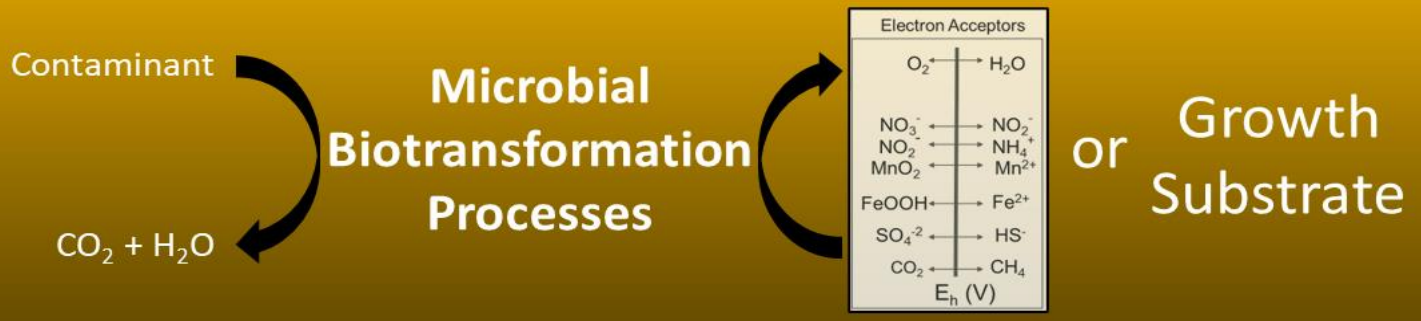
SMWG WHITE PAPER (MAY 2022)

Application of Molecular Biological Tools to Assess, Monitor, or Enhance Biodegradation and Biotransformation at Sediment Sites



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Scope: Literature review to explore potential for field-scale use of molecular biological tools (MBTs) to support use of monitored natural recovery (MNR) or enhanced MNR (EMNR) as an appropriate remedial alternative for sediment sites

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1.0 INTRODUCTION

1.1 Overview and Objectives

Assessment of natural recovery mechanisms to guide sediment site management strategies has traditionally focused on physical and chemical attenuation processes (e.g., burial, dilution, precipitation, adsorption, volatilization, hydrolysis). While physical and chemical processes are important for risk reduction and natural recovery, biologically-mediated attenuation processes (i.e., biodegradation and biotransformation[1]) can play a substantial role in governing natural recovery mechanisms (ESTCP, 2009) and are appropriate to be considered as a remedial alternative. The limited or absent assessment of biological attenuation processes in many contaminant sediment site investigations leads to uncertainties regarding contaminant fate (ITRC, 2013). This is not to say that sediment practitioners do not recognize the importance of biologically-mediated processes on contaminant fate, but that application of technologies to directly assess these processes are not traditionally performed. Rather, biological processes are typically inferred through contaminant trends, biogeochemical conditions, or chemical forensics, introducing uncertainties in the conclusions reached and project decisions informed by these conclusions (Lawson *et al*., 2019; Rittmann and McCarty, 2020; Magar and Wenning, 2006; Murphy and Morrison, 2007; Stout *et al*., 2001, 2004). Decades of laboratory and field studies have shown that microorganisms indigenous to the subsurface can biodegrade or biotransform a variety of contaminants, including petroleum hydrocarbons, chlorinated solvents, munitions, pesticides, PCBs, heavy metals (e.g., mercury, arsenic, chromium, etc.) and many other compounds (Bombach *et al*., 2010; Bouwer & Zehnder 1993; Wiedemeier *et al*., 1999).

Due to the technical advances and reduced costs with molecular biological tools (MBTs), such as quantitative polymerase chain reaction (qPCR), microbial-mediated contaminant attenuation of sediment contaminants can now be measured rather than relying on conceptualizations or inferences. While these tools have been applied at upland sites with increasing regularity, far fewer case studies or applications at contaminated sediment sites are cited in the literature.

This white paper presents an overview of the scientific basis for MBTs, applications to date, and how MBTs may be applied to advance sediment site characterization, reduce uncertainties related to biodegradation and biotransformation, aid in discussions with regulators/stakeholders, and guide remedial decision-making to advance a project towards cleanup or closure. An assessment of the state of knowledge and practice for application of MBTs to key contaminants at sediments is presented.

1.2 Molecular Biological Tools (MBTs)

Biological analytical techniques, collectively termed MBTs, are available to environmental practitioners to facilitate the identification, contaminant-degrading capabilities, and activities of microorganisms present in the environment. Over just the past two decades, MBTs have improved understanding of biotic attenuation processes and thereby decreased uncertainties of effectiveness, giving stakeholders greater confidence in making management decisions at upland sites (Beller *et al*., 2002; Cupples, 2008; Madsen, 2000; Wilson *et al*., 1999; Winderl *et al*., 2007). MBTs consists of assays to assess microbial biomolecules (e.g., DNA, RNA, phospholipids) or stable isotopes indicative of biotransformation, and can complement traditional data by providing direct measurement of

[1] For the purposes of this white paper biodegradation will be used strictly to mean complete mineralization to CO_2 and H_2O, whereas biotransformation means yield organic metabolites that may or may not be able to be further transformed (Kiel and Engesser, 2015).

the presence or activity of contaminant-degrading microorganisms and their biodegradation/biotransformation processes.

