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Bioremediation of Organic and Metal Co-contaminated Environments: Effects of Metal Toxicity, Speciation, and Bioavailability on Biodegradation

Todd R. Sandrin and Douglas R. Hoffman

Department of Biology and Microbiology, University of Wisconsin Oshkosh, Oshkosh, Wisconsin, USA, Email: sandrin@uwosh.edu

1. Introduction

Forty percent of the hazardous waste sites on the U. S. Environmental Protection Agency's National Priority List (NPL) are co-contaminated with metal and organic pollutants (Sandrin et al. 2000). Metals most frequently found at Superfund sites include arsenic, barium, cadmium, chromium, lead, mercury, nickel and zinc. Common organic co-contaminants include petroleum, chlorinated solvents, pesticides and herbicides. Conventional approaches to removing the organic pollutants at these sites, such as pump and treat, are costly and often ineffective (NRC 1994). Bioremediation is a viable alternative to conventional technologies, but metal toxicity at co-contaminated sites may limit its utility. Many studies report that metals inhibit general microbial activity (e.g., litter decomposition, methanogenesis, acidogenesis, nitrogen transformation), but a few have specifically investigated the impact of metals on organic pollutant biodegradation. The fact, that metals affect a myriad of microbial activities suggests that metals have the potential to affect the biodegradation of organics in co-contaminated environments. In some studies, metals have no impact or have a stimulatory effect on microbial activity. Thus, the effect of metals on organic pollutant biodegradation remains poorly characterized. This review discusses: 1) the toxicity of metals to microorganisms, 2) the roles metal speciation and bioavailability play in governing the extent to which metals affect organic pollutant biodegradation, 3) reported effects of metals on aerobic and anaerobic biodegradation, 4) patterns in which metals affect biodegradation, and 5) approaches to increasing organic biodegradation in co-contaminated systems.

2. Metal Toxicity to Microorganisms

An understanding of mechanisms of metal toxicity is essential in anticipating to what extent, metals will inhibit pollutant biodegradation by a particular population of microorganisms. A lucid and comprehensive understanding of modes of metal toxicity may lead to the development of novel technologies to mitigate metal toxicity in metal and organic co-contaminated environments. Mechanisms of metal toxicity to microorganisms have been studied extensively, and several excellent reviews are now available (Nies 1992; Rouch et al. 1995a; Ji and Silver 1995; Silver and Phung 1996; Rosen 1996; Silver 1996; Nies 1999). Despite this sizable body of work, the precise mechanisms of the toxicity of many metals remain unclear. Hence Nies so astutely observed in his review of microbial metal toxicity and resistance, “We are just beginning to understand the metabolism of heavy metals” (Nies 1999).

2.1 Metal Chemistry

Incompletely filled d-orbitals allow metals to form complex compounds with organic ligands, such as the proteins (Nies 1999), nucleic acids, and cell wall materials of microorganisms (Toth and Tomasovicova 1989). This binding is beneficial in the case of some metals such as calcium, magnesium, manganese, copper, and zinc. These metals serve as enzyme co-factors in complex biochemical processes; however, at high concentrations, the same essential metals can form non-specific complexes with organic ligands. This leads to toxicity. In addition, some metals, such as mercury, cadmium, and silver, form such strong complexes with organic ligands that they are rarely used in biochemical process (Nies and Silver 1995). For example, only one enzyme, carbonic anhydrase utilized by a marine diatom, is known to use cadmium as a cofactor (Lane and Morel 2000; Lane et al. 2005).

Metals bind to functional groups of biological molecules with varying affinities and can be classified as either hard or soft. Hard metals (e.g., sodium, potassium, magnesium, calcium, manganese and iron) are small cations that are not readily polarizable, while soft metals (e.g., copper, lead, cadmium, mercury, and silver) are larger cations that are very polarizable due to their large number of electrons (Hughes and Poole 1991). Hard metals prefer to bind to ligands containing oxygen, such as carboxylic acid, sulfate, and phosphate functional groups. In contrast, soft metals preferentially bind to ligands containing sulfur, such as the sulfhydryl ($-SH_2$) groups found in proteins.

2.2 Heavy Metal Uptake

Of course, for a metal to bind to an essential protein, nucleic acid or membrane component, the metal must first be taken up by the cell. Differentiating between

toxic and non-toxic metals is a complex cellular process. The structures of many metals, toxic and non-toxic, are remarkably similar. For instance, manganese, iron, cobalt, nickel, copper and zinc have ionic diameters which vary by less than 14% (from 138-160 pm) (CRC 1991). In addition, each of these cations is divalent. Serving as further disguise, some metals can coordinate with oxygen in such a way as to resemble common innocuous molecules. Arsenate (AsO_4^{3-}) resembles phosphate (PO_4^{3-}), while chromate (CrO_4^{2-}) is remarkably similar to sulfate (SO_4^{2-}). Evolution has endowed microorganisms with effective mechanisms to distinguish between toxic and non-toxic metals. Two general types of uptake mechanisms have been described: 1) selective, substrate-specific uptake systems that are slow and require considerable energy (ATP) and 2) substrate-non-specific, fast systems that transport metals using a chemiosmotic gradient rather than ATP (Nies and Silver 1995). Fast, nonspecific uptake systems are constitutively expressed, while slower, specific, energy-consuming uptake systems are inducible (Nies and Silver 1995).

An example of a fast, non-specific uptake system is the magnesium uptake system, CorA, found in Gram negative bacteria, archaea and baker's yeast. This system is responsible for the uptake of a variety of cations in addition to magnesium, including nickel, cobalt, zinc, and manganese. Two common fast transport systems that heavy metals often exploit to enter cells are Pit (phosphate inorganic transport) and the sulfate transport system. Arsenate is able to enter via Pit, while chromate can infiltrate cells via the sulfate transport system (Nies 1999). Slow, specific metal uptake systems include the P-type ATPases that transport zinc, manganese, cadmium, magnesium, calcium, potassium, copper, lead and silver (Fagan and Saier 1994).

2.3 Interaction of Heavy Metals with Cellular Components

Even highly evolved, substrate-specific uptake mechanisms may not prevent entry of a toxic metal into a cell. Once inside, metal cations can interact with various cellular components including cell membranes, proteins, and nucleic acids. Interactions of metals with these cellular components have been linked to toxicity (Toth and Tomasovicova 1989). Baath (1989) reported that copper and zinc disrupt the cell membrane. Furthermore, an early step in metal uptake may be binding of the metal to the cell surface. The outer membrane of Gram negative bacteria effectively complexes metals including sodium, calcium, magnesium, strontium, nickel, manganese, lead, and iron. In addition, the thin layer of peptidoglycan of Gram negative bacteria can bind metals, albeit not nearly as effectively as the thick layer of peptidoglycan of Gram positive bacteria which contain teichoic acid, a potent metal chelator (Beveridge and Doyle 1989).

The ability of cell surfaces to complex metals lies in their net negative charge at normal growth pH. In Gram negative bacteria, the net negative charge of the cell surface results from the phosphate and carboxyl groups of

lipopolysaccharide molecules (Goldberg et al. 1983; Volesky 1990), while the negative charge in Gram positive bacteria results largely from teichoic acid. A more negative cell surface charge may more effectively attract and bind toxic metal cations, thus rendering the cell more susceptible to the toxic effects of the metal (Rai et al. 1996).

Interactions of metals with cellular proteins are more commonly implicated in causing toxicity than interactions of metals with membranes. Toxic metals readily bind to sulfhydryl groups of proteins. As mentioned above, soft cations, such as cadmium and lead, preferentially bind sulfur-containing ligands over oxygen-containing ones. This binding affects the structure and function of the protein. Interestingly, the dissociation constants of soft metals complexed to sulfhydryl groups correlate well with the minimum inhibitory concentration (MIC) of the same metals. This illustrates the importance of the ability of a metal to bind to proteins in determining its toxicity (Nies 1999).

2.4 Substitution for Essential Metabolites

If both hard and soft cations are present, soft cations will replace hard cations on ligands. This can lead to substitution of an essential metabolite by a toxic metal. The resemblance of some deleterious heavy metals to essential metals not only allows them to enter the cell, but also to exert their toxic effects via substitution. For example, chromate is often mistakenly used as sulfate, arsenate is mistaken for phosphate, cadmium is used as an enzyme co-factor instead of zinc or calcium, nickel and cobalt replace iron, and zinc is commonly mistaken for magnesium. All of these mistaken identities result in the construction of an unstable, inhibited, or non-functional enzyme or other biological molecule (Nies and Silver 1995; Nies 1999).

