminated locations and also from extreme environments, such as strain ECP37 related to the type strains of the species *Chryseobacterium antarcticum C. jeonni* and *C. marinum*, which were isolated from terrestrial samples from Antarctic (Yi *et al*., 2005; Lee *et al*, 2007; Kämpfer *et al*, 2009). However some of the closest relatives of the representative strains isolated in this study had diverse origins, such as sea water, human skin and meat just to name a few (Table 3.3). This indicates that many bacterial divisions have representatives that are not confined to live in pristine environments but can also thrive in contaminated environments and that represent a reservoir of resistant organisms to pollution.

The phyla detected in contaminated soils includes groups commonly found in pristine soils, however the proportion of phylotypes detected within these phyla differed (Buckley and Schmidt, 2002). Bacteria have a wide array of metabolic activities, with an inherent ability to survive complex and challenging environments. The metal resistance systems inherent or ubiquitous in microorganisms (Bruce, 1997) may explain the readiness of some bacterial groups to survive the addition of metal contaminants over time.

The bacteria isolated in this study can be seen as a broad group of opportunistic heterotrophs, which have evolved to exploit a wide variety of environmental niches.

This study has detected families within the phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria* that are frequently associated with contaminated environments (i.e. resistance to heavy metals, reduction of nitrate and heavy metals, degradation of aromatic compounds and polychlorinated biphenyls) (Dhakephalkar and Chopade, 1994; Boswell *et al*., 2001; Goris *et al*., 2001; Nogales *et al*., 2001; Kanaly *et al*., 2002; Konstantinidis *et al*., 2003; Mergeay *et al*., 2003; Fields *et al*., 2005). The majority of unpolluted soils are typically near neutral pH and are rich in organic matter, whereas metal contaminated soils are lower in organic matter, have higher nitrate concentrations, a wider pH range, and have high toxic metal concentrations (Jardine *et al*., 2003; Dell'Amico *et al*., 2005; Moon *et al*., 2006; Pérez-de-Mora *et al*., 2006). Phylotypes closely related to taxa adapted for growth in metal contaminated environments (i.e. *Bacillus, Microbacterium, Arthrobacter, Pseudomonas, Acinetobacter*) were frequently detected in the soils of the study site. These genera have also been detected in relative abundance from other contaminated sites (Jackson *et al*., 2005; Dell'Amico *et al*., 2005) and during bioremediation experiments (Chen *et al*., 2005a; 2005b; Abou-Shanab *et al*., 2007; Ansari and Malik, 2007). Thus the phylogenetic analysis of the study sites indicates the presence of a microbial community adapted to the particular characteristics of this site. In agreement with the high metal concentrations of the contaminated Estarreja sediments, isolated strain members of the *Proteobacteria* (including the genera *Pseudomonas, Acinetobacter, Sphingomonas, Alcaligenes*), *Bacilli* (i.e. *Bacillus*) and *Actinobacteria* (including *Microbacterium, Arthrobacter*), showed a high relative abundance in the total and metabolically active fractions of the microbial community. Interestingly, members of the *Pseudomonas, Acinetobacter* and *Bacillus* groups are typically resistant to metals, (Cu, Ni, Cd, and Zn) (Dhalkephalkar and Chopade, 1994;

Brim *et al*., 1999; Boswell *et al*., 2001; Abou-Shanab *et al*., 2007; Ansari and Malik, 2007), suggesting that the abundance of toxic heavy metals in the contaminated region of Estarreja may have selected for these lineages.

It was noticed in this study that some strains isolated from this particular environment did not grow with high concentrations of metals, or had difficulties in growing under laboratory conditions in culture media adjusted with metals, with the exception of Zn. However, this effect is somewhat common, and most of the isolates from metal contaminated environments only proliferate under laboratory conditions when no metal or small amounts of metal are used (Gadd, 1990). The fact that these isolates did not grow when metal concentrations similar to those found in their environment can also be explained probably due to the fact that the composition of the media and other conditions of incubation were decisive for the determination not only of the metal tolerance but also for the determination of the optimal pH and temperature for growth (Horikoshi, 1999; Krulwich, 2000). However, since all strains were isolated from highly contaminated soils, they should have been stress adapted and able to proliferate, or at least survive in such an environment. Although some studies have shown detailed mechanisms of heavy metal tolerance for some bacterial species, for others these mechanisms are not yet very well known (Tsai *et al*., 2005). The ability to cope under this metal-contaminated environment may depend on genetic and/or physiological adaptation (Gadd, 1990; Jjemba, 2004). Moreover, bacterial populations present at Estarreja may be partially unaffected by the metal concentration of the environment. Therefore the presence of certain bacterial strains that are not adapted to live in metal contaminated environments may be sustained or induced by the activity of other bacterial strains or at a given concentration, a metal

may be toxic to one species while serving as a growth stimulant to others (Tsai *et al*., 2005). Heavy metals are more than likely able to affect the microbial populations in a given environment by reducing abundance and species diversity and selecting for a resistant population (Gadd, 1990). Tolerance to heavy metals may result from intrinsic properties of the microorganism, e.g. producing extracellular mucilage or polysaccharide or an impermeable cell wall (Gadd, 1990). Likely in this type of extreme environment, one of the most important factors that explains the presence or absence of a given population may well be the relationships that exist between the whole bacterial community.

From the seventeen strains found to be relatively resistant to heavy metals, the majority of them were Gram-negative. In general, Gram-negative bacteria are more tolerant to metals than gram positive bacteria. This is due to the fact that gram negative bacteria have a complex three layered cell wall, that immobilizes metals more effectively (Madigan *et al*., 2003; Jjemba, 2004), opposite to the Gram-positive cell wall composed mainly by a thick layer of peptidoglycan.

This study points to several groups of metabolically active bacterial lineages with capabilities that are adapted to contaminated sediments. The microbial community analysis in conjunction with potential rates of microbial activity suggests that these groups have a high potential for bioremediation and should be explored further.

Chapter 4: Isolation of heavy metal tolerant bacteria from rhizosphere soil at Estarreja in northern Portugal

4.1 Introduction

Soil contamination with heavy metals has become a worldwide problem, leading to losses in agricultural yield and hazardous health effects as they enter the food chain. Heavy metals cannot be degraded to be harmless and hence persist in the environment for long periods of time (Yang *et al*., 2002). Metals can exist in soil as free cations (e.g. Cd^{2+}, Zn^{2+}), as soluble complexes with organic or inorganic ligands (e.g. metal citrates, $CdCl_3^-$) and associated with colloid material. Moreover, heavy metal toxicity is affected by the physico-chemical nature of the environment and chemical behavior of the metal species (Sandifer and Hopkin 1996; Sivakumar and Subbhuraam, 2005). To clean up soils contaminated with heavy metals by traditional physiochemical methods can be very costly, and also destructive to the soil.

Several studies have demonstrated the toxic effect of heavy metals on microbial diversity, biomass and activity (Kamnev *et al*., 2005; Wani *et al*., 2007). However, bacterial strains isolated from heavy metal polluted environments are tolerant to higher concentrations of metals than those isolated from unpolluted areas. In addition to this, after the addition of heavy metals, metal tolerance is increased in bacterial communities by the disappearance of sensitive species and strains and subsequent competition and adaptation of surviving bacteria (Díaz-Raviña and Bååth, 1996).

Bacterial populations inhabiting the rhizosphere can enhance biomass production and heavy metal tolerance of plants in polluted environments (Sheng and Xia, 2006; Dell'Amico *et al*., 2008a). Thus, an alternative way to reduce the toxicity of heavy metals, especially to plants, is by using the rhizosphere microorganisms (Burd *et al*., 2000). Certain heavy metal resistant bacteria have the ability to promote plant growth by various mechanisms such as nitrogen fixation, solubilisation of minerals, production of siderophores and phytohormones, and transformation of nutrients (Glick et at, 1999). The production of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase, an enzyme that modulates ethylene levels in plants (Glick *et al*., 1998), may also be a contributing factor to heavy metal tolerance in plants. Furthermore it has been reported that ACC producing bacteria could promote plant growth and protect plants against heavy metal toxicity in contaminated sediments (Burd *et al*., 2000; Belimov *et al*., 2005; Madhaiyan *et al*., 2007). These organisms can also increase plant tolerance to flooding (Grichko and Glick, 2001), salt stress (Mayak *et al*., 2004a) and water stress (Mayak *et al*., 2004b). Plant growth promoting bacteria are not only significant from an agricultural point of view, but may also play an important role in soil remediation strategies, not only by enhancing growth and successful establishment of plants in polluted soils, but also by increasing the availability of contaminants. For example, heavy metal tolerance of Zn and Ni, in *Thlaspi caerulescens* (Whiting *et al*., 2001) and in *Alyssum murale* and *Thlaspi goesingense* (Abou-Shanab *et al*., 2003; Idris *et al*., 2004). Therefore, such organisms, with this natural heavy metal tolerance are of practical importance for remediation of metal contaminated environments.

A large industrial complex, composed essentially of industrial chemical facilities, surrounds the area considered in the present study. For many years, several of these industries have discharged their solid residues in an improvised sediment basin in the surrounding area, and released its wastewaters into a nearby stream - "Esteiro de Estarreja" - which is a small and almost stagnated watercourse (Oliveira *et al*., 2001). Because of the high permeability of the site, the percolates from this improvised sediment basin contaminate the soil of the surrounding area. Therefore, the levels of heavy metals in the sediments of this stream, to a depth of 50 cm, are above the limits established by EC Directive 86/278/EC (Atkins, 1999).

The area near the former exit of contaminated wastewaters is the most polluted zone (to a distance of ca 50 m further from the exit), although the contamination along the banks and between the banks is very heterogeneous, and mainly occurs in the top 20 cm layer of the soil (Atkins, 1999; Oliveira *et al*., 2001). The banks of the stream, with ca. 2 m width, are periodically flooded with rainwater, from late October to late February, and the ditch of the stream (with ca. 1.5 m depth) remains almost dry during the remaining months. Despite the high levels of metals in the sediments, the vegetation on the banks of the stream remains prolific, yet heterogeneously distributed.

The aim of this study was to (a) isolate heavy-metal tolerant bacteria using culture dependent methods from the rhizosphere in contaminated soils, (b) analyze the bacterial population composition on site, and (c) characterize the isolates and select the most resistant strains which might be useful for bioremediation of contaminated soils.

4.2 Materials and methods

4.2.1 Samples and sampling site

Soil samples were collected from Estarreja, Portugal. A large industrial complex surrounds the area considered in the present study. The site has a long history of metal contamination, due to the industrial activity in the surrounding area. There is a high presence of metals: average levels of 835 mg Pb kg^{-1}, 66 mg Hg kg^{-1}, 26 mg Cr kg^{-1}, 37 mg Ni kg^{-1}, 16 800 mg Fe kg^{-1} and 3620 mg Zn kg^{-1} (total Zn) (Marques *et al*., 2007). However, the area still has significant vegetation (Oliveira *et al*., 2001). Soil was collected near a stream "Esteiro de Estarreja" in two different locations. The site characteristics have been described previously in Chapter 2, section 2.1.1 and Chapter 3, section 3.2. Due to the fact that no viable organisms were recovered on previous sampling occasions it was decided not to survey site 2 referred to in Chapter 2, section 2.1.1 and Chapter 3, section 3.2.

Sub-site 1: located on the banks of a stream, mainly contaminated with Zinc (Zn), Arsenic (As) and Lead (Pb).

Sub-site 3: located upstream site 1, periodically flooded soil with a pH of 4.5/5 to a depth of 10 cm. It shows high levels of contamination by As, Zn and Mercury (Hg).

Soil samples for bacterial analysis were recovered directly from the two locations on two sampling periods in September 2006. Soil samples were then transported to the laboratory at 4 ºC and were assayed within 12 h of sampling.

4.2.2 Enumeration and isolation of the heterotrophic populations

For the isolation and enumeration of the bacterial populations, samples were serially diluted and plated onto different isolation media, and the plates incubated at 30 ºC for up to 10 days. The cultures were observed daily for colony growth, and all different morphological colony types were isolated. Isolates were purified by sub-culturing on the corresponding isolation media and were stored at –80 ºC in freezing media with 15 % (vol/vol) glycerol.

4.2.3 Isolation media

The following media was used: plate count agar (PCA) (Pronadisa), and trypticase soy agar (TSA) (Pronadisa). The media was modified by adjusting the pH of each of the basic composition to three different pH values 5, 6 and 7, by using buffer solutions at a final concentration of 100 mM. Solutions with heavy metals were added to the media to select environmental resistant bacterial strains.

4.2.3.1 Media composition and preparation

PCA, and TSA were prepared according to the manufacturer's instructions, but 100 ml of a defined buffer solution at a concentration of 1 M was added in a final volume of 1 liter of media. Both media were supplemented with Cd, Zn and As, supplied as $CdCl_2$ (Sigma), $ZnCl_2$ (Sigma) and KH_2AsO_4 (Sigma) from sterile stock solutions, to obtain final concentrations ranging from 250 to 1000 mg L^{-1}. Higher metal concentrations than those present in both sites were used in order to isolate the most resistant bacterial strains. The metal stock solutions contained 10000 mg L^{-1} of $CdCl_2$ (Sigma), $ZnCl_2$ (Sigma) and KH_2AsO_4 (Sigma).

The metal-modified PCA and TSA media were prepared exactly in the same way but metal solutions were added to obtain the target final concentrations. The metal solutions were mixed after the media was autoclaved. The buffer solutions were autoclaved separately from the other components of the different media and mixed after cooling to 45 ºC. All the other steps were as described previously for media without metals.

4.2.3.2 Buffer solutions

Different buffer solutions were used to adjust the media to different pH values. All solutions were prepared according to Gomori (1990) and were autoclaved separately. The following buffer solutions were used to adjust the media to the different pH values: citrate buffer to adjust the different media to pH 5 and 6, and phosphate buffer to adjust the different media to pH 7.

4.2.4 RAPD analysis of isolates

Random amplified polymorphic DNA (RAPD) analysis was used as a primary method to group the isolates. Crude cell lysates were used as DNA templates, as described by Widmann-al-Ahmad *et al*. (1994). The RAPD protocol is described in Chapter 2, section 2.6.1.

4.2.5 Morphological and biochemical characteristics of representative strains

Cell morphology was examined by phase-contrast microscopy after cultivation on PCA and TSA. Gram staining was determined as described by Doestch (1981), cytochrome-oxidase and catalase activities were determined as described by Smibert and Krieg (1981).

4.2.6 Phylogenetic analysis of representative bacterial strains

The extraction of genomic DNA for 16S rRNA gene sequence determination, and PCR amplification of the 16S rRNA gene were carried out as described by Rainey *et al*. (1996). The 16S rRNA gene amplification protocol is also described in detail in Chapter 2, section 2.6.2. Sequencing of the purified PCR products was performed by Macrogen Inc. (Seoul, Republic of Korea).

For phylogenetic analyses, the protocol is described in detail in Chapter 3, section 3.2.6.

4.2.7 Determination of heavy metal tolerance of the representative strains of the isolated population

The heavy metal range for growth was determined on solid media. For comparison purposes, a medium on which all of the strains could grow was chosen. For this reason TSA buffered to pH 7 was used. Media preparation was carried out as described previously.

For determination of the quality of growth, 24 h cultures were ressuspended in saline solution (0.85 %, w/v, NaCl) to a turbidity equivalent to a McFarland no. 2 standard (Smibert and Krieg, 1981). 0.1 ml were spread onto TSA agar adjusted to pH 7 and then incubated at 30 ºC for 72 h. Growth was then quantitatively verified as follows: -, no growth; +, poor growth; ++, good growth; and +++, exuberant growth.

4.3 Results

4.3.1 Enumeration of the microbial populations

The number of heterotrophic bacterial populations recovered was dependent on the media composition, and the pH of the media used. No isolates were recovered at pH 5. pH 7 yielded a higher number of isolates and only 10 strains were isolated at pH 6. The number of bacterial strains isolated was also higher on TSA.

The Colony Forming Units (CFU) for sub-site 1 varied between 7.19 CFU Log_{10}/g (for PCA medium + As) and 7.55 CFU Log_{10}/g (for PCA + Zn). No viable isolates were recovered when a mixed metal mixture were used and when PCA media were supplemented with Cd. Surprisingly no viable isolates were isolated in the second sampling period in any of the metal combinations used (Table 4.1).

CFU's at sub-site 3 varied from 5.18 CFU Log_{10}/g (for TSA medium + Zn) to 6.65 CFU Log_{10}/g (for TSA medium + As). These values were determined for the first isolation period. During the second isolation period CFU counts were higher than in the first sampling period. Similarly to sub-site 1, no viable counts were recovered with any of the media when a metal mixture was used. As in sub-site 1, when PCA + Cd media were used no viable bacterial population were recovered (Table 4.1). The highest levels of recovery reached 7.55 and 7.49 CFU Log_{10}/g, and were verified on PCA and TSA in sub-site 1, during the first sampling period.

Table 4.1 Microbial CFU's per gram soil obtained from the contaminated sites sampled

Media	Sub-Site 1 CFU_{10}/g		Sub-Site 3 CFU_{10}/g	
	Samplings			
	1st	2nd	1st	2nd
PCA + Cd	NG	NG	NG	NG
PCA + As	7.19	NG	6.18	7.27
PCA + Zn	7.55	NG	5.54	6.69
PCA + Metal mix	NG	NG	NG	NG
TSA + Cd	7.49	NG	5.45	NG
TSA + As	7.47	NG	6.65	7.26
TSA + Zn	7.35	NG	5.18	6.39
TSA + Metal mix	NG	NG	NG	NG

NG, no growth detected

Throughout the sampling periods, a total of 42 bacterial strains were recovered from site 1 and 3. From these, 25 strains originated from site 1 (labelled 1Z, 1A and 1C, for Zn, As and Cd, respectively), and 17 strains from site 3 (labelled 3Z, 3A and 3C, for Zn, As and Cd respectively). Interestingly, as in Chapter 3, section 3.1.1, the only media capable of supporting the growth of the all the isolates was TSA, so for comparative purposes this medium was used to grow the isolates for further characterisation.

4.3.2 Grouping the isolates

Primary grouping of the 42 isolates was performed by RAPD analysis in an attempt to find similar strains that may have been isolated several times. Using this approach 40 different RAPD profiles were recognized (Fig 4.1). This procedure together with analysing the morphological and biochemical characteristics of the isolates, made it possible to identify strains with the same RAPD patterns. Interestingly, only 4 isolates had similar RAPD profiles. As was mentioned in the

previous Chapter most strains had similar morphological and biochemical characteristics, but had distinct RAPD patterns. In order to choose representative strains of the isolates recovered and to ensure the preservation of the diversity, further characterization of all of the types with different RAPD profiles was made.

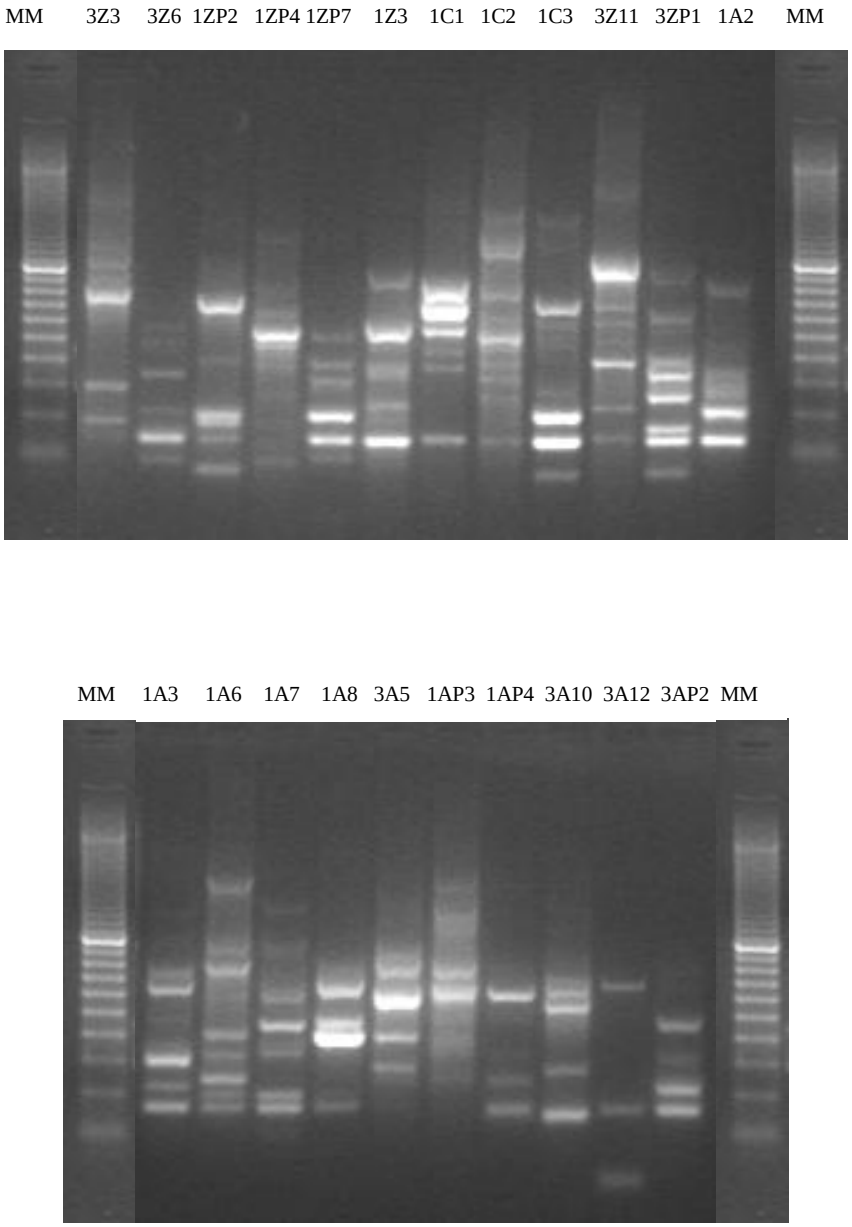


Figure 4.1 RAPD profiles of the isolated populations (not all RAPD profiles are shown) – MM, molecular ruler marker. Site 1: 1Z, 1A and 1C. Site 3: 3Z, 3A and 3C.

4.3.3 Characterisation of the recovered populations

During the different sampling periods the main goal was to isolate the highest possible amount of different morphotypes able to grow in a high concentration of heavy metals. As shown in Chapter 3 the diversity found in cell and colony morphology and pigmentation was very discrete (Table 4.3). Of the representative strains of the populations isolated about half were Gram-negative (20 phylotypes) and slightly more were Gram-positive (21 phylotypes). All of the isolated strains were from media at pH 7. This contrasted with the previous survey where a substantial part of the microbial populations were isolated at pH 6.

4.3.4 Phylogenetic analysis of the recovered populations

The Gram-positive isolates recovered were related to class Bacilli and Actinobacteria (Fig. 4.2). Class Bacilli isolates were clustered into class *Bacilli*, with strains associated to *Bacillus*. Class Actinobacteria on the other hand had a more diverse composition as isolates were associated with the genera *Arthrobacter*, *Streptomyces*, *Rhodococcus*, *Oerskovia*, *Microbacterium* and *Curtobacterium* (Fig. 4.3). *Bacillus* was the most common Gram-positive genera isolated in both sub-sites. The values of 16S rRNA gene sequence similarity between the isolated Gram-positive populations the described taxa were generally high (98-100 %) (Table 4.2).

In the neighbour-joining phylogenetic tree (Fig. 4.4) of the Gram-positive isolates formed monophyletic groups with different groups of bacteria supported by high bootstrap values (61 to 99 %). The other phylogenetic trees showed essentially the same topology (data not shown). These results showed, beyond any doubt, that the isolated strains belong to 6 different branches.