MBTs can be further grouped as genetic- (or nucleic acid-) based tools or isotope-based tools.

1.2.1 Genetic- or Nucleic Acid-Based Tools

Nucleic acid-based tools are analyses that probe the genetics of microorganisms including deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). These tools are used to detect or quantify genes associated with microorganisms. These tools can be designed to target specific functional genes that encode enzymes implicated in contaminant biodegradation or can be applied in a non-targeted approach to assess the composition of the microbial community.

Polymerase Chain Reaction (PCR)

What it is: a laboratory method used to make copies of a specific DNA segment extracted from an environmental sample or microbial culture to identify specific organisms and functional genes.

Example application: In PCR reactions, a target gene in a sample is located using short segments of DNA called primers. Many copies of the target gene are then generated.

Quantitative Polymerase Chain Reaction (PCR)

What it is: a laboratory analytical technique for quantification of a target gene based on DNA PCR technology.

Example application: Detect and quantify the presence of a specific gene(s) to assess the presence and abundance of contaminant-degrading microorganisms or functional genes. The abundance of the genes can be monitored over space and time.

Reverse Transcriptase qPCR (RT-qPCR)

What it is: a laboratory analytical technique for quantification of an expressed target gene based on complementary DNA (cDNA) transcribed from RNA that indicates if microorganisms are actively expressing specific genes.

Example application: While qPCR quantifies the DNA of genes having the potential to biodegrade contaminants, the genes may be present but not expressed. RT-qPCR can assess the degree to which genes associated with contaminant biodegradation are being actively expressed.

Next Generation Sequencing (NGS) targeting 16S rRNA genes

What it is: a DNA sequencing technology that identifies the presence and relative abundance of microorganisms in environmental samples. NGS targeting 16S rRNA genes is used to evaluate and identify prokaryotes (bacteria and archaea).

Example application: DNA sequencing is useful when the identities of microorganisms responsible for contaminant biodegradation or biotransformation at a site are unknown.

At present, MBTs based on nucleic acids are not covered by USEPA, ASTM, or other standards. As such, there is variability in methods that have been applied in different laboratories. The lack of method standardization may complicate comparisons between studies and between sites. It should also be noted that as new information from research findings becomes available, it may be necessary to reevaluate conclusions from previous analyses. Data

interpretation and reinterpretation requires that assays are standardized or information about assays be fully documented by service providers. In the case of PCR, qPCR, and RT-qPCR, this includes documenting the sequences of primers, probes, thermal programs, and reaction chemistry. At the time of this white paper development, an ASTM Standard is being developed to standardize the application and methods of gene-based MBTs.

1.2.2 Isotope-Based Tools

Isotope-based tools are analytical methods that measure stable isotope levels as evidence of biodegradation or biotransformation of a specific contaminant. These methods may be employed to measure isotope ratios (e.g., ^{13}C:^{12}C normalized to a standard carbonate mineral, Pee Dee Belemnite) or track isotope fate.

Compound Specific Isotope Analysis (CSIA)

What it is: an analytical method that determines the ratio of naturally occurring stable isotopes of select elements (typically $^{13}C/^{12}C$, $^{2}H/^{1}H$, or $^{37}Cl/^{35}Cl$) in a particular compound following separation from other compounds in a sample matrix.

Example application: For some compounds, the ratio of $^{13}C/^{12}C$, $^{2}H/^{1}H$, and/or $^{37}Cl/^{35}Cl$ can serve to differentiate biotic and abiotic reactions and can serve to demonstrate a dominant fate process.

Stable Isotope Probing (SIP)

What it is: a method which uses synthetic ^{13}C-labeled contaminant of interest, called a stable isotope probe. Methods track the environmental fate of a ^{13}C-labeled contaminant.

Example application: Recovery of ^{13}C in the form of $^{13}CO_2$ or ^{13}C-labeled DNA or phospholipids can provide proof that microbes indigenous to a site have the capacity to transform a contaminant. Methods to identify ^{13}C-labeled DNA segments can allow identification of degrading microbes when the contaminant is assimilated as a carbon source.

2.0 APPLICABILITY OF MBTS TO CONTAMINATED SEDIMENT MANAGEMENT

2.1 Successful MBTs Application at Upland Sites

For some pollutants, the application of MBTs has become well established to complement traditional site characterization parameters (e.g., measurement of contaminant concentrations and geochemical parameters) in remedy selection, and performance monitoring at upland sites (Beller *et al*., 2002; Cupples, 2008; Madsen, 2000; Wilson *et al.*, 1999; Winderl *et al*., 2007). Several publications and guidance documents have been written to present the advantages of employing MBTs at upland sites in concert with traditional analyses to reduce attenuation uncertainties and better characterize subsurface microbiology (Amos et al., 2008; Bombach et al., 2010; Bouchard *et al*., 2018; Busch-Harris *et al*., 2008; Lawson *et al*., 2019; Rittmann & McCarty, 2020; Zhang *et al*., 2016). MBTs have been applied as complementary tools at each stage of the contaminated upland site project lifecycle as an additional line of evidence to: (a) develop or refine biogeochemical processes within the conceptual site model (CSM) (b) reduce uncertainty of biological processes associated with remedy design, (c) monitor remedy performance and differentiate biological processes from chemical or physical processes, and (d) support communications with stakeholders (ITRC, 2013).

