

# Application of Diagnostic Tools to Evaluate Remediation Performance at Petroleum Hydrocarbon-Impacted Sites

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## Abstract

In situ treatment technologies for petroleum hydrocarbon-impacted sites (e.g., multiphase extraction, air sparging, soil vapor extraction, or in situ chemical oxidation) usually rely on a specific chemical, microbial, or physical contaminant removal process. However, target contaminant concentrations can also vary due to other co-occurring processes (e.g. delivery of remedial fluids, natural variability), which can confound the ability to demonstrate treatment efficiency. This technical note proposes a methodology that integrates several diagnostic tools to assess treatment performance. Stable isotope methods and biomarkers were selected because they provide process-specific and, often, also compound-specific information on contaminant removal. The isotope tools include compound-specific isotope analysis that can be used to discriminate between a broad range of removal processes, and isotope analysis of oxidants and degradation end products to assess overall transformation of hydrocarbons. The biomarkers cover characteristic metabolites and functional genes on a mRNA rather than DNA level to understand biological activity more carefully. This technical note integrates information from laboratory and field studies, especially controlled-field experiments where the tools have been evaluated side-by-side for different treatment methods. A tiered approach is proposed to deploy the tools in a step-wise manner until sufficient information is obtained to confidently identify the mass removal processes of interest and demonstrate efficacy of the intended treatment mechanism. The order of tool application considers the type of information that can be gained, the level of certainty, and the ease of implementation. The objective of this technical note is to enable widespread use of these diagnostic tools with the motivation to improve the efficacy of in situ treatment systems.

## Introduction

Achieving cost-effective site cleanup is one of the key goals of an engineered in situ treatment system (Suthersan and Payne 2005). In situ treatment of petroleum hydrocarbon (PHC)-impacted sites usually relies upon a specific intended chemical, microbiological, or physical contaminant removal process. However, in complex subsurface systems, it can be difficult to unequivocally demonstrate the occurrence of the intended contaminant removal process (Madsen 1991). The effects of treatment might be overwhelmed, especially in the early phase, by naturally occurring concentration fluctuations or displacement of contaminants by the injection of remedial fluids (e.g., dissolved oxidants to promote biodegradation or chemical oxidation). If treatment processes are solely assessed based on contaminant concentrations, then treatment effectiveness can be either over- or underestimated. A concentration decrease due to injection of treatment fluids can be mistaken as an effect of

the intended removal process, thus overestimating treatment effectiveness. Alternatively, if a nonaqueous phase liquid (NAPL) is present, a treatment process might effectively remove mass although no concentration change is observed due to continual dissolution. The effect of treatment may also be underestimated because the contribution of co-occurring process, such as biodegradation during air sparging (Johnson 1998), are overlooked. Such oversights may lead to the prolonged operation of ineffective treatment systems or the premature termination of an apparent ineffective method. Thus, there is a need for diagnostic tools to identify contaminant removal processes and attempt to assess their actual effectiveness related to the intended behavior. Such a toolbox is particularly valuable for strategies that increasingly rely on combinations of treatment methods making it necessary to evaluate the expected sequential occurrence of contaminant removal processes over time.

For monitored natural attenuation (MNA), the need for methodologies to differentiate destructive contaminant removal from mere physical attenuation is well recognized (Wiedemeier et al. 1999), and guidelines for integrating multiple lines of evidence to demonstrate contaminant removal have been developed (ASTM 2015). Methods based on stable isotope analysis (Aelion et al. 2010) and biomarkers (Griebler et al. 2004) have become particularly common. A similar systematic approach is generally lacking for engineered in situ

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remediation. During engineered in situ remediation, a broader range of contaminant removal processes are relevant compared to MNA. These processes can be reactive, such as biodegradation and chemical oxidation, or non-reactive, such as volatilization (Table 1). A treatment can involve two intended processes (e.g., volatilization and aerobic biodegradation in the case of air sparging), but it remains unclear if both processes occur simultaneously. If a treatment targets only one removal process, but additional processes co-occur that influence contaminant concentrations, then the additional processes can either confound the assessment of the intended process (e.g., due to dilution) or contribute to contaminant removal. Based on Table 1, there is a need for tools to differentiate between: (1) a destructive process (biotic or abiotic) versus dilution, (2) biodegradation versus volatilization, (3) chemical oxidation versus biodegradation, and (4) biodegradation associated with the consumption of specific electron acceptors.

In this technical note, we propose a methodology for assessing these co-occurring contaminant removal processes during the treatment of PHC-impacted sites that incorporates various diagnostic tools. The proposed approach is based on several controlled-release experiments (Bouchard et al. 2018; Shayan et al. 2018; Wei et al. 2018) in which these tools were tested side-by-side. We present selected diagnostic tools that are suitable to identify and differentiate among the removal processes and discuss their application principles. Based on a discussion of the advantages and limitations of the different tools, we propose a tiered approach for the systematic deployment of the diagnostic tools addressing co-occurring contaminant removal processes.

## Diagnostic Tools

A critical requirement for diagnostic tools is that they are process-specific (i.e., suitable to differentiate the contaminant removal processes listed in Table 1) thus overcoming

the limitations of contaminant concentration data, which could reflect multiple processes. If possible, the diagnostic tools should also be compound-specific. For example, it is usually important to know if specific contaminants of concern, which are the regulatory drivers (e.g., benzene), are removed and not just the bulk hydrocarbon mass (e.g., total petroleum hydrocarbons [TPH]). When considering the sets of co-occurring processes discussed above, there is frequently a need for tools to identify contributions by biodegradation. This reflects the fact that biodegradation of PHCs is a ubiquitous process that occurs under a wide range of redox conditions, whether intended or not. The proposed diagnostic tools consist of a combination of stable isotope tools and biomarkers (Table 2 and Figure 1) that are described in the following sections.