2.5 Heavy Metal Induced Oxidative Stress

The toxicity of heavy metals to Gram negative bacteria is due, in part, to oxidative stress (Kachur et al. 1998). Metal cations may bind two glutathione molecules, forming a bis-glutathione molecule that reacts with diatomic oxygen to yield oxidized bis-glutathione, the metal cation, and hydrogen peroxide. The oxidized bis-glutathione must be reduced using NADPH; however, the metal cation released in the process is once again free to re-initiate this process and continue imposing considerable oxidative stress on the cell (Nies 1999).

3. Metal Speciation and Bioavailability

Despite the substantial information concerning mechanisms of metal toxicity, meaningful quantitative data on responses of pollutant-degrading

microorganisms to metals is still lacking. This is largely due to the fact that making comparisons between concentrations of metals that inhibit biodegradation reported by different studies is exceedingly difficult. For example, five orders of magnitude separate literature reports of concentrations of zinc that inhibit biodegradation (Table 1). While it should be noted that not all studies attempted to pinpoint the lowest concentration that inhibits biodegradation, many disparities likely result from variations in metal bioavailability between studies.

Most commonly, metal inhibition of biodegradation has been related to the total metal concentration in a system. This may not be the most appropriate predictor of metal toxicity, as suggested by the wide range of total metal concentrations reported to inhibit biodegradation (Table 1). The concentration of the most bioavailable form (i.e., species) of the metal (commonly held to be the free, ionic, solution-phase metal species) is likely a better indicator of the extent to which a metal will inhibit biodegradation. In media commonly used to study metal toxicity, metals exist in a number of different species in addition to the free, ionic species. Depending on medium characteristics described below, metals can exist as free ions (possibly with different oxidation states), hydroxo-complexes, or be complexed to organic or inorganic ligands (Hughes and Poole 1991; Twiss et al. 2001). The distribution of these different metal forms is referred to as metal speciation.

3.1 Factors Affecting Metal Speciation and Toxicity

It is well-established that different metal species vary in their biological reactivity (Hughes and Poole 1991; Traina and Laperche 1999; Twiss et al. 2001; Behra et al. 2002). Certain metal species are more likely than others to associate with biochemically active sites (e.g., enzymes) and initiate biological responses. In this review, we define bioavailability as the ability of a metal species to access these sites. In the case of organic-degrading microbes, interactions of metals with enzymes results in the inactivation of enzymes necessary for biodegradation (e.g., monoxygenases, dioxygenases) or of enzymes used in the general metabolism (Nies 1999; Baldrian et al. 2000; Sandrin and Maier 2003). There is still some debate as to which metal species are most bioavailable. Currently, though, there is a considerable amount of evidence suggesting that free, ionic, solution-phase metal species are most bioavailable (Angle and Chaney 1989; Traina and Laperche 1999; Behra, et al. 2002). Despite being highly bioavailable, the free ionic metal concentration may represent only a small fraction of the total metal species distribution in a solid or aqueous medium. For these reasons, it is of paramount importance to understand what properties of metal toxicity test systems impact metal speciation and metal bioavailability. Two of the most important of these properties are medium chemical composition and pH.

Table 1. Reported metal concentrations that inhibit aerobic (A) and anaerobic (B) biodegradation and/or transformation of organic pollutants

A.

Metal	Organic	Lowest metal conc. reported to reduce biodegradation	Microbe(s) Studied	Environment	pH	Reference
Cd^{2+}	2,4-D	0.060 mg/g[a]	*Alcaligenes eutrophus* JMP134	soil microcosms	8.2	Roane et al. (2001)
Cd^{2+}	2,4-D	0.060 mg/g[a]	*Alcaligenes eutrophus* JMP134	field-scale bioreactors	8.2	Roane et al. (2001)
Cd^{2+}	2,4-DME	0.100 mg/l[a]	indigenous community	sediment (microcosm)	6.5	Said and Lewis (1991)
Cd^{2+}	2,4-DME	0.629 mg/l[a]	indigenous community	aufwuchs[e] (microcosm)	5.6	Said and Lewis (1991)
Cd^{2+}	PHEN	1 mg/l[b]	indigenous community	soil microcosms	7.6	Maslin and Maier (2000)
Cd^{2+}	NAPH	1 mg/l[b]	*Burkholderia* sp.	dilute mineral salts medium containing 1.4 mM phosphate	6.5	Sandrin et al. (2000)
Cd^{2+}	diesel fuel	1.1 mg/l[a]	Enrichment culture	MES-buffered mineral salts medium containing 0.33 mM phosphate	6.8	Riis et al. (2002)
Cd^{2+}	2,4-D	>3 mg/l[a]	*Alcaligenes eutrophus* JMP134	mineral salts medium	6.0	Roane et al. (2001)
Cd^{2+}	2,4-D	24 mg/l[a]	*Alcaligenes eutrophus* JMP134	mineral salts medium containing cadmium resistant isolate	6.0	Roane et al. (2001)

Cd^{2+}	4CP, 3CB, 2,4D, XYL, IPB, NAPH, BP	<25.3 - 50.6 mg/l[a,c]	*Alcaligenes* spp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael et al. (1993)
Cd^{2+}	TOL	37 mg/l[a]	*Bacillus* sp.	mineral salts medium containing 36 mM phosphate	5.9	Amor et al. (2001)
Cd^{2+}	EDTA	562 mg/l[d]	Enrichment culture	MOPS-buffered minimal medium	7.0	Thomas et al. (1998)
Co^{2+}	4CP,3CB,2,4D, XYL, IPB, NAPH, BP	<13.3 - 1,330 mg/l[a, c]	*Alcaligenes* spp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael et al. (1993)
Co^{2+}	NTA	116.9 mg/l[d]	enrichment culture	PIPES-buffered mineral salts medium	7.0	White and Knowles (2003)
Co^{2+}	EDTA	292 mg/l[d]	Enrichment culture	MOPS-buffered minimal medium	7.0	Thomas et al. (1998)
Cr^{3+}	2,4-DME	0.177 mg/l[a]	indigenous	aufwuchs[e] (microcosm)	6.1	Said and Lewis (1991)
Cr^{6+}	diesel fuel	2.32 mg/l[a]	Enrichment culture	MES-buffered mineral salts medium containing 0.33 mM phosphate	6.8	Riis et al. (2002)
Cr^{6+}	4CP, 3CB, 2,4D, XYL, IPB, NAPH, BP	<131 mg/l[a c]	*Alcaligenes* spp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael et al. (1993)
Cu^{2+}	PH	0.01 mg/l[a]	*Acinetobacter calcoaceticus* AH strain	bioreactor medium containing 0.15 mM phosphate	7.8	Nakamura and Sawada (2000)
Cu^{2+}	2,4-DME	0.027 mg/l[a]	indigenous community	aufwuchs[e] (microcosm)	5.0	Said and Lewis (1991)
Cu^{2+}	2,4-DME	0.076 mg/l[a]	indigenous community	sediment (microcosm)	6.1	Said and Lewis (1991)

Cu^{2+}	NTA	3.18 mg/l[d]	*Chelatobacter heintzii* ATCC 29600	PIPES-buffered mineral salts medium	7.0	White and Knowles (2000)
Cu^{2+}	crude oil	6.30 mg/l[a]	*Pseudomonas* sp.	mineral salts medium containing 31 mM phosphate	7.2	Benka-Coker and Ekundayo (1998)
Cu^{2+}	diesel fuel	6.35 mg/l[a]	Enrichment culture	MES-buffered mineral salts medium containing 0.33 mM phosphate	6.8	Riis et al. (2002)
Cu^{2+}	PHB	8 mg/l[b]	*Acidovorax delafieldii*	agar plates containing 4.70 mM phosphate	6.9	Birch and Brandl (1996)
Cu^{2+}	crude oil	11.25 mg/l[a]	*Micrococcus* sp.	mineral salts medium containing 31 mM phosphate	7.2	Benka-Coker and Ekundayo (1998)
Cu^{2+}	4CP, 3CB, 2,4-D, XYL, IPB, NAPH, BP	<14.3 -71.6 mg/l[a,c]	*Alcaligenes* sp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael et al. (1993)
Cu^{2+}	NTA	127.1 mg/l[d]	enrichment culture	PIPES-buffered mineral salts medium	7.0	White and Knowles (2003)
Cu^{2+}	NTA	127.1 mg/l[d]	*Mesorhizobium* sp. NCIMB 13524	PIPES and phosphate-buffered mineral salts media	7.0	White and Knowles (2003)
Cu^{2+}	EDTA	318 mg/l[d]	Enrichment culture	MOPS-buffered minimal medium	7.0	Thomas et al. (1998)
Hg^{2+}	2,4-DME	0.002 mg/l[a]	indigenous community	aufwuchs[e] (microcosm)	6.8	Said and Lewis (1991)
Hg^{2+}	diesel fuel	4 mg/l[a]	Enrichment culture	MES-buffered mineral salts medium containing 0.33 mM phosphate	6.8	Riis et al. (2002)
Hg^{2+}	4 CP, 3 CB, 2,4-D, XYL, IPB, NAPH, BP	<45.2 - 226 mg/l[a,c]	*Alcaligenes* sp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael et al. (1993)

