Cometabolic Bioremediation

Terry C. Hazen

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Abstract

Cometabolic bioremediation is probably the most underappreciated bioremediation strategy currently available. Cometabolism strategies stimulate only indigenous microbes with the ability to degrade the contaminant and cosubstrate, e.g., methane, propane, toluene, and others. This highly targeted stimulation insures that only those microbes that can degrade the contaminant are targeted, thus reducing amendment costs, well and formation plugging, etc. Cometabolic bioremediation has been used on some of the most recalcitrant contaminants, e.g., PCE, TCE, MTBE, TNT, dioxane, atrazine. Methanotrophs have been demonstrated to produce methane monooxygenase, an oxidase that can degrade over 1000 compounds. Cometabolic bioremediation also has the advantage of being able to degrade contaminants to trace concentrations, since the biodegrader is not dependent on the contaminant for carbon or energy. In the Gulf of Mexico and

T. C. Hazen (✉)
Department of Civil & Environmental Engineering, Microbiology, Earth & Planetary Sciences & Institute for Secure and Sustainable Environments, University of Tennessee, Knoxville, TN, USA

Oak Ridge National Laboratory, Knoxville, TN, USA
e-mail: tchazen@utk.edu

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in the Arctic Tundra, we have recently found that natural attenuation can be a cometabolic process also. Increasingly we are finding that in order to protect human health and the environment that we must remediate to lower and lower concentrations, especially for compounds like endocrine disrupters and trace organics, thus cometabolism may be the best and may be the only possibility that we have to bioremediate some contaminants.

1 Introduction

Cometabolism is the process by which a contaminant is fortuitously degraded by an enzyme or cofactor produced during microbial metabolism of another compound. Typically, there is no apparent benefit to the microorganism involved. Bioremediation strategies that use electron donors that only stimulate a specific group of microorganisms that can degrade the contaminants of concern are ideal for many applications. Many electron donors used as amendments for bioremediation can broadly stimulate many members of the indigenous microbial community, most of which do not have the ability to degrade or completely degrade the contaminants of concern. Indeed, this often creates problems of excess biomass (e.g., plugging the aquifer around the injection site), incomplete degradation of contaminants, transformation of contaminants to more recalcitrant or toxic daughter products, higher costs (amendment/contaminant), and inability of the amendment to stimulate biodegradation at low contaminant concentrations. Cometabolic bioremediation enables remediation strategies that stimulate biodegradation of the contaminants at contaminant concentrations that are way below the concentration that could be of carbon or energy benefit to the biodegrader. Thus, cometabolic bioremediation has the added advantage of allowing scrubbing of environmental contaminants down to undetectable concentrations, e.g., <parts per trillion. Cometabolic bioremediation has been applied both aerobically and anaerobically to a wide variety of contaminants in different environments. The first mention of cometabolic bioremediation was by Wilson and Wilson (1985) and was later defined by McCarty (1987). Cometabolic bioremediation has been used in the field for more than 30 years on some of the most recalcitrant contaminants, e.g., chlorinated alkenes, PAHs, halogenated aliphatic and aromatic hydrocarbons, MTBE, explosives, dioxane, PCBs, pesticides, and pharmaceuticals.

Microorganisms are versatile in their ability to exist in a variety of habitats and live in hostile environments having a wide range of pH, temperature, heavy metal concentrations, oxygen concentrations, barometric pressures, salinity, and radiation. Under these diverse conditions, a number of microbial types have been isolated that cometabolize contaminants and their daughter products. Ensley (1991) demonstrated a linkage between TCE degradation and aromatic metabolism in *P. cepacia* G4, *P. mendocina*, and *P. putida*. Ensign et al. (1992) reported that pure cultures of *Xanthobacter* sp. cometabolized TCE with the utilization of propylene as a substrate using the enzyme alkene monooxygenase. It is well recognized that TCE and other