## Stable Isotope Tools

We put an emphasis on stable isotope tools because several types of contaminant removal processes can be differentiated (chemical, biological, and physical) making them broadly applicable. In the case of biodegradation, isotope methods can be applied to different compounds involved in PHC degradation (i.e., the contaminant itself, oxidants, and reaction by-products, Aelion et al. 2010).

### Compound-Specific Isotope Analysis

Among the isotope tools, compound-specific isotope analysis (CSIA) provides the most direct insight into the removal of specific contaminant by a specific process (US EPA 2008). During biodegradation or chemical oxidation of organic compound, bonds with light isotopes (for instance  $^{12}\text{C}$ - $^1\text{H}$ ) react slightly faster than those with a heavier isotope (e.g.,  $^{13}\text{C}$ - $^1\text{H}$ ,  $^{12}\text{C}$ - $^2\text{H}$ ) (US EPA 2008). Such reactive processes enrich heavier isotopes in the residual unreacted contaminant mass. The isotope ratio  $^{13}\text{C}/^{12}\text{C}$  and  $^2\text{H}/^1\text{H}$  are

**Table 1**

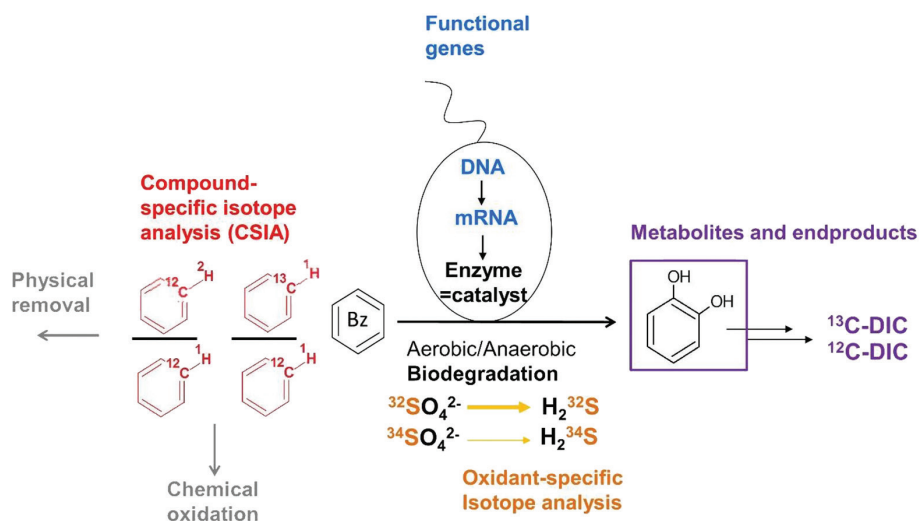
**Intended and Co-occurring Contaminant Removal Process During the Lifecycle of Some Remediation Technologies**

Technology	Contaminant Removal Process		Remarks
	Intended	Possible/Co-occurring	
In situ air sparging (IAS)	Volatilization, aerobic biodegradation	—	Aerobic biodegradation may be induced due to injected ambient air
Soil vapor extraction (SVE)	Volatilization	Aerobic biodegradation, anaerobic biodegradation	Aerobic biodegradation may be induced due to changes in air flow patterns, or background anaerobic biodegradation may continue to persist
Multi-phase extraction (MPE)	Volatilization	Aerobic biodegradation, anaerobic biodegradation	Aerobic biodegradation may be induced due to changes in air flow patterns, or background anaerobic biodegradation may continue to persist
In situ chemical oxidation (ISCO) using persulfate	Chemical oxidation	Anaerobic biodegradation	Anaerobic biodegradation may occur following the degradation of persulfate as a result of $\text{SO}_4^{2-}$ formation
Application of limiting electron acceptors ( $\text{O}_2$ , $\text{NO}_3^-$ , $\text{SO}_4^{2-}$ )	Aerobic biodegradation ( $\text{O}_2$ ), anaerobic biodegradation ( $\text{SO}_4^{2-}$ , $\text{NO}_3^-$ )	Dilution	Dilution from injected or infiltrating reagent solution. Aerobic biodegradation can also occur from dissolved oxygen in injected or infiltrating solution during land application of $\text{SO}_4^{2-}$ or $\text{NO}_3^-$

**Table 2**  
**Overview of Diagnostic Tools**

Name	Parameter That Is Evaluated	Principle	Type of Removal Processes That Can Be Evaluated	Specificity
CSIA	Stable carbon and/or hydrogen isotope composition of the target compounds present in aqueous or vapor phase	Each of the contaminant removal processes (except for dilution) alters the isotope composition (carbon and hydrogen isotopes) of contaminants in a predictable manner	Biological, chemical and physical	Process and compound-specific
<sup>13</sup> C in DIC or CO <sub>2</sub>	Measurement of <sup>13</sup> C in DIC in aqueous phase or <sup>13</sup> C in CO <sub>2</sub> in vapor phase	Biodegradation of PHCs leads to the formation of DIC and CO <sub>2</sub> with an isotopic signature ( <sup>13</sup> C) similar to that of the degraded hydrocarbon pool (more depleted in <sup>13</sup> C than the initial pool)	Biological, chemical	Process-specific <sup>1</sup> but not compound/group-specific
<sup>34</sup> S in sulfate	Measurement of <sup>34</sup> S in sulfate dissolved in water	During sulfate reduction, the abundance of heavy isotopes ( <sup>34</sup> S) in the remaining sulfate increases, as molecules with the light isotope ( <sup>32</sup> S) are consumed more rapidly than those with the heavy isotope	Biological	Process-specific but not compound/group-specific
Metabolites	Metabolites (intermediate, characteristic byproducts) dissolved in water that are formed during biodegradation of contaminants	Biodegradation of aromatic hydrocarbons leads to the temporary production of metabolites that are characteristic of biodegradation of target compounds under aerobic or anaerobic conditions	Biological	Process-specific and compound or group-specific
Functional genes (mRNA)	mRNA associated with genes that encode enzymes necessary for contaminant degradation (=functional genes)	The detection of mRNA demonstrates that the functional gene has been activated and biodegradation is ongoing	Biological	Process-specific and compound or group-specific

<sup>13</sup>C in CO<sub>2</sub>/DIC can be used to demonstrate hydrocarbon biodegradation. However, it is not possible to distinguish aerobic from anaerobic biodegradation using <sup>13</sup>C/DIC alone.