Mn^{2+}	crude oil	28.2 mg/l[a]	*Micrococcus* sp.	mineral salts medium containing 31 mM phosphate	7.2	Benka-Coker and Ekundayo (1998)
Mn^{2+}	crude oil	317.0 mg/l[a]	*Pseudomonas* sp.	mineral salts medium containing 31 mM phosphate	7.2	Benka-Coker and Ekundayo (1998)
Ni^{2+}	4 CP, 3 CB, 2,4-D, XYL, IPB, NAPH, BP	5.18 - 10.3 mg/l[a,c]	*Alcaligenes* sp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael et al. (1993)
Ni^{2+}	diesel fuel	5.9 mg/l[a]	Enrichment culture	MES-buffered mineral salts medium containing 0.33 mM phosphate	6.8	Riis et al. (2002)
Ni^{2+}	TOL	20 mg/l[a]	*Bacillus* sp.	mineral salts medium containing 36 mM phosphate	5.9	Amor et al. (2001)
Ni^{2+}	NTA	117.4 mg/l[d]	*Mesorhizobium* sp. NCIMB 13524	PIPES and phosphate-buffered mineral salts media	7.0	White and Knowles (2003)
Ni^{2+}	EDTA	293 mg/l[d]	Enrichment culture	MOPS-buffered minimal medium	7.0	Thomas et al. (1998)
Pb^{2+}	crude oil	1.41 mg/l[a]	*Micrococcus* sp.	mineral salts medium containing 31 mM phosphate	7.2	Benka-Coker and Ekundayo (1998)
Pb^{2+}	crude oil	2.80 mg/l[a]	*Pseudomonas* sp.	mineral salts medium containing 31 mM phosphate	7.2	Benka-Coker and Ekundayo (1998)
Pb^{2+}	diesel fuel	41.4 mg/l[a]	Enrichment culture	MES-buffered mineral salts medium containing 0.33 mM phosphate	6.8	Riis et al. (2002)
Zn^{2+}	2,4-DME	0.006 mg/l[a]	indigenous community	sediment (microcosm)	6.4	Said and Lewis (1991)
Zn^{2+}	2,4-DME	0.041 mg/l[a]	indigenous community	aufwuchs[e] (microcosm)	5.6	Said and Lewis (1991)

Zn^{2+}	crude oil	0.43 mg/l[a]	*Pseudomonas* sp.	mineral salts medium containing 31 mM phosphate	7.2	Benka-Coker and Ekundayo (1998)
Zn^{2+}	crude oil	0.46 mg/l[a]	*Micrococcus* sp.	mineral salts medium containing 31 mM phosphate	7.2	Benka-Coker and Ekundayo (1998)
Zn^{2+}	TOL	2.8 mg/l[a]	*Bacillus* sp.	mineral salts medium containing 36 mM phosphate	5.9	Amor et al. (2001)
Zn^{2+}	pH	10 mg/l[a]	*Acinetobacter calcoaceticus* AH strain	bioreactor medium containing 0.15 mM phosphate	7.8	Nakamura and Sawada (2000)
Zn^{2+}	4 CP, 3 CB, 2,4-D, XYL, IPB, NAPH, BP	<29.5 - 736 mg/l[a,c]	*Alcaligenes* sp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael et al. (1993)
Zn^{2+}	diesel fuel	65.4 mg/l[a]	Enrichment culture	MES-buffered mineral salts medium containing 0.33 mM phosphate	6.8	Riis et al. (2002)
Zn^{2+}	NTA	130.8 mg/l[d]	*Mesorhizobium* sp. NCIMB 13524	PIPES-buffered mineral salts media	7.0	White and Knowles (2003)

Abbreviations:
2,4D, 2,4-dichlorophenoxy acetic acid; 2,4-DME, 2,4-dichlorophenoxy acetic acid methyl ester; BP, biphenyl; CB, chlorobenzoate; CP, chlorophenol; EDTA, ethylenediaminetetraacetic acid; IPB, isopropylbenzene; MES, morpholinoethane sulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; NAPH, naphthalene; NTA, nitrilotriacetic acid; PH, phenol; PHB, poly (3-hydroxybutyrate); PHEN, phenanthrene; PIPES, Piperazine-N,N'-bis(2-ethanesulfonic acid); TOL, toluene; XYL, xylene

[a] value represents total metal added to system

[b] value represents solution phase concentration of metal present in system

[c] value represents Minimum Inhibitory Concentration (MIC) calculated by multiplying Maximum Tolerated Concentration (MTC) by a factor of 2.25. MIC = MTC*2.25.

[d] metal was complexed to a biodegradable organic (NTA or EDTA)

[e] floating algal mats

B.

Metal	Organic	Lowest metal conc. reported to reduce biodegradation	Microbe(s) Studied	Environment	pH	Reference
Cd^{2+}	HCB	0.001 mg/g[a]	indigenous community	microcosms containing contaminated sediment	NR	Jackson and Pardue (1998)
Cd^{2+}	TCA	0.01 mg/l[b]	indigenous community	laboratory soil microcosms containing rice paddy and bottomland hardwood soils	6.9-7.4	Pardue et al. (1996)
Cd^{2+}	TCA	0.2 mg/l[b]	indigenous community	laboratory soil microcosms containing organic matter-rich soil	6.8	Pardue et al. (1996)
Cd^{2+}	2CP, PH, BEN, 3CB	0.5-1.0 mg/l[a]	indigenous community	aqueous sediment enrichment in anaerobic growth medium	7.0	Kuo and Genthner (1996)
Cd^{2+}	TCE	5 mg/l[a]	*Burkholderia picketti* PK01	mineral salts medium containing 44 mM phosphate; denitrifying conditions	NR	Degraffenreid and Shreve (1998)
Cd^{2+}	2CP, 3CP	20 mg/l[a]	indigenous community	sediment slurry	7.0	Kong (1998)
Cr^{6+}	2CP, PH, BEN, 3CB	0.01-0.5 mg/l[a]	indigenous community	aqueous sediment enrichment in anaerobic growth medium	7.0	Kuo and Genthner (1996)
Cr^{6+}	2CP, 3CP	20 mg/l[a]	indigenous community	sediment slurry	7.0	Kong (1998)
Cr^{c}	OD	5,000 µg/g[a]	indigenous community	clay-containing sediment slurry	6.5	DeLaune et al. (1998)
Cu^{2+}	2CP, PH, BEN, 3CB	0.1-1.0 mg/l[a]	indigenous community	aqueous sediment enrichment in anaerobic growth medium	7.0	Kuo and Genthner (1996)
Cu^{2+}	2,4-DANT, RDX	4 mg/g[a]	indigenous community	soil slurry containing 50 mM phosphate buffer	6.5	Roberts et al. (1998)

Cu^{2+}	4-ADNT	8 mg/g[a]	indigenous community	soil slurry containing 50 mM phosphate buffer	6.5	Roberts et al. (1998)
Cu^{2+}	2CP, 3CP	20 mg/l[a]	indigenous community	sediment slurry	7.0	Kong (1998)
Hg^{2+}	2CP, PH, BEN, 3CB	0.1-1.0 mg/l[a]	indigenous community	aqueous sediment enrichment in anaerobic growth medium	7.0	Kuo and Genthner (1996)
Pb^{2+}	HCB	0.001 mg/g[a]	indigenous community	microcosms containing contaminated sediment	NR	Jackson and Pardue (1998)
Pb^{2+}	2,4-DANT, RDX	>1 mg/g[a]	indigenous community	soil slurry containing 50 mM phosphate buffer	6.5	Roberts et al. (1998)
Zn^{2+}	2,4-DANT	1.5 mg/g[a]	indigenous community	soil slurry containing 50 mM phosphate buffer	6.5	Roberts et al. (1998)
Zn^{2+}	PCP	2 mg/l[a]	indigenous community	anaerobic digester sludge in a liquid medium containing 0.6 mM phosphate	NR	Jin and Bhattacharya (1996)
Zn^{2+}	PCP	8.6 mg/l[a]	indigenous community	anaerobic enrichment cultures in serum bottles	NR	Majumdar et al. (1999)
Zn^{2+}	NB	10 mg/l[a]	indigenous community	anaerobic enrichment cultures in serum bottles	NR	Majumdar et al. (1999)

Abbreviations:
2,4-DANT, 2,4-diamino-6-nitrotoluene; 4-ADNT, 4-amino-2,6-dinitrotoluene; BEN, benzoate; CB, chlorobenzoate; CP, chlorophenol; HCB, hexachlorobenzene; NB, nitrobenzene; NR, not reported; OD, octadecane; PCP, pentachlorophenol; PH, phenol; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; TCA, trichloroaniline; TCE, trichloroethylene.

