In situ detection of an aerobic alkane metabolites in subsurface environments

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Alkanes comprise a substantial fraction of crude oil and refined fuels. As such, they are prevalent within deep subsurface fossil fuel deposits and in shallow subsurface environments such as aquifers that are contaminated with hydrocarbons. These environments are typically anaerobic, and host diverse microbial communities that can potentially use alkanes as substrates. Anaerobic alkane biodegradation has been reported to occur under nitrate-reducing, sulfate-reducing, and methanogenic conditions. Elucidating the pathways of anaerobic alkane metabolism has been of interest in order to understand how microbes can be used to remediate contaminated sites. Alkane activation primarily occurs by addition to fumarate, yielding alkylsuccinates, unique anaerobic metabolites that can be used to indicate in situ anaerobic alkane metabolism. These metabolites have been detected in hydrocarbon-contaminated shallow aquifers, offering strong evidence for intrinsic anaerobic bioremediation. Recently, studies have also revealed that alkylsuccinates are present in oil and coal seam production waters, indicating that anaerobic microbial communities can utilize alkanes in these deeper subsurface environments. In many crude oil reservoirs, the in situ anaerobic metabolism of hydrocarbons such as alkanes may be contributing to modern-day detrimental effects such as oilfield souring, or may lead to more beneficial technologies such as enhanced energy recovery from mature oilfields. In this review, we briefly describe the key metabolic pathways for anaerobic alkane (including n-alkanes, isoalkanes, and cyclic alkanes) metabolism and highlight several field reports wherein alkylsuccinates have provided evidence for anaerobic in situ alkane metabolism in shallow and deep subsurface environments.

Keywords: anaerobic, metabolites, alkylsuccinates, alkanes, subsurface
such as alkanes under a variety of electron-accepting conditions is important for both bioremediation and microbial-based energy recovery applications.

**PATHWAYS OF ALKANE BIODEGRADATION**

Alkanes have long been known to be biodegradable under aerobic conditions by microorganisms. In addition to utilizing oxygen as an electron acceptor, aerobes use oxygen as a co-reagent to activate the stable carbon-carbon bonds of alkanes using monooxygenases (Rojo, 2009). The monooxygenases incorporate oxygen into the alkane molecule typically at the terminal carbon, forming an alcohol. This metabolite is then transformed to an aldehyde, which is subsequently converted to a carboxylic acid. The latter product is then readily metabolized via β-oxidation reactions to products that can enter central metabolic pathways (Rojo, 2009). The reader is referred to other papers in this volume for more details on the aerobic microbial oxidation of alkanes.

The anaerobic oxidation of alkanes has also been documented in a few early reports (ZoBell, 1946; Muller, 1957; Jack et al., 1985) but was largely discounted due to the overwhelming evidence for and widespread prevalence of aerobic alkane biodegradation processes that can readily occur in surface soils or in aerated environments. However, mounting evidence over the last two decades has shown that petroleum mixtures spilled into natural environments such as groundwater aquifers results in a rapid depletion of oxygen, creating anaerobic conditions. Thus, much attention has now also been focused on examining the potential for anaerobic hydrocarbon metabolism primarily in the interest of applying bioremediation for site cleanup.