2.2 Application of MBTs to Sediment Sites to Minimize Uncertainty

Sediment environments are generally complex settings where physical, chemical and biological processes interact to directly or indirectly affect contaminant fate and transport and exposure risk to potential receptors. Because of these complexities, cleanup considerations for sediment sites should be based on multiple lines of analyses and characterizations that develop the basis of a sound conceptual site model (CSM). Key to developing this understanding is knowledge of how natural processes influence the natural recovery of contaminated sediments. Further, with the increased focus on in-place sediment management approaches by regulators and industry, natural recovery (i.e., Monitored Natural Recovery [MNR]) or combined approaches that integrate MNR with capping, dredging, or technologies to accelerate natural recovery are remedy approaches for managing long-term environmental risk.

Published literature and guidance from federal agencies (e.g., USDoD, USEPA) consistently recognize that the appropriateness, effectiveness, and permanence of natural recovery as a stand-alone or component of the remedy should be evaluated using multiple lines of evidence to minimize uncertainties to the extent possible (consistent with Section 4.4 of USEPA's *Contaminated Sediment Remediation Guidance for Hazardous Waste Sites* [USEPA, 2005]). While empirical measurements of physical natural recovery processes are collected to develop conclusions, when biological attenuation processes in sediment environments are evaluated, contaminant biodegradation/biotransformation is usually inferred based on a limited suite of biogeochemical parameters and/or contaminant trends. This can result in uncertainty in determining the occurrence and effect of biodegradation/biotransformation related to natural recovery timeframes. As a result, these large uncertainty bounds can minimize predicted natural recovery performance trajectories or result in the omission of biodegradation/biotransformation during remedy selection and can result in an overly conservative remedial strategy.

While application of MBTs at upland sites has become more common, MBT applications to assess microbiological processes at sediments sites by the sediment cleanup community has not expanded substantially beyond focused research and development applications[2] for reasons unclear to the authors. Application of MBTs to sediments have the potential to provide supplementary empirical data to reduce uncertainty related to contaminant fate and biodegradation by:

- Assessing presence and abundance of microorganisms capable of degrading contaminants
- Assessing contaminant degrading activity of microorganisms
- Assessing occurrence of contaminant biodegradation/biotransformation pathway(s)

[2] Example applications include qPCR analyses to assess the mercury methylation (Podar et al. 2015) and microbial transformations of heavy metals (Sun et al. 2021).

3.0 REVIEW OF STATE OF KNOWLEDGE AND STATE OF PRACTICE ON BIODEGRADATION OF CHLORINATED ETHENES, PCBs, AND PAHs

3.1 Scope

Because successful application of MBTs has been demonstrated at upland sites and industry standardization efforts are underway[3], there is an opportunity to develop similar strategies for applying MBTs at sediment sites. To assess this opportunity specific to sediment sites, a literature review was performed to assess the state of knowledge and practice for application of MBTs to key sediment contaminants. This search aimed to identify which target contaminant(s) can be utilized in future applied research to demonstrate the application of MBTs to assess microbial degradation in sediments. As part of this review, the Contaminants of Concern (COCs) for consideration[4] included:

- Chlorinated Volatile Organic Compounds (CVOCs), specifically chlorinated ethenes
- Polychlorinated Biphenyls (PCBs)
- Polycyclic Aromatic Hydrocarbons (PAHs), specifically naphthalene and methylnaphthalenes

As part of the literature review, evaluation of each COC was conducted to assess the current state of knowledge of biodegradation to identify a COC recommended for focus during potential, future applied research applications of MBTs. The key considerations used to screen each COC include:

- Are biodegradation or biotransformation pathways established?
- Have sediment and/or porewater laboratory-scale biodegradation studies (e.g., microcosms, columns) been documented?
- Are MBTs established to monitor biodegradation or biotransformation in sediments at laboratory-scale or field-scale?

A summary of the literature review for each COC suite is provided in the following sections and associated summary tables.

3.2 Chlorinated Ethenes

3.2.1 Lab-Scale Biotransformation Studies & Associated Pathways

Under aerobic conditions, TCE, *cis*-1,2-DCE, and vinyl chloride can be cometabolically[5] transformed by bacteria that grow on a variety of hydrocarbons including methane, ethene, propane, and toluene through processes employing mono- or dioxygenase enzymes (McCarty *et al.*, 1998; Mattes *et al*., 2010). The intermediate products can vary depending on the primary substrate and microorganism. For example, toluene 2-monooxygenase from *Burkholderia cepacia* G4 and soluble methane monooxygenase from *Methylosinus trichosporium* OB3b both transform TCE to the unstable intermediate TCE epoxide which undergoes spontaneous reactions to form

[3] An ASTM work group is currently drafting a guide for application of MBTs at contaminated sites to promote standardization of applications, data evaluation, sampling and laboratory procedures, data quality, and data usability.

[4] Additional COCs identified as less appropriate for immediate consideration but suggested for future consideration included heavy metals (mercury, lead, copper), pesticides (DDTx, BHC), and dioxins and furans.