[a] value represents total metal added to system

[b] value represents solution phase concentration of metal present in system

[c] oxidation state not specified

3.1.1 Chemical Composition

To accurately characterize metal speciation, the chemical composition of the medium must be known. This requires use of a chemically defined medium to ensure that all components capable of interacting with metals are taken into consideration (Hughes and Poole 1991; Twiss et al. 2001). Many complex microbiological media contain extracts (e.g., yeast extract and beef extract) that vary in their precise chemical composition. Common to many studies investigating organic pollutant biodegradation, a minimal medium is often used. Minimal media typically consist of a solution of mineral salts amended with an organic pollutant targeted for degradation as the sole source of carbon (Springael et al. 1993; Benka-Coker and Ekundayo 1998; Amor et al. 2001; Roane et al. 2001; Sandrin and Maier 2002). Some studies have also used sediment or soil slurries taken directly from the environment to monitor the biodegradation of an added organic, while others have used a combination of these approaches by placing a defined amount of sediment or soil into a minimal medium containing an organic pollutant (Said and Lewis 1991; Pardue et al. 1996; Delaune et al. 1998; Kong 1998; Roberts et al. 1998; Maslin and Maier 2000). Regardless of the type of medium, the buffering system has a dramatic impact on metal speciation and bioavailability. Because buffers are often present at higher concentrations than other medium components and may contain agents that reduce metal bioavailability, their impact on metal speciation and bioavailability must be considered (Hughes and Poole 1991; Teresa et al. 2000; Vasconcelos and Leal 2002).

A variety of buffers have been used in studies examining effects of metals on biodegradation. Phosphate buffers, probably among the most common buffers used in microbiology, have been used in the majority of studies (Birch and Brandl 1996; Benka-Coker and Ekundayo 1998; Amor et al. 2001; Nakamura and Sawada 2000). Phosphate readily sequesters metals and reduces their bioavailability via the formation of insoluble metal-phosphate species. In fact, phosphate is so efficient at metal sequestration that it has been used as a metal-complexing agent in a few studies to reduce free ionic metal concentrations (Ruby et al. 1994; White and Knowles 2000). The remarkable ability of phosphate to reduce bioavailable metal concentrations is illustrated in Figure 1A, which shows predicted concentrations of free ionic metals as a function of phosphate concentration in a medium commonly used in biodegradation studies, Bushnell Haas medium (Difco™, Sparks, MD). With a relatively low phosphate concentration of 2.27 mM, 44% less free ionic cadmium exists in the medium containing phosphate than in the same medium not containing phosphate. Some metals are more sensitive to phosphate precipitation than others. As shown in Figure 1A, cobalt bioavailability is predicted to remain high (95% remains in the free, ionic form) as the phosphate concentration is raised to 15 mM, but the concentration of free, ionic nickel is predicted to fall to 21% of its concentration in the medium free of phosphate. Metal-phosphate species are quite insoluble, even at neutral to mildly acidic pH values.

The ability of phosphate buffers to precipitate metals has been taken for granted in several metal toxicity studies. In their review of metal speciation, Hughes and Poole (1991) describe the difficulty of detecting metal precipitates in a turbid culture. Metal-phosphate precipitates can present many problems, especially if culture turbidity is used as the measure of growth and biodegradation. Precipitates can easily be misinterpreted as cell biomass, making growth measurements misleading and inaccurate. In their study of nitrilotriacetic acid (NTA) biodegradation, White and Knowles avoided this problem by acidifying their samples prior to measuring culture turbidity (White and Knowles 2000; 2003). Lowering the pH dissolved any metal-phosphate precipitates present in the samples. Other techniques have been developed to overcome problems with phosphate precipitation. For example, Malakul *et al.* (1998) replaced phosphate with glycerophosphate. In this form, phosphate will not readily bind metals and cause precipitation. Glycerophosphate, though, can potentially serve as a carbon source for organic-degrading microbes, thus decreasing the effectiveness of pollutant biodegradation and confounding interpretation of biodegradation data based solely on biomass measurements. Metal-phosphate precipitation can also be reduced by decreasing the phosphate concentration. This allows higher metal levels to be tested while reducing precipitation and increasing bioavailability. Though, caution should be exercised as the buffering capacity of the medium will be compromised as the phosphate concentration is reduced.

Metals tend to remain more bioavailable in the presence of zwitterionic buffers (such as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MES (morpholinoethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), and PIPES (1,4-piperazinebis(ethanesulfonic acid)) than in the presence of phosphate buffers. This is due to the fact that these buffers do not interact with metals as strongly as phosphate buffers. At pH 7.2, Mash *et al.* (2003) reported that MES and MOPS buffers (each at 50 mM) did not complex copper, while HEPES (35 mM) showed some copper complexation. PIPES buffer (0.8 mM) did not complex copper (Vasconcelos et al. 1998). Despite its frequent use in metal toxicity studies, little metal complexation data is available for Tris-base (2-amino-2-(hydroxymethyl)-1,3-propanediol). Available data, however, suggest that Tris-base is capable of complexing many metals, though to what extent is not clear (Twiss et al. 2001). Because of their limited interaction with metals, many have recommended the use of MES, MOPS, and PIPES in metal toxicity studies, presuming studies are conducted in the operational pH range of the buffers (6.1-7.5) (Twiss et al. 2001; Mash et al. 2003).

While some buffers do not complex metals, many inorganic ligands, such as Cl^-, NO_3^-, OH^-, SO_3^- and SO_4^{2-}, have strong metal-complexing capabilities and high affinities for many metals. Metals complexed with these ligands usually remain soluble; however, their bioavailability is thought to be lower than free, ionic metals (Reed and Nonavinakere 1992; Janos 1993; Bianchini and Bowles 2002).

3.1.2 pH

Metal speciation and bioavailability are also dependent on pH. In general, metals are more bioavailable at acidic pH values (Hughes and Poole 1991; Twiss et al. 2001). Under acidic conditions, free ionic metal species are thought to be more prevalent, likely from the saturation of metal binding sites with protons (H^+). This saturation limits interactions between metals and potential metal-complexing ligands. Also, under basic conditions, metals tend to form hydroxy-metal complexes. Figure 1B illustrates the predicted pH-dependent loss of free ionic metal species in Bushnell Haas medium amended with a total concentration of 100 µM of one of several metals. Depending on the particular metal, hydroxo-metal complexes may be soluble (e.g., $CdOH^+$, $NiOH^+$, $ZnOH^+$) or insoluble (e.g., $Cr(OH)_3$, $Fe(OH)_3$). The dependence of metal bioavailability on pH varies between different metals. For example, at pH 7, 68 µM cobalt is predicted to exist in the free, ionic form, whereas only 4.1 µM nickel remains in the same form. Free, ionic concentrations of lead, copper, cadmium, and zinc are predicted to be considerably lower.

Medium pH acts in conjunction with phosphate content to profoundly impact free ionic metal levels. Figure 1C shows predicted free ionic concentrations of cadmium in Bushnell-Haas medium initially amended with 100 µM total cadmium, adjusted to different pH values, and containing variable amounts of phosphate. Small changes in pH or phosphate concentration can have large effects on free ionic metal concentrations. For example, Cd^{2+} levels decreased dramatically as pH and phosphate concentration increased. At pH 7 in the presence of 0, 0.15, 1.5, 15, 30, and 50 mM phosphate, Cd^{2+} levels were predicted to be 66, 64, 17, 1.5, 0.93, and 0.65 µM, respectively. Of the studies summarized in Table 1, nine used a medium containing a mean phosphate concentration of ~19 mM and were adjusted to a mean pH of 6.8. Thus, bioavailable concentrations of metals in studies cited in Table 1 are likely much lower than the reported total metal concentrations.

Because pH strongly influences free ionic levels of metals and their bioavailability, maintaining pH throughout the duration of an experiment is necessary. This requires selection of an appropriate buffering system. Biodegradation studies are typically conducted at neutral to mildly acidic pH values. Use of buffers whose operational pH range lies in this region is recommended to avoid dramatic changes in pH. The operational pH range of a buffer is dependent on the pKa of the weak acid(s) used to buffer the medium. A buffer's pKa value represents the pH at which one-half of the buffering agent is protonated. Using a buffer at a pH significantly higher or lower than its pKa will result in a poorly buffered medium. Excretion of acidic metabolic end products by microbes can reduce the pH of marginally buffered media and can result in unanticipated metal speciation events (Hughes and Poole 1991; Twiss et al. 2001).

