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Technical Communication

Microbial Growth and Action: Implications for Passive Bioremediation of Acid Mine Drainage

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Abstract. Due to the variable environmental nature of mine water, several species of bacteria are important in the generation of acid mine drainage (AMD) and in bioremediation treatment technology. Enzymatic metal transport and transformation allow bacteria to survive in high-metal environments and to oxidize, reduce, and exude metals. For example, the enzymes Cr (VI) reductase and cytochrome-c3 hydrogenase allow *Pseudomonas sp.* to reduce Cr (VI) to less toxic Cr (III). Much more toxic organomercuric compounds are transformed by *Pseudomonas fluorescens* and *Escherichia coli*, using the enzymes organomercurial lyase and mercuric reductase. The role of bacteria in the AMD environment is not yet fully understood and consequently researchers should pay attention in this field.

Key words: Acid mine drainage; *Acidithiobacillus ferrooxidans*; enzyme; enzyme kinetics; *Escherichia coli*; metal biodegradation; microorganism

Introduction

Microorganisms carry out unique and essential services for the living world: decomposing organic matter, photosynthesizing, mineralizing macro- and micro-nutrients, cycling nutrients, and producing and consuming compounds that are life supporting and climate defining. Their biochemical diversity and adaptability also allows them to process waste products from human and natural systems, degrade toxic xenobiotics, and produce an astonishing variety of life-sustaining products through traditional and biotechnological manipulations. Bacteria have a relatively simple cell structure, lacking a cell nucleus and organelles such as mitochondria and chloroplasts. Multi-cellular eukaryotic organisms mostly utilize O_2 as an electron acceptor for respiration, while microorganisms are known to use over 20 elemental systems. Of these, only six are known to be respired in solid form: S, As, Se, U, Fe, and Mn (Ruebush et al. 2006); of these, the ability to reduce iron is the most common (Lovely 2000). The direction and kinetics of these reversible reactions are controlled by enzymes.

The mineralogy of ochreous precipitates formed in AMD may be correlated with microbial preferences. The common end products are jarosite [$RFe_3(SO_4)_2(OH)_6$, where R generally stands for potassium, sodium, hydronium and/or lead], schewertmannite [$Fe_8O_8(OH)_6SO_4$], goethite [α-FeOOH], and ferrihydrite [$Fe_5HO_8{\bullet}H_2O$] (Schewertmann et al. 1995). When conditions are highly acidic, schewertmannite formation is favoured, but over time, schewertmannite transforms into more stable goethite and ferrihydrite, releasing substantial quantities of sulfate, with much of the process being microbially controlled (Murad and Roj 2005).

Acidophilic Bacteria

The cytoplasm of bacteria plays an important role in metal metabolism. Both organic and inorganic nutrients and reserves can be stored in the cytoplasm as glycogen, lipids, polyphosphate, or in some cases, sulfur or nitrogen**.** The transfer of nutrients from the aqueous media usually takes place through the outer plasma membrane, which is a lipid bilayer much like the cytoplasmic (plasma) membrane of other animal cells. Proteins moving within or upon this layer are primarily responsible for transport of ions, nutrients, and waste across the membrane**.**

The first microbe isolated from AMD (Colmer et al. 1950) was an iron-oxidizing rod-shaped acidophilic microbe called *Thiobacillus ferroxidans* (subsequently renamed *Acidithiobaciilus ferroxidans*). Acidophiles are categorized based on phenotypic traits, such as temperature (as mesophiles, moderate thermophiles, and thermophiles), pH optima for growth, and the basis of cellular carbon acquisition (autotrophs assimilate CO_2, heterotrophs assimilate organic carbon, and mixotrophs use both). Hallberg and Johnson (2005) outlined the various acidophilic microorganisms that grow in acid waters (Table 1).

At. ferrooxidans has historically been described as the major microbe in acidic environments, but this may be an experimental artefact and not reflect the true

importance of this microbe in mine water (Hallberg and Johnson 2005; Johnson 1998). Enrichment cultures with ferrous iron have been used in many studies to enumerate microbial population, and *At. ferrooxidans* usually dominates such cultures. In other cases, the culture media used to determine the importance of iron-oxidizing microbes do not support any domination (e.g. Kirby et al. 1999).