**Figure 1. Overview of diagnostic tools in the context of contaminant removal processes.**

usually reported in the  $\delta$  notation (accordingly  $\delta^{13}\text{C}$  and  $\delta^2\text{H}$ ) relative to an international standard as a reference,

$$\delta = \left( \frac{R_{\text{sample}}}{R_{\text{reference}}} - 1 \right), \quad (1)$$

where  $R_{\text{sample}}$  and  $R_{\text{reference}}$  is the <sup>13</sup>C/<sup>12</sup>C or <sup>2</sup>H/<sup>1</sup>H ratio of the measured sample and the reference material, respectively.  $\delta$  is usu-

ally multiplied by 1000 to express the result in per mil (‰). The difference in reactivity among molecules with light and heavy isotopes can be expressed by the isotope fractionation factor ( $\alpha$ ),

$$\alpha = {}^h k / {}^l k, \quad (2)$$

where  ${}^h k$  and  ${}^l k$  represent the reaction rates of the light and heavy molecule, respectively, for a given chemical or micro-

bial transformation process. For convenience,  $\alpha$  is commonly reported as the enrichment factor ( $\epsilon$ ),

$$\epsilon = (\alpha - 1). \quad (3)$$

Analogous to the delta notation, enrichment factors are expressed in per mil (‰). The isotope enrichment or fractionation factor describes how much the isotope ratio changes in relation to the progress of the reaction, as described by the Rayleigh equation. In its simplest form, it corresponds to,

$$\Delta\delta^{13}\text{C} = \epsilon \cdot \ln f, \quad (4)$$

where  $\Delta\delta^{13}\text{C}$  is the difference in the isotope ratio at time  $t$  relative to its initial value and  $f$  is the fraction of compound remaining. Isotope fractionation is usually much larger for hydrogen than carbon due to the larger relative mass difference between the isotopes of hydrogen as compared to carbon (Hunkeler and Morasch 2010). The magnitude of isotope fractionation tends to decrease with molecule size. For biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX), which are often of main interest, generally both carbon and hydrogen isotope fractionation occurs, while for larger molecules, such as naphthalene, only hydrogen isotope fractionation is detectable (Hunkeler and Morasch 2010).

CSIA provides insight into whether biodegradation occurs and often under which redox conditions it takes place, which can be of major interest for engineered in situ treatment. To differentiate among biodegradation under different conditions, the analysis of both carbon and hydrogen isotopes is required (Elsner et al. 2005). Isotope data of two elements are usually visualized by plotting carbon versus hydrogen isotope on a so-called dual-element isotope or 2D-plot. Bacteria make use of a range of different mechanisms to degrade a certain compound leading to different dual-element isotope slopes in such plots. To assess reactive processes with dual-element isotope plots, reference zones

are defined by compiling the respective range of dual-element isotope slopes associated with the different processes. The reference zones are based on reported dual-element isotope slopes from laboratory studies (Table 3), usually given by the symbol lambda ( $\Lambda$ ). In some studies, only isotope enrichment factors are reported but not dual-element isotope slopes. In this case,  $\Lambda$  can be estimated based on

$$\Lambda = \frac{\epsilon_H}{\epsilon_C}. \quad (5)$$

However, only values originating from the same experiment (thus from the same microbial strain or population) should be combined. Furthermore, the uncertainty associated with  $\Lambda$  calculated according to Equation 5 tends to be higher due to uncertainty propagation from the isotope enrichment factors. The reference zones correspond to the envelop comprising all dual-element isotope slopes for the process of interest. For some important contaminants of concern (e.g., benzene), the slopes separate in two groups associated with aerobic and anaerobic biodegradation, while for others (e.g., toluene) the aerobic and anaerobic zones overlap. If biodegradation under certain conditions can be excluded or biodegradation under certain redox conditions is of main interest (e.g., sulfate-reducing conditions resulting from gypsum land application), more specific reference zones can be defined. In Figure 2, the resulting reference zones are illustrated for chemical oxidation, aerobic biodegradation and anaerobic biodegradation of benzene.

In contrast to reactive processes, physical processes in groundwater such as dissolution, sorption, advective-dispersive transport, and aqueous phase diffusion only lead to detectable changes in isotope ratios in exceptional situations (Hunkeler et al. 2004; Kopinke et al. 2005; Wanner and Hunkeler 2015). This is the basis for differentiating biodegradation from physical attenuation during MNA in groundwater (US EPA 2008). However, under partially saturated conditions, isotope fractionation associated with

**Table 3**

**Dual-Element Isotope Slopes  $\Lambda^{1,2}$  and Isotope Enrichment Factors (Hydrogen/Carbon) Associated with Different Benzene and Toluene Removal Processes (Biological, Physical, and Chemical)**