Research conducted in the 1990’s examining the anaerobic biodegradation of alkylbenzenes such as toluene and xylene using samples collected from hydrocarbon-contaminated sites revealed that anaerobes activate hydrocarbons by a novel carbon-carbon addition mechanism known as “fumarate addition.” In this mechanism, the methyl group of an alkylbenzene such as toluene is added to fumarate (a C4-dicarboxylic acid present in central microbial metabolic pathways) forming benzylsuccinic acid (Biegert et al., 1996; Beller and Spormann, 1997). The enzyme carrying out this reaction, benzylsuccinate synthase, is known to be part of the radical SAM superfamily of enzymes (Frey, 2001; Widdel and Grundmann, 2010). Following this discovery, many more details regarding the mechanism, enzymes, and genes involved in this activation step (and further downstream metabolic reactions) have been elucidated for many aromatic hydrocarbons, including methylated mono- and polycyclic aromatic hydrocarbons [e.g., reviewed by Focht (2008)]. Subsequently, investigators examining anaerobic n-alkane metabolism found that this class of hydrocarbons could also be activated by carbon-carbon bond addition to fumarate, forming alkylsuccinates (Kropp et al., 2000; Rabus et al., 2001). Fumarate addition occurs primarily at the C2 subterminal carbon position of a given n-alkane (Figure 1A). Alkylsuccinates are then further transformed by a postulated carbon skeleton rearrangement followed by decarboxylation yielding branched fatty acids that can readily enter β-oxidation and other central metabolic pathways (Wilkes et al., 2002; Cravo-Laureau et al., 2005; Davidova et al., 2005; Callaghan et al., 2006; Widdel and Grundmann, 2010) (Figure 1A). Recently, the genes responsible for the initial “alkylsuccinate synthase” reaction were identified, known as the masD or assA genes (Callaghan et al., 2008; Grundmann et al., 2008). Laboratory-based studies examining the anaerobic degradation of pure n-alkanes have demonstrated that alkanes ranging from C3 to C16 are susceptible to fumarate addition, as the corresponding alkylsuccinates have been identified in culture fluids (Table 1). A recent laboratory study has also shown that n-alkanes up to C26 in whole crude oil can be degraded via fumarate addition under sulfate-reducing conditions (Aitken et al., 2013). Further, the assA gene has been detected in anaerobic cultures incubated with pure n-alkanes as short as C3 and as long as C28 (Callaghan et al., 2010). It has been suggested that fumarate addition may even be possible for anaerobic methane oxidation (Thauer and Shima, 2008; Beasley and Nanny, 2012). An alternate mechanism to fumarate addition has also been suggested involving carboxylation at the C3 position of an n-alkane, followed by ethyl group removal by an unknown mechanism (So et al., 2003). Studies with a nitrate-reducing enrichment culture degrading n-hexadecane suggested a similar mechanism (Callaghan et al., 2009). Even though recent research has shown that alkanes are biodegraded under methanogenic conditions in laboratory incubations (e.g., Zengler et al., 1999; Siddique et al., 2006; Gieg et al., 2008; Jones et al., 2008; Siddique et al., 2011; Zhou et al., 2012; Aitken et al., 2013; Cheng et al., 2013), alkylsuccinates have not been detected as metabolites during the metabolism of n-alkanes, prompting the hypothesis that a different pathway may be occurring under these highly reduced conditions (Mbadinga et al., 2011; Aitken et al., 2013).

A few studies have now shown that cyclic alkanes may be also be activated by fumarate addition (Figure 1B). In studies examining the biotransformation of hydrocarbons in whole crude oil by a nitrate-reducer (strain HxN1) capable of utilizing C6–C8 n-alkanes, Wilkes et al. (2003) detected metabolites showing that cyclopentane and methylcyclopentane were biotransformed to the corresponding fumarate addition products in co-metabolic reactions with hexane as the primary carbon source. Using sediments from a gas condensate-contaminated site, Rios-Hernandez et al. (2003) demonstrated sulfate-dependent ethylcyclopentane metabolism, and identified a putative fumarate addition metabolite from this cyclic alkane. In anoxic laboratory incubations prepared from the same contaminated sediments amended with sulfate and methylcyclohexane-δ14, the corresponding δ-labeled fumarate addition metabolite was also tentatively identified from this cyclic alkane (Gieg et al., 2009). Unfortunately, the exact location on the alkylated cyclic alkanes to which fumarate was added (e.g., alkyl group or ring carbon) could not be determined in the studies. Musat et al. (2010) showed that the model unsubstituted cyclic alkane cyclohexane was also activated by a carbon-carbon addition to fumarate under nitrate-reducing conditions, a process that was also coupled to anaerobic ammonium oxidation. A proposed pathway for the anaerobic biodegradation of a model cyclic alkane (cyclohexane) via fumarate addition and subsequent reactions (Rios-Hernandez et al., 2003; Musat et al., 2010) is shown in Figure 1B.

Comparatively little has been reported on the anaerobic biotransformation of isoalkanes. Branched alkanes such as pristane...
and phytane have frequently been used as biomarkers with which to compare the extent of biodegradation of alkanes in crude oils, as these kinds of compounds are typically more recalcitrant than \( n \)-alkanes (Huang and Larter, 2005). However, there is some evidence that branched alkanes can be utilized under anaerobic conditions. Two studies carried out under anaerobic conditions showed that the branched alkane pristane was susceptible to biodegradation under nitrate-reducing or methanogenic conditions but no metabolites were reported (Bregnard et al., 1997; Grossi et al., 2000). Using oil sands tailings ponds samples as an inoculum, Abu Laban et al. (2012) recently showed that \( C_7 \) and \( C_8 \) isoalkanes could also be metabolized under sulfate-reducing or methanogenic conditions. In the study, metabolites consistent with the mass spectral (MS) profiles of the corresponding alkyl-succinates were detected, showing for the first time that isoalkanes are also susceptible to fumarate addition (Figure 1A).