Another iron-oxidizing acidophilic species, *Leptospirillum ferrooxidans*, is understood to contribute in generating acidic mine waters. Johnson (2005) described Leptospirillum as highly motile vibrios and spirilla that oxidize ferrous iron but not sulphur, and which do not show turbidity when grown in ferrous iron/yeast extract medium.

The Afon Goch (a stream draining the former Parys Mountain copper mine in north Wales; pH 2.2-2.8) was found to contain about 10^3/mL of both *At. ferrooxidans* and *L. ferrooxidans* (Walton and Johnson 1992). The relative numbers of these iron oxidizers changed with distance from the discharge adit and appeared to correlate with changing concentrations of ferrous iron; *L. ferrooxidans* has a higher affinity for Fe (II) and were more numerous than *At. ferrooxidans* when Fe (II) concentrations were < 10 mg/L. In contrast, *At. ferrooxidans* was noted to be the most numerous iron oxidizer present in AMD draining the abandoned Cae Coch pyrite mine in North Wales, where Fe (II) concentrations were often > 500 mg/L and the pH was 2.3-2.5 (McGinness and Johnson 1993). As at Parys Mountain, *L. ferrooxidans* dominated over *At. ferrooxidans* in Iron Mountain mine water (Schrenk et al. 1998). Further work at Iron Mountain revealed the presence of a microbe related to *L. ferrooxidans*, which on occasion represented nearly 100% of the total microbial population (Bond et al. 2000a; Bond et al. 2000b).

Schrenk et al. (1998) also cast doubt on the popular concept that *At. ferrooxidans* were acid-generating bacteria, suggesting that at Iron Mountain, they may be essentially opportunistic, deriving metabolic energy but contributing little to acid generation. They found that at Iron Mountain, the principal geochemical

Table 1. Acidophilic prokaryotic microorganisms associated with mine water (Hallberg and Johnson 2005)

Mineral-degrading acidophiles	Thermal Classification*	Phylogenetic affiliation
Iron-oxidizers		
Leptospirillum ferrooxidans	Meso	Nitrospira
L. ferriphilum	Meso	Nitrospira
L. thermoferrooxidans	Mod Thermo	Nitrospira
"Thiobacillus ferrooxidans" m^{-1}	Meso	Beta-*Proteobacteria*
"Ferrimicrobium acidiphilum	Meso	*Actinobacteria*
Ferroplasma acidiphilum	Meso	*Thermoplasmales*
"Fp. acidarmanus"	Meso	*Thermoplasmales*
Sulfur-oxidizers		
Acidithiobacillus thiooxidan	Meso	Beta/Gamma *Proteobacteria*
At. Caldus	Mod Thermo	Beta/Gamma-*Proteobacteria*
Thiomonas cuprina	Meso	Beta –*Proteobacteria*
Hydrogenobacter acidophilus	Mod Thermo	*Aquifacales***
Iron- and sulfur-oxidizers		
Acidithiobacillus ferrooxidans	Meso	Beta/Gamma-*Proteobacteria*
Iron-reducers		
Acidiphilium spp.	Meso	alpha-*Proteobacteria*
Iron-oxidizers/reducers		
Acidimicrobium ferrooxidans	Meso	*Actinobacteria*
Iron-oxidizers/reducers and sulfur-oxidizers		
Sulfobacillus spp.	Meso and Mod Thermo	*Firmicutes*
Heterotrophic acidophiles (non mineral-degrading)		
Acidocella spp.	Meso	alpha-*Proteobacteria*
Acidisphaera rubrifaciens	Meso	alpha-*Proteobacteria*
Acidobacterium capsulatum	Meso	Acidobacterium
Acidomonas methanolica	Meso	alpha –*Proteobacteria*
Alicyclobacillus spp.	Meso	*Firmicutes*
Picrophilus spp.	Mod Thermo	*Thermoplasmales*
Thermoplasma spp.	Mod Thermo	*Thermoplasmales*

* Meso – mesophiles ($T_{optimum} < 40^{\circ}C$); Mod Thermo – moderate thermophiles ($T_{optimum}$= 40-60°C); ** inferred ability to oxidize minerals (via production of sulfuric acid)

impact of *At. ferrooxidans* was the precipitation of ferric iron that followed their oxidation of ferrous iron.