[5] Cometabolic biodegradation is a non-growth linked process that occurs when microorganisms are utilizing other substrates for metabolic energy gain and growth and produced enzymes fortuitously degrade the contaminant.

glyoxylic and formic acids and carbon monoxide (Fox *et al*., 1990; Newman and Wackett, 1997). The latter enzyme also leads to minor production of chloral (trichloacetaldehyde) and dichloroacetate not detected for the former (Fox *et al*., 1990). Regardless, the various intermediates can be further oxidized under aerobic conditions (ultimately to CO_2, water, and chloride). Isolates from some bacterial species (e.g., *Mycobacterium*, *Pseudomonas*, *Nocardioides*, *Ochrobactrum*, and *Ralstonia*) are able to use vinyl chloride as a carbon and energy source under aerobic conditions (Hartmans and de Bont, 1992; Coleman *et al*., 2002; Danko *et al*., 2004; Elango *et al.*, 2006).

The **pathway for anaerobic biotransformation** of tetrachloroethene (PCE) has been extensively studied with intermediates unambiguously determined (Maymó-Gatell *et al*., 1997). Bacteria belonging to a variety of genera can sequentially dehalogenate PCE to trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-1,2-DCE) or trans-1,2-dichloroethene (*trans*-1,2-DCE), and vinyl chloride (Table 1). The halogenated compounds serve as terminal electron acceptors and carbon is not assimilated. Strains from only two genera, *Dehalococcoides* and *Dehalogenimonas*, are known to carry out dechlorination of the carcinogen vinyl chloride to the non-toxic final product ethene (Maymó-Gatell *et al*., 1997; Löffler *et al*., 2013; Yang *et al*., 2017). Because these genera are not known to grow in the absence of halogenated organic compounds, their presence is indicative of the metabolic potential to transform halogenated organics.

Table 1: Bacterial 16S rRNA genes targeted by MBTs for chlorinated ethenes (anaerobic)

Genus	Relevance	References
Dehalococcoides	Reductively dechlorinates PCE, TCE, all DCE isomers, vinyl chloride	Löffler *et al*. (2000); Hendrickson *et al*. (2002); Dennis *et al*. (2003); Duhamel *et al*. (2004); Cupples (2008)
Dehalogenimonas	Dechlorination of *trans*-1,2-DCE to vinyl chloride and vinyl chloride to ethene	Yang *et al*. (2017); Molenda *et al*. (2016); Moe *et al*. (2009); Yan *et al*. (2009); Chen *et al*. (2014)
Dehalobacter	Partial dechlorination of PCE and TCE to *cis*-1,2-DCE	Holliger *et al*. (1998), Maillard *et al*. (2003)
Desulfuromonas	Partial dechlorination of PCE to *cis*-1,2-DCE	Sung *et al*. (2003)
Geobacter	Partial dechlorination of PCE to *cis*-1,2-DCE	Sung *et al*. (2006a)
Desulfiltobacterium	Partial dechlorination of PCE and TCE to *cis*-1,2-DCE	Gerritse *et al*. (1996)

A variety of laboratory-scale enrichment cultures and column studies have demonstrated anaerobic dehalogenation of chlorinated ethenes by bacteria in freshwater sediments (De Bruin *et al*., 1992; Qiu *et al*., 2020), brackish sediments (Aulenta et al. 2002), and marine sediments (Kittelmann & Friedrich, 2008; Futagami *et al*., 2013; Matturro *et al*. 2016).

3.2.2 Established MBTs

MBTs targeting non-specific oxygenases that can cometabolically transform TCE and lower chlorinated alkenes under **aerobic conditions** have been developed (Table 2). Rate constants calculated based on qPCR assays have been found to correlate with rate constants derived from ^{14}C assays (Wilson *et al*., 2019); though additional work is being performed to assess applications to assess rates at field-scale. MBTs targeting genes associated with vinyl chloride assimilation (*etnC* and *etnE*) have also been developed (Table 2).

Table 2: Functional genes targeted by nucleic-acid based MBTs for aerobic cometabolism or utilization of chlorinated ethenes

Gene / Symbol	Function	References
mmoX	Subunit of soluble methane monooxygenase	Paszczynski *et al*. (2011); Tentori and Richardson (2020)
prmA	Subunit of propane monooxygenase	Sharp *et al*. (2007)
TOD	Toluene dioxygenase	Baldwin *et al*. (2003)
RMO	Toluene monooxygenase	Baldwin *et al*. (2003)
RDG	Toluene monooxygenase	Baldwin *et al*. (2003)
etnC	subunit of alkene monooxygenase	Coleman and Spain (2003); Jin and Mattes (2010, 2011)
etnE	subunit of epoxyalkane:coenzyme M transferase (EaCoMT)	Coleman and Spain (2003); Jin and Mattes (2010, 2011)

PCR and qPCR methods for detection and enumeration of bacterial genera known to participate in the **anaerobic** reductive dechlorination process are well established (Table 1) and have been widely applied to aid in decision making for contaminated soil and groundwater (Fennell *et al*., 2001; Major *et al*., 2002). *Dehalococcoides* concentrations on the order of 10^4 16S rRNA gene copies per mL groundwater and higher have been proposed as leading to dechlorination rates at the field-scale that are greater than a “generally useful” rate of 0.3 per year (Lu *et al*., 2006). For sites with lower concentrations, bioaugmentation and/or biostimulation may be more appropriate than MNA.