	Biodegradation						Air-Water Partitioning	NAPL-Vapor Equilibration	ISCO
	Aerobic		Anaerobic		Min	Max			
	Min	Max	Min	Max					
Benzene	3.3 (-11.2/-3.4)	13 (-17.5/-1.35)	12.1 (-29/-2.4), <i>17.0<sup>10</sup></i> (-56/-3)	<b>39.0</b> (-34/-0.8)	$\infty$ (-6 <sup>3</sup> /ns <sup>3</sup> )	16.3 (3.1 <sup>8</sup> /0.17 <sup>7</sup> )	0 <sup>6</sup> (ns <sup>6</sup> -1.7 <sup>6</sup> )		
Toluene	<b>1.0</b> (-2/-1.8)	63.6 (-159/-2.5)	<b>11.0</b> (-78/-5.7) <b>20.0<sup>9</sup></b> (-126/-6.7)	<b>41.0<sup>9</sup></b> (-107/-2.5)	$\infty$ (-5 <sup>3</sup> /ns <sup>3,4</sup> )	28.1 (6.9 <sup>5,8</sup> /0.2 <sup>7</sup> )	35.0 <sup>6</sup> (-20.3 <sup>6</sup> -0.64 <sup>6</sup> )		

Note: For biodegradation, minimum and maximum values are reported for construction of reference zones on dual-element isotope plots. For anaerobic biodegradation, separate slope and isotope enrichment factors are given for generally reducing conditions (values in normal font) and strongly reducing conditions by excluding enrichment factors for denitrification (values in italic). ns, not significant.

<sup>1</sup>Normal font:  $\Lambda$  is the  $\epsilon_H/\epsilon_C$  ratio. Bold font:  $\Lambda$  is the slope from dual hydrogen-carbon isotope plots.

<sup>2</sup>When no reference is specified, value was taken from Aelion et al. (2010). For replicas, the mean value is reported.

<sup>3</sup>Bouchard et al. (2018).

<sup>4</sup>Slater et al. (1999).

<sup>5</sup>Kuder et al. (2009).

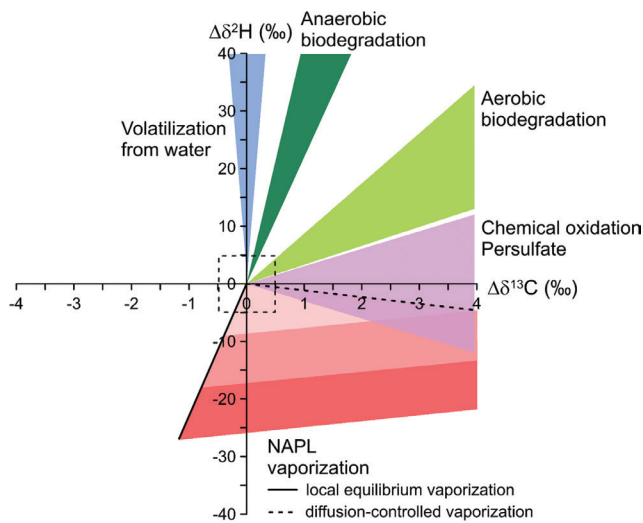
<sup>6</sup>Solano et al. (2018).

<sup>7</sup>Harrington et al. (1999).

<sup>8</sup>Wang and Huang (2003).

<sup>9</sup>Herrmann et al. (2009).

<sup>10</sup>Bergmann et al. (2011).



**Figure 2. Dual carbon and hydrogen isotope plot for benzene illustrating expected isotope trends for biodegradation, chemical oxidation, and physical removal processes. For NAPL volatilization, the trends for volatilization under local equilibrium conditions followed by advective removal (solid line) and for volatilization controlled by diffusion in the soil gas (dashed line) are shown. The red zones in between the lines correspond to intermediate situations. The different shades of red illustrate the isotope change per order of magnitude of contaminant removal. The dashed box represents the typical analytical uncertainty of carbon (0.5‰) and hydrogen (5‰) isotope analysis.**

physical processes becomes relevant and provides opportunities to evaluate physical contaminant removal (Buscheck et al. 2009). Molecules with heavy carbon and hydrogen isotopes in NAPL are more volatile than those with light isotopes (inverse isotope effect), leading to depletion of heavy isotope with increasing NAPL volatilization, opposite to the trend for reactive processes (Table 3 and solid line in Figure 2). In porous media, this effect can be modified by air phase diffusion away from the NAPL, which is more rapid for light compared to heavy molecules (normal isotope effect). For carbon, the diffusion effect is larger than the volatilization effect, leading to a net shift in the positive direction, while for hydrogen the opposite is the case (dashed line in Figure 2). Depending on how strongly NAPL removal is limited by diffusion, the isotope ratios are expected to lay in the enveloped between the two lines (Figure 2). During volatilization from water (e.g., air sparging), molecules with light hydrogen isotopes are removed preferentially compared to those with heavy isotopes, while carbon isotope ratios do not change significantly (Figure 2; Buscheck et al. 2009; Kuder et al. 2009).

In summary, volatilization from NAPL and water lead to distinct isotope trends, that also differ from those for biodegradation and chemical oxidation as illustrated for benzene in Figure 2. This allows us to differentiate among a broad range of processes during engineered in situ treatment using dual-element isotope plots. Isotope fractionation is generally smaller for physical than reactive processes. A significant change of the isotope ratios due to physical processes is only expected if a large fraction of NAPL or dissolved compounds is removed. This is illustrated by

indicating the expected isotope shift for each order of magnitude of contaminant removal from NAPL in Figure 2 as different shades of color. Furthermore, the shift is expected to be smaller under field than laboratory conditions. Under the frequently heterogeneous field conditions, isotope shifts due to NAPL vaporization can be partly masked by mixing with compounds from zones where NAPL vaporization has proceeded less far and thus isotope ratios have not changed significantly yet.