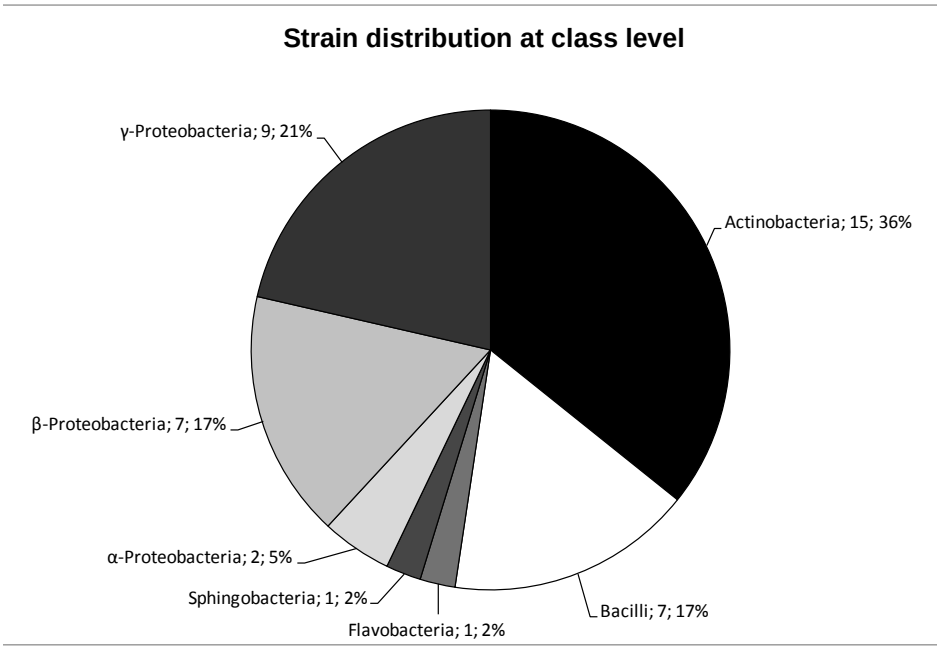


Figure 4.2 Proportion on a class level of the bacterial strains isolated. The number of isolated strains and their percentage per taxa are given together with the class.

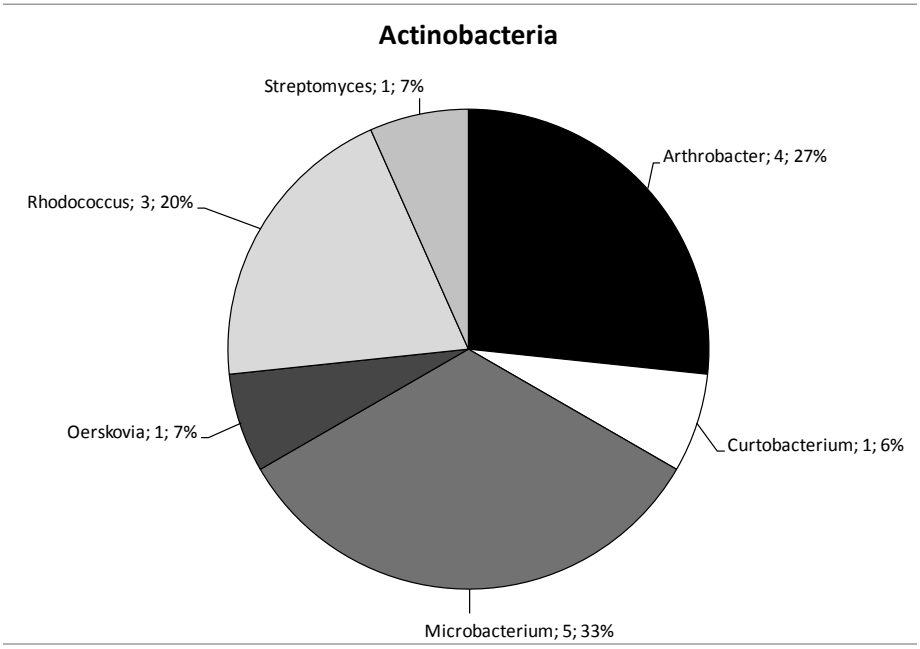


Figure 4.3 Proportions of taxonomic groups represented by the Gram-positive bacterial strains isolated. Class Bacilli included only one representative strain and are not shown as a separate pie. The number of isolated strains and their percentage per taxa are given together with the genus.

Table 4.2 List of the representative isolates and their closest relatives identified by 16S rRNA

Isolate[1]	*Phylogenetic group*		Closest relatives		
	Class	**Genus**	**Identification (NCBI/BLAST assession number)**	**Source**	**Similarity (%)**
1A1	*Actinobacteria*	*Microbacterium*	*Microbacterium oxydans* strain2350 (EU714340)	Clinical specimen	100
1A2	*Actinobacteria*	*Rhodococcus*	*Rhodococcus wratislaviensis* FPA1 (FM999002)	Groundwater contaminated with s-simazine	99
1A3	*Actinobacteria*	*Microbacterium*	*Microbacterium* sp. LJH-17 (GU332500)	Mud volcano	99
1A4	γ-*Proteobacteria*	*Pseudomonas*	Uncultured *Pseudomonas* sp. clone 54 (EU135788)	Skin	99
1A5	*Actinobacteria*	*Arthrobacter*	*Arthrobacter* sp. C6 (FN392690)	Water	99
1A6	γ-*Proteobacteria*	*Mycoplana*	*Mycoplana bullata* strain F8 (EU977700)	Space Center clean-room floor	99
1A7	*Actinobacteria*	*Oerskovia*	*Oerskovia paurometabola* DSM 14281 (NR_025471)	Unavailable	100
1A8	α-*Proteobacteria*	*Novosphingobium*	*Novosphingobium* sp. N8 (GU086416)	Rape leaves	99
1AP1	*Actinobacteria*	*Microbacterium*	*Microbacterium* sp. LJH-17 (GU332500)	Mud volcano	99
1AP2	β-*Proteobacteria*	*Achromobacter*	*Achromobacter* sp. THG 42 16S (GU138383)	Ginseng field soil	99
1AP3	γ-*Proteobacteria*	*Pseudomonas*	*Pseudomonas* sp. M49-6.2 (FJ015028)	Turbot larval rearing unit, tank water	99
1AP4	*Actinobacteria*	*Rhodococcus*	Actinobacterium TB3-11-I (AY599740)	Fungal ascocarp	100
1C1	β-*Proteobacteria*	*Burkholderia*	Uncultured bacterium clone C132 (FJ466234)	Kilauea volcanic deposit	98
1C2	β-*Proteobacteria*	*Cupriavidus*	*Cupriavidus* sp. 2CSa-12 (GU167923)	Rhizosphere of *Commelina communis* in copper mining area	100
1C3	*Actinobacteria*	*Rhodococcus*	*Rhodococcus* sp. LKE-021 (GU944775)	Soil	99
1Z1	*Bacilli*	*Bacillus*	*Bacillus pumilus* BHK6 (AB298784)	Compost	100
1Z2	*Bacilli*	*Bacillus*	*Bacillus aryabhattai* strain LS11 (GU563347)	Rhizosphere soil	100
1Z3	*Bacilli*	*Bacillus*	*Bacillus* sp. A15 (EU621382)	Mud from cage culture zones	99
1ZP2	*Bacilli*	*Bacillus*	*Bacillus subtilis* strain S64 (FJ763648)	Wastewater of silk industry	100
1ZP3	*Actinobacteria*	*Microbacterium*	*Microbacterium oxydans* strain 2350 (EU714340)	Clinical specimen	100
1ZP4	*Sphingobacteria*	*Sphingobacterium*	*Sphingobacterium* sp. MG2 (AY556417)	Unknown	99
1ZP5	*Bacilli*	*Bacillus*	*Bacillus pumilus* CH4 (AM711564)	Faeces and water	99
1ZP6	*Bacilli*	*Bacillus*	*Bacillus* sp. T105 (GU991819)	Rice rhizosphere	99
1ZP7	β-*Proteobacteria*	*Variovorax*	*Variovorax paradoxus* rif200835 (FJ527675)	Legume nodule	99
1ZPX	β-*Proteobacteria*	*Variovorax*	Bacterium PSB-1-33 (AY822567)	Dune plant *Calystegia soldanella*	99

3A5	γ-*Proteobacteria*	*Stenotrophomonas*	*Stenotrophomonas maltophilia* DN1.1 (EU034540)	Unknown	99
3A10	*Flavobacteria*	*Chryseobacterium*	*Chryseobacterium* sp. 1YB-R12 (EU834239)	Soil	96
3A12	α-*Proteobacteria*	*Mesorhizobium*	*Mesorhizobium* sp. IAFILS10 (EU430057)	Consortium degrading PHAs	98
3AP1	*Bacilli*	*Bacillus*	*Bacillus megaterium* DS08 (EU834239)	Soil	100
3AP2	γ-*Proteobacteria*	*Stenotrophomonas*	*Stenotrophomonas maltophilia* H177 (EF204218)	Raw milk	100
3AP3	γ-*Proteobacteria*	*Stenotrophomonas*	*Stenotrophomonas* sp. Enf29 (DQ339605)	Unknown	99
3AS1	β-*Proteobacteria*	*Alcaligenes*	*Alcaligenes* sp. 80 (AY672759)	Unknown	99
3C5	*Actinobacteria*	*Arthrobacter*	Uncultured *Arthrobacter* sp. (AM936058)	Hydrocarbon-contaminated soil	100
3C7	*Actinobacteria*	*Arthrobacter*	Uncultured *Arthrobacter* sp. (AM936058)	Hydrocarbon-contaminated soil	100
3Z3	γ-*Proteobacteria*	*Brevundimonas*	*Brevundimonas* sp. NCCP-147 (AB549434)	Rice leaves	100
3Z6	*Actinobacteria*	*Streptomyces*	*Streptomyces caniferus* NRRL B-16358 (EF654099)	Unknown	99
3Z7	γ-*Proteobacteria*	*Stenotrophomonas*	*Stenotrophomonas* sp. Pm3 (GU391493)	Roots of *Platanthera minutiflora*	100
3Z10	γ-*Proteobacteria*	*Frateuria*	*Frateuria* sp. Ni-H2-1 (EU170476)	Soil	99
3Z11	β-*Proteobacteria*	*Castellaniella*	*Castellaniella defragrans* PD-19 (AB195161)	Soil	98
3ZP1	*Actinobacteria*	*Curtobacterium*	*Curtobacterium flaccumfaciens* 2P04PE (EU977762)	Space Center clean-room floor	100
3ZP2	*Actinobacteria*	*Microbacterium*	*Microbacterium* sp. JJD-1 (FJ765512)	Slaughterhouse waste soil	99
3ZP3	*Actinobacteria*	*Arthrobacter*	Uncultured bacterium clone AKAU3746 (DQ125674)	Uranium contaminated soil	99

[1]Strains from **Site 1** (1A's; 1C's; 1Z's); from **Site 3** (3A's; 3C's; 3Z's).

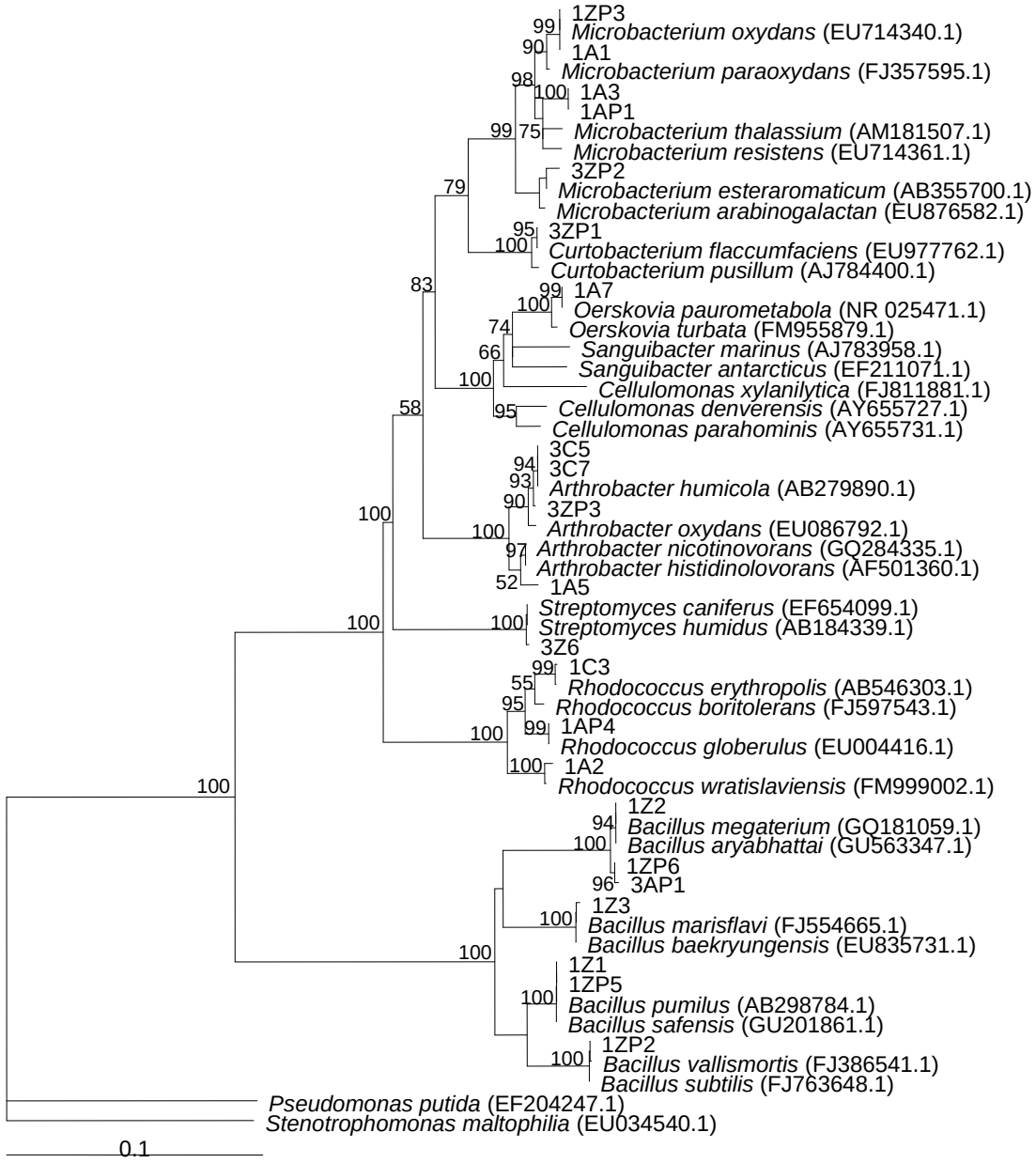


Figure 4.4 Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between sequences of the Gram-positive representatives isolated and some of their closest phylogenetic relatives. The tree was created by the neighbour-joining method. Bootstrap values >80 % are shown at nodes. *Pseudomonas putida* (EF204247.1) and *Stenotrophomonas maltophila* (EU034540.1) were used as outgroup. Bar, 0.1 substitutions per nucleotide position.

The Gram-negative isolates were related to classes *γ*-Proteobacteria, *β*-Proteobacteria, *α*-Proteobacteria, Sphingobacteria and Flavobacteria (Fig 4.2). The most frequently class was *γ-Proteobacteria*, related with some genera within the families *Pseudomonadaceae, Caulobacteraceae, Brucellaceae* and *Xantomonadaceae,* with a relationship with species of genera *Pseudomonas, Brevundimonas, Frateuria, Mycoplana* and *Stenotrophomonas* (Fig. 4.5-A). Values of 16S rRNA gene sequence similarity of the isolated strains of this class and the described taxa were high, ranging from 99 to 100 % (Table 4.2).

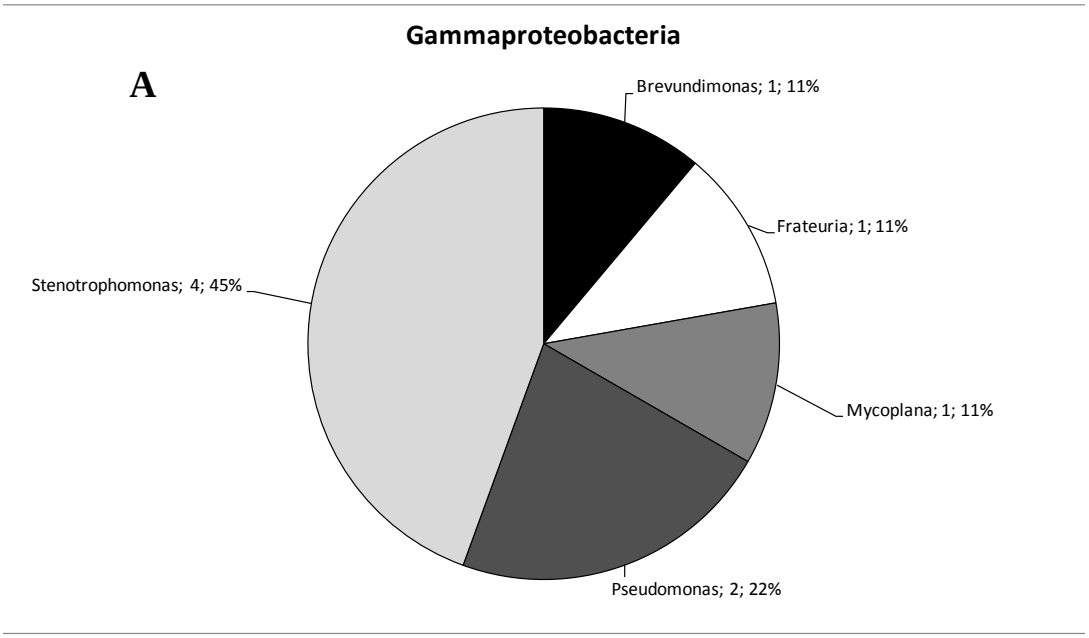
β-Proteobacteria was the Gram-negative class with the second highest number of isolates recovered. *β-Proteobacteria* isolates were related to the families *Alcaligenaceae, Comamonadaceae* and *Bulkholderiaceae* and closely related to the lineage containing *Alcaligenes, Achromobacter, Burkholderia, Castellaniella, Cupriavidus* and *Variovorax* (Fig. 4.5-B). Values of 16S rRNA gene sequence similarity between the isolates of this class and described taxa ranged from 98 to 100 % (see Table 4.2). *α-Proteobacteria* had representatives of families *Phyllobacteriaceae* and *Sphingomonadaceae,* related to some genera within the genera *Mesorhizobium*, and *Novosphingobium* (Fig. 4.5-C). 16S rRNA gene similarity with described taxa was also high, in line with previous classes (see Table 4.2).

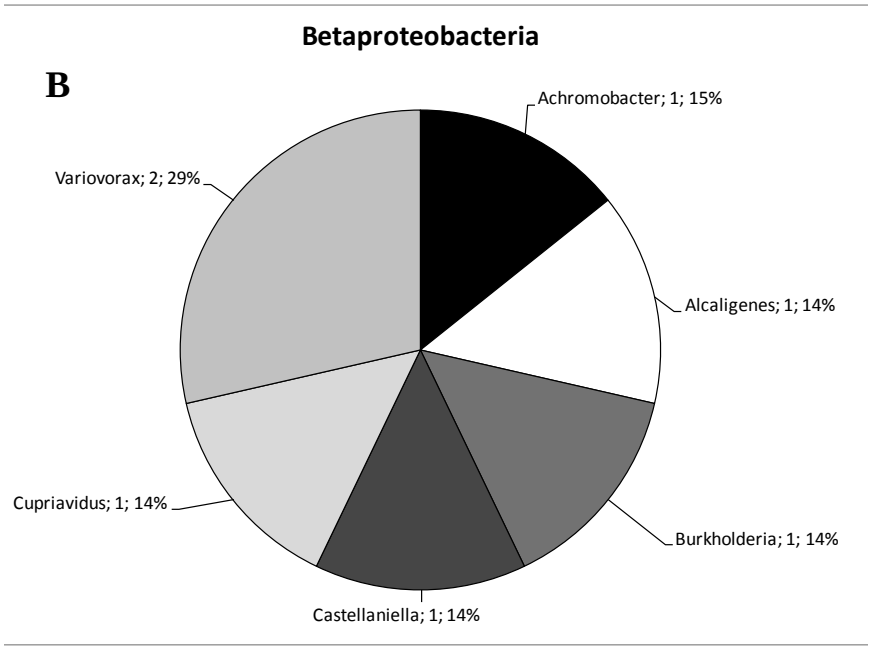
Flavobacteria and *Sphingobacteria* were the least represented class in the population with only one isolated recovered for each class. The mentioned isolates were related to families *Flavobacteriaceae* and *Rhodothermaceae,* and they were represented by isolates affiliated to genera *Chryseobacterium* and *Sphingobacterium*.

Values of 16S rRNA gene sequence similarity between isolate 3A10 with other *Flavobacteria* from the genus *Chryseobacterium* was only ~96 % (Table 4.2).

In the neighbour-joining phylogenetic tree (Fig. 4.6) the Gram-negative strains isolated formed also monophyletic groups with different groups of bacteria supported by high bootstrap values (61 to 99 %). The other phylogenetic trees showed essentially the same topology (data not shown). These results show beyond doubt that the isolated strains belong to 6 different branches as for the Gram-positives.

Interestingly some genera had only representatives in one sub-site, which can be caused by the fact that so few isolates were recovered, perhaps not showing the real composition of the bacterial populations.





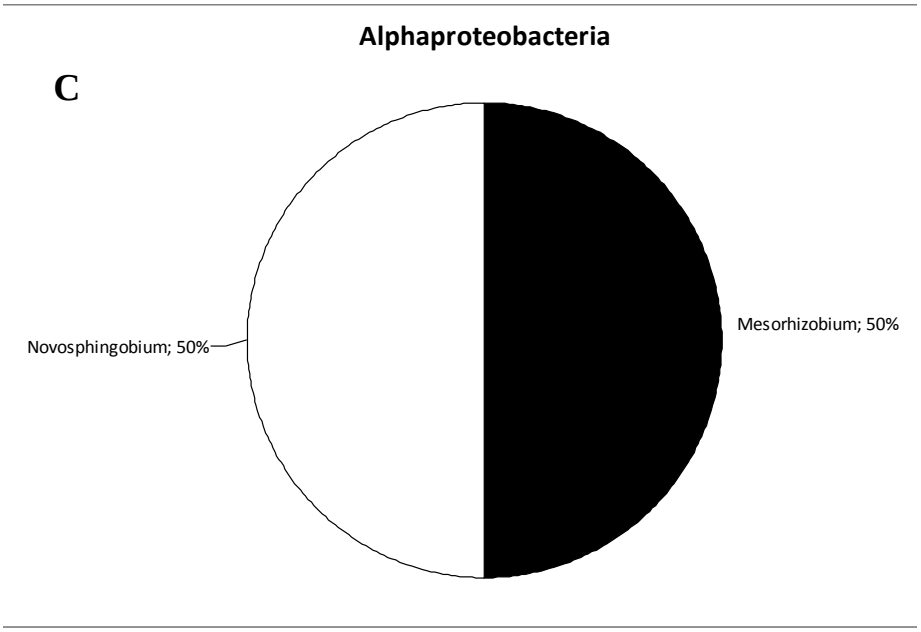


Figure 4.5 Proportions of taxonomic groups represented by the bacterial strains isolated. Class Flavobacteria and Sphingobacteria included only one representative strain and are not shown as a separate pie. The number of the isolated strains and their percentage per taxa are given together with the genus.