**SIGNATURE ANAEROBIC METABOLITES AS INDICATORS OF IN SITU HYDROCARBON BIODEGRADATION**

Given the overwhelming evidence that microbes are able to biodegrade hydrocarbons, relying on such activity for the remediation of contaminated sites is an attractive and relatively inexpensive clean-up option. However, documenting that such anaerobic hydrocarbon biodegradation is occurring in fuel-contaminated sites or in fossil energy reservoirs remains a challenge. For example, multiple lines of evidence, both chemical and

FIGURE 1 | The anaerobic biodegradation of alkanes via fumarate addition and subsequent reactions. (A) Pathway for the biodegradation of \( n \)-alkanes and potentially for isoalkanes (dotted lines) based on literature reports [e.g., as overviewed by Widdel and Grundmann (2010)]. (B) Proposed pathway for the biodegradation of cyclic alkanes using (after Rios-Hernandez et al., 2003; Musat et al., 2010). Compounds marked with asterisks indicate fumarate addition metabolites that are most diagnostic of in situ anaerobic biodegradation of alkanes.
addition metabolites of alkylbenzenes (e.g., toluene, ethyl-
benzene, xylene, and trimethylbenzene) can be detected in
hydrocarbon-contaminated groundwater [see reviews by Beller
(2000); Gieg and Suflita (2005), and Callaghan (2012)]. With
the discovery that methylcyclohexane and other multi-ringed
aromatics (including heterocycles) can also be metabolized by
fumarate addition, the corresponding succinates have also been
detected in field studies (Griebler et al., 2004; Bombach et al.,
2010; Jobelius et al., 2011; Morasch et al., 2011), attesting to
the usefulness of this method for demonstrating the in situ anaero-
bic biodegradation of many hydrocarbon classes. Furthermore,
primer sets based on the catabolic gene(s) encoding benzylsuccin-
ate synthase have been developed by several investigators and
have been used successfully to indicate the metabolic poten-
tial for microbial communities in contaminated field samples to
anaerobically biodegrade alkylbenzenes (e.g., Beller et al., 2002,
2008; Winderl et al., 2007; Callaghan et al., 2010; Oka et al.,
2011; von Netzer et al., 2013). It should be noted that down-
stream metabolites of anaerobic hydrocarbon biodegradation can
also be indicators of in situ biodegradation, especially if they
are detected in contaminated but not in unrelated, uncontamin-
ated groundwater samples (Cozzarelli et al., 1995; Gieg and
Suflita, 2002). However, these compounds are typically not spe-
cific to a parent substrate (for example, benzoate, often detected
in contaminated groundwater, is a central intermediate in the
anaerobic metabolism of many aromatic compounds) or may
be produced via aerobic or anaerobic processes (such as tolu-
ates from xylene) (Beller, 2000; Gieg and Suflita, 2005). In
anaerobic alkane metabolism, downstream metabolites include
fatty acids, which may be produced via a variety of other cellu-
lar pathways. Thus, the fumarate addition metabolites are most
diagnostic of the in situ anaerobic metabolism of specific hydro-
carbons including alkanes. It should be noted, however, that
there are some disadvantages to using signature metabolites as
sole indicators of in situ bioremediation (Morasch et al., 2011).
For example, these products may be below analytical detection
limits, knowledge of biodegradation pathways for various hydro-
carbons is required (e.g., in order to determine which metabolites
to look for), and commercially-available authentic standards for
many of the fumarate addition products are not available for
analytical comparisons with metabolites found in field samples.
Thus, using signature fumarate addition metabolites to determine
whether in situ hydrocarbon metabolism is occurring at a given
site should ideally be done in conjunction with other methods of
site assessment (Bombach et al., 2010; Morasch et al., 2011).