Three distinct genes encoding enzymes that catalyze dechlorination of vinyl chloride to ethene have been identified to date, two from *Dehalococcoides* (*vcrA* and *bvcA*) and one from *Dehalogenimonas* (*cerA*). PCR and qPCR methods for the detection of these functional genes have been developed (Table 3). Application of PCR and qPCR targeting genes for vinyl chloride dehalogenation as well as genes encoding upper portions of the PCE dechlorination pathway (Table 1) are routinely assayed.

Table 3: Functional genes targeted by nucleic-acid based MBTs for chlorinated ethenes (anaerobic pathways)

Gene	Function	References
tceA	Trichloroethene reductive dehalogenase	Magnuson *et al*. (2000)

bvcA	Vinyl chloride reductase (BAV1)	Krajmalnik-Brown *et al*. (2004)
vcrA	Vinyl chloride reductase (VS)	Holmes *et al*. (2006)
cerA	Vinyl chloride reductase (GP)	Yang *et al*. (2017)
TdrA	*Trans*-1,2-Dichloroethene reductase	Molenda *et al*. (2016)

Stable isotope fractionation patterns have been applied for chlorinated alkenes transformed under aerobic and anaerobic conditions (Hirschorn *et al*., 2007; Fletcher *et al*., 2011; Schmidt *et al*. 2014; Franke *et al*., 2020).

3.3 Polychlorinated Biphenyls (PCBs)

3.3.1 Lab-Scale Biotransformation & Associated Pathways

Under **aerobic conditions**, several bacteria have the ability to grow using PCB congeners with one or two chlorines as sole sources of carbon and energy (Masse *et al*., 1984; Furukawa and Miyazaki, 1986; Abramowicz, 1990). Also under aerobic conditions, several bacteria are capable of cometabolizing lower chlorinated PCBs when provided with biphenyl as the primary substrate. Cometabolizing strains include a diverse assortment of both Gram positive and negative genera including *Acinetobacter, Alcaligenes, Achromobacter, Burkholderia, Comamonas, Corynebacterium, Pseudomonas, Ralstonia, Rhodococcus, Sinorhizobium and Sphingomonas* (Furukawa, 2000; Pieper, 2005; Field and Sierra-Alvarez, 2008; Tu *et al*., 2011). Isolates vary with regard to the type and extent of PCB congeners metabolized, with some strains having a narrow spectrum and others able to transform a broader range of congeners. In the case of aerobic biodegradation and cometabolic biotransformation of lower chlorinated PCBs, the best characterized pathway is the *php* pathway which is initiated by the enzyme biphenyl-2,3-dioxygenase (Masse *et al*., 1984; Erb & Wagner-Döbler, 1993; Furukawa, 2000; Pieper, 2005; Field and Sierra-Alvarez, 2008). Depending on the number and position of chlorine substituents, a variety of products may be formed, some of which are relatively recalcitrant and others of which can be mineralized (Pieper, 2005).

The pathways for **anaerobic biotransformation** of polychlorinated biphenyls (PCBs) have been extensively studied. A variety of isolates from the genus *Dehalococcoides* have been unequivocally demonstrated to reductively dechlorinate PCBs in processes that replace chlorine with hydrogen on the biphenyl ring. The isolates markedly differ with respect to which of the 209 possible congeners they are able to dehalogenate, and because chlorines may be removed from *meta* or *para* positions, the final dechlorination products can vary between strains (Fennell *et al*., 2004; Adrian *et al*., 2009; LaRoe *et al*., 2014; Wang *et al*., 2014, 2015). Commercial PCB mixtures are dechlorinated to a variety of lower chlorinated PCBs. In addition to *Dehalococcoides*, an anaerobic bacterial strain referred to as "*Dehalobium chlorocoercia*" strain DF-1 has been demonstrated to utilize some doubly flanked PCB congeners as the sole electron acceptor, for example, dechlorinating 2,3,4,5-tetrachlorobiphenyl to 2,3,5-trichlorobiphenyl (Wu *et al*., 2000; May *et al*. 2008; Lombard *et al*., 2014). Though not yet demonstrated in pure cultures, a combination studies indicate that representatives from the genera *Dehalogenimonas* (Wang and He, 2013a; Liang *et al*., 2015; Xu *et al*., 2022) and *Dehalobacter* (Yan *et al*., 2006a; Yoshida *et al*., 2009, Wang and He, 2013b) can also dechlorinate higher chlorinated PCBs to less chlorinated PCBs (Table 4).

Table 4: Bacterial 16S rRNA genes targeted by MBTs for PCBs (anaerobic)

Genus	Relevance	References
Dehalococcoides	Dechlorinates some PCB congeners	Löffler *et al*. (2000); Hendrickson *et al*. (2002); Dennis *et al*. (2003); Duhamel *et al*. (2004); Cupples (2008)
Dehalogenimonas	Dechlorinates some PCB congeners	Yang *et al*. (2017); Molenda *et al*. (2016); Moe *et al*. (2009); Yan *et al*. (2009); Chen *et al*. (2014)
Dehalobacter	Dechlorinates some PCB congeners	Holliger *et al*. (1998); Maillard *et al*. (2003)

Laboratory studies have demonstrated aerobic biodegradation of lower chlorinated PCBs in river sediments (Williams & May, 1997; Sul *et al*., 2009). Several studies have also documented anaerobic dechlorination of higher chlorinated PCBs in microcosms and enrichment cultures derived using freshwater sediments (Liang *et al*., 2014; Yan *et al*., 2006a,b; Ewald et al., 2020; Xu et al., 2022) and marine sediments (Fava *et al*., 2003; Yan *et al*., 2006b; Nuzzo *et al*., 2017).