#### CSIA Field Data Interpretation

Interpretation of field data is best performed when several measurements are available for a specific sampling point over time. The first sampling event should be taken as the initial condition ( $t=0$ ), irrespectively of whether the site is already under active treatment or not. The subsequent  $\delta^{13}\text{C}$  and  $\delta^2\text{H}$  values are then compared to the initial value to evaluate the isotope shift ( $\Delta\delta^{13}\text{C}$  and  $\Delta\delta^2\text{H}$ ). Field data can potentially plot in between two representative zones, which may suggest the occurrence of two processes. For example, in case of benzene, a dataset might plot in the zone of anaerobic biodegradation due to a combination of aerobic biodegradation and volatilization from water. In this case, additional information (e.g., redox-sensitive parameters such as ORP and/or concentrations of dissolved sulfate or methane) should be incorporated using a multiple lines of evidence approach. The reference zones are only representative if the shift starts from the origin and proceeds in a fixed direction. In some case, the direction of change in the isotope ratios might change over time or even reverse. In this case, the isotope trend should be evaluated relative to the previously altered isotope signature rather than from the origin. When applying these general principles, site-specific factors that can influence isotope ratios should be considered. NAPL dissolution can revert isotope shifts back to the source signature. At heterogeneous sites, reactive processes may only occur in certain horizons. If long-screen wells are used for sampling, water from different zones mix, which complicates the identification of reactive processes and can lead to their underestimation. In addition to reactive processes, multiple sources or spill events can influence isotope ratios as well.

#### Stable Isotope Analysis of Oxidants and End Products

Stable isotope analysis can also be applied to the electron acceptor (e.g., nitrate or sulfate) or biodegradation end-product ( $\text{CO}_2/\text{DIC}$ ) providing process but not compound-specific information about biodegradation (Clark and Fritz 1997; Aelion et al. 2010). In the case of electron acceptors, a typical motivation for isotope analysis is to differentiate concentration changes due to biodegradation from those due to mixing (Schroth et al. 2001). As only one reactive versus physical process is differentiated and because the physical process does not fractionate isotopes, it is sufficient to analyze the isotope ratio of a single element, generally N in case of nitrate (Spence et al. 2005) or S in case of sulfate (Schroth et al. 2001). An enrichment of the heavy isotopes ( $^{15}\text{N}$  or  $^{34}\text{S}$ ) indicates that the electron acceptor is consumed while constant isotope ratios along with a decrease in electron acceptor concentrations suggest dilution by mixing.

The occurrence and extent of overall PHC biodegradation can be evaluated by analyzing the carbon isotope composition of dissolved inorganic carbon (DIC) (Bolliger et al. 1999; Hunkeler et al. 1999). The method relies on differences between the isotopic signature of background DIC versus DIC/CO<sub>2</sub> produced by PHC mineralization, which can vary among sites. A major part of background DIC usually originates from soil CO<sub>2</sub> (Clark and Fritz 1997). Its isotope composition reflects the biomass signature, which varies among the common C3 (−24 to −30‰) and the rarer C4 plants (e.g., corn, sugarcane; −10 to −16‰). Background DIC is however, usually enriched in <sup>13</sup>C relative to these values, due to the preferential dissolution of heavier inorganic carbon in water, and, depending on the geology, due to dissolution of carbonates, which are enriched in heavy isotopes (δ<sup>13</sup>C ≈ 0‰ for marine carbonates) relative to biomass (Clark and Fritz 1997). As a result, the isotopic composition of background DIC typically ranges from −10 to −15‰. PHCs typically have a δ<sup>13</sup>C in the range of −23 to −30‰ and the produced CO<sub>2</sub> (or DIC) might be slightly depleted in <sup>13</sup>C relative to that range (Aggarwal and Hinchee 1991). Thus, biodegradation of PHC under non-methanogenic (e.g., nitrate, sulfate, or iron reducing) conditions often leads to a shift in δ<sup>13</sup>C of DIC in the negative direction (Bolliger et al. 1999). For methanogenic conditions, the opposite trend occurs (Landmeyer et al. 1996), because the carbon partitions between isotopically light CH<sub>4</sub> and CO<sub>2</sub>/DIC enriched in <sup>13</sup>C. If the methane is subsequently oxidized, the DIC trend can be reversed toward a depletion of <sup>13</sup>C in DIC because the isotopically light carbon is released from methane. Ethanol, a common gasoline oxygenate additive, is usually more enriched in <sup>13</sup>C (−11 to −13‰) compared to PHC due to its origin from corn, a C4 plant. During its methanogenic degradation, inorganic carbon enriched in <sup>13</sup>C relative to

typical background values is produced (Freitas et al. 2010). Mineralization of natural organic carbon in aquifers leads to CO<sub>2</sub> with a similar isotope signature as CO<sub>2</sub> from PHC and thus can confound the data interpretation. The contribution of inorganic carbon from PHCs versus natural organic matter can be differentiated by <sup>14</sup>C (radiocarbon) measurement because PHCs are depleted in <sup>14</sup>C relative to the younger soil natural organic carbon (Aelion et al. 1997).

## Biomarkers

### Metabolites

During organic compound biodegradation, a series of intermediary compounds (called metabolites) are successively formed as the carbon atoms of the contaminant flow through cellular metabolism toward a combination of new microbial cells and CO<sub>2</sub> as final products (Alexander 1999). The study of microbial metabolism has revealed pathways that can feature “diagnostic” or “signature” intermediate metabolites. These can be specific to both the parent compounds and the ambient physiological conditions (e.g., aerobic versus anaerobic) that support biodegradation processes (Weelink et al. 2010; El-Naas et al. 2014). For diagnostic purposes, the most informative metabolites are those whose molecular structure still strongly resembles that of the parent contaminant; these occur early in the metabolic pathways. Among early metabolites (Table 4), some are uniquely of microbial origin (e.g. *cis*-dihydrodiols and benzylsuccinates), and unambiguously document in situ biodegradation processes of aromatic compounds (Beller et al. 1992; Gibson and Parales 2000). The other early compound-specific metabolites listed in Table 4 document in situ biodegradation only if it can be proven that they were absent from the original mix of contaminants released and were not detected in upgradient groundwater samples (such as *o*-, *m*- and *p*-cresol).