### ANAEROBIC ALKANE METABOLITES IN HYDROCARBON-CONTAMINATED AQUIFERS

In field bioremediation studies, the biodegradation of BTEX
compounds has been a focus because these compounds are
highly water-soluble (150–1780 mg/L; Heath et al., 1993) thus can
rapidly migrate away from the spill source. Furthermore, ben-
zene is among the most highly regulated hydrocarbons due to its
carcinogenicity (Maltoni et al., 1989). However, in many fuel
mixtures that spill into aquifers (such as gasoline, diesel, gas con-
densate, jet fuel), the saturated fraction (alkanes, isoalkanes, and
cyclic alkanes) is the most abundant (e.g., up to 80% by wt., Heath

### Table 1 | Overview of literature studies wherein fumarate addition
metabolites (alkylsuccinates) were detected in anaerobic laboratory
incubations with n-alkanes added as pure substrates.

| n-alkane | Electron-accepting condition and culture | Literature references |
|---------|------------------------------------------|-----------------------|
| C₃      | Sulfate-reducing—strain BuSS            | Kniemeyer et al., 2007 |
|        | Sulfate-reducing—enrichment culture     | Savage et al., 2010   |
| C₄      | Sulfate-reducing—strain BuSS            | Kniemeyer et al., 2007 |
| C₆      | Nitrate-reducing—strains HxN1, OcN1     | Rabus et al., 2001    |
|        | Sulfate-reducing—strain ALDC (Desulfoglaeba alkanexedens) | Zedelius et al., 2011 |
| C₈      | Nitrate-reducing—strain HxN1, OcN1, HdN1| Rabus et al., 2001    |
|        | Sulfate-reducing—strain ALDC (Desulfoglaeba alkanexedens) | Davidova et al., 2005 |
| C₁₀     | Sulfate-reducing—strain ALDC (Desulfoglaeba alkanexedens) | Davidova et al., 2005 |
|        | Sulfate-reducing—strain TD3             | Rabus et al., 2011    |
| C₁₂     | Sulfate-reducing—strain ALDC (Desulfoglaeba alkanexedens) | Kropp et al., 2000    |
|        | Sulfate-reducing—strain ALDC (Desulfoglaeba alkanexedens) | Davidova et al., 2005 |
| C₁₅     | Sulfate-reducing—Desulfatbacillus aliphaticovorans CV2803 | Cravo-Laureau et al., 2005 |
| C₁₆     | Sulfate-reducing—Desulfatbacillus aliphaticovorans CV2803 | Cravo-Laureau et al., 2005 |
|        | Sulfate-reducing—Desulfatbacillus aliphaticivorans AK-01 | Callaghan et al., 2006 |

microbiological, are required to offer support that in situ hydro-
carbon bioremediation is occurring in contaminated aquifers
(NRC, 1993; Gieg and Suflita, 2005; Beller et al., 2008; Weiss
and Cozzarelli, 2008; Bombach et al., 2010; Morasch et al., 2011;
Jeon and Madsen, 2012). In conjunction with the elucidation
of anaerobic hydrocarbon metabolic pathways, the idea of using
anaerobic metabolites to indicate that in situ anaerobic hydro-
carbon biodegradation is occurring emerged as a powerful tool
for proving in situ bioremediation. Beller et al. (1995) first pro-
posed that these metabolites can be effective indicators of in situ
anaerobic hydrocarbon biodegradation since they are uniquely
anaerobic and are specific to their hydrocarbon substrate; indeed
they detected these metabolites in anoxic hydrocarbon-impacted
groundwater but not in uncontaminated areas. Beller (2000) pro-
posed a list of criteria that define a signature metabolite for use as
an in situ bioremediation indicator including that the metabolite
should be (1) actively produced during biodegradation, (2) rela-
tively chemically and biologically stable so that it can be detected,
(3) a true intermediate rather than a cometabolic by-product, (4)
absent in the contaminant mixture, (5) absent in uncontaminated
environments, and (6) be of no commercial use.

In addition to the initial reports by Beller and col-
leagues, many other studies have now shown that fumarate
et al., 1993). Since many alkanes have appreciable water solubilities (in the tens of mg/L), some alkanes can also migrate in groundwater away from the spill source. Many alkanes have known toxicity (Ritchie et al., 2001), thus their presence in contaminated aquifers is also of concern and their levels are often regulated (Nascarella et al., 2002).