3.3.2 Established MBTs

Nucleic acid-based methods targeting genes segments encoding enzymes for the **aerobic** PCB biodegradation pathway (Table 5) have been applied to quantify or characterize aerobic PCB degradation in laboratory enrichments and environmental samples.

Table 5: Functional genes targeted by nucleic-acid based MBTs for aerobic PCB transformation

Gene	Function	References
bphA	biphenyl-2,3-dioxygenase	Hoostal *et al*. (2002); Demnerová *et al*. (2005); Petrić *et al*. (2011); Zubrova *et al*. (2021)
bhpC	2,3-dihydroxybiphenyldioxygenase	Erb and Wagner-Döbler (1993); Cao *et al*. (2021)

Nucleic-acid-based tools targeting 16S rRNA gene sequences unique to the **anaerobic** genera *Dehalococcoides*, *Dehalogenimonas*, and *Dehalobacter* (Table 4) are well established. *Dehalococcoides* and *Dehalogenimonas* phylotypes present in anaerobic cultures reductively dechlorinating PCBs reported to date contain large numbers of putative reductive dehalogenase encoding genes. Further research is needed to characterize the specific functional roles that these play. Nevertheless, there are a growing number of genes that have been implicated in PCB dechlorination reactions (Table 6), and nucleic acid based MBTs targeting these genes have been applied in a limited number of cases for enrichment cultures established using marine sediments (e.g., La Spezia harbor,

Italy) and freshwater sediments (e.g., Taihu Lake, China), with increased gene expression correlating with PCB dechlorination (Matturro *et al*. 2016a; Xu *et al*., 2022).

Table 6: Functional genes targeted by nucleic-acid based MBTs for anaerobic PCB transformation

Gene	Function	References
ardA	Reductive dehalogenase	Xu *et al*. (2022)
*rdh*12	PCB reductive dehalogenase	Park *et al*. (2011); Xu *et al*. (2022)
*pcb*A1	PCB reductive dehalogenase	Wang *et al*. (2014); Matturro *et al*. (2016a); Xu *et al*. (2022)
*pcb*A4	PCB reductive dehalogenase	Wang *et al*. (2014); Matturro *et al*. (2016a); Chen and He (2018); Xu *et al*. (2022)
*pcb*A5	PCB reductive dehalogenase	Wang *et al*. (2014); Matturro *et al*. (2016a); Xu *et al*. (2022)
SKFPat9	DF1 reductive dehalogenase	Payne *et al*. (2013)

SIP using ^{13}C-labeled PCBs has allowed identification of PCB degrading bacteria in aerobic systems through incorporation of the ^{13}C label into phospholipids and DNA (Tillmann *et al*., 2005; Leigh *et al*., 2007).

3.4 Polycyclic Aromatic Hydrocarbons (PAHs)

3.4.1 Lab-Scale Biotransformation & Associated Pathways

Under **aerobic conditions,** numerous bacterial species are capable of transforming two-, three-, and four-ring PAHs to non-toxic end products such as water and carbon dioxide (i.e., PAH mineralization) and partially degrading five- and six-ring PAHs to intermediate compounds (Cerniglia, 1992). Naphthalene is the simplest structure PAH (two-ring) and has broadly served as a model compound to study metabolic pathways, enzymes, and their regulation (Phale *et al*., 2020). Aerobic naphthalene degradation pathways and their enzymes have been extensively studied in mesophilic bacteria including *Pseudomonas* species. Based on enzyme induction and regulation studies, the naphthalene degradation pathway is segmented into upper pathway (naphthalene to salicylate, *nah* operon) and lower pathway (salicylate to central carbon pathway either via catechol*, sal* operon or gentisate, *gen/sgp* operon) (Phale *et al*. 2019; Miyazawa *et al*., 2020). These operons are induced by salicylic acid and its analogues (Shamsuzzaman & Barnsley 1974; Park *et al*., 2002). Both the upper pathway and lower pathway include multiple intermediates. The initial step of aerobic naphthalene biodegradation upper pathway is the addition of dioxygen to naphthalene by the multimeric enzyme naphthalene 1,2-dioxygenase (NahA) (Eaton and Chapman, 1992). Aerobic degradation pathways have been elucidated for many other PAHs, with initial reactions involving mono-or di-oxygenation (Schneider *et al*., 1996; Krivobok *et al*., 2003; Payne *et al*., 2013).