Acid Coal Mine Drainage

Interestingly, acid coal mine drainage shows less diversity of microorganisms than acid metal mine drainage, possibly due to lower metal enrichment and other factors like temperature, ionic strength, pH, and likely redox conditions. Two studies employing denaturing gradient gel electrophoresis and fluorescent in situ hybridization by Nicomrat et al. (2006 a, b) report that the most numerous bacterial species in a successive alkalinity producing (SAPS) wetland, in Carbondale, Ohio, was *At. ferroxidans*, comprising 37% of the bacterial population. *Acidithiobacillus thioxidans* were also abundant. Chemolithoheterotrophs in the Acidiphilium genus totalled 20% of the bacterial population. Combined, they represented 91% of the total bacterial count in these samples. *L. ferroxidans* was below the level of detection. Microbial populations peaked in oxic surface zones. Oxidation of Fe (II) was not restricted to the periods of O_2 availability at the water-sediment interface. During periods of O_2 availability, Fe (II) can be oxidized close to the water sediment interface at high rates, allowing schwertmannite to precipitate in the sediment, where it is readily available for microbial reduction due to its poor crystalline structure (Murad and Roj 2005). Their findings, though not corroborated by secondary sources, appear to indicate potential design improvements to SAPS in terms of selecting the proper substrate and operating conditions like residence time, flow rate, and alkalinity.

Metal Biodegradation Pathway Map

There may be several tens of millions of organic and inorganic compounds that could be bioremediated (Dagley 1987), but natural degradation pathways are known for only a few thousand. Understanding the complete metabolic pathways is necessary for at least two reasons. First, co-metabolic processes represent a metabolic burden for a microorganism and therefore need an input of energy; if we are to facilitate the process, we need to know how to optimize it. Second, the end product of metabolites produced by incomplete pathways may be themselves toxic or subject to further transformations by other microorganisms; unforeseen consequences could be a significant problem (Tyson et al. 2004).

Johnson et al (2005) claimed to have developed protocols that include all documented groups of acidophilic iron-oxidizing bacteria, including some that have yet to be named. A great source for such information in the public domain is the University of Minnesota Biocatalysis/Biodegradation Database. Some metal biodegradation pathways are discussed below. It should be understood that these pathways are mostly modelled heuristically, though analytical results are used to validate the models. In some cases, a complete pathway for a particular substance may not exist in a single organism; partial and complementary pathway segments may exist in different organisms.

Biodegradation pathway prediction is based on biochemistry and "metabolic logic" (Wackett and Ellis 1999), and requires knowledge of:

- Organic functional groups to match a new chemical structure with one whose metabolism is already known.
- Intermediary metabolism pathways to deduce how a new biodegradation can funnel a metabolite into a common pathway most efficiently.
- Microbial enzymatic reactions to match a given reaction with a known enzyme.
- Organic chemistry reactions to deduce what new reactions are chemically plausible to decompose a compound when precedents are not available.

Pyrite Oxidation by Iron (III) Reduction

In heterotrophic acidophilic bacteria, the electron transfer and reductase pathways involved in oxidation of pyrite by Fe (III) are not fully resolved (Kusel et al. 2002; Ram et al. 2005; Tyson et al. 2004). Facultative anaerobic Fe (III)-reducing bacteria, like the neutrophile *S. putrefaciens*, display negligible capacity to reduce Fe (III) in the presence of O_2 (Arnold et al. 1990). Working on the nearly complete gene inventories for the five dominant members of the microbial community growing on the biofilms of flowing AMD in Richmond mine at Iron Mountain, California, Tyson et al. (2004) and Ram et al. (2005) advanced some findings that may contribute to the Fe (III) reduction pathway map, particularly for *Leptospirillum* group II. The biofilm samples grew on the surface of acidic (pH $\approx$ 0.8) solutions that contained near molar concentrations of Fe and millimolar concentration of Zn, Cu, and As. They detected 2,033 proteins from the five most abundant species in the biofilm, including 48% of the predicted proteins from the dominant biofilm organism Leptospirillum group II.

All genomes recovered from the AMD system contained formate hydrogenlyase complexes. These, in combination with carbon monoxide dehydrogenase,

may be used for carbon fixation via a reductive acetyl coenzyme A pathway by some or all organisms.