To date, though, relatively few studies have reported on the detection of signature anaerobic alkane metabolites indicative of in situ anaerobic alkane in hydrocarbon-contaminated near surface environments. Gieg and Suflita (2002) documented that alkylsuccinates can be detected in hydrocarbon contaminated aquifers alongside benzylsuccinates, providing evidence that alkanes are susceptible to in situ anaerobic biodegradation and that alkylsuccinates can also be used as indicator metabolites. In 5 of the 6 sites examined for these signature compounds, alkylsuccinates were detected in contaminated groundwater samples, ranging from C₃ to C₁₁ alkylsuccinates depending on the site interrogated (Gieg and Suflita, 2002; Table 2). Several of the detected metabolites had MS fragments with two mass units less than those predicted to arise from straight chain (or branched) alkane metabolism, suggesting the formation of fumarate addition products from cyclic alkanes. Using sediments from a gas condensate-contaminated site, Rios-Hernandez et al. (2003) demonstrated that ethylcyclopentane could be biodegraded under sulfate-reducing conditions in laboratory incubations, and identified a putative fumarate addition metabolite that formed during

| Field location                        | Hydrocarbon contaminant | Electron accepting condition(s) of field Site | Alkylsuccinates (and/or assA gene detected) | References                                      |
|---------------------------------------|-------------------------|----------------------------------------------|---------------------------------------------|------------------------------------------------|
| Weld County, CO, USA                  | Gas condensate          | Sulfate-reducing, methanogenic              | Saturated: C₆–C₉ Unsaturated: C₆–C₉ assA gene detected | Gieg and Suflita, 2002; Rios-Hernandez et al., 2003; Callaghan et al., 2010 |
| Wise County, TX, USA                  | Natural gas liquids     | Sulfate-reducing                            | Saturated: C₃–C₆ Unsaturated: C₆             | Gieg and Suflita, 2002                          |
| Sedgewick County, KS, USA             | Gasoline range organics | Nitrate-reducing, sulfate-reducing, methanogenic | Saturated: C₅ Unsaturated: C₅ | Gieg and Suflita, 2002                          |
| West central AB, Canada               | Flare pit site, variable hydrocarbons | Sulfate-reducing, methanogenic         | Saturated: C₅–C₁₁ Unsaturated: C₇           | Gieg and Suflita, 2002                          |
| East central AB, Canada               | Gas condensate          | Nitrate-reducing, Fe- and Mn-reducing, sulfate-reducing | Saturated: C₄, C₆, C₈ Unsaturated: C₈, C₇ | Gieg and Suflita, 2002                          |
| Casper, WY, USA                       | Former refinery site, variable hydrocarbons | Sulfate-reducing, methanogenic          | Saturated: C₅–C₆ Unsaturated: C₅–C₇         | Parisi et al., 2009                             |
| Hickam AFB, HI, USA                   | Jet fuel                | Sulfate-reducing, methanogenic             | Saturated: C₅–C₉ Unsaturated: C₇             | Gieg et al., 2009                               |
| Passaic River, NJ, USA                | Hydrocarbon-contaminated sediments | Not reported | assA gene detected | Callaghan et al., 2010                          |
| Newtown Creek, NY, USA                | Hydrocarbon-contaminated sediments | Not reported | assA gene detected | Callaghan et al., 2010                          |
| Arthur Kill Waterway, NY/NJ, USA      | Hydrocarbon-contaminated sediments | Not reported | assA gene detected | Callaghan et al., 2010                          |
| Gowanus Canal, NY, USA                | Hydrocarbon-contaminated sediments | Not reported | assA gene detected | Callaghan et al., 2010                          |
| Rhine River Valley (Flingern aquifer), Germany | Former gas works site, tar oil-contaminated | Primarily sulfate-reducing | assA gene detected | von Netzer et al., 2013                         |

*Deep-sea marine sediments rather than groundwater aquifer sediments.
detecting anaerobic alkane metabolites in situ}.

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Detecting anaerobic alkane metabolites in situ