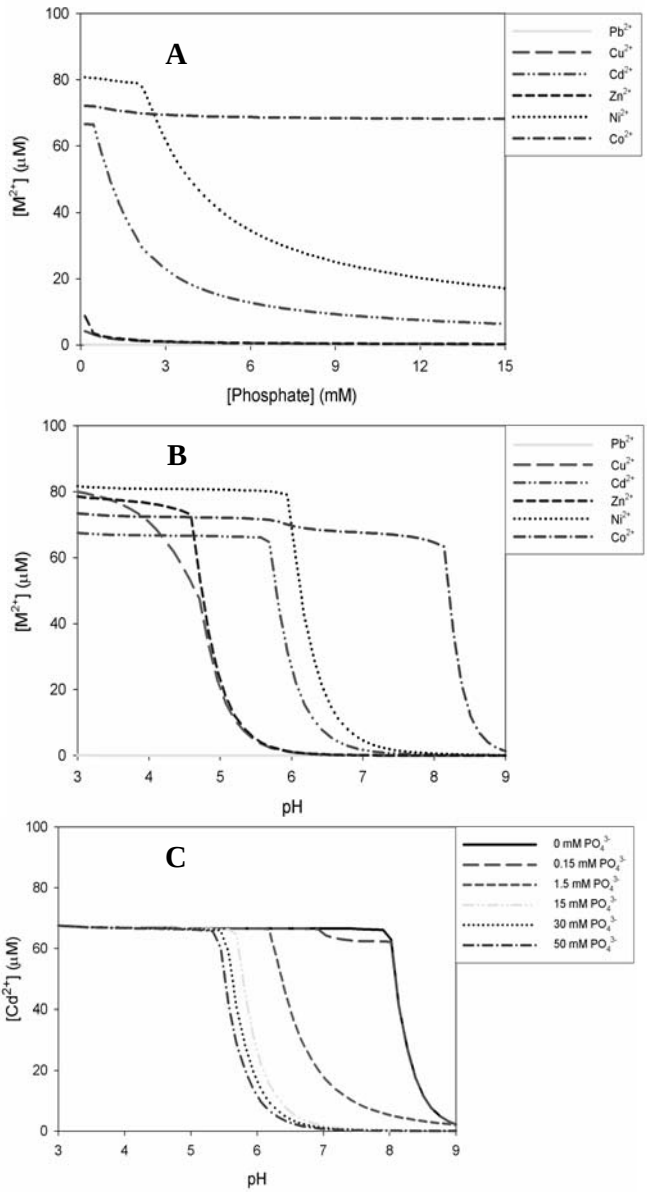

Fig. 1. Effect of phosphate concentration (A), pH (B), and interactions of phosphate and pH (C) on solution-phase, ionic metal ([M^{2+}]) and cadmium ([Cd^{2+}]) concentrations as predicted by MINEQL+ geochemical modeling software (Environmental Research Software, Hallowell, ME, USA) in Bushnell-Haas broth (Difco, Sparks, MD) amended with 100 µM total lead, copper, cadmium, zinc, nickel, or cobalt. When prepared according to the manufacturer's specifications, Bushnell-Haas broth contains 15 mM pH and has a pH of 7.0 ±0.2. The pH of the medium in (A) was set at 6.5

3.2 Metal Speciation and Bioavailability to affect Biodegradation in Soil

Metal bioavailability is often low in soil systems. This is due largely to the composition and pH of many soils studied. For example, in soil systems used to investigate effects of cadmium on phenanthrene biodegradation, 394 mg total cadmium/kg were added, but only 3 mg cadmium/L were actually bioavailable (Maslin and Maier 2000). Similarly, only 1% of the total zinc used in the work of Majumdar et al. (1999) was in the aqueous phase. Kong (1998) found that soluble metal concentrations in treatments initially amended with 20 mg total metal/L were below detection limits of 0.03-0.04 mg/L. At 100 mg total metal/L, only 1 mg cadmium/L and less than 0.12 mg copper and chromium/L were found in the aqueous phase.

In the soil environment, organic matter and clay mineral content are important factors that can reduce metal bioavailability. Thus, as increasing amounts of metal are added, toxicity is observed only after binding sites on organic matter become saturated with metal cations. For instance, Pardue *et al.* (1996) found that only 0.01 mg solution phase cadmium/L was required to inhibit trichloroaniline dechlorination in a mineral dominated soil, while 0.2 mg solution phase cadmium/L was required for inhibition in an organic matter dominated soil. This increase in tolerance to cadmium was correlated to saturation of metal binding sites on the organic matter. Similarly, only bioavailable cadmium has been reported to inhibit dehalogenation in microcosms containing cadmium-contaminated sediment (Jackson and Pardue 1998). Furthermore, Said and Lewis (1991) reported that biodegradation of a common herbicide, 2,4-dichlorophenoxy acetic acid methyl ester (2,4-DME), was much more sensitive to metal inhibition in aufwuchs (floating algal mats) than in sediments. The authors suggested that this was due to higher metal binding by sediments than by aufwuchs. Roberts et al.. (1998) observed inhibition of 2,4-diamino-6-nitrotoluene biodegradation at an undetectable concentration of soluble lead (below 1 mg/L) in treatments initially containing 10,000 mg total lead/kg. The phosphate buffer in this study may have caused this large reduction in lead bioavailability. Clay minerals have also been shown to reduce metal bioavailability. Clays with high cation exchange capacities, such as montmorillonite, appear to reduce metal bioavailability and toxicity most (Babich and Stotzky 1977). In fact, the profound impacts of clays on the bioavailability of toxic metals have prompted investigations into the use of clays to reduce metal toxicity as described later in this review.

3.3 Measurement of Bioavailable Metal

Reporting of bioavailable metal concentrations is a vital step towards standardizing experiments to determine effects of metals on organic pollutant biodegradation. Bioavailable metal concentrations can be estimated from

solution phase metal concentrations using tools such as ion selective electrodes, which measure only ionic solution phase metals. A number of promising tools are in development that use biological systems to quantify solution phase and bioavailable metal concentrations. One of the most attractive aspects of these tools is that they can be used in complex systems, such as microbiological media and soil. The first such tool is the immunoassay which can detect solution phase metal concentrations in low μg/L range. Immunoassays have been developed for cadmium, lead, cobalt, nickel, and zinc. An immunoassay for mercury is commercially available (Blake et al. 1998; Khosraviani et al. 1998). A second tool is the use of bioreporters. These are whole cells that produce a protein with measurable activity (e.g., LacZ) or light in response to bioavailable metal. Bioreporters for detection of mercury have been created using both the *lacZ* system (Rouch et al. 1995b) and the luminescent *lux* system (Selifonova et al. 1993; Corbisier et al. 1999). While a bioreporter measures bioavailable metal, it should be emphasized that depending on the metal resistance mechanisms of the bioreporter system used, measurement of bioavailable metal can vary. A review of applications, advantages and limitations of immunoassays and bioreporters for metal detection is available (Neilson and Maier 2001).

In addition to biological-based approaches, geochemical modeling software (e.g., MINTEQA2, MINEQL+) can be used to predict metal speciation as a function of pH and ionic strength (Pardue et al. 1996). At least three computational models have been developed to predict the impact of metals on organic biodegradation (Jin and Bhattacharya 1996; Nakamura and Sawada 2000; Amor et al. 2001). These models account for metal inhibition by adding metal inhibition constants (e.g., K_i) to conventional microbial growth and/or degradation equations. For instance, Amor *et al.* (2001) used a form of the Andrew's equation (often used to describe microbial growth with inhibition) to model effects of cadmium, zinc, and nickel on rates of alkylbenzene biodegradation:

$$\mu = \mu_{max}\, S/(K_s + S + S^2/K_i), \qquad (1.1)$$

Where μ is the alkylbenzene biodegradation rate
μ_{max} is the maximum alkylbenzene biodegradation rate
S is the alkylbenzene concentration
K_s is the alkylbenzene concentration that yields $\frac{1}{2}\mu_{max}$
K_i is the metal inhibition constant.

None of these models incorporates metal speciation and bioavailability. Thus, data generated by these models may only be meaningful for the medium or soil that was used to develop the model. For example, the medium used by Nakamura and Sawada (2000) was adjusted to a pH of 7.8 and contained 0.147 mM phosphate. Likewise, the medium used by Amor et al. (2001) was adjusted to a pH of 5.9 and contained 36 mM phosphate. In both media, much of the metal may precipitate. Thus, these models are likely to underpredict metal toxicity in systems that have a lower pH and/or less phosphate.

4. Metal Inhibition of Biodegradation

The impacts of metals on many general microbial activities including litter decomposition, methanogenesis and acidogenesis, nitrogen transformation, biomass generation, and enzymatic (e.g., dehydrogenase) activity have been studied extensively (Mosey 1976; Doelman and Haanstra 1979a; Doelman and Haanstra 1979b; Capone et al. 1983; Pankhania and Robinson 1984; Babich and Stotzky 1985; Rogers and Li 1985; Kouzelikatsiri et al. 1988; Baath 1989; Hickey et al. 1989; Nandan et al. 1990; Burkhardt et al. 1993; Lin 1993; Bardgett and Saggar 1994; Masakazu and Itaya 1995; Knight et al. 1997). Metals including copper, zinc, cadmium, chromium (III and VI), nickel, mercury, and lead have been reported to inhibit each of these processes. In contrast, some metals have been observed to stimulate activity. For example, Baath (1989) noted that both inhibitory and stimulatory effects of lead on carbon mineralization have been observed. Equally perplexing, the addition of some metals including mercury, lead, nickel, cadmium, and copper, stimulated methanogenesis in anoxic salt sediments (Capone et al. 1983) and nickel (< 300 mg total nickel/L) stimulated acidogenesis (Lin 1993). As illustrated below, available data on the effect of metals on organic pollutant biodegradation is not extensive, but demonstrates that metals have the potential to inhibit pollutant biodegradation under both aerobic and anaerobic conditions.

4.1 Effects of Metals on Aerobic Biodegradation

Metals have been shown to inhibit the aerobic biodegradation of a variety of organic pollutants (Table 1A). For example, copper, cadmium, mercury, zinc and chromium (III) were found to inhibit the aerobic biodegradation of 2,4-DME in lakewater samples inoculated with either a sediment or an aufwuch (floating algal mat) sample (Said and Lewis 1991). Zinc, with a minimum inhibitory concentration (MIC) of 0.006 mg total Zn/L, was most toxic in sediment samples; however, in aufwuch samples, mercury was most toxic with an MIC of 0.002 mg total Hg/L. A pure culture study using a naphthalene-degrading *Burkholderia* sp. reported an MIC of 1 mg bioavailable cadmium/L (Sandrin et al. 2000). This MIC was in the same range as the MICs reported by Said and Lewis (1991) for cadmium (0.1 mg total cadmium/L for sediment samples and 0.629 mg/L for aufwuch samples). The fact, that different microorganisms were used in each study likely accounts for differences between the reported MICs.

Springael et al. (1993) also showed that metals inhibited biodegradation of a variety of organic contaminants by several bacterial genera in pure culture. Reported MICs were 2 to 4 orders of magnitude higher than those reported by Said and Lewis (1991) (see Table 1A). The large discrepancies between MICs reported by these two studies are likely due to differences in the test system

used in each study. Springael *et al.* (1993) quantified metal toxicity on solid agar media, while Said and Lewis quantified metal toxicity in liquid culture. Colony growth, that occurs on solid media, may have aided in protection against metal toxicity and resulted in higher MICs.

Metal inhibition has also been observed in soil systems. For example, 60 mg total cadmium/kg, inhibited the biodegradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in a soil system that was inoculated with the 2,4-D-degrader *Alcaligenes eutrophus* JMP134 (Roane et al. 2001). This study was performed both in small-scale microcosms and larger 5-gallon mesocosms showing similar metal sensitivity. Experiments have also been performed investigating the impact of metals on biodegradation by the indigenous soil community (Maslin and Maier 2000). In this case, the impact of cadmium on phenanthrene degradation in two desert soils was measured over a nine-day period. Results showed a 5-day increase in lag period for phenanthrene degradation in the presence of 1 and 2 mg bioavailable cadmium/L and complete inhibition at 3 mg bioavailable cadmium/L.

Effects of metal toxicity on biodegradation are not only limited to aromatic contaminants. The impact of copper toxicity on biodegradation of a polymer commonly used for medical, agricultural, and industrial purposes, polyhydroxybutyrate (PHB), has also been investigated (Birch and Brandl 1996). The polymer is used in agriculture both as a film mulch and as a long-term delivery device for fertilizers. In both applications, the material is expected to biodegrade after it has served its purpose; however, treatment of agricultural fields with sewage sludge (which is often rich in copper) can increase the soil metal content. To determine the impact of copper toxicity on PHB biodegradation, a PHB-containing agar overlay was placed on media containing a concentration gradient of copper. Plates were inoculated with a PHB-degrading strain of *Acidovorax delafieldii*. The concentration of copper along the gradient was determined by measuring copper in filter paper that was in contact with the gradient. Using this novel approach, the authors found that 8 to 15 mg bioavailable copper/L were required to inhibit PHB biodegradation.

Not all studies have investigated the impact of single metals on biodegradation of only a single, pure organic. Benka-Coker and Ekundayo (1998) investigated the impact of zinc, lead, copper and manganese on crude oil biodegradation by a *Micrococcus* sp. and a *Pseudomonas* sp. Biodegradation was reduced most by zinc (concentrations as low as 0.43 mg total zinc/L) and least by manganese (concentrations as low as 28.2 mg total manganese/L). Interestingly, combinations of metals were reported to be less toxic than some single metals. For instance, toxicity of 0.5 mg total zinc/L was mitigated by addition of 0.5 mg total copper, lead, and manganese/L. Most recently, Riis *et al.* (2002) reported inhibition of diesel fuel biodegradation in liquid cultures by combinations of metals, including copper, nickel, and zinc.

Some readily biodegradable organic pollutants, such as ethylenediaminetetraacetic acid (EDTA) and NTA, interact strongly with metals. Despite the

ubiquity of these compounds in wastewater, there is a paucity of information in the literature regarding the biodegradability of metal-organic complexes. Biodegradation of several EDTA-metal complexes, including complexes containing cadmium, nickel, cobalt, and copper, has been reported to be much slower than biodegradation of EDTA alone (Thomas et al. 1998). Similarly, *Chelatobacter heintzii* ATCC 29600 readily degraded free NTA, but was unable to degrade NTA complexed by copper, nickel, or cobalt (White and Knowles 2000). Complexation of NTA by the same metals reduced NTA biodegradation by *Mesorhizobium* sp. NCIMB 13524 (White and Knowles 2003). Additional organic pollutants capable of complexing and interacting with metals do exist. For this reason and the fact that the bioavailability of metals complexed to various organic ligands has not been well-characterized, more research in this area is warranted.

4.2 Effects of Metals on Anaerobic Biodegradation

Anaerobic catabolic pathways often represent the sole process for biodegradation of highly halogenated organics such as trichloroethene (TCE) and perchloroethene (PCE) (Alexander 1999). Many of these solvents have been discarded with metals. For this reason, several studies have addressed the effects of metal toxicity on the biodegradation of organic pollutants by anaerobic bacterial consortia (Table 1B).

Only 5 mg total cadmium/L has been reported to reduce TCE biodegradation (Degraffenreid and Shreve 1998). Representative of additional solution studies, Kuo and Genthner (1996) investigated the impact of cadmium, copper, chromium, and mercury on dechlorination and biodegradation by an anaerobic bacterial consortium isolated from an aquatic sediment. The consortium was capable of completely degrading 2-chlorophenol (2CP), 3-chlorobenzoate, phenol and benzoate. Results showed that different activities (e.g., dehalogenation, biodegradation, and methanogenesis) were affected differently by each metal. For example, biodegradation of 3-chlorobenzoate was inhibited most by cadmium and chromium, biodegradation of benzoate was most sensitive to copper, and phenol biodegradation was most reduced by mercury. In general, the addition of low levels of metals (0.1-2.0 mg total metal/L) lengthened acclimation periods and decreased dechlorination and biodegradation rates. Concentrations from 0.5-5 mg total metal/L completely inhibited either dechlorination or biodegradation. Similar results have been reported elsewhere. Kamashwaran and Crawford (2001) found that cadmium reduced pentachlorophenol biodegradation rates. Kuo and Genthner (1996) point out that their results suggest that, in addition to adversely affecting degraders in a consortium, metals may affect non-degrading consortium members that play a vital but indirect role in the degradation process. For instance, members of the consortium that produce reducing equivalents for reductive dehalogenation or

remove dechlorinated products from the system to allow further dehalogenation may be deleteriously impacted, thus reducing the overall rate and extent of biodegradation.

Such an indirect mode of toxicity has also been implicated in the mechanism by which metals inhibited the anaerobic biodegradation of trinitrotoluene (TNT) metabolites (Roberts et al. 1998). Copper, zinc, and lead did not affect establishment of anaerobic conditions in a bioreactor, nor did these metals impact loss of the parent TNT compound; however, subsequent removal of TNT degradation intermediates was reduced by each of the metals. For instance, lead (total concentrations > 1000 mg/kg) delayed degradation of a TNT biodegradation intermediate (2,4-diamino-6-nitrotoluene) by as many as nine days. Zinc (1500 mg total zinc/kg) delayed degradation of the same intermediate by eight days. Copper (4000 and 8000 mg total copper/kg) completely inhibited removal of this intermediate. Thus, it is important to consider the effects of metals on populations of microorganisms other than those biodegrading the parent compound.

Soil type affects the extent to which metals inhibit biodegradation. For example, Pardue et al. (1996) examined the impact of cadmium on reductive dehalogenation of trichloroaniline in different soils. As described above, in microcosms containing two mineral-dominated soils, only 0.01 mg solution phase cadmium/L was required to inhibit reductive dehalogenation. In microcosms containing an organic matter-dominated soil, more than an order of magnitude higher cadmium concentration (0.2 mg solution phase cadmium/L) was required to inhibit dehalogenation. Furthermore, results showed that the dehalogenation pathway expressed in soil exposed to cadmium was different than the pathway expressed in cadmium-stressed soil. This suggests that cadmium stress selected for a different, dominant dehalogenating population than was found in the cadmium-free soil. Sediments have also been shown to mediate metal toxicity. The impact of metals on reductive dehalogenation of hexachlorobenzene in a waste lagoon sediment co-contaminated with cadmium and lead has been investigated (Jackson and Pardue 1998). In this study, cadmium and lead inhibited reductive dehalogenation, but only when not bound to sediment material.

4.3 Relationships between Metal Concentration and Inhibition of Biodegradation

It should be noted that the literature contains reports that metals do not inhibit some biodegradative processes. For example, cadmium (≤500 mg total cadmium/L) and mercury (≤100 mg total mercury/L) did not affect biodegradation of a variety of polycyclic aromatic hydrocarbons (PAHs) by the fungus *Pleurotus ostreatus* in soil (Baldrian et al. 2000). Similarly, Delaune et al. (1998) investigated the effects of chromium and lead on crude oil biodegradation. Those metals did not affect overall total hydrocarbon

biodegradation, chromium (5,000 µg total chromium/g) reduced biodegradation of a constituent hydrocarbon of the oil, octadecane. This reduction occurred only under reducing conditions. Similarly, a suite of metals (copper, nickel, and zinc, at 31.8, 29.3, and 32.7 mg total metal/L, respectively) had no effect on diesel fuel biodegradation in soil slurries by an indigenous community of degraders (Riis et al. 2002); however, the same metals at 25-fold lower concentrations inhibited diesel fuel degradation in liquid culture by a community of degraders extracted from the soil. As with several other studies described throughout this review (Said and Lewis 1991; Pardue et al. 1996), the low bioavailability of metals in these studies may account for the fact that inhibitory effects were not observed. Furthermore, metal toxicity in the study conducted by Baldrian *et al.* (2000) may have been ameliorated by the acidity of the soil in which the experiments were conducted, as has been described previously (Franklin et al. 2000; Sandrin and Maier 2002).

When metals inhibit biodegradation, their effects are not always dose-dependent. The data presented thus far suggest that there is a direct, dose-dependent relationship between the amount of toxic metal in a co-contaminated environment and the extent of metal inhibition of organic biodegradation (Fig. 2A); however, there is an evidence for two semi-dose dependent patterns of metal effects on organic biodegradation.

4.3.1 Semi-Dose Dependent Pattern 1

The results of several studies suggest that metals stimulate activity until a maximum level of stimulation is reached. Thereafter, metal toxicity increases with increasing metal concentration (Fig. 2B). All of these studies used consortia, not single isolates. For this reason, it is likely that this pattern results from differential toxicity effects, wherein one population that is sensitive to metal stress competes in some way with another, metal-tolerant population expressing the activity of interest (e.g., biodegradation). Inhibition of the more sensitive population reduces competition for resources needed by the metal tolerant population expressing the activity of interest. Capone *et al.* (1983) provide an evidence supporting this view point. Methanogenesis was stimulated by the addition of some metals. As the authors suggested, this may have resulted from differential inhibition of the methane and non-methane producing microorganisms. Metals may have selected for a metal-resistant, methanogenic population and reduced competition from a metal-sensitive, non-methanogenic population. Similarly, Kuo and Genther (1996) reported that low concentrations of metals stimulated biodegradation. Hexavalent chromium (0.01 mg total chromium/L) increased the biodegradation rate of phenol by 177% and that of benzoate by 169% over controls containing no metals. Other metals exhibited similar effects. Copper and cadmium (both at 0.01 mg total metal/L) increased the benzoate biodegradation rate 185% and the 2-chlorophenol biodegradation rate by 168%. Mercury (1-2 mg total

mercury/L) increased the biodegradation rates of 2-chlorophenol and 3-chlorophenol by 133-154%.

Other studies report similar results with various consortia (Sterritt and Lester 1980; Hughes and Poole 1989). These groups suggested the stimulatory effect may be due to metals reducing competition for reducing equivalents or nutrients between metal-resistant degraders and metal-sensitive non-degraders. As in the work of Capone et al. (1983), Kuo and Genthner (1996), and Roberts et al. (1998), the impact of metals on microbially mediated processes in these studies may be mainly due to effects of metals on a population other than the one carrying out the process of interest, the existence of this semi-dose dependent pattern of metal effects underscores the importance of considering not only the physiological impact of a toxic metal on a degrading population of interest, but also the ecological impact of the toxic metal.

4.3.2 Semi-Dose Dependent Pattern 2

The second semi-dose dependent pattern is one in which low concentrations of metals increasingly inhibit activity until a maximum level of inhibition is reached and, thereafter, metal toxicity decreases with increasing metal concentration (Fig. 2C). The data of Said and Lewis (1991) generally shows that 2,4-DME biodegradation decreased in a dose-dependent fashion; however, a closer examination of these data reveals that the maximal degradation rate (V_{max}) of 2,4-DME was less in the presence of 10 μM cadmium (0.61 ± 0.03 μg 2,4-DME/L/min) than in the presence of 100 μM cadmium (0.74 ± 0.00 μg 2,4-DME/L/min). In a subsequent study, a similar pattern of inhibition was observed as populations of 2,4-D degraders in a cadmium contaminated soil were more resistant to cadmium toxicity at a higher concentration of cadmium (40 mg total cadmium/L) than at a lower concentration of cadmium (20 mg total cadmium/L) (Roane and Pepper 1997). Pattern 2 responses to metals might be explained by microbial community dynamics. High metal concentrations may create selective pressure for metal-resistant, organic-degrading microorganisms that reduced competition from metal-sensitive non-degrading microorganisms, thus increasing biodegradation at higher metal concentrations. At the level of single cells, though, it is possible that high metal concentrations may more rapidly induce a metal resistance mechanism important in cadmium detoxification (e.g., an efflux pump) than low metal concentrations.

In summary, the existence of semi-dose dependent patterns of metal effects on biodegradation complicates understanding and predicting metal toxicity in the environment. As demonstrated by the patterns described above, metals may impact both the physiology and ecology of pollutant degrading microorganisms. For this reason, models designed to predict the impact of metals on biodegradation may fail to do so accurately unless they include both physiological and ecological effects of metals on organic-degrading microorganisms.

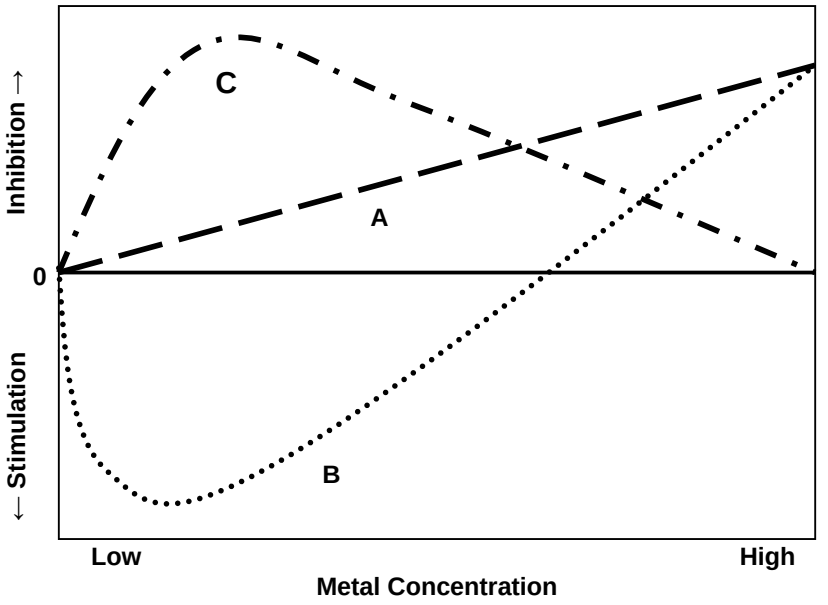

Fig. 2. Reported patterns in which metals affect organic pollutant biodegradation: the most commonly reported, dose-dependent pattern (A), semi-dose-dependent pattern 1 (B), and semi-dose-dependent pattern 2 (C)

5. Strategies to Enhance Biodegradation in Co-contaminated Environments

Several approaches have been described to reduce the extent to which metals inhibit organic biodegradation. Specifically, each approach has involved lowering metal bioavailability and/or increasing metal resistance to facilitate biodegradation. Approaches include inoculation with metal resistant microorganisms and the addition of materials that can reduce metal bioavailability including calcium carbonate, phosphate, clay minerals, and surfactants.