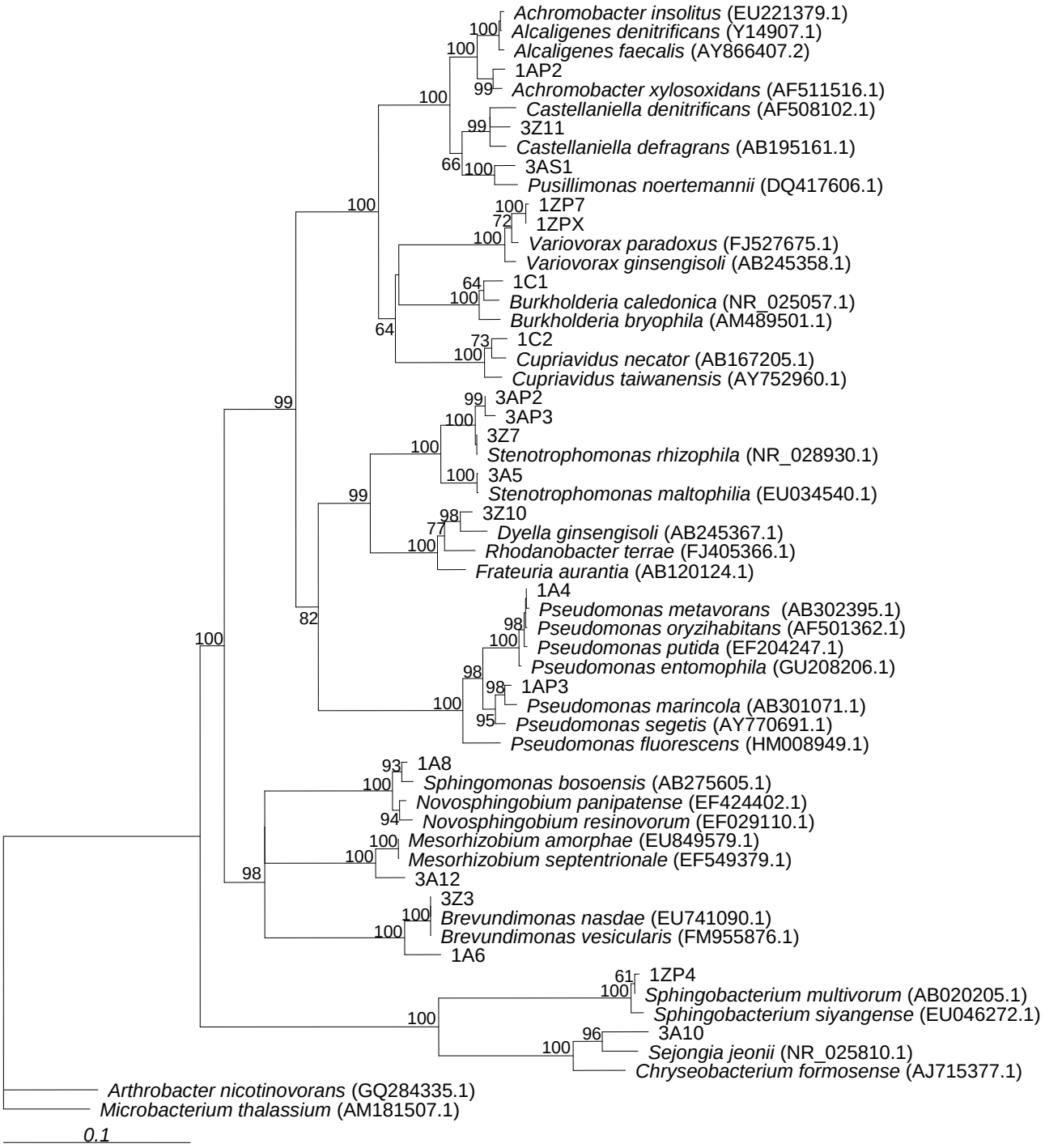


Figure 4.6 Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between sequences of the Gram-negative representatives isolated and some of their closest phylogenetic relatives. The tree was created by the neighbour-joining method. Bootstrap values >80 % are shown at nodes. *Arthrobacter nicotinovorans* (GQ284335.1) and *Microbacterium thalassium* (AM181507.1) were used as outgroup. Bar, 0.1 substitutions per nucleotide position.

4.3.5 Metal tolerance for growth

Despite the high levels of metal contamination present in the environment, and during the isolation periods, few isolates were able to grow at the metal levels tested, and even showed poor growth when the isolation setup was tested (Table 4.3). The representative populations isolated on both sub-sites apparently showed the same levels of heavy metal tolerance. It was impossible to distinguish whether any of the sub-sites has a more tolerant bacterial population than the other. This is a clear contrast to what was shown in Chapter 3 where the bacterial populations isolated in sub-site 3 were more tolerant to the different heavy metal combinations tested. None of the isolated populations were able to grow when a metal mixture was tested (Table 4.3); similarly, 2 isolates had no apparent metal tolerance and did not grow in the presence of any of the metals tested either alone or mixtures. Comparatively to what happened in the previous sampling occasion (see Chapter 3), the majority of the isolates grew well when Zn was present (Table 4.3). None of the strains recovered in this survey had been isolated in the previous sampling. Less than half of the isolates had limited growth or growth when Cd was tested (Table 4.3). The majority of the isolates grew when As was present until 500 ppm (Table 4.3). Eight strains were capable of growing in high concentration of As, Zn and Cd, six of these strains were Gram-positive and only two were Gram-positive (Table 4.3).

Table 4.3 Characteristics of representative strains of the isolated population (most tolerant strains shown in bold).

Strain[2]	*Colony pigmentation*	*Cell morphology*	*Gram*	**Metal tolerance (growth at ppm)**[1]									
				Cd			**Zn**			**As**			
				250	**500**	**1000**	**250**	**500**	**1000**	**250**	**500**	**1000**	**Mix**
1A1	Yellow	Rod	+	-	-	-	+++	-	-	++	+	+	-
1A2	**White**	**Coccus**	**+**	**+**	**+**	**+**	**++**	**++**	**+**	**++**	**++**	**-**	**-**
1A3	Yellow	Rod	+	-	-	-	+	-	-	+++	+	+	-
1A4	White	Rod	-	-	-	-	++	+	-	+	-	-	-
1A5	Yellow	Round rod	+	+	-	-	++	+	+	+	+	-	-
1A6	White	Short rod	-	-	-	-	++	++	+	+++	+++	+	-
1A7	Pale yellow	Rod	+	+	+	-	++	++	-	++	++	+	-
1A8	White	Rod	-	+	+	-	+	++	++	+	++	+	-
1AP1	Beige	Rod	+	-	-	-	+	-	-	+++	+	+	-
1AP2	Yellow	Rod	-	-	-	-	-	-	-	-	-	-	-
1AP3	White	Rod	-	-	-	-	-	-	-	+	-	-	-
1AP4	White	Coccus	+	+	+	-	++	++	++	++	+	++	-
1C1	**White**	**Rod**	**-**	**++**	**++**	**+**	**++**	**++**	**+**	**+**	**+**	**+**	**-**
1C2	**White**	**Short rod**	**-**	**++**	**++**	**-**	**+++**	**++**	**+**	**++**	**+**	**+**	**-**
1C3	**Yellow**	**Coccus**	**+**	**++**	**++**	**-**	**++**	**++**	**++**	**++**	**+**	**+**	**-**
1Z1	White	Rod	+	+	-	-	++	++	+	+	-	-	-
1Z2	White	Rod	+	-	-	-	++	++	+	-	-	-	-
1Z3	White	Rod	+	+	-	-	+++	+++	+++	++	+	+	-
1ZP2	White	Rod	+	-	-	-	++	+++	+++	++	++	+	-
1ZP3	Yellow	Short rod	+	-	-	-	+++	+	+	++	-	-	-
1ZP4	**Yellow**	**Large rod**	**-**	**+**	**+**	**-**	**+++**	**+++**	**++**	**++**	**+**	**+**	**-**
1ZP5	White	Rod	+	-	-	-	++	++	+	+	+	-	-
1ZP6	Beige	Rod	+	-	-	-	++	++	+	+	+	-	-
1ZP7	White	Rod	-	++	-	-	++	++	++	++	+	+	-
1ZPX	White	Rod	-	++	-	-	++	++	++	++	+	+	-
3A5	White	Rod	-	-	-	-	-	-	-	-	-	-	-
3A10	**Yellow**	**Rod**	**-**	**+**	**+**	**-**	**++**	**+**	**++**	**++**	**++**	**-**	**-**
3A12	**White**	**Rod**	**-**	**+**	**+**	**-**	**++**	**+**	**++**	**++**	**++**	**-**	**-**
3AP1	Beige	Rod	+	-	-	-	+++	+	-	++	-	-	-

3AP2	Yellow	Rod	-	+	-	-	+++	++	+	++	+	-	-
3AP3	Yellow	Rod	-	+	-	-	+++	++	+	++	+	-	-
3AS1	White	Rod	-	-	-	-	++	++	+	+++	+	-	-
3C5	White	Rod	+	+	-	-	++	+	+	+	+	-	-
3C7	White	Rod	+	+	-	-	++	+	+	+	+	-	-
3Z3	White	Rod	-	+	-	-	++	++	-	-	-	-	-
3Z6	White	Rod	+	-	-	-	++	+++	++	-	++	++	-
3Z7	White	Rod	-	+	-	-	+++	++	+	++	+	-	-
3Z10	Yellow	Rod	-	+	-	-	++	++	++	++	++	+	-
3Z11	**White**	**Short rod**	-	++	+	-	++	+++	++	++	++	+	-
3ZP1	Yellow	Rod	+	+	-	-	+	+	+	+	-	-	-
3ZP2	Yellow	Rod	+	+	-	-	-	+++	+	+	++	-	-

[1] **-,** no growth; +, poor growth; ++, good growth; +++ exuberant growth.

[2] Strains from **sub-site 1** (1A's; 1C's; 1Z's); from **sub-site 3** (3A's; 3C's; 3Z's).

4.4 Discussion

Bioremediation can be complicated by spatial heterogeneity in the composition and metabolic activities of the indigenous microbial populations (Whitley and Bailey, 2000). Therefore, in order to develop effective strategies for the bioremediation of heavy metal contaminants, a better understanding of the composition and metabolic potential of microbial communities in contaminated soils is required. This study presents the first characterization of the bacterial of the rhizosphere in the industrial area of Estarreja, Portugal. Using cultivation-dependent methods and heavy metal supplemented media 42 strains were isolated. Culture dependent techniques and molecular techniques rRNA targeted were used in order to describe the rhizosphere microbial communities of the study site.

Many studies exist regarding the metal tolerance and resistance of bacteria. However, it is difficult to make a comparison with the results in the literature because of the diversity of growth media and incubation conditions and also of the metal contaminated environments screened reported by other authors. Heavy metals exert their toxic effects on microorganisms through different mechanisms. Even at micromolar concentrations, heavy metals inhibit the growth of most bacteria and are tolerated by only a minority of microorganisms. Metal tolerant bacteria can survive in heavy metal contaminated environments and can be isolated and selected for their potential application in bioremediation (Piotrowska-Seget, *et al*., 2005). Long term effects of heavy metals on bacterial populations in contaminated environments have been shown, and in comparison to “clean” soils, a pronounced reduction of bacterial

diversity as well of changes in bacterial community structure have been described, even when relatively low amounts of metals are present (Sandaa *et al*., 1999).

The heavy metal tolerance of the populations isolated in this study may have been acquired by adaptation, a genetically altered tolerance, or to a shift in species composition, where organisms already tolerant became more competitive (Li *et al*., 2006). This high tolerance to heavy metals could be attributed to the fact that these bacteria were isolated from a contaminated area containing high levels of heavy metals (Abou-Shanab *et al*., 2007; Pal *et al*., 2005). Therefore in this environment soil bacteria would have been exposed to heavy metals once the metals are in the forms that are available either in solution or adsorbed on soil colloids (Giller *et al*., 1998), and metal exposure led this way to the selection of tolerance among members of the bacterial population. A hypothesis for this fact is that through the evolutionary process, bacteria have been improving their heavy metal resistance mechanisms to adapt to adverse environments. Products encoded by these heavy metal resistant genes can reduce or even eliminate heavy metal toxicity (Wei *et al*., 2009). On the other hand, previously dominant groups may loose their advantage compared to the majority of individuals becoming uncompetitive thus lowering the bacterial diversity (Giller *et al*., 1998). Microorganisms isolated from contaminated environments with heavy metals often exhibit tolerance to multiple pollutants as they have adapted to these environments (Abou-Shanab *et al*., 2007; Pal *et al*., 2005). Bacterial metal tolerance and antibiotic resistance have been reported before (Hassen *et al*., 1998; Verma *et al*., 2001), and it has been suggested that under conditions of enforced stress, metal resistance in microorganisms possibly helps them to adapt spontaneously instead of via genetic mutations and natural selection (Rosen, 1996; Silver and Misra 1988).

Heavy metal resistant bacteria belonging to different genera such as *Pseudomonas, Mycobacterium, Agrobacterium, Arthrobacter, Achromobacter, Sphingomonas* and *Microbacterium* have been found to potentially promote plant growth and reduce stress symptoms in plants (Abou-Shanab *et al*., 2007; Dell'Amico *et al*., 2005; Jiang *et al*., 2008; Ma *et al*., 2009). During this survey *Bacillus, Microbacterium, Achromobacter* and *Pseudomonas* strains were very well represented within the bacteria recovered. It was reported that *Bacillus* sp. had a higher relative abundance in the most heavy metal contaminated soils (Ellis *et al*., 2003), however this was not asserted in this study. β-*Proteobacteria* have been identified with a putative *arsC* gene, but there have been few studies on the arsenic resistance in this group. An arsenite oxidizing strain of *Variovorax paradoxus* has been isolated from arsenic amended soil columns (Jackson *et al*., 2005), a *Variovorax* sp. isolated in this study also showed resistance to As and Zn.

Macur *et al*., (2004), also isolated an arsenate resistant species of *Flavobacterium* from soil columns, which appears to be the only arsenic resistant *Flavobacteria* that has been characterized (Jackson *et al*., 2005). During this survey a Flavobacteria strain was isolated that not only showed resistance to the three metals tested, but also proved to be a bacterial species previously unknown. Characterisation of this strain is detailed in Chapter 5.

Actinobacteria and Bacilli also have representatives that contain species that have putative arsenic resistance genes. Similarly, arsenic resistance and metabolism has been shown in various members of the α- and γ-*Proteobacteria,* (Jackson *et al*., 2005), taking this in consideration it will not be a surprise to find As resistant representatives in the isolated strains.

Several of the isolates recovered were identified as *Stenotrophomonas*. Other studies have mentioned the peculiar characteristics of some members of this genus for cadmium and antibiotic resistance. (Chien *et al*., 2007). It has also been noticed that *Stenotrophomonas* sp. isolated from heavy metal contaminated soil showed a much higher tolerance to heavy metals than those obtained from culture collections (Chien *et al*., 2007).

The most heavy metal tolerant isolates recovered were Gram-negative. Many studies have shown that Gram-negative bacteria are more tolerant to heavy metals than Gram-positive, this metal tolerance can be attributed to the interactions between the bacterial cell wall and the metal ions resulting in their detoxification.

Regarding toxicity it was shown in this study that for all isolated strains the order of toxicity of the metals tested was found to be Cd > As > Zn.

The bacterial ability to promote plant growth in metal contaminated environments makes them a primary target for microbial assisted phytoremediation studies. Bacteria may produce indole acetic acid (IAA) in the presence of heavy metals can be exploited to promote the growth of plants under metal stress. Previously, metal resistant bacterial isolates belonging to genera such as *Pseudomonas* and *Bacillus* were reported to produce IAA and aid plant growth (Rajkumar *et al*., 2006; Zaidi *et al*., 2006). The fact that these genera were present in the isolated populations possibly indicates that the strains may produce IAA. Different metal resistant bacteria have shown their capabilities in improving host plant growth and development in metal contaminated soils by ways of mitigating the heavy metal toxic effects in plants; siderophores producing bacteria and containing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase protects the plants against Ni, Pb and Zn toxicity In addition, various

N_2-fixing and auxin-producing bacteria immobilised Cd and promoted growth and plant nutrient uptake in the presence of toxic Cd concentrations (Belimov *et al*., 2004). Sheng and Xia (2006) reported that the addition of Cd-resistant bacterial strains to *Brassica napus* grown in metal contaminated soil significantly increased the plant uptake of Cd when compared with the non-inoculated controls. Belimov *et al*., (2005) have isolated cadmium resistant *Variovorax paradoxus* from the rhizosphere of *Brassica juncea* that promotes plant growth.

Doelman (1985) has reported that the efficiency of revegetation and phytoremediation of heavy metal-contaminated sites is closely related to the presence of higher proportions of metal resistant microbial populations in the soil, which likely conferred a better nutritional assimilation effect on plants, by mechanisms previously described (eg. IAA, auxin and ACC production). Bacteria possibly affect trace metal availability in the rhizosphere and subsequently to the plant through the release of chelating substances, acidification of the microenvironment, and by influencing changes in redox potential (Smith and Read, 1997). Soil bacteria have been known to exude biosufactants, organic acids and to produce siderophores that stimulate metal bioavailability in soil and thereby facilitate the uptake of various metal ions, including Fe^{2+} (Crowley *et al*., 1991) and Cd^{2+} (Salt *et al*., 1995).

Although the microbial activity strongly influences metal speciation and transport in the environment, further studies including detailed knowledge on the bacterial metabolites and microbial analysis in the rhizosphere are required to better understand the interactions between microorganisms and heavy metal accumulating plants and to elucidate the mechanisms on how bacteria can promote heavy metal accumulation and translocation in plants.

The results obtained in this work indicate that heavy metal resistant bacteria can survive in the soils with high amount of Cd, Zn and As, showing a high potential for bioremediation that should be explored further. It was noticed in the results section that some of the bacteria did not show resistance to some of the heavy metals or to some of the concentrations used so it is recommended that for future diversity studies in this site or to "domesticate" new bacteria resistant to heavy metals the appropriate level of heavy metals should be used.

Analysis of the most abundant strains strongly indicate that further efforts have to be made to fully understand this metal contaminated environment. This includes further isolation surveys and detailed characterization of the isolated populations in order to define their ecophysiological roles.

Chapter 5: *Chryseobacterium humi* sp. nov. and *Chryseobacterium palustre* sp. nov., isolated from industrially contaminated sediments

5.1 Introduction

The genus *Chryseobacterium* was first proposed by Vandamme *et al.* (1994). It belongs to the family *Flavobacteriaceae* and currently comprises 37 species with valid names (Kämpfer *et al*., 2009). *Chryseobacterium* species may be found in soil and water environments and in clinical and dairy sources (Bernardet *et al.*, 2006). Three flavobacteria previously classified as *Sejongia antarctica*, *S. jeonii* (Yi *et al.*, 2005) and *S. marina* (Lee *et al.*, 2007), have been recently transferred to the genus *Chryseobacterium* (Kämpfer *et al*., 2009). Recently the bacterial diversity of sediments collected from an industrially polluted site at Estarreja, in northern Portugal was investigated. Those sediments present high levels of contamination, especially by heavy metals (Costa & Jesus-Rydin, 2001; Oliveira *et al*., 2001; Carvalho *et al*., 2002). Two different sampling occasions yielded two unidentified organisms phylogenetically related to the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). In the study presented here, a detailed classification of these two strains is provided on the basis of a polyphasic analysis, which includes a thorough analysis of morphological and physiological characteristics, cellular fatty acid profiling, DNA-DNA hybridization experiments and phylogenetic analysis of 16S rRNA gene sequences(IJSEM-description of a new taxon). On the basis of the results obtained, strains 3A10^{T} and ECP37^{T} represent new species of the genus *Chryseobacterium* for which the names *Chryseobacterium palustre* sp. nov. (3A10^{T} = LMG 24685^{T} = NBRC 104928^{T}) and

Chryseobacterium humi sp. nov (ECP37^T = LMG 24684^T = NBRC 104927 ^{T}) are proposed. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 3A10^T and ECP37^T are EU360967 and EU360966, respectively.

5.2 Materials and methods

5.2.1 Bacterial strains and culture conditions

Sediment samples were serially diluted in saline solution (0.85% (w/v) NaCl) and inoculated on trypticase soy agar (TSA; Oxoid) adjusted to pH 7.0 at 30 ºC. Strains 3A10^T and ECP37^T were selected on the basis of colony morphology and colour, purified by subculturing and preserved at -80 ºC in modified Luria-Bertani broth (MLB) (Tiago *et al.*, 2004), supplemented with 15% (v/v) glycerol. Unless otherwise stated, all morphological and tolerance tests were performed on TSA.

5.2.2 Morphological, physiological and biochemical tests

Cell morphology and gliding motility were examined by phase-contrast microscopy. Flagellar motility was tested as described by Alexander & Strete (2001). Gram staining and the catalase and cytochrome oxidase tests were performed as described by Murray *et al*. (1994) and Smibert & Krieg (1994). Cell dimension was determined using a Leica DM4000B light microscope equipped with the Leica Applied Suite Software. Presence of flexirubin pigments (carotenoids located in the Gram-negative outer membrane) was investigated using the bathochromatic test with a 20 % (w/v) KOH solution as described by Bernardet *et al.*, (2002).

The phenotypic tests listed below were performed on strains 3A10^T and ECP37^T and on their closest phylogenetic neighbours *Chryseobacterium marinum* NRBC

103143^T, *C. antarcticum* AT1013^T and *C. jeonii* AT1047^T. The pH range for growth was determined in buffered trypticase soy broth (TSB) adjusted at pH 3-10 (at 1 pH unit intervals). The turbidity of the cultures grown in an orbital shaker at 25 ºC was measured at 610 nm. All buffer solutions used to adjust the pH of TSB were prepared from 1 M stock solutions according to Gomori (1990). Citrate buffer was used for pH 3-6, phosphate buffer for pH 7, Tris buffer for pH 8, and a carbonate-bicarbonate buffer for pH 9 and 10. Growth temperature ranges were determined on TSA incubated at 4, 10, 15, 20, 25, 30, 37 and 50 ºC.

Growth in the presence of 0-20 % (w/v) NaCl (at 1 % intervals up to 10 %, then 12, 15 and 20 %) was examined in TSB adjusted to pH 7 and incubated at 25 ºC. The ability to grow under anaerobic conditions was evaluated by incubating TSA plates in an anaerobic jar (with 9-13 % CO_2) in O_2-deprived atmosphere using Anaero*Gen*^{TM} (Oxoid) at 25 ºC for 7 days.

Acid production from carbohydrates was examined in API 50 CH test strips using the API 50 CHB/E medium (bioMérieux, France), according to the instructions of the manufacturer. Results were recorded after 24, 48, 72 and 120 h of incubation at 25 ºC. Single carbon source assimilation was also determined in API 50 CH test strips (bioMérieux, France), using cells suspended in 0.1 M phosphate buffer (pH 7) supplemented with 0.7 % yeast nitrogen base (Difco) and 0.05 % NH_4Cl_2 (Tiago *et al.*, 2005; 2006). Bacterial cells were suspended in sterilized water to reach a turbidity corresponding to McFarland No. 6 standard. Cell suspensions (3 ml) were then added to 60 ml of the medium and inoculated in the API 50 CH test strip wells, as recommended by the manufacturer. Results were recorded at 24, 48, 72 and 120 h of incubation at 25 ºC. Nitrate reduction, indole production and presence of β-galactosidase, L-arginine dihydrolase and urease activities were determined using API 20 NE strips and the API

AUX medium (bioMérieux, France). Results were recorded after 24, 48, 72 and 120 h of incubation at 25 ºC. Hydrolysis of arbutin, gelatin, casein, Tween 20, Tween 80 and starch, were tested on TSA as described by Hudson *et al.* (1986) and Smibert & Krieg (1994). Antibiotic susceptibility was examined on TSA at 25 ºC for 72h with disks (Oxoid) containing penicillin G (10 µg), erythromycin (15 µg), ceftazidime (30 µg), cephalothin (30 µg), tetracycline (30 µg), amoxicillin (25 µg), ciprofloxacin (5 µg), colistin sulphate (50 µg), ticarcillin (75 µg), sulfamethoxazole-trimethoprim (25 µg), vancomycin (30 µg), sulphamethoxazole (25 µg), methicillin (5 µg), meropenem (10 µg), gentamicin (10 µg), streptomycin (10 µg), lincomycin (2 µg), rifampicin (30 µg), cefoxitin (30 µg), cephalothin (30 µg), amoxicillin/clavulanic acid (20/10 µg), chloramphenicol (30 µg), polymixin B (300 µg) and ampicilin (10 µg), following the interpretation criteria proposed by the Comité de l'Antibiogramme de la Société Française de Microbiologie (1998).

5.2.3 Determination of G+C content of DNA and 16S rRNA gene sequence determination and phylogenetic analysis

The genomic DNA for the determination of the G+C content was obtained as described by Cashion *et al.* (1977). The G+C ratios were estimated by HPLC as described by Mesbah *et al.* (1989), by the Identification Service of the DSMZ (Braunschweig, Germany). Fatty acid methyl esters (FAMEs) were obtained from fresh wet biomass, grown on TSA at 28 ºC for 24 h, by saponification, methylation, and extraction as described previously by Kuykendall *et al.* (1988) and the fatty acids were separated, identified, and quantified according to the protocol of the Microbial Identification System, Sherlock version 4.6 (MIS-MIDI). The closest phylogenetic neighbors of strains 3A10^T and ECP37^T were not included in the fatty acid analysis

because they could not be grown under the same conditions as the isolates. Analysis of the respiratory quinones was also carried out at the DSMZ.

Extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described by Rainey *et al.* (1996). Cloning of the amplicons into pGEM T-Easy vector (Promega) and cycle-sequencing were performed at Macrogen Inc. (Seoul, Republic of Korea), using 16S universal bacterial primers (f27, f357, f519, f1114, r519, r800, r1056, r1492) (Lane, 1991). For phylogenetic analyses, the sequences were aligned using the BioEdit program (version 7.0.5.3) (Hall, 1999) and analysed via the DNAML, SEQBOOT, DNAPARS, DNADIST (Kimura two-parameter correction), NEIGHBOR, FITCH and CONSENSE programs of the PHYLIP package (Felsenstein, 1995). 16S rRNA gene sequences of other members of the family *Flavobacteriaceae* were obtained from the National Center for Biotechnology Information database (Benson *et al.*, 2007). A manually corrected and degapped alignment of 31 sequences of 1249 nucleotides was used. The robustness of the tree was confirmed by using bootstrap analysis based on 100 resamplings of the sequences (1000 for the neighbour-joining analysis). Non-homologous and ambiguous nucleotide positions were excluded from the calculations.

(i) 3A10^T 16S rRNA gene sequence:

ttgagtttgattctggctcaggatgaacgctagcgggaggcctaacacatgcaagccgag
cggtatgagtagcttgctactcagagagcggcgtacgggtgcgtaacacgtgtgcaacct
acctttatcaggggaatagcctttcgaaaggaagattaatactccataatatatttgatg
gcatcattagatattgaaaactccggtggatagagatgggcacgcgcaagattagatagt
tggtgaggtaacggctcaccaagtcgatgatctttaggggtcctgagagggagatccccc
acactggtactgagacacggaccagactcctacgggaggcagcagtgaggaatattggac
aatgggtgagagcctgatccagccatcccgcgtgaaggatgacggtcctatggattgtaa
acttcttttgtacagggataaacccagatacgtgtatctggctgaaggtactgtacgaat
aagcaccggctaactccgtgccagcagccgcggtaatacggagggtgcaagcgttatccg
gatttattgggtttaaagggtccgtaggcggaccaataagtcagtggtgaaatctcatag
cttaactatgaaactgccattgatactgttggtcttgagtaaattagaggtagctggaat
aagtagtgtagcggtgaaatgcatagatattacttagaacaccaattgcgaaggcaggtt
accatgatttaactgacgctgagggacgaaagcgtgggtagcgaacaggattagataccc
tggtagtccacgctgtaaacgatgctaactcgtttttggagcgcaagcttcagagaccaa
gcgaaagtgataagttagccacctggggagtacgttcgcaagaatgaaactcaaaggaat
tgacgggggcccgcacaagcggtggattatgtggtttaattcgatgatacgcgaggaacc
ttaccaagacttaaatgggaaatgacaggtttagaaatagacccttcttcggacattttt

caaggtgctgcatggttgtcgtcagctcgtgccgtgaggtgttaggttaagtcctgcaac
gagcgcaacccctgtcactagttgctaacattcagttgaggactctagtgagactgccta
cgcaagtagagaggaaggtggggatgacgtcaaatcatcacggcccttacgtcttgggcc
acacacgtaatacaatggccggtacagagggcagctaccacgcgagtggatgcgaatctc
gaaagccggtctcagttcggattggagtctgcaactcgactctatgaagctggaatcgct
agtaatcgcgcatcagccatggcgcggtgaatacgttcccgggccttgtacacaccgccc
gtcaagccatggaagtttggggtacctgaagtcggtgaccgaaaaggagctgcctagg

(i) ECP37^{T} 16S rRNA gene sequence:

ttgagtttgatcctggctcaggatgaacgctagcgggaggcctaacacatgcaagccgag
cggtatagaatcttcggattttagagagcggcgtacgggtgcgtaacacgtgtgcaacct
acctttatctgggggatagcctttcgaaaggaagattaataccccataatatattaaatg
gcatcatataatattgaaaactccggtggatagagatgggcacgcgcaagattagatagt
tggtgaggtaacggctcaccaagtctgtgatctttaggggcctgagagggtgatccccc
acactggtactgagacacggaccagactcctacgggaggcagcagtgaggaatattggac
aatgggtgagagcctgatccagccatcccgcgtgaaggacgacggccctatgggttgtaa
acttcttttgtataggaataaacctagatacgtgtatctagctgaaggtactatacgaat
aagcaccggctaactccgtgccagcagccgcggtaatacggagggtgcaagcgttatccg
gatttattgggtttaaagggtccgtaggcggacctgtaagtcagtggtgaaatctcatag
cttaactatgaaactgccattgatactgcaggtcttgagtgaatttgaggtagctggaat
aagtagtgtagcggtgaaatgcatagatattacttagaacaccaattgcgaaggcaggtt
accaagattcaactgacgctgagggacgaaagcgtggggagcgaacaggattagataccc
tggtagtccacgctgtaaacgatgctaactcgtttttgggtcttcgggctcagagaccaa
gcgaaagtgataagttagccacctggggagtacgttcgcaagaatgaaactcaaaggaat
tgacgggggcccgcacaagcggtggattatgtggtttaattcgatgatacgcgaggaacc
ttaccaagacttaaatgggaaatgacagatttagaaataaatccttcttcggacattttt
caaggtgctgcatggttgtcgtcagctcgtgccgtgaggtgttaggttaagtcctgcaac
gagcgcaacccctgtcactagttgctaccattaagttgaggactctagtgagactgccta
cgcaagtagagaggaaggtggggacgacgtcaaatcatcacggcccttacgtcttgggcc
acacacgtaatacaatggccggtacagagggcagctaccatgtgaatggatgcaaatctc
gaaagccggtctcagttcggattggagtctgcaactcgactctatgaagctggaatcgct
agtaatcgcgcatcagccatggcgcggtgaatacgttcccgggccttgtacacaccgccc
gtcaagccatggaagtctggggtacctgaagtcggtgaccgtaaaaggagctgcctaggg
taaaacaggtaactagggctaagtcgtaaacaaggtaaag

For DNA-DNA hybridization experiments, the genomic DNAs of strains 3A10^{T} and ECP37^{T} were hybridized and each of them was hybridized with the DNA of the type strains of their closest phylogenetic neighbors, *Chryseobacterium antarticum* AT1013^{T}, *C. jeonii* AT1047^{T}, and *C. marinum* NRBC 103143^{T}. DNA-DNA hybridization was performed at the DSMZ, as described by De Ley *et al*. (1970) with the modifications described by Huss *et al*. (1983) using a model Cary 100 Bio UV/visual spectrometer equipped with a Peltier-thermostated 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al*. (1977).

5.3 Results

5.3.1 Phenotypic characteristics

The phenotypic characteristics of strains 3A10^T and ECP37^T are given in Table 5.1 and in section 5.4.1 and 5.4.2.

Table 5.1 Differential characteristics of strains 3A10^T and ECP37^T and related species of the genus *Chryseobacterium*

Strains: 1, strain 3A10^T; 2, strain ECP37^T; 3, *C. marinum* NRBC 103143^T; 4, *C. antarcticum* AT1013^T ; 5, *C. jeonii* AT1047^T. All data from this study. +, Positive; -, negative; +/- weak positive. Data in parentheses are those reported in the original descriptions of the species.

Characteristic	**1**	**2**	**3**	**4**	**5**
Colony pigmentation on TSA	Yellow	Yellow	Yellow	Pale yellow	Yellow
Cell size (length × width, μm)	1.5-2.1 × 0.6	1.6-2.5 × 0.5-0.6	0.6-1.4 × 0.5-0.7	1.3-3.1 × 0.4-0.6	1.0-2.9 × 0.4-0.5
Catalase test	+	+/-	+	+	+
Oxidase test	+	+	-	+	+
Growth temperature (ºC)					
Range	10-37	4-37	5-25	5-25	5-25
Optimum	30	25-30	15	20	20
Growth at pH					
Range	6-9	6-9	7-9	6-10	6-10
Optimum	7	7-8	7	7-8	7-8
Growth with NaCl (% w/v)					
Range	0-6	0-7	0-3	0-3	0-2
Optimum	2-3	2	0-1	1	0
Indole production	-	-	-	+	- (+)
Hydrolysis of					
Casein	+	+	-	- (+)	+/-
Starch	+	+	+	+/-	+
Gelatin	+	+	+	- (+)	- (+)
Arbutin	+	+	-	-	-
Tween 20	-	-	+	-	+
Tween 80	-	-	- (+)	- (+)	+
Acid production from					
Galactose	+/-	-	+	+ (-)	-
Lactose	-	+/-	-	-	-
Gluconate	-	-	+	-	-
L-Arabitol	-	-	+	-	-
D-Cellobiose	-	+	-	-	-
D-Mannose	+/-	-	-	-	- (+)
Starch	+/-	+	+	+	+
Glycogen	-	+	-	+	+
Amygdalin	-	+	-	-	-
Arbutin	-	+/-	-	-	-
Salicin	-	+/-	-	-	-
5-Ketogluconate	-	-	+	-	-
Xylitol	-	+/-	-	-	-
Gentiobiose	-	+	-	-	-
DNA G + C content (mol %)	43	34	35	34	36

5.3.2 Chemotaxonomic characteristics and G+C content of the DNA

The major respiratory lipoquinone of both strains was menaquinone 6 (MK-6), which is in line with all other members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2006). The fatty acid profile of strains 3A10^T and ECP37^T, like those of other species of the genus *Chryseobacterium*, was dominated by branched fatty acids, namely iso-$C_{15:0}$, iso-$C_{17:1}$ *ω9c*, anteiso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. Table 5.2 compares the fatty acid profile obtained during this study for strain 3A10^T and ECP37^T with those reported in the literature for related *Chryseobacterium* species (Yi *et al*., 2005; Lee *et al.*, 2007). The G+C content of the DNA of isolates 3A10^T and ECP37^T was 43 and 34 mol%, respectively. G+C content of strain ECP37^T is in line with those reported for other *Chryseobacterium* species (Yi *et al*., 2005; Bernardet *et al*., 2006; Lee *et al*., 2007), but that of strain 3A10^T is significantly higher (Bernardet *et al*., 2006), being similar to that of *Kaistella koreensis* (Kim *et al*., 2004).

Table 5.2 Fatty acid contents (%) of strains 3A10^T and ECP37^T and of the type strains of related *Chryseobacterium* species

Strains: 1, strain 3A10^T; 2, strain ECP37^T; 3, *C. marinum* NRBC 103143^T (data from Lee *et al*., 2007); 4, *C. antarcticum* AT1013^T (data from Yi *et al*., 2005); 5, *C. jeonii* AT1047^T (data from Yi *et al*., 2005). All strains were not cultivated under the same conditions. Cellular fatty acids that amount to less than 1 % of the total fatty acid content in all strains are not shown. -, Not detected; tr, traces (< 0.5 %).

Fatty acid	1	2	3	4	5
iso-$C_{12:0}$	-	-	-	0.5	1.0
iso-$C_{13:0}$	3.1	5.8	0.6	2.5	2.9
anteiso-$C_{13:0}$	tr	tr	tr	tr	tr
iso-$C_{14:0}$	0.9	0.7	0.8	1.5	5.0
$C_{15:0}$	-	-	-	2.6	1.5
$C_{15:0}$ 2-OH	0.8	1.0	2.9	1.9	1.9
iso-$C_{15:0}$	40.0	45.0	17.0	13.6	12.2
iso-$C_{15:0}$ 3-OH	2.0	6.4	3.2	1.0	1.3
anteiso-$C_{15:0}$	17.0	8.0	33.8	15.2	24.2
anteiso-$C_{15:1}$	-	-	-	6.6	-
iso-$C_{16:0}$	tr	tr	0.6	2.8	5.7
iso-$C_{16:0}$ 3-OH	1.4	1.0	2.9	5.1	9.0
iso-$C_{16:1}$ H	tr	-	2.3	3.6	9.1
$C_{16:1}\omega 5c$	tr	tr	-	-	-
$C_{17:0}$ 2-OH	0.8	0.5	5.9	3.3	2.3
iso-$C_{17:0}$ 3-OH	6.0	10.8	5.0	5.6	4.4
iso-$C_{17:1}\omega 9c$	18.7	8.7	8.8	21.3	8.9
anteiso-$C_{17:1}\omega 9c$	tr	-	2.3	2.5	1.9
$C_{18:1}\omega 5c$	1.5	1.1	1.5	1.5	0.8
unknown 11.543*	tr	tr	-	-	-
unknown 13.565*	0.7	1.4	-	0.7	0.4
unknown 16.582*	0.7	1.0	-	0.5	tr
Summed Feature 3†	3.8	6.8	-	-	-
Summed Feature 4†	0.5	0.6	-	2.7	2.6

* Unknown fatty acid; numbers indicate equivalent chain length.

† Summed features are group of 2-3 fatty acids that cannot be reliably separated by GLC with the MIDI system. Summed feature 3 comprised iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega 7c$; Summed feature 4 comprised iso-$C_{17:1}$ and/or anteiso-$C_{17:1}$B

5.3.3 16S rRNA gene sequence comparison and DNA-DNA hybridization

In the neighbour-joining phylogenetic tree (Fig. 5.1) strains 3A10^T and ECP37^T formed a monophyletic group with *Chryseobacterium antarcticum* AT1013^T, *C. jeonii* AT1047^T and *C. marinum* NRBC 103143^T supported by high bootstrap values (82 to 99%). The other phylogenetic trees showed essentially the same topology (data not

shown). These results show beyond any doubt that the new strains belong to the genus *Chryseobacterium*. Isolate 3A10^T shared pairwise sequence similarity of 97.2 and 96.6 % to the type strains of *C. antarcticum* and *C. jeonii*, respectively, while strain ECP37^T showed a sequence similarity of 97.3 % to *C. marinum*.

DNA–DNA hybridization experiments revealed levels of relatedness of 15.2, 29.5 and 21.8 % between strain 3A10^T and *Chryseobacterium marinum* NBRC 103143^T, *C. antarcticum* AT1013^T and *C. jeonii* AT1047^T, respectively. The DNA–DNA relatedness values between strain ECP37^T and *C. marinum* NBRC 103143^T, *C. antarcticum* AT1013^T and *C. jeonii* AT1047^T were 10.1, 11.0 and 7.4 %, respectively. DNA–DNA relatedness between strains 3A10^T and ECP37^T was 19.8 %.

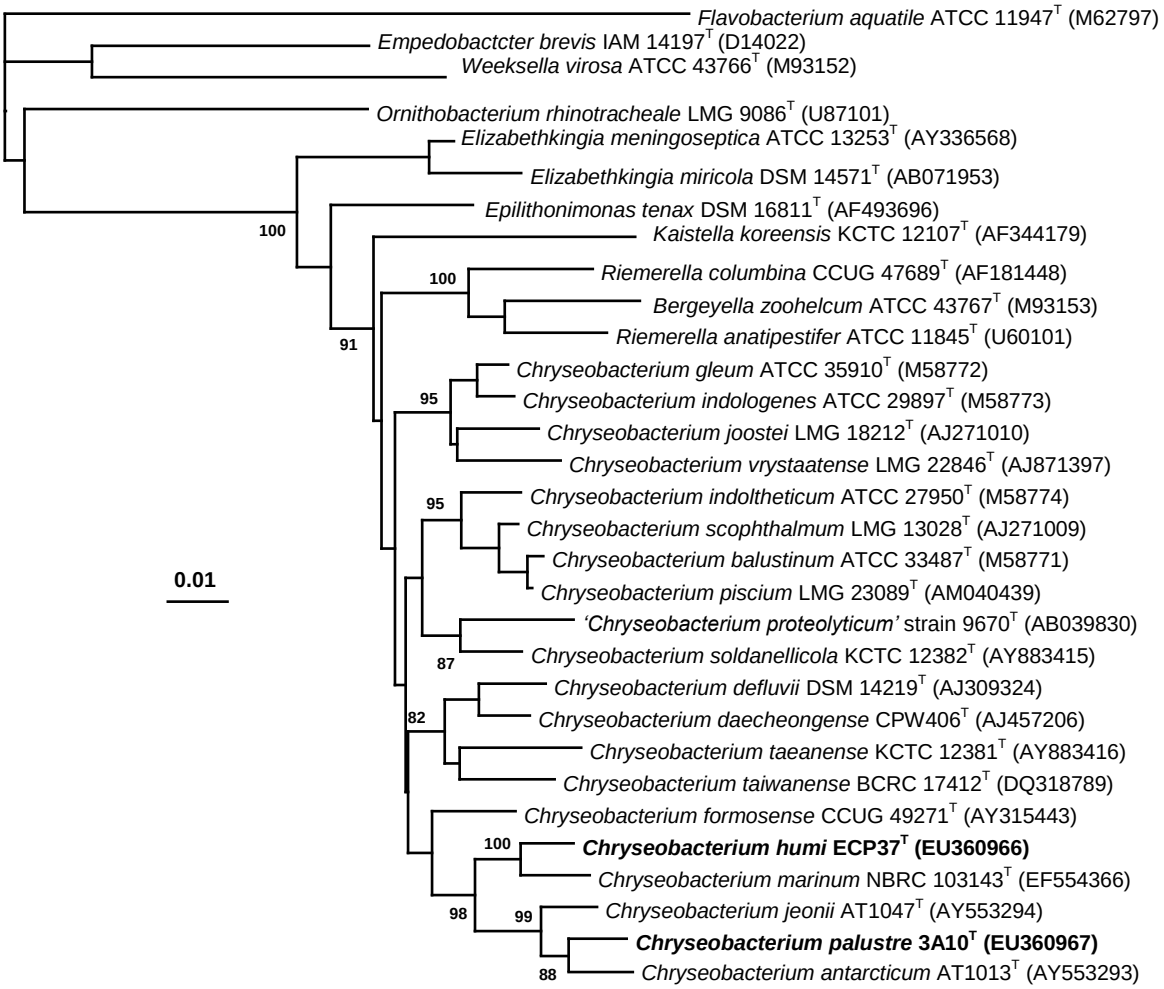


Figure 5.1 Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strains 3A10^T and ECP37^T and representative members of the family *Flavobacteriaceae*. Bootstrap values >80 % are shown at nodes. *Flavobacterium aquatile* ATCC 11947^T (M62797) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

5.4 Discussion

Phylogenetic and DNA data demonstrate that strains 3A10^T and ECP37^T represent two novel species of the genus *Chryseobacterium*, for which the names *Chryseobacterium palustre* sp. nov. and *Chryseobacterium humi* sp. nov., respectively,

are proposed. The new isolates can be differentiated from closely related species by a combination of phenotypic features (Tables 5.1 and 5.2).

5.4.1 Description of *Chryseobacterium palustre* sp. nov. *Chryseobacterium palustre* (pa.lus'tre. L. neut. adj. *palustre* pertaining to a marsh).

Cells are rods 1.5-2.1 µm in length and 0.6 µm in diameter, Gram-staining-negative, aerobic and chemoheterotrophic. Devoid of flagellar and gliding motility. Colonies grown on TSA for 3 days are 0.3-1.2 mm in diameter, circular with regular edges and yellow in colour. Flexirubin type pigments are absent. Oxidase- and catalase-positive. Growth occurs at 10-37 ºC (optimum, about 30 ºC), at pH 6.0-9.0 (optimum, pH 7) and in the presence of 0-6.0 % NaCl (optimum, 2-3 %). Nitrite and nitrate are not reduced. Casein, gelatin, arbutin, esculin and starch are hydrolyzed. Positive for the Voges-Proskauer test (API 20NE). Urease and *β*-galactosidase activities are absent. D-glucose, L-arabinose, D-mannose, *N*-acetyl-glucosamine, D-maltose, adipic acid, malic acid, trisodium citrate, L-xylose, D-fructose, L-sorbose, L-rhamnose, inositol, D-mannitol, D-sorbitol, D-lactose, D-saccharose, glycogen, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate are assimilated. Acid is produced from galactose, D-glucose, D-mannose, D-maltose and starch. Assimilation or acid production is negative for the other carbon sources in API 50CH and API 20NE strips. Growth occurs in the presence of penicillin G (10 µg), polymyxin B (300 µg), gentamicin (10 µg), sulphamethoxazole (25 µg), colistin sulphate (50 µg) and methicillin (5 µg). Growth does not occur in the presence of erythromycin (15 µg), ceftazidime (30 µg), cephalothin (30 µg), tetracycline (30 µg), amoxicillin (25 µg), ciprofloxacin (5 µg), ticarcillin (75 µg), sulfamethoxazole-trimethoprim (25 µg),

vancomycin (30 µg), meropenem (10 µg), streptomycin (10 µg), lincomycin (2 µg), rifampicin (30 µg), cefoxitin (30 µg), cephalothin (30 µg), amoxicillin/clavulanic acid (20/10 µg), chloramphenicol (30 µg) and ampicilin (10 µg). The major respiratory lipoquinone is MK-6. The major (≥ 6 %) cellular fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{17:0}$ 3-OH. Other cellular fatty acids are listed in Table 2. The G+C content of the genomic DNA of strain 3A10^T is 43 mol%.

The type strain, 3A10^T (=LMG 24685^T = NBRC 104928^T), was isolated from a rhizosphere sediment sample collected near a stream at a polluted site located in the industrial complex of Estarreja, northern Portugal.

5.4.2 Description of *Chryseobacterium humi* sp. nov.

Chryseobacterium humi (hu´mi. L. gen. n. *humi* of earth, soil).

Cells are rods 1.6-2.5 µm in length and 0.5-0.6 µm in diameter, Gram-staining-negative, aerobic and chemoheterotrophic. Devoid of flagellar and gliding motility. Colonies grown on TSA for 3 days are 0.2-1.4 mm in diameter, circular with regular edges and yellow in colour. Flexirubin type pigments are absent. Oxidase- and catalase-positive. Growth occurs at 4-37 ºC (optimum, 25-30 ºC), at pH 6.0-9.0 (optimum, pH 7.0-8.0) and in the presence of 0-7.0 % NaCl (optimum, 2 %). Nitrite and nitrate are not reduced. Casein, gelatin, arbutin, esculin and starch are hydrolyzed. Positive for the Voges-Proskauer test (API 20NE). Urease and *β*-galactosidase activities are absent. D-glucose, L-arabinose, D-mannose, D-maltose, potassium gluconate, amygdalin, D-cellobiose, maltose, glycogen and β-gentiobiose are assimilated. Acid is produced from lactose, D-glucose, D-cellobiose, starch, glycogen, amygdalin, arbutin, salicin, xylitol and gentiobiose. Assimilation or acid production is negative for the other carbon

sources in API 50CH and API 20NE strips. Growth occurs in the presence of: sulphamethoxazole (25 µg), vancomycin (30 µg), methicillin (5 µg), gentamicin (10 µg), polymyxin B (300 µg) and penicillin G (10 µg). Growth does not occur in the presence of erythromycin (15 µg), ceftazidime (30 µg), cephalothin (30 µg), tetracycline (30 µg), amoxicillin (25 µg), ciprofloxacin (5 µg), colistin sulphate (50 µg), ticarcillin (75 µg), sulfamethoxazole-trimethoprim (25 µg), meropenem (10 µg), streptomycin (10 µg), lincomycin (2 µg), rifampicin (30 µg), cefoxitin (30 µg), cephalothin (30 µg), amoxicillin/clavulanic acid (20/10 µg), chloramphenicol (30 µg) and ampicilin (10 µg). The major respiratory lipoquinone is MK-6. The major (≥ 8 %) cellular fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, iso-$C_{17:1}$ *ω9c*, and anteiso-$C_{15:0}$. Other cellular fatty acids are listed in Table 2. The G+C content of the genomic DNA of strain ECP37^T is 34 mol%.

The type strain, ECP37^T (=LMG 24684^T = NBRC 104927 ^{T}), was isolated from a soil sample collected at a polluted site located in the industrial complex of Estarreja, northern Portugal.

Chapter 6: Removal of heavy metals using different polymer matrixes as support for bacterial immobilisation

6.1 Introduction

Heavy metal pollution is one of the most important environmental problems today, especially in relation to water contamination. Several industries (mining and smelting), as well as production of fuel, energy, fertilizers, metallurgy, electroplating, electrolysis, leatherworking and photography (Wang and Chen, 2009) produce waste and wastewaters that are discharged in water courses threatening the ecosystems and ultimately human health. Traditional methods of metal removal generally consist of physical and/or chemical approaches which are often expensive, with high energy and chemical requirements, producing high amounts of residues (Quintelas *et al*., 2009) and sometimes not effective especially for low to moderate metal concentrations (Volesky, 2001). In this context, the search for new, inexpensive methods is necessary to reduce heavy metal contamination in waste water to environmentally acceptable levels. Nevertheless, biologically-based, eco-friendly and economically more attractive technologies exist.