the biodegradation of this compound. The gas chromatographic (GC) retention time and MS profile of this incubation-derived metabolite matched with that of a GC peak detected in several of the samples from the contaminated site, supporting the notion that cyclic alkanes can be anaerobically biotransformed in situ. Parisi et al. (2009) also detected alkysuccinates from C$_5$ to C$_9$ n- or cyclic alkanes in groundwater samples collected from an aquifer contaminated with a variety of fuel mixtures while Gieg et al. (2009) detected C$_5$ to C$_9$ alkysuccinates in several anoxic groundwater samples prior to conducting push-pull tests in a jet fuel contaminated aquifer, further attesting to the in situ anaerobic biodegradation of alkanes (Table 2). In a gas condensate-contaminated site wherein alkysuccinates were detected in groundwater samples (Gieg and Sulflita, 2002; Rios-Hernandez et al., 2003), Callaghan et al. (2010) detected the presence of the alkysuccinate synthase gene (assA gene), augmenting the metabolite findings. Primer sets for this gene were also used to probe samples collected from a handful of other hydrocarbon-contaminated aquifers in the US and in Germany, and indeed this gene was detected (Table 2; Callaghan et al., 2010; von Netzter et al., 2013). Recently, assA gene sequences were also detected in deep sea core samples collected from areas near the Deepwater Horizon oil spill, indicating that the extant organisms in deep-sea sediments have the potential to biodegrade alkanes and can potential contribute to bioremediation of such oil spills (Kimes et al., 2013). Although the alkysuccinates themselves were not detected in these latter field sites, the detection of the gene catalyzing their formation in contaminated sites reveals a widespread potential for in situ anaerobic alkane biodegradation and usefulness of probing for the requisite metabolic genes. However, there are currently some limitations in the use of the assA gene. For example, this gene appears to be highly diverse, as a single primer set has not yet been designed that can be used to detect/amplify this gene (Callaghan et al., 2010; Aitken et al., 2013; von Netzter et al., 2013). Further, the assA genes show high similarity with the bssA genes, thus primer pairs designed to detect assA may also amplify the bssA genotypes (Callaghan et al., 2010). However, as stated above, such limitations can be overcome by coupling gene analysis with metabolite analysis and other indicators of biodegradation at field sites to provide strong evidence for intrinsic bioremediation of hydrocarbons like alkanes at contaminated sites. Table 2 summarizes the reports to date wherein alkysuccinates (and/or alkysuccinate synthase genes) have been detected in hydrocarbon-contaminated sites, providing evidence in support of in situ anaerobic alkane biodegradation.

**ANAEROBIC ALKANE METABOLITES IN FOSSIL ENERGY RESERVORIES**

Geochemical data suggesting the biodegradation of crude oil in reservoirs over geological time generating heavy oil has prompted an examination of these oils and fluids of oil reservoirs in general for evidence of anaerobic hydrocarbon metabolites. Upon examining 77 crude oils from across the globe, Aitken et al. (2004) detected known anaerobic metabolites of naphthenalenes in 52 samples (including 2-naphthoic acid; 5,6,7,8-tetralhydro-2-naphthoic acid; decahydro-2-naphthoic acid), offering the first metabolic evidence that anaerobic in situ biodegradation of crude oil can occur in reservoirs. Although alkysuccinates were also sought, none were detected in the assays. In a later study, Duncan et al. (2009) examined the metabolite profiles of several produced water samples (e.g., water removed along with oil from production wells during oil recovery) collected from high temperature Alaska North Slope (ANS) oilfields. The authors were able to detect some low molecular weight alkysuccinates derived from C$_1$–C$_4$ alkanes in the produced water samples (Table 3). Downstream, branched alkanoic acids related to these alkysuccinates were also detected (Duncan et al., 2009). Operationally, these oilsfields were pressurized with natural gas to aid in oil recovery, providing the oilfield microbial community with a continuous source of low molecular weight alkanes that were likely ultimately biotransformed to the corresponding alkysuccinates (Duncan et al., 2009). In a subsequent study, Gieg et al. (2010) examined samples collected from two different high temperature ANS oilfields, and also detected several fumarate addition metabolites including C$_1$–C$_3$ alkysuccinates (Table 3). This latter study further showed that the methanogenic microbial communities present in crude oil reservoirs are able to subsist on hydrocarbons supporting the notion that oil biodegradation within fossil energy reservoirs can occur under anaerobic conditions.

A low temperature (∼30°C) heavy oil field in Alberta, Canada (Medicine Hat Glaucotic C field) has been under study for over 5 years to test the effects of nitrate treatment on souring caused by sulfate-reducing bacteria (Lambo et al., 2008; Voordouw et al., 2009; Callbeck et al., 2011; Agrawal et al., 2012). Oil analysis conducted on various samples from this field showed that toluene served as a major electron donor driving the microbial activities within the subsurface reservoir environment (Lambo et al., 2008). This result was subsequently confirmed in laboratory studies conducted under nitrate-reducing and sulfate-reducing conditions using produced water and heavy crude oil from the field. However, the laboratory incubation tests also showed that other alkylbenzenes and alkanes present in the oil served as substrates for the oilfield microbial community (Agrawal et al., 2012). Notably, several alkanes were depleted in sulfate-reducing enrichments, and numerous metabolites indicative of fumarate addition to both aromatics and alkanes were detected, including methyl- and dimethylbenzysuccinates and alkysuccinates derived from C$_6$–C$_9$ alkanes, confirming the anaerobic biodegradation of hydrocarbons in the oil. These laboratory-based studies prompted an investigation of produced water samples from the heavy oil field for the presence of alkysuccinates, and indeed several of these signature anaerobic alkane metabolites were detected, ranging from C$_5$ to C$_8$ alkysuccinates (Table 3). These data suggested that the anaerobic microbial communities present within this low temperature reservoir can utilize alkanes as a carbon and energy source, potentially leading to souring when sulfate is present. Although alkysuccinates were not specifically identified in other oilfields (Li et al., 2012; Zhou et al., 2012; Cheng et al., 2013), recently identified alkysuccinate synthase genes in incubations prepared from oilfield fluids, showing that microbes residing in the sampled oilfield harbor the potential for in situ anaerobic alkane
metabolism, again attesting to the usefulness of catabolic gene probes for determining in situ alkane biodegradation potential (Table 2).