5.1 Metal Resistant Bacteria

Microorganisms employ a variety of mechanisms to cope with toxic metals. These have been reviewed thoroughly elsewhere (Nies 1992; Ji and Silver 1995; Nies and Silver 1995; Rosen 1996; Silver 1996; Silver and Phung 1996; Nies 1999). Resistance mechanisms include intracellular and extracellular metal sequestration, metal reduction, metal efflux pumps, and production of metal chelators such as metallothioneins and biosurfactants. Despite the ubiquity and

efficacy of microbial metal resistance mechanisms, a few studies have attempted to exploit them to increase pollutant biodegradation in co-contaminated systems.

Introduction of metal-resistance mechanisms into pollutant-degrading bacteria may represent a viable strategy to mitigate metal-inhibition of organic pollution biodegradation. Springael *et al.* (1993) showed that metal inhibition of biodegradation could be reduced by the introduction of metal resistance genes into biodegrading microorganisms. For example, strains containing metal resistance genes degraded both polychlorinated biphenyls (PCBs) and 2,4-D in the presence of either 1 mM nickel or 2 mM zinc. Biodegradation of these compounds by organisms without introduced resistance genes was inhibited at the same metal concentrations.

A single study has investigated inoculation of metal-contaminated soil with metal-resistant bacteria to enhance organic contaminant biodegradation (Roane et al. 2001). In this study, soil microcosms were co-contaminated with 2,4-D (500 mg/kg) and cadmium (60 mg total cadmium/kg). Inoculation with *Alcaligenes eutrophus* JMP134, a 2,4-D degrader, was required because this soil did not contain an active 2,4-D-degrading population. JMP134, though, was sensitive to cadmium. To achieve rapid degradation of the 2,4-D, it was necessary to inoculate the metal-contaminated soil with both JMP134 and a cadmium resistant isolate, *Pseudomonas* H1, which accumulates cadmium intracellularly. These results suggest that inoculation with metal-sequestering microorganisms will foster increased biodegradation in the presence of a toxic metal.

5.2 Treatment Additives

Treatment additives, such as calcium carbonate, phosphate, cement, manganese oxide and magnesium hydroxide can reduce metal bioavailability and mobility in metal-contaminated sites (Ruby, et al. 1994; Traina and Laperche 1999; Hettiarachchi et al. 2000). In spite of this, only a single study has examined the impact of such reductions on metal toxicity to soil microorganisms. Jonioh et al. (1999) examined the effect of calcium carbonate on the toxicity of lead to microorganisms isolated from a military rifle range soil contaminated with lead and other heavy metals. Calcium carbonate was found to reduce lead toxicity when added at 1, 2.5, 5, and 10% (w/w). Toxicity was determined using the Microtox® assay (which uses a luminescence assay to determine viability). The effective concentration of contaminated soil required for a 50% reduction in loss of luminescence (EC50) increased from 14% in the absence of calcium carbonate to 75% in the presence of 10% calcium carbonate. Calcium carbonate decreased lead leachability and raised the soil pH. Because lead bioavailability typically decreases as pH increases, the additive likely reduced lead toxicity by reducing its bioavailability. Such promising results suggest that treatment

additives may play key roles in future viable approaches to remediating metal and organic co-contaminated sites.

5.3 Clay Minerals

Clay minerals can reduce metal bioavailability and toxicity. The addition of kaolinite (1 to 20%) or montmorillonite (1 to 5%) to an agar medium containing cadmium reduced the toxicity of cadmium to several fungi including *Aspergillus niger* and *Trichoderma viride*, to bacteria including *Bacillus megaterium*, *Agrobacter tumefaciens*, and to an actinomycete, *Nocardia corallina* (Babich and Stotzky 1977). Similarly, in solution studies, bentonite and vermiculite (at 3% each) reduced the toxicity of 150 mg total cadmium/L to *Streptomyces bottropensis* (Kamel 1986). Kaolinite also reduced cadmium toxicity, but more was required (6% vs. 3%) and less protection was afforded than with the other clays. In general, protection increased with clay concentration. The protective ability of each clay correlated well with its cation exchange capacity (CEC). For example, the most effective clay, vermiculite, had a CEC of 108.7 meq/g, while the least effective clay, kaolinite, had a CEC of only 4.8 meq/g.

The effect of clay addition on metal toxicity was less pronounced in soil than in the plate and solution studies described above. Babich and Stotzky (1977) found that 3 to 12% montmorillonite was required to reduce cadmium toxicity to various fungi in soil; however, kaolinite failed to reduce toxicity. The low CEC of kaolinite appeared to explain its failure to reduce metal bioavailability and hence toxicity, as in the results of plate studies.

5.4 Chelating Agents

Chelating agents have been used to mitigate metal toxicity to organic-degrading microorganisms. EDTA has been shown to reduce the toxicity of cadmium to *Chlorella* sp. of nickel to algae (Spencer and Nichols 1983) and an actinomycete (Babich et al. 1983), and of copper to bacteria and algae (Sunda and Guillard 1976); however, the toxicity of EDTA to many microorganisms and its limited biodegradability may reduce its suitability for application to the bioremediation of co-contaminated environments (Braide 1984; Ibim et al. 1992; Borgmann and Norwood 1995; Ogundele 1999). In addition, biodegradation of metal-EDTA complexes may be slow (Thomas et al. 1998). Thus, the use of other chelating agents to reduce metal toxicity is of interest.

A commercially available chelating resin (Chelex 100) and surfactant-modified clays reduced cadmium toxicity during biodegradation of naphthalene (Malakul et al. 1998). Clays were modified by adsorbing a cationic surfactant to the clay surface to which various metal-binding ligands (e.g. palmitic acid) were attached via hydrophobic interactions. Naphthalene biodegradation occurred at

higher cadmium concentrations in the presence of the modified clays than in controls containing either no clay or unmodified clay. The abilities of the resin and the modified clays to reduce cadmium toxicity were quantitatively related to the metal adsorption characteristics of the two chelating agents.

Biosurfactants (i.e., microbially produced surfactants) show promise for enhancing organic biodegradation in metal and organic co-contaminated environments. Sandrin et al. (2000) showed that a rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* reduced cadmium toxicity during naphthalene biodegradation by a *Burkholderia* sp. in solution studies. The mechanism by which the biosurfactant reduced cadmium toxicity appeared to involve a combination of rhamnolipid complexation of cadmium and rhamnolipid-induced lipopolysaccharide release from the outer membrane of the degrader (Leive 1965; Goldberg et al. 1983; Al-Tahhan et al. 2000). Later, Maslin and Maier (2000) used the same biosurfactant to reduce cadmium toxicity during biodegradation of phenanthrene by indigenous populations in two soils co-contaminated with phenanthrene and cadmium. Serial additions of rhamnolipid increased phenanthrene mineralization from 7.5 to 35% in one soil and from 10 to 58% in the second soil. Serial applications were necessary due to biodegradation of rhamnolipid which occurred in two to three weeks. The possibility for *in situ* biosurfactant production is being investigated to make this approach more cost-effective.

6. Conclusions and Future Directions

The current body of knowledge concerning metal effects on biodegradation is still in its infancy, yet the timely and cost-effective remediation of metal and organic co-contaminated sites will require a lucid understanding of factors important in determining the extent to which toxic metals inhibit organic biodegradation. Past attempts to measure impacts of metals on biodegradation are difficult to interpret, because they have generally been based on total metal rather than solution phase or bioavailable metal concentrations. This has resulted in reported inhibitory concentrations of metals that vary by as many as 5 orders of magnitude. A critical first step will be to consistently report solution phase or bioavailable metal concentrations so that legitimate comparisons of biodegradation behaviors in co-contaminated sites can be made. Currently, a useful approximation is to measure and use solution phase metal data; however, new methods of defining and determining bioavailable metal are rapidly being developed. Despite the enormous variance among reported inhibitory concentrations of metals, it remains clear that metals have the potential to inhibit organic biodegradation in both aerobic and anaerobic systems. The mechanisms and patterns by which metals inhibit biodegradation vary with the composition and complexity of each system and include both physiological and ecological components. A more thorough understanding of these systems,

taking into account various levels of complexity, is needed to develop new approaches to bioremediate co-contaminated sites. Nevertheless, there already exist several approaches including addition of metal resistant microorganisms and additives that reduce metal bioavailability. Field trials are needed to validate these approaches.

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