chlorinated aliphatic compounds can be degraded by selected methanogens (Bouwer and McCarty 1984), methanotrophs (Little et al. 1988), species of *Pseudomonas* (*P. cepacia, P. mendocina* and *P. putida*), and nitrifiers (Vannelli et al. 1990; Hyman et al. 1988) capable of degrading aromatic compounds (Nelson et al. 1988). Additionally, aerobic conditions do not appear to support the formation of undesirable metabolites, such as c-DCE, t-DCE or VC that are dehalogenation products of anaerobic degradation of TCE. Mahendra et al. (2007) demonstrated that monooxygenase-containing bacteria could degrade 1,4-dioxane. Methyl tert-butyl ether (MTBE) has also been remediated cometabolically (Chen et al. 2006), as has TNT (Yasin et al. 2008), PCBs (Lajoie et al. 1994), and atrazine (Ghosh and Philip 2004) (Table 1). More recently emerging trace organic contaminants (Liu et al. 2015), carbazole (Shi et al. 2015), dibenzofuran (Shi et al. 2013), pharmaceuticals (Gauthier et al. 2010), 1,1,2,2-tetrachloroehane (Cappelletti et al. 2018), lincomycin (Li et al. 2016), tetrabromobisphenol (Gu et al. 2016), and decolorization of textile dyes (Karim et al. 2017) have all been shown to biodegrade cometabolically.

The aerobic cometabolic biodegraders are dependent upon oxygenases, e.g., methane monooxygenase, toluene dioxygenase, toluene monooxygenase, and ammonia monooxygenase. These enzymes are extremely strong oxidizers, e.g., methane monooxygenase is known to degrade over 1000 different compounds. However, like any bioremediation process, the proper biogeochemical conditions are necessary to maximize and maintain biodegradation, e.g., maintaining oxygen levels or other terminal electron acceptors that the cometabolic biodegrader is dependent (Table 2 and Hazen 1997; Hazen and Sayler 2016, and *Chapter in this book on in situ groundwater bioremediation*). In addition, cometabolic biostimulation may require pulsing of electron donor or electron acceptor to reduce competitive inhibition between the substrate the microbe can use and the contaminant. Pulsing of methane was found to significantly improve biodegradation of TCE rates by methanotrophs (Hazen et al. 2009). Indeed, during the Deep Water Horizon (DWH) leak (Hazen et al. 2010), there was evidence that in the Gulf of Mexico where episodic releases methane have occurred for millions of years from natural seeps that this pulsing of methane may be degrading oil and other organics via cometabolic biodegradation. The methane oxidizers bloomed during the DWH leaked above 400 m once the well was capped (Dubinsky et al. 2013; Redmond and Valentine 2012; Reddy et al. 2012). This suggests that intrinsic cometabolic bioremediation or cometabolic natural attenuation may be a serious phenomenon in the ocean and in arctic tundra (Stackhouse et al. 2017). It has also been found that significant background biodegradation reactions can occur during injection of terminal electron acceptors like oxygen. Enzien et al. (1994) demonstrated that in a bulk aerobic environment being injected with methane and air significant amount of reductive dechlorination of PCE to TCE could occur in anaerobic niches in the aquifer sediment. Rates of PCE and TCE oxidation are inversely different depending on the number of Cl (Fig. 1).

Given the diverse body of literature on cometabolic bioremediation processes, we will focus in detail on the two groups that have been most well studied, i.e., methanotrophs and ammonium oxidizers.

Table 1 Cometabolic bioremediation substrates, enzymes, and contaminants

Cosubstrates	Methane, methanol, propane, propylene biphenyl (aerobic)	Ammonia, nitrate (aerobic)	Toluene, butane, phenol, citral, cumin aldehyde, cumene, and limonene (aerobic)	Methanol (anaerobic)	Glucose, acetate, lactate, sulfate, pyruvate (anaerobic)
Enzymes (microbes)	Methane monooxygenase, methanol dehydrogenase, alkene monooxygenase, catechol dioxygenase (*Methylosinus, Ralstonia, Rhodococcus*)	Ammonia monooxygenase (*Nitrosomonas, Nitrobacter, ammonia oxidizing archaeal*)	Toluene monooxygenase, toluene dioxygenase (*Rhodococcus, Pseudomonas, Arthrobacter, Comamonas*)	Alcohol dehydrogenases (*Pseudomonas, Streptomyces, Corynebacterium*)	Dehalogenase, AtzA, dichloromethane dehalogenase (*Dehalococcoides, Dehalobacter, Methanogens, Desulfovibrio, Clostridium, Geobacter, Clavibacter, Aspergillus*)
Contaminants	TCE, DCE, VC, PAHs, PCBs, MTBE, creosote, 1,4 dioxane, >1000 different compounds	TCE, DCE, VC, TNT, emerging trace organic contaminants	TCE, DCE, VC, 1,1-DCE, 1,1,1-TCA, MTBE, TBBPA, carbazole, dibenzofuran, pharmaceuticals	PCE, TCE, DCE, VC, hexachloro-cyclohexane	BTEX, PCE, PAHs, pyrene, atrazine, TNT, lincomycin decolorization, etc.