Nitrogen fixation is another process essential to the community. There is no evidence to suggest that Ferroplasma type I or II fix nitrogen. It is likely that Ferroplasma species obtain nitrogen fixed by other organisms from solutions using numerous amino acid transporters and ammonia permeases. Leptospirillum group II is likely to be the primary nitrogen fixer (Tyson 2004).

Leptospirillium group II seems to dedicate a comparatively large number and variety of genes for cell membrane biosynthesis, suggesting a complex cell wall structure. Many signature enzymatic proteins have recently been identified (Ram et al. 2005), and research on Fe (III) reduction pathway mapping (Banfield 2006) continues. Most of these communities possess genes for resistance to copper, chromium, cobalt, arsenite, mercury, zinc, silver, and cadmium (Tyson et al. 2004), but iron-oxidizing acidophilic bacteria may not be involved in the metabolism of these ions in water.

It is extremely difficult, indeed impractical in many situations, to pinpoint in a natural setting the set or sets of reactions that might have led to produce particular product(s). But they can be better explained probabilistically, knowing that the reactions and the product preferences and processes are random or stochastic in nature. In the absence of laboratory or field verifications of a definitive map, stochastic pathway maps are constructed to provide a near-real view of the processes. We will discuss some stochastic reduction pathway maps for other metals to suggest how they may look.

Chromium (VI) Reduction Pathway Map

Many contaminated sites, viz. groundwater aquifers, lake and river sediments, soils contain the most toxic form of chromium i.e. hexavalent chromium, Cr(VI), co-existing with a variety of aromatic compounds. The major industries responsible for such organic co-pollutants are petroleum refining, agriculture, leather tanning, car manufacturing, wood preservation, and photographic-film making. According to Cervantes et al. (2001), Cr (VI) reduction to the less toxic Cr (III) is commonly done through the formation of intermediate unstable Cr (V). A variety of Cr-resistant microorganisms is supposed to carry out this reduction. Cr(VI) reduction by E. coli ATCC 33456 was a largely soluble reductase activity (Shen and Wang 1993). Chromium uptake by Cr-resistant, sulphate-reducing bacteria is diagrammed in Figure 1. Though it is less common, Nkhalambayausi-Chirwa and Wang (2001) observed concomitant Cr (VI) reduction and phenol degradation by a co-culture of *Pseudomonas putida* DMP-1 and *Escherichia coli* ATCC 33456 in a fixed-film bioreactor. Among the two bacteria, *Pseudomonas sp.* is an obligate aerobe in nature and can biodegrade phenol (Wang and Qu 1992) with the interaction of different substrates like lactose and acetate, while *E. coli* performs the Cr (VI) reduction under both aerobic and anaerobic condition (Shen and Wang 1993). *E. coli* may enhance Cr (VI) reduction in a spatially and physiologically heterogeneous form due to the formation of biofilms when co-cultured with *P. putida,* supporting the latter's growth. Bacteria that are believed to carry out the Cr (VI) reduction reactions along with the path of transformation are shown in Figure 1. However, there may also be other microbes that carry out chromium reduction by different pathways (Dommer 2006).

Organomercury Pathway Map

Mercury can enter watercourses as a leachate from gold mining and its processing. It may also come from the atmosphere due to emissions from coal-fired power plants. The degree of mercury toxicity depends on its speciation. In this context, the well known toxic species of mercury are elemental mercury (Hg^0), mercuric ion (Hg^{2+}), or organic mercuric compounds. Depending on the environment where it resides, different species of mercury can be interconverted depending on physicochemical parameters like temperature, pH, ionic strength, pressure, etc. Mercury poisoning in humans is due to its absorption by the digestive tract, respiratory tract, or skin. Mercury forms a tight coordinate bond with the sulfhydril (-SH) functional group of enzymes, leading to the disruption of enzymatic systems in several organs like kidney, brain, and lung. Methylmercury, a dangerous species of mercury that can accumulate in aquatic organisms, have the potential to poison humans through the ingestion of contaminated fish, as occurred in the Minamata Bay in Japan in 1956.