**Table 4**  
**Metabolites Used in Assessment of In Situ Biodegradation**

Compound	Redox Condition	
	Aerobic	Anaerobic
Benzene	<b>Benzene-cis-dihydrodiol*</b> <i>Phenol</i>	<i>Benzoic acid</i> <i>Phenol</i> <i>4-Hydroxybenzoate</i>
Toluene	<b>Toluene-cis-dihydrodiol*</b> <b><i>o</i>-Cresol</b> <b><i>m</i>-Cresol</b> <b><i>p</i>-Cresol</b> <b>Benzyl alcohol</b> Benzaldehyde <i>Benzoic acid</i>	<b>Benzylsuccinate*</b>
Ethylbenzene	<b>Ethyl benzene-cis-dihydrodiol*</b>	1-Phenylethylsuccinate
Xylene	<b>2,3-Dimethylphenol</b> <b>3,4-Dimethylphenol</b> <i>o</i> -Toluic acid	<b>2-Methyl-benzylsuccinate*</b>
Naphthalene	<b>Naphthalene-cis-dihydrodiol*</b> Salicylaldehyde Salicylic acid 2-Naphthol	<b>5,6,7,8-tetrahydro-2-naphthoic acid*</b> 2-Naphthoic acid

Note: Listed are metabolites arranged according to redox conditions. Metabolites occurring early in pathways and formed exclusively via microbial metabolism are shown in bold with an asterisk (\*). Metabolites occurring early in pathways, but may not be strictly of microbial origin, are shown in bold. Compounds appearing late in pathways are not in bold. Metabolites in italic are not compound-specific or redox condition-specific.

Metabolites of BTX are generally chemically or biologically unstable; thus, they represent a transient metabolite pool. Due to this transient aspect, metabolites are not expected to significantly migrate with groundwater. Detection of these highly unstable metabolites can thus serve as local evidence for real-time, in situ metabolism of PHC compounds. Detection of a particular metabolite can also indicate the local geochemical conditions prevailing in the aquifer (Weelink et al. 2010; El-Naas et al. 2014). Detection of multiple biomarkers can also demonstrate that aerobic and anaerobic biodegradation occurs simultaneously. Detection of key metabolites can thus inform appropriate site management decisions and guide the effectiveness of treatment designs. In a management context, failure to detect metabolites may indicate inefficiency of the remedial strategy; however, negative findings are always difficult to interpret, especially because the metabolite may be present, but be below limits of detection for the chosen analytical technique. Furthermore, the low stability of metabolites increases the risk of concentration changes during sampling and storage.

Table 4 provides a list of compound-specific metabolites formed under specific redox condition (aerobic or anaerobic) for BTEX. These metabolites can be detected by standard GC-MS instruments after solid phase extraction of stabilized samples. For toluene, any of the six aerobic metabolites shown in Table 4 can be utilized in site-assessment procedures, as all associated enzymatic pathways have the potential to be present and active at contaminated sites. Under anaerobic conditions only a single signature toluene metabolite (benzylsuccinate) has so far been identified (Beller et al. 1992). Assessment of ethylbenzene, xylene, and naphthalene degradation can also occur via metabolite analysis. For benzene, a compound-specific metabolite has been identified for aerobic conditions (Gibson and Parales 2000), but the few identified anaerobic metabolites produced from benzene, that is, benzoic acid and phenol (Abu Laban et al. 2010; Zhang et al. 2013; Zhang et al. 2014), can have many sources and therefore, are considered ambiguous biomarkers. As such, detection of benzoic acid and phenol by itself is not sufficient to demonstrate anaerobic benzene biodegradation. It is important to note that the information in Table 4 is evolving because ongoing research continues to reveal new findings on how microorganisms metabolize contaminants. For example (Zhang et al. 2013; Zhang et al. 2014) have recently confirmed that phenol is an early metabolite during anaerobic benzene biodegradation, whereas Dong et al. (2017) recently identified benzoyl-CoA as a metabolite of anaerobic benzene degradation.

#### *Functional Genes (DNA and mRNA)*

Microbial DNA contains the genetic blueprint for cellular production of biodegradation enzymes. A number of genes (DNA) that encode different enzymes involved in the biodegradation of PHCs are known (Varjani 2017). Because microorganisms with the genetic potential for contaminant biodegradation can be present but inactive (dormant) at field sites, finding DNA encoding biodegradation genes is not sufficient for documenting the occurrence of biodegradation in groundwater. To document biodegradation as an active

process, the biomarker known as “mRNA” (messenger RNA) is far more convincing. Documenting the occurrence of mRNA encoded by biodegradation genes in groundwater shows that microorganisms hosting the DNA are metabolically active and engaged in the biodegradation process (Bombach et al. 2010). The mRNA molecule (evidence of the expressed, or transcribed, gene) conveys information encoded in DNA to the cellular site of enzyme production, the ribosome. The ribosome translates the mRNA into the proteins that catalyze biodegradation pathways. It can be quantified by methods such as quantitative reverse transcription polymerase chain reaction (RT-qPCR). Detection of mRNA transcripts provides robust evidence for in situ biodegradation processes. Whereas some genes have been shown to encode enzymes able to degrade a variety of compounds, some genes encode enzymes that are compound-specific (Gibson and Parales 2000).