Table 2 Performance monitoring parameters for cometabolic biodegradation

Performance parameter	Method	Data use	Performance expectation	Recommended frequency of analysis
Chemicals of concern (CoCs)	EPA SW-846: 8260B (VOC) or 8270D (SVOC) (laboratory). Field gas chromatography (GC) or GC/mass spectroscopy (MS)	Background and source/plume for comparison following treatment. Also used to determine if there are compounds that may inhibit the cometabolic process	CoCs and degradation products are expected to decline to below regulatory compliance levels within the treatment zone after substrate addition	Baseline and recommended for each groundwater sampling round
Primary substrate	EPA SW-846: 8260B (VOC) or 8270D (SVOC) (laboratory). Field gas chromatography (GC) or GC/mass	Used to determine the extent and availability of substrate for consumption by bacteria	Downward trend in substrate concentrations should track downward trend in CoC concentrations	Site specific – baseline and all sampling events thereafter
Appropriate cometabolic degrading microorganisms	Quantified by molecular techniques such as polymerase chain reaction – specialty laboratory	Used to determine presence and quantity of appropriate microorganism at baseline period or after bioaugmentation	Appropriate microorganisms will be detected and increase as a consequence of adding electron acceptors and/or donors	Baseline prior to remedy initiation and quarterly. Once a high titer is measured and growth is ensured, the test is not critical
Oxygen	Bacharach Fyrite® Gas Analyzer (soil gas). DO meter (APHA 1992: 4500-O G) (field). Downhole probe or flow-through cell (groundwater)	For aerobic cometabolism determines aerobic conditions exist. For anaerobic cometabolism determines absence of oxygen	Determines if consumption of oxygen requires supplementation or if increase in oxygen will require substrate to reduce it	Site specific – baseline and as appropriate thereafter
pH	Field probe with direct-reading meter (APHA 1992: 4500-H+ B)	Used to confirm pH conditions are stable or to identify trends of concern (EPA 2004)	Enhanced aerobic bioremediation pH range of 5–9 pH units (EPA 2004)	For active systems daily for the startup phase (7–10 days) and weekly to monthly

(*continued*)

Table 2 (continued)

Performance parameter	Method	Data use	Performance expectation	Recommended frequency of analysis
				thereafter (EPA 2004)
Carbon dioxide	APHA et al. 1992: 4500-CO_2 C (titrimetric) or 4500-CO_2 D (calculation requiring total alkalinity and pH)	Used as an indicator that microbial activity has been stimulated	Indicator parameter	Optional for active system (ITRC 2008)
Oxidation reduction potential (ORP)	Direct-reading meter, A2580B, or USGS A6.5 (field)	Used with other geochemical parameters to determine if groundwater conditions are optimal for aerobic or anaerobic biodegradation	Positive ORP values (>0.0 mV) with elevated DO and absence of TOC/DOC can indicate that additional substrate is needed for anaerobic biodegradation	Baseline and typically measured at the wellhead using a flow-through cell to protect samples from exposure to oxygen

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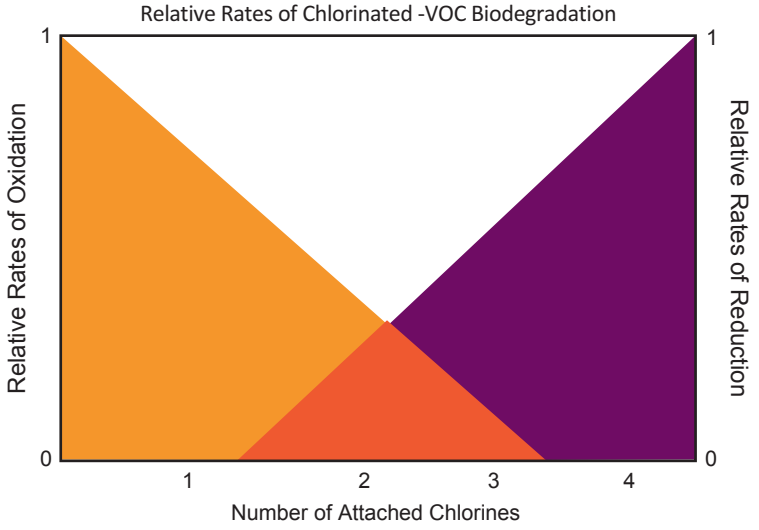