Under **anaerobic conditions,** degradation of two- and three-ring PAHs has been documented under nitrate (Mihelcic and Luthy, 1988; McNally *et al.,* 1998; Rockne and Strand, 1998; Zhang *et al*., 2020), iron (Coates *et al*., 1996b; Anderson &, Lovley, 1999), sulfate-reducing (Coates *et al*., 1996a,b; Mueller *et al*., 1989; Meckenstock *et al*., 2000; Kummel *et al*., 2015), and methanogenic conditions (Chang *et al*., 2006; Toth *et al*., 2018). Although mineralization has been demonstrated in many cases, anaerobic pure culture isolates are limited (Table 7), and

full pathways for biodegradation with unequivocal identification of intermediates (and responsible enzymes) are lacking. The initial step in the anaerobic naphthalene pathway for sulfate reducing *Deltaproteobacteria* strains NaphS2, NaphS3, and NaphS6 involves carboxylation (Meckenstock and Mouttaki, 2011; Mouttaki *et al*., 2012; Meckenstock *et al*., 2016), as does the initial step in transformation of phenanthrene by the sulfate reducing *Geobacter sulfurreducens* PheS2 (Zhang *et al*., 2021b) and strain PheS1 (Zhang *et al*., 2021a). The initial step in the anaerobic degradation of 2-methylnapthalene is activated by the addition of fumarate (Meckenstock *et al*., 2004; Selesi *et al.*, 2010). Aside from the recently reported anaerobic transformation of benz[a]anthracene (Zhang *et al*., 2021a,b), anaerobic degradation of PAHs has generally been limited to PAHs with two or three rings (Himmelberg *et al*., 2018).

Table 7: Pure cultures capable of anaerobic PAH degradation

Bacterium	Substrates	References
Deltaproteobacteria strain NaphS2	Naphthalene 2-Methylnaphthalene	Galushko *et al*. (1999)
Deltaproteobacteria strain NaphS3	Naphthalene 2-Methylnaphthalene	Musat *et al*. (2009)
Deltaproteobacteria strain NaphS6	Naphthalene 2-Methylnaphthalene	Musat *et al*. (2009)
Strain PheS1	Naphthalene Phenanthrene Anthracene Benz[a]anthracene	Zhang *et al*. (2021a)
Geobacter sulfurreducens strain PheS2	Naphthalene Phenanthrene Anthracene Benz[a]anthracene	Zhang *et al*. (2021b)
Achromobacter denitrificans strain PheN1	Phenanthrene	Zhang *et al*. (2020)

Multiple laboratory-scale studies demonstrating biodegradation of naphthalene and other PAHs have been reported in enrichment cultures derived from freshwater and marine sediments. These include aerobic biodegradation as well as biodegradation under nitrate-reducing (Langenhoff *et al*., 1996; Rockne and Strand, 1998, 2001; Lu *et al*., 2012; Dou *et al*. 2009), sulfate-reducing conditions (Langenhoff *et al*. 1996; Coates *et al*. 1996a,b; Zhang and Young, 1997; Rockne and Strand, 1998; Galushko *et al*., 1999; Rothermich *et al*., 2002; Lu *et al*., 2012) and methanogenic conditions (Chang *et al*., 2006; Li et al., 2015).

3.4.2 Established MBTs

A variety of PCR and qPCR methods have been developed to detect and quantify concentrations of *nahAc* gene which codes for a subunit of naphthalene 1,2-dioxygenase which initiates the first step of **aerobic** naphthalene biodegradation (Mawad *et al*. 2020; Park and Crowley, 2006; Salminen *et al*., 2008; Tuomi *et al*., 2004; Cébron *et al*., 2008; Iwai *et al*., 2011). Multiple studies have reported a positive correlation between the abundance of *nahAc* gene copies and degradation of naphthalene under **aerobic** conditions (Tuomi *et al*., 2004; Nyyssönen *et al*. 2006; Salminen *et al*., 2008). In addition to initiating aerobic naphthalene metabolism in bacteria, naphthalene

dioxygenase has many other catalytic abilities, allowing biotransformation of several additional PAHs including anthracene, phenanthrene, acenaphthylene, and fluorene (Jerina *et al*., 1976; Resnick and Gibson, 1996a, 1996b; Selifonov *et al*., 1996). As such, nucleic acid-based MBTs targeting the *nahAc* gene may also serve as a general indication of the metabolic potential for transforming additional PAHs.

PCR primers targeting dioxygenases that act on higher molecular weight PAHs, notably the *nidA* gene which encodes the large subunit of a dioxygenase that acts on pyrene and phenanthrene (Khan *et al*., 2001; Stingley *et al*., 2004), have also been developed and applied to environmental samples (DeBruyn *et al*, 2007; Peng *et al*., 2010). Previous studies have found the degradation of pyrene is usually positively related to the abundance and expression of *nidA* (Zhou *et al*. 2008; Peng *et al*., 2010). More general PCR primers targeting genes that encode the alpha subunit of the PAH-ring hydroxylating dioxygenases involved in the initial step of the aerobic metabolism of PAHs in Gram positive and Gram negative bacteria have also been established (Cébron *et al*., 2008). PCR primers targeting the *pahE* gene which codes for a later transformation step in the aerobic upper PAH biodegradation pathway have also been reported (Table 8).

Table 8: Functional genes targeted by nucleic-acid based MBTs for aerobic naphthalene and other PAH biodegradation

Gene targets	Function	References
nahAc	Subunit of naphthalene-1,2-dioxygenase	Mawad *et al*. (2020); Park and Crowley (2006); Salminen *et al*. (2008); Tuomi *et al*., (2004)
PAH-RDH$_{\alpha}$ GN	PAH-ring hydroxylating dioxygenases of Gram negative bacteria	Cébron *et al*. (2008)
PAH-RDH$_{\alpha}$ GP	PAH-ring hydroxylating dioxygenases of Gram positive bacteria	Cébron *et al*. (2008)
nidA	PAH-ring hydroxylating dioxygenase that acts on pyrene and phenanthrene	DeBruyn *et al.* (2007); Peng *et al*. (2010)
pahE	PAH hydratase-aldolase	Liang *et al*. (2019)

As noted above, **anaerobic** transformation pathways for PAHs are much less well characterized than **aerobic** pathways. Consequently, nucleic acid-based MBTs are relatively limited (Table 9). PCR primers and methods for targeting the napthoyl-CoA reductase (initiates a first step in anaerobic naphthalene biodegradation) and naphthl-2-methylsuccinate synthase (which initiates transformation of methylnaphthalene) have been developed (Morris *et al*., 2014; von Netzer *et al*., 2013).