Analogous to metabolites discussed above, gene expression (in the form of mRNA) generally occurs under redox-specific conditions. Thus, documenting mRNA for particular genes in groundwater can be diagnostic of both contaminant-specific degradation and the prevalence of aerobic or anaerobic conditions in situ (Bombach et al. 2010). Clearly, detection of specific mRNA transcripts in high abundance indicates whether or not remediation strategies aimed at enhancing biodegradation have been successful although it is not possible to conclude on its rate. Interpretation of negative mRNA assays (like those of metabolites) is difficult due to the often low abundance and low stability of mRNA. Thus, mRNA may remain undetected although a process is ongoing. Furthermore, samples must be frozen directly in the field which makes the method application more challenging.

Currently, only few biodegradation genes and their corresponding mRNA transcripts have been characterized rigorously enough to qualify as robust, compound-specific genetic biomarkers (Table 5). The enzymes and underlying DNA and mRNA involved in aerobic biodegradation of BTEX are so similar that compound-specific mRNA assays have not been devised (Table 5). For example, assays detecting mRNA of the *todC* (toluene dioxygenase) gene are positive during the biodegradation of three aromatic compounds (BTE) under aerobic conditions (Liu et al. 2011). Regarding anaerobic benzene biodegradation, to date, the genetic basis for only a single metabolic pathway associated with anaerobic benzene biodegradation has been reported, the *abcA* gene (anaerobic benzene carboxylase, responsible for converting benzene to benzoic acid). This gene is associated with benzene biodegradation under iron-reducing and likely other anoxic conditions (Abu Laban et al. 2010). Recently, an alternate pathway has been described (Dong et al. 2017) whose genetic underpinnings remain to be elucidated.

Regarding anaerobic degradation of methylated aromatics, the *bssA* (benzylsuccinate synthase) gene (and corresponding mRNA) encodes for biodegradation of TEX under anaerobic conditions (Table 5). Also, a specific variant of the *bssA* gene, *bssA*-SRB, has been shown to be carried by bacteria that are active in anaerobic TEX metabolism under sulfate-reducing conditions (Beller et al. 2008). Thus, detecting mRNA of the *bssA*-SRB gene links biodegradation of TEX to

**Table 5**

**Genes (Detected as Expressed mRNA) That Are Diagnostic of In Situ Biodegradation**

Compound	Biodegradation Gene	Encoded mRNA and Enzyme	Redox Condition
Benzene	<i>abcA</i>	Anaerobic benzene carboxylase	Anaerobic
Benzene, toluene, ethylbenzene	<i>todC</i>	Toluene dioxygenase	Aerobic
Toluene, ethylbenzene, xylene	<i>bssA</i> (various electron acceptors)	Benzylsuccinate synthase	Anaerobic
Naphthalene and other PAHs	<i>nahAc</i>	Naphthalene dioxygenase	Aerobic
Sulfate	<i>dsrA</i> , <i>dsrB</i>	Dissimilatory sulfate reductase	Anaerobic
Toluene, ethylbenzene, xylene	<i>bssA<sub>srb</sub></i> (sulfate as electron acceptor)	Benzylsuccinate synthase (used by sulfate reducing bacteria)	Anaerobic

Note: Shown is a selection of contaminant compounds relevant to groundwater sites and the relevant biodegradation genes and corresponding enzymes whose expression as mRNA can assist in proving metabolism of PHCs.

populations carrying out sulfate reduction. Table 5 also lists an mRNA assay for detecting sulfate reduction, an anaerobic respiration (electron-accepting) process that can assist in site-management decisions. It is important to note that the information in Table 5 represents our current understanding, but ongoing research continues to reveal new findings pertinent to how microorganisms metabolize contaminants.

#### *Biomarker Field Data Interpretation*

Interpretation of metabolites and mRNA is typically an assessment of the presence/absence of a process while it is not possible to conclude on its rate. The presence of targeted biomarkers and/or mRNA will directly indicate that biodegradation of the targeted contaminant is occurring, often also shedding light on the prevailing redox conditions. However, the absence of targeted biomarkers is not direct evidence that a specific biodegradation process is not occurring. In fact, biodegradation can be occurring via uncharacterized microbial populations (Abu Laban et al. 2009). Qualitative interpretations can be performed by reporting metabolite or mRNA results relative to the highest measurement observed. A spatial evaluation can be carried out for a specific contaminated area or a temporal evaluation in selected sampling wells. In addition, concentration of BTEX should also be integrated, as low BTEX concentrations are likely to produce low biomarker concentrations.

#### **Tiered Approach**

A three-tiered approach is presented in Table 6 to assist practitioners in the selection of the appropriate diagnostic tools. The choice of the diagnostic tools depends on the set of co-occurring processes that are expected for the given treatment method and the contaminant(s) of interest. The Tier 1 level consists of reviewing and interpreting the data already available. While concentration time series provide important background information, additional insight can often be gained through time series of proportions among contaminants, for example, toluene to benzene ratios (Wilson et al. 2016), ternary diagrams for BTX (Lipson and Siegel 2000), or ratios among all BTEX compounds through tetrahedral analysis (Sra et al. 2017). While dilution changes the concentration of different compounds proportionally, removal processes favor some compounds over others due to differences in physico-chemical properties or degradability.