Some bacteria are resistant to inorganic mercuric salts and methylmercury and can convert mercury using two enzymes. The organomercurial lyase enzyme cleaves the C-Hg bond, releasing Hg (II) and the respective organic compound (Begley et al. 1986), while mercuric reductase reduces Hg (II) to Hg^0, which is less toxic and reactive, and escapes out of the medium due its high volatility (Schiering et al. 1991). Biological mercury transport occurs through the coordination of mercury with cellular thiols (Figure 2), where thiolate acts as ligand to the mercury and mercury shuttles on and off the surface of the

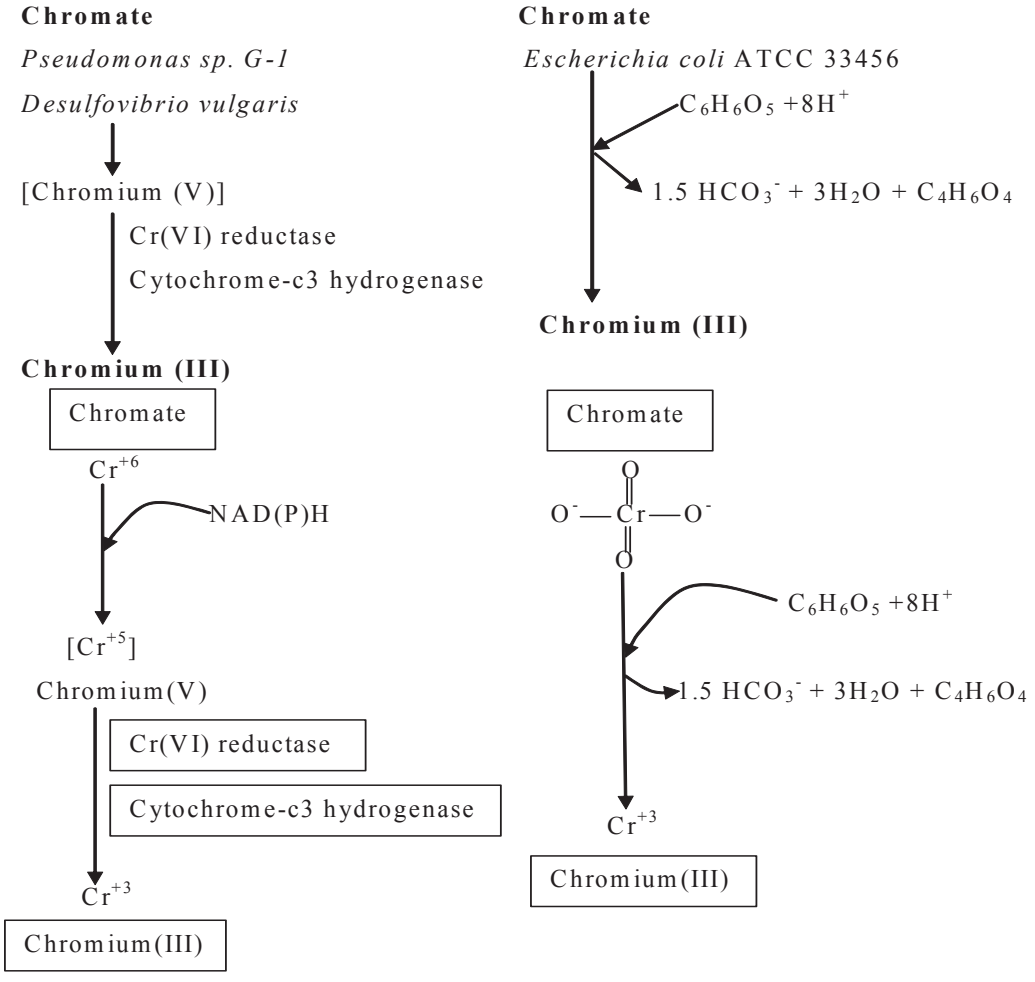

Figure 1. Chromate reduction pathway (note: it not possible to create a complete pathway at this time)

organomercurial lyase and mercury reductase enzymes (Nasevicius et al. 2006).

Modelling Enzyme Kinetics

Heuristic rules are derived from compiled biodegradation information. Additional rules are generated by deconstructing compounds into the 40 most common organic functional groups. The rules consist of deriving biochemically-plausible catabolic reactions for each of the functional groups. More complex compounds, containing multiple functional groups, are analyzed using higher order rules requiring prioritizing enzymatic attack and reaction cleaving functional groups. Understandably, mimicking the natural process is very complex but this kind of prediction models will increasingly be called upon to bridge the gap between the data currently existing and the millions of organic compounds for which the data is lacking (Wackett and Ellis 1999).