Wawrik et al. (2012) recently conducted a study examining the biological conversion of coal to methane in a major coal-bed methane producing region in the USA (San Juan Basin) using a series of enrichments, functional gene assays, hydrocarbon metabolite analyses, and microbial community profiling of produced water samples. Coal is a complex, organic carbon-rich mixture derived from ancient wetland plants that were buried and transformed by heat and pressure over geological time (Strapoć et al., 2011). While methane produced from coal beds is typically thermogenic, increasing evidence (such as the isotopic signature of methane) is showing that biologically produced methane is also occurring in many coal-bed regions (Strapoć et al., 2011). Although not as clearly defined as crude oil, coal can contain many substrates such as alkanes (Orem et al., 2007; Strapoć et al., 2011) that may be used by microbial communities living in coal beds to produce methane in real time, presumably via pathways similar to those shown in Figure 1. Using a functional gene array (GeoChip 3.1), Wawrik et al. (2012) detected the assA gene in several produced water samples collected from the San Juan Basin, showing that the indigenous microbial community has the metabolic potential for in situ anaerobic alkane biodegradation. In addition, they also detected C1–C3, C7, and C8 alkylsuccinates in several of the produced waters (Table 3). These data collectively showed for the first time that alkanes (and other substrates) associated with the coal can be metabolized by the associated microbial community, presumably leading to biological methane production in such fossil energy resources (Wawrik et al., 2012).

### Table 3 | Summary of studies reporting the detection of alkylsuccinates [and/or alkylsuccinate synthase (assA) gene] in fossil energy reservoir samples.

| Field location | Field details | Electron accepting condition(s) | Alkylsuccinates (and/or assA gene detected) | References |
|----------------|---------------|---------------------------------|---------------------------------------------|------------|
| Alaska North Slope, USA | High temperature oilfield (50–60°C) | Sulfate-reducing, methanogenic | Saturated: C1–C4 | Duncan et al., 2009 |
| Alaska North Slope, USA | High temperature oilfield (50–60°C) | Primarily sulfate-reducing | Saturated: C1–C3 | Gieg et al., 2010 |
| Alaska North Slope, USA | High temperature oilfield (50–60°C) | Primarily methanogenic | Saturated: C1–C3 | Gieg et al., 2010 |
| SE Alberta, Canada | Low temperature oilfield (~30°C); oil produced via PWRI1, nitrate treatment for souring | Nitrate-reducing, sulfate-reducing, methanogenic | Saturated: C6–C8; Unsaturated: C6–C8 | Agrawal et al., 2012 |
| Huabei Oilfield, China | Low temperature oilfield (37°C) | Not reported but incubations prepared with nitrate, sulfate, and under methanogenic conditions | assA gene detected in methanogenic incubations from production waters | Li et al., 2012 |
| Shengli Oilfield, China | High temperature oilfield (70°C) | Not reported but incubations prepared under methanogenic conditions | assA gene detected in methanogenic incubations (55°C) from production waters | Zhou et al., 2012 |
| Shengli Oilfield, China | High temperature oilfield (70°C) | Not reported but incubations prepared under methanogenic conditions | assA gene detected in methanogenic hexadecane-degrading enrichment | Cheng et al., 2013 |
| San Juan Basin (Fruitland Coal Formation, CO and NM), USA | Coal bed methane site | Methanogenic | Saturated: C1–C3, C8; Unsaturated: C7–C8 assA gene detected | Wawrik et al., 2012 |