In exceptional cases, a Tier 1 analysis might be sufficient to discriminate among the co-occurring contaminant removal processes, but in many cases a Tier 2 tool might be required. The Tier 2 level consists of one (or more) diagnostic tools that is considered the most appropriate to assess the co-occurring processes for a selected compound (Table 6). In most cases Tier 2 consists of CSIA rather than biomarkers for several reasons. With CSIA, a broad range of removal processes can be differentiated. For some biodegradation processes, the range of biomarkers is still limited, making the interpretation of absence of biomarkers ambiguous. Furthermore, for CSIA, standard VOC sampling and shipping procedures can be used, whereas, given the transient nature of biomarkers, shipping requires rigorous procedures to preserve the sample integrity, especially for mRNA, which is thus only proposed in Tier 3. For some removal processes (e.g., aerobic biodegradation of toluene), the CSIA reference zone is broad and thus might overlap with the reference zone of a second process of interest. If the role of the co-occurring contaminant removal processes is identified successfully with the Tier 2 tools, the assessment may be terminated; however, it may be necessary for several reasons to proceed to Tier 3. In the presence of NAPL, the CSIA trends associated with reactive removal processes may be masked by ongoing dissolution contributing to the original isotopic signature. Furthermore, in situations with more than two contaminant removal processes, the same isotope pattern might be created by a single process or a combination of two. In general, biomarkers can be added in Tier 3 to specifically address the contribution of biodegradation. Depending on the availability of a compound-specific indicator, either metabolites and/or mRNA is proposed. In some cases, it can also be more straightforward to first employ a process-specific tool (e.g., <sup>34</sup>S to demonstrate sulfate reduction) before proceeding to a compound-specific tool.

In the following, the proposed assignment of diagnostic tools to different tiers is explained further for each specific set of co-occurring removal processes (Table 6). For chemical or biological removal versus dilution, CSIA is proposed as a Tier 2 diagnostic tool. In case of chemical removal, CSIA is the only tool that provides compound-specific information. Furthermore, dilution generally does not alter isotope signatures, and thus CSIA is a robust way to differentiate dilution versus reactive processes. In the case of biodegradation versus volatilization, CSIA is proposed as a Tier 2 diagnostic tool as well because biodegradation leads to isotope shifts

**Table 6**

**Tiered Approach for Diagnostic Tool Selection According to Intended and Co-occurring Contaminant Removal Processes and Compound Targeted in Groundwater Samples**

Contaminant Removal Processes	Technology Involved	Tier	Targeted Compounds	Proposed Tool
Chemical or biological removal versus dilution	ISCO <sup>1</sup> Delivery of limiting electron acceptors (e.g., sulfate)	Tier 1: use available data (including groundwater geochemistry)	BTEX	Concentration time series Compound proportions
		Tier 2: use of one diagnostic tool		CSIA
		Tier 3: use a complementary diagnostic tool		<sup>34</sup> S in sulfate <sup>2,3</sup> <sup>13</sup> C in DIC Metabolites and/or mRNA <sup>3</sup>
Biodegradation versus volatilization	Air sparging Multi-phase extraction SVE	Tier 1: use available data	BTEX	Concentration time series Compound proportions
		Tier 2: use of one diagnostic tool	BTEX, Naphthalene	CSIA
		Tier 3: use a complementary diagnostic tool	BTEX, Naphthalene	Metabolites and/or mRNA
Biodegradation versus chemical oxidation	ISCO <sup>1</sup>	Tier 1: use available data	BTEX	Concentration time series Compound proportions
		Tier 2: use of one diagnostic tool	BTEX	CSIA and <sup>34</sup> S in sulfate <sup>2</sup>
		Tier 3: use a complementary diagnostic tool	BTEX	Metabolites and/or mRNA
Aerobic versus anaerobic biodegradation	Air sparging Delivery of electron acceptors	Tier 1: use available data	BTEX	Concentration time series Compound proportions
		Tier 2: use of one diagnostic tool	Benzene, Toluene, Ethylbenzene, Xylenes, Naphthalene	CSIA and <sup>34</sup> S in sulfate <sup>2</sup> Metabolites
		Tier 3: use a complementary diagnostic tool	Benzene, Toluene Ethylbenzene, Xylenes, Naphthalene	Metabolites and/or mRNA CSIA Metabolites and/or mRNA

<sup>1</sup>For unactivated persulfate.

<sup>2</sup>In some cases, it can be useful to first evaluate if sulfate reduction occurs before using compound-specific diagnostic tools.

<sup>3</sup>For biological removal only.

that are distinctly different from those for volatilization. For biodegradation versus chemical oxidation by persulfate, it can be useful to first evaluate if sulfate reduction occurs, before investigating the degradation of specific contaminants. Thus, Tier 2 might either consist of <sup>34</sup>S of sulfate and/or CSIA. Finally, for aerobic versus anaerobic biodegradation, the proposed Tier 2 diagnostic tool depends on the compound. For some compounds, the CSIA reference zones for aerobic and anaerobic degradation are distinctly different allowing their discrimination, while for others they overlap or dual element isotope slopes are not known, and thus metabolites are proposed as a Tier 2 tool rather than CSIA.

Examples for the application of multiple diagnostic tools to differentiate between contaminant removal processes can be found in this issue. Bouchard et al. (2018) applied CSIA, <sup>13</sup>C in DIC and biomarkers to differentiate among aerobic biodegradation, anaerobic biodegradation and physical contaminant removal during air sparging. Shayan et al. (2018) made use of CSIA, <sup>34</sup>S in sulfate, <sup>13</sup>C in DIC, and biomarkers to distinguish chemical oxidation from anaerobic biodegradation following persulfate injection. Wei et al. (2018)

evaluated anaerobic biodegradation after sulfate application using CSIA, <sup>34</sup>S in sulfate, <sup>13</sup>C in DIC, and biomarkers.

**Conclusions**

This technical note is aimed at synthesizing the recent knowledge on the application of diagnostic tools (isotope tools and biomarkers) to assess the performance of in situ treatment systems at PHC-impacted sites. Based on results from laboratory and controlled field studies, a tiered approach was proposed to prioritize the diagnostic tools depending on co-occurring processes and contaminants of interest. This tiered approach is intended to aid practitioners diagnose if the intended contaminant removal processes are taking place, whether system optimization has improved the removal of a compound of concern, or whether a system had reached its limits of technical effectiveness. Each diagnostic tool contributes to a “multiple lines of evidence” approach that improves the understanding of mass removal processes. Better process understanding will make in situ remediation systems more effective and efficient.

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