Biosorption is a method that involves the use of biological materials that form complexes with metal ions using their functional groups (Krishnani *et al*., 2008). In the process, a chemical link between functional groups on the biosorbent and the metal ions present in solution or an ion-exchange reaction due to the high ion-exchange capacity of the biosorbent may occur (Unuabonah *et al*., 2007). Bacteria have a high surface area-to-volume ratio and can thus provide a large contact surface, which allows the interaction with metals in its surroundings (Zouboulis *et al*., 2004). However, studies demonstrated that sometimes living systems had been unreliable, especially when using

freely suspended biomass. In fact, although freely suspended biomass can have higher contact with the contaminants during the removal, it is usually not a practical form to apply directly in the clean-up process (Chen *et al*., 2005a). Biopolymers are non-toxic, selective, efficient and inexpensive and their use in immobilizing biomass may help improve biomass performance, biosorption capacity and facilitate biomass separation from metal bearing solutions (rendering the process non-destructive if necessary and allowing the regeneration of biosorbents for multiple uses), as well as increasing biomass concentration (Aksu *et al*., 1998; Akhtar *et al*., 2007). The ion-exchange process that occurs in such polymers when exposed to water contaminated with metals (Khotimchenko *et al*., 2008) is complemented with the biosportion capacity of the immobilized microorganisms. On the other hand, the synthesis of synthetic polymers that can control or promote bio-adhesion such as bacterial attachment is also of considerable industrial and clinical importance. Potential applications for synthetic materials that are bio-adherent or bio-compatible are widespread (de las Heras Alarcón *et al*., 2005). Synthesis of functional cross-linked polymeric materials involves the use of a monomer to impart the desired characteristics to the final material (such as acid, base, hydrophobic, hydrophilic) and a cross-linker which will give the necessary rigidity to the polymer network. Polymerisation is usually initiated via free-radical initiators which decompose thermally or by exposure to UV radiation. The main advantages of using these materials are the possibility to fine-tune the final properties by varying polymer composition, robustness and stability under a wide range of chemical and physical conditions.

Amongst these polymer matrices used to support microorganisms – hydrogels (Degiorgi *et al*., 2002), activated alumina and charcoal (Mor *et al*., 2005), kaolin (Quintelas *et al*., 2009), polyacrylonitrile (Vilar *et al*., 2009) alginate and pectate are the

most widespread. Alginate is a linear polysaccharide that can be found in many algal species (Stokke *et al*., 1991) which has been used extensively, to remove Pb, Zn (Arica *et al*., 2003), Cd and Hg (Kacar *et al*., 2002) as a support to the fungus *Phanerochaete chrysosporium*, to the bisorption of Cu when immobilizing the algae *Chara vulgaris* (Aksu *et al*., 1998) or of Pb, Cd and Hg when immobilizing the cyanobacterium *Microcystis aeruginosa* (Chen *et al*., 2005a). Pectate is a pectin compound which has been used to remove Zn in aqueous solutions by Khotimchenko *et al*. (2008). Nevertheless, synthetic responsive polymers have also been used successfully to control the attachment of bacterial cells to surfaces. Ista *et al* (1999) demonstrated that attachment of *Hallomonas* and *Staphylococcus* strains to substrates could be controlled by surface-grafted synthetic polymers.

The generality of biosorption studies have focused on single metal ion removal with one type of organism. Nevertheless, the situation of a single metal ion in the contaminated matrix rarely exists, and an approach to remove metal mixtures would be more realistic. The objectives of this study were to compare the use of alginate, pectate and a porous synthetic cross-linked polymer, to absorb Cd and Zn alone or when present as mixture for immobilization by different metal resistant bacterial species. Bacteria have been successfully used as biosorbents (Mameri *et al*., 1999; Nakajima and Tsuruta, 2004; Selatnia *et al*., 2004; Ziagova *et al*., 2007) because of their small size, their ubiquity, ability to grow under controlled conditions and resilience to a wide range of contaminants (Urrutia, 1997). The aim of the work was to assess the effect of the presence of the bacteria in metal removal, as well as to compare the efficiency of all polymer and bacterial combinations in order to understand what combination was more appropriate for further use in the clean-up of Cd and Zn contaminated waters. An additional objective of the work was to ascertain if Cd and Zn adsorption capacities of

the chosen bacteria varied when they were exposed to individual or mixtures of these metals.

6.2 Materials and Methods

6.2.1 Isolation and selection of heavy metal resistant bacterial strains

The bacterial strains were isolated from the non-rhizosphere and rhizosphere soils at Estarreja, Northern Portugal, as described in Chapter 3 and 4. Bacterial strains were screened for metal resistance. To check the extent of the resistance, 30 bacterial isolates previously tested for metal tolerance were further studied. Due to the number of isolates recovered from the non-rhizosphere and rhizosphere it was decided to study in more detail only the isolates that had visible growth in the presence of cadmium (Cd), metal combinations and/or showed a low phylogenetic similarity with known taxa. The strains selected were:

(i) from first survey (Chapter 3): EC1B, EC2, EC3, EC4, EC6, EC10, EC15, EC17, EC21, EC29, EC30, EC31, EC33, EC35, ECP2, ECP37, ED50, EDP28, S3X, EAL, EAPAA, EAR and EAXY4;

(ii) from the second survey (Chapter 4): 1A2, 1C1, 1C2, 1C3, 1ZP4, 3A10, 3A12 and 3Z11.

Bacterial strains were grown in trypticase soy agar (TSA) (Pronadisa) containing different concentrations of Cd and Zn alone and in combinations, ranging from 50 to 1000 ppm. The detailed protocol is referred to in Chapter 3, section 3.2.7.

As a complementary resistance test, the strains were inoculated in 25 ml Universal bottles, in 5 ml trypticase soy broth (TSB) (Pronadisa), supplemented with two metals at the following concentrations: Cd^{2+}, 25, 50, 100, 250, 500, 1000 and 2000 ppm; Zn^{2+}, 25, 50, 100, 250, 500, 1000. The cultures were incubated at 30 ºC and agitated at 150 rpm for 5 days. The cultures were then checked daily for turbidity as an indication of growth. All tests were done in triplicate.

10 isolates were then selected for their metal resistance. The selected isolates were EC15, EC30, ECP37, EAXY4, 3A10, 3Z11, 1A2, 1ZP4, 1C2 and 1C3. For further characterization, genomic DNA was isolated and the 16S rRNA gene was amplified by PCR. The partial 16S rDNA sequences obtained were matched to nucleotide sequences present in GenBank using the BLASTn program (Altschul *et al*., 1990). For comparison purposes sequences were aligned with previous data obtained in Chapters 3, 4 and 5.

6.2.2 Effect of metals on bacterial growth

300 ml Erlenmeyer flasks containing 100 ml TSB supplemented with heavy metals at the concentration of 50, 100 mg l^{-1} (Cd^{2+}), 100, 250 mg l^{-1} (Zn^{2+}) and metal mixtures of 200 mg l^{-1} ([100 mg l^{-1} (Cd^{2+}) + 100 mg l^{-1} (Zn^{2+})]) were inoculated with the bacterial strains in logarithmic growth phase. All the cultures including controls (in triplicate) were incubated at 30 ºC for 24 h at 150 rpm. Bacterial growth was monitored at defined time intervals by measuring the optical density at 610 nm. The specific growth rate (μ) was obtained using the following formula.

$$\mu = \frac{\ln OD_t - \ln OD_0}{(T_t - T_0)}$$

Where μ h^{-1} denotes the specific growth rate of the initial bacterial concentration over a given period of time; OD_t and OD_0 represent the optical density (610 nm) of the

cultures at time t and o; T_t and T_0 represent corresponding times (h). Specific growth rate of each strain was determined and those with the maximum mean specific growth rate were selected for uptake tests. The strains with the highest μ were EC30, 1ZP4 and 1C2 and were selected for further characterization and for the uptake tests.

6.2.3 Physiological and biochemical characteristics of the most resistant bacterial strains

A detailed study of the physiological and biochemical characteristics of the selected strains was done in order to better understand their biology to enhance the immobilisation experiments.

Cell morphology was tested as described by Alexander & Strete (2001). Gram staining and the catalase and cytochrome oxidase tests were performed as described by Murray *et al*. (1994) and Smibert & Krieg (1994).

The pH range for growth was determined in buffered TSB adjusted to pH 3-10 (at 1 pH unit intervals). The turbidity of the cultures grown in an orbital shaker at 30ºC was measured at 610 nm. All buffer solutions used to adjust the pH of TSB were prepared from 1 M stock solutions according to Gomori (1990). Citrate buffer was used for pH 3-6, phosphate buffer for pH 7, Tris buffer for pH 8, and a carbonate-bicarbonate buffer for pH 9 and 10. Growth temperature ranges were determined on TSA incubated at 4, 10, 15, 20, 25, 30, 37 and 50 ºC.

Growth in the presence of 0-20% (w/v) NaCl (at 1 % intervals up to 10 %, then 12, 15 and 20 %) was examined in TSB adjusted to pH 7 and incubated at 30 ºC. Acid production from carbohydrates was examined in API 50 CH test strips using the API 50

CHB/E medium (bioMérieux, France), according to the instructions of the manufacturer. Results were recorded after 24, 48, 72 and 120 h incubation at 30 ºC. Single carbon source assimilation was also determined in API 50 CH test strips (bioMérieux, France), using cells suspended in 0.1 M phosphate buffer (pH 7) supplemented with 0.7 % yeast nitrogen base (Difco) and 0.05 % NH_4Cl_2 (Tiago *et al.*, 2005; 2006). The protocol was the same as that described in Chapter 5, section 5.2.2.

Nitrate reduction, indole production and presence of *β*-galactosidase, L-arginine dihydrolase and urease activities were determined using API 20 NE strips and the API AUX medium (bioMérieux, France). Results were recorded after 24, 48, 72 and 120 h of incubation at 30 ºC.

6.2.4. Synthetic cross-linked polymer synthesis

Chemicals. Ethylene glycol dimethacrylate, N, N-diethylamino ethyl methacrylate, 1,1‘-azobis(cyclohexanecarbonitrile), N, N-dimethylformamide were purchased from Aldrich (UK). Polyethylene glycol 35000 was supplied by Fluka. All chemicals and solvents were analytical or HPLC grade and were used without further purification.

Polymers were prepared by mixing in a 100 ml glass bottle 40 g ethylene glycol dimethacrylate, 0.37 g N, N-diethylamino ethyl methacrylate, 2 g polyethylene glycol 35000, 40.37 g N, N-dimethylformamide and 0.8-0.9 g 1,1'-azobis cyclohexanecarbonitrile. The mixture was bubbled with nitrogen for 5 min and sealed with teflon coated caps. Polymerisation took 20 min and was initiated using an UVAPRINT 100 CVI UV source with a 0.163 W/cm^2 intensity (Dr. Hönle) (Piletsky *et al.*, 2005). The resulting polymer monolith was crushed manually in a mortar with a

pestel and the particles in the range 200-500 μm collected using sieves from Endecotts, UK. Polymers were then washed with methanol overnight in a sohxlet apparatus in order to remove any unreacted monomers and the polyethylene glycol and after dried at 60 ºC during 6 hours. Porous synthetic cross-linked polymers were produced with weak alkaline monomers in order to promote bacterial adhesion. The composition of the polymer was adapted from Barral *et al*. (2010).

6.2.5 Bacterial inoculum preparation

The bacterial strains (EC30, 1ZP4 and 1C2) were grown in 300 ml Erlenmeyer flasks containing 100 ml TSB until reaching 1.0 of optical density (610 nm). Cells were harvested by centrifugation at 6000 rpm for 15 min and the bacterial pellet was weighted and washed using sterile ultra-pure water. The harvested biomass was re-suspended in 25 ml sterile universal bottles containing 5 ml of saline solution (0.85 % w/v).

6.2.6 Immobilisation tests

6.2.6.1 Ca-Alginate and Ca-Pectate

Under aseptic conditions, the bacterial inoculum with 1.5 g of bacterial pellet was immobilised by encapsulating the bacterial cells in alginate and pectate using the method described by Escamilla *et al*. (2000) and Montes and Magaña (1991) with some modifications. The inoculum was adjusted in a volumetric cylinder to 1:1 inoculum/polymer ratio by using either polygalacturonic (Sigma) or alginic acid (Sigma) 4 % (w/v) concentrated. The solution was homogenized and forced though a needle template (gauge for ± 3 mm beads) with a peristaltic pump (Watson-Marlow Bredel, Wilmington, Mass.) flowing at 10 ml m^{-1}, and the droplets were collected in a

sterile gel inducer solution of 3.5 % (w/v) $CaCl_2$. (Sigma) After soaking for 1 h, the liquid was decanted and the spherical beads were washed with sterile ultra pure water. In aseptic conditions 3 g the beads were then packed into sterile 6 ml fritted SPE tubes (Supelco) with a filter. An adaptor cap (Phenomenex) was fitted to each of the tubes, including controls.

6.2.6.2 Synthetic cross-linked polymer

(i) Under aseptic conditions 1 g of the synthetic polymer was packed in sterile 6 ml fritted SPE tubes (Supelco) containing a filter. 1.5 g of Bacterial biomass was then added to the tube. An adaptor cap (Phenomenex) was fitted to each of the tubes, including control. Tubes were then left to settle for 1 h at room temperature.

(ii) The bacterial strains were grown in 300 ml Erlenmeyer flasks containing 100 ml TSB and 3 g of the synthetic polymer until reaching the growth exponential peakn of 1.0 of optical density (610 nm). Cells and polymer were then harvested by centrifugation at 6000 rpm for 15 min and the bacterial and polymer pellet was weighted. Under aseptic conditions 1.5 g of the pellet containing the bacterial biomass and the synthetic polymer was packed in sterile 6 ml fritted SPE tubes (Supelco) with filter. An adaptor cap (Phenomenex) was fitted to each of the tubes, including control. Tubes were then left to settle for 1 h at room temperature.

6.2.7 Heavy metal uptake tests

For metal uptake batch experiments 5 ml of a solution containing 100 mg l^{-1} of Cd^{2+}, Zn^{2+} or a metal mix solution containing 100 mg l^{-1} of each of the referred metals was added to the polymer packed tubes. Outlet metal solution was recovered, filtered using a Puradisc 25 Syringe Filter (Whatman) and then stored at 4 ºC for further analysis. Three 5 ml trials were tested per tube for each bacterial strain. The testes were

done in triplicate. The experimental set-up experiemt is shown in figure 6.1. Uptake tests with As were not carried out due to equipment limitations to anaylise the different oxidation states of this heavy metal.

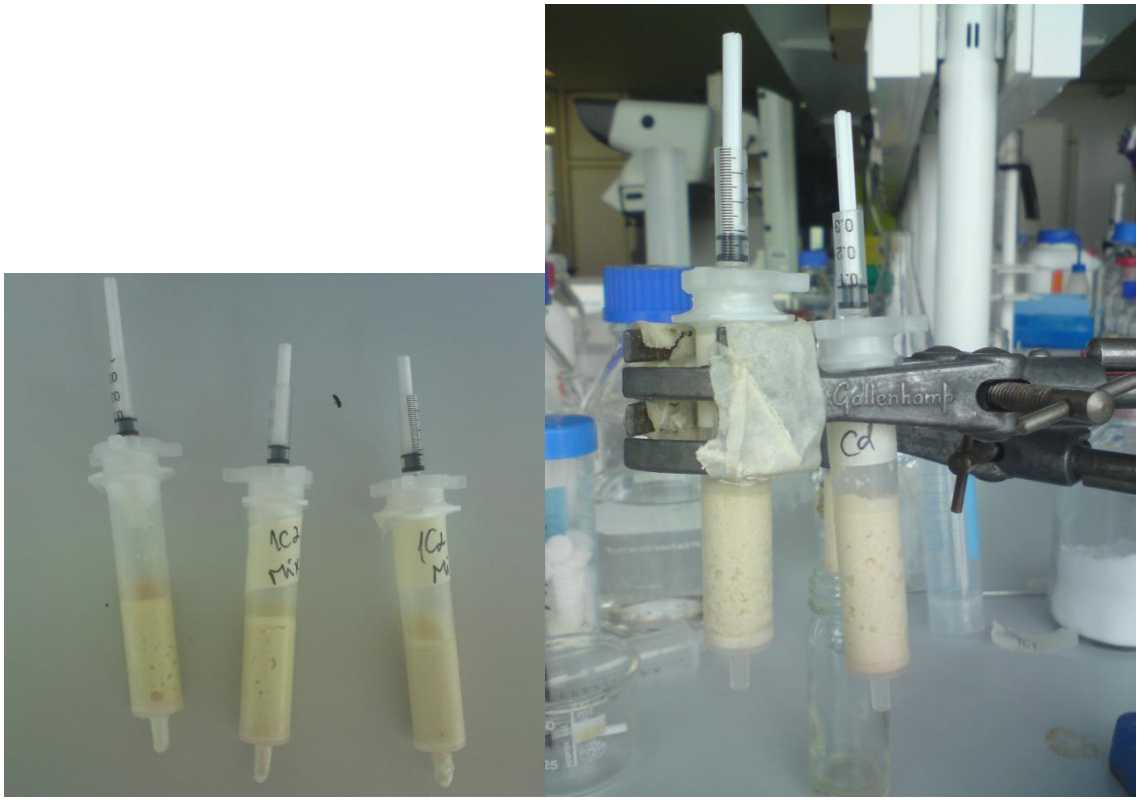


Figure 6.1 Experimental set-up showing the polymer packed columns.

The amount of residual metal present in the recovered outlet metal solution was measured by atomic absorption spectrophotometry. Total biosorbed metal values were calculated by taking the difference between metal contents in the inlet solution at time zero and at the time of sampling at the exit.

After each uptake experiment – 3 cycles each – the synthetic cross-linked polymer was washed with 1 % (w/v) NaOH (Sigma) to remove biological material and

with a 0.1 M HCl solution to remove any metals entrapped by the polymer. Polymer was then dried in a oven at 50 ºC and re-used as for new uptake tests.

6.2.8 Statistical analysis

Each test comprised 3 replicates. Statistical analysis was performed using the SPSS program (SPSS Inc., Chicago, IL Version 15.0). The data were analysed through analysis of variance (ANOVA). To detect the statistical significance of differences ($P<0.05$) between means, the Tukey test was performed.

6.3 Results

6.3.1 Screening of heavy metal resistant strains

Among the 30 bacterial strains, isolated on two different sampling occasions, the majority of them gave similar results as in previous resistance tests performed in Chapters 3 and 4, when metal tolerance in TSA with different combinations were tested. The majority of strains did not grow when metal concentrations in TSB were >100 mg L^{-1} (100 ppm) of Cd and in metal mixtures containing Cd. The ranking of the inhibitory effects of the two heavy metals tested on the majority of the bacterial strains was Cd^{2+} > Zn^{2+}. Remarkably only two of the strains, EC30 and 1ZP4, grew in all combinations tested, except for metal mixtures higher than 500 ppm. Only 9 of the initially selected isolates screened for metal tolerance in liquid media were selected for further study. The selected strains were: EC15, EC30, ECP37, EAXY4, 3A10, 1A2, 1ZP4, 1C2 and 1C3.

These experiments were used to determine the most heavy metal resistant strains, based on growth. The mean specific growth rates were calculated using the formula described previously in section 6.2.2. According to the specific growth rates,

the two metals, alone and in mixtures, differed in their inhibitory effects on the strains (Table 6.1). The raking of toxicity of the heavy metal tested was Cd^{2+} > Metal Mix > Zn^{2+} for all concentrations tested, although that was not evident for strain EC30. Surprinsingly Cd alone was more toxic than in mixtures with Zn. The strains with the highest specific growth rates in the presence of the heavy metals - EC30, 1ZP4 and 1C2 - were selected to be the bacterial strains with maximum heavy metal resistance and were used for further study.

Table 6.1 Specific growth rate of resistant strains in different heavy metal stress. The ones in bold were used for further study.

Strain	**Phylogenetic group**	**Specific growth rate (μ h^{-1})**					
		No metal	**Cd^{2+} mg L^{-1}**		**Zn^{2+} mg L^{-1}**		**Mix mg L^{-1}**
			50	**100**	**100**	**250**	**100+100**
EC15	*Bacillus*	0.36	-0.08	-0.06	0.31	0.21	-0.03
EC30	***Alcaligenes***	**0.58**	**0.53**	**0.46**	**0.44**	**0.23**	**0.37**
ECP37	*Chryseobacterium*	0.26	0.20	0.14	0.21	0.20	0.19
EAXY4	*Acinetobacter*	0.29	0.21	0.01	0.16	0.14	0.16
3A10	*Chryseobacterium*	0.23	0.17	0.08	0.24	0.20	0.15
1A2	*Rhodococcus*	0.21	0.17	0.14	0.22	0.13	0.13
1ZP4	***Sphingobacterium***	**0.52**	**0.32**	**0.23**	**0.44**	**0.40**	**0.22**
1C2	***Cupriavidus***	**0.58**	**0.31**	**0.28**	**0.31**	**0.25**	**0.26**
1C3	*Rhodococcus*	0.31	0.20	0.18	0.22	0.21	0.19

6.3.2 Physiological and biochemical characteristics

The physiological and biochemical characteristics of strains EC30, 1ZP4 and 1C2 are shown in Table 6.2. All strains were able to grow over a wide range of temperature and pH levels. All tests were done in triplicate (see Table 6.2).

Table 6.2 Differential characteristics between strains 1ZP4, EC30 and 1C2

Strains: 1ZP4; EC30; 1C2. +, positive; -, negative; w, weakly positive. Strain 1ZP4 was not able to produce acid from the carbon sources: L-Xylose, Methyl-xyloside, Esculine, Inuline, Tagatose, Fucose, Arabitol, Gluconate, 2-Ceto-gluconate and 5-Ceto-gluconate. Strain EC30 did not produce acid from any of the carbon sources tested. Strain 1C2 was not able to produce acid from the carbon sources: N-Acetyl-glucosamine, Arbutine, Gluconate and 2-Ceto-gluconate. All strains are aerobic.

Characteristic	1ZP4	EC30	1C2
Colony pigmentation	White	White	Pearly white
Cell morphology	Rod	Rod	Rod
Gram	-	-	-
Catalase test	+	+	+
Oxidase test	+	+	+
Growth temperature (° C)			
Range	10-40	10-40	10-40
Optimum	25-30	30	25
pH for growth			
Range	5-9	5-9	5-9
Optimum	7	7-8	6-7
Nitrate reduction	-	+	+
Urease	+	+	-
Voges-Proskauer reaction	w	-	+
Indole production	-	-	-
Hydrolise of			
Gelatin	+	-	-
Esculin	+	-	-
Assimilation of			
D-glucose	+	+	+
L-arabinose	+	+	+
D-mannose	+	+	+
D-mannitol	-	+	+
N-Acetyl-Glucosamine	+	+	+
D-maltose	+	+	+
Potassium gluconate	-	+	+
Capric acid	-	+	+
Adipic acid	-	+	+
Malic acid	-	+	+
Trisodium citrate	-	+	+
Phenlyacetic acid	-	+	+

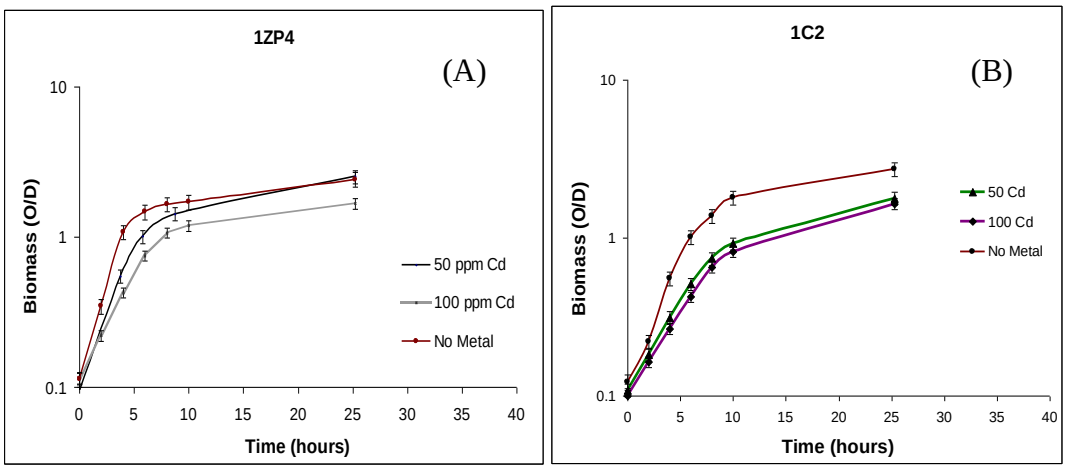
Characteristic	1ZP4	EC30	1C2
Acid production from:			
Galactose	+	-	+
Glucose	+	-	+
Frutose	+	-	+
Mannose	+	-	+
Amygdalin	+	-	+
Arbutin	-	-	-
Salicin	+	-	+
Cellobiose	+	-	+
Maltose	+	-	+
Lactose	+	-	+
Saccharose	+	-	+
Starch	+	-	+
Glycogen	+	-	+
Xylitol	+	-	+
Gentiobiose	+	-	+

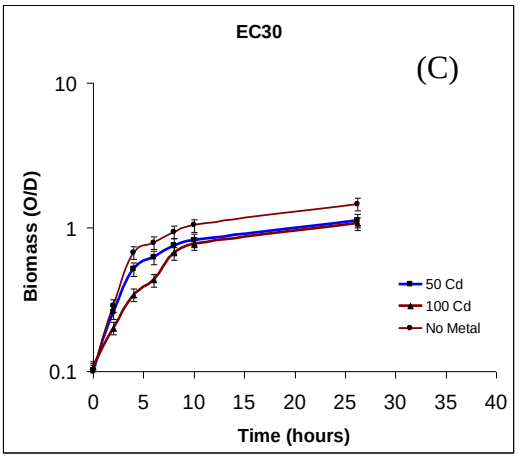
6.3.3 Growth of 1ZP4, EC30 and 1C2 in the presence of heavy metals

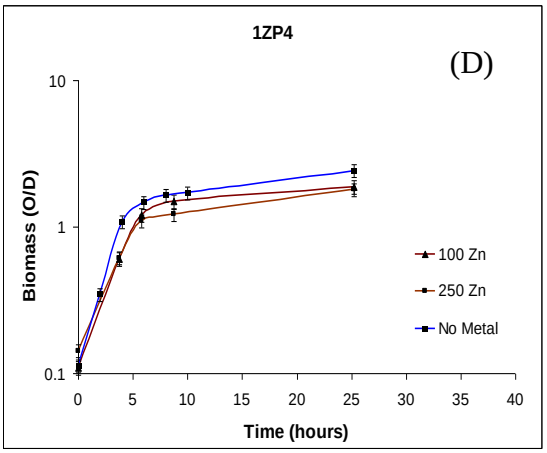
The growth of strain 1ZP4, EC30 and 1C2 was significantly reduced when TSB medium contained Cd^{2+}, indicating that this metal ion had a high degree of toxicity towards the strains. 1C2 was the strain most affected by the presence of Cd. Remarkably none of the tested strains needed a significant lag phase. The final biomass was significantly lower with 100 mg l^{-1} of Cd^{2+} compared to the TSB medium without metals (Fig. 6.2-A-C).

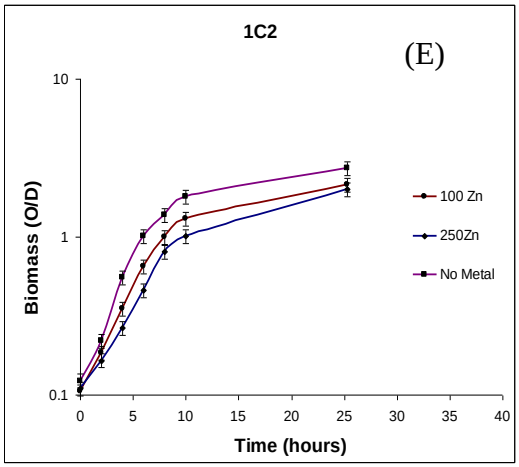
Growth curves for strains 1ZP4, EC30 and 1C2 in the presence of Zn^{2+} are shown in Fig. 6.2-D-E. At the concentrations tested Zn^{2+}, had only a small effect on the growth of the strains.

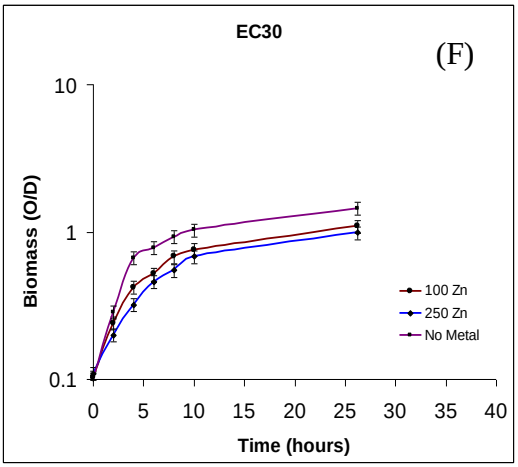
When a metal mixture was present the growth of strain 1C2 was visibly reduced. This can possibly be attributed to the presence of Cd (Fig. 6.2-H). On the other hand, in strains EC30 and 1ZP4 the metal mixture had a small effect on their growth. For strain EC30 part of the exponential growth rate was similar to the curve corresponding to absence of metals (Fig. 6.2-I), however strain 1ZP4 had a higher measured biomass when compared to EC30 (Fig. 6.2-G).

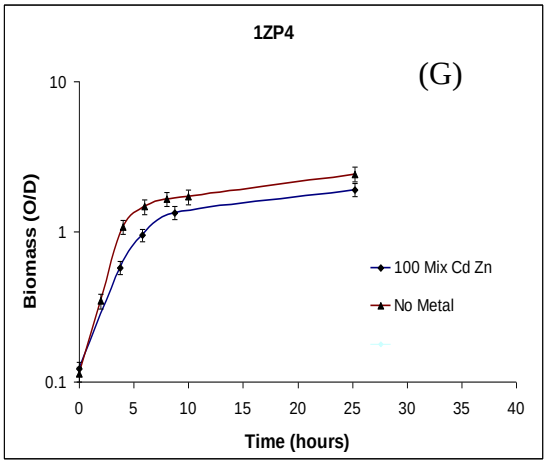


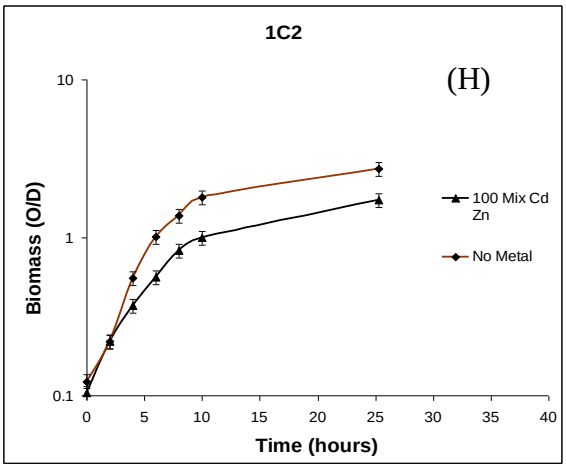

EC30
(C)
Biomass (O/D)
Time (hours)
50 Cd
100 Cd
No Metal


1ZP4
(D)
Biomass (O/D)
Time (hours)
100 Zn
250 Zn
No Metal


1C2
(E)
Biomass (O/D)
Time (hours)
100 Zn
250Zn
No Metal


EC30
(F)
Biomass (O/D)
Time (hours)
100 Zn
250 Zn
No Metal


1ZP4
(G)
Biomass (O/D)
Time (hours)
100 Mix Cd Zn
No Metal


1C2
(H)
Biomass (O/D)
Time (hours)
100 Mix Cd Zn
No Metal

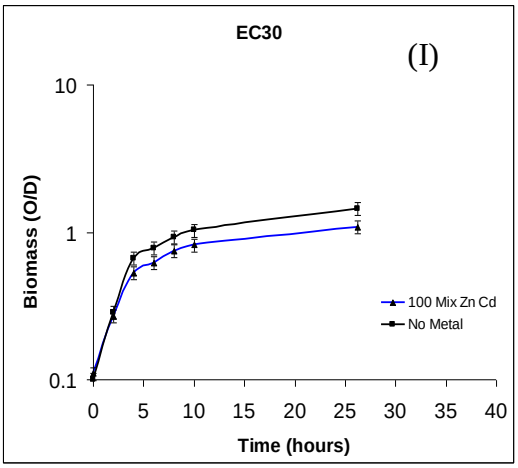


Figure 6.2 Growth of strains 1ZP4, 1C2 and EC30 in the presence of different heavy metal concentrations

6.3.4. Removal of metals in single solutions by different matrices and immobilised bacterial strains

6.3.4.1. Removal of Zn

In order to evaluate the influence of the matrix type, bacterial strain, and of their interaction on the efficacy of the Zn removal (i.e. Zn level at the outlet), a two-way ANOVA was performed. It was possible to conclude that the influence of the matrix was significant ($P<0.05$), as well as the effect of bacteria. Generally, the treatments that included bacteria showed significantly ($P<0.05$) better Zn removal (i.e. significantly lower concentrations of Zn in the outlet). Test results were, in summary, after the first removal phase, $F_{Zn(matrix)}=434$ ($P<0.001$), $F_{Zn(bacteria)}=1124$ ($P<0.001$) and $F_{Zn(matrix*bacteria)}=154$ ($P<0.001$); for the 2^{nd} cycle $F_{Zn(matrix)}=446$ ($P<0.001$), $F_{Zn(bacteria)}=725$ ($P<0.001$) and $F_{Zn(matrix*bacteria)}=253$ ($P<0.001$); and for the 3^{rd} cycle $F_{Zn(matrix)}=69.4$ ($P<0.001$), $F_{Zn(bacteria)}=175$ ($P<0.001$) and $F_{Zn(matrix*bacteria)}=58.5$ ($P<0.001$).

For each specific matrix (alginate, pectate, polymer and incubated polymer), the levels of Zn in the outlet were analysed, and the effect of the bacterial application was determined using one way ANOVA; the effect of applying multiple cycles through the matrix combinations, to examine the temporal effect was also assessed via one-way ANOVA. In the alginate matrix, generally EC30 was shown to be the bacterial treatment that significantly ($P<0.05$) enhanced the matrix performance (Table 6.3); the removal behaviour of these combinations of alginate-bacteria varied significantly ($P<0.05$) with time, showing that a clear relationship between the repeated use and the removal efficiency cannot generally be drawn for alginate. For pectate-based treatments, generally strain 1ZP4 was shown to be the bacterium that most significantly ($P<0.05$) enhanced the matrix performance– with the exception of data from the 3rd cycle when the best results were observed for the control, indicating that none of the bacterial combinations immobilised with pectate would have a long term effect (Table 6.3). Indeed, with the exception of the control (only pectate), in which the performance increased throughout the cycles, the remaining treatments removal efficiency was significantly ($P<0.05$) similar between cycles of treatment. The levels of Zn at the outlet of the polymer matrix based treatments are also shown in Table 6.3. In general the application of 1C2 appeared to be the treatment that most improved the performance of the polymer matrix. In spite of this, all the treatments showed a significant ($P<0.05$) decrease in the removal efficiency of Zn throughout the cycles, suggesting a saturation of the matrix. When the bacteria were previously incubated with the polymer before packing, again 1C2 seems to be the treatment that most significantly ($P<0.05$) enhanced Zn removal by this matrix (Table 6.3) and the efficiency of this matrix and its treatments decreased throughout the cycles.

To further understand which combination of matrix and bacteria would improve Zn removal, based on measurements at the outlet, each combination was evaluated as a specific treatment. Results for Zn levels at the outlet are shown in Table 6.3. With the exception of the 3rd cycle, PY+1C2 was the treatment that significantly ($P<0.05$) outperformed the others. Effective results were also observed for PY+EC30 and for both these combinations when bacteria were previously incubated with the polymer (PYInc+1C2 and PYInc+EC30).

6.3.4.2. Removal of Cd

The influence of the matrix, bacterial strain, and their interaction on Cd removal was evaluated using a 2-way ANOVA. For all treatments, the influence of the matrix was significant ($P<0.05$), as well as the effect of bacterial strain. In fact, in all cycles it was observed that the treatments that included bacteria showed significantly ($P<0.05$) better Cd removal (i.e. significantly lower concentrations of Cd at the outlet). Test results were for the 1st cycle $F_{Cd(matrix)}=756$ ($P<0.001$), $F_{Cd(bacteria)}=1524$ ($P<0.001$) and $F_{Cd(matrix*bacteria)}=135$ ($P<0.001$); for the 2nd cycle $F_{Cd(matrix)}=185$ ($P<0.001$), $F_{Cd(bacteria)}=630$ ($P<0.001$) and $F_{Cd(matrix*bacteria)}=272$ ($P<0.001$); and for the 3rd cycle $F_{Cd(matrix)}=45.2$ ($P<0.001$), $F_{Cd(bacteria)}=645$ ($P<0.001$) and $F_{Cd(matrix*bacteria)}=209$ ($P<0.001$).

Table 6.3 Levels of Zn in the outlet for each treatment (mg Zn L^{-1})

Treatment	Round 1		Round 2		Round 3	
	100 mg Zn/L	100 mgZn + 100 mgCd / L	100 mg Zn/L	100 mgZn + 100 mgCd / L	100 mg Zn/L	100 mgZn + 100 mgCd / L
A	97.4 ± 0.1 ^{gh,D}	92 ± 2 $^{ef,C\neq}$	84 ± 2 ^{ef,B}	84 ± 6 ^{fg,BC}	82 ± 1 ^{ef,B}	76 ± 0 ^{abc,AB}
A + 1C2	83.4 ± 0.5 ^{ef,C}	81 ± 2 ^{de,B}	87.8 ± 0.3 ^{f,B}	89.8 ± 0.8 $^{g,C\neq}$	87,1 ± 0.6 ^{f,C}	83 ± 2 ^{abc,C}
A + 1ZP4	52 ± 3 ^{c,A}	66 ± 5 $^{c,A\neq}$	84 ± 2 ^{efv,B}	75.7 ± 0.09 $^{df,AB\neq}$	79 ± 4 ^{def,B}	79 ±2 ^{abc,BC}
A + EC30	64.9 ± 0.2 ^{d,B}	70 ± 2 $^{cd,A\neq}$	69 ± 2 ^{d,A}	74 ± 3 ^{d,A}	71.0 ± 0.8 ^{cd,A}	73.9 ± 0.8 $^{abc,A\neq}$
	****F=513*	****F=44.2*	****F= 67.1*	***F=14,5*	****F=35.6*	***F=16.3*
P	91 ± 1 ^{gh,C}	99 ± 2 $^{f,C\neq}$	79.4 ± 0.7 ^{e,B}	77 ± 4 ^{df,A}	74 ± 2 ^{cde,A}	65 ± 0 $^{a,A\neq}$
P + 1C2	79 ± 2 ^{e,B}	77.8 ± 0.4 ^{d,B}	80 ± 3 ^{e,B}	83.5 ± 0.6 ^{fg,B}	79 ± 6 ^{def,A}	82.425 ± 0.005 ^{abc,D}
P + 1ZP4	41 ± 2 ^{b,A}	44 ± 2 ^{b,A}	68 ± 3 ^{d,A}	74 ± 2 $^{cd,A\neq}$	79.9 ± 0.8 ^{defg,A}	75.5 ± 0.6 $^{abc,B\neq}$
P + EC30	80.28 ± 0.03 ^{e,B}	74 ± 3 $^{cd,B\neq}$	80 ± 1 ^{e,B}	76.8 ± 0.3 $^{df,A\neq}$	77 ± 2 ^{de,A}	79.7 ± 0.5 $^{abc,C\neq}$
	****F=588*	****F=386*	****F=21.0*	***F=10.4*	*^{NS}F=2.14*	**** F=734*
PY	102.05 ± 0.05 ^{h,C}	101.4 ± 0.6 ^{f,C}	105.5 ± 0.8 ^{h,D}	106.2 ± 0.6 $^{h,D\neq}$	109.0 ± 0.4 ^{g,B}	108.6 ± 0.3 ^{c,A}
PY + 1C2	26 ± 4 ^{a,A}	22 ± 6 ^{a,A}	31.9 ± 0.5 ^{a,A}	42,0 ± 0.1 $^{a,A\neq}$	74 ± 8 ^{cde,A}	72.3 ± 0.2 ^{abc,A}
PY + 1ZP4	44 ± 4 ^{b,B}	46 ± 2 ^{b,B}	68 ± 2 ^{d,C}	73 ± 2 ^{cd,C}	99 ± 1 ^{g,B}	101 ± 1 ^{abc,A}
PY + EC30	22 ± 2 ^{a,A}	35 ± 12 ^{b,AB}	50 ±2 ^{c,B}	64 ± 5 ^{bc,B*}	65 ± 4 ^{c,A}	76 ± 6 ^{abc,A}
	****F=477*	****F=82.6*	****F=1118*	****F=305*	****F=64.6*	*^{NS}F=1.41*
PYInc	96 ± 4 ^{gh,C}	101 ± 1 ^{f,C}	96 ± 1 ^{g,C}	103.99 ± 0.06 ^{h,C}	101 ± 2 ^{g,D}	106 ± 4 ^{bc,C}
PYInc + 1C2	28 ± 4 ^{a,AB}	18.9 ± 0.5 $^{a,A\neq}$	41 ± 4 ^{b,A}	48 ± 7 ^{a,A}	51 ± 3 ^{b,B}	67 ± 2 $^{ab,A\neq}$
PYInc + 1ZP4	37 ± 4 ^{b,B}	44 ± 3 ^{b,B}	47.5 ± 0.4 ^{c,B}	44 ± 4 ^{a,A}	79 ± 3 ^{def,C}	79 ± 3 ^{abc,B}
PYInc + EC30	25 ± 3 ^{a,A}	21 ± 1 ^{a,A}	38.7 ± 0.4 ^{b,A}	60 ± 2 $^{b,B\neq}$	39 ± 4 ^{a,A}	70 ± 2 ^{abc,A}
	****F=277*	****F=1503*	****F=520*	****F=140*	****F=254*	****F=118*
	*** (F=404)	*** (F=172)	*** (F=387)	*** (F=108)	‘*** (F=84)	* (F=2.52)

Results are expressed as mean ± S.D. (n = 3). Means for each treatment in the same column with different lowercase letters are significantly different from each other (P < 0.05) according to the Tukey test. For each round, the test results are shown with the test statistics and as: NS, non-significant at the level P < 0.05; *significant at the level P < 0.05; **significant at the level P < 0.01; ***significant at the level P < 0.001.
For each matrix (alginate, pectate, polymer and incubated polymer) results of one way ANOVA are also shown with the test statistics and as: NS, non-significant at the level P < 0.05; *significant at the level P < 0.05; **significant at the level P < 0.01; ***significant at the level P < 0.001. Means for the same matrix type in the same round with different uppercase letters are significantly different from each other (P < 0.05) according to the Tukey test.
Results of the comparison between results for different effluents (Zn and Zn+Cd) for each treatment are shown and when means of Cd+Zn in each round have a ≠ signal they are significantly different from means of outlet Zn (P < 0.05) according to the t-test.

As for Zn, Cd removal, based on Cd levels at the outlet, was analysed for each specific matrix treatment (alginate, pectate, polymer and incubated polymer), in the presence and absence of the bacterial strains, using one way ANOVA. The temporal effects for the three cycles was also assessed. In the alginate matrix, EC30 was shown to be the treatment that significantly (P<0.05) most improved the matrix performance (Table 6.4). The behaviour of these combinations of alginate-bacteria was also analysed throughout the cycles and it generally varied with time, with significant (P<0.05) differences in the removal efficiencies between the 3 cycles. For pectate based treatments, generally strains 1ZP4 and 1C2 were shown to be the bacteria that most significantly (P<0.05) improved Cd removal efficiency of the matrix. The behaviour of these pectate-bacteria combinations varied throughout the cycles. EC30 seems to be the treatment that most significantly (P<0,05) enhanced Cd removal by the polymer; additionally, all the treatments showed a significant (P<0.05) decrease of removal efficiency of Cd throughout the cycles similarly to what happened for Zn (Table 6.4). When the bacteria were incubated with the polymer prior to packing, no specific treatment was found to be more effective than the others in all cycles of metal treatments in the effluents. However, strains EC30 and 1C2 gave the best results in enhancing the removal abilities of the polymer (Table 6.4); again, the efficiency of this matrix and its treatments decreased throughout time.

To further understand which combination of matrix and bacteria would enhance Cd removal, each combination was considered as a specific treatment (Table 6.4). For all cycles, the treatment that generally significantly ($P<0.05$) outperformed the others was PY+EC30; good results were also obtained for this combination when previous incubation of the bacteria with the polymer was made (PYInc+EC30).

6.3.5 Removal of binary mixtures of metals by matrices and immobilised bacterial strains

6.3.5.1 Removal of Zn from binary mixture

A two-way ANOVA was used to analyse the treatment effects. This showed that the influence of matrix and bacterial strain were significant ($P<0.05$) (with the single exception of the concentration of Zn in the outlet in the 3rd cycle, which was not affected by the matrix type). In most cases, matrixes with immobilised bacteria showed better Zn removal. Test results for the 1st cycle were $F_{Zn+Cd(matrix)}=196$ ($P<0.001$), $F_{Zn+Cd(bacteria)}=467$ ($P<0.001$) and $F_{Zn+Cd(matrix)}=66.2$ ($P<0.001$); for the 2nd cycle $F_{Zn+Cd(matrix)}=68.1$ ($P<0.001$), $F_{Zn+Cd(bacteria)}=204$ ($P<0.001$) and $F_{Zn+Cd(matrix)}=88.7$ ($P<0.001$); and for the 3rd cycle $F_{Zn+Cd(matrix)}=0.485$ ($P>0.05$), $F_{Zn+Cd(bacteria)}=3.09$ ($P<0.05$) and $F_{Zn+Cd(matrix)}=3.01$ ($P<0.05$).

Table 6.4 Levels of Cd in the outlet for each treatment (mg Cd L^{-1})

Treatment	Round 1		Round 2		Round 3	
	100 mg Cd/L	100 mgZn + 100 mgCd / L	100 mg Cd/L	100 mgZn + 100 mgCd / L	100 mg Cd/L	100 mgZn + 100 mgCd / L
A	88 ± 2 ^{f,C}	85 ± 2 ^{h,A}	61.5 ± 0.3 ^{f,B}	61 ± 2 ^{def,A}	63.1 ± 0.1 ^{e,B}	60 ±1 $^{a,AB\neq}$
A + 1C2	67.9 ± 0.2 ^{e,B}	72 ± 1 ^{gB}	68 ± 2 ^{fg,C}	65 ± 2 ^{ef,A}	69 ± 1 ^{e,B}	68.0 ± 0.9 ^{bcde,C}
A + 1ZP4	63.3 ± 0.4 ^{de,B}	58.47 ± 0.05 $^{d,A\neq}$	64 ± 1 ^{fg,BC}	61 ± 2 ^{def,A}	66 ± 5 ^{e,B}	58 ± 3 ^{a,A}
A + EC30	47 ± 4 ^{c,A}	60 ± 1 $^{de,A\neq}$	45 ± 2 ^{d,A}	63 ± 1 $^{ef,A\neq}$	48 ± 1 ^{cd,A}	62.7 ± 0.4 $^{abc,B\neq}$
	****F=147*	****F=239*	****F=144*	^{NS} *F=3.18*	****F=35.6*	****F=22.4*
P	92 ±3 ^{f,C}	86 ± 1 $^{h,B\neq}$	54 ± 2 ^{e,A}	58 ± 3 ^{de,A}	65.7 ± 0.3 ^{e,A}	64 ± 7 ^{abcd,AB}
P + 1C2	63 ± 1 ^{de,AB}	64.1 ± 0.4 ^{ef,A}	69.2 ± 0.7 ^{g,B}	69 ± 1 ^{f,C}	65 ± 1 ^{e,A}	69.6 ± 0.2 $^{de,B\neq}$
P + 1ZP4	58 ± 3 ^{d,A}	61.9 ± 0.8 ^{def,A}	64.8 ± 0.8 ^{fg,B}	64.9 ± 0.5 ^{ef,B}	65 ± 2 ^{e,A}	57 ± 3 $^{a,A\neq}$
P + EC30	68 ± 4 ^{e,B}	64 ± 3 ^{f,A}	64 ± 5 ^{fg,B}	59.92 ± 0.07 ^{def,A}	64 ± 2 ^{e,A}	61 ± 1 ^{a,AB}
	****F=87.7*	****F=76.0*	****F=18.7*	****F=34.8*	^{NS} *F=1.03*	**F=6.32*
PY	91.9 ± 0.3 ^{f,C}	98.24 ± 0.03 $^{i,C\neq}$	92.46 ± 0.07 ^{h,D}	96.9 ± 0.2 $^{g,C\neq}$	95.7 ± 0.5 ^{f,C}	100 ± 2 $^{f,C\neq}$
PY + 1C2	21 ± 1 ^{b,B}	40 ± 1 $^{c,B\neq}$	36 ± 3 ^{c,}	63.8 ± 0.9 $^{ef,B\neq}$	49 ± 3 ^{cd,B}	69 ± 2 $^{cdeB,\neq}$
PY + 1ZP4	6 ± 2 ^{a,A}	38.41 ± 0.05 $^{c,B\neq}$	25 ± 4 ^{bc,B}	33 ± 4 ^{a,A}	46 ± 4 ^{b,B}	61.7 ± 0.7 $^{ab,A\neq}$
PY + EC30	5 ± 1 ^{a,A}	23 ± 1 $^{b,A\neq}$	15.8 ± 0.7 ^{a,A}	38 ± 6 $^{a,A\neq}$	31 ± 1 ^{a,A}	58 ± 3 $^{a,A\neq}$
	****F=3860*	****F=5269*	****F=680*	****F=431*	****F=353*	****F=295*
PYInc	101.65 ± 0.05 ^{g,C}	101.25 ± 0.05 $^{i,C\neq}$	107.6 ± 0.2 ^{i,C}	106 ± 3 ^{g,C}	105.3 ± 0.7 ^{g,D}	105 ± 1 ^{f,D}
PYInc + 1C2	18 ± 3 ^{b,AB}	18.5 ± 0.5 ^{ab,A}	25 ± 3 ^{b,A}	46 ± 7 $^{bc,AB\neq}$	30 ± 2 ^{a,A}	69 ± 1 $^{cde,B\neq}$
PYInc + 1ZP4	19 ± 5 ^{b,B}	37 ± 3 $^{c,B\neq}$	37 ± 5 ^{c,B}	37 ± 3 ^{ab,A}	54.6 ± 0.8 ^{d,C}	72.2 ± 0.7 $^{e,C\neq}$
PYInc + EC30	11 ± 3 ^{a,A}	16.4 ± 0.5 $^{a,A\neq}$	22 ± 2 ^{ab,A}	52 ± 7 $^{cd,B\neq}$	41 ± 2 ^{b,B}	61.6 ± 0.6 $^{ab,A\neq}$
	****F=528*	****F=2052*	****F=476*	****F=96.3*	****F=1607*	****F=1112*
	*** (F=404)	*** (F=172)	*** (F=387)	*** (F=108)	‘** (F=84)	* (F=2.52)

Results are expressed as mean ± S.D. (n = 3). Means for each treatment in the same column with different lowercase letters are significantly different from each other ($P < 0.05$) according to the Tukey test. For each round, the test results are shown with the test statistics and as: NS, non-significant at the level $P < 0.05$; *significant at the level $P < 0.05$; **significant at the level $P < 0.01$; ***significant at the level $P < 0.001$.
For each matrix (alginate, pectate, polymer and incubated polymer) results of one way ANOVA are also shown with the test statistics and as: NS, non-significant at the level $P < 0.05$; *significant at the level $P < 0.05$; **significant at the level $P < 0.01$; ***significant at the level $P < 0.001$. Means for the same matrix type in the same round with different uppercase letters are significantly different from each other ($P < 0.05$) according to the Tukey test.
Results of the comparison between results for different effluents (Cd and Zn+Cd) for each treatment are shown and when means of Cd+Zn in each round have a ≠ signal they are significantly different from means of outlet Cd ($P < 0.05$) according to the t-test.

For each specific matrix, the levels of Zn removal were also analysed for the binary mixture, and the effect of the bacteria application was again determined using one way ANOVA. The effect of applying multiple cycles to the matrix combinations – time effect - was also assessed. In the alginate matrix, generally strain EC30 gave better immobilisation (P<0.05; see Table 6.3), while 1ZP4 was the one showing the best metal immobilisation for pectate treatments. Isolate 1C2 was the species that most significantly (P<0.05) increased the performance of the polymer matrix, even when prior incubation to packing occurred – in this case, EC30 incubated polymer also showed good results. Generally, there was a decrease in removal efficiency over each cycle. When looking at each combination as a specific treatment, it was observed that PY+1C2 was the one that significantly (P<0.05) outstood the others. Good results were also observed for the combinations when bacteria have been previously incubated with the polymer (Table 6.3).

6.3.5.2 Removal of Cd from binary mixture

A two-way ANOVA was used to analyse the treatment effects. For all cases the influence of the matrix was significant (P<0.05), as well as the effect of bacteria. In fact, in all cycles the treatments that included bacteria shown significantly (P<0.05) lower concentrations of Cd in the outlet. Test results showed that in the 1st cycle

$F_{Zn+Cd(matrix)}$=875 ($P<0.001$), $F_{Zn+Cd(bacteria)}$=2740 ($P<0.001$) and $F_{Zn+Cd(matrix)}$=319 ($P<0.001$); for the 2nd cycle $F_{Zn+Cd(matrix)}$=7.74 ($P<0.01$), $F_{Zn+Cd(bacteria)}$=228 ($P<0.001$) and $F_{Zn+Cd(matrix)}$=104 ($P<0.001$); and for the 3rd cycle $F_{Zn+Cd(matrix)}$=124 ($P<0.001$), $F_{Zn+Cd(bacteria)}$=223 ($P<0.001$) and $F_{Zn+Cd(matrix)}$=81.1 ($P<0.001$).

For all matrixes, the effect of the bacteria application was determined using one way ANOVA (Table 6.4.); the time effect was again also assessed. For most matrixes (with the exception of previously incubated polymer, in which no significant differences were observed), EC30 showed to be the bacterial treatment that most significantly ($P<0.05$) enhanced the matrix performance (Table 6.4); additionally, all the treatments showed significant ($P<0.05$) variations in the removal efficiencies of Cd throughout the cycles. When considering all cycles and combinations of matrix and bacterial isolate, the treatment that generally significantly ($P<0.05$) outperformed the others was PY+EC30; good results were also obtained for this combination when previous incubation of the bacteria with the polymer was made (PYInc+EC30).

6.3.6 Differential uptake by the binary mixture vs single metal

The effect of using a mixture of contaminants in the effluent was also assessed to determine the effect of having another ion present in the uptake of each one of the metals. Zn removal in single (Zn) and binary (Zn+Cd) solutions in each treatment were compared pair wise using the t-test (Table 6.3). For all matrixes and cycles, differences in the ability to remove Zn were observed between simple and binary contamination scenarios, which seem to indicate that the performance of the treatments is influenced not only by the concentration but also by the mixture composition. The same procedure was taken for Cd removal in single (Cd) and binary (Zn+Cd) solutions (Table 6.4). As

in the case of Zn, for all matrixes and cycles, differences in Cd removal were observed between simple and binary contamination scenarios.

The affinity of the tested matrix-bacteria combinations to remove either of the metals was also assessed and Cd and Zn removal in the binary mixture were compared using the t-test. Results are shown in Figure 6.3 for alginate, and indicate that with few exceptions (in which levels of both metals were similar) levels of Cd in the outlet were significantly ($P<0.05$) lower than those of Zn. For pectate based combinations, the same trend was observed, with the exception of the treatment with 1ZP4 in which levels of Zn were significantly ($P<0.05$) lower than those of Cd (Figure 6.4). With the exception of 1C2 incubated polymer, that presented significantly ($P<0.05$) higher levels of Zn in rounds 1 and 2, generally levels of Cd were always significantly ($P<0.05$) lower than those of Zn in the incubated polymer based treatments (Figure 6.5). As seen in Figure 6.6, levels of Cd in the outlet of the binary mixture were generally lower than those of Zn, and in the majority of cases this trend is significant ($P<0.05$). It seems thus that generally the tested bacteria-matrix combinations had higher affinity for Cd when a binary mixture is to be treated.

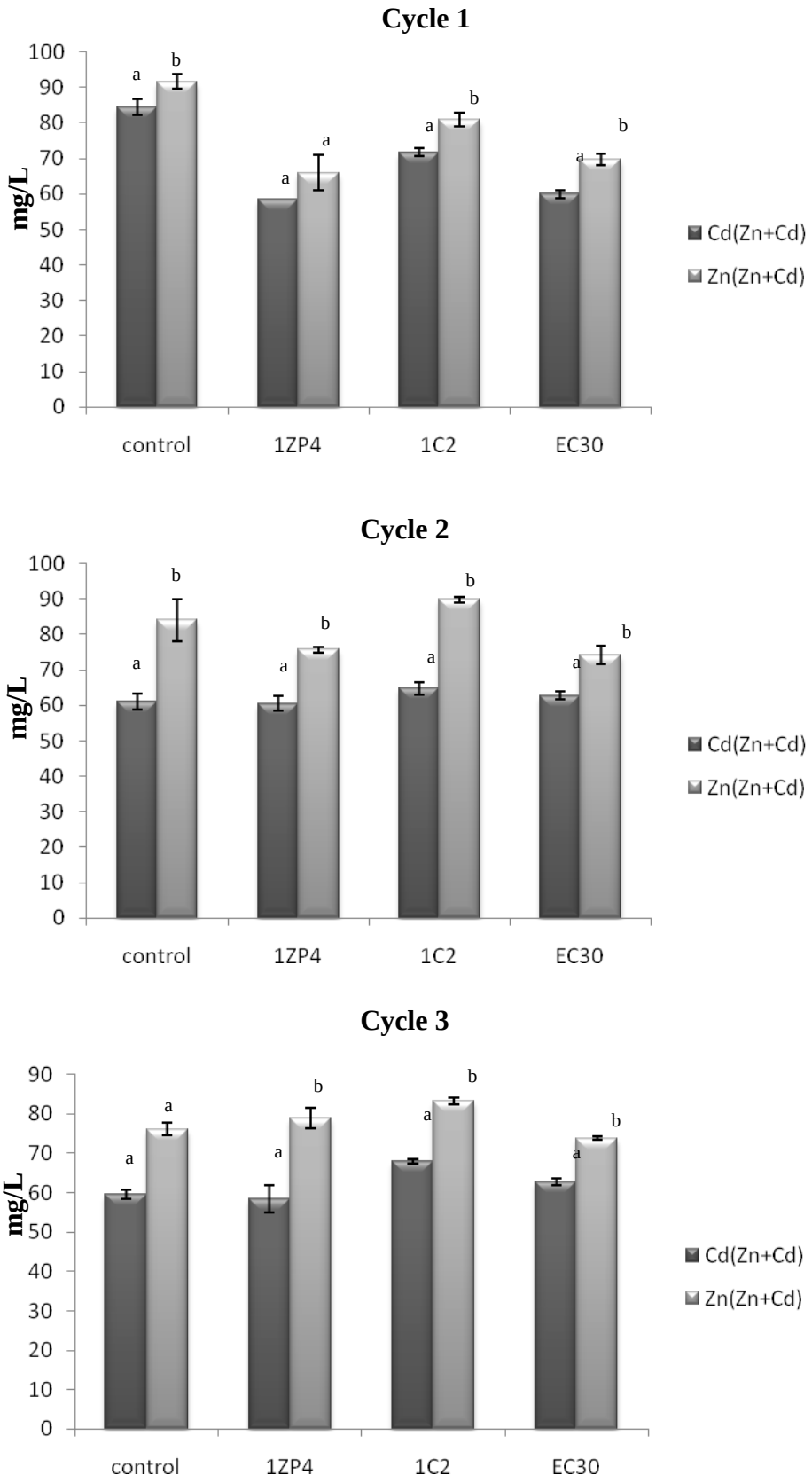


Figure 6.3 Zn and Cd levels in the combined outlet (Zn+Cd) in the alginate matrix with different bacterial applications (mg/L). Results are expressed as mean ± S.D. (n = 3). Means for the same bacterial treatment in each round with different letters are significantly different from each other (P < 0.05) according to the t-test.

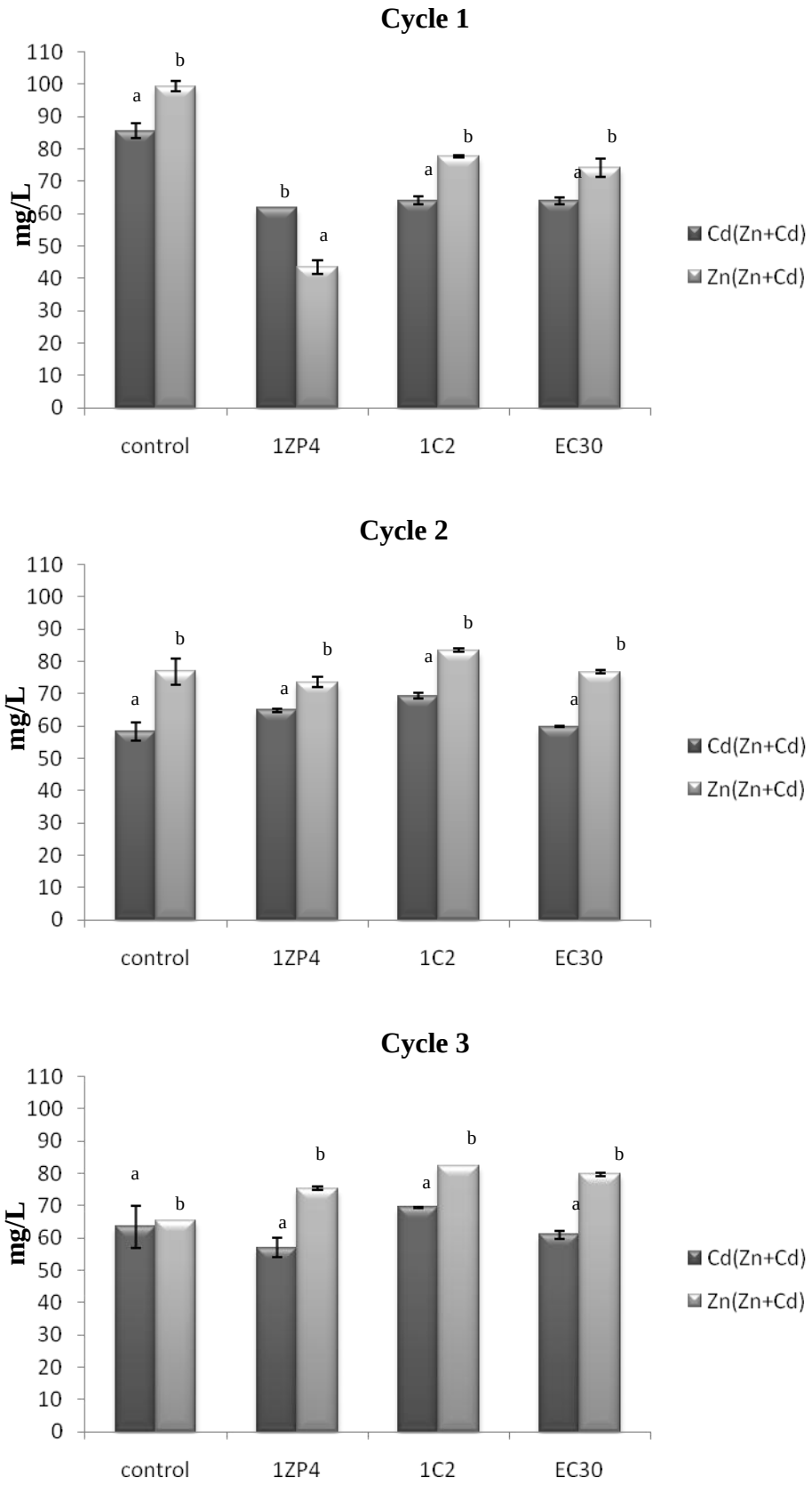


Figure 6.4 Zn and Cd levels in the combined outlet (Zn+Cd) in the pectate matrix with different bacterial applications (mg/L). Results are expressed as mean ± S.D. (n = 3). Means for the same bacterial treatment in each round with different letters are significantly different from each other (P < 0.05) according to the t-test.

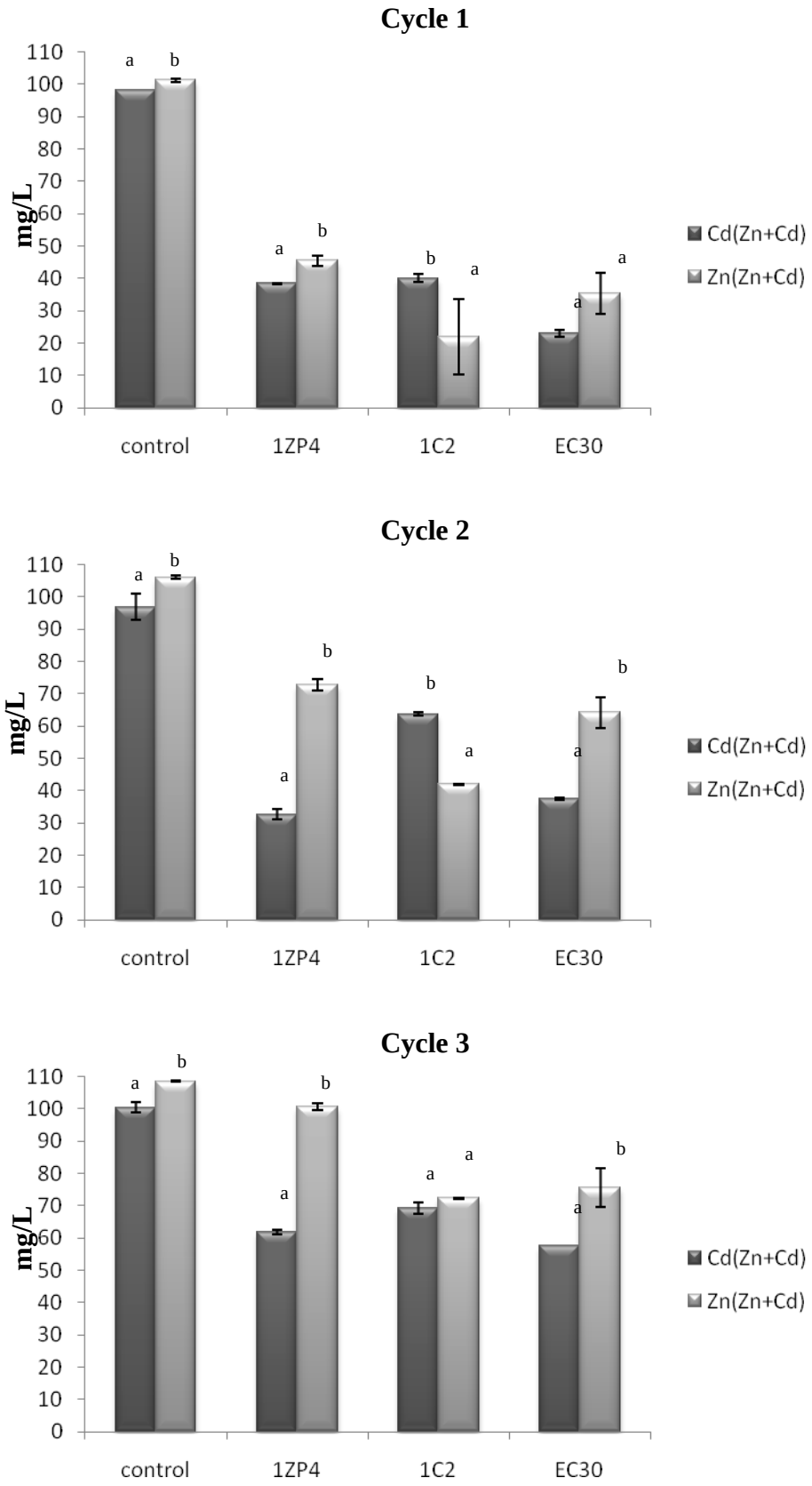


Figure 6.5 Zn and Cd levels in the combined outlet (Zn+Cd) in the polymer matrix with different bacterial applications (mg/L). Results are expressed as mean ± S.D. (n = 3). Means for the same bacterial treatment in each round with different letters are significantly different from each other (P < 0.05) according to the t-test.

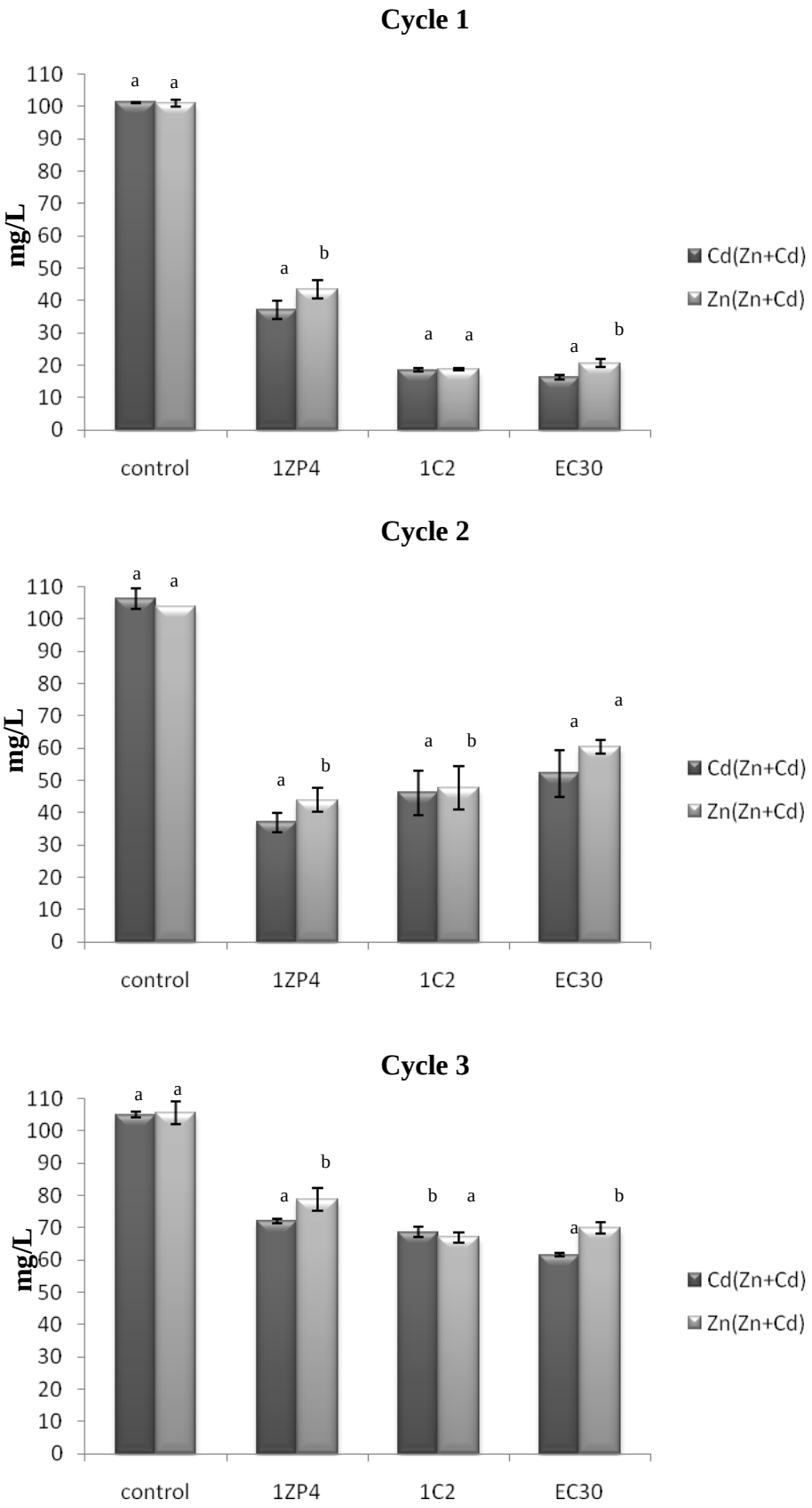


Figure 6.6 Zn and Cd levels in the combined outlet (Zn+Cd) in the incubated polymer matrix with different bacterial applications (mg/L). Results are expressed as mean ± S.D. (n = 3). Means for the same bacterial treatment in each round with different letters are significantly different from each other ($P < 0.05$) according to the t-test.

6.4 Discussion

The aim of the work was to assess the effect of the presence of the bacteria in metal removal, and to compare the efficiency of polymer and bacteria combinations in order to understand what combination seems more appropriate for further use in the clean-up of Cd and Zn contaminated waters.

6.4.1 Immobilisation of individual metals by different matrices and bacterial strains

Metal sequestration by a sorbent may be due to one or a combination of the following processes: ion exchange, physical adsorption, chemisorptions, complexation or microprecipitation (Utgikar *et al*., 2000). In the case of alginate and pectate, it seems that the process of ion-exchange takes place when metals bind to this matrix (Myklestad, 1968; Paskins-Hurlburt *et al*., 1977). Despite this adsorption capacity of the polymers, the present study showed that the immobilization of bacteria increased the removal abilities of all the matrices (alginate, pectate and the synthetic polymer). Bacteria are known to produce extracellular polymeric substances which are composed of proteins, polysaccharides, humic substances and uronic acid. These substances contain several functional groups like carboxyl, phosphoric, amine and hydroxyl groups (Van Hullebush *et al*., 2003; Omoike and Chorover, 2004). Both the phosphoryl and carboxyl groups of the peptide chains in bacterial cell walls provide negatively charged sites in Gram-positive bacteria. For Gram-negative bacteria, such as 1ZP4, EC30 and 1C2, the phosphate groups within the net negative charge of lipopolysaccharides of their outer membrane are the primary sites for metal interaction, with only one of the carboxyl group in this net being free to interact with metals (Urrutia, 1997). The process of binding of metal ions to bacteria involves

electrostatic interaction between metal ions and the biomass (Krishnani *et al*., 2008) as bacteria have a negative charge that favours the biosorption of metal (Silva *et al*., 2008), as observed in the present work. Further studies have shown a similar pattern when comparing the use of polymers alone and when immobilizing microorganisms: Sag *et al*. (1995) have shown that when aqueous solutions of Cu were treated with Ca-alginate immobilized *Zooglea ramigera*, an increase in Cu removal occurred from 64%, for the treatment with only Ca-alginate, to 94%. Aksu *et al*. (1998) have also shown that after long periods the adsorption capacity of alginate immobilized *C. vulgaris* exceeded that of alginate itself. However, the amount of biosorbent, initial concentration of metal, existence of further contaminants in the aqueous solutions, structural properties of both the support matrix and the biosorbent material, etc., affect the biosorption rate (Arica *et al*., 2003), rendering it difficult to compare results of different reports, and thus the main focus of this report is not to attempt such a comparison. The 3 selected strains – 1C2, 1ZP4 and EC30 – exhibited high resistance to Cd and Zn and all showed high specific growth rates when these heavy metals were present at different concentrations. Strains 1C2, 1ZP4 and EC30 are all Gram-negative and affiliated to genera *Cupriavidus*, *Sphingobacterium* and *Alcaligenes*, respectively. Many reports have shown that Gram-negative are more tolerant to heavy metals than Gram-positive bacteria. This metal tolerance can be attributed to the interactions between microbial cell wall components and heavy metal ions both contributing for metal detoxification (Frostegard *et al*., 1993; Roane and Kellog, 1996; Pennanen *et al*., 1996). In the biosorption of complex solutions, different metal ions may compete for the active sites existing on the support matrix and/or on the cell wall of the biomass. Consequently, the preference of the biomass for some metals is

an important issue (Romera *et al*., 2008), and thus the knowledge of the growth and metal resistance patterns of the used bacterial species is of great importance.

Measurement of the growth of the selected strains in the presence of Cd and Zn indicated differences in toxicity to the bacteria among the heavy metals. Specifically the presence of Cd^{2+} inhibited the growth of the strains tested, except for strain EC30 that showed a remarkable capacity to tolerate Cd in solution, with only a 15-20 % biomass reduction. Zn^{2+} presence caused also a reduction in biomass production, however in a less significant scale when compared to Cd. Strain EC30 apparently was more sensitive to Zn^{2+} than to Cd^{2+}. When metal mixtures were present, the growth rate was in between of the growth rate observed when Zn and Cd were tested alone. The decrease in biomass observed whenever metals were present possibly results from a decrease in the substrate utilization efficiency due to a higher energy cost of microorganisms subject to metal stress (Giller *et al*., 2009).

In the present study 1C2, a species from the *Cupriavidus* genera, was generally the one that most increased the removal performance of Zn (in single and binary solutions), especially when associated with the synthetic polymer. On the other hand, EC30, a bacteria from the *Alcaligenes* family, showed amongst the most promising results concerning Cd removal in single and binary mixtures, especially when combined with the synthetic polymer. In fact, EC30 has also shown to be the most resistant to Cd in the tolerance study performed which can explain this affinity Mondal *et al*. (2008) reported the use a species from the *Ralstonia* family, phylogenetically related to *Cupriavidus*, *Ralstonia eutropha*, for the elimination of Fe, Mn, Cu, As and Zn, with removals of up to 65.2, 72.7, 98.6, 8% and 99.3% respectively from metal contaminated water. Species from the genera *Alcaligenes* (such as EC30) have also been reported by Chang and Tseng (1998) as important in

immobilized biomass strategies, and Diels *et al*. (1995) have studied the application for heavy metal removal of composite membrane reactor immobilized *Alcaligenes eutrophus* bacteria with a reduction of metals such as Cd, Zn, Cu, and Pb in solution from 100 ppm to less than 50 ppb. As for strain 1ZP4, belonging to genera *Sphingobacterium*, there is also a study from Bootham *et al*. (2006) describing *Sphingobacterium mizutatae* as being part of a bacterial consortium used to treat metal contaminated effluents.

6.4.2 Immobilisation of binary mixtures of metals by matrices / bacterial strains

Although Fan *et al*. (2008) have shown that when using binary mixtures of Cd and Zn, the biosportion capacity of either metal was lower than that found in non competive conditions, this did not always happen in the present study. In one hand, in some cases there was an increase in the removal abilities of either of the tested metals in binary solution when compared to single solution. Such phenomenon may be explained by the hypothesis that the sorption of the other metallic contaminants in solution altered the conformation of the metal binding sites and increased the affinity of sites for that particular metal adsorption in that specific combination of matrix, bacteria and usage (Chen *et al*., 2005a). On the other hand some reverse data indicate that there was a decrease in Cd or Zn removal capacities of specific matrix-bacteria combinations. The most likely reason for this antagonistic effect may be the completion for adsorption sites on the cell and polymer surfaces. Chen *et al*. (2005a) also found that Cd uptake capacity was slightly inhibited by the presence in solution of Pb and Hg, suggesting that in Ca-alginate immobilized *Microcystis aeruginosa* most Cd adsorption sites were specific, whereas some of these Cd binding sites were

also capable of binding further metals. Despite these variations in the removal of metals in the binary mixture, for the generality of matrices-bacteria combinations, levels of Cd at the outlet of the binary mixture were generally lower than those of Zn, and in the majority of cases this trend has demonstrated to be significant. The preference of a sorbent for a metal may be explained on the basis of electronegativity of the metal ions (Cd=1,69 and Zn= 1,65, according to the Pauling scale), molecular weight (Cd=112,4 and Zn=65,4) and ionic radius (Cd=95 and Zn=74), with the first being positively related to the adsorption capacity, and the second and third being inversely related with it (Quintelas *et al*., 2009). In the present study, electronegativity seems to play an important role in the affinity of the tested combinations to Cd, but other conditions such as ionization energy can have contributed to influence the adsorption behavior of the metals (Ekop and Eddy, 2009).

Chapter 7: General discussion and conclusions

Agricultural and industrial activities have led to the release of enormous amounts of heavy metals, whose levels in soils pose significant hazards to human and animal health and to the ecosystems (Blaylock *et al*., 2000). Contaminated soils harbour organisms able to deal with pollution. Metals can generally exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions or modifying the active conformations of proteins and nucleic acids (Bruins *et al*., 2000). However, the ability to grow at high metal concentration is found in many microorganisms and may be the result of intrinsic or induced mechanisms that may reduce metal toxicity. Microbial survival in heavy metal polluted soils depends on intrinsic biochemical properties, physiological and/or genetic adaptation, as well as environmental modifications of metal speciation (Abou-Shanab *et al*., 2007). Long term exposure to metals leads to the selection/adaptation of a microbial community which then thrives in polluted soils (Pérez-de-Mora *et al*., 2006; Dell'Amico *et al*., 2007). Bacteria that survive and indeed flourish in such environments have developed or acquired genetic systems that counteract the effects of high metal ions concentrations and should be studied in detail.

7. 1 Microbial diversity from contaminated sub-sites 1 and 3 (see Chapters 3 and 4)

This study has presented the first attempt to characterise the bacterial diversity of the microbial communities from the contaminated site located within the industrial complex of Estarreja, northern Portugal.

To fully characterise the most active bacterial populations present in contaminated environments and to select microorganisms with capacity to be used in bioremediation strategies, knowledge of the heterotrophic culturable bacteria is essential. The presence of heavy metal contamination in soils greatly affects the microbial populations and diversity when compared to uncontaminated soils (Tsai *et al*., 2005). Overall, 278 isolates were recovered during the bacterial survey described in Chapter 3. They constituted an important fraction of the heterotrophic microbial populations present in the study site. Approx. 103 groups were characterized which corresponded most likely to the same number of bacterial species. However, these populations were clustered within a very small number of genera, confirming the idea that the bacterial diversity may have been affected by the contamination present in this polluted site (Giller *et al*., 1998; Buckley and Schmidt, 2002; Tsai *et al*., 2005) when compared to non-contaminated soils which generally have a higher bacterial diversity with more phylogentic groups present. Some of the isolates recovered had their closest phylogenetic relatives isolated in extreme environments, such as the coastal seawater of the Antarctic sub-continent (Lee *et al*., 2007), metal contaminated environments and from other contamination sources (see table 3.3).

The results presented in this study show that an active microbial community adapted to living in the presence of heavy metals and other xenobiotic compounds exist and became well established. This suggests that the selective pressure enabled over a period of time an ecological community able to not only survive but actively grow in such an ecosystem (Gadd, 1990; Tsai *et al*., 2005).

A significant number of bacterial isolates recovered were found to have resistance to various heavy metals tested at different concentrations. However, a few

isolates were sensitive to heavy metals perhaps due to differences between lab and soil conditions or because of interactions with other members of the bacterial community that may stimulate or sustain their activity and growth in this type of environment (Gadd, 1990; Horikoshi, 1999; Krulwich, 2000), another possible explanation may be related to the availability of metals, that may be higher in lab environment.

Some of the most resistant isolates recovered were Gram-negative. This confirms other studies in the literature (Madigan *et al*., 2003, Jjemba, 2004). The most metal tolerant strains isolated were Gram-negative. Studies have shown that these bacteria are more tolerant to heavy metals than Gram-positive ones (Madigan *et al*., 2003; Bennisse *et al*., 2004; Jjemba, 2004), probably because of the composition of the cell wall and interactions with metal ions. However, some Gram-positive strains were also shown to be very resistant to high concentrations of heavy metals. The data obtained in this study enabled the selection of potential for the capacity to tolerate high concentrations of heavy metals, making them effective candidates for bioremediation studies.

The bacterial survey of the rhizosphere soil was also performed during this study. The rhizospheric bacteria were isolated using metal-based media to provide some selective pressure to facilitate isolation of strains which were culturable, but with better resistance to heavy metals. This strategy yielded 42 strains. During this survey bacterial groups not previously isolated were recovered and some of these groups belonged to genera associated with metal contamination (Abou-Shanab *et al*., 2007; Pal *et al*., 2005). It is likely that the presence of metals during isolation may

have selectively resulted in isolation of bacteria with some kind of resistance/tolerance to heavy metals. These bacteria come from a metal contaminated environment having been exposed to metals either in solution or adsorbed to soil colloids leading to metal tolerance and/or resistance (Giller *et al*., 1998). Culture-dependent methodologies can underestimate the occurrence and diversity of microorganisms (Chee-Sanford *et al*., 2001; Schwartz *et al*., 2003). In this study, only culture-dependent approaches were used and it is possible that higher numbers of phylogenetically different bacteria would be found using culture-independent approaches. Moreover, the molecular diversity within each bacterial population would be higher among sequences retrieved by the culture-independent methodology (Handelsman, 2004). On the other hand, despite their well known limitations, culture-dependent approaches gave an insight into the phylogenetic affiliations of selected microorganisms showing heavy metal resistance and also to characterise the culturable fraction of the heterotrophic population.

Some of the strains isolated in this survey belong to taxa that potentially have the capacity to promote plant growth and reduce metal stress in plants, making them good candidates for use in phytoremediation strategies (Belimov *et al*., 2005; Rajkumar *et al*., 2006; Zaidi *et al*., 2006). Other strains isolated belonged to bacterial groups reported as having genes that confer metal resistance to heavy metals (Jackson *et al*., 2005; Chien *et al*., 2007). Once the genes conferring resistance to metals are closely related to antibiotic resistance (Alonso *et al*., 2001; Chien *et al*., 2007) these bacterial communities need to be studied in detail to evaluate the antimicrobial-resistance patterns for the surveillance, prevention and control of the risk that that

genes and/or organisms might constitute to human health and to ecosystem destabilization.

42 isolates recovered belonged to the most active prokaryotes in the soil rhizosphere and provided an insight into biological responses to heavy metal contamination. Results suggest the existence of phylogenetically diverse communities throughout this contaminated environment or alternatively the existence of communities with high metabolic adaptability to extremely contaminated soils (Li *et al*., 2006). Additionally, detailed insights into the phylogenetic diversity are essential to understand the shifts in the composition of prokaryotic populations and the links between composition and metabolic activity. The data obtained indicated that bacteria from the study site can thrive when high amounts of different heavy are present (Pal *et al*., 2005; Abou-Shanab *et al*., 2007), and represent a reservoir of metal resistant organisms showing a high potential for their use in bioremediation strategies.

7.2 Phylogenetic characterization of two new bacterial species isolated from the study site

During the two sampling occasions two strains were isolated that, after being characterised phylogenetically, showed low similarity to previously described taxa. These strains were Gram-negative, and designated as 3A10 and ECP37. These two organisms were rod-shaped, non-motile, aerobic, catalase- and oxidase-positive and formed yellow-coloured colonies. The predominant fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{17:0}$ 3-OH. The G+C content of the DNA was 43 and 34 mol% for $3A10^T$ and $ECP37^T$, respectively. The major isoprenoid quinone of both

strains was MK-6. 16S rRNA gene sequence analysis revealed that strains 3A10^T and ECP37^T were members of the family *Flavobacteriaceae* and were phylogenetically related to the genus *Chryseobacterium*. Strain 3A10^T presented sequence similarity values of 97.2 and 96.6 % to the type strains of *C. antarcticum* and *C. jeonii* (Yi *et al*., 2005), respectively, while strain ECP37^T showed a similarity of 97.3 % to the type strain of *C. marinum* (Lee *et al*., 2007). DNA-DNA hybridization experiments revealed levels of relatedness <70% between strains 3A10^T and ECP37^T and the type strains of *C. marinum, C. antarcticum* and *C. jeonii*, justifying their classification as two novel species within the genus *Chryseobacterium*, for which the names *Chryseobacterium palustre* sp. nov. and *Chryseobacterium humi* sp. nov. were proposed.

These two new bacterial strains showed resistance to heavy metals and were studied in detail for possible use in immobilised systems for heavy metal uptake. It seems very likely that more detailed studies onto the culturable fraction of heterotrophic bacterial populations of Estarreja will provide additional new bacterial species/strains that may show a high capacity for metal uptake. The discovery of new bacterial species is not only exciting but also suggests the potential for finding species previously unknown and metabolically active in heavy metal contaminated environments with encoding genes for metal resistance (Malik, 2004). They could be of interest either for biotechnological or bioremediation applications.

7.3 Removal of Zinc and Cadmium by immobilised strains EC30, 1ZP4 and 1C2

The aim of the work was to assess the effect of the presence of the bacteria on metal removal, as well as to compare the efficiency of all polymers and bacterial combinations in order to understand what combinations were more appropriate for further use in the clean-up of Cd and Zn contaminated waters. The bacterial strains selected came from the two samplings described in Chapters 3 and 4. They showed high levels of activity when heavy metals where present. They also had a high tolerance to pH and NaCl, features that probably are linked to their capacity to tolerate high concentrations of heavy metals.

The immobilization of bacteria in alginate and in pectate, naturally occurring support matrices, and onto a synthetic polymer increased the removal abilities of all the matrices (alginate, pectate and the synthetic polymer). 1C2, a species from the *Cupriavidus* genus, was generally the strain that increased the removal rate of Zn (in single and binary solutions), especially when associated with the synthetic polymer; EC30, a bacterial strain from the *Alcaligenes* family, showed promising results with regard to Cd removal in single and binary mixtures, especially when combined with the synthetic polymer. Thus, the combinations that could be recommended for further study with clean-up systems for aqueous solutions contaminated with Zn or Cd would be respectively PY+1C2 and PY+EC30 with the second one also showing good results for the removal of Zn.

The relative adsorption capacities for Cd and Zn varied when they were added singly or in combination. It was possible to conclude that the removal capacities

varied, but with no clear patterns – increases and decreases where shown in the binary mixture when compared to single solution results. Additionally, the study indicated that, for the analyzed combinations of matrix and bacterial species, the conditions are more favorable for the removal of Zn in binary mixtures of the metals than with Cd.

7.4 Conclusions

The main conclusions drawn for this thesis are:

- A range of heterotrophic culturable bacteria were obtained from the metal contaminated site, mostly belonging to γ-*Proteobacteria*, *Bacilli* *Actinobacteria* and β-*Proteobacteria*.
- Unusually tolerant bacterial strains, resistant to high levels of Cd, Zn and As, were isolated from metal contaminated sediments, with different taxa recovered in the absence of metals.
- The selective pressure over a period of time enabled a bacterial community to survive and thrive in such a contaminated ecosystem.
- Two new bacterial species, *Chryseobacterium palustre* and *Chryseobacterium humi*, belonging to phyla Bacteroidetes, class Flavobacteria, were isolated from the contaminated site, depicting the potential for finding species previously unknown in heavy metal contaminated environments.
- The most tolerant strains, 1C2 – *Cupriavidus* sp. – , 1ZP4 – *Sphingobacterium* sp.– and EC30 – *Alcaligenes* sp. –, were successfully applied in immobilised cartridges enhancing metal removal from aqueous solutions contaminated with single and binary mixtures of Zn and Cd.

- Effective bacterial candidates suitable for bioremediation were recovered, highlighting the potential for finding new species metabolically active in heavy metal contaminated environments.

7.5 Suggestions for future work

The most tolerant microbial strains should continue to be examined in detail. The rates of uptake and saturation should be determined in detail and the kinetics modeled. Studies must involve determination of the ratio of active transport vs passive binding to live biomass and comparison with dead biomass. Comparisons of dead and live biomass also needs to be evaluated. Immobilisation of the microbial biomass onto synthetic polymers must be investigated in more detail. Synthetic polymers are promising matrices and should be explored further in immobilised microbial cartridges because of the results obtained and their ability for reuse. Other polymeric matrices should also be evaluated, to assess their possible use in immobilised microbial cartridges. The rates of saturation should also be compared and used to determine the size of immobilised columns and the flow rate for effective use of such columns. These studies should be developed using statistical methods which would enable the optimised immobilisation system to be predicted based on using orthogonal design and surface response approaches (Parra *et al*., 2005). The relative economics of using such systems must be evaluated.

The study presented here raises a number of questions that remain open concerning the microbial communities present in the contaminated soils of Estarreja.

Despite the fact that important insights into the phylogenetic diversity of these communities were obtained, additional studies are needed to quantify the most prevalent phylogenetic groups. This can be achieved using quantitative analysis, such as fluorescent in situ hybridization (FISH) or quantitative real time polymerase chain reaction (qRT-PCR) (Amann *et al*., 1995; Zhang and Fang, 2006). Also links between phylogenetic and metabolic diversities must be established in order to attribute the measured microbial processes to a specific phylogenetic group.

Further investigations are also needed to completely evaluate the potential of the contaminated soils of Estarreja to constitute a reservoir of heavy-metal resistant microorganisms or heavy-metal resistance/tolerance genes. The search must be extended to genetic determinants conferring resistance to several heavy metals. Culture dependent and culture independent methodologies must be combined to accomplish this objective. Novel culturing procedures must be applied to assess a higher diversity of culturable bacteria and to determine not only resistance phenotypes but also their genetic content. Culture independent methodologies based on RNA analysis would be useful to quantify the expression of heavy metal resistance genes in the environment. Culture independent methodologies should be used in sub-site 2, where no viable bacterial isolates were recovered. After analyzing the results a new screen should be carried out using different culture-dependent strategies in order to assess the culturable bacterial fraction that must exist.

It would also be interesting to evaluate the potential of the bacterial resistant strains isolated in this study to promote plant growth. These strains could be screened for their plant growth promotion abilities, assessing indole acetic acid (IAA), siderophores, hydrogen cyanide, 1-amino-cyclopropane-1-carboxylate (ACC)

deaminase activity and ammonia production. Plant growth promoting bacteria are not only significant from an agricultural point of view, but may also play an important role in soil remediation strategies, not only by enhancing growth and successful establishment of plants in polluted soils, but also by increasing the availability of contaminants, as reported for heavy metals, namely Zn and Ni, in *Thlaspi caerulescens* (Whiting *et al*., 2001) and in *Alyssum murale* and *Thlaspi goesingense* (Abou-Shanab *et al*., 2003; Idris *et al*., 2004).

The most metal resistant bacteria could also be analysed in terms of metal distribution in the cell which can reveal the relative importance of intracellular and extracellular mechanisms that help to cope with metal stress. Cd and Zn distribution in selected isolates should be investigated (Lima *et al*., 2006). Histochemical staining trough autometallography processes (Danscher, 1984) could also be applied to assess localisation of the metals in the bacterial cells by electron microscopy. The operons involved on the active export of heavy metals should also be studied. The criteria used in metal-resistance genes primer design would be the conservation of homologous sequences. Bacteria with known metal resistance genes could also be used as sources for probes.

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Appendix: Published works

a. Publications

Pires, C., Marques, A.P.G.C., Guerreiro. A., Magan, N. & Castro, P.M.L. (2010). Removal of heavy metals using different polymer matrixes as support for bacterial immobilisation. Journal of Hazardous Materials. Vol. XXX, Pages xx-xx (Submitted)

Marques A. P. G. C., Pires, C., Moreira, H., Rangel, A. O. S. S. & Castro, P.M.L. (2010). Assessment of the plant growth promotion abilities of six bacterial species using *Zea mays* as indicator plant. Soil Biology & Biochemistry. **42**, 1229-1235.

Pires, C., Carvalho, M.F., Magan, N. & Castro, P:ML. (2010). *Chryseobacterium palustre* sp. nov. and *Chryseobacterium humi* sp. nov., isolated from industrially contaminated sediments. International Journal of Systematic and Evolutionary Microbiology. **60**, 402-407.

b. Announcements

C. Pires, N. Magan & and P.M.L. Castro. "*Bacterial Diversity in a Heavily Contaminated Site in Estarreja, Northern Portugal*"; BioMicroWorld2007, II International Conference on Environmental, Industrial and Applied Microbiology. Seville (Spain), 28 November to 1 December 2007.

C. Pires, N. Magan & P.M.L. Castro. "*Bacterial Diversity and Heavy Metal Resistance in a Contaminated Site in Portugal*"; 4th European Bioremediation Conference. Chania, Greece, 3-6 September, 2008.

C. Pires, A. R. Guerreiro, N. Magan & P.M.L. Castro. "*Use of Immobilised Microbial Systems for Heavy Metal Removal*"; Microbiotec 09, Vilamoura, Portugal. 28-30 November 2009.

C. Pires, *Viva voce* examination's presentation. 18/11/2010, Cranfield Campus, Cranfield Health, U.K.