Table 9: Functional genes targeted by nucleic-acid based MBTs for anaerobic naphthalene biodegradation

Gene	Function	References
ncr	Napthoyl-CoA reductase	Morris *et al*. (2014)
nmsA	Naphthyl-2-methyl-succinate synthase	von Netzer *et al*. (2013)

Sieradzki *et al*. (2021) and others have employed stable isotope probing to identify aerobic naphthalene degraders based on incorporation of ^{13}C labeled PAHs into 16S rRNA genes (Singleton *et al*., 2005; Rochman *et al*., 2017). Carboxylation as the initial step in anaerobic naphthalene and phenanthrene biotransformation has allowed use of reverse stable isotope labeling (incorporation of ^{13}C bicarbonate) rather than use of ^{13}C labeled PAH for assessment of anaerobic PAH transformation (Dong *et al*., 2017; Zhang *et al*., 2021a,b). Carbon and hydrogen CSIA fractionation has also been successfully applied with anaerobic 2-methylnaphthalene biodegradation (Marozava *et al*., 2019).

3.5 Summary of Review Considerations

The literature review detailed in the previous sections highlights the current state of knowledge of biodegradation for reviewed COCs ranges from moderately characterized biodegradation mechanisms with limited laboratory-scale testing to well-characterized biodegradation mechanisms with extensive laboratory testing. Based on the results of the literature review, a comparative summary of each COC is presented in Table 10.

The results of the literature can be summarized as follows:

- ***Are biodegradation or biotransformation pathways established?***
  - With the exception of high molecular weight (HMW) PAHs, the biodegradation or biotransformation pathways are well-established for chlorinated ethenes, PCBs and low molecular weight (LMW) PAHs.
- ***Have sediment and/or porewater laboratory-scale biodegradation studies been documented?***
  - With the exception of HMW PAHs, laboratory-scale biodegradation studies have been documented for each class of COCs. Lab-scale studies with chlorinated ethenes and LMW PAHs are the most well-documented of the COCs considered here.
- ***Are MBTs established to monitor lab-scale and potentially field-scale biodegradation or biotransformation in sediments?***
  - MBTs to assess biodegradation of chlorinated ethenes have been established for the reviewed COCs. While MBTs for chlorinated ethene biodegradation are well-established and have been applied at the lab-scale using sediments, there is uncertainty of the specificity of the individual MBT to assess individual biodegradation mechanisms of PCBs and PAHs.

Table 10: Comparative summary of the results of the literature review for each COC. The shading presents the interpreted state of knowledge or extent of documented studies. Green shading indicates a mature state of knowledge or numerous documented studies. Orange indicates an established, but comparatively less mature state of knowledge and few documented studies. Light red indicates limited state of knowledge and limited documented studies. Dark red indicates an absence of knowledge, MBTs, or documented studies.

Criteria	Biogeochemical Conditions	CVOCs - Chlorinated Ethenes	PCBs	PAHs	
				LMW	HMW
Are biodegradation or biotransformation pathways established?	Aerobic	**High** Well-established (except for PCE)	**High** Well-established	**High** Well-established	**Low** Few pathways characterized / established
	Anaerobic	**High** Well-established	**High** Well-established	**High** Well-established	**Low** Few pathways characterized / established
Have biodegradation lab-scale studies been documented?	Aerobic	**High**	**Medium**	**High**	**Medium**
	Anaerobic	**High**	**Medium**	**Medium**	**Absent**
Are MBTs established to monitor lab-scale and potentially field-scale biodegradation or biotransformation in sediments?	Aerobic	**High** Well-established and applied at field-scale	**Low** Limited MBTs focused on two gene targets	**High** Well-established targets for *nahAc* gene and applied at field scale	**Low**
	Anaerobic	**High** Well-established and applied at field-scale	**Medium** Further research necessary to establish functional roles that these play	**Low-Medium** MBTs are limited (two function gene targets) and are an active area of ongoing research	**Absent** No MBTs available/identified

Overall, the literature review highlighted that the COCs of interest are known to biodegrade in the environment under aerobic and anaerobic conditions present in the sediment environment and that a suite of MBTs can be utilized to measure and characterize biodegradation processes.

4.0 CONCLUSIONS

Application of MBTs to contaminated sediment sites can supplement traditional data analyses with empirical evidence of biodegradation/biotransformation natural recovery mechanisms to reduce CSM uncertainty and support remedial decisions and strategies. MBTs to assess biodegradation/biotransformation processes for key sediment COCs including CVOCs, PCBs, and LMW PAHs have been established and applied at laboratory-scale; though field-scale application, the number of applications, and maturity of the state of knowledge for each COC varies.

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