Fig. 1 Aerobic and anaerobic biodegradation rates

2 Methanotrophs

Methanotrophs, methane-oxidizing bacteria, oxidize methane via a series of enzymes that are unique to this group (Koh et al. 1993). The primary enzyme in this oxidation chain is methane monooxygenase. Methane monooxygenase is an extremely powerful oxidizer, thus giving it the capability of oxidizing a wide variety of normally recalcitrant compounds including TCE (Cardy et al. 1991). Wackett (Newman and Wackett 1991; Tsien et al. 1989) and others (Chaudhry and Chapalamadugu 1991; Wilson and Wilson 1985; Fogel et al. 1986; Little et al. 1988) demonstrated that soluble methane monooxygenase induces formation of TCE-epoxide from TCE. TCE-epoxide is extremely unstable and therefore spontaneously breaks down to simpler compounds like formate, etc. All of the daughter compounds are either unstable or small and easily metabolizable compounds, thus making the final and almost immediate end products of TCE-epoxide formation, carbon dioxide, and chloride salts, unlike anaerobic dechlorination which can stall at daughter products like vinyl chloride which are more toxic than the original contaminant, e.g., PCE and TCE (Fig. 2).

Methanotrophic bacteria (methanotrophs) are bacteria that use methane as a sole source of carbon. The first enzyme involved in the oxidation of methane to methanol by methanotrophs is methane monooxygenase (MMO). Two forms of MMO have been reported: soluble methane monooxygenase (sMMO), found mainly in the