1 PWRI, Produced water re-injection.
methyltion or silylation prior to analysis by GC-MS (Gieg and Suflita, 2002). Depending on the method of derivatization, alkylsuccinates have diagnostic MS fragment ions that can be used to readily determine their presence in a given water sample. For example, trimethylsilyl esters of alkylsuccinates have diagnostic fragment ions of m/z 262, 217, 172, 147, and 73 (Gieg and Suflita, 2002, 2005). Metabolite concentrations can be estimated by comparison to calibration curves prepared from commercially available or readily synthesized alkylsuccinates (e.g., methylsuccinate or n-octylsuccinate). Detection limits for alkylsuccinates by GC-MS analysis are in the nanomolar range (Gieg and Suflita, 2002). Hydrocarbon metabolite analysis by LC-MS-MS analysis has the advantage of requiring little to no sample preparation prior to analysis thus is far less labor-intensive (Beller, 2002). Hydrocarbon metabolite analysis by LC-MS-MS analysis has the advantage of requiring little to no sample preparation prior to analysis thus is far less labor-intensive (Beller, 2002). Further, the technique requires only small amounts of sample (~1 mL). Parent and/or daughter ions of various metabolites are typically monitored (Beller, 2002; Alumbaugh et al., 2004). Beller (2002) found the detection limits for benzylsuccinates to be ~0.3 μg/L using LC tandem MS, while Alumbaugh et al. (2004) found that sample pre-treatment using solid-phase extraction lowered the LC-MS-MS detection level of benzylsuccinates by at least an order of magnitude (0.006–0.029 μg/L). Although high sensitivity LC-MS techniques (e.g., LC/MS-ESI-Q-TOF) have been used to successfully detect benzylsuccinates in environmental samples (Jobelius et al., 2011; Kimes et al., 2013), the detection of alkylsuccinates remains elusive, possibly because these metabolites are present at levels below detection limits (Kimes et al., 2013). Other analytical techniques such as 2-dimensional GC analysis (GC × GC) coupled with MS, currently used for separating and quantifying complex mixtures of crude oil components and related compounds (such as naphthenic acids) (e.g., Rowland et al., 2011; Reddy et al., 2012), may find future application for hydrocarbon metabolite analysis.

CONCLUSIONS AND FUTURE DIRECTIONS

Research on the topic of anaerobic alkane metabolism has increased in the last dozen years, revealing that alkanes can be activated by fumarate addition reactions as well as by other proposed mechanisms (yet to be understood). Of the saturate fraction of fuels, the anaerobic metabolism of n-alkanes has been the most frequently studied while less is known about how isoalkanes and cyclic alkanes are metabolized anaerobically, representing a knowledge gap that can be addressed by future research. As knowledge is gained regarding the key metabolic pathways for anaerobic alkane biodegradation, the identified metabolites can readily be sought in the natural environment to indicate biodegradation. Further, as newer fuel mixtures such as biofuels become more commonly used, metabolic insights gained from the study of alkanes and other hydrocarbons may help predict the kinds of metabolites that can serve as diagnostic indicators for the biotreatment of such alternative fuels. To date, a metabolite profiling approach using robust tools of analytical chemistry (GC-MS, LC-MS-MS) has been widely been used to garner evidence for the in situ anaerobic metabolism of aromatic hydrocarbons in contaminated aquifers, but has been used to a lesser extent for determining for in situ alkane biodegradation. Since alkanes (including n-alkanes, isoalkanes, and cyclic alkanes) comprise substantial components of many fuel mixtures that are spilled into aquifers, looking for their metabolic by-products should be included in more future site assessments to determine the prospects for in situ alkane bioremediation; details on how to look for such metabolites in environmental samples have been described (e.g., Gieg and Suflita, 2002; Duncan et al., 2009). Parallel assessments of the catabolic gene (e.g., assA gene) in subsurface microbial populations along with metabolite profiling offers very strong evidence for the in situ anaerobic biodegradation of alkanes (Callaghan et al., 2010; Wawrik et al., 2012), an approach that should also be part of future site assessments. Although many hydrocarbon biodegradation studies have been carried out in the context of petroleum contaminated site bioremediation, understanding anaerobic hydrocarbon biodegradation in fossil energy reservoirs has enormous economic implications related to detrimental effects (e.g., heavy oil generation and souring) and beneficial technologies (e.g., microbially enhanced energy recovery). Thus, more extensive research into hydrocarbon-related microbial metabolic processes including anaerobic alkane biodegradation in fossil energy resources such as crude oil reservoirs, and coal and shale deposits is warranted.

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