In the earlier discussion about the role of enzymes, the process of defining a preferred course for the reactants and products was addressed. To do this, it is particularly important to study specific operational parameters such as maximum rate, temperature, pH, and redox dependence. The nature of how the rate of enzyme-catalyzed reactions changes in response to experimental conditions is known as enzyme kinetics and is used in understanding the mechanism by which an enzyme carries out its catalytic activity. These in-vitro experiments provide clues to selectively energize specific reactions in field applications.

To determine the maximum rate of an enzyme-mediated reaction, the substrate concentration is increased until a constant rate of product formation is achieved. This is the maximum reaction rate or velocity of the enzyme. In this state, enzyme active sites are saturated with substrate. The effect of the substrate concentration (S) on the initial rate of an enzyme–catalyzed reaction (V) is a central concept in enzyme kinetics. The speed V means the number of reactions per second that are catalyzed by an enzyme. With increasing substrate concentration [S], the enzyme asymptotically approaches its maximum speed, V_{max}. According to the Michaelis and Menten

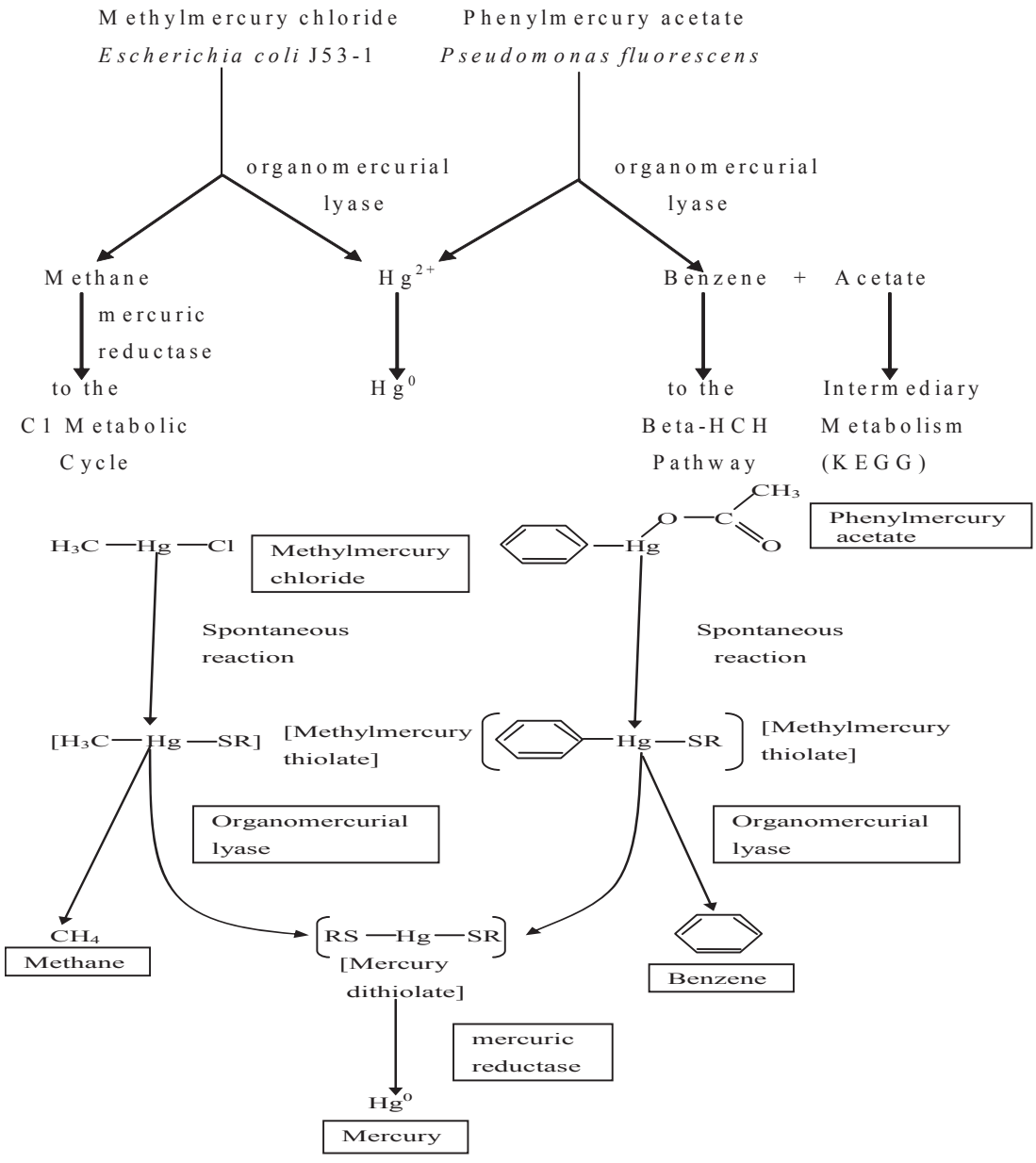

Figure 2. Organo-mercury reduction pathway

Equation, $V = \frac{V_{max}[S]}{K_M + [S]}$

where K_m is known as the Michaelis constant and V_{max} is the maximum rate of reaction. The relationship between V and S of many enzymatic reactions exhibit a rectangular hyperbolic curve.

There are few instances of *in vitro* analyses of media supporting the culture of iron-reducing bacteria. Kaksonen et al. (2003) reported K_m values of 4.3 - 7.1 mg L^{-1} and V_{max} values of 0.19 -0.22 mg gVS^{-1} min^{-1} in a fixed-batch reactor experiment treating metallic wastewater using an ethanol substrate. These results are applicable in understanding the phenomenon, though not, at this stage of research, directly applicable to field conditions.

Recently, *in vitro* solid-phase mineral-oxide reduction under anoxic conditions was reported on by Ruebush et al. (2006). They reported V_{max} and K_m values for formate (electron source), goethite, and hematite (electron acceptor) substrates. The concentration of formate was varied (0 - 10 mM) with 20 mg/mL goethite and 0.1 mg total membrane sample (TM)/mL in each reaction at 23°C. The rate of goethite reduction by the membrane culture was measured as a function of formate concentration. The Michaelis-Menten equation yielded a K_m of 9.724 ± 2.3 mg L^{-1} for sodium formate ($NaCHO_2$) and Vmax of 0.27 ± 12 mg gVS^{-1} min^{-1} in the formate substrate.

The V_{max} from such in vitro experiments provides a maximum limit of reaction for what would occur in a

more natural setting (*in vivo*) since inoculated cultures of microbes in *in vitro* experiments do not face competition from other microorganisms. In an *in vivo* experimental arrangement, species competitively feed on a natural or mixed substrate, thus inhibiting each other and increasing the overall reaction time. Ruebush et al. (2006) observed by experimentation that under similar situations, one microbial population (*S. oneidensis*) performed 157-197 reactions against a combined population doing only up to a maximum of 140 reactions and averaging only up to 70-100 reactions. For details and other results regarding the specific study, please see Ruebush et al. (2006).

Conclusions

Passive and semi-passive treatment is now a popular alternative to active treatment of AMD from abandoned as well as active mines. In studying bioremediation techniques and particularly, in increasing the rate and efficiency of metal removal, knowledge of microbial activity and reactions will be crucial.

The two studies cited here on in-vitro analyses of reaction dynamics suggest that microbes respond favourably to the reduction of mineral oxides, though the processes require optimization. Inhibitory characteristics have also been observed and require detailed investigation. At the same time, however, it appears that enzymatic systems can kinetically account for biological reduction of minerals, given that microbes have different tolerance levels and preferences. Proper knowledge and application of these aspects have the potential to dramatically improve bioremediation, which is normally associated with low speeds of reaction, periods of microbial inactivity, and deviation from the targeted outcome. The information presented in this study should be considered in exploring the nature of a community metabolic network, in defining conditions for cultivating new organisms, to monitor community structural differences, and to understand substrate preferences. Despite genetic similarities, microbes at different sites can show certain evolutionary changes in their types and behaviour, much of which is still unknown. A limiting aspect of all the reported studies is that they concern only AMD in isolation, not in mixtures with other pollutants like organics, and are thus only useful for mine discharges.

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