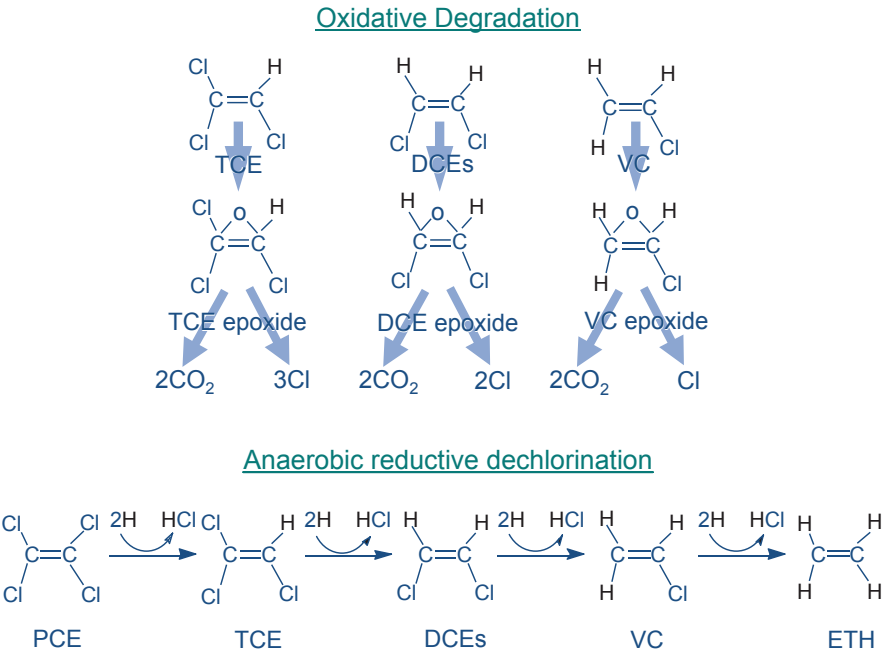

Fig. 2 Aerobic and anaerobic cometabolic pathways

cytoplasm and particulate methane monooxygenase (pMMO) which is associated with the cell membrane. Studies related to these two enzymes have mainly been studied in two methanotrophs, namely, *Methylococcus capsulate* (Bath) and *Methylococcus trichosporium* OB3b. Numerous groups have studied sMMO in great detail with regard to isolation and characterization as well as crystal structure. Since pMMO is membrane bound, this enzyme losses activity upon lysis making it difficult to isolate and purify resulting in fewer details regarding this enzyme. The two enzymes can coexist in methanotrophs; however, their activities have been directly reported to be dependent on the copper ion to biomass ratio in *M. capsulate* (Bath). A low copper ion to biomass ratio expresses sMMO, while a high copper ion to biomass ratio expresses pMMO (Stanley et al. 1983). While pMMO is found in most methanotrophic bacteria, sMMO is present only in a few select methanotrophs. Both MMOs oxidize methane to methanol and are capable of cometabolizing chlorinated aliphatic hydrocarbons, namely, chloroform, dichloromethane, *trans*-dichloroethene, *cis*-1,2-dichloroethene, 1,1-dichloroethene, trichloroethene at various rates and to different extents. Therefore, methanotrophs are a useful tool for commercial purposes mainly cleanup of sites contaminated with toxic pollutants. However, sMMO being nonspecific has a broader substrate specificity in comparison to pMMO, some substrates like cyclohexane or naphthalene cannot be oxidized by pMMO, and both enzymes do not oxidize perchloroethylene. Methanotrophs have also been reported to be useful for production of bulk chemicals and as methane sinks (Oremland and Culbertson 1992). Mixed cultures expressing pMMO have shown to degrade t-DCE, VC, c-DCE, TCE, and 1,1-DCE. Transformation of t-DCE and VC by pMMO was 20 times greater than those reported for sMMO, while transformation of the other three compounds was either similar or less, indicating the importance of this enzyme over sMMO for bioremediation.

One of the many uses of methanotrophs has been in the bioremediation of trichloroethylene (TCE), which is most commonly found in groundwater along with other halogenated compounds. The first product formed in the oxidation of TCE is an epoxide which is then converted to glyoxylic acid with chloride being released. Glyoxylic acid is then oxidized to carbon dioxide. Although TCE is known to be degraded by several other bacteria, e.g., various species of *Pseudomonas*, containing oxygenases, the rate of degradation by methanotrophs expressing sMMO is many times faster than pMMO and other oxygenases making it favorable for use in bioremediation. For efficient bioremediation, it is important to optimize enzyme/enzymes activity responsible for the transformation, as well as to maintain the activity for extended period of time. This has been studied in detail for *M. trichosporium* OB3 by Sayler et al. (1995). Their study showed that specific sMMO activity was directly proportional to the concentration of dissolved methane. Addition of formate (20 mM) significantly increased sMMO activity. Nitrate, phosphate, iron, and magnesium also had remarkable effect on growth as well as sMMO activity. Addition of vitamins also effected sMMO activity; however, excessive vitamins proved to be harmful. Such studies are necessary and prove useful when designing a bioremediation process.

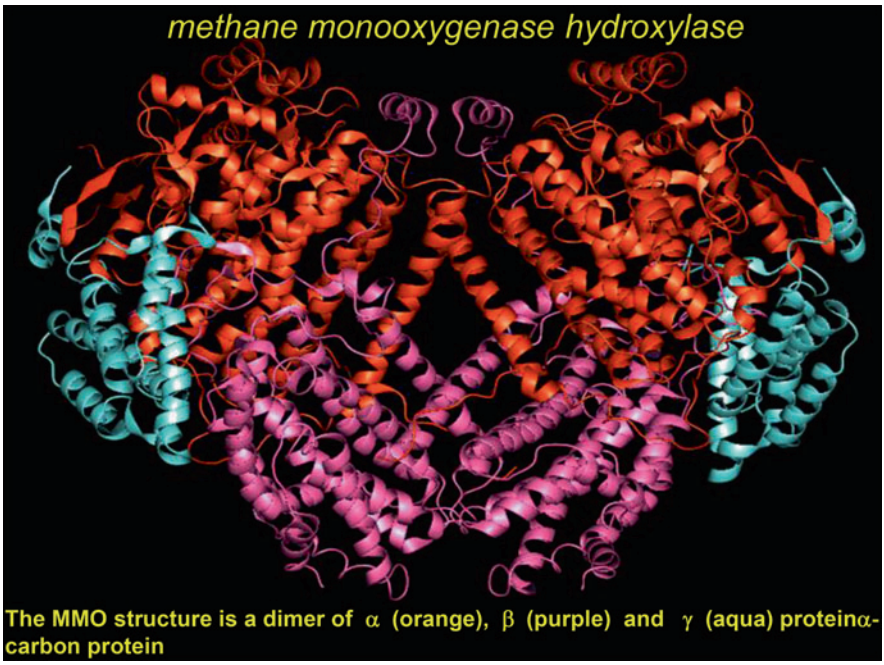

Fig. 3 Methane monooxgenase 3D molecular structure

sMMO from *M. capsulatus* (Bath) and *M. trichosporium* OB3b was shown to consist of three components: protein A, a hydroxylase made-up of three subunits α, β, γ, of molecular masses 60, 45, and 20 kDa, respectively; protein B which is 16 kDa, a regulatory protein; and protein C 39 kDa, a reductase (Paulsen et al. 1994). The crystal structure of sMMO hydroxylase has also been determined (Rosenzweig et al. 1993) (Figs. 3 and 4). In both organisms, the genes encoding for soluble methane monooxygenase enzyme complexes have been found to be clustered on the chromosome. The complete DNA sequences of both gene clusters have been determined and they show considerable homology (Murrell 1992). Detailed studies of the genes encoding sMMO, the DNA sequence, have led to the development of sMMO probes which have been used to detect MMO gene-specific DNA and methanotrophs in mixed cultures and in natural environmental samples (Hazen et al. 2009). The genes for Protein B and Protein C of *Methylococcus* have been expressed in *E. coli* and the proteins obtained were functionally active. Cloning of sMMO genes has led to construction of sMMO mutants of *M. trichosporium* OB3b.

Anderson and McCarty (1997) have reported higher yields of t-DCE and VC degradation by methanotrophs expressing pMMO as compared to sMMO. Also the fact that pMMO are present in most methanotrophs seems logical to develop systems that can enhance this activity for the purpose of treatment of sites contaminated with these compounds. Although sMMO and pMMO are known to coexist in

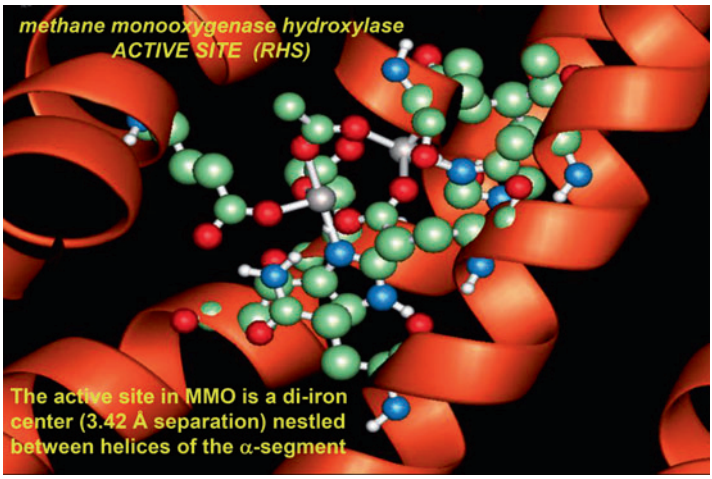

Fig. 4 MMO reaction site

methanotrophs, the fact that pMMO is membrane bound has made it difficult to purify this enzyme unlike sMMO and perform detailed studies like sMMO. Several groups have attempted and are still pursuing this aspect of pMMO and to date only a few reports are available.

Isolation of active pMMO from methanotrophs has been difficult since it loses activity once it has been separated from the membrane. The loss of pMMO activity has been reported to be overcome by addition of a nonionic detergent followed by removal of the detergent and reconstitution of lipid vesicle. Activity of pMMO in the membrane fraction was also stabilized by increasing the concentration of copper in growth medium. Other factors favoring pMMO activity were increased iron and copper concentration, maintaining the pH of buffer at 7.0 and anaerobic conditions during solubilization. Addition of copper ions has resulted in enhanced pMMO activity; however, it has not prolonged the activity nor does it reactivate the enzyme once activity is lost (Zahn et al. 1996). The isolation and characterization of pMMO from *M. capsulatus* (Bath) have been reported by Nguyen et al. (1998). They have obtained active stable pMMO from *M. capsulatus* (Bath) by maintaining high copper levels and methane stress conditions in growth medium. Membrane solubilization was achieved under anaerobic conditions and by addition of dodecyl beta-D-maltoside. The active extract was then purified by chromatography. By switching the growth conditions to favor pMMO activity over sMMO, the same group has reported three polypeptides of 46, 35, 26 kDA and has shown a trinuclear copper center in pMMO by EPR. They have reported pMMO to be copper requiring and sensitive to dioxygen similar to the results of Zahn et al. (1996). The switch between sMMO and pMMO gene expression has been suggested to involve a common regulatory pathway. Chan et al. (2004) have shown pMMO from *M. capsulatus* (Bath) to be a copper-containing three-subunit enzyme. The role of copper in pMMO has been reported to be in the active site of pMMO rather than a structural one.

3 Ammonium Oxidizers

Nitrification is the bacterial mediated process in which ammonia is oxidized sequentially to nitrite then to nitrate. In soils and fresh and saline waters, ammonia is oxidized to nitrite by nitrite-oxidizing bacteria such as the chemolithoautotrophic bacterium, *Nitrosomonas europaea*. Nitrite is oxidized to nitrate by nitrate-oxidizing bacteria such as *Nitrobacter agilis* and *N. winogradskyi* (Fliermans et al. 1974). Nitrifying bacteria are ubiquitous components of the soil and sediment microbial populations. Their activities are stimulated in agricultural soils following the application of ammonia- or urea-based fertilizers.

The oxidation of ammonia to nitrite by *Nitrosomonas europaea* is initiated by the enzyme ammonia monooxygenase (AMO). Because of the broad substrate range of AMO (Arciero et al. 1989), nitrifiers such as *N. europaea* can be used in the bioremediation of contaminated soils, sediments, and groundwaters (Yang et al. 1999). AMO catalyzes the oxidation of ammonia to hydroxylamine which is subsequently oxidized to nitrite (NO_2) by hydroxylamine oxidoreductase (Wood 1986) with the release of four electrons. Two of the electrons are transferred to AMO in order to activate the O_2 and maintain a steady state for ammonia oxidation. AMO in *Nitrosomonas europaea* also catalyzes the oxidation of several alternate substrates including hydrocarbons and halogenated hydrocarbons (Rasche et al. 1990). These oxidations require a reductant which can be supplied by the simultaneous oxidation of ammonia.

Both CH_4 and C_2H_4 competitively inhibit ammonia oxidation by *N. europaea*, since it appears that these compounds bind predominantly to the same binding site as ammonia (Keener and Arp 1993). The competitive character of the inhibition of CH_4, C_2H_4, C_2H_6, CH_3Cl, and CH_3Br is supported by the optimal N_2H_4 requirements that decrease with increasing concentrations of ammonia. Thus, it is not likely that the stimulation of TCE degrading bacteria of the genus *Nitrosomonas* would occur with the injection of methane or other substrates that were competitively inhibitory to the AMO enzyme. Under bioremediation techniques that injected methane, a loss of the *Nitrosomonas* population that has the ability to degrade TCE would be inhibited. Such a phenomenon was observed through the use of species-specific fluorescent antibodies (Fliermans et al. 1994; Hazen et al. 1994).

The AFCEE *IRP Aerobic Cometabolic In Situ Bioremediation Technology Guidance Manual and Screening Software User's Guide* provides specific guidance on well placement and technology design.

See Hazen and Sayler (2016) for examples and methods for Environmental Systems Biology of Contaminated Sites.

4 Research Needs

Cometabolic bioremediation is extremely underappreciated as a bioremediation strategy, though it has been used for an extremely wide variety of contaminants in different environments with different cosubstrates. Indeed, it has also been

underappreciated as a natural attenuation phenomenon as recently demonstrated by studies in the arctic tundra and the Gulf of Mexico (DWH). Much more research needs to be done on modeling life cycle costs of various remediation strategies, including treatment trains and grading into natural attenuation or intrinsic bioremediation. These models need to be tested and verified in full-scale deployments. Cometabolic processes quite often can easily be graded into natural attenuation, e.g., air injection alone at sites with methane or other cometabolic substrate to increase degradation rate and transition into a stable aerobic or microaerophilic environment that can sustain natural attenuation of any residual contaminant. Research on bioaugmentation strategies using cometabolic biodegraders and synthetic biology to produce unique, high rate, and highly specific biodegraders could vastly improve our environmental stewardship in the future.

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