

Natural attenuation of fatty acid methyl esters (FAME) in soil and groundwater



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Abstract: Fatty acid methyl esters (FAME) are a group of organic compounds that can be synthesized through the process of esterification of fatty acids with methanol. With the increasing use of FAME in biodiesel, there is interest in the fate and effects of FAME in the environment. Single FAME compounds are of low aqueous solubility, low volatility and low mobility but the mechanisms of autoxidation and hydrolysis may result in the generation of more mobile but equally biodegradable components. The FAME types that have been studied in the peer-reviewed literature do not appear to enhance the solubility of hydrocarbons. FAME are widely reported to be readily biodegradable under both aerobic and anaerobic conditions, although rates may vary from site to site. In the majority of studies, biodiesel FAME biodegradation occurred more rapidly than petroleum diesel biodegradation. At sites with limited electron acceptors and macronutrients, microorganisms that degrade FAME have the potential to deplete available electron acceptors and nutrients, resulting in an extended time for diesel biodegradation. As with other labile biofuels, anaerobic biodegradation of FAME may result in significant methane generation. Overall, natural attenuation would appear to be significant in controlling the fate, behaviour and potential risks posed by biodiesel.

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Fatty acid methyl esters (FAME) are of considerable environmental and economic importance as they are used as an alternative renewable fuel (biodiesel) either in the neat form or more typically blended with varying proportions of conventional oil-derived diesel (Ginn *et al.* 2009).

In the most common process, FAME are produced via the transesterification of feedstock material through reaction with methanol in the presence of a catalyst. The resulting mixture contains FAME and glycerine (glycerol) and the latter is separated from the FAME prior to use. FAME may be sourced from a variety of feedstocks including vegetable oils (rapeseed, soy, palm, sunflower and maize), animal fats (tallow, lard, and poultry and fish oils) and waste oils and fats (used cooking oils) (CONCAWE 2009; Ginn *et al.* 2009).

Fuels containing FAME are recognized by the percentage of FAME in the mixture using the letter B followed by the percentage in the fuel. B100 is pure FAME and, although B100 can be used directly as a fuel, it is usually blended with crude oil-derived diesel fuels to produce an amended biofuel (Prince *et al.* 2008). Typically blends are 5 and 20% v/v biodiesel and are designated B5 and B20, respectively. Biodiesel is internationally recognized as an alternative to conventional fuel, and a number of standards have been developed that specify the key properties of biodiesel (e.g. ASTM D6751-09 (ASTM 2009); BS EN 14214:2012+A1:2014 (BSI 2013)). For the purposes of this review, the term biodiesel is used to describe both pure FAME (B100) feedstock and blends of FAME with petroleum diