MICROBIAL DIVERSITY AND ZINC TOXICITY TO *PSEUDOMONAS* SP. FROM COEUR d'ALENE RIVER SEDIMENT

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Sutapa Barua

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APPROVAL

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Sutapa Barua

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Dr. Brent M Peyton (Chair of Committee)

Approved for the Department of Chemical and Biological Engineering

Dr. Ron Larsen (Department Head)

Approved for the Division of Graduate Education

Dr. Carl A. Fox (Vice Provost)

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Sutapa Barua

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DEDICATION

This thesis is dedicated to my mother, Smrity Kana Barua and father, Jinadatta Barua.

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ABSTRACT

Coeur d'Alene River (CDAR) in northern Idaho is one of the metal contaminated rivers in US. The sediments of the river are enriched with As, Cd, Cu, Pb, and Zn which are toxic metals to humans and animals. It is hypothesized that microorganisms living in this river sediment can remove the metals and thus detoxify their environment. The objective of this work is to investigate the microbial communities existing in CDAR sediment using 16S ribosomal RNA (rRNA) gene sequencing and 16S rRNA gene microarray (PhyloChip) analysis. According to our phylogenetic analysis, the CDAR clones fell into 13 distinct phylogenetic classes including 2 environmental samples, 1 uncultured bacterium, and an unclassified *Chloroflexi*. The major representative genera found were *Thiobacillus* (7 of 91), *Azoarcus* (7/91), *Acidobacterium* (6/91), *Burkholderia* (5/91), *Flavobacterium* (5/91) and *Janthinobacterium* (5/91). PhyloChip data showed the presence of 1551 operational taxonomic units (OTUs). 97% of the clone library sequences matched at various taxonomic levels with the microarray results. The results from the clone library and PhyloChip have provided the broad picture of the microorganisms found in the metal polluted CDAR sediment.

To better understand the metal toxicity kinetics of specific isolates, *Pseudomonas* sp. strain JM001 from the CDAR sediment was selected for further study. Cell growth and aqueous Zn removal rate of strain JM001 was observed in batch kinetic experiments. It was found that cell growth rate depends on substrate and nutrient concentration and incubation temperature. The minimum inhibitory concentration (MIC) of Zn to the cells was 0.024 mM and the 50% inhibition in specific growth rate was found at 0.011 mM Zn when cells were grown in nutrient limited defined media at 22 ºC. The MIC was 1 mM Zn and 50% inhibition occurred at 0.515 mM Zn in minimal salts medium with vitamin solutions at 35 ºC. The results are significant to develop a dose-response model that will quantify the effects of toxic metals on microbial growth and inhibition in complex CDAR environment.

CHAPTER ONE

INTRODUCTION

Overview

Maintaining soil and water quality is one of the most formidable global challenges in the 21st century. The western U.S. has a rich mining history that has resulted in vast amounts of water and soil contaminated with toxic metals. In northern Idaho, mining of precious metals since the late 1800's has left Coeur d'Alene River (CDAR) sediments heavily contaminated with toxic metals (e.g. Ag, As, Cd, Cu, Pb, and Zn). These heavy metals are toxic to humans and animals. Thermal treatment [Ho et al., 1995], landfill [Yanful et al., 1988], and soil capping [Harris et al., 2004] are expensive and environmentally destructive methods of removing metals. However, microorganisms that live in these metal-contaminated sediments are capable of detoxifying their environment [Cummings et al., 2003; Fortin et al., 2000; Niggemyer et al., 2001; Sani et al., 2001; Viamajala et al., 2002] and thus constitute an important component in the biogeochemical cycling of these metals.

The objective of this thesis is to describe the diversity of microorganisms present in CDAR sediments and to understand the effect of zinc (Zn) stress on one species, *Pseudomonas* sp. strain JM001, isolated from the sediment. The organization of the thesis is based on the Montana State University manuscript format. Two manuscripts (chapters two and three) will be submitted for publication in peer-reviewed journals. In addition, metal inhibited growth of *P.* sp. strain JM001 with flocs is described in chapter four, the

future work (chapter five) and an appendix of supporting data are presented at the end of the thesis.

Background

Coeur d'Alene River, Idaho

The Coeur d'Alene River (CDAR) located in northern Idaho (U.S.) flows from the Silver Valley into Lake Coeur d'Alene (LCDA) and drains a substantial portion of wastes from mining that were disposed directly to the South Fork of the CDAR [Horowitz et al., 1995]. The South Fork of the CDAR starts at the Idaho-Montana border and the river flows westward to join the North Fork near Pinehurst, ID [NRC, 2005]. There are three basins (upper, middle and lower) in the South Fork of the CDAR [Meckel Engineering et al., 1983]. The upper basin has steep stream gradients and limited floodplains [NRC, 2005]. The middle basin of the CDAR has wider floodplain areas and less steep river gradient [NRC, 2005]. The lower basin, containing the main stem of the CDAR, runs from Cataldo to Harrison [Meckel Engineering et al., 1983]. The river system in this basin is deltaic containing wetlands, lateral lakes, and agricultural lands [NRC, 2005; Sprenke et al., 2000]. At the bottom of the lower basin, the river drains into LCDA which also fed by the St. Joe River (SJR) in the south [Javorka, 1991; Meckel Engineering et al., 1983] and drained by the Spokane River in the north [Horowitz et al., 1995].

The Coeur d'Alene mining district in the Silver Valley operated since 1880 [Bender, 1991] was one of the highest producers of Ag, Pb, and Zn in the world [Sprenke

et al., 2000]. Historic mining and ore processing practices in the mining district for more than a century produced an estimated of 63.5 to 90.7 million metric tons of mining waste that are carried throughout the region by streams, rivers, floodplains, and lakes [Long, 1998] as well as the CDAR. Mining and ore processing occurring at a number of mines and mills throughout the Silver Valley [Harrington et al., 1998] has contaminated CDAR sediments with Ag, As, Cd, Cu, Fe, Hg, Mn, Pb, Sb, and Zn [Horowitz et al., 1992]. In addition, a large amount of mining, milling, and ore processing wastes enriched with trace metals from the Bunker Hill Superfund side, about 50 km upstream of LCDA, have been deposited throughout the lower CDAR and LCDA [Horowitz et al., 1995]. Among these different kinds of toxic heavy metals, Zn is of interest because of its elevated concentrations in this pH neutral river [Kuwabara et al., 2003].

Major Trace Elements in the Delta Region of Coeur d'Alene River (CDAR)

The CDAR delivers its metal contaminated sediments into the lateral lakes, marshes and LCDA during floods [NRC, 2005]. Horowitz et al. [1995] estimated that metal rich sediment deposition rate near the CDAR delta is about 2.1 to 1.3 cm/yr and 75 million metric tons of metal contaminated sediments from this region had been deposited on the bottom of LCDA since the onset of mining. Sprenke et al. [2000] showed that the sediments of lateral lakes that lie within the flood plain of the CDAR have heavy metal contamination with As, Cd, Pb and Zn. Metal concentrations in LCDA sediments near the CDAR inlet, four lateral lakes (Rose, Medicine, Black, and Anderson Lakes) and metal enriched and unenriched LCDA lakebed sediments are shown in Table 1.1.

Table 1.1. Metal concentrations in LCDA and other lateral lakes' sediments near CDAR as found by earlier researchers.

Metals	Maximum concentrations in LCDA sediments near CDAR (mg/kg of dry sediment); [Harrington et al., 1998]	Mean concentrations in LCDA sediments near CDAR (mg/kg dry sediment); [Harrington et al., 1998]	Median concentrations in sub-bottom interstitial water of four lateral lakes (mg/l); [Sprenke et al., 2000]	Median concentrations in metal enriched LCDA sediments (mg/kg of dry sediment); [Woods et al., 1997]	Median concentrations in metal unenriched LCDA sediments (mg/kg of dry sediment); [Woods et al., 1997]
Fe	123,200	82,486		-	-
Mn	9,200	5,953		-	-
Pb	21,493	3,820	3.32	-	-
Zn	11,169	2,995	10.09	3,500	110
As	568	201	0.42	1,800	24
Cd	-	-	1.12	56	2.8

- 'not analyzed'

Most of these metals are immobile in subsurface systems due to precipitation, adsorption, ion exchange reactions or complexation with sorbed organic matter [Evanko et al., 1997]. In LCDA, most of the metals are predominantly associated with sulfidic and amorphous oxide phases [Harrington et al., 1998; Toevs et al., 2006]. Redox active Fe, As and Mn are available at the redox boundary of the sediment-water interface, while the less redox sensitive elements Pb and Zn are found in deeper sediments [Harrington et al., 1998]. The distribution pattern of the metals in the sediments reveals the cycling of the metals by oxidation-reduction reactions. The solubility, mobility and thus geochemical cycling of these trace elements are highly dependent on biological conditions encountered within the sediment environment [Ehrlich, 1981; Lovely, 1991]. Details of Pb and Zn speciation and biological activities in the metal contaminated area are discussed later.

Impacts of Metals in the Coeur d'Alene Area

Metals in the CDAR sediments are the contaminants of greatest concern, particularly As, Cd, Pb and Zn. Most of these metals have potential toxic effects on biota [Sprenke et al., 2000], including humans. The effects of toxic metals depend on their concentration, bioavailabiliy and speciation [Deheyn et al., 2004]. Pb and Zn compounds are biologically available metals, which can pose great risks to ecosystems [Kuwabara et al., 2003; Horowitz et al., 1992; Horowitz et al., 1995; Sprenke et al., 2000; Woods et al., 1997]. For example, Pb contamination is thought to be the most significant health threat in the Coeur d'Alene district. Very young children (less than 5 years old) are most susceptible to the neurological effects of Pb [Koller et al., 2004]. The Pb-blood level concentrations in children living in the district were 10-15 mg/L in the 1980s [EPA 1989] which exceeded the EPA's standard of no more than 5% exceeding 10 mg/L blood Pb level for children [EPA, 1994]. Children are sensitive to Pb because intestinal absorption rates of Pb are higher in children and Pb ions can penetrate the blood brain barrier of the developing nervous system and the placenta. In adults, Pb can attack the peripheral and central nervous systems, the kidneys and the reproductive system.

Although Zn is an essential trace metal for terrestrial and aquatic organisms, it can be toxic at excessive concentrations. It is a metal of primary concern in the CDAR basin because of its toxicity to aquatic ecosystems and people [Koller et al., 2004]. Some varieties of fish such as rainbow trout and cutthroat trout as well as tundra swans are affected by aqueous phase Zn [Davies et al., 1976]. The Idaho Department of Health and Welfare and the Coeur d'Alene Tribe advise members of the general public, especially

pregnant women, breastfeeding mothers, and children under six-years-old to limit the number of fish they eat from the CDAR basin.

Lead and Zinc Speciation

The health impacts of metals on humans and other biota are highly dependent on the speciation of the metal. Pb is present as a solid phase (often PbS) and more soluble phase such as $PbCO_3$ in CDAR sediments [NRC, 2005]. The US Geological Survey (USGS) estimates that the river bed contains 51% of the Pb in the entire lower basin of CDAR (Bookstrom et al., 2001). Pb is also associated with Mn and Fe oxides [Balistrieri et al., 2002]. While the primary phase for Pb in the sediments is the solid phase rather than the dissolved phase, Zn is often in the dissolved form [Balistrieri et al., 2002]. The most available state of Zn in the environment is +2 oxidation state [Lindsay, 1979]. It is partitioned into sediments in aerobic waters through sorption onto hydrous Fe and Mn oxides, clay minerals, and organic material [Lindsay, 1979]. The sorption of Zn from solution depends on concentration, pH, redox potential (Eh), salinity, the nature and concentrations of complexing ligands, and cation exchange capacity. Zn exists predominantly in mineral form of particulate sphalerite [Martin et al., 2003] in anaerobic environments and in the presence of sulfide ions. Precipitation of Zn as ZnS in reduced sediments limits its mobility. It can also exist in other mineral forms often altered from sphalerite such as smithsonite ($ZnCO_3$), in a completely dissociated ionic state (Zn^{2+}), as hydrated forms of Zn^{2+}, or in a dissolved form complexed with other inorganic or organic solutes [NRC, 2005; Sprenke et al., 2000].

In the solution phase, Zn^{2+} is a reactive molecule and undergoes a variety of interactions with other ions and dissolved organic matter, affecting the solubility of the complex. Solubility of Zn is dynamic and reversibly controlled by pH, concentration, oxic state, temperature, and moisture content of the environment [NRC, 2005; Sprenke et al., 2000]. At neutral pH, Zn is present as soluble compounds in surface or groundwaters, while at higher pH and under reducing conditions it can precipitate as $Zn(OH)_2$, $ZnCO_3$, and ZnS [Evanko et al., 1997]. Zn may coprecipitate with hydrous oxides of Fe or Mn [Smith et al., 1995]. It associates with ferric oxyhydroxides in LCDA sediments [Harrington et al., 1998]. Under suboxic conditions, Fe and Mn (hydr)oxides release Zn into the aqueous phase by reductive dissolution; the persistence of suboxic conditions may then lead to a repartitioning of Zn into sulfide and carbonate solids. It can also associate with inorganic species, or dissolved or particulate organic matter [Martin et al., 2003; Sprenke et al., 2000].

Soluble Zn within the CDAR basin may interact with biotic and abiotic components in the water column, affecting the transport of the metal. For instance, soluble Zn coming from the CDAR may associate with phytoplankton (and become sorbed to the organic matrix of the cell or incorporated into the silica in diatom frustules) [NRC, 2005]. Upon dying, the phytoplanktons settle out of the water column and become incorporated in sediments. The Environmental Protection Agency (EPA) estimated that in the year 1999, approximately 50% of the dissolved Zn input was converted into the particulate form within the lake [URS Greiner, Inc. and CH2M Hill 2001k], which presumably settles to the lake bottom or is further transported downstream. A dynamic

redox environment of LCDA sediments controls the partitioning of metals along the gradient of oxic conditions [Toevs et al., 2006]. The gradient exists at the sediment-water interface to anoxic conditions below 15 cm [Toevs et al., 2006] of sediments which are the primary sink for Zn [Sprenke et al., 2000]. Thus, the partitioning of Zn exists in a dynamic redox environment of CDAR depending on local environmental factors.

Microorganisms in Sediments from Coeur d'Alene River Area

Microorganisms have evolved several mechanisms to tolerate heavy metals by efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration [Spain et al., 2003]. The effects of metals on microorganisms have been studied by several researchers [Lugauskas et al., 2005; Meharg et al., 1991; Sani et al., 2001; Tuovinen, 1971; Viamajala et al., 2002]. The activity of microorganisms has been reported for mining impacted sediments [Castro-Silva et al., 2003; Cummings et al., 2003; Cummings et al., 1999; Küsel et al., 2000; Niggemyer et al., 2001]. Results of these studies suggest that microbes may play a major role in the biogeochemical cycling of toxic heavy metals in natural sediments and can be used for remediating metal contaminated environments [Bruneel et al., 2006]. Due to the presence of heavy metals (mainly Pb, Zn, Cd, As, and Hg) in the delta region of CDAR sediments, scientists have been studying microbes that oxidize or reduce heavy metals in order to understand and perhaps remediate metal-contaminated CDAR sites. In the last few years, a number of iron reducing bacteria (IRB) *Geobacter* spp. [Cummings et al., 2003], *Ferribacterium limneticum* [Cummings et al., 1999] and As reducing bacteria

(AsRB) [Cummings et al., 1999] strain GBFH [Niggemyer et al., 2001] were found in the sediments from the delta region of CDAR at Harrison, ID. IRB and sulfate reducing bacteria (SRB) may participate in Fe and S cycling in CDAR environment. SRB can promote the precipitation of metal sulfides, which in river sediments can affect the solubility of other metals (Zn, Pb, Cu, Cd) [Morse et al., 1999]. Pb resistant bacteria, *Pseudomonas marginalis* and *Bacillus megaterium* in soils from Siver Valley, ID were identified by Roane [1999]. The metal resistant bacteria are important since they tolerate high concentrations of metals in the environment.

The investigations cited above have focused on the identification of particular metabolic groups of bacteria in sediments near the CDAR upper and lower basin rather than focusing on characterized bacterial community diversity at the delta region of CDAR which is one of the high metal content areas near LCDA. The present study aims to investigate the microbial communities which exist in CDAR sediment using 16S ribosomal RNA (rRNA) gene sequencing and 16S rRNA gene microarray analysis.

Metal Toxicity Behavior of Different *Pseudomonas* spp.

Zn inhibition on *Pseudomonas* sp. was studied because high concentrations of Zn are harmful to aquatic life. Zn concentrations observed in the middle and lower basin of CDAR are 110 and 3.4 mg/L respectively [NRC, 2005]. *Pseudomonas* sp. respond to heavy metals by several processes, including uptake [Chen et al., 2005; Mago et al., 1994; Teitzel et al., 2003], biosorption to cell walls [Chen et al., 2005; Komy et al., 2006;

Uslu et al., 2006], entrapment in extracellular capsules [Cooksey et al., 1994; Toner et al., 2005], and oxidation–reduction reactions [Outten et al., 2000].

The uptake of Zn by *P*. sp. strain UDG26 was described by Mago et al. [1994]. Uptake studies of *Pseudomonas* induced to resist Zn revealed that cells of a Zn resistant strain accumulates more Zn on its surface than a Zn sensitive strain [Mago et al., 1994]. The resistant cells exhibit a rapid initial Zn^{2+} uptake followed by a gradual stable phase [Mago et al., 1994]. A Zn-sensitive variant took up significantly less metal ion than the resistant one. Zn accumulation by cells was less in the sensitive strain than that of resistant, induced cells [Mago et al., 1994]. *P. putida* CZ1 showed high tolerance to Cu and Zn [Chen et al., 2005], where results demonstrated that about 40–50% of Cu and Zn were actively taken up by *P. putida* CZ1. The remainder was passively bound to the bacterium [Chen et al., 2005]. Biosorption of Cu and Zn to *P*. sp. CZ1 involved surface binding, intracellular uptake and storage via active cationic transport systems [Chen et al., 2006]. Carboxyl functional groups are the major ligands responsible for metal binding in *P. putida* CZ1 [Chen et al., 2007]. The affinity of Cu^{2+}, Zn^{2+}, Cd^{2+} for the functional group present on the cellular surface of *P. putida* is strongly dependent on the pH and ionic attraction [Pardo et al., 2003]. At low pH values the inactivated cell surface becomes more positively charged, reducing the attraction between metal ions and functional groups on the cell wall [Sadowski, 2001]. In contrast, when the pH increases the cell surface is more negatively charged and the process of retention is favored [Sadowski, 2001]. *P. marginalis* isolated from Siver Valley region, ID showed high Pb resistance (2.5 mM Pb) at pH 7.32. The cells sequestered Pb extracellularly [Roane,

1999]. *P. marginalis* produced extracellular polymers even at neutral pH and high metal stress conditions [Roane, 1999]. Metals were bound to the bacterial exopolymers due to ionic interactions [Roane, 1999].

Komy et al. [2006] investigated and modeled the ability of *P. aeruginosa* to accumulate Cd^{2+} from wastewater industries. It was found that maximum Cd biosorption capacities, affinity of Cd^{2+} to adsorptive sites of biomass, and equilibrium times were different for different *Pseudomonas* strains depending on the differences in chemical composition between biomasses of the strains [Komy et al., 2006]. In the gram-negative species, different families of transporters coordinate transport of metal ions out of the cytosol and periplasmic space of the cells [Cooksey et al., 1994; Outten et al., 2000; Teitzel et al., 2003]. The *cop* system (efflux system) of *P. syringae* contains the structural genes *copABCD* and is homologous to the *pco* system in *Escherichia coli* [Teitzel et al., 2003]. The *copB* and *copD* genes are involved in the transport of Cu^{2+} across the membrane, while the products of *copA* and *copC* genes are outer membrane proteins that bind Cu^{2+} in the periplasm, protecting the cell from Cu^{2+} [Cooksey et al., 1994]. These previous studies reveal that various species of *Pseudomonas* spp. efficiently remove heavy metals in the bioaccumulation procees. The toxic effects of Zn on the growth of *P.* sp. strain JM001 isolated from CDAR sediments are described in chapter three.

16S rRNA Gene Sequencing Analysis

The use of 16S rRNA gene sequencing has proven to be a useful technique for inferring phylogenetic relationships between microorganisms, because of the high information content in DNA sequence, conservative nature, and universal distribution

[Lane et al., 1985]. 5S rRNA fragments are too small (approximately 120 nucleotides) to resolve phylogenetic relationships of a complex system, whereas 16S rRNA is generally thought to be adequate (length of 1500 nucleotides) to resolve most relationships for reliable phylogenetic analyses [Amann et al., 1995]. The major advantage of 16S rRNA gene detection is that the use of a 'universal' primer allows recognition of a large range of bacteria [Gutell et al., 1994]. The presence of universally conserved sequences at the 5' and 3' ends [Edwards et al., 1989] allows both the recovery of rRNA sequences as complementary DNA (cDNA) and amplification of nearly complete 16S rRNA genes from DNA extracted from natural samples [Britschgi et al., 1991; Head et al., 1998].

Although the 16S rRNA analysis provides information about the taxa present in an environment [Torsvik et al., 1990], it is important to keep in mind that 16S rRNA genes may not represent a complete or accurate picture of the bacterial community. There are limits to this analysis. Firstly, the species diversity is so great that PCR amplified 16S rRNA genes may not represent the broad-scale microhabitats in CDAR [Curtis et al., 2002; Torsvik et al., 1990]. The efficiencies of nucleic acid extraction may be different for different bacteria due to differences in 16S rRNA genes in bacterial cells [Janssen, 2006]. Some sequences may arise from contaminating DNA and may not represent actual environmental bacteria that are being studied [Tanner et al., 1998]. In addition, the rRNA gene phylogenies will not reveal cell functions, which drive the evolution of microbial groups and the important biochemical activities of these organisms in the environment [Rappé et al., 2003]. However, the rRNA gene based phylogenetic trees are used extensively as the critical source of information to identify groups of microorganisms in

nature and to organize relationships among them [Pace, 1997]. Scientists continue to utilize rRNA approaches widely because populations of cells in nature correlate well with rRNA sequences [Rappé et al., 2003]. The Ribosomal Database Project (RDP) contained 262,030 aligned and annotated rRNA sequences as of release 9.42 (September, 2006) [Cole et al., 2007]. Of the sequences, 84,442 were from cultivated bacterial strains and 177,588 were derived from environmental samples [Cole et al., 2007]. More than 240,000 named organisms were published in 2007 in GenBank which is a comprehensive database containing publicly available DNA sequences [Benson et al., 2007]. The available libraries of 16S rRNA genes permit an initial survey of the global soil bacterial community structure [Janssen, 2006] with other communities like marine and freshwater communities.

Using 16S rRNA sequence analysis, Gremion et al. [2003] found 282 clones in heavy metal contaminated soils. Clones were affiliated with five major phylogenetic groups, the *Actinobacteria*, α-*Proteobacteria*, β-*Proteobacteria*, *Acidobacteria* and the *Planctomycetales*. Hanbo et al. [2004] isolated 60 *Arthrobacter* species from mining tails by analyzing their partial 16S rRNA gene sequences. Phylogenetic diversity of the Fe-reducing bacteria *Geobacter* and *Ferribacterium* spp. in sediments near delta region of CDAR was investigated by Cummings et al. [2003, 1999]. In addition, partial gene fragment sequencing reveals that there are As-reducing bacteria strain GBFH sp. related to *Desulfitobacterium hafniense*, and *Desulfitobacterium frappieri* [Niggemyer et al., 2001] from CDAR delta, and that a close relative of *Shewanella alga* strain BrY was found in LCDA sediments [Cummings et al., 1999]. In this study, presence of a clone

library of 95 clones in CDAR sediment was investigated using the diversity of 16S rRNA genes. In chapter two of this thesis, the sediment microbial community in CDAR characterized by 16S rRNA gene sequence and microarray analysis is described.

Microarray Analysis

A microarray gene chip is a two dimensional array on a glass, filter or silicon wafer upon which unique portions of single genes of known sequences are arrayed by covalent attachment to chemically suitable matrices. Affymetrix's GeneChip array is one of the commercially produced and available microarrays. Hundreds to thousands of 16S rRNA genes or various functional genes can be spotted on the solid surface of the chip and the array of nucleic acids can be hybridized simultaneously [Brodie et al., 2007]. The affixed gene is called the probe. Multiple DNA probes are arrayed for detecting different species in parallel. Probes complementary to target sequences are called perfect matched (PM) probe [DeSantis et al., 2007]. PM probes pair with mismatched probes (MM) and constitute a probe pair. Probe pairs (PM, MM) score positive, if the fluorescent intensity from the PM is at least 1.3 times higher than the intensity from MM and (PM-MM)>130N, where N is the noise value due to the variation in pixel intensity signals observed by the scanner as it read the array surface) [DeSantis et al., 2007]. For each probe, the positive fraction (pf) is calculated which is the number of positive probe pairs divided by the total number of probe pairs in a probe set [DeSantis et al., 2007]. In the Affymetrix GeneChip designed by the Lawrence Berkeley National Laboratory (Berkeley, CA, U.S.) 30,627 16S rDNA genes are clustered into 8,988 Operational Taxonomic Units (OTUs) [DeSantis et al., 2007]. For each OTU, a set of 11 or more

specific 25-mers (targets) are used that are prevalent in members of a given OTU, but are dissimilar from sequences outside the given OTU. Probes presumed to cross-hybridize are those 25-mers that contain a central 17-mer matching sequences in more than one OTU [Urakawa et al., 2002]. As each PM probe is chosen, it is paired with a control 25-mer, identical in all positions except the 13th base (MM probe) [DeSantis et al., 2007]. The MM probe does not contain an internal 17-mer complimentary to sequences in any OTU. When the PCR products are loaded and processed on a GeneChip for several hours, the array elements bind specifically to labelled molecules (the targets) present in complex molecular mixtures. Fluorescent signals are generated revealing the identity of the interacting labeled species [Kelly, 2003]. An OTU is considered present when >90% of its probe pairs were positive, i.e., its pf value is greater than 0.92 [DeSantis et al., 2007] depending on the number of active probe pairs.

This technique has been used to identify microbial communities in various environmental conditions [Brodie et al., 2007; Peplies et al., 2006; Sanguin et al., 2006]. Brodie et al. [2007] identified about 1800 diverse bacterial types in urban aerosol using high density DNA PhyloChip. Its great advantage of providing information about the occurrence of thousands of microorganisms has made it favorable over other taxonomic nucleic acid-based assays [Kelly, 2003; Peplies et al., 2006], which are limited by the rate at which sequences can be analyzed, especially in a complex system like metal contaminated CDAR sediments. Despite the great potential of microarrays, the technology has some obstacles that can complicate the use and overshadow the results obtainable [Spiegelman et al., 2005]. Physical and biochemical properties of the chip and

the bound oligonucleotides can conspire to create false negative and positive results [Spiegelman et al., 2005]. False results can also be generated due to secondary structures of target oligos and steric hindrance based on spot density and other hybridization conditions [Peplies et al., 2003]. Cross-hybridization of multiple probes to a single target may lead to increased signal intensity for a species [Spiegelman et al., 2005]. This may overestimate the abundance of the species. Hybridization sensitivity is another drawback in case of low concentrations of DNA [Spiegelman et al., 2005]. In this case, the target DNA must be amplified using PCR. PCR products can introduce significant biases that might increase the risk of false-negative results [Spiegelman et al., 2005]. Compared with sequencing a 16S rDNA clone library, the microarray cannot recognize novel prokaryotic families [DeSantis et al., 2007]. Though the technology has its own limitations, microarray analysis may be useful for environmental microbiology because it can screen a microbial community using labeled targets for taxonomic genes of interest [Spiegelman et al., 2005] within several hours. A library consisting of 10,000 clones can be screened in a single assay, while the clone library requires labor intensive analysis of individual hundreds or thousands of clones.

In this study, a microbial community of phylogenetically distinct microorganisms present in the CDAR sediment was identified by 16S rRNA gene sequencing and PhyloChip analysis. The microorganisms may constitute an important driving component in the biogeochemical cycling of the metals in CDAR. Accordingly, the aim of this study is to focus on the kinetic study of Zn inhibited growth of *P.* sp. strain JM001, isolated from CDAR sediment. The influence and inhibitory effects of Zn on the cell growth was

examined in different batch experiments and different media. The toxicity study helped to understand the microbe-metal interactions in a natural environment like CDAR. The interactions between heavy metals and microorganisms are important because the microbiological processes on the metal contamination of the environment are of economic and environmental significance [Gadd, 1993, 2001]. The details of the microbial diversity and kinetic experiments are described in chapters two and three respectively.

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CHAPTER TWO

MICROBIAL DIVERSITY IN METAL CONTAMINATED COEUR d'ALENE RIVER SEDIMENT

Abstract

The sediments of Coeur d'Alene River (CDAR) in northern Idaho are contaminated with toxic metals such as Ag, As, Cd, Cu, Pb, and Zn due to mining since the late 1800's. It is hypothesized that microorganisms in this river sediments play an important role in biogeochemical cycling of metals. To characterize microbial diversity in metal contaminated CDAR sediments, a clone library of 16S rRNA genes was analyzed by 16S rRNA gene sequencing and with 16S rRNA gene microarray (PhyloChip) analysis. The CDAR clones observed fell into 13 distinct phylogenetic classes with major representative genera of *Thiobacillus* (7 of 91), *Azoarcus* (7/91), *Acidobacterium* (6/91), *Burkholderia* (5/91), *Flavobacterium* (5/91), and *Janthinobacterium* (5/91). The remaining 56 clones showed significant genetic diversity. Microarray analysis of PCR amplicons of the same sediment sample showed the presence of 1551 operational taxonomic units (OTUs). Of the clone library sequences, 97% were detected with the PhyloChip. Clone library and PhyloChip characterization have provided a broad picture of microbial diversity in the metal contaminated CDAR sediment.

Key words: CDAR, 16S rRNA gene, clone library, phylogenetic analysis, PhyloChip

Introduction

The Coeur d'Alene River (CDAR) located in northern Idaho (U.S.) flows westward from Silver Valley into Lake Coeur d'Alene (LCDA), ID. Three basins (upper, middle and lower) in the South Fork of the CDAR [Meckel Engineering et al., 1983] drain a substantial portion of mining wastes that were disposed to the South Fork of CDAR [Horowitz et al., 1995]. The lower basin at Harrison, containing the main stem of the CDAR, forms a deltaic region with wetlands and lateral lakes [NRC, 2005; Sprenke et al., 2000]. At the bottom of the lower basin, the river drains into the LCDA [Javorka, 1991; Meckel Engineering et al., 1983]. The sediments of CDAR and LCDA are heavily contaminated with high levels of As, Fe, Mn, Pb, Zn and other elements [Harrington et al., 1998; Sprenke et al., 2000]. Measured maximum toxic metal concentrations in LCDA sediment in mg/kg of dry weight sediment are Pb, 21493; Zn, 11169; and As, 568 [Harrington et al., 1998]. River and pore water analysis of CDAR showed Pb and Zn concentrations of 0.003 and 0.16 mg/L in river water and 0.13 and 2.7 mg/L in pore water, respectively [Moberly, 2006]. Balistrieri et al. [1999] reported 0.001 and 0.458 mg/L of Pb and Zn respectively in CDAR water. Sprenke et al. showed 3.32, 10.09, 0.42, and 1.12 mg/L maximum concentrations of Pb, Zn, As and Cd respectively in water and its underlying sediments of four lateral lakes (Rose, Medicine, Black, and Anderson Lakes) near CDAR. The speciation of metals largely depends on the seasonal changes of the river temperatures and pH [Balistrieri et al., 1999, 2002; Sprenke et al., 2000]. X-ray fluorescence spectroscopic analysis of CDAR sediment in April, 2005 showed that trace metals contained 7470 and 4670 mg/kg of dry sediment of ZnO and PbO respectively

[Moberly, 2006]. These oxides are transformed by metal sulfide precipitation in July to October [Hamilton-Taylor et al., 1996] when oxygen diffusion rate is limited.

The transport and fate of metals in the CDAR system involves both biotic and abiotic mechanisms [Tebak et al., 2005]. The sediments near the delta region of CDAR show a wide range of microbial cell concentrations with 10^4- 10^8 g^{-1} (wet weight) of sediment [Cummings et al., 2000; Harrington et al., 1998]. Despite heavy metal contamination, microorganisms play an important role in the biogeochemical cycling of metal ions [Harrington et al., 1998; Spain et al., 2003]. Cummings et al. found a new genus *Ferribacterium* (CdA-1) of Fe(III)-reducing bacterium (FeRB) [1999] and two new strains (CdA-2 and CdA-3) [2003] of dissimilatory FeRB of the *Geobacteraceae* family (class δ-*Proteobacteria*) from LCDA sediments near the CDAR delta. They recovered approximately 1.4×10^6 *Geobacteraceae* genomes g^{-1} sediment by most probable number (MPN) analyses. Their results showed that Fe(III)-reducing *Geobacteraceae* were diverse and abundant in LCDA sediments regardless of the metal content of the sediments in which they colonize [Cummings et al., 2003; Lloyd et al., 2000]. Niggemyer et al. [2001] isolated an Fe(III)- and As(V)-reducing bacterium and As(V)-respiring strain GBFH from LCDA sediment close to CDAR delta which were closely related to *Desulfitobacterium hafniense* and *Desulfitobacterium frappieri*. Roane [1999] isolated two lead-resistant microorganisms, *Pseudomonas marginalis* and *Bacillus megaterium*, from metal-contaminated Silver Valley soil located in northern Idaho. The Coeur d'Alene mining district in the Silver Valley is one of the highest Pb producers in the world [Sprenke et al., 2000] and Pb minerals from the Silver Valley area are discharged to tributaries of the

CDAR [NRC, 2005]. Pb resistant microorganisms in the Silver Valley may affect Pb bioavailability by bioaccumulation and sequestration of Pb from the aqueous phase into sediments.

Previous studies of microbial diversity in sediments of the CDAR have focused on microorganisms with specific capabilities (e.g., As(V) reduction, Fe(III) reduction and Pb resistance), rather than obtaining a broad community level observation such that the microbial community present in metal-contaminated CDAR sediments remains largely uncharacterized. The objective of our work was to characterize the microbial community which had been exposed to multiple toxic metals. The diversity of microorganisms was examined by 16S rRNA gene sequencing and Phylochip microarrays to identify the bacterial groups present.

Materials and Methods

Sampling

Water and sediment samples were collected in April 2005 from the CDAR just upstream of the delta region of LCDA near Harrison, Idaho (N 47°28'43.8", W 116°43'59.6") (Fig. 2.1), an area highly contaminated with heavy metals [Horowitz et al., 1993, Niggemyer et al., 2001, Sprenke et al., 2000]. The dissolved oxygen and temperature in the water were measured at the site using a portable DO meter (Extech Instruments Model 407510).Cores were obtained in 15 cm PVC pipe (5.08 cm inner diameter, schedule 20) with removable caps on both ends. The pipes were pre-washed

with ethanol in the laboratory. The cap on one side was removed and the PVC pipes were pressed into the sediment. The cap was replaced after filling the pipes with sediment.

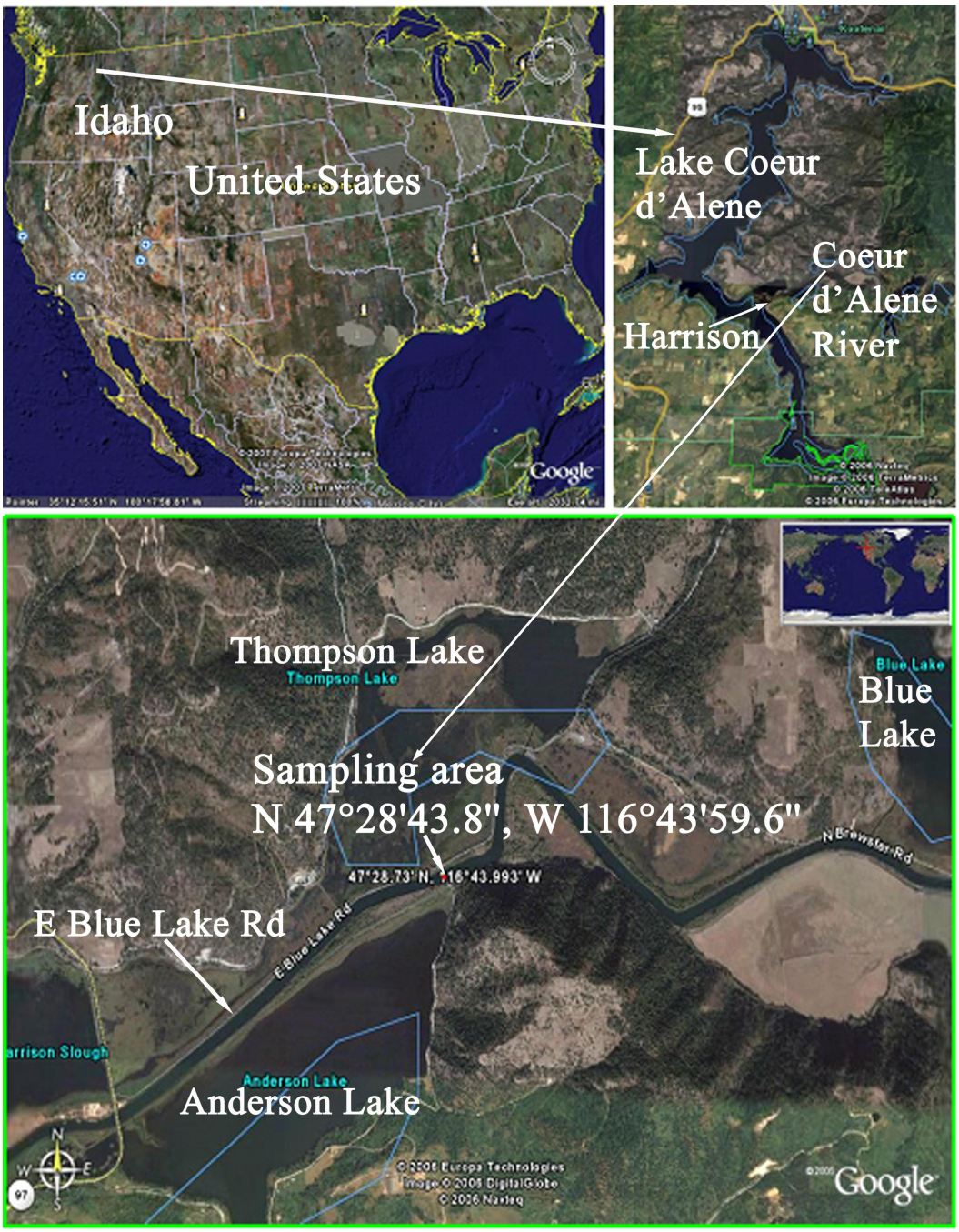

Figure 2.1. Location of the sampling site near Harrison, Idaho on the Coeur d'Alene River.

The samples were transferred to plastic bags and kept on ice in the field until they were transported to the laboratory approximately 2 h later. In the laboratory, cores were stored at -25 ºC until they were thawed and homogenized manually with a sterile spatula in an anaerobic chamber (Forma Scientific Inc. Model 1025) [Moberly et al., 2006]. After homogenizing, sediments for 16S rRNA analyses were transferred to sterile 50-ml centrifuge tubes and stored at -25 ºC until DNA extraction. The homogenized sediments from a single centrifuge tube were sub-sampled for all analyses.

DNA Extraction and PCR Amplification

To characterize the CDAR sediment microbial community DNA was extracted in duplicate from 500 mg sediment samples using the FastDNA SPIN Kit for Soil (BIO 101, Inc., La Jolla, CA). The gene coding for the 16S rRNA was amplified for the generation of gene fragments for cloning [Polz et al., 1997]. Bacterial DNA was amplified in two consecutive optimum (empirically determined) polymerase chain reactions (PCR) using the universal bacterial primers 8F (5′-AGT TTG ATC CTG GCT CAG-3′) and 1492R (5′-ACC TTG TTA CGA CTT-3′) in the first PCR reaction and 338F (5′-CTC CTA CGG GAG GCA GCA G-3′) and 907R (5′-CCG TCA ATT CCT TTR AGT TT-3′) (Integrated DNA Technologies, Inc.) in the second PCR reaction [Nevin et al., 2003]. Nested PCR was performed for each of the duplicate DNA extracts to recover 25 ng/µl of PCR product. The use of 8F and 1492R primers resulted in products that encompassed all but 56 of the 1,542 bases of the 16S rRNA gene (*Escherichia coli* standard) [Polz et al., 1997]. The 338F and 907R primer set was used to amplify bases 338 to 907 of the 16S

rRNA gene (*E. coli* standard) [Koizumi et al., 2003]. Each 50 µl PCR reaction mixture contained the reagents presented in Table 2.1.

Table 2.1. PCR reagents required to prepare a 50 µl reaction mixture (stock concentrations are in parentheses).

10X PCR Buffer[i] (-$MgCl_2$)	$MgCl_2$[ii] (50mM)	dNTP[i] (10 mM each)	BSA[ii] (10 mg/ml)	Primer[iii] (12.5 µM)	*Taq* DNA polymerase[i] (5 U/µl)	Molecular biology-grade water[iv]
2.5 µl	2 µl	1 µl	2 µl	2 µl	0.25 µl	37.25 µl

[i]Invitrogen, Carlsbad, CA;

[ii]New England BioLabs, Ipswich, MA;

[iii]Integrated DNA Technologies, Coralville, IA;

[iv]Fisher Scientific International

One microliter of extracted DNA (approx. 50 ng) was added to the above mixture as template in the first PCR and 1 µl of amplified DNA from the first PCR was used as template in the second PCR. PCRs were performed with the mastercycler gradient thermal cycler (Eppendorf, Westbury, NY). PCR cycles contained a 94 ºC incubation for 4 min, followed by 30 cycles of 94 ºC denaturation for 1min, 55 ºC annealing for 1min, and 72 ºC elongation for 1.5 min. A final extension of 72 ºC for 20 min was used. Each amplification was confirmed with UV transillumination with ethidium bromide staining in 1% agarose gel electrophoresis (1X TAE buffer, 1.5h, 80V).

Clone Library Construction and
Screening of 16S rRNA Amplicons

The 16S rRNA fragments of each of the duplicates were purified by StrataPrep® PCR Purification Kit (La Jolla, CA) and ligated into the pGem-T Easy Vector (Promega,

Madison, WI) according to the manufacturer's instructions. *E. coli* JM109 cells were transformed with the ligated vector and spread onto two S-Gal^{TM}/LB Agar (Sigma Aldrich, St. Louis, MO) plates with ampicillin (sodium salt, 100 µg/ml). The S-Gal^{TM}/LB Agar blend contained the autoclavable chromogenic β-galactosidase substrate S-Gal, IPTG and LB Agar. The plates were incubated overnight at 37 ºC. The plates with higher single white colonies from the duplicate reactions were selected for further analysis.

In total, ninety-five white colonies, each transformed with an *E. coli* cell, were used directly as the template in whole-cell PCR. The inserts were amplified by PCR with primers M13F (5′-GTT TTC CCA GTC ACG AC-3′) and M13R (5′-CAG GAA ACA GCT ATG AC-3′) (Promega, Madison, WI) flanking the insert on the vector [Polz et al., 1997]. Each of 95 clones was transferred with an autoclaved toothpick to 2.5 µl 10X PCR buffer (-MgCl2) (Invitrogen, Carlsbad, CA) and 22.5 µl of DNA-free water (Fisher Scientific International) pre-mixture. The PCR pre-mixtures were placed in the thermocycler at 99 ºC for 15 min to lyse the cells, followed by 80 ºC for 5 min when the PCR post mixture (prepared as described in Table 2.1) was added for PCR amplification as described above.

Sequencing and Phylogenetic Analysis of 16S rRNA Amplicons

PCR products of 95 clones were purified and sequenced using M13F & M13R primers (Promega, Madison, WI) at TGEN Research Institute (Phoenix, Arizona). Sequencing reactions used the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and automated DNA sequencer

(Model 3730, Applied Biosystems). The 16S rRNA gene sequences amplified by the forward and reverse primers were aligned with the corresponding primer sequences using Sequencher 4.5 software (Gene Codes Corporation, Ann Arbor, MI). The primer sequences were cut from the 16S rRNA gene sequences. Then the forward and reverse sequences of each clone were aligned with each other using Sequencher 4.5 software. 99% identities between the forward and reverse sequences of 91 clones were found and the conserved residues were saved as 'consensus sequences'. Four clones containing non-homologous base pairs were no longer used for analysis. The consensus sequences were then compared to known sequences by using the nucleotide-nucleotide BLAST (blastn) search algorithm [McGinnis et al., 2004; Tatusova et al., 1999] provided by the National Center for Biotechnology Information (NCBI). Sequences were aligned to their closest relatives in GenBank by using BioLign alignment version 4.0.6.2 [Cork et al., 2005]. Only one sequence, among the CDAR clones showing similarity score[†] of ≥97%, was selected for drawing the phylogenetic tree.

The sequences and their closely related representative sequences from GenBank were aligned with ClustalX 1.83 [Chenna et al., 2003; Thompson et al., 1997] to get the NEXUS format [Maddison et al., 1997] for compatibility with PAUP*4.0.b10 [Yang, 1996]. In ClustalX, sequences were aligned using a slow-accurate method with a gap opening penalty of 10.00 and a gap extension penalty of 0.10 [Hall, 2005].

[†] Similarity score is a standard technique of alignment. A similarity score can be defined as a sum of cost values assigned to identities and replacements, minus a sum of penalty values assigned to gaps within alignments [Mückstein, 2001]. The general formula of the similarity score s is as follows:

$$s = \sum \text{costs}_{\text{identities, replacements}} - \sum \text{penalties}_{\text{gaps}}$$

The homologous regions of the sequences were considered to draw the phylogenetic tree, and gaps were treated as missing nucleotides. Distances were calculated using the Jukes-Cantor algorithm [Jukes et al., 1969]. Neighbor-joining analysis was performed using parsimony (PAUP*) version 4.0.b10 [Yang, 1996] on 100 replicates [Hall, 2005]. *Parachlamydia acanthamoebae* was defined as the outgroup sequence. In the phylogenetic tree, bootstrapping was used to check the level of support for individual nodes [Harshman, 1994], i.e., the bootstrap value of 99 for a certain clade indicated that 99 out of 100 bootstrap trees generated randomly contained this group. A bootstrap value of ≥50% was selected for the tree. The resulting tree (phylogram) was drawn using the TreeView program [Page, 1996].

16S rRNA Gene Based Microarray Assay

DNA extracts were amplified using 8F and 1492R primers and concentrated to 0.05 µg/µl using the StrataPrep® PCR Purification Kit (La Jolla, CA). Duplicate samples were sent to the Lawrence Berkeley National Laboratory (CA, USA) for the microarray analysis. PCR amplicons of both samples were loaded and processed on a 16S rRNA gene microarray called the PhyloChip [Brodie et al., 2007]. The bacterial 16S rRNA gene sequences were grouped into operational taxonomic units (OTUs), which were each identified on the PhyloChip using 11 sets of 25-mer probes [DeSantis et al., 2007]. Each probe set consisted of one perfect match (PM) and one mismatched (MM) probe. A PM probe had the exact sequence of the target molecule, whereas the MM probe had the middle base changed. This MM probe purposefully did not match perfectly with any of the PM sequences for any OTU. Arrays were scanned using a GeneArray Scanner

(Affymetrix) and the pixel image was captured from the scanner using standard Affymetrix software (GeneChip Microarray Analysis Suite, version 5.1). If the fluorescent intensity of the PM probe was at least 1.3 times greater than the MM probe, the probe pair was scored positive. The presence of an OTU was considered by the positive fraction (pf) of each probe set which was the number of positive probe pairs divided by the total number of probe pairs in a probe set [DeSantis et al., 2007]. The higher the pf value, the more conservative the results would be. A cut off value of 0.9 pf was used for the CDAR samples. The OTU was considered to be present in CDAR sediment if the pf value was greater than 0.9. OTUs with pf values less than 0.9 were considered absent in the samples.

Diversity Indices

To characterize the diversity of represented phylogenetic groups, two common diversity indices, Shannon index (H) and Simpson's index of diversity (1-D) were calculated using the PAST (PAleontological STatistics) software v. 1.67 [Hammer et al., 2001].

$$H = -\sum(p_i \ln p_i) \qquad (2.1)$$

$$D = -\sum p_i^2 \qquad (2.2)$$

where p_i is the fraction of individuals that belong to i-th species. These parameters were calculated for the clone library and PhyloChip data. Also calculated were parameters of evenness and dominance of microbial class for CDAR communities.

Results

Partial 16S rRNA gene sequences were obtained for all 95 CDAR clones. Four sequences were discarded because they contained ambiguous and nonhomologous base positions. Among the remaining 91 sequences, 72 clones had ≥95% similarity with the sequences of representative bacteria from GenBank and 19 clones had similarity <95%. The CDAR clones fell into 13 distinct phylogenetic classes (Table 2.2). Two clones (CDAR 18 and CDAR 85) had >97% similarity with 'environmental samples' (GenBank accession numbers were AB243673 and AF314435 respectively). CDAR 82 was an 'uncultured bacterium' (accession number AY050595) and CDAR 80 was an unclassified *Chloroflexi* (accession number DQ811877). The relative abundance of 13 bacterial classes is shown in Figure 2.2.

β-*Proteobacteria* was the most abundant (56%) phylogenetic class in terms of clone numbers. Out of 7 *Thiobacillus* clones, four clones (CDAR 25, CDAR 57, CDAR 61, and CDAR 88) were closely related (99% similarity) to *Thiobacillus plumbophilus* and three clones (CDAR 15, CDAR 65, and CDAR 73) were most similar (>97% similarity) to *Thiobacillus denitrificans* (Table 2.3). The major genera which are known to exist in metal contaminated environments [Bruneel et al., 2006; Nies, 2000; Templeton et al., 2003] and also present in our clone libraries are *Ralstonia* (CDAR 86 with 95% similarity), *Gallionella* (CDAR 43 and CDAR 90 with ≥98% similarity), *Burkholderia* (CDAR 37, CDAR 40 and CDAR 83 with ≥98% similarity), and *Desulfobacca* (CDAR 60 with 98% similarity).

Table 2.2. Thirteen different bacterial classes found in CDAR sediment using 16S rRNA gene sequencing and phylogenetic analysis.

Proteobacteria				*Acidobacteria*	*Actinobacteria*	*Flavobacteria*	*Bacteroidetes*	*Chloroflexi*	*Sphingobacteria*	*Chlorobia*	*Cyanobacteria*	*Clostridia*
β -	α -	δ -	γ -									
51	4	3	2	6	6	5	3	3	1	1	1	1

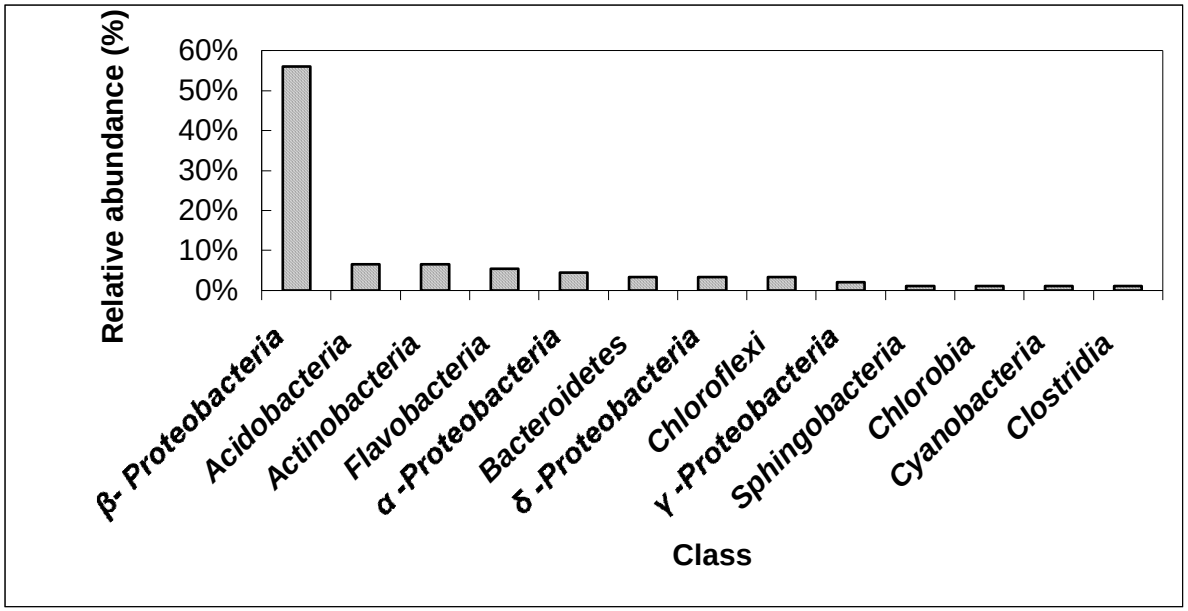

Figure 2.2. Relative abundance of class level phylogenetic diversity identified in 16S rRNA of CDAR clone library.

Table 2.3. Classification of the sequences at the genus level (Continued next page).

Genus no.	Genus (% similarity)	Number of clones from genera	Class	Metabolism of interest	References
1	*Thiobacillus* (≥97)	7	β -*Proteobacteria*	Sulfur oxidizer	a-c
2	*Azoarcus* (≥95)	7	β-*Proteobacteria*	Degrades aromatic	d, e
3	*Acidobacterium* (≥98)	6	*Acidobacteria*	--	--
4	*Burkholderia* (98)	5	β-*Proteobacteria*	Pb sorption, Pathogen	f
5	*Flavobacterium* (≥94)	5	*Bacteroidetes*	--	--
6	*Janthinobacterium* (≥93)	5	β-*Proteobacteria*	--	--
7	*Chloroflexi* (≥94)	4	*Chloroflexi*	Dechlorination, S oxidizer	g
8	*Arthrobacter* (≥99)	3	*Actinobacteria*	Resistant to Cr, Pb, Zn & Cu	h- j
9	*Nitrosospira* (≥95)	3	β-*Proteobacteria*	Ammonia oxidizer	k
10	*Rhodoferax* (≥96)	3	β-*Proteobacteria*	Fe reducer	l
11	*Sterolibacterium* (≥93)	3	β-*Proteobacteria*	Denitrifier	m
12	*Bacteroidetes* (99)	3	*Bacteroidetes*	--	--
13	*Bacterium Ellin* (97)	2	β-*Proteobacteria*	--	--
14	*Bradyrhizobium* (≥95)	2	α-*Proteobacteria*	Metal resistant	n
15	*Dechloromonas* (95)	2	β-*Proteobacteria*	Chlorate reducer	o
16	*Gallionella* (≥97)	2	β-*Proteobacteria*	Iron oxidizer	p
17	*Legionella* (93)	2	γ-*Proteobacteria*	Pathogen	--
18	*Methylophilus* (99)	2	β-*Proteobacteria*	Methylotroph	q
19	*Polaromonas* (98)	2	β-*Proteobacteria*	--	--
20	*Comamonadaceae* (≥97)	2	β-*Proteobacteria*	--	--
21	*Rhodocyclaceae* (97)	2	β-*Proteobacteria*	--	--
22	*Anaeromyxobacter* (100)	1	δ-*Proteobacteria*	Fe reducer	r
23	*Beijerinckia* (98)	1	α-*Proteobacteria*	--	d
24	*Cellulomonas* (97)	1	*Actinobacteria*	--	--
25	*Desulfobacca* (98)	1	δ-*Proteobacteria*	Sulfate reducer	s
26	*Desulfuromonas* (96)	1	δ-*Proteobacteria*	Carbon oxidizer	t
27	*Devosia* (97)	1	α-*Proteobacteria*	Nitrifying bacteria	d
28	*Flavobacteria* (≥95)	1	*Bacteroidetes*	--	--
29	*Herbaspirillum* (98)	1	β-*Proteobacteria*	Nitrogen fixing bacteria	u
30	*Massilia* (98)	1	β-*Proteobacteria*	--	--
31	*Paucibacter* (97)	1	β-*Proteobacteria*	--	--
32	*Ralstonia* (95)	1	β-*Proteobacteria*	Ni, Cd, Zn, and Cr resistant	v- x
33	*Sphingobacterium* (98)	1	*Bacteroidetes*	--	--
34	*Sporichthya* (99)	1	*Actinobacteria*	--	--
35	*Thermaerobacter* (98)	1	*Clostridia*	--	--
36	*Actinobacterium* (99)	1	*Actinobacteria*	--	--
37	*Chlorobi* (98)	1	*Chlorobia*	--	--
38	*Zoogloea* (97)	1	β-*Proteobacteria*	--	--
	Total number of sequences	89			

a: Harshman, 1994; b: Janssen, 2006; c: Environmental microbiology: Thiobacillus, 1998; d: Tarlera e al., 2003; e: Valverde et al., 2003; f: Jones et al., 1997; g: Häggblom et al., 1999; h: Grass et al., 2000; i: Anton et al., 1999; j: Konstantinidis et al., 2003; k: He et al., 2003; l: Finneran et al., 2003; m: Hullar et al., 2006; n: Coates et al., 1995; o:

Mechichi et al., 2002; p: Van Nstrand et al., 2005; q: Achenbach et al., 2001; r: Anderson et al., 2003; s: White et al., 1997; t: Shivaji et al., 1991; u: Kanokratana et al., 2004; v: Mergeay et al., 1985; w: Nies, 2000; x: Grass et al., 2000.

The most frequently detected 16S rRNA gene sequences in the clone library were sulfur oxidizers (Table 2.3). We also observed sequences showing ≥97% similarity to known iron oxidizers, iron reducers, a sulfate reducer, several metal resistant bacteria, nitrogen fixing bacteria, nitrogen oxidizing bacteria, and heterotrophs in the CDAR clone library. A phylogenetic tree was constructed (Fig 2.3) using CDAR clone sequences (one sequence was selected for the sequences having ≥99% sequence similarity) and their representative members from BLAST search. Figure 2.3 illustrates the phylogenetic diversity of the 53 unique CDAR clones and their closest match representatives. The accession numbers assigned by GenBank of closely related bacteria are shown in parentheses in the tree. Matching of the clones with the PhyloChip at different taxonomic level is annotated by alphabetic symbols in parentheses. Phylogenetic analysis of the clones presented in the tree has shown that the clones belong to 6 different phyla. The clone library results were compared with the PhyloChip results from PCR products of the same sediment. Comparison of the two approaches was made at phylum and class levels (Table 2.4), as well as at the genus and subfamily levels (Fig. 2.3) where a subfamily is a taxonomic level below family and above genus classification. The PhyloChip detected the presence of 1551 OTUs from 25 different phyla, and 43 different classes were identified with pf values greater than 0.9. The class most frequently detected by the PhyloChip was α-*Proteobacteria* (Table 2.4) while β-*Proteobacteria* was the most frequently detected class in the clone data (Fig. 2.2).

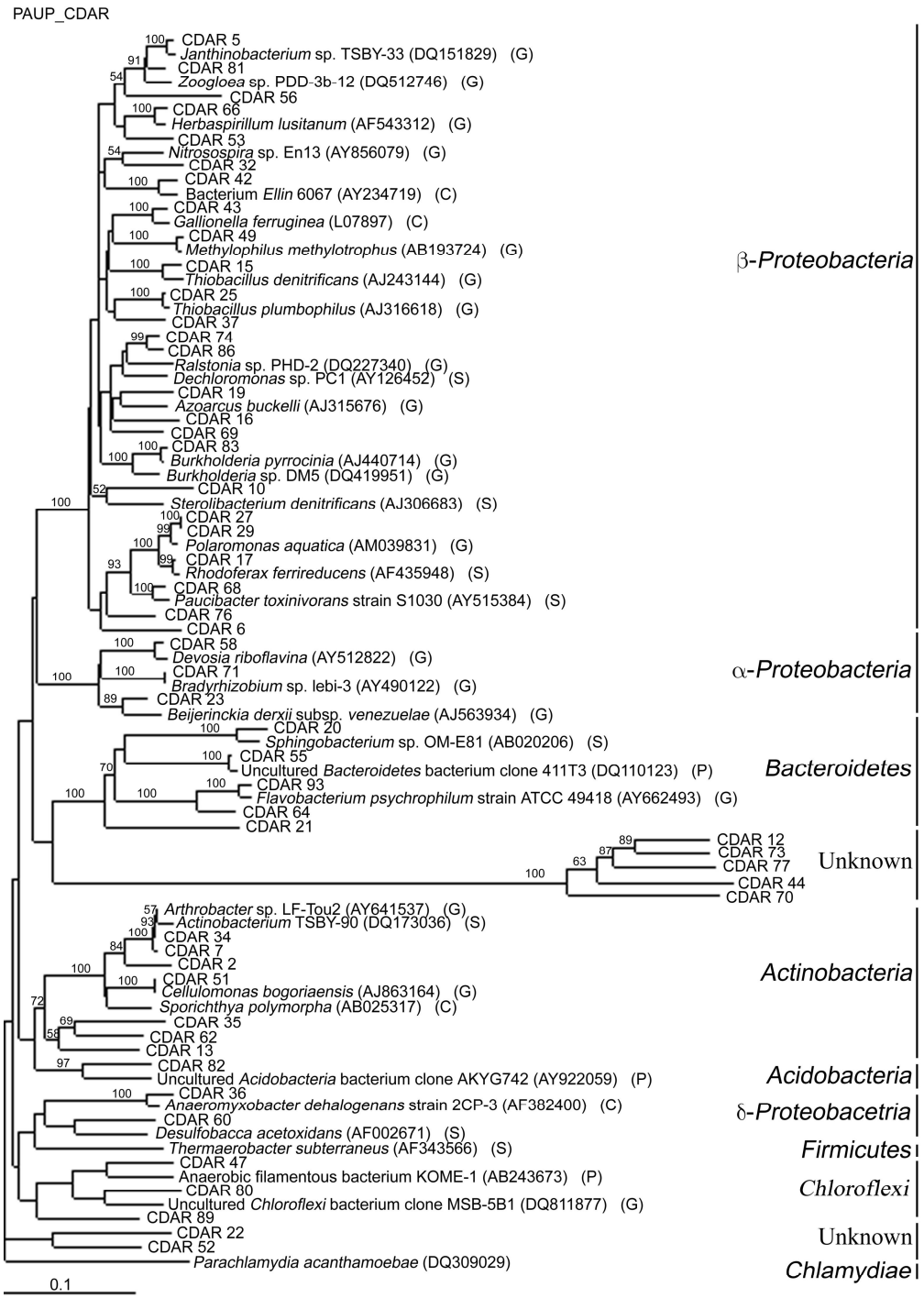

Figure 2.3 Neighbor-joining tree showing the phylogeny of bacterial 16S rRNA sequences from CDAR sediment DNA. Sequences were aligned with Clustal X; Bootstrap values (100 replicates) above 50% are shown. The scale bar shows 10% sequence divergence. *Parachlamydia acanthamoebae* was used as outgroup.

G = Representative organisms matched at genus level with the PhyloChip data;
S = Representative organisms matched at subfamily level with the PhyloChip data;
C = Representative organisms matched at class level with the PhyloChip data;
P = Representative organisms matched at phylum level with the PhyloChip data.

Table 2.4. Comparison of clone and PhyloChip results for CDAR sediment samples.

Phylum no.	Phylum as described in PhyloChip	Class as described in PhyloChip	No. of class represented on PhyloChip data	No. of class represented on Clone data
1	*Proteobacteria*	α-*Proteobacteria*	245	4
2	*Proteobacteria*	γ-*Proteobacteria*	224	2
3	*Actinobacteria*	*Actinobacteria*	157	6
4	*Proteobacteria*	β-*Proteobacteria*	149	51
5	*Firmicutes*	*Clostridia*	140	1
6	*Coprothermobacteria*	Unclassified	104	-
7	*Firmicutes*	*Clostridia*	98	-
8	*Proteobacteria*	δ-*Proteobacteria*	94	3
9	*Acidobacteria*	*Acidobacteria*	48	6
10	*Bacteroidetes*	*Bacteroidetes*	43	3
11	*Bacteroidetes*	*Sphingobacteria*	38	1
12	*Proteobacteria*	ε-*Proteobacteria*	36	-
13	*Bacteroidetes*	*Flavobacteria*	33	5
14	*Cyanobacteria*	*Cyanobacteria*	23	1
15	*Chloroflexi*	*Anaerolineae*	20	-
16	*Verrucomicrobia*	*Verrucomicrobiae*	18	-
17	*Planctomycetes*	*Planctomycetacia*	11	-
18	*Spirochaetes*	*Spirochaetes*	9	-
19	*Chloroflexi*	*Dehalococcoidetes*	7	-
20	*Firmicutes*	*Catabacter*	5	-
21	*Firmicutes*	*Mollicutes*	5	-
22	*Nitrospira*	*Nitrospira*	5	-
23	TM7	TM7-3	5	-
24	*Firmicutes*	*Desulfotomaculum*	4	-
25	*Chlorobi*	*Chlorobia*	3	1
26	*Actinobacteria*	BD2-10 group	2	-
27	OP10	CH21 cluster	2	-
28	*Chloroflexi*	*Chloroflexi*-3	2	4
29	*Chloroflexi*	*Chloroflexi*-4	2	-
30	*Firmicutes*	*gut clone group*	2	-
31	OP9/JS1	OP9	2	-
32	*Acidobacteria*	*Solibacteres*	2	-
33	*Firmicutes*	*Symbiobacteria*	2	-
34	*Chloroflexi*	*Thermomicrobia*	2	-
35	*Aquificae*	*Aquificae*	1	-
36	*Chlamydiae*	*Chlamydiae*	1	-
37	*Deferribacteres*	*Deferribacter*	1	-
38	*Bacteroidetes*	KSA1	1	-
39	marine group A	mgA-2	1	-
40	NC10	NC10-1	1	-
41	NC10	NC10-2	1	-
42	OD1	OP11-5	1	-
43	*Thermodesulfobacteria*	*Thermodesulfobacteria*	1	-
		Total	1551 OTUs	88 sequences

The majority of the clones from the CDAR heavy metal contaminated sediment were found to be *Proteobacteria*. Twenty five CDAR clones in the tree appeared to be related to different species of β-*Proteobacteria*. The long branch of CDAR clones 12, 44, 70, 73, and 77 grouped with *Bacteroidetes* represents a significant difference of their nucleotides from the surrounding sequences. CDAR clone 55 formed the same group with uncultured *Bacteroidetes*; no specific representative organism, other than this, in the BLAST search had high similarity for CDAR 55. Similarly, CDAR clones 80 and 82 represented uncultured *Chloroflexi* and *Acidobacteria* respectively.

Table 2.4 and Fig. 2.3 demonstrated that 97% of the cloned sequences matched at the class level with the PhyloChip results while the PhyloChip identified 2.3 fold more diversity showing 1551 OTUs whose pf values were greater than 0.9. Out of the 38 different genera (Table 2.3) in clone library results, 21 genera (53.8%) matched at the genus level with OTUs on the PhyloChip, 13 (33.3%) matched at the subfamily level with PhyloChip and 12.9% matched at the class or phylum level (Fig. 2.3).

Diversity indices were calculated for both the clone library and PhyloChip data of the CDAR sediment (Table 2.5). The Shannon index and Simpson's index of diversity calculated from PhyloChip data were higher than the indices from the clone library because a much larger number of taxonomic phyla, classes and OTUs were detected in the microarray analysis (Table 2.5). Higher Simpson's index of diversity for PhyloChip results mean higher probability of two randomly selected OTUs belonging to different species. In the clone library, the probability is less; therefore the sample diversity is smaller. Evenness and dominance of the species for both analyses are shown in the Table.

Table 2.5. Comparison of sequence diversity between two molecular techniques for CDAR sediment.

				Diversity estimate			
Community analysis by	No. of distinct sequences	No. of distinct OTUs	No. of Phyla	Shannon (H)	Simpson (1-D)	Evenness	Dominance
Clone library	91	--	16	1.783	0.667	0.372	0.333
PhyloChip	--	1551	25	2.719	0.911	0.3525	0.089

Discussion

Comparison of Clone Library and PhyloChip

Clone library sequencing and PhyloChip analysis of metal contaminated CDAR sediment showed that this metal containing environment is the habitat of highly diverse microorganisms. The sediment provides a wealth of microorganisms which likely play an important role in geochemical cyling of the metals. Trends of bacterial clone numbers in CDAR sediment community analysis are shown in Table 2.2 and 2.4, and Figure 2.2. The variation in the abundance of members of different phyla and classes in the sediment was identified by the variation in the 16S rRNA gene bacterial sequences. The most abundant phyla of the CDAR clone library are *Proteobacteria, Bacteroidetes, Acidobacteria* and *Actinobacteria* whereas for the PhyloChip, the order is *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* [Table 2.4]. The results are consistent with the general trends often observed in other soil clone libraries [Janssen et

al., 2006]. The microarray results showed greater abundance than clone library data because a clone library of 95 sequences was insufficient to adequately determine diversity in the CDAR sediments. Analysis of an increasing number of clones would have likely revealed further diversity in this area. On the other hand, high-density microarrays target the most unique portions of the 16S rRNA gene of a whole microbial community at once in a high-throughput manner [DeSantis et al., 2007].

The calculated diversity indices, Shannon's index, H, and Simpson's index, D, were higher for PhyloChip results than the clone library results because the array detected the presence of more phyla, classes, and orders than the clone library. These indices were chosen because they reflect different aspects of diversity: H is sensitive to rare species, whereas D is more sensitive to changes in abundance of more common species. H is affected by both the number of species and their evenness. The relative abundance of species as measured by evenness was almost the same in both analyses. The calculated evenness value dictated that different species have similar abundance in the clone library and PhyloChip. A greater number of species in the PhyloChip data increased diversity as measured by H [Pielou, 1974]. The maximum diversity of a sample is found when all species are equally abundant [Pielou, 1975]. (1-D) value gives the probability of any two individuals drawn at random from an infinitely large community belonging to different species. In this case, it indicates dominance of species, so if D increases, sample diversity increases and dominance of a species decreases [Jost, 2006]. Relatively higher (1-D) value for higher diversity in PhyloChip results (Table 2.5) means less dominance of the

species. Higher dominance of species (lower 1-D) was calculated in comparatively less diverse clone library data.

Though the 16S rRNA sequences cannot represent the whole microbial distribution [DeSantis et al., 2007], it gives an idea of existence of phylogenetically distinct groups present in CDAR sediment. Also the clone library allows the detection of novel taxa, whereas the PhyloChip is limited to known sequences. Microarray analysis is a time saving method to confirm the presence of phyla, class, and genera identified by the clone library. Identification of the seqeuences and OTUs of several sulfur-oxidizing, metal-oxidizing/reducing, sulfur-reducing or metal-resistant bacteria in the clone library and PhyloChip, respectively, are of interest because they can lead to metal cycling or immobilization of heavy metals in CDAR sediments.

Sulfur-oxidizing Bacteria

Members of the genus *Thiobacillus* (Table 2.3) appeared in greatest numbers in the CDAR clone library. An OTU, *Thiobacillus thiooxidans* of the same genus was found in the PhyloChip. *Thiobacillus* spp. are known to leach Cu, Au and other metals from ores [Ehrlich, 1996] and are able to oxidize reduced sulfur compounds [Drobner et al., 1992]. Microorganisms can potentially utilize the sulfide minerals in CDAR sediments which could result in the release of metals and sulfate through oxidative reactions [Balistrieri et al., 2002; Balistrieri et al., 2003]. *Thiobacillus* occur widely in terrestrial and aquatic habitats wherever inorganic sulfur and iron are present. For example, *T. plumbophilus* (CDAR 25, 57, 61, 88 in the clone library) can grow aerobically on natural and synthetic PbS as sole energy source [Drobner et al., 1992]; *T. denitrificans* (CDAR

15, 65, 73) grows by coupling the oxidation of inorganic sulfur compounds (such as hydrogen sulfide, thiosulfate, and minerals like pyrite, FeS_2 and FeS) to denitrification, and it was recently found to couple the anaerobic oxidation of Fe to denitrification as well [Beller et al., 2006]. *T. plumbophilus* can also grow in the presence of Ag, As, Cd, Co, Cu, Mo, Ni, Sb and Zn ions [Drobner et al., 1992]. The presence of all these metals as metal sulfides in CDAR and LCDA sediments [Harrington et al., 1998; Horowitz et al., 1995; NRC, 2005; Toevs et al., 2006] provide energy as electron donors for microorganisms during respiration [Rawlings, 2005]. FeS and FeS_2 can be biologically oxidized by the *Thiobacillus* spp. in anoxic and suboxic zones of sediments and move from the anoxic to oxic zone in the water column.

Ammonia-oxidizing and Nitrifying Bacteria

During the sulfur oxidation reaction, *Thiobacillus* organisms could promote denitrification by transforming nitrate (NO_3^-) produced by ammonia oxidizing or nitrifying bacteria to N_2 ($NO_3^- + 6H^+ \rightarrow 0.5N_2 + 3H_2O$) [Tebak et al., 2005]. NO_3^- is available in the overlying water column or occasionally in interstitial water near the sediment-water interface in LCDA sediments [Toevs et al., 2006]. Recent studies of *T. denitrificans* have shown that it is a natural agent for bioremediation of NO_3^- [Beller et al., 2006]. The diversity and abundance of ammonia oxidizing bacteria such as *Nitrosospira* spp. (CDAR 32, 53, 67) both in clone library and PhyloChip data in the sediment suggest that these ammonia oxidizing bacteria [Avrahami, 2007] may interact with the *Thiobacillus* bacterial biomass to consume NO_3^- produced and play key roles in nitrogen cycling [Smith et al., 1977]. *Devosia riboflavina* (CDAR 58) in the class α-

Proteobacteria (Fig. 2.3) is a nitrifying bacterium [Vanparys et al., 2005] found in the clone library and PhyloChip results.

Iron-oxidizing and reducing Bacteria

Identification of bacteria related to the iron-oxidizing *Gallionella ferruginea* (CDAR 43 and CDAR 90) and iron reducers *Anaeromyxobacter dehalogenans* (CDAR 36) and *Rhodoferax ferrireducens* (CDAR 17 and CDAR 18) in the clone library are significant, though none of them was identified at the genus level in PhyloChip data. The bacteria matched at their respective class levels, β- and δ-*Proteobacteria* with the PhyloChip data. *G. ferruginea* is a lithoautotroph that can grow aerobically on Fe(II) as an energy source and CO_2 as a carbon source, although mixotrophic growth is possible in the presence of glucose [Hallbeck et al., 1991]. It is reported to play an important role in oxidizing Fe(II) in acid mine drainage [Bruneel et al., 2006; Kim et al., 2003]. It has been shown to efficiently oxidize Fe(II), As(III) and As(V) in water and thus the oxidized products are removed from water by subsequent precipitation [Katsoyiannis et al., 2004]. Since the average pH of the CDAR sediment is usually lower at the surface than in the deeper sediments [Harrington et al, 1998], the Fe precipitating bacterium *G. ferruginea* may conduct the biotic iron oxidation/precipitation process near the sediment-water interface. An environmental scanning electron microscopy (ESEM) technique used by Hallberg et al. [2004] revealed that iron precipitated in form of flakes and formed fibers on *Gallionella* in a hydrated state.

On the other hand, iron reducing bacteria such as *A. dehalogenans* and *Rhodoferax ferrireducens* grow by coupling Fe(III) reduction to the oxidation of acetate

[Finneran et al., 2003; He et al., 2003]. Some iron reducing bacteria can use toxic metals or metalloids such as As(V), Se(VI), and U(VI) as terminal electron acceptors [Tebak et al., 2005]. Niggemyer et al. [2001] isolated an dissimilatory As(V)-respiring strain GBFH from sediments at delta region of CDAR which could reduce Fe (III) and therefore could contribute to the mobilization of arsenic as As(V). A new genus and species of Fe-reducing bacterium, *Ferribacterium limneticum* was isolated from the sediment of CDAR delta by Cummings et al. [1999]. Cummings et al. [2003] also found Fe(III)-reducing *Geobacter* spp. and genera of *Anaeromyxobacter* in the same location of delta which contained 2% Pb and >10% Fe with elevated concentrations of dissolved metals in the porewaters. These bacteria appeared to be involved in both the sequestration and remobilization of Fe(III) oxide-associated trace metals such as As, Zn, Co, and Ni [Cummings et al., 1999; Islam et al., 2005; Zachara et al., 2001]. The *Geobacteraceae* family is also an important humic-reducing organism in sediments [Coates et al., 1998].

Sulfate-reducing Bacteria

The sulfate-reducing bacteria (SRB) found in the clone library and detected at the subfamily level, *Desulfomicrobiaceae*, in the PhyloChip results mediate the direct and indirect reduction of heavy metals and metalloids [Hao, 2000; White et al., 1997]. SRB can immobilize metals as sulfides through the release of H_2S and can grow using organics and other different terminal electron acceptors, like Fe(III), Mn(IV) and U(VI) [Tebo et al., 1998]. SRB have been observed in mining environments, for example, in several Au and Cu-Zn mine tailings [Fortin et al., 2000]. Earlier investigations [Harrington et al., 1998; NRC, 2005; Toevs et al., 2006] showed that there are significant amounts of Fe,

Mn, Pb, Zn and As associated with sulfidic minerals in CDAR and LCDA sediment. CDAR clone 62 (*Desulfobacca acetoxidans*) represents the only member of the *Desulfobacca* group that reduces sulfate and eventually promotes the precipitation of metal sulfides. *Desulfobacca* belongs to the δ-*Proteobacteria*; the closest relatives are *Desulfosarcina variabilis*, *Desulfacinum infernum,* and *Syntrophus buswellii*. Elferink et al. [1999] isolated and characterized *D. acetoxidans* (a sulfate-reducing bacterium) as a sulfate reducer from granular sludge of a laboratory-scale anaerobic sludge bed reactor. They found that sulfate and other inorganic sulfur compounds, but not elemental sulfur, serve as terminal electron acceptor for *Desulfobacca* and are reduced to H_2S. Fortin et al. [2000] showed that degradation of existing microbial mass, mainly Fe-oxidizing bacteria, or the excretion of metabolic products, could supply the electron donors to SRB. H_2S produced by SRB can lead to the formation of FeS, FeS_2 (pyrite), PbS, ZnS, CdS and CuS. Also, FeS and FeS_2 affect the solubility of other dissolved metals (such as Cu, Zn, Pb, Cd, etc.) through sorption reactions and co-precipitation [Morse et al., 1999]. Sulfide precipitation is an efficient means of removing toxic metals from solution. Presence of various metal sulfides in the CDAR sediments indicates the active microbial sulfate reduction by SRB is an important biogeochemical process for the precipitation of metals in cooperation with Fe and S cycling.

Metal-resistant Bacteria

Characterization of the microbial community based on 16S rRNA genes identified the sequences and OTUs of *Burkholderia* sp. in CDAR sediment (CDAR 12, 37, 40, 77, 83). *B.* spp. are known to be metal-resistant i.e., *B. cepacia* has been shown to uptake 10^{-5}

M Pb by simultaneous sorption and biomineralization processes [Templeton et al., 2003]. Van Nostrand et al. [2005] observed the growth of *B. cepacia* PR1 in the presence of metals (Ni, Co, Cd and Zn) at various pH values. Their studies suggest that at neutral pH, *B. cepacia* PR1 is sensitive to Ni^{2+} and other divalent cations, however, at lower pH values it can grow in the presence of high concentrations of Ni (3.41×10^{-3} M at pH 5), Co (4.24×10^{-3} M at pH 5 and 6), Cd (2.22×10^{-3} M at pH 5 and 6), or Zn (3.82×10^{-3} M at pH 5 and 6) [Van Nostrand et al., 2005].

Clone CDAR 86 is most closely related to *Ralstonia* sp. [Anton et al., 1999; Grass et al., 2000; Mergeay et al., 1985; Nies, 2000] with 95% similarity. *Ralstonia* spp. have been reported to live in heavy metal contaminated environments [Nies, 2000]. Fourteen *Ralstonia* OTUs were identified in the microarray analysis. Konstantinidis et al. [2003] characterized *Arthrobacter* and *Ralstonia* in copper contaminated sediments of Torch Lake, Michigan, USA. They found that Cu resistant *Ralstonia* isolates were also resistant to Cd, Ni, and Zn showing two patterns of phenotypic resistance to these three metals. CDAR clones 2, 7, and 34 showed two different strains of *Arthrobacter* spp.which are reported to be resistant to Cd, Co, Cu, Pb, and Zn [Hanbo et al., 2004] and capable of low temperature Cr(VI) reduction [Horton et al., 2006]. It is possible that the heavy metal resistance of *Arthrobacter* sp. is related to megaplasmid mediated resistance mechanisms [Hanbo et al., 2004] as found in *Ralstonia* sp. Because of heavy metal detoxification by ion efflux systems, both *Ralstonia* sp. and *Arthrobacter* sp. may be important bacteria of interest in CDAR environment where substantial portions of sediments are enriched with Cu, Cd, and Zn [Horowitz et al., 1995].

There are two clones in the library and three OTUs in the PhyloChip data from the genus *Bradyrhizobium* (CDAR clone 8 and 71) present which has been shown to tolerate high concentrations of Cu, Cd, and Zn sulfates [Matsuda et al., 2002]. There are 7 clones related to *Azoarcus* sp. (2 *A. buckelii*, *A. toluclasticus* and *A. denitrificians*, as well as 3 other strains of *Azoarcus* sp.) in the CDAR clone library. Two OTUs, *A. denitrificans* and *A. anaerobius* were found in the PhyloChip. *Azoarcus* spp. were found in terrestrial and marine ecosystems [An et al., 2004], anoxic and oxic soil, and sediment [Mechichi et al., 2002].

Besides the relatives of metal-tolerant species in the CDAR clone library and PhyloChip data, there are several other clones e.g., *Sterolibacterium denitrificans* (CDAR 6, 10, 92), *Herbaspirillum lusitanum* (CDAR 66), *Janthinobacterium* sp. (CDAR 5, 24, 31, 56, & 76), *Chloroflexi* (CDAR 1, 47, 59, & 80), *Dechloromonas* sp. (CDAR clones 74 & 84), bacterium *Ellin* (clone 13 and 42), *Sporichthya polymorpha* (CDAR 62), *Paucibacter toxinivorans* (CDAR 68), *Desulfuromonas palmitatis* (CDAR 35), *Cellulomonas bogoriaensis* (CDAR 51), *Massilia* sp. Tibet-IIU65 (CDAR 14) and *Methylophilus methylotrophus* (CDAR 49). The phylogenetic relationships of clones CDAR 47 (environmental sample), CDAR 85 (environmental sample) and clone 82 (uncultured bacterium) remained unclear. In Fig. 2.3, CDAR 12, 73, 77, 44, and 70 and CDAR 22 and 52 grouped together without showing any closest matched representative microorganisms in the tree. This might be because 16S rRNA gene sequences of these clones were not available in the NCBI's largest databank, GenBank, of nucleotide sequences.

Summary

Owing to the heavy metal tolerance of microorganisms and the ability to transform metals to less toxic forms, bacteria in CDAR sediment may be useful in toxic metal cycling. Identification of clone squences and OTUs of known metal reducers, oxidizers, sulfate reducer, oxidizers, and metal resistant bacteria is important to understand the continuous cyling of Fe, Pb, Zn, Cu, Cd, S, N and C in the neutral pH environment of CDAR sediments. Iron-reducing microorganisms support their growth by the reduction of Fe(III) to Fe(II) with the oxidation of acetate or lactate or elemental sulfur in acidic environements [Lovely, 1993; Tebak et al., 2005] depending on the availability of nutrients in their habitats. Iron oxidizers transfer back Fe(II) to Fe(III) using CO_2 or glucose as the carbon source and O_2 or NO_3^- as electron acceptor. Ammonia oxidizers and nitrifying bacteria can oxidize NH_3 and NO_2^- respectively to NO_3^- in the nitrogen cycle. Other divalent cations like Zn^{2+}, Cu^{2+} and Cd^{2+} react with H_2S generated by SRB in the anoxic sediment and produce metal sulfides. Thus, the microorganisms in CDAR sediment facilitate a dynamic process of metal cycling.

To compare the community with the other CDAR microbes collected at different time or from different sites, the calculated diversity indices would be a good source to compare the abundance and richness of data. The indices' values depend on robust sampling of diversity [Cummings et al., 2003]. It is important that comparisons of diversity involve either temporal or spatial variation. It is necessary to balance a broad survey of microbial diversity in the river with sequencing efforts [Martin, 2001] to

compare the abundance, dominance and distribution of microorganisms and estimate their role in biogeochemical cycling of metals.

Conclusions

A significant microbial diversity was detected in CDAR sediments which permit major advances in understanding of potential microbial functional roles in environmental geochemical processes. The application of 16S rRNA gene microarray analysis (PhyloChip) was a time saving technology for comparison with clone library data of CDAR sediment samples. 97% of the clone library data sequences matched with the PhyloChip results at different taxonomic levels (genus, subfamily and class) with the addition of 2.3 times more OTUs in the PhyloChip data. A high diversity among the sequences was found in both types of analysis that revealed the existence of microorganisms in the long term mine impacted CDAR sediment. Biological activities in the microbial community at this metal contaminated region can reduce, eliminate, contain or transform the metals. Different microbial activities (Fe-reducer, Fe-oxidizer, SRB, sulfur-oxidizers, metal-resistant bacteria) in the CDAR environment likely play an integral role in the biogeochemical cycling of metals, nitrogen, sulfur, other nutrients, and trace elements depending on pH and redox environment at different depths of sediments. It is difficult to analyze the role of all individual species or their integral role in the complex natural environment of the CDAR, however functionally important isolates can be selected and focused on for further study. A combined study of molecular phylogenetics and functional genomics will provide information to understand the

structure and function of microbial ecosystems in CDAR sediments. Studies like this are an important first step for further studies and experiments aimed at obtaining reliable data about the diversity, abundance, and activity of microbial populations in metal contaminated CDAR sediments.

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CHAPTER THREE

ZINC TOXICITY TO *PSEUDOMONAS* SP. FROM COEUR d'ALENE RIVER SEDIMENT

Abstract

The inhibitory effects of zinc (Zn) were studied with the *Pseudomonas* sp. strain JM001, isolated from the metal contaminated Coeur d'Alene River (CDAR) sediment, in order to test metal toxicity to cell growth in two different liquid media. It is found from this study that the cell kinetic rate depends on substrate and nutrient concentration and incubation temperature. The minimum inhibitory concentration (MIC) was measured 0.024 mM Zn in the nutrient limited defined medium (medium A) incubated at 22 ºC while it was 1 mM Zn in the minimal salts medium with vitamin solutions (medium B) at 35 ºC. The 50% inhibition in specific growth rate was found at 0.011 mM and 0.515 mM Zn in media A and B respectively. The maximum specific growth rate, μ_{max}, and Michaelis-Menten constant, K_S, were higher in medium B than medium A. Kinetic parameters were determined for the metal free control as μ_{max}= 0.284 and 0.325 h^{-1}, K_S= 1.4 and 4.62 mM in medium A and B respectively. The kinetic parameters for metal inhibited cell growth revealed that cell kinetic rates depend on the nutrient availability and temperature of the natural habitat. The results from this study will be used in a dose-response model to understand the kinetics of toxic metal-microbe interactions in complex CDAR environment.

Keywords: CDAR, *Pseudomonas,* specific growth rate, yield, growth kinetics

Introduction

The Coeur d'Alene River (CDAR) flows from Silver Valley in northern Idaho, U.S., runs westward and drains into the Lake Coeur d'Alene (LCDA) at the bottom part of the CDAR lower basin [Horowitz et al., 1995; NRC, 2005]. The lower basin at Harrison, containing the main stem of the CDAR [Meckel Engineering et al., 1983], forms a deltaic region with wetlands and lateral lakes [NRC, 2005; Sprenke et al., 2000]. CDAR delivers its metal contaminated sediments into the lateral lakes, marshes and LCDA during floods [NRC, 2005]. As a result, the sediments of the CDAR lower basin, LCDA and the lateral lakes are enriched with As, Cd, Pb, and Zn [Horowitz et al., 1995; Sprenke et al., 2000]. Zn is the most abundant divalent cation found in the environment, as well as in CDAR sediment [Balistrieri et al., 2002; Reece et al., 1978]. Analysis of aqueous geochemistry of CDAR sediments showed that pore and shore water contained 0.0412 mM (2.7 mg/L) and 0.0024 mM (0.16 mg/L) Zn respectively [Moberly, 2006]. Sprenke et al. [2000] found 0.154 mM maximum concentrations of Zn in water and its underlying sediments of four lateral lakes (Rose, Medicine, Black, and Anderson Lakes) near CDAR. Previous studies of the maximum, mean and median Zn concentrations in terms of mg/kg of dry sediment in metal enriched lakebed sediments are 11,169 [Harrington et al., 1998], 2,995 [Harrington et al., 1998] and 3,500 [Woods et al., 1997] respectively. Zn concentration of the collected sediment was detected as 7,470 mg /kg of dry sediment [Moberly, 2006] using X-ray fluorescense spectroscopic analysis. High concentrations of Zn in sediment, pore and shore water are toxic to humans and microorganisms though it is an essential nutrient to microorganisms and cofactor in

microbial metabolism at nanomolar concentration [Nies, 1992]. However, microorganisms living in this metal contaminated sediment develop several mechanisms at the cellular and molecular levels to overcome high concentrations of heavy metals in their environment [Lloyd et al., 2001; Ramos et al., 2002].

Heavy metal resistance in microorganisms can occur by a variety of mechanisms, including physical sequestration, exclusion, efflux, reduced uptake, detoxification, and synthesis of binding proteins [Robinson et al., 1984]. Since most metal-microbe interactions are initiated at the level of uptake [Chen, 2006; Eilmaz, 2003], the uptake and efflux mechanisms are likely to be closely linked to the mechanism of metal ion balance in microorganisms at low concentrations [Choudhury et al., 2001]. Regulation of Zn has been observed in a wide range of organisms, e.g., *Escherichia coli*, *Synechococcus* and *Saccharomyces cerevisiae* [Heuchel et al., 1994; Outten et al., 2001; Singh et al., 1999; Zhao et al., 1997]. Zn containing transcription factor proteins that control Zn uptake, storage, deployment, and export (collectively called homeostasis) in these organisms regulate the levels of Zn uptake and efflux transporters [Heuchel et al., 1994; Outten et al., 2001; Singh et al., 1999; Zhao et al., 1997]. In *E. coli*, metalloregulatory proteins ZntR and Zur sense Zn concentrations in the cell during transcription and repress Zn uptake systems (Zur) or activate Zn export systems (ZntR) accordingly [Outten et al., 2001]. The Zn resistance mechanism in *Pseudomonas putida* strain S4, a multiple metal-resistant strain, consists of three components: uptake, efflux, and binding [Choudhury et al., 2001]. Like *E. coli*, strain S4 applies a dual strategy of

efflux and binding of Zn to maintain the proper level of Zn in the cells [Choudhury et al., 2001].

Zn sorption to a biofilm of *P. putida* involves organic metal-complexing functional groups that play an important role in metal cycling in natural and contaminated environments [Chen et al., 2007; Toner et al., 2005]. The functional groups like carboxyl or phosphoryl groups bind biosorbed Zn forming Zn-phosphoryl or carboxyl type complexes [Chen et al., 2007; Toner et al., 2005] that are important for the efficient removal of metals from aqueous phases. The overall response of a common soil bacterium *P. fluorescens* was monitored by Rossbach et al. [2000] at elevated Zn concentrations. Their results showed that genes responding with induction to the presence of Zn include a P-type ATPase and pyoverdine synthesis genes. In this study, the kinetics of Zn biosorption and effects of environmental factors on cell growth kinetics were examined. The toxicity of Zn to the growth of the isolate, *P.* sp. strain JM001, from CDAR sediment was studied in batch kinetic experiments in the presence of various Zn concentrations and different culture media. The effect of Zn on cell growth was monitored by measuring absorbance, analyzing the lag time in presence and absence of Zn, reduction in specific growth rates, and measuring the change in acetate and Zn concentrations with time. The minimum inhibitory concentration (MIC) of Zn in the growth cycle of *P.* sp. strain JM001 was observed to be highly dependent on the composition of culture media and incubation temperature.

Methods

Isolation and Characterization of Strain JM001

Sediments were collected in April 2005, from the metal polluted delta region of Harrison, Idaho (N 47°28'43.8", W 116°43'59.6") near the CDAR. The sediments were collected in ethanol washed PVC (PolyVinyl Chloride) pipes (5.08 cm internal diameter, schedule 20) with removable caps on both ends and transported on ice to the laboratory. A sediment core was taken, thawed, and homogenized. Homogenized sediments were added (5.24±0.28 g of sediment) to autoclaved modified metal toxicity medium [Moberly, 2006] in serum bottles. The bottles were sealed with sterile cotton plugs for aerobic media. Cell concentrations from aerobic batch cultures were diluted 10,000 and 100,000 fold in a sterile 0.89% NaCl solution. 50 µl of this salt solution was plated onto agar plates consisting of modified metal toxicity medium with 15 g agar per liter (Invitrogen). Plates were incubated at 22 ºC. Seven species were isolated from the CDAR sediment [Moberly, 2006]. Single colonies were picked and transferred into serum bottles containing metal toxicity medium and the process was repeated to ensure purity of cultures. The cultures were preserved in a modified metal toxicity medium with 40% glycerol at -80ºC. For further identification, genomic DNA was isolated using Promega Wizard® Genomic DNA purification kit and the 16S rRNA gene was amplified by PCR using two universal bacterial 16S rRNA primers, 8F (5′-AGT TTG ATC CTG GCT CAG-3′) and 1492R (5′-ACC TTG TTA CGA CTT-3′). Amplified 16S rRNA gene was purified with gel filtration cartridges (Performa® DTR Edge Biosystems) as per

BigDye™ protocol which was used as the sequencing template. Sequencing was carried out as described by Moberly [2006] using an ABI 373 automated DNA sequencer at Washington State University's DNA sequencing facility. The determined sequences were compared with 16S rRNA gene sequences obtained from GenBank. A phylogenetic tree was constructed with *Pseudomonas* sp. strain JM001 and its closest matches using phylogenetic analysis using parsimony (PAUP*) version 4.0.b10. Molecular phylogenetic analysis showed that *P.* sp. strain JM001 was closely related to *P. putida* and *P. fluorescens.*

Culture Preparation for
Pseudomonas sp. strain JM001

The isolate *P.* sp. strain JM001 was selected for this study since *Pseudomonas* are known as metal resistant species [Mago et al., 1994, Lloyd et al., 2001, Ramos et al., 2002, Choudhury et al., 2001]. To see the effect of growth media and incubation conditions, cells were grown in the following two media at different Zn concentrations.

Medium A: It is a growth medium described by Moberly [2006]. The medium was prepared by adding 2 ml of mineral solution, and 100 µl of trace metal solution to 1L of basal medium. One liter of basal medium contained 0.246 g CH_3COONa (JT Baker), 0.06 g Na_2SO_4 (Fisher), 0.02 g $NaHCO_3$ (Fisher), 0.004 g NaH_2PO_4 (Fisher), 0.016 g NH_4Cl (Fisher), 0.02 g yeast extract (Difco), and 1.73 g of 1,4-piperazinediethanesulfonic acid (PIPES) (Fisher) buffer. A liter of trace metal solution was composed of 0.006 g $NiCl_2.6H_2O$ (Fisher), 0.3 g $FeCl_3.6H_2O$ (Spectrum), 0.32 g $MnCl_2.4H_2O$ (Fisher), 0.115 g $CoCl_2$ (Fisher), 1.742 g $Na_2MoO_4.2H_2O$ (Fisher), and 0.0004 g $C_6H_6NO_6Na_3.H_2O$

(trisodium nitriloacetic acid monohydrate) (Acros). One liter of mineral solution was made of 0.798 g H_3BO_3 (Merck), 1.05 g $MgCl_2.6H_2O$ (Fisher), 2.75 g $CaCl_2.2H_2O$ (JT Baker), and 0.25 g KCl (Fisher). After mixing these three solutions, the pH was adjusted to 7.0 with 6 N HCl.

Medium B: This is a modified version of minimal salts vitamin medium (MSV) as described by Teitzel et al. [2003] at pH 7.0, which contained (per liter) 1.36 g of CH_3COONa (JT Baker), 1 g of $(NH_4)_2SO_4$ (Fisher), 0.06 g of $MgSO_4.7H_2O$ (Fisher), 0.06 g of $CaCl_2$ (JT Baker), 0.02 g of KH_2PO_4 (Fisher), 0.03 g of $Na_2HPO_4.7H2O$ (Fisher), 2.383 g of HEPES (JT Baker), 1 ml of 10 mM $FeSO_4$ (Fisher), and 1 ml of a trace vitamin solution. The trace vitamin solution contained (per liter) 20 mg of biotin, 20 mg of folic acid, 50 mg of thiamine HCl, 50 mg of D-(+)-calcium pantothenate, 1 mg of vitamin B_{12}, 50 mg of riboflavin, 50 mg of nicotinic acid, 100 mg of pyridoxine HCl, and 50 mg of *p*-aminobenzoic acid.

100 ml of each of the medium was transferred to each 250-ml shaker flask (Pyrex™ Shaker Flasks with Extra-Deep Baffles). The flasks were previously washed with 3% HNO_3. Then the flasks containing media were autoclaved and cooled down to room temperature in a laminar flow hood. No precipitation was found after autoclaving the media. The vitamin solution was filtered through 0.2 µm filters (Fisher) and added aseptically to autoclaved medium B. The inoculum was examined under transmittance microscope prior to inoculation and the cells were found to be motile. 2 ml of *P.* sp. strain JM001 inoculum from its 3rd generation of growth were transferred aseptically to the autoclaved media in flasks. A stock solution of 1 mM of $ZnCl_2$ was filtered through 0.2

μm filters (Fisher) and added to the medium A to give the desired metal concentrations (varied from 0.008 to 0.024 mM). The concentration range was selected by preliminary screening tests (same batch kinetic study done in 96-well plate reader and in 250 ml shaker flasks). After the addition of metal solution and inoculum in medium A, the flasks were incubated at 22°C, 100 rpm. Samples were collected and analyzed for cell growth at an absorbance of 610 nm (medium A), total number of cells (medium A), and aqueous Zn concentrations. For the inoculum in medium B, a stock solution of 50 mM $ZnSO_4$ was sterilized using 0.2 μm sterile filters (Fisher) and added to the medium B varying the concentrations from 0.1 mM to 1mM. The cultures were then incubated at 35°C, 100 rpm. The cell growth in medium B was measured as total cell protein concentration because of initial instant precipitation of Zn in 1mM Zn supplemented medium. For both media, cell free control media supplemented with metal were used as controls. Samples for acetate concentrations were collected at different stages and frozen for analysis at the end of each batch experiment. The aqueous acetate concentrations were measured for each sampling point. Each experiment was carried out in duplicate and repeated for the set of conditions.

Determination of Live/Dead Cell
Counts, Cell and Protein Concentrations

The optical density (O.D.) of each sample in medium A was measured at average 2 h intervals using the Genesys™ 10 Series Spectrophotometer (Thermo Electron Corporation). The total number of cells in medium A was measured using a LIVE/DEAD Baclight bacterial viability kit (Molecular Probes, Eugene, OR). Cells were collected on

0.22 µm membrane filters (Polycarbonate, Black, 25 mm, Osmonic Inc.) using vacuum filtration. The filter was subjected to epifluorescence microscopy (Nikon Eclipse E800) with excitation filters of 425-475 nm and 541-551 nm and emission filters of 600-660 nm and 590 nm. The LIVE/DEAD Baclight bacterial viability kit includes mixtures of the green fluorescent nucleic acid stain SYTO 9 and the red fluorescent nucleic acid stain propidium iodide. The SYTO 9 stain generally labels all bacteria in any population, both live and dead bacteria [Jin et al., 2005; Leuko et al., 2004]. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Damaged cells were counted by their red color and were scored as having damaged membranes (dead) [Boules et al., 1999]. Live cell counts of selected samples were plotted corresponding to their absorbances. The linear correlation (R^2= 0.88) between the live cells and absorbance was used to calculate the cell growth concentration, X in medium A during the 88 h observation period. Cell protein was determined for the growth in medium B at different stages of 65 h using a quantitative colorimetric Coomassie assay method (Pierce, Rockford, Ill.) as described by Sani et al. [Aug., 2001]. The absorbance of protein concentration was measured at 595 nm. The bovine serum albumin (BSA) protein standard curve (R^2= 0.99) was used for calibration.

Comparison of Biomass Concentrations in Two Different Media

To compare the cell and protein concentrations in media A and B respectively, a graph of $X_{max,Zn}/X_{Zn=0}$ versus Zn concentrations was plotted where $X_{max,Zn}$ = maximum

biomass concentrations at any Zn concentration and $X_{Zn=0}$ = biomass concentrations in metal free control. The cell concentrations in medium A and protein concentrations in medium B were considered as biomass concentrations in these two media respectively.

Determination of Acetate and Aqueous Zn Concentrations

Samples for acetate were filtered (IC Millex ®-LG; pore diameter, 0.2 µm), and concentrations were determined using a Dionex Ion Chromatograph (DX-500) equipped with IonPac AS11 Column (4 mm×25 cm), Ultra II suppressor, and a conductivity detector (CD-20). Elution was carried out using a potassium tetraborate gradient (3.5 to 100 mM). Samples for aqueous Zn concentrations were prepared by filtering through 0.2 µm pore-size membrane filter (Fisher), and determined using a quantitative colorimetric method (Zincon, Hach Company, CO) modified to reduce the required sample volume. The absorbance was measured at 620 nm and compared to a standard curve generated from known concentrations of Zn. This method gave a detection limit of 1.376×10^{-4} mM.

Calculation of Specific Growth Rate and Kinetic Parameters

The specific growth rate was calculated from equation (3.2):

$$X = X_{0,\,exp}e^{\mu t} \qquad (3.1)$$

$$\text{Or, } \mu = \left(\frac{1}{t}\right)\ln\left(\frac{X}{X_{0,exp}}\right) \qquad (3.2)$$

where X is the cell (or protein) concentration at time,t , $X_{0,\,exp}$ is the cell (or protein) concentrations at the beginning of exponential growth, and µ is the specific growth rate.

The specific growth rate was a time dependent parameter. So, the specific growth rate at different time during the exponential growth phase of each sample was first calculated using Equation (3.2) and then the average of these specific growth rates was considered as μ.

For determination of maximum specific growth rates, $\frac{1}{\mu}$ vs. $\frac{1}{S}$ (S is the acetate concentration) curve (Lineweaver-Burke plot) were drawn for each case. The reciprocal of the intercept $\left(\frac{1}{\mu_{\max}}\right)$ from these curves gave the maximum specific growth rate, $\mu_{\max}$ and the slope $\left(\frac{K_S}{\mu_{\max}}\right)$ gave the Michaelis-Menten constant, K_S for acetate.

The percent inhibition in specific growth rate was calculated by Equation (3.3):

$$\text{Inhibition (\%)} = 100 \times \left(1 - \frac{\mu_{\max .sample}}{\mu_{\max, mfc}}\right) \qquad (3.3)$$

where, $\mu_{max,sample}$ is the maximum specific growth rate for a given concentration and $\mu_{max,mfc}$ is the maximum specific growth rate for the metal free control.

The Lineweaver-Burke curves for all cases had R^2>0.98. The yield, Y was calculated by Equation (3.4):

$$Y = -\left(\frac{X - X_0}{S - S_0}\right) \qquad (3.4)$$

where, X and S are the concentrations of cells (or proteins) and acetate, respectively, at any time, t. X_0 and S_0 are the concentrations of cells (or proteins) and acetate at t =0.

Results

To determine the phylogenetic relationship between the bacterium and the known bacteria, a phylogenetic tree was constructed comparing the nucleotide sequence of the bacterium with available 16S rRNA sequences provided by the National Center for Biotechnology Information (NCBI) GenBank (Figure 3.1). The tree clearly shows that *Pseudomonas* sp. strain JM001 is closely related to the two species, *P. putida* and *P. fluorescens.*

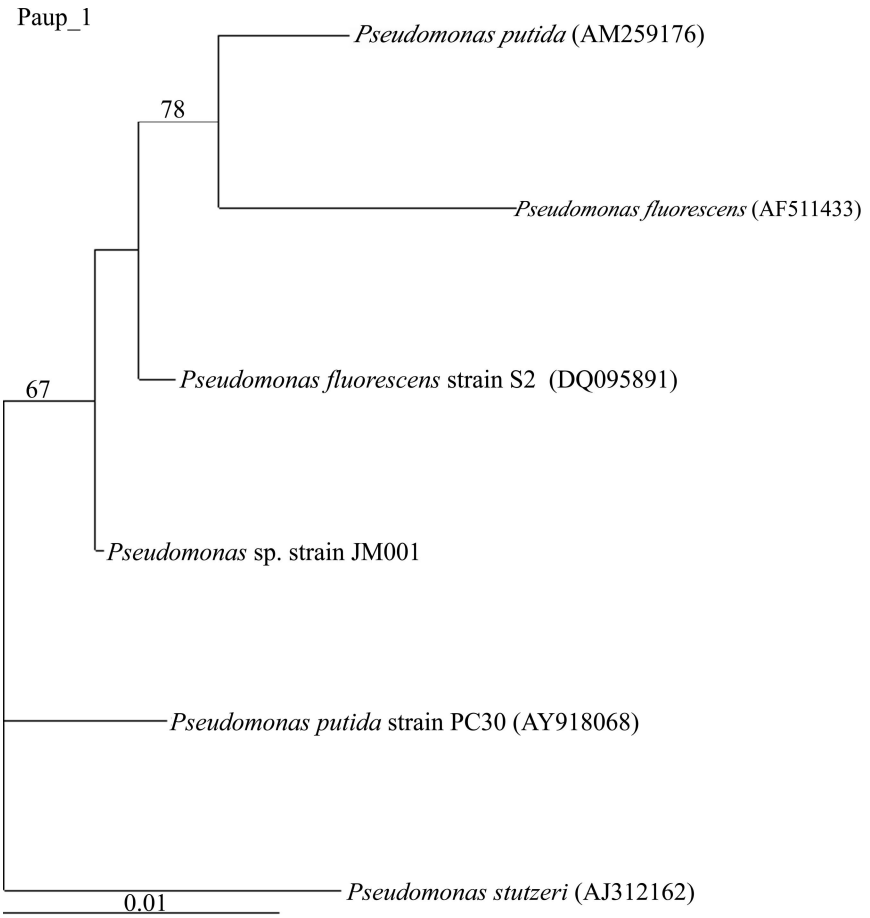
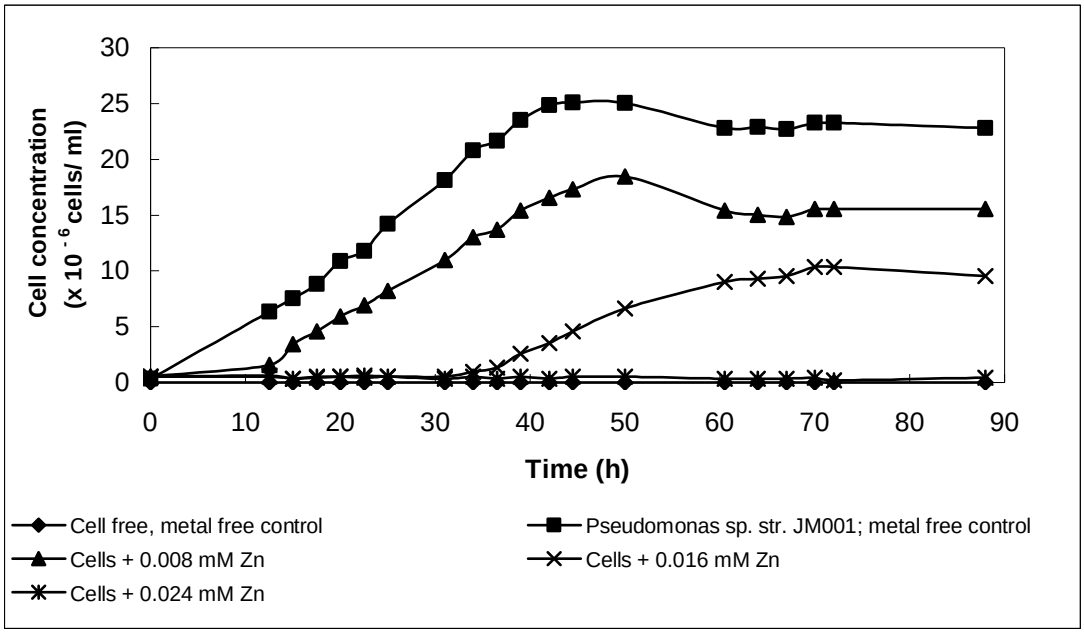
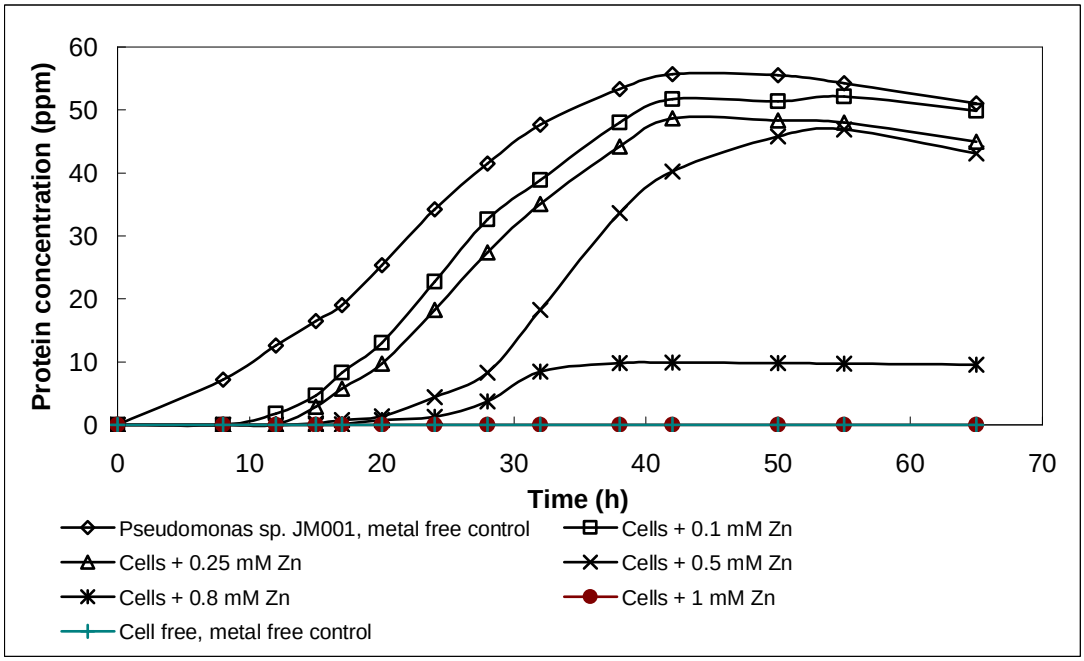


Figure 3.1. Neighbor-joining tree (unrooted) derived from NCBI's GenBank data and other related species showing closest matches of *Pseudomonas* sp. strain JM001 isolated from CDAR sediment. Bootstrap values greater than 50% are shown. The scale bar corresponds to 1% nucleotide sequence difference.

Assessment of Zn toxicity was quantified as the inhibition of *P*. sp. strain JM001 growth based on cell concentration in medium A, total protein concentration in medium B, lag phase, and the reduction in specific growth rates at different Zn concentrations. Growth profiles of *P*. sp. strain JM001 in both media at various Zn concentrations are shown in Figure 3.2. Growth was completely inhibited at 0.024 mM Zn in medium A (Figure 3.2 (i)), and 1 mM Zn in medium B (Figure 3.2 (ii)). The differences in composition of the growth media and incubation conditions significantly affect the minimum inhibitory concentration (MIC) of Zn to the cell growth. As shown in Figure 3.2, in the presence of Zn, cell concentration increases with time until the Zn concentration reaches the MICs. The final cell concentrations were always lower than the metal free control. No lag phase was observed for the Zn free control, but lag times increased as toxicity of Zn increased. Comparatively shorter lag phase was found in medium B where the cells showed more resistant to Zn than in medium A. The lag phase of 12 h was observed for 0.008 mM Zn in medium A and 0.1 mM Zn in medium B. It can be concluded that the toxicity of Zn to *P*. sp. strain JM001 in terms of cell (or protein) concentrations and lag times are dependent on combination of media composition and incubation temperatures.



(i)



(ii)

Figure 3.2. Effects of Zn on the growth of *Pseudomonas* sp. strain JM001 in (i) medium A at 22 ºC; the cell concentration was measured in terms of absorbance at 610 nm, and (ii) medium B at 35 ºC; the total cell protein was measured at absorbance 595 nm. Data points are averages of duplicates. Error bars are not shown because they are smaller than the symbols.

Comparison of relative biomass concentrations in two different media (Figure 3.3) shows that the relative biomass was higher in medium B even at higher Zn concentrations. In medium B, acetate concentration was three times higher than in medium A that might help the cells to grow providing more C substrates to them. Also cells in medium B were incubated at higher temperature than in medium A. It is seen that the combination of medium type and growth temperature can alter the cell growth rate and metal sensitivity behavior of the cells.

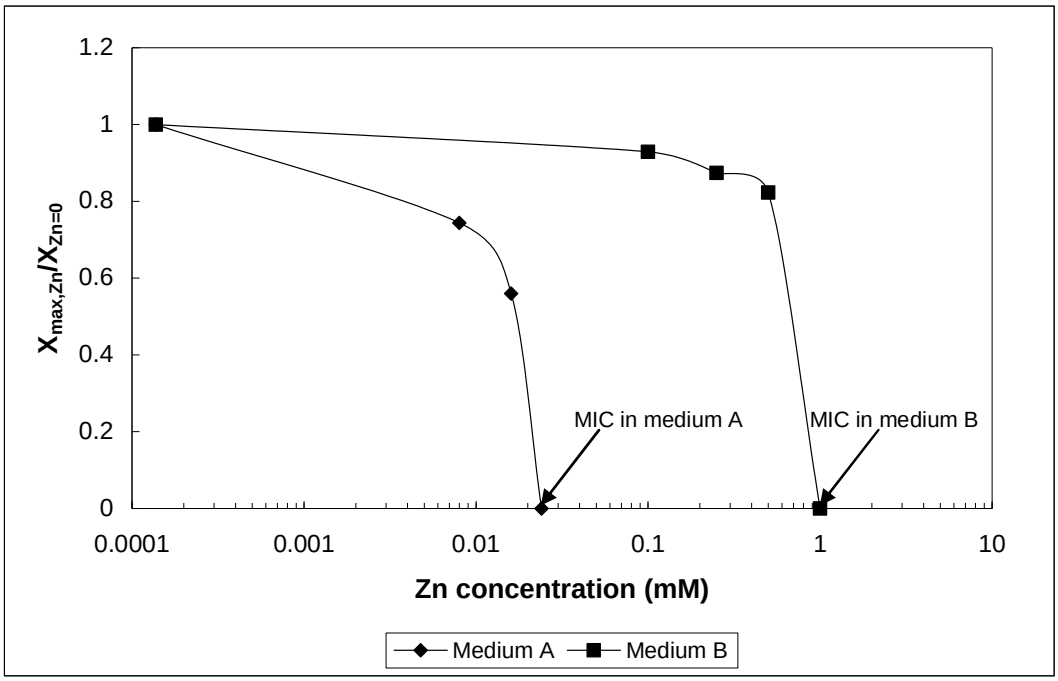
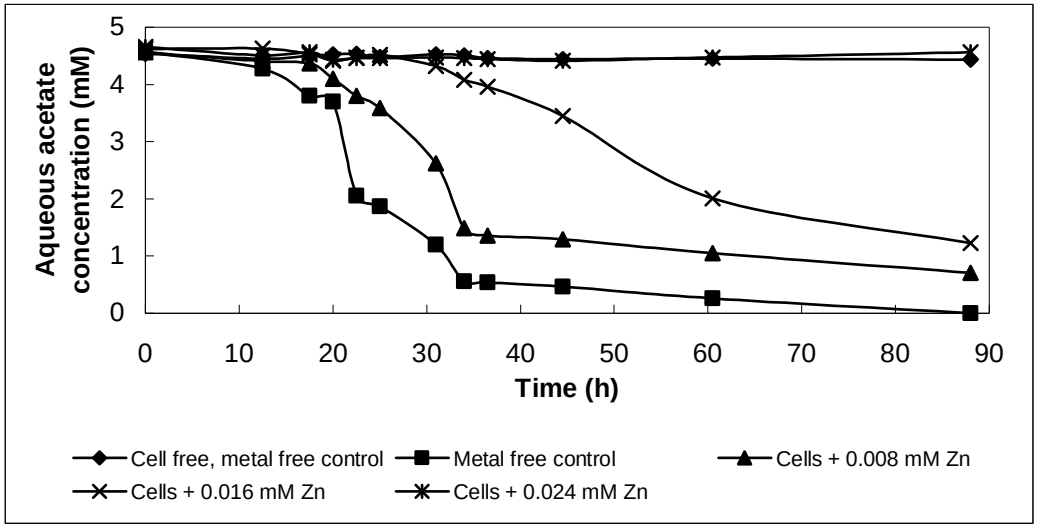


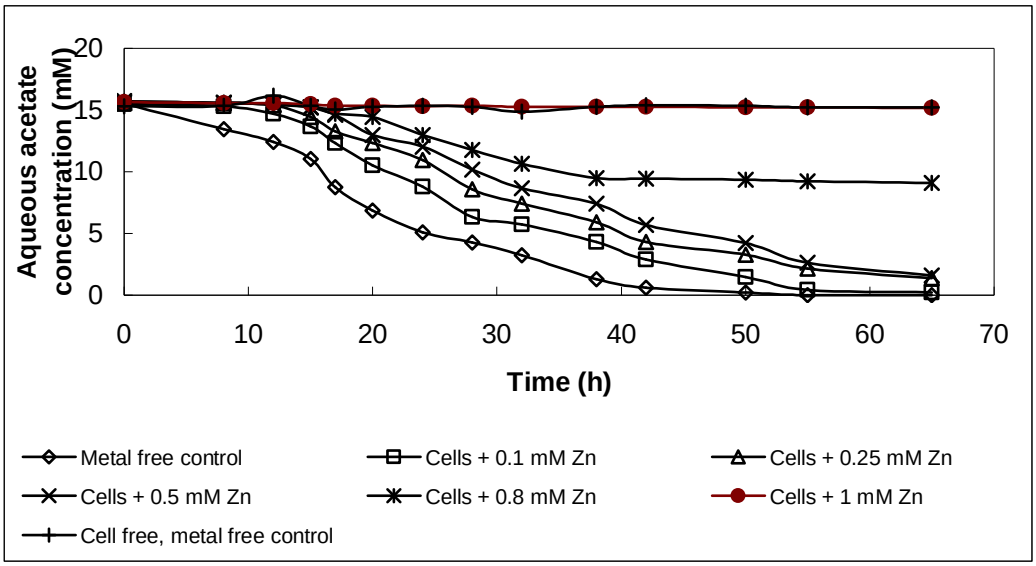
Figure 3.3. Comparison of relative biomass concentrations of *Pseudomonas* sp. strain JM001 in medium A at 22 °C and medium B at 35 °C in the presence of various Zn concentrations. $X_{max,Zn}$ = Maximum biomass concentrations at any Zn concentration and $X_{Zn=0}$ = biomass concentrations in metal free control. Biomass concentrations were measured as cell and protein concentrations in media A and B respectively. Data points are averages of duplicates.

Figure 3.4 (i) shows the decrease in acetate concentrations with time during the cell growth in medium A treated with different Zn concentrations. The acetate

concentrations decreased earliest for the metal free control, and showed a sharp decline in acetate concentration from 12.5 to 34 h. The acetate concentration reached zero between 60 and 88 h. In the presence of 0.008 and 0.016 mM Zn acetate concentrations decreased after a longer lag time than the Zn-free control, indicating active utilization of acetate. Similarly, in medium B (Figure 3.4 (ii)), the consumption of acetate was slower in the presence of Zn than in the metal free control. All the acetate was used by the cells when there was no metal present despite the fact that the acetate concentration was 3 times higher in this medium than in medium A. 76.3 % of the initial acetate was utilized by the cells for their growth in presence of 0.1 mM Zn while only 41.8% was comsumed in presence of 0.8 mM Zn. No change in acetate concentrations was observed for cell free controls.

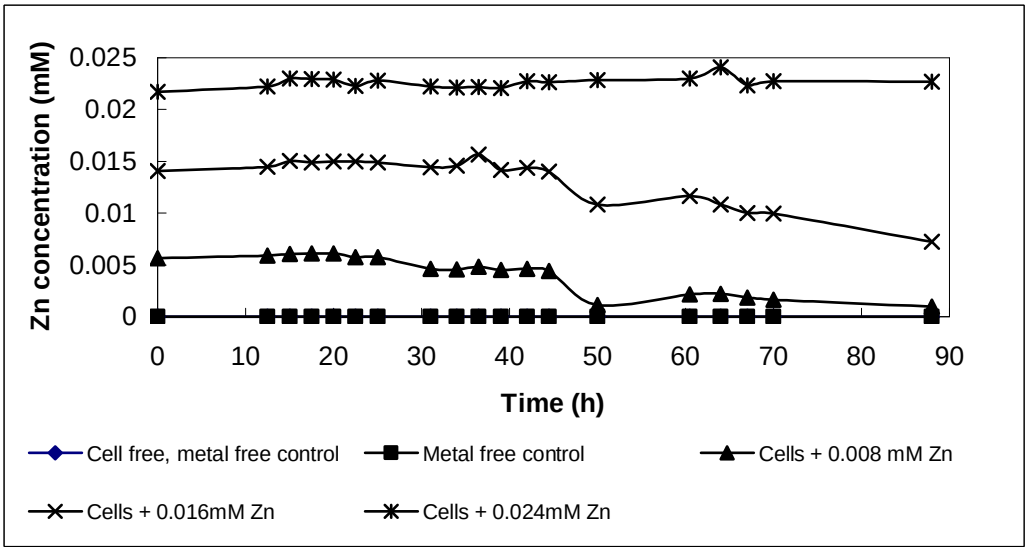


(i)

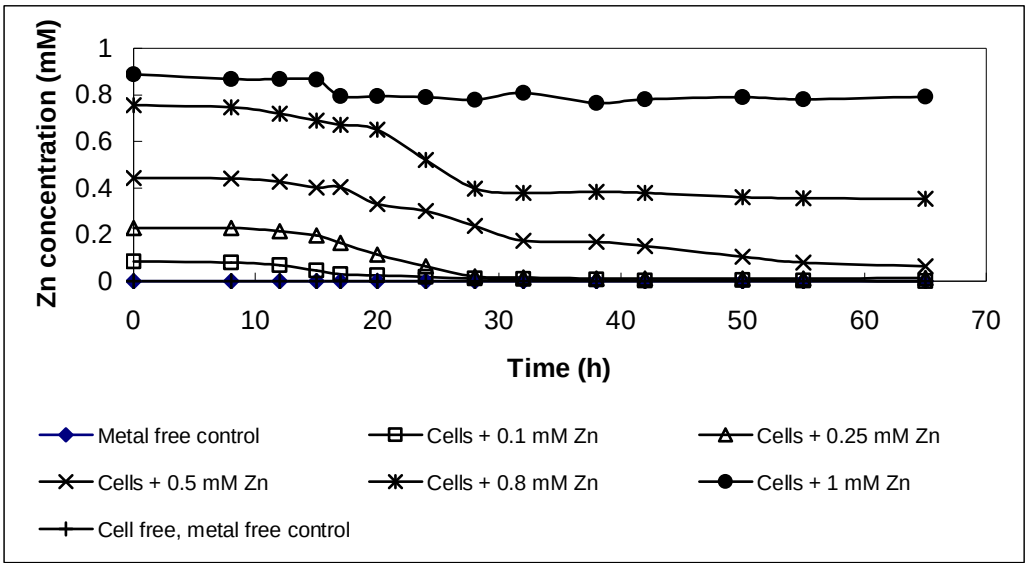


(ii)

Figure 3.4. Aqueous acetate concentrations during the growth of *Pseudomonas* sp. strain JM001 in presence of Zn in (i) medium A, and (ii) medium B.



(i)



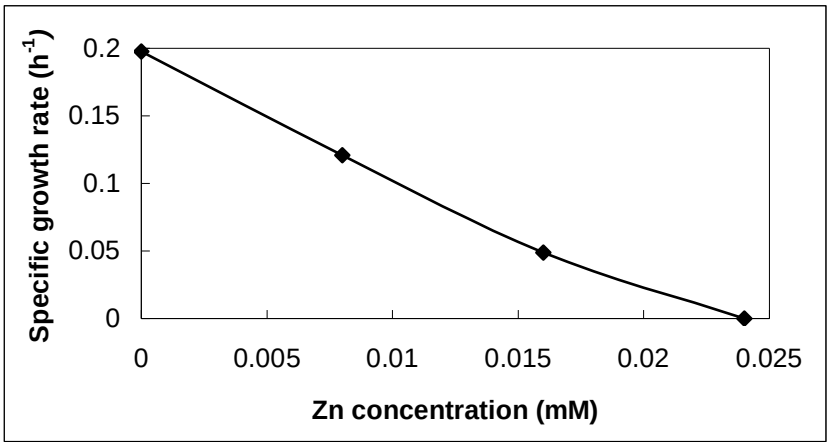
(ii)

Figure 3.5. Aqueous Zn concentrations during the growth of *Pseudomonas* sp. strain JM001 for (i) 0 to 0.024 mM Zn in medium A, and (ii) 0 to 0.1 mM Zn in medium B. The points are the averages of duplicates, and error bars are not shown since they are smaller than the symbols.

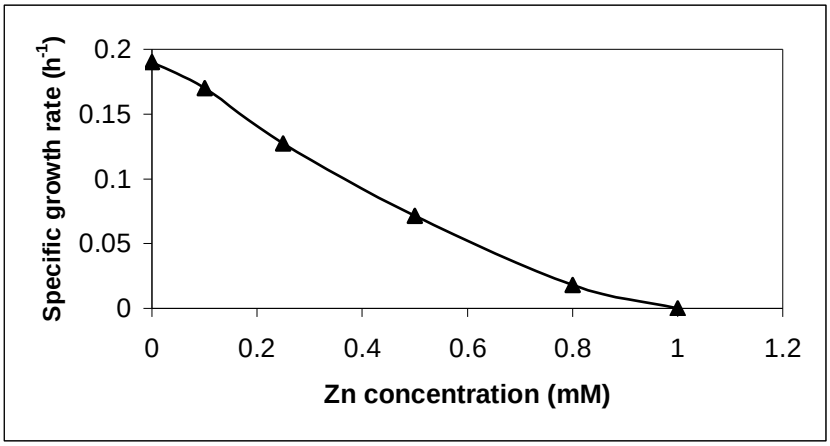
The aqueous Zn concentrations (Figure 3.5 (i)) for 0.008 mM Zn in medium A decreased slightly at 25 h (mid-exponential phase in cell growth) and then decreased to zero after 44.5 h (end of exponential phase). In the same medium, final Zn concentration reached 0.007 mM for 0.016 mM initial Zn concentration. The concentrations decreased from 44.5 h which was the mid-exponential phase of the cell growth in presence of 0.016 mM initial Zn. Zn concentrations remained constant for initial Zn concentration of 0.024 mM (Figure 3.5 (i)). At time, t = 0, there was approximately 0.002 mM difference between the measured and target Zn concentrations for each sample. No precipitation in the flasks or decrease in Zn concentrations in the cell free control media (data are not shown) was found at the end of the experiment. In medium B, there was also an average of 0.02 mM difference in initial Zn concentrations observed (Figure 3.5 (ii)) as it was

found in medium A. There were no significant differences in initial concentrations between the target and measured Zn concentraions for the cell free controls (data are not shown) except for 1mM Zn. In the flasks of 1mM Zn, precipitation was observed as soon as the filtered Zn was added to the inoculated media. The concentrations of Zn up to 0.8 mM decreased with time and cell growth where most of the metals were removed in the late exponential growth phase. Zn was completely removed at 0.1 and 0.25 mM concentrations. In case of 0.5 and 0.8 mM Zn, 85.5% and 53.2% of the initial concentrations were removed.

Zn inhibitions in the specific growth rate are shown in Figure 3.6. Specific growth rate was highest for the metal free control and decreased with the addition of Zn to the media.



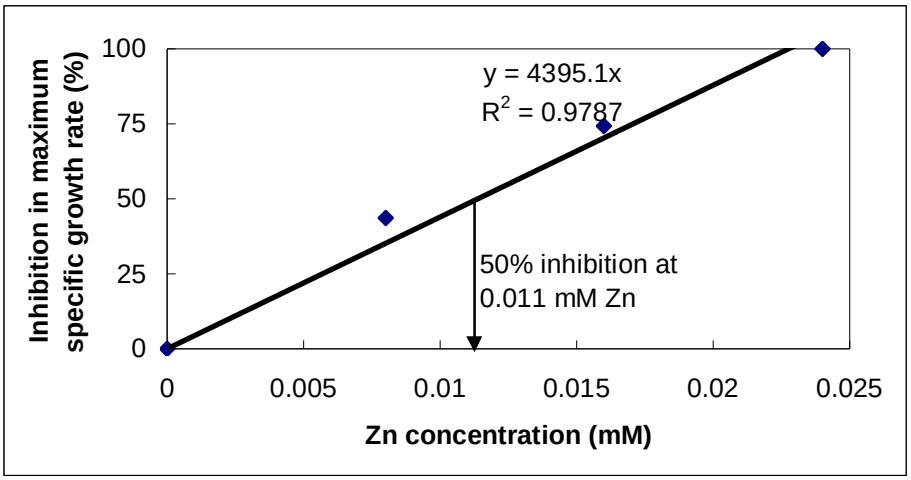
(i)



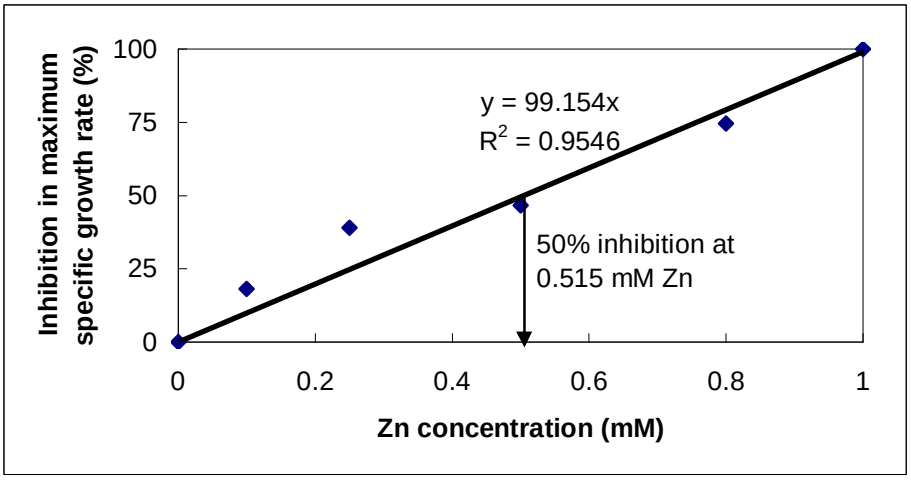
(ii)

Figure 3.6. Specific growth rates of *Pseudomonas* sp. strain JM001 in (i) medium A, and (ii) medium B as a function of Zn concentrations. Data points are averages of duplicates, and error bars are not shown because they are smaller in size than the symbols.

To quantify Zn toxicity, the inhibition in maximum specific growth rate at different Zn concentrations was calculated and plotted as shown in Figure 3.7. A least-square line was drawn (R^2 =0.978 and 0.954 in (i) and (ii) repectively) and used to estimate 50% inhibition in specific growth rate as 0.011 mM and 0.515 mM Zn in medium A and B respectively. The concentration was about 40 times higher in medium B than in A.



(i)



(ii)

Figure 3.7. Inhibition of maximum specific growth rate of *Pseudomonas* sp. strain JM001 as a function of Zn concentration in (i) medium A, and (ii) medium B.

The kinetic parameters, μ_{max}, K_S and the yield, Y are shown in Table 3.1. Obviously, lower specific growth rate, μ, μ_{max}, K_S, and Y are found at higher Zn concentrations in each medium. The specific growth rate for metal free controls was almost the same in both media but the maximum specific growth rate for metal free controls was higher in medium B than in medium A. Even at higher Zn concentrations in

medium B, μ, μ_{max}, and K_S showed higher values than the cells in medium A with less Zn.

Table 3.1. Summary of kinetic parameters for Zn inhibited growth of *P.* sp. strain JM001.

Media	Zn	μ	μ_{max}	K_S	Y
	mM	h^{-1}	h^{-1}	mM	cells/mmoles acetate
Medium A	0	0.197	0.284	1.40	4.91×10^9
	0.008	0.120	0.161	1.40	3.88×10^9
	0.016	0.048	0.074	1.27	2.66×10^9
	0.024	0	0	0	0
	mM	h^{-1}	h^{-1}	mM	mg protein/mmoles acetate
Medium B	0	0.190	0.325	4.69	3.29
	0.1	0.170	0.266	4.39	3.26
	0.25	0.127	0.198	3.54	3.14
	0.5	0.071	0.173	3.48	3.04
	0.8	0.018	0.083	0.21	1.46
	1.0	0	0	0	0

Zn = Zinc concentration, μ= Specific growth rate from experiment, μ_{max}= Maximum specific growth rate from Lineweaver-Burke plot, K_S = Michaelis-Menten constant for acetate from Lineweaver-Burke plot, Y = Yield

Discussion

Pseudomonas sp. strain JM001 cells, isolated from metal polluted CDAR sediment, showed less cell growth rate and more Zn toxicity in growth medium A than in medium B [Figure 3.3]. It is found from the results of this study that Zn toxicity and cell growth rate depends on the combination of media composition, substrate concentration, and incubation temperature. Bacterial cell growth and cell division in aquatic systems is

highly dependent on the availability of phosphate, substrate C and mineral nutrients [Giorgio et al., 1998]. Medium B contained the vitamin solution, more phosphate and acetate than the nutrient limited medium A. The approximately four times higher acetate concentration used in medium B increased the maximum specific growth rate in this medium [Table 3.1]. A study of growth of fungi from metal contaminated soils by Fomina et al. [2003] showed a decrease in Cd and Cu toxicity to the soil fungi with increasing concentration of available carbon source. Fungal branch formation utilizes all the substrates that were explored by the hyphal extension in presence of the metals.

Zn can be removed from the media by chelation to various components of the media, formation of complexes with other compounds in the media or binding of metal to the microorganisms. Phosphate ions show high ability to bind divalent cations [Fernandez-Pifias, 1991]. Zn may bind with phosphate in medium B and form $Zn_3(PO_4)_2$ or $Zn_3(PO_4)_2.4H_2O$ complex which showed more Zn resistant behavior of cells in medium B. A number of researchers have proposed that metals are toxic to the planktonic cells in the free cationic form and compounds capable of reducing free ion activity reduce the metal toxicity [Trevors et al., 1986; Vymazal, 1987]. Also higher biomass concentrations in medium B might provide more Zn binding sites in the cell cultures [Fernandez-Pifias, 1991]. Cells might compete with the components in the medium for Zn binding sites. In medium B, Zn can form more metal complexes and help the cells to grow in higher acetate concentrations compared to that in medium A. The cells then provide more Zn binding sites for Zn and thus show less toxic to Zn in medium B. The MIC values and 50% inhibition in specific growth rate of *P*. sp. strain JM001 are

compared with other *Pseudomonas* species grown in different media and substrate in Table 3.2.

Table 3.2. Comparison of MIC values of different *Pseudomonas* species grown in presence of Zn in different media and incubation conditions.

Species	MIC (mM)	50% inhibition (mM)	pH	T (ºC)	Metal solution used	Medium	Substrate	Reference
Pseudomonas sp. strain JM001	0.024	0.011	7	22	$ZnCl_2$	Medium A: Nutrient limited	Acetate	This study
Pseudomonas sp. strain JM001	1	0.515	7	35	$ZnSO_4$. $7H_2O$	Medium B: Minimal salts with vitamin	Acetate	This study
Pseudomonas sp. PLK5	1.53	0.301	6	20	$ZnSO_4$. $7H_2O$	Nutrient broth	Glucose	Nweke et al., 2006
Pseudomonas putida CZ1	3 to 5	-	5	30	$Zn(NO_3)_2.6H_2O$	Nutrient and mineral salts	Glucose	Chen et al., 2006
Pseudomonas aeruginosa	8	-	6.5	37	$ZnSO_4$. $7H_2O$	Minimal salts vitamin solution	Glucose	Teitzel et al., 2003
Pseudomonas gladioli	10	-	7	27	$ZnSO_4$. $7H_2O$	Nutrient agar	-	Piotrowska-Seget et al., 2005

Previous studies [Chen et al., 2006; Nweke et al., 2006; Teitzel et al., 2003] show relatively higher MIC for *Pseudomonas* when it is grown on glucose substrate at higher temperatures. This may be because yields on sugars are higher than organic acids [Narang et al., 1997] and the affinity of microorganisms for substrate increases as temperature reaches the optimum temperature for growth [Nedwell, 1999]. The growth rate of bacteria decline if temperatures move away from the optima for each type of bacteria in laboratory studies [Payne et al., 1978]. Uslu et al. [2006] showed that biosorption kinetics and equilibrium uptake of Pb and Cu by *P. putida* was affected by temperature. The highest Cu ion uptake by *P. putida* was exhibited at 30 ºC. Optimum

temperature of growth is required because, below the optimum temperature, stiffening of the lipids of the membrane lead to decreased efficiency of transport proteins embedded in the membrane [Nedwell, 1999]. The effect of decreased substrate affinity at low temperature may have profound implications on the availability of substrates in CDAR environment with the seasonal temperature change. It might happen that during the winter and early spring, the *Pseudomonas* isolate are unable to sequester substrates from their environment because of less affinity to substrate at low temperature, thereby showing more sensitivity to Zn. In the summer time, when the temperature of the lake water rises, the isolate may become more resistant to Zn depending on the quality and quantity of the carbon substrates. The mean total organic carbon in surface and subsurface of LCDA sediments are 25 and 21 mg/g sediment respectively [Horowitz et al., 1995]. Analysis of CDAR water and pore water of the collected sample in April, 2005 showed the presence of 170 and 1.6 ppm of total organic carbon [Moberly, 2006], and 10 mg phosphorus/g dry sediment as P_2O_5. *Pseudomonas* in the trace element rich CDAR sediments [Horowitz et al., 1995] can use multiple organic C-substrates simultaneously and may become metal sensitive or resistant depending on other available nutrients and its environment.

There was a small concentration difference between the measured and target Zn concentrations [Fig. 3.5] at time, t = 0 in both media. The Zn concentrations did not change with time for the cell free control media (data are not shown). Also no precipitation was found in the flasks for Zn concentrations less than 1 mM in medium B. So the most probable mechanisms of biotic Zn removal are accumulation and biosorption

on the cell surface. Divalent metal accumulation and sequestration at the *Pseudomonas* cell surface has been reported by several researchers [Chen et al., 2006; Mago et al., 1994; Roane et al., 1999; Sandrin et al., 2003; Teitzel et al., 2003]. *Pseudomonas marginalis*, a Pb resistant bacterium, was isolated from metal contaminated soils of Silver Valley region in northern Idaho by Roane [1999]. It showed extracellular Pb exclusion in presence of high Pb concentration (MIC 2.5 mM) at pH 7.32. *P*. H1 accumulates Cd intracellularly and thus reduces bioavailable metal concentrations via sequestration [Sandrin et al., 2003]. Biosorption of Zn to *Pseudomonas* involves not only surface binding but also intracellular uptake and storage via active cationic transport systems [Chen et al., 2006]. Carboxyl groups in *P. putida* CZ1 bind Cu^{2+} from aqueous solutions [Chen et al., 2007]. Zn^{2+} uptake in *P*. sp. strain UDG26 involves the synthesis of protein(s) that regulates net accumulation [Mago et al., 1994]. The *Zur* protein is involved in the regulation of Zn uptake in *P. putida* [Cánovas et al., 2003].

Nies D. H. [1992] reported that Zn resistance includes only complexation and active efflux for Zn^{2+} since NAD(P)H dependent reduction of the divalent metal cations is not energetically favored. The efflux mechanism of Zn^{2+} in metal-resistant *P. putida* strain S4 studied by Choudhury et al. [2001] showed that it did not eject Zn^{2+}out of the cell but stored them in the outer membrane and periplasm, which provided the binding sites. Though efflux is an important mechanism of bacterial metal resistance, Zn sensitive *P*. sp. strain UDG86 lacks efflux of metal [Mago et al., 1994]. From these above discussions, it can be said that depending on the water temperatures and nutrient availability, *P*. sp. strain JM001 in CDAR environment can remove Zn from sediment

and water via uptake and adsorption to the extracellular surface by regulating the proteins in their cell membrane. Periplasmic sequestration of Cu was observed in *P. putida* strain S4 by Saxena et al. [2002]. They said that the effluxed Cu was not thrown out of the cell, but remained in a bound form to a protein in the periplasm. Thus, in a similar way, a balance between the intracellular level (to fulfill the metabolic requirements), and the periplasmic sequestration (to evade toxicity) of Zn could be maintained by *P*. sp. strain JM001 inhabiting the metal contaminated lake.

The Michaelis-Menten constant for acetate, K_S, in medium B as shown in Table 3.1 is close to the value of Michaelis-Menten constant, K_m= 4.62 mM, for *P*. sp. strain UDG86 grown in succinate medium in the presence of 0.1 mM of Zn as shown by Mago et al. [1994]. The results of the kinetics of Zn inhibited cell growth in two different media with single substrate showed that the combination of nutritional composition and temperature of the media of *P*. sp. strain JM001 has a profound effect on its sensitivity to metals. The kinetic parameters are not constants but can vary depending on growth conditions. The cells can grow on a wide variety of substrates in CDAR sediments where there is a continuous influx of trace metals from CDAR source [Harrington et al., 1998]. The results from this study will be used to develop a dose-response kinetic model. It is important to know the response of the microbial community to metal toxicity in the river. Moreover, biofilm and planktonic cells have distinct metal toxicity behavior which is important to understand the microbial ecology of heavy metal-affected environments [Teitzel et al., 2003]. The rate of metal consumption by microbes depends on the total microbial concentration, their distribution in sediments, transport through the porous

media, availability and concentration of substrate, electron donor and acceptor, growth yield of the biomass and the minerals present in the system [Liu et al., 2002]. In the batch study, the nutrients for microbial growth are readily available to every microbial cell, whereas in soil microcosms the availability of nutrients and oxygen (or other electron acceptors) is subject to convective mass transfer limitation [Zhang et al., 1995]. In addition, oxygen limited conditions can exist in biofilms due to mass transfer effect and as a result of oxygen consumption by other aerobic microorganisms [Chen, 1996; Patel et al., 1991]. Observation of metal inhibited cell growth in multiple substrates will be helpful to demonstrate the growth kinetics in multiple nutrient controlled growths as found in a natural river. To better understand the temperature effect on the cell growth rate, a batch experiment in medum B at 22 ºC should be done. Electron microscopy and quantitative analysis of the cells should be done at the end of the metal inhibition growth to understand the mechanism of their toxic metal resistance.

Conclusion

It is seen from the batch kinetic experiments that the metal inhibition growth of *Pseudomonas* sp. strain JM001 was highly dependent on the combination of growth media composition and temperature. The growth was completely inhibited in the presence of 0.024 mM Zn in a nutrient limited medium at 22 ºC and at 1 mM Zn in a vitamin solution supplemented nutrient medium at 35 ºC. Though the mechanism of Zn resistance behavior in the isolate was not verified, the results of this study show the potential applicability of an isolate from CDAR sediment in the treatment of heavy metal

containing solutions or soil. The kinetic parameters obtained here are important to understand the kinetics of metal inhibited microbial growth in presence of a single substrate and metal-microbe interaction in a complex environment such as CDAR. Further studies into the mechanisms and reactions involved in Zn inhibition, effect of different substrates, and batch studies with mixed consortia in the presence of a single metal or multiple metals would fundamentally improve the understanding of metal removal processes in the CDAR environment.

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CHAPTER FOUR

METAL RESISTANCE BEHAVIOR OF *PSEUDOMONAS* SP. STRAIN JM001 FLOCS IN 96-WELL CELL CULTURE - A HYPOTHESIS

To study the effects of Pb, Zn, and Cu individually or in combination to *Pseudomonas* sp. strain JM001, an experiment was done in 300 µl of liquid media in a 96-well cell culture (Corning Inc., NY) without shaking. *Pseudomonas* cells were grown in an aerobic modified metal toxicity medium (MTMA) [Sani et al., 2001]. One liter of MTMA contained 0.41 g CH_3COONa (JT Baker), 0.83 g $MgCl_2$ (Fisher), 0.06 g $CaCl_2.2H_2O$ (JT Baker), 1 g NH_4Cl (Fisher), 0.05 g yeast extract (Difco), 0.5 g of tryptone (Fisher), and 10.93 g of PIPES (Fisher) buffer. The pH of the medium was adjusted to 7.0 and then autoclaved. After autoclaving, the media was cooled to room temperature (22°C) in a laminar flow hood. Then the medium was transferred into the wells of the 96-well cell culture cluster in such a way so that the total volume of solution would be 300 µl. 6 µl of *P*. sp. strain JM001 inoculum from its 3rd generation of growth were transferred aseptically to the autoclaved media in wells. A stock solution of 1 mM of $ZnCl_2$, $PbCl_2$, and $CuSO_4$ was filtered through 0.2 µm filters (Fisher) and added to the solution in the wells to give the metal concentrations of 0.01-0.2 mM Pb, 0.002-0.06 mM Zn, 0.001-0.004 mM Cu or in combination of any two metals. The concentration range was selected arbitrarily. After adding metal solution and inoculum, the cluster plate was put on the plate of the spectrophotometer (Multiskan® Spectrum, Thermo Electron Corporation, Finland). A kinetic loop was generated in the software of the

spectrophotometer so that the cell growth (absorbance at 610 nm) could be measured automatically every 2 h for a 58 h time period.

Results from this experiment showed that the growth of the culture increased with time in the presence of Pb, Zn and Cu whereas planktonic cells of the same organism in batch kinetic experiment showed its growth only in presence of Zn (chapter three). The heavy metal toxicity trend was seen as Pb< Zn< Cu. The absorbance of the cell culture was measured in the presence of 0.01-0.2 mM Pb (Figure 4.1), 0.002-0.06 mM Zn (Figure 4.2), 0.001-0.004 mM Cu (Figure 4.3), and in combination of Pb-Cu (Figure 4.4) and Cu-Zn (Figure 4.5). The minimum inhibitory concentration (MIC) of Pb was estimated to be 0.2 mM. The lag phase was 12 h in presence of all Pb concentrations. After 12 h, the cultures grew exponentially. In the presence of Zn, the cultures showed toxicity at 0.06 mM Zn with a 10 h lag phase. Cu was highly toxic to the cell culture; the MIC for Cu was 0.004 mM. The lag phase for Cu was the same as Zn. For Pb-Cu combination, the Cu concentrations used were 100 times less than the Pb concentrations because Cu was shown to be more toxic to the culture (Figure 4.4). For Cu-Zn combination, the concentrations of both metals were varied from 0.002-0.004 mM (Figure 4.5). Though MIC for Zn was 0.06 mM, the cells could not grow in the presence of both Cu and Zn concentrations $\geq$ 0.004 mM

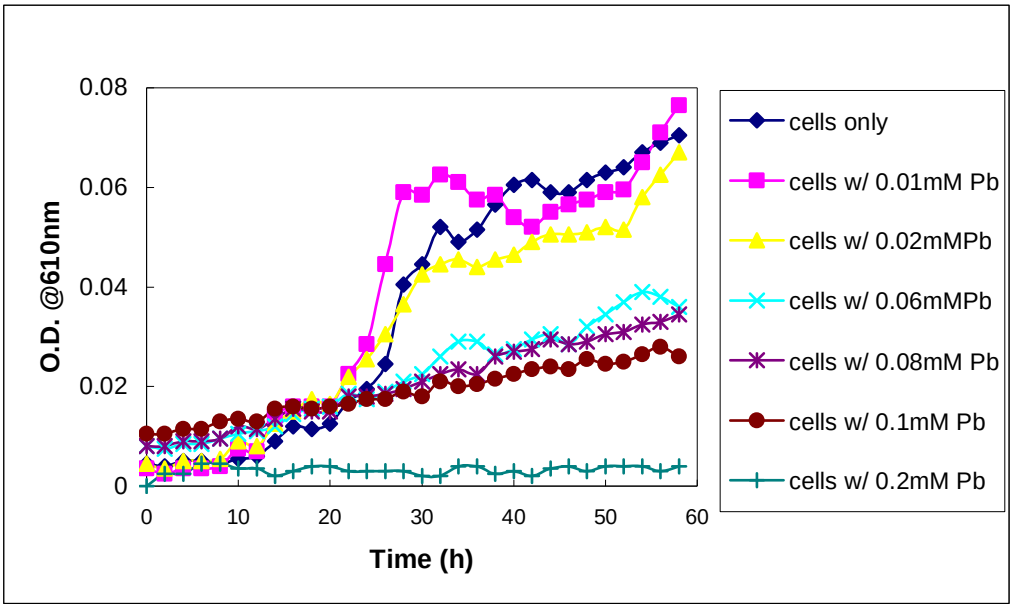

Figure 4.1. Effect of Pb on the growth of *Pseudomonas* sp. strain JM001 in the 96-well cell culture. The cell concentration was measured in terms of absorbance at 610 nm. The points are the averages of duplicates.

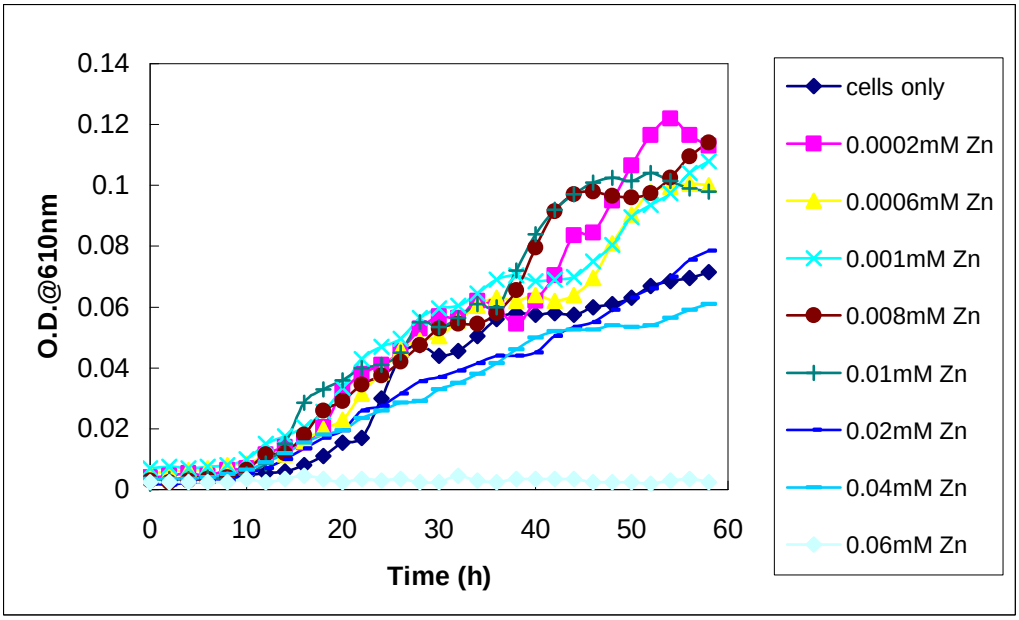

Figure 4.2. Effect of Zn on the growth of *Pseudomonas* sp. strain JM001 in 96-well cell culture cluster. The cell concentration, M was measured in terms of absorbance at 610 nm. The points are the averages of duplicates.

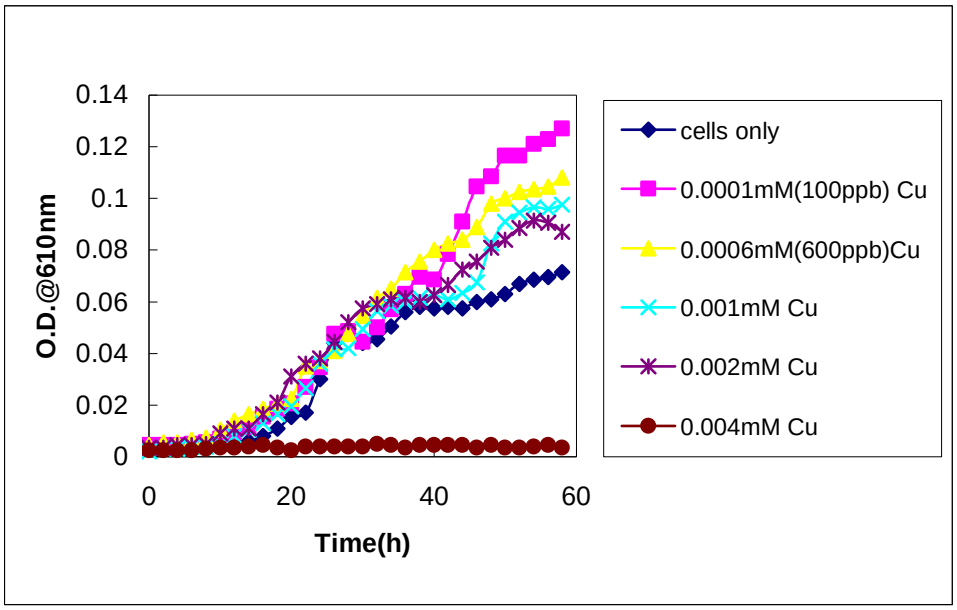

Figure 4.3. Effect of Cu on the growth of *Pseudomonas* sp. strain JM001 in the 96-well cell culture cluster. The cell concentration, M was measured in terms of absorbance at 610 nm. The points are the averages of duplicates.

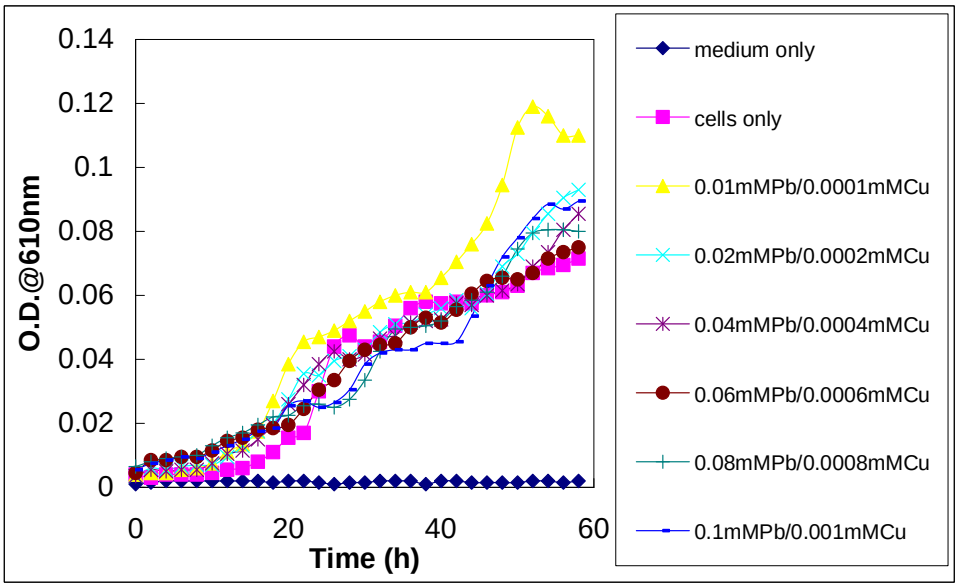

Figure 4.4. Effect of both Pb and Cu on the growth of *Pseudomonas* sp. strain JM001 in 96-well cell culture cluster. The cell concentration, M was measured in terms of absorbance at 610 nm. The points are the averages of duplicates.

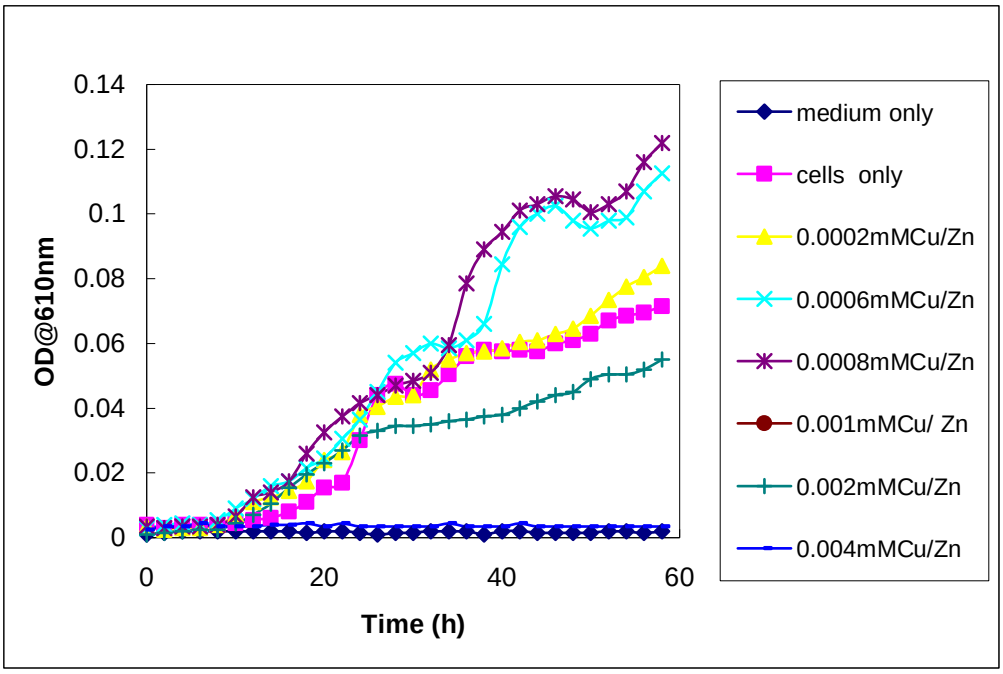

Figure 4.5. Effect of both Cu and Zn on the growth of *Pseudomonas* sp. strain JM001 in 96-well cell culture cluster. The cell concentration, M was measured in terms of absorbance at 610 nm. The points are the averages of duplicates.

Comparing these results with the results of planktonic cells (chapter three), it can be hypothesized that since shaking was absent in the plate reader, most of the inoculated cells settled down to the bottom of the wells rather than remaining in the aqueous phase. The cells may have formed flocs adherent to each other and to the surfaces or interfaces of the wells. As a result, flocs associated with *P*. sp. strain JM001 showed more resistance to Pb, Zn, and Cu than planctonic cells. It is seen that the flocs grew even in the presence of dual combination of metals while planktonic cells grew only in presence of less than 0.024 mM Zn (chapter three).

In this small volume experiment, it is anticipated that flocs may have helped cells be more resistant to Pb, Zn, and Cu. The formation of flocs may have enhanced the cell resistance to metals. This may be due to transport resistance slowing metal transport and

lowering the concentration to the cells at the bottom of the well. Some cells could die when they were exposed to heavy metal stress and so there would be layers of dead cells along with the live cells and flocs. Thus flocs with dead cells may protect live cells from heavy metal stress by binding heavy metals and retarding the metal diffusion within flocs and dead cells. This phenomenon might be a representative view of what can be happenning in natural environment of CDAR. The microorganisms in nature grow on a surface like sediment or rocks or on the surface of any minerals or inside a porous surface where the transport is diffusion limited. Most of the bacteria in natural environment like CDAR are not in a shaking condition. Relatively few bacteria are suspended in the lake water. The surface attached bacteria form a community like flocs. These flocs associated with microbial cells may play a bigger role than the free streaming microbes to remove the metal in CDAR system. Further experiments should be carried out to reveal the presence of flocs by electron microscopy. This will provide a new idea of the role of flocs along with microorganisms in removing toxic metals at CDAR atmosphere.

Reference

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CHAPTER FIVE

FUTURE WORK

This thesis focused on the identification of microbial diversity by 16S rRNA gene sequencing and microarray (PhyloChip) analysis from Coeur d'Alene River (CDAR) sediment, and the zinc inhibition of *Pseudomonas* sp. strain JM001 growth. In light of previous and on-going studies regarding the microorganisms inhabiting CDAR sediments and zinc inhibited growth of *Pseudomonas* sp. strain JM001, the following recommendations are made for future work.

1. Microorganisms from different sites of CDAR should be analyzed using quantitative molecular techniques. Functional genes from CDAR sediments are important as well as distribution of microbial populations. This will help to understand the types of microorganisms and their bioremediation capabilities in the metal polluted area. In the current project, the sediment was collected near Harrison, ID on the CDAR delta in April, 2005 and September, 2004 [Appendix B]. The microbial diversity from the sediments collected at two different time of the year was analyzed by 16S rRNA technique. The data for April, 2005 sediment is presented in chapter two of the thesis. Approximately 104 clones were obtained from the September, 2004 sediment [Appendix B]. The diversity indices of the clone data obtained from the same site but at two different seasons of the year should be compared in terms of Shannon and Simpson indices.
2. Kinetic studies of other isolates from the sediment should be done in presence of a single metal, e.g. Zn, or in combination of metals, e.g. Zn and Pb, or Zn and Cu,

or Cu and Pb, or Zn, Pb and Cu. Results from the batch experiments should be evaluated with a dose-response kinetic model to understand the metal inhibited cell growth. Growth kinetics of a consortium in the presence of single metals or combinations of metals should be developed to better understand the microbe-metal interactions in a complex environment such as CDAR sediment.

3. In this work, mechanisms involved in cell to decrease metal concentrations were not studied. In future batch kinetic studies, metal reduction mechanisms should be examined using transmission electron microscopy (TEM) and elemental analysis of the cell-associated metal precipitates.
4. Batch experiments should also be conducted in the presence of a mineral phase such as goethite or quartz, to observe sediment metal interactions with microbial growth. This will attempt to understand a natural microbial community's response to heavy metal toxicity. This work should be assimilated with the numerical model to develop a kinetic model of interest.

APPENDICES

APPENDIX A

CLONE LIBRARY DATA BY BLAST SEARCH FOR

CDAR SEDIMENT COLLECTED IN APRIL, 2005

Table A1. Clone libraries from Coeur d' Alene River sediment (April, 2005) by 16S rRNA technique.

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
1	746	Uncultured *Chloroflexi* bacterium clone AKYG628	gi|60266590|gb|AY922044.1|	94	*Chloroflexi*	*Chloroflexi*
2	746	*Arthrobacter* sp. LCKW-Isolate25N	gi|71840362|gb|DQ129877.1|	99	*Actinobacteria*	α- *Proteobacteria*
3	739	Uncultured *Chlorobi* bacterium clone BDC3LB10	gi|58199730|gb|AY689768.1|	98	*Chlorobi*	*Chlorobia*
4	760	Uncultured *Bacteroidetes* bacterium	gi|71041256|gb|DQ110123.1|	99	*Bacteroidetes*	*Bacteroidetes*
5	767	*Janthinobacterium agaricidamnosum* strain SAFR-022	gi|27497658|gb|AY167838.1|	98	*Proteobacteria*	β-*Proteobacteria*
6	741	*Sterolibacterium denitrificans*	gi|13940381|emb|AJ306683.1|SDE306683	96	*Proteobacteria*	β-*Proteobacteria*
7	673	*Arthrobacter* sp. Tibet-IIR23	gi|75860314|gb|DQ177473.1|	99	*Actinobacteria*	α- *Proteobacteria*
8	403	*Bradyrhizobium japonicum* strain HHB-02	gi|99030352|gb|DQ517954.1|	94	*Proteobacteria*	α- *Proteobacteria*
9	535	*Flavobacterium xinjiangense* AS1.2749	gi|17016962|gb|AF433173.1|	94	*Bacteroidetes*	*Flavobacteria*
10	661	*Sterolibacterium denitrificans*	gi|13940381|emb|AJ306683.1|SDE306683	93	*Proteobacteria*	β-*Proteobacteria*

Table A1 Continued

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
11	752	*Oryza sativa* (indica cultivar-group)	gi|47824756|emb|AJ699155.1|	97	*Eukaryota*	
12	767	*Burkholderia* sp. 418	gi|48994246|gb|AY580068.1|	92	*Proteoacteria*	β-*Proteobacteria*
13	609	Bacterium Ellin6090	gi|38564760|gb|AY234742.1|	96	*Actinobacteria*	
14	532	*Massilia* sp. Tibet-IIU65	gi|75860322|gb|DQ177481.1|	94	*Proteobacteria*	β-*Proteobacteria*
15	788	*Thiobacillus denitrificans* partial 16S rRNA gene, strain NCIMB 9548	gi|5139662|emb|AJ243144.1|TDE243144	98	*Proteobacteria*	β-*Proteobacteria*
16	768	*Azoarcus buckelii*	gi|18072936|emb|AJ315676.1|ASP315676	98	*Proteobacteria*	β-*Proteobacteria*
17	775	*Rhodoferax ferrireducens* DSM 15236	gi|89343559|gb|CP000267.1|	99	*Proteobacteria*	β-*Proteobacteria*
18	703	Anaerobic filamentous bacterium KOME-1	gi|21068594|emb|AJ278174.1|UBA278174	97	environmental samples	
19	740	*Azoarcus toluclasticus* strain MF63	gi|90296962|gb|DQ404614.1|	99	*Proteobacteria*	β-*Proteobacteria*

Table A1 Continued

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
20	766	*Sphingobacterium* sp. OM-E81 DNA	gi|3970887|dbj|AB020206.1|	98	*Bacteroidetes*	*Sphingobacteria*
21	760	Flavobacteria symbiont 5 of Acromyrmex otcospinosus	gi|32400895|gb|AF491884.1|	96	*Bacteroidetes*	*Flavobacteria*
22	740	Uncultured *Acidobacteria* bacterium clone AKYG1698	gi|60266519|gb|AY921973.1|	98	*Acidobacteria*	*Acidobacteria*
23	752	*Beijerinckia derxii* subsp. venezuelae	gi|47457724|emb|AJ563934.1|BDE563934	98	*Proteobacteria*	α-*Proteobacteria*
24		*Janthinobacterium* sp. Antarctic IS0	gi|88909679|gb|DQ341421.1|		*Proteobacteria*	β-*Proteobacteria*
25	772	*Thiobacillus plumbophilus*	gi|21538794|emb|AJ316618.1|TPL316618	99	*Proteobacteria*	β-*Proteobacteria*
26	630	Uncultured Acidobacteria bacterium clone VC47	gi|28804158|gb|AY211077.1|	99	*Acidobacteria*	*Acidobacteria*
27	767	*Polaromonas aquatica*	gi|68051131|emb|AM039831.1|	98	*Proteobacteria*	β-*Proteobacteria*
28	278	*Polaromonas* sp. 'hydrogenovorans'	gi|70610322|gb|DQ094183.1|	98	*Proteobacteria*	β-*Proteobacteria*
29	779	Uncultured *Comamonadaceae* bacterium	gi|32452513|gb|AF523045.1|	98	*Proteobacteria*	β-*Proteobacteria*

Table A1 Continued

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
30	644	*Rhodocyclaceae* bacterium FTL11	gi|91982977|gb|DQ451827.1|	98	*Proteobacteria*	β-*Proteobacteria*
31	556	*Janthinobacterium* sp. TSBY-33	gi|73624705|gb|DQ151829.1|	97	*Proteobacteria*	β-*Proteobacteria*
32	776	*Nitrosospira* sp. En13	gi|57232110|gb|AY856079.1|	93	*Proteobacteria*	β-*Proteobacteria*
33	806	*Azoarcus denitrificians*	gi|1785601|gb|U82665.1|ADU82665	93	*Proteobacteria*	β-*Proteobacteria*
34	750	*Arthrobacter* sp. Tibet-IIR23	gi|75860314|gb|DQ177473.1|	100	*Actinobacteria*	α-*Proteobacteria*
35	741	*Desulfuromonas palmitatis*	gi|886802|gb|U28172.1|DPU28172	86	*Proteobacteria*	δ-*Proteobacteria*
36	785	*Anaeromyxobacter dehalogenans* strain 2CP-3	gi|14485223|gb|AF382400.1|	95	*Proteobacteria*	δ-*Proteobacteria*
37	770	*Burkholderia* sp. Ellin155	gi|33309397|gb|AF408997.1|		*Proteoacteria*	β-*Proteobacteria*
38	684	*Flavobacterium xinjiangense* AS1.2749	gi|17016962|gb|AF433173.1|	96	*Bacteroidetes*	*Flavobacteria*
39	516	Uncultured *Acidobacteriaceae* bacterium clone VHS-B3-4	gi|89001450|gb|DQ394942.1|	94	*Acidobacteria*	*Acidobacteria*
40	718	*Burkholderia* sp. Yws-12	gi|47109364|emb|AJ704385.1|	92	*Proteoacteria*	β-*Proteobacteria*
41		TOO SHORT LENGTH				

Table A1 continued…

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
42	769	Bacterium Ellin6067	gi|38564757|gb|AY234719.1|	97	*Proteobacteria*	β-*Proteobacteria*
43	725	*Gallionella ferruginea*	gi|1171632|gb|L07897.1|GLLRGDA	98	*Proteboacteria*	β-*Proteobacteria*
44	705	Uncultured *Acidobacteria* bacterium clone AKYG742	gi|60266605|gb|AY922059.1|	92	*Acidobacteria*	*Acidobacteria*
45	596	*Azoarcus* sp. 16S rRNA gene, strain T3	gi|1845157|emb|Y11041.1|AS16RNAT3	95	*Proteobacteria*	β-*Proteobacteria*
46	766	*Azoarcus* sp. 16S rRNA gene, strain T4	gi|1845157|emb|Y11041.1|AS16RNAT3	94	*Proteobacteria*	β-*Proteobacteria*
47	742	Anaerobic filamentous bacterium KOME-1	gi|83305692|dbj|AB243673.1|	99	environmental samples	
48	766	Uncultured *Comamonadaceae* bacterium	gi|32452513|gb|AF523045.1|	98	*Proteobacteria*	β-*Proteobacteria*
49	557	*Methylophilus methylotrophus*	gi|54695302|dbj|AB193724.1|	99	*Proteobacteria*	β-*Proteobacteria*
51	676	*Cellulomonas bogoriaensis*	gi|97955105|emb|AJ863164.1|	97	*Actinobacteria*	*Actinobacteria*

Table A1 Continued

Clone ID	Len-gth of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
52	744	Uncultured *Acidobacteria* bacterium clone AKYH527	gi|60266680|gb|AY922134.1|	99	*Acidobacteria*	*Acidobacteria*
53	773	*Nitrosospira* sp. En13	gi|57232110|gb|AY856079.1|	94	*Proteobacteria*	β-*Proteobacteria*
54	597	*Rhodoferax ferrireducens* DSM 15236	gi|89343559|gb|CP000267.1|	98	*Proteobacteria*	β-*Proteobacteria*
55	680	Uncultured *Bacteroidetes* bacterium clone 411T3	gi|71041256|gb|DQ110123.1|	99	*Bacteroidetes*	*Bacteroidetes*
56	769	*Janthinobacterium* sp. TSBY-33	gi|73624705|gb|DQ151829.1|	97	*Proteobacteria*	β-*Proteobacteria*
57	720	*Thiobacillus plumbophilus*	gi|21538794|emb|AJ316618.1|TPL316618	99	*Proteobacteria*	β-*Proteobacteria*
58	741	*Devosia riboflavina*	gi|41323203|gb|AY512822.1|	97	*Proteobacteria*	α-*Proteobacteria*
59	614	Uncultured *Chloroflexi* bacterium clone 276	gi|62866070|gb|AY935667.1|	96	*Chloroflexi*	*Chloroflexi*
60	773	*Desulfobacca acetoxidans*	gi|4100902|gb|AF002671.1|AF002671	97	*Proteobacteria*	δ-*Proteobacteria*
61	777	*Thiobacillus plumbophilus*	gi|21538794|emb|AJ316618.1|TPL316618	99	*Proteobacteria*	β-*Proteobacteria*

Table A1 Continued

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
62	666	Sporichthya polymorpha gene for 16S ribosomal RNA	gi|6009629|dbj|AB025317.1|	99	*Actinobacteria*	*Actinobacteria*
63	782	*Rhodoferax antarcticus* strain Fryx1	gi|47716702|gb|AY609198.1|	96	*Proteobacteria*	β-*Proteobacteria*
64	751	*Flavobacterium* sp. WB 2.4.4 partial 16S rRNA gene, strain WB 2.4.4	gi|83318973|emb|AM167564.1|	96	*Bacteroidetes*	*Flavobacteria*
65	766	*Thiobacillus denitrificans*	gi|5139662|emb|AJ243144.1|TDE24314	97	*Proteobacteria*	β-*Proteobacteria*
66	767	*Herbaspirillum lusitanum*	gi|26794026|gb|AF543312.1|	98	*Proteobacteria*	β-*Proteobacteria*
67	534	*Nitrosospira sp. En13*	gi|57232110|gb|AY856079.1|	95	*Proteobacteria*	β-*Proteobacteria*
68	714	*Paucibacter toxinivorans* strain S1030	gi|46309765|gb|AY515384.1|	97	*Proteobacteria*	β-*Proteobacteria*

Table A1 Continued

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/abovclass.html)	Class (Source:http://www.bacterio.cict.fr/abovclass.html)
69	762	*Azoarcus buckelii*	gi|18072936|emb|AJ315676.1|ASP315676	92	*Proteobacteria*	β-*Proteobacteria*
70	768	Uncultured *Acidobacteria* bacterium clone Spr10	gi|45738839|gb|AY568890.1|	89	*Acidobacteria*	*Acidobacteria*
71	741	*Bradyrhizobium* sp. lebi-3	gi|45686240|gb|AY490122.1|	99	*Proteobacteria*	α-*Proteobacteria*
73	738	*Thiobacillus denitrificans* ATCC 25259	gi|74055513|gb|CP000116.1|	97	*Proteobacteria*	β-*Proteobacteria*
74	771	*Dechloromonas* sp. PC1	gi|22530862|gb|AY126452.1|	94	*Proteobacteria*	β-*Proteobacteria*
75	782	Uncultured *Acidovorax* sp.	gi|82940442|emb|AM161161.1|	96	*Proteobacteria*	β-*Proteobacteria*
76	766	*Janthinobacterium* sp. WSH04-01	gi|54111791|gb|AY753304.1|	93	*Proteobacteria*	β-*Proteobacteria*
77	772	*Burkholderia* sp. DM5	gi|89741425|gb|DQ419951.1|	88	*Proteobacteria*	β-*Proteobacteria*
78	767	Bacteria from anoxic bulk soil	gi|6273406|emb|AJ229237.1|BSA229237	94	*Bacteroidetes*	
79	769	*Azoarcus* sp. 16S rRNA gene, strain T3	gi|1845157|emb|Y11041.1|AS16RNAT3	92	*Proteobacteria*	β-*Proteobacteria*

Table A1 Continued

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
80	741	Uncultured *Chloroflexi* bacterium clone MSB-5B1	gi|110747042|gb|DQ811877.1|	89	*Chloroflexi*	*Chloroflexi*
81	767	*Zoogloea* sp. PDD-3b-12	gi|94451289|gb|DQ512746.1|	97	*Proteobacteria*	β-*Proteobacteria*
82	710	Uncultured bacterium clone GOUTB8	gi|15912174|gb|AY050595.1|	92	*uncultured bacterium*	
83	747	*Burkholderia pyrrocinia* partial 16S rRNA gene, strain R13058	gi|27524918|emb|AJ440714.1|BPY440714	99	*Proteobacteria*	β-*Proteobacteria*
84	771	*Dechloromonas* sp. PC1	gi|22530862|gb|AY126452.1|	94	*Proteobacteria*	β-*Proteobacteria*
85	758	Uncultured bacterium PHOS-HE36	gi|11141789|gb|AF314435.1|	99	environmental samples	
86	769	*Ralstonia* sp. PHD-2	gi|77999233|gb|DQ227340.1|	95	*Proteobacteria*	β-*Proteobacteria*
87	771	Uncultured *Rhodocyclaceae* bacterium clone KRA34	gi|51317257|gb|AY689089.1|	97	*Proteobacteria*	β-*Proteobacteria*

Table A1 Continued

Clone ID	Length of sequence	Nearest neighbour by BLAST search	Accession no. of nearset neighbour	% similarity	Phylogenetic group (Source:http://www.bacterio.cict.fr/aboveclass.html)	Class (Source:http://www.bacterio.cict.fr/aboveclass.html)
88	771	*Thiobacillus plumbophilus*	gi|21538794|emb|AJ316618.1|TPL316618	94	*Proteobacteria*	β-*Proteobacteria*
89	768	*Thermaerobacter subterraneus*	gi|18462164|gb|AF343566.1|	98	*Firmicutes*	*Clostridia*
90	769	*Gallionella ferruginea*	gi|1171632|gb|L07897.1|GLLRGDA	97	*Proteobacteria*	β-*Proteobacteria*
91	780	*Legionella gresilensis*	gi|4836458|gb|AF122883.1|AF122883	93	*Proteobacteria*	γ-*Proteobacteria*
92	752	*Sterolibacterium denitrificans*	gi|13940381|emb|AJ306683.1|SDE306683	89	*Proteobacteria*	β-*Proteobacteria*
93	741	*Flavobacterium psychrophilum* strain ATCC	gi|56131575|gb|AY662493.1|	98	*Bacteroidetes*	*Flavobacteria*
95	764	*Legionella gresilensis*	gi|4836458|gb|AF122883.1|AF122883	93	*Proteobacteria*	γ-*Proteobacteria*

APPENDIX B

CLONE LIBRARY DATA BY RIBOSOMAL DATABASE PROJECT (RDP) SEARCH FOR CDAR SEDIMENT COLLECTED IN SEPTEMBER, 2004

Table B1. Clone libraries from Coeur d' Alene River sediment (Sep., 2004) by 16S rRNA technique.

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
7	S000020495	*Proteobacteria*	α-*Proteobacteria*	0.648	*Sphingomonas sp*. K6; AJ000918
	S000012774			0.634	*Sphingomonas asaccharolytica*; IFO 10564-T; Y09639
	S000278677			0.867	*Afipia broomeae*; C-12; AY568503
19	S000019779	*Proteobacteria*	α-*Proteobacteria*	0.852	uncultured eubacterium WD275; AJ292603
	S000393796			0.845	*Bradyrhizobium japonicum*; DASA03066; AF417546
28	S000354851	*Proteobacteria*	α-*Proteobacteria*	0.937	uncultured bacterium; UP4; AY080911
	S000338803			0.904	
	S000429024			0.899	*Methylocystis parvus*; pAMC269; AF150805
					Methylosinus pucelana; MTS; AF107461
31	S000437300	*Proteobacteria*	α-*Proteobacteria*	0.265	*Wolbachia pipientis* (T); U23709
40	S000493194	*Proteobacteria*	α-*Proteobacteria*	0.878	uncultured alpha proteobacterium; AKYG1560; AY921960
	S000392541			0.804	*Nordella oligomobilis*; N21; AF370880
	S000000623			0.645	*Hyphomicrobium vulgare*; IFAM MC-750, ATCC 27500; Y14302
	S000437754			0.645	*Mesorhizobium loti*; ICMP3153, USDA 3455, 261; U50166
42	S000483994	*Proteobacteria*	α-*Proteobacteria*	0.268	*Pseudomonas* sp. An19; AJ551157
	S000126944	*Proteobacteria*	ε-*Proteobacteria*	0.268	proteobacterium BHI60-11; AJ431219
56	S000376367	*Proteobacteria*	α-*Proteobacteria*	0.257	*Reclinomonas americana*; ATCC50394; AF007261
63	S000089362	*Proteobacteria*	α-*Proteobacteria*	0.364	*Methylobacterium* sp. LMG 19419; LMG-19419; AJ276806

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
	S000018317			0.785	*Novosphingobium hassiacum* (T); W-51; AJ416411
67	S000002972	*Proteobacteria*	α-*Proteobacteria*	0.772	*Novosphingobium aromaticivorans*; IFO 16084; AB025012
	S000345720			0.766	uncultured Sphingomonas sp.; KL-2-4-7; AF408323
68	S000340064 S000392541	*Proteobacteria*	α-*Proteobacteria*	0.749	uncultured sludge bacterium H34; AF234750
				0.702	*Nordella oligomobilis*; N21; AF370880
86	S000004163	*Proteobacteria*	α-*Proteobacteria*	0.623	*Methylocystis* sp. KS8a; AJ458493
94	S000492994	*Proteobacteria*	α-*Proteobacteria*	0.532	uncultured alpha proteobacterium; AKYH1490; AY921760
	S000372776			0.421	*Ochrobactrum* sp. AS12; AY662685
95	S000329056	*Proteobacteria*	α-*Proteobacteria*	0.442	uncultured alpha proteobacterium; EB1032; AY395351
107	S000401976	*Proteobacteria*	α-*Proteobacteria*	0.366	uncultured bacterium; 164ds20; AY212616

Article II. **Total α-*Proteobacteria* = 16**

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
4	S000387097	*Proteobacteria*	γ-*Proteobacteria*	0.381	*Pasteurella pneumotropica*; CNP 160; AF012090
10	S000483980	*Proteobacteria*	γ-*Proteobacteria*	0.269	*Pseudoalteromonas* sp. An2; AJ551143
24	S000356722	*Proteobacteria*	γ-*Proteobacteria*	0.288	uncultured bacterium; KD9-118; AY218652
32	S000386646	*Proteobacteria*	γ-*Proteobacteria*	0.877	*Pseudomonas putida* KT2440; AE016775
41	S000355848	*Proteobacteria*	γ-*Proteobacteria*	0.202	endosymbiont of Cosmopolites sordidus; AY126632

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
46	S000455571	Unclassified *Gammaproteobacteria*		0.275	uncultured Flexibacter sp.; F3C16A; AY794151
50	S000007554	*Proteobacteria*	γ-*Proteobacteria*	0.261	*Xanthomonas campestris*; XCC15; AF123092
51	S000427525	*Proteobacteria*	γ-*Proteobacteria*	0.244	Y-symbiont of Anomoneura mori; AB013087
65	S000386989	*Proteobacteria*	γ-*Proteobacteria*	0.311	*Shewanella algae*; ATCC 8073; AF005250
80	S000386989	*Proteobacteria*	γ-*Proteobacteria*	0.311	*Shewanella algae*; ATCC 8073; AF005250
81	S000399536 S000354965	*Proteobacteria*	γ-*Proteobacteria*	0.659 0.659	*Stenotrophomonas maltophilia*; 15; AY169434 uncultured bacterium; KRA30-21; AY081992
83	S000459803	*Proteobacteria*	γ-*Proteobacteria*	0.228	uncultured bacterium; GZKB126; AJ853618
89	S000141133	*Proteobacteria*	γ-*Proteobacteria*	0.292	*Pseudomonas filiscindens*; ATCC BAA-697; AY259924
101	S000386989	*Proteobacteria*	γ-*Proteobacteria*	0.281	*Shewanella algae*; ATCC 8073; AF005250
104	S000355852	*Proteobacteria*	Unclassified γ-*Proteobacteria*	0.204	endosymbiont of Rhynchophorus palmarum; AY126636
			Total γ-*Proteobacteria* =	**15**	
Clone no.	**RDP sequence identifier**	**Phylum**	**Class**	**Similarity score**	**Name of Microorganisms**
5	S000370418	*Actinobacteria*	*Actinobacteria*	0.585	uncultured bacterium; Amsterdam-1B-31; BC20-1B-31; AY592335

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
14	S000344716 S000089215 S000018062 S000006621	*Actinobacteria*	*Actinobacteria*	0.842 0.799 0.793 0.789	uncultured actinobacterium; GCP18; AF387313 Tetrasphaera elongata; ASP12; AB051430 *Candidatus Nostocoida limicola*; Ben18; X85212 Janibacter limosus (T); DSM 11140T; Y08539
17	S000394831	*Actinobacteria*	*Actinobacteria*	0.850	uncultured bacterium; ARKCH2Br2-66; AF468240
36	S000005373	*Actinobacteria*	*Actinobacteria*	0.500	*Micrococcus luteus*; IFO 16250; AB023371
38	S000008956	*Actinobacteria*	*Actinobacteria*	0.461	*Streptomyces thermovulgaris* (T); DSM 40444 (type strain); Z68094
44	S000407868 S000432315	*Actinobacteria*	*Actinobacteria*	0.470 0.461	*Actinomyces* sp. oral clone GU067; AY349362 *Actinomyces naeslundii*; ChDC B204; AF543282
47	S000125312 S000440166	*Actinobacteria*	*Actinobacteria*	0.333 0.333	*Actinomyces vaccimaxillae*; R10176T; AJ427451 *Arthrobacter kerguelensis*; type strain: KGN 15; AJ606062
49	S000131289	*Actinobacteria*	*Actinobacteria*	0.362	*Amycolatopsis methanolica*; IMSNU 20055T; AJ249135
54	S000329080	*Actinobacteria*	*Actinobacteria*	0.740	uncultured Rubrobacteridae bacterium; EB1056; AY395375
62	S000012597	*Actinobacteria*	*Actinobacteria*	0.316	*Cryptobacterium curtum* (T); ATCC700683; 12-3; AB019260
72	S000403485 S000379948	*Actinobacteria* *Proteobacteria*	*Actinobacteria* *Deltaproteobacteria*	0.618 0.586	bacterium Ellin5201; AY234552 uncultured Desulfuromonas sp.; M76; AY692042
73	S000352751	*Actinobacteria*	*Actinobacteria*	0.439	*Propionibacterium jensenii*; type strain: DSM 20535; AJ704571
			Total *Actinobacteria* =	**12**	

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
12	S000355710	*Proteobacteria*	β-*Proteobacteria*	0.721	uncultured bacterium; a13103; AY102310
22	S000019559 S000441779	*Proteobacteria*	β-*Proteobacteria*	0.776 0.670	beta proteobacterium A0837; AF236012 uncultured beta proteobacterium; LiUU-9-233; AY509483
33	S000345582	*Proteobacteria*	β-*Proteobacteria*	0.787	uncultured bacterium; RA13C6; AF407405
52	S000130867 S000459805 S000331824 S000427718	*Aquificae* *Proteobacteria* *Spirochaetes*	*Aquificae* β-*Proteobacteria* *Spirochaetes*	0.298 0.298 0.298 0.298	Coprothermobacter sp. BHI60-1; AJ431251 uncultured bacterium; GZKB128; AJ853620 uncultured spirochete; LH014; AY605167 Treponema sp. I:G:C1; AF023052
64	S000354952	*Proteobacteria*	β-*Proteobacteria*	0.354	uncultured bacterium; KRA30+11; AY081979
71	S000335688	*Proteobacteria*	β-*Proteobacteria*	0.788	uncultured bacterium; MIZ16; AB179507
75	S000017877	*Proteobacteria*	β-*Proteobacteria*	0.322	Candidatus Tremblaya princeps; AF476092
76	S000409628	*Proteobacteria*	β-*Proteobacteria*	0.748	uncultured bacterium; mv13.2; AY424823
88	S000134525	*Proteobacteria*	β-*Proteobacteria*	0.242	*Burkholderia* sp. M14; AY307366
98	S000410841	*Proteobacteria*	β-*Proteobacteria*	0.333	uncultured bacterium; oc9; AY491560
102	S000413720 S000252528	*Proteobacteria* *Bacteroidetes*	β-*Proteobacteria* *Flavobacteria*	0.264 0.264	*Actinobacillus pleuropneumoniae* (T); Shope4074; D30030 uncultured marine bacterium ZD0203; AJ400340
			Total β-*Proteobacteria*=	**11**	

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
3	S000100213	*Firmicutes*	*Clostridia*	0.238	uncultured bacterium; C13-5; AJ308396
9	S000113994 S000260778 S000022934	*Cyanobacteria* *Firmicutes* *Chlamydiae*	*Cyanobacteria* *Clostridia* *Chlamydiae*	0.364 0.364 0.364	uncultured Synechococcus sp.; A715014; AY125370 Clostridium tetani (T); NCTC 279; X74770 Chlamydophila pneumoniae; TW183; L06108
30	S000351774 S000000658 S000004840	*Firmicutes*	*Clostridia*	0.581 0.596 0.598	*Propionispora hippei*; type strain: KS; 5; AJ508927 *Sporomusa malonica* (T); DSM 5090 Type; AJ279799 *Sporomusa sphaeroides* (T); DSM 2875 Type; AJ279801
35	S000414971	*Firmicutes*	*Clostridia*	0.444	unidentified bacterium; JTB326; AB015272
66	S000365164	*Firmicutes*	*Clostridia*	0.378	uncultured rumen bacterium; F24-F11; AB185632
84	S000326495	*Firmicutes*	*Clostridia*	0.255	Selenomonas genomosp. C1; C5AKM062; AY278627
100	S000384062 S000414423	*Firmicutes*	*Clostridia*	0.450 0.450	uncultured bacterium; BCf5-21; AB062828 Gemella morbillorum (T); L14327
108	S000260540	*Firmicutes*	*Clostridia*	0.746	*Clostridium aminobutyricum*; DSM 2634; X76161
			Total *Clostridia* =	**8**	
Clone no.	**RDP sequence identifier**	**Phylum**	**Class**	**Similarity score**	**Name of Microorganisms**
6	S000485009	*Firmicutes*	*Bacilli*	0.858	*Bacillus smithii*; R-7170; AY373319
11	S000001560	*Firmicutes*	*Bacilli*	0.754	*Bacillus smithii* (T); DSM 4216; Z26935
21	S000001560 S000130886	*Firmicutes*	*Bacilli*	0.830 0.674	*Bacillus smithii* (T); DSM 4216; Z26935 *Bacillus firmus* (T); NCIMB 9366; X60616

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
34	S000470212	*Firmicutes*	*Bacilli*	0.368	*Bacillus clausii*; M-29-A; AB201800
43	S000148054	*Proteobacteria*	β-*Proteobacteria*	0.316	uncultured beta proteobacterium; S15B-MN110; AJ583170
	S000484021	*Firmicutes*	*Bacilli*	0.316	*Pediococcus claussenii*; type strain: DSM 14800; AJ621555
45	S000148943	*Firmicutes*	*Bacilli*	0.907	*Alicyclobacillus pomorum*; 3A; AB089840
	S000002313			0.834	*Alicyclobacillus hesperidum*; FR-1B; AJ133632
48	S000352853	*Firmicutes*	δ-*Proteobacteria*	0.228	*Desulfobulbus* sp. oral clone CH031; AY005036
	S000120376	*Proteobacteria*	*Mollicutes*	0.228	Candidatus Phytoplasma cynodontis; BGWL-C2; AJ550985
	S000437151			0.228	*Bulleidia extructa*; W1365; 1365-12; U13036
	S000016658		*Bacilli*	0.228	*Weissella kandleri*; NCDO 2753; X52570
53	S000370876	*Firmicutes*	*Bacilli*	0.283	uncultured bacterium; Napoli-4B-79; BC07-4B-79; AY592793

Article III. **Total *Bacilli* = 8**

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
1	S000271692	*Bacteroidetes*	*Sphingobacteria*	0.507	uncultured *Bacteroidetes* bacterium; SW36; AJ575722
2	S000425016	*Bacteroidetes*	*Sphingobacteria*	0.743	uncultured *Bacteroidetes* bacterium; S1-4-CL9; AY728066
15	S000335703	*Bacteroidetes*	*Sphingobacteria*	0.722	uncultured bacterium; MIZ31; AB179522
	S000384749			0.621	*Flexibacter japonensis*; IFO 16041; AB078055

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
25	S000440940 S000490075 S000380249	*Bacteroidetes*	*Sphingobacteria*	0.788 0.561 0.541	uncultured bacterium; Hot Creek 2; AY168735 *Tyrosinophaga indica*; GPTSA100-15; AY904352 *Algoriphagus yeomjeoni*; MSS-160; AY699794
26	S000440940 S000380249	*Bacteroidetes*	*Sphingobacteria*	0.770 0.523	uncultured bacterium; Hot Creek 2; AY168735 *Algoriphagus yeomjeoni*; MSS-160; AY699794
78	S000261025	*Bacteroidetes*	*Sphingobacteria*	0.642	uncultured bacterium; S9A-31; AB154301
85	S000383914	*Bacteroidetes*	*Sphingobacteria*	0.245	*Saprospira* sp. SS90-1; AB058899
			Total *Sphingobacteria* =	**7**	

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
16	S000452794	unclassified_ Bacteria	unclassified_Bacteria	0.374	uncultured bacterium; TANB37; AY667257
18	S000126884	unclassified_ Bacteria	unclassified_Bacteria	0.622	uncultured bacterium SHD-245; AJ278174
37	S000116489	unclassified_ Bacteria	unclassified_Bacteria	0.633	uncultured bacterium SJA-15; AJ009453
79	S000447472	unclassified_ Bacteria	unclassified_Bacteria	0.309	uncultured delta proteobacterium; HMMVPog-18; AJ704673
103	S000148742	unclassified_ Bacteria	unclassified_Bacteria	0.874	uncultured bacterium; S15B-MN72; AJ583209
106	S000469795	unclassified_ Bacteria	unclassified_Bacteria	0.335	uncultured bacterium; KNA6-NB10; AB179666
			Unclassified bacteria =	**6**	

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
29	S000379948 S000440962 S000115088	*Proteobacteria*	δ-*Proteobacteria*	0.507 0.483 0.480	uncultured *Desulfuromonas* sp.; M76; AY692042 *Geopsychrobacter electrodiphilus*; A1; AY187303 *Melittangium boletus* (T); Me b8; AJ233908
39	S000392793	*Proteobacteria*	δ-*Proteobacteria*	0.858	Anaeromyxobacter dehalogenans; 2CP-C; ATCC BAA-259;
90	S000115749	*Proteobacteria*	δ-*Proteobacteria*	0.570	*Chondromyces lanuginosus*; Sy t2; AJ233939
99	S000417333 S000344591	*Proteobacteria*	δ-*Proteobacteria*	0.383 0.383	*Byssophaga cruenta*; "type strain: By c2 = DSM 14553"; AJ833647 uncultured bacterium; ZA3704c; AF382126
			Total δ-*Proteobacteria* =	**4**	
Clone no.	**RDP sequence identifier**	**Phylum**	**Class**	**Similarity score**	**Name of Microorganisms**
27	S000340041 S000006614	*Planctomycetes*	*Planctomycetacia*	0.692 0.583	uncultured sludge bacterium A12; AF234727 *Gemmata obscuriglobus* (T); UQM 2246; mp18 HH1, mp19 HH2
105	S000376582	*Planctomycetes*	*Planctomycetacia*	0.546	uncultured *planctomycete*; DSP19; AJ290174
			Total *Planctomycetacia* =	**2**	

Table B1 Continued

Clone no.	RDP sequence identifier	Phylum	Class	Similarity score	Name of Microorganisms
8	S000087507	*Deinococcus-Thermus*	*Deinococci*	0.428	*Thermus* sp. RH-914; AF521186
	S000021615	*Nitrospira*	*Nitrospira*	0.416 0.602	*Nitrospira* sp.; Y14639
13	S000382272	*Bacteroidetes*	*Bacteroidetes*	0.793	*Cytophaga* sp.; BD1-16; AB015525
70	S000018502	*Acidobacteria*	*Acidobacteria*	0.521	Bacteria; kb2426; Z95732
	S000346305	Putative_Chimera	Putative_Chimera	0.515	uncultured soil bacterium; 384-2; AF423257
74	S000320820	*Spirochaetes*	*Spirochaetes*	0.232	uncultured Treponema clone RFS102; AF068415
82	S000004843	*Cyanobacteria*	*Cyanobacteria*	0.237	*Stanieria* sp. PCC 7301; AB039009
93	S000393473	*Thermodesulfobacteria*	*Thermodesulfobac-teria*	0.386	*Geothermobacterium ferrireducens*; FW-1a; AF411013
97	S000389154	*Proteobacteria*	Unclassified *Proteobacteria*	0.319	*Candidatus Carsonella ruddii*; AF211128
109	S000115377	*Acidobacteria*	*Acidobacteria*	0.727	uncultured bacterium SJA-149; AJ009495
110	S000012815	Unclassified *Deferribacterales*		0.754	*Phormidium autumnale*; UTCC471; AF218371
			Miscellaneous =	**9**	

Total = 98

APPENDIX C

COMPARISON OF TWO CLONE LIBRARIES FROM CDAR

Table C1. Comparison of the 'Class' in two different CDAR sediments from Harrison site, ID, USA.

Class	**Availability in sediment collected in April, 2005 by BLAST search**	**Availability in sediment collected in Sep., 2004 by RDP search**
Bacteroidetes	7	1
Acidobacteria	6	1
Actinobacteria	2	12
Bacilli	---	8
Chlorobi	1	---
Chloroflexi	3	---
Clostridia	---	8
Cyanobacteria	1	1
α-Proteobacteria	5	16
β-Proteobacteria	51	11
δ-Proteobacterai	3	4
γ-Protebacteria	2	15
Environmental Sample	2	---
Flavobacteria	5	---
Plactomycetacia	---	2
Sphingobacteria	1	7
Uncultured Bacteria	1	6
Miscellaneous	---	6
Total	**90**	**98**

APPENDIX D

SEQUENCES OF THE CLONES OBTAINED FROM CDAR SEDIMENT (APRIL, 2005) USING 16S rRNA TECHNIQUE

>CDAR1
TAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCAT
GGCGGCCGCGGGAATTCGATTCTCCTACGGGAGGCAGCAGTCGGGAATTTTGCTCAATGGGC
GAAAGCCTGAAGCAGCAACGCCGCGTGAGGGATGAAGGCCTTCGGGTTGTAAACCTCTTTTTC
CAGGGACGATAATGACGGTACCTGAAGAATAAGTCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGACAAGCGTTGTCCGGATTTACTGGGCGTAAAGAGCGCGCAGGCGGTCG
TTCGAGTCGAGTGTGAAAGCCCCCGGCTCAACTGGGGAGGGTCATTCGATACTGATCGACTCG
AAGGCAGGAGAGGGAAGCGGAATTCCCGGTGTAGTGGTGAAATGCGTAGATATCGGGAGGA
ACACCAGTGGCGAAGGCGGCTTCCTGGTCTGCTTCTGACGCTAAGAGGCGAAAGCTAGGGGA
GCGAACGGGATTAGAAACCCCGGTAGTCCTAGCCATAAACGATGGATACTAGGTGTTGGTGG
TCCTAACCCCATCAGTGCCGAAGCTAACGCGTTAAGTGTCCCGCCTGGGGAGTACGGCCGCAA
GGCTGAAACTCAAAGGAATTGACGGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACC
ATATGGGAGAGCTCCCAACCGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAA

>CDAR2
ATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTTCGCATGCTCCCGGCCGCCATG
GCGGCCGCGGGAATTCGATTCTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCG
AAAGCCTGACGCAGCAACGCCGCGTGAGCGAAGAAGACCTTCGGGTTGTAAAGTTCTTTTCTG
AAGGACGAGGAAGGACGGTACTTCAGGAATAAGGATCGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGATCCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTG
TCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGATCTGCGGTGGGTACGGGCAGACTAG
AGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAA
CACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAG
CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGAC
ATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAA
GGCTAAAACTCAAAGGAATTGACGGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACC
ATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAA

>CDAR3
ATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGG
CGGCCGCGGGAATTCGATTCTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGGGA
AACCCTGACGCAGCAACGCCGCGTGGAGGATGAAGCCCCTTGGGGCGTAAACTCCTTTCGAC
CAAGACGATAATGACGGTACTGGTGGAAGAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGG
TAATACGTAGGGGGCAAACGTTGTCCGGATTTACTGGGTGTAAAGGGTGCTCAGGCGGTTTTG
TAAGTCAGAAGTGAAATCCTAGAGCTTAACTCTGGAACTGCTTTTGATACTGCAAGGCTTGAA
TGTGGAAGAGGAGGATGGAATTTCTGGTGTAGCAGTGAAATGCGTAGATATCAGAAAGAACA
CCAGTGGCGAAGGCGATCCTCTGGTCCACTATTGACGCTAAAGCACGAAAGCGTGGGGAGCA
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APPENDIX E

DATA OBTAINED FROM KINETIC STUDY OF *PSEUDOMONAS* SP. STRAIN JM001 IN MEDIUM A

Table E1. Measurement of optical density at different time in presence of Zn concentrations in medium A.

Time (h)	Absorbance @ 610nm								
	No cells, no metal	Cells only, no metal	Cells+ 8 µM Zn	Cells+ 16 µM Zn	Cells+ 24 µM Zn	Cells+ 32 µM Zn	Cells+ 40 µM Zn	Cells+ 60 µM Zn	Cells+ 100 µM Zn
0	0	0.002	0.003	0.003	0.003	0.003	0.002	0.003	0.003
12.5	0	0.036	0.009	0.004	0.003	0.004	0.003	0.004	0.003
15	0	0.043	0.020	0.002	0.002	0.002	0.002	0.004	0.002
17.5	0	0.050	0.026	0.003	0.003	0.002	0.003	0.003	0.003
20	0	0.062	0.034	0.003	0.003	0.002	0.003	0.003	0.003
22.5	0	0.067	0.039	0.003	0.004	0.002	0.003	0.003	0.003
25	0	0.081	0.047	0.003	0.003	0.002	0.003	0.004	0.003
31	0	0.103	0.062	0.003	0.002	0.003	0.002	0.003	0.002
34	0	0.118	0.074	0.006	0.003	0.003	0.003	0.003	0.003
36.5	0	0.123	0.078	0.008	0.002	0.002	0.002	0.003	0.003
39	0	0.133	0.087	0.015	0.003	0.002	0.003	0.004	0.003
42	0	0.141	0.094	0.020	0.002	0.002	0.003	0.003	0.003
44.5	0	0.142	0.098	0.026	0.003	0.002	0.003	0.004	0.003
50	0	0.142	0.105	0.038	0.003	0.003	0.003	0.003	0.003
60.5	0	0.129	0.087	0.051	0.002	0.002	0.002	0.002	0.002
64	0	0.130	0.085	0.053	0.002	0.002	0.002	0.002	0.002
67	0	0.129	0.084	0.054	0.002	0.002	0.002	0.002	0.002
70	0	0.132	0.088	0.059	0.003	0.002	0.002	0.002	0.003
72	0	0.132	0.088	0.059	0.001	0.002	0.002	0.002	0.002
88	0	0.129	0.088	0.054	0.003	0.002	0.003	0.003	0.003

The contents in two ZincoVer 5 reagent powder pillow was added to 20 ml nanopure water. One mililiter of this solution was added to 1 ml of filtered sample. Then 100 µl of cyclohexanone were used and was vortexed for 20 s, and left for 3 min reaction time.

Table E2. Measurement of Zn concentrations by ZincOver method.

Time (h)	Absorbance @ 620nm								
	No cells, no metal	Cells only, no metal	Cells+ 8 µM Zn	Cells+ 16 µM Zn	Cells+ 24 µM Zn	Cells+ 32 µM Zn	Cells+ 40 µM Zn	Cells+ 60 µM Zn	Cells+ 100 µM Zn
0	0	0	0.069	0.147	0.219	0.295	0.386	0.574	0.921
12.5	0	0	0.071	0.151	0.223	0.293	0.385	0.567	0.918
15	0	0	0.073	0.156	0.231	0.301	0.389	0.569	0.937
17.5	0	0	0.073	0.155	0.230	0.295	0.383	0.563	0.915
20	0	0	0.073	0.156	0.230	0.299	0.388	0.572	0.922
22.5	0	0	0.070	0.156	0.224	0.295	0.386	0.563	0.906
25	0	0	0.070	0.155	0.229	0.295	0.384	0.567	0.899
31	0	0	0.059	0.151	0.223	0.293	0.385	0.572	0.922
34	0	0	0.059	0.152	0.222	0.294	0.381	0.568	0.910
36.5	0	0	0.061	0.162	0.223	0.297	0.381	0.556	0.912
39	0	0	0.058	0.148	0.222	0.291	0.387	0.560	0.915
42	0	0	0.059	0.150	0.228	0.296	0.380	0.564	0.926
44.5	0	0	0.057	0.147	0.227	0.300	0.384	0.567	0.926
50	0	0	0.027	0.117	0.199	0.267	0.360	0.538	0.913
60.5	0	0	0.036	0.125	0.231	0.296	0.384	0.567	0.912
64	0	0	0.037	0.117	0.241	0.308	0.397	0.591	0.938
67	0	0	0.033	0.109	0.224	0.287	0.387	0.562	0.921
70	0	0	0.031	0.109	0.228	0.294	0.387	0.580	0.902
88	0	0	0.025	0.084	0.228	0.299	0.387	0.575	0.917

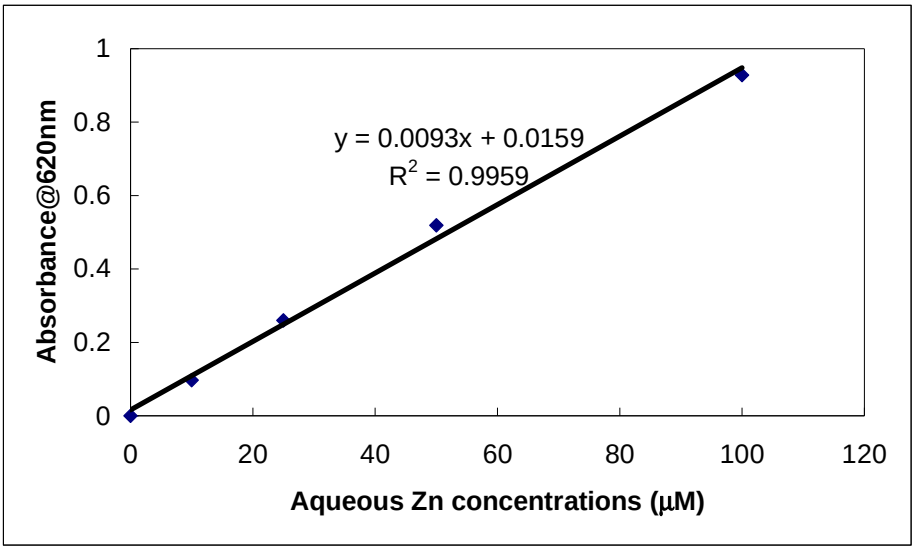


Figure E1. Calibration curve for measuring Zn concentration by Zincon method (HACH).

Table E3. Zn concentrations in μM using the calibration curve (Figure E1).

Time (h)	No cells, no metal	Cells only, no metal	Cells+ 8 μM Zn	Cells+ 16 μM Zn	Cells+ 24 μM Zn	Cells+ 32 μM Zn	Cells+ 40 μM Zn	Cells+ 60 μM Zn	Cells+ 100 μM Zn
0	0	0	5.6428	14.0641	21.734	29.941	39.649	59.8180	97.0434
12.5	0	0	5.9110	14.4932	2.2172	29.673	39.542	59.0671	96.7752
15	0	0	6.0719	15.0296	23.021	30.531	39.971	59.2816	98.7598
17.5	0	0	6.1255	14.8686	22.968	29.941	39.381	58.6916	96.4534
20	0	0	6.1255	14.9759	22.914	30.316	39.864	59.6571	97.1507
22.5	0	0	5.7500	14.9759	22.270	29.887	39.703	58.6380	95.4879
25	0	0	5.7500	14.8686	22.807	29.887	39.488	59.0671	94.6833
31	0	0	4.6236	14.4395	22.217	29.673	39.596	59.6571	97.1507
34	0	0	4.5700	14.6005	22.109	29.833	39.113	59.1743	95.8633
36.5	0	0	4.8382	15.6732	22.163	30.102	39.167	57.9406	96.0779
39	0	0	4.5163	14.1713	22.056	29.458	39.810	58.3161	96.4534
42	0	0	4.6236	14.385	22.753	29.994	39.059	58.7989	97.5798
44.5	0	0	4.4091	14.010	22.646	30.477	39.435	59.0671	97.5798
50	0	0	1.1371	10.845	19.642	26.883	36.860	55.9560	96.1852
60.5	0	0	2.1562	11.650	23.021	30.048	39.435	59.0671	96.0779
64	0	0	2.2099	10.845	24.094	31.282	40.883	61.6954	98.9207
67	0	0	1.8344	9.9875	22.324	29.029	39.810	58.5843	97.0434
70	0	0	1.6198	9.9339	22.753	29.780	39.810	60.4617	95.0587
88	0	0	0.9762	7.2519	22.699	30.370	39.757	59.9253	96.6143

Table E4. Calculation of specific growth rate, μ for *Pseudomonas* sp. strain JM001 in medium A.

Time (h)	M_t			$\mu = \frac{1}{M_t}\ln\left(\frac{M_t}{M_0}\right)$		
	Metal free control	Cells+ 8 µM Zn	Cells+ 16 µM Zn	Metal free control, M_0= 0.002	Cells+ 8 µM Zn, M_0=0.003	Cells+ 16 µM Zn, M_0=0.003
12.5	0.036			0.231		
15	0.043	0.020		0.204	0.125	
17.5	0.050	0.026		0.184	0.123	
20	0.062	0.034		0.171	0.121	
22.5		0.039			0.114	
50			0.038			0.051
60.5			0.051			0.047
Specific growth rate (taking the average)				0.198	0.121	0.049

Table E5. Aqueous acetate concentrations using ion chromatography.

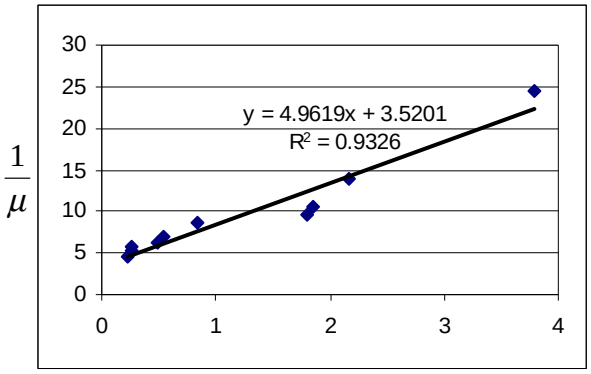
Time (h)	No cells, no metal	Cells only, no metal	Cells+ 8 µM Zn	Cells+ 16 µM Zn	Cells+ 24 µM Zn	Cells+ 32 µM Zn	Cells+ 40 µM Zn	Cells+ 60 µM Zn	Cells+ 100 µM Zn
0	4.537	4.573	4.568	4.617	4.658	4.437	4.524	4.536	4.529
12.5	4.449	4.280	4.544	4.624	4.512	4.566	4.583	4.532	4.519
7.5	4.498	3.800	4.534	4.524	4.559	4.444	4.454	4.524	4.556
20	4.529	3.698	4.436	4.414	4.436	4.490	4.437	4.434	4.495
22.5	4.532	2.056	4.308	4.468	4.463	4.437	4.425	4.412	4.544

Table E5 Continued…

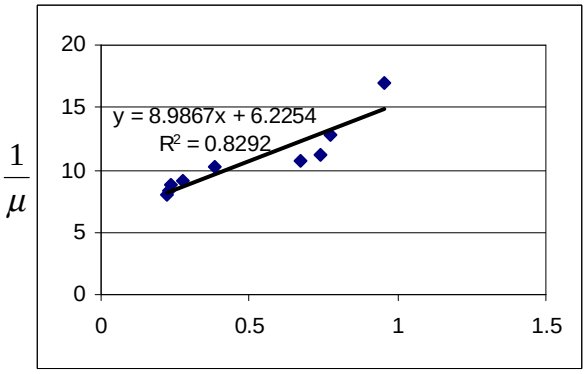
Time (h)	No cells, no metal	Cells only, no metal	Cells+ 8 µM Zn	Cells+ 16 µM Zn	Cells+ 24 µM Zn	Cells+ 32 µM Zn	Cells+ 40 µM Zn	Cells+ 60 µM Zn	Cells+ 100 µM Zn
25	4.478	1.864	3.588	4.515	4.459	4.500	4.558	4.442	4.578
31	4.529	1.202	2.625	4.488	4.473	4.424	4.485	4.549	4.488
34	4.510	0.554	1.490	4.415	4.464	4.508	4.532	4.527	4.519
36.5	4.459	0.541	1.354	4.471	4.439	4.547	4.498	4.427	4.520
44.5	4.444	0.463	1.290	3.446	4.419	4.527	4.458	4.466	4.553
60.5	4.451	0.264	1.047	2.010	4.473	4.476	4.419	4.502	4.429
88	4.432	0.000	0.702	1.229	4.568	4.468	4.469	4.425	4.432

Table E6. Data for Lineweaver-Burke plot.

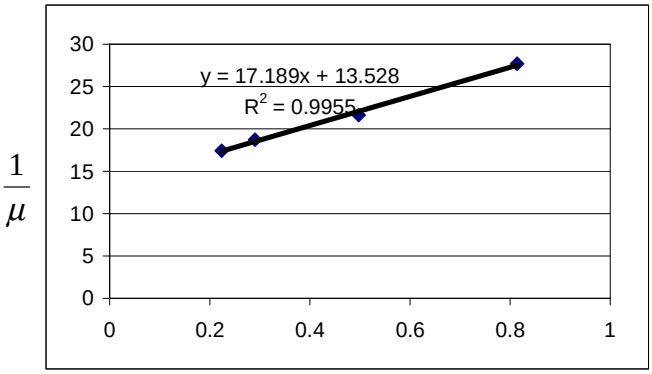
	Metal free control				Cells+ 8 µM Zn				Cells+ 16 µM Zn			
Time (h)	A (mM)	μ (h^{-1})	$\frac{1}{A}$	$\frac{1}{\mu}$	A (mM)	μ (h^{-1})	$\frac{1}{A}$	$\frac{1}{\mu}$	A (mM)	μ (h^{-1})	$\frac{1}{A}$	$\frac{1}{\mu}$
12.5	4.279	0.225	0.233	4.443	4.544	0.136	0.220	7.350				
17.5	3.8	0.188	0.263	5.308	4.533	0.124	0.220	8.023				
20	3.698	0.172	0.270	5.803	4.435	0.119	0.225	8.381				
22.5	2.055	0.157	0.486	6.343	4.308	0.114	0.232	8.756				
25	1.864	0.144	0.536	6.933	3.588	0.109	0.278	9.148				
31	1.201	0.116	0.832	8.584	2.625	0.098	0.380	10.16				
34	0.554	0.104	1.804	9.552	1.489	0.093	0.671	10.70				
36.5	0.540	0.095	1.849	10.44	1.354	0.089	0.738	11.18	4.471	0.057	0.223	17.42
44.5	0.462	0.072	2.161	13.88	1.289	0.077	0.775	12.86	3.445	0.053	0.290	18.72
60.5	0.264	0.040	3.782	24.53	1.047	0.058	0.954	17.02	2.010	0.046	0.497	21.62
88									1.228	0.036	0.813	27.70



(A)



(B)



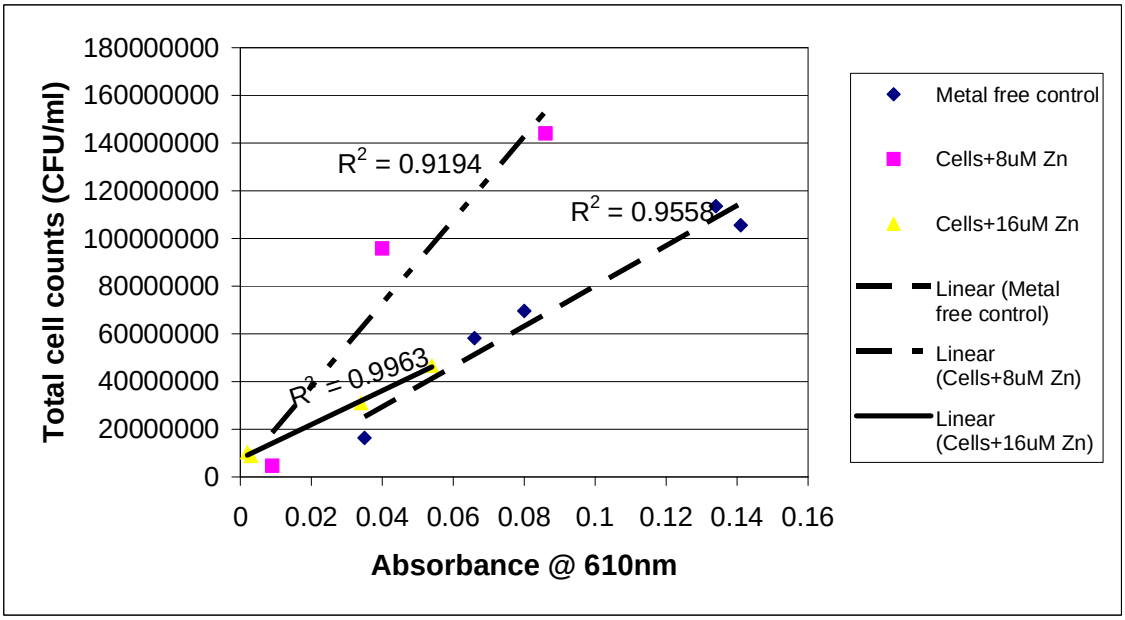
(C)

Figure E2. Determination of μ_{max} and K_S from Lineweaver and Burke plot for *Pseudomonas* sp. strain JM001 in presence of (A) 0, (B) 8 μM, and (C) 16 μM of zinc in medium A.

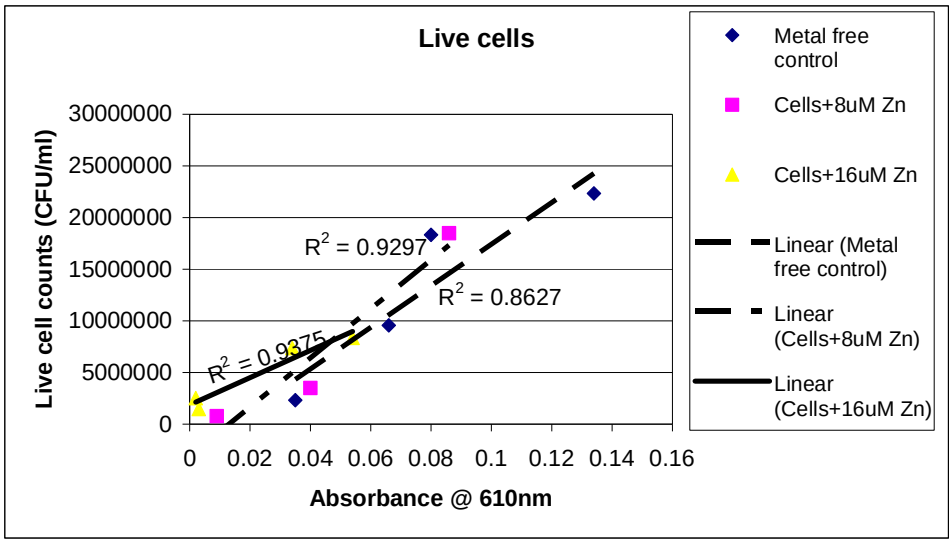
Intercept of each plot= $\frac{1}{\mu_{max}}$ and slope= $\frac{K_S}{\mu_{max}}$.

Table E7. Calculation of yield for metal free control and 8 and 16 µM Zn concentrations.

Sample ID	(Absorbance, M at t=88h) –(Absorbance, M_0 at t=0)	(A_0 at t=0)- (Concentration of acetate, A at t=88h)	$Y = \frac{(M - M_0)}{(A_0 - A)}$
Metal free control	0.127	4.572	0.027
Cells+ 8 µM Zn	0.085	3.866	0.021
Cells+ 16 µM Zn	0.051	3.388	0.015



(A)



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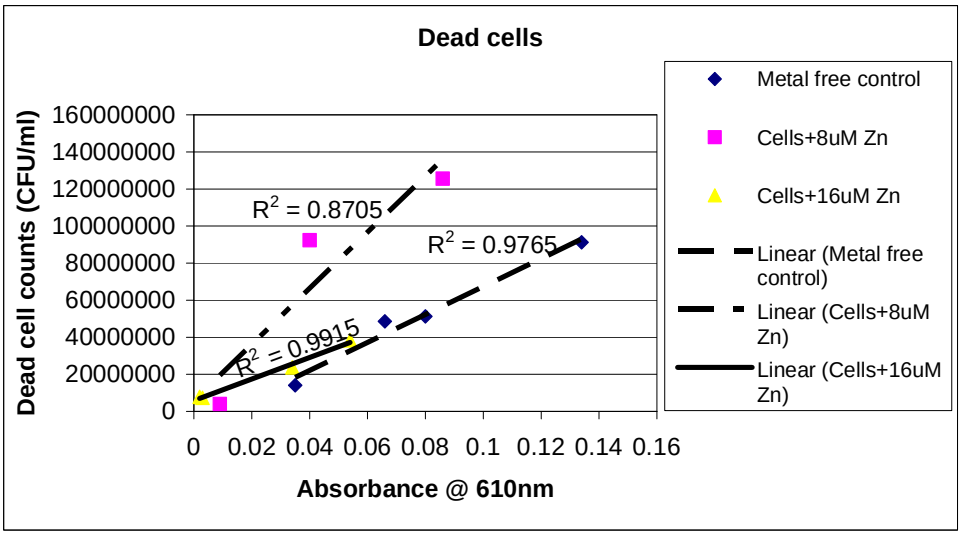

(C)

Figure E3. (A) The total cell numbers, (B) The number of live cells only, and (C) The number of dead cells only as a function of absorbance for *Pseudomonas* sp. strain JM001 grown in medium A.

APPENDIX F

DATA OBTAINED FROM KINETIC STUDY OF *PSEUDOMONAS* SP. STRAIN JM001 IN MEDIUM B

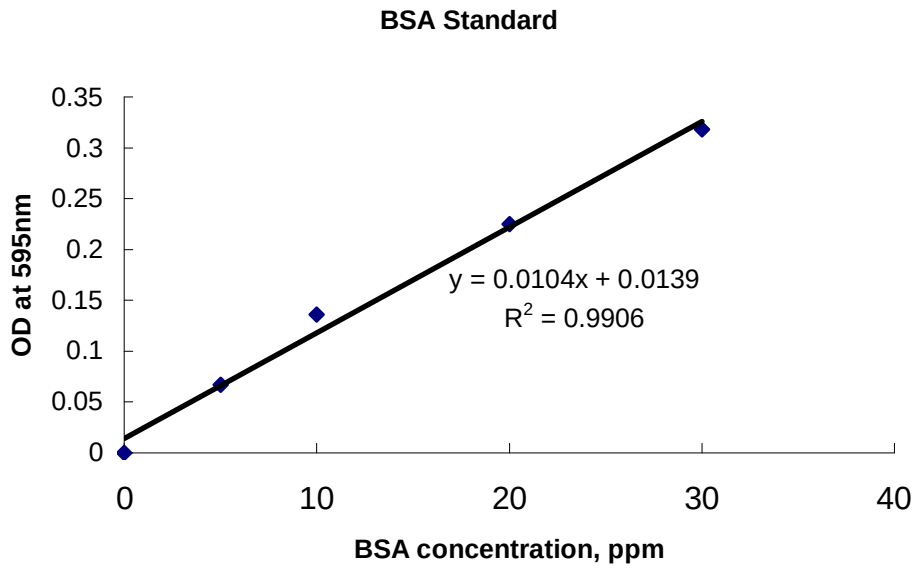

Figure F1. Bovine serum albumin protein standard curve used to convert absorbance at 595 nm to protein concentration for *Pseudomonas* sp. strain JM001 grown in medium B.

Table F1. Protein concentration of *Pseudomonas* sp. strain JM001 in medium B at 35°C.

	Proetin concentration (ppm)					
time (h)	Cells only control	Cells + 0.1mM Zn	Cells + 0.25mM Zn	Cells + 0.5mM Zn	Cells + 0.8mM Zn	Cells + 1mM Zn
0	0.000	0.000	0.000	0.000	0.000	0
8	7.173	0.000	0.000	0.000	0.000	0
12	12.606	1.740	0.106	0.000	0.000	0
15	16.452	4.673	2.894	0.250	0.000	0
17	19.000	8.279	5.731	0.779	0.106	0
20	25.394	13.038	9.721	1.356	0.731	0
24	34.192	22.750	18.279	4.385	1.260	0
28	41.452	32.654	27.365	8.279	3.760	0
32	47.654	38.856	35.106	18.279	8.471	0
38	53.327	48.038	44.192	33.663	9.769	0
42	55.683	51.740	48.663	40.202	9.913	0
50	55.538	51.404	48.375	45.827	9.769	0
55	54.288	52.125	47.990	46.885	9.721	0
65	51.019	49.817	44.962	43.135	9.577	0

Table F2. Zn concentrations in mM for *Pseudomonas* sp. strain JM001 in medium B at 35°C.

	Zn concentration (mM)					
time (h)	Cells only control	Cells +0.1mM Zn	Cells +0.25mM Zn	Cells +0.5mM Zn	Cells +0.8mM Zn	Cells +1mM Zn
0	0	0.084	0.229	0.443	0.755	0.888
8	0	0.081	0.228	0.435	0.746	0.867
12	0	0.068	0.214	0.427	0.720	0.868
15	0	0.047	0.197	0.401	0.689	0.865
17	0	0.029	0.164	0.403	0.670	0.795
20	0	0.024	0.114	0.331	0.652	0.795
24	0	0.019	0.063	0.302	0.520	0.790
28	0	0.012	0.020	0.237	0.396	0.779
32	0	0.009	0.015	0.174	0.379	0.809
38	0	0.006	0.012	0.170	0.383	0.766
42	0	0.002	0.010	0.150	0.379	0.782
50	0	0.004	0.010	0.105	0.360	0.790
55	0	0.002	0.011	0.080	0.356	0.782
65	0	0.001	0.013	0.064	0.353	0.793

Table F3. Aqeous acetate concentrations using ion chromatography for *Pseudomonas* sp. strain JM001 in medium B at 35°C.

	Acetate concentrations (mM)					
time (h)	Cells only control	Cells + 0.1mM Zn	Cells + 0.25mM Zn	Cells + 0.5mM Zn	Cells + 0.8mM Zn	Cells + 1mM Zn
0	15.515	15.475	15.675	15.739	15.610	15.651
8	13.461	15.295	15.576	15.563	15.586	15.553
12	12.403	14.688	15.380	15.441	15.481	15.569
15	11.037	13.651	14.427	15.231	15.203	15.454
17	8.742	12.342	13.281	14.627	14.698	15.363
20	6.837	10.525	12.342	12.990	14.424	15.325
24	5.092	8.786	10.966	12.044	12.963	15.292
28	4.258	6.329	8.566	10.156	11.773	15.332
32	3.247	5.732	7.410	8.661	10.651	15.271
38	1.292	4.315	5.915	7.403	9.498	15.261
42	0.586	2.908	4.315	5.692	9.427	15.244
50	0.234	1.481	3.281	4.241	9.363	15.224
55	0.000	0.424	2.142	2.620	9.217	15.203
65	0.000	0.220	1.369	1.586	9.075	15.193

Table F4. Protein concentration of *Pseudomonas* sp. strain JM001 in medium B at 22°C.

	Proetin concentration (ppm)						
time (h)	Cells only control	Cells + 0.1mM Zn	Cells + 0.25mM Zn	Cells + 0.5mM Zn	Cells + 0.8mM Zn	Cells + 1mM Zn	Cells + 1mM Zn
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
10	9.000	3.471	0.779	0.000	0.000	0.000	0.000
12.5	13.038	4.817	1.019	0.058	0.000	0.000	0.000
15	15.827	7.029	1.788	0.394	0.000	0.000	0.000
17.5	22.654	10.442	2.510	0.731	0.000	0.000	0.000
19.5	27.558	11.788	3.760	1.115	0.058	0.000	0.000
22.5	35.010	18.423	4.913	1.260	0.298	0.000	0.000
25	38.567	26.212	6.644	1.740	0.298	0.000	0.000
28	44.288	32.606	9.096	2.942	0.731	0.000	0.000
32	48.327	41.452	16.885	7.077	0.923	0.000	0.000
35	48.615	44.529	27.942	9.721	4.000	0.000	0.000
41.5	48.135	45.490	37.558	19.865	10.779	0.202	0.000
44.5	48.038	45.106	39.577	24.769	12.798	2.558	0.000
47.5	47.990	44.625	41.788	31.740	12.269	2.606	0.000
50	47.365	44.481	43.087	32.846	12.654	2.510	0.000
71	47.173	44.096	43.808	33.279	12.221	0.875	0.000

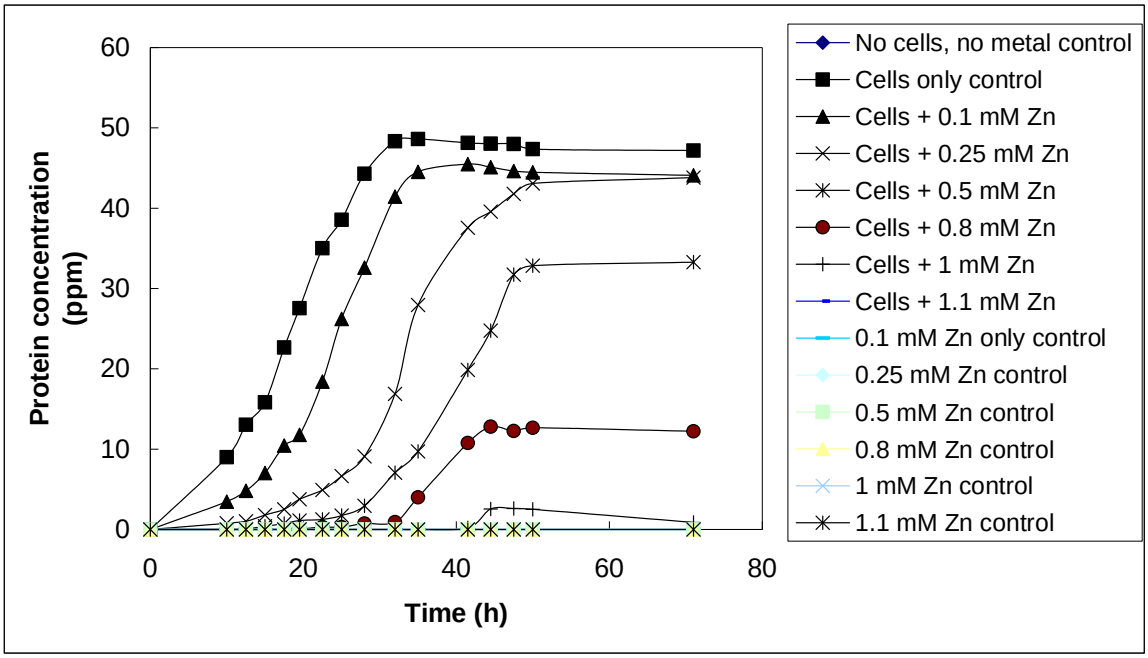

Figure F2. Effects of Zn on the growth of *Pseudomonas* sp. strain JM001 in medium B at 22 ºC; the total cell protein was measured at absorbance 595 nm. Data points are averages of duplicates.

Table F5. Zn concentrations in mM for *Pseudomonas* sp. strain JM001 in medium B at 22°C.

	Zn concentration (mM)						
time (h)	Cells only control	Cells + 0.1mM Zn	Cells + 0.25mM Zn	Cells + 0.5mM Zn	Cells + 0.8mM Zn	Cells + 1mM Zn	Cells + 1mM Zn
0	0.000	0.082	0.209	0.467	0.714	0.956	1.074
10	0.000	0.082	0.214	0.429	0.671	0.935	1.015
12.5	0.000	0.082	0.187	0.397	0.655	0.913	0.972
15	0.000	0.082	0.198	0.375	0.644	0.908	0.924
17.5	0.000	0.081	0.182	0.365	0.623	0.848	0.838
19.5	0.000	0.078	0.182	0.354	0.639	0.838	0.768
22.5	0.000	0.074	0.155	0.332	0.526	0.687	0.703
25	0.000	0.065	0.133	0.311	0.478	0.671	0.666
28	0.000	0.060	0.123	0.311	0.456	0.628	0.628
32	0.000	0.054	0.096	0.289	0.429	0.607	0.617
35	0.000	0.054	0.107	0.279	0.392	0.580	0.607
41.5	0.000	0.051	0.107	0.160	0.375	0.547	0.601
44.5	0.000	0.035	0.080	0.112	0.354	0.494	0.558
47.5	0.000	0.010	0.064	0.096	0.354	0.445	0.547
50	0.000	0.001	0.047	0.074	0.338	0.381	0.526
71	0.000	0.000	0.004	0.064	0.327	0.370	0.542

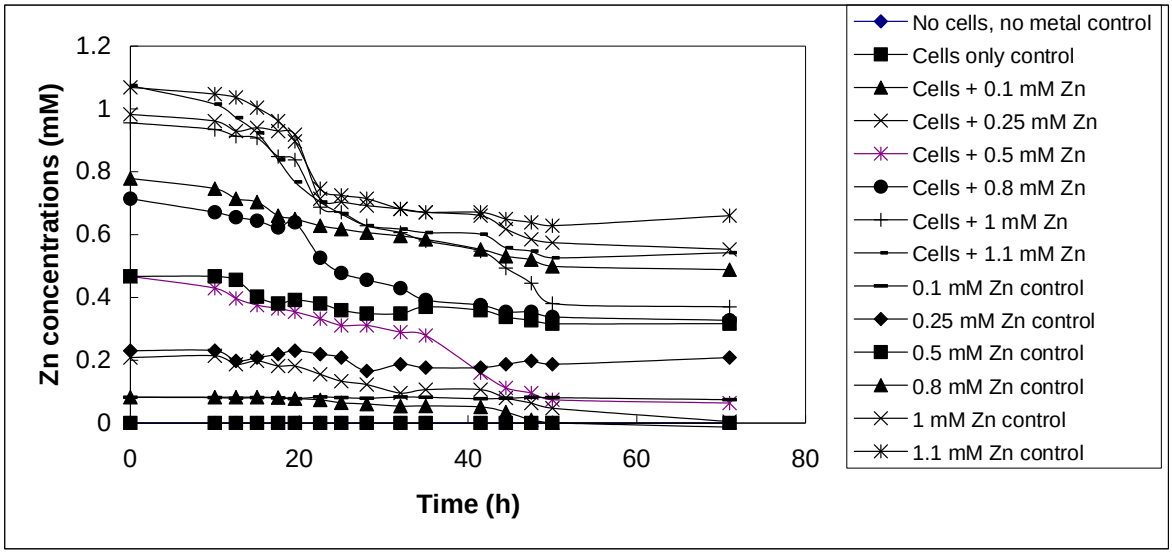

Figure F3. Aqueous Zn concentrations during the growth of *Pseudomonas* sp. strain JM001 for in medium B at 22°C. The points are the averages of duplicates.

Table F6. Aqeous acetate concentrations using ion chromatography for *Pseudomonas* sp. strain JM001 in medium B at 22°C.

	Acetate concentrations (ppm)						
time (h)	Cells only control	Cells + 0.1mM Zn	Cells + 0.25mM Zn	Cells + 0.5mM Zn	Cells + 0.8mM Zn	Cells + 1mM Zn	Cells + 1mM Zn
0	15.934	15.922	15.775	15.966	15.954	15.851	15.841
10	13.310	14.529	15.566	15.820	15.915	15.808	15.751
12.5	12.705	14.198	15.002	15.641	15.851	15.768	15.724
15	12.246	13.886	14.676	15.571	15.739	15.753	15.666
17.5	8.583	13.437	14.192	15.185	15.547	15.727	15.631
19.5	7.180	12.742	13.731	14.956	15.183	15.675	15.685
22.5	5.681	10.227	13.275	14.636	14.969	15.653	15.741
25	4.912	8.785	12.420	14.454	14.825	15.480	15.686
28	4.161	7.129	10.434	13.276	14.500	15.427	15.624
32	3.269	5.685	8.398	11.044	13.380	15.180	15.654
35	2.181	4.546	6.564	9.783	12.224	14.910	15.663
41.5	1.500	2.315	4.024	7.547	10.369	14.624	15.593
44.5	0.517	1.449	3.188	6.390	9.612	14.137	15.634
47.5	0.268	0.575	2.395	5.273	9.085	13.802	15.688
50	0.012	0.023	1.653	4.619	8.822	13.642	15.673
71	0.000	0.000	0.564	3.410	8.563	13.525	15.683

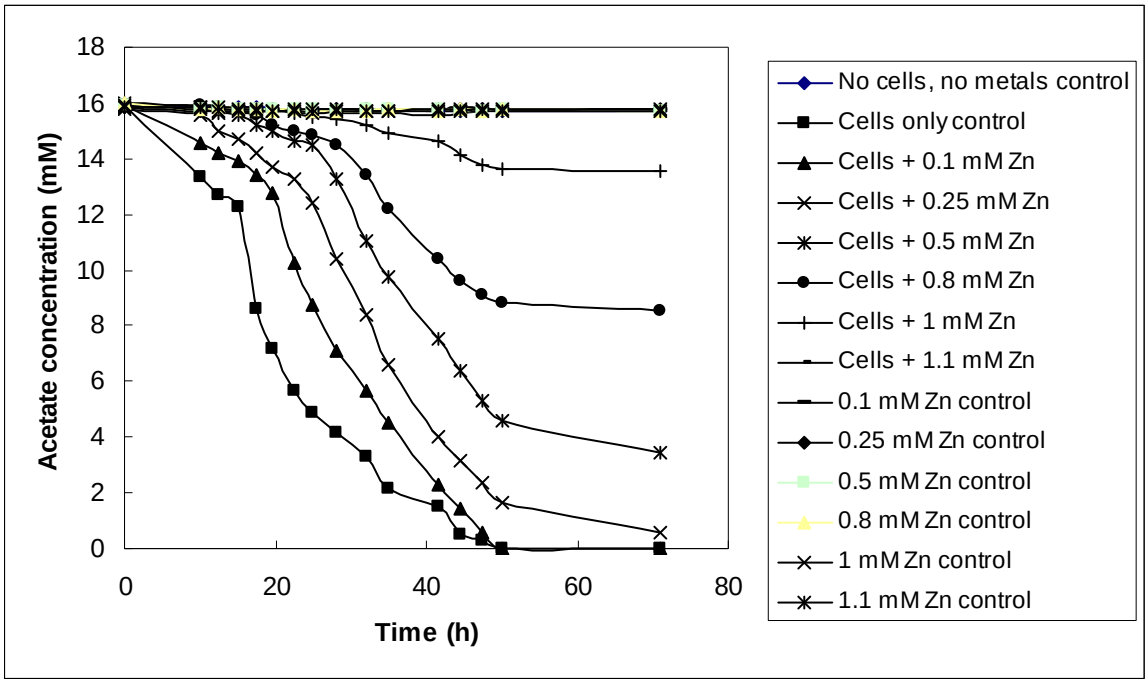

Figure F4. Aqueous acetate concentrations during the growth of *Pseudomonas* sp. strain JM001 in presence of Zn in medium B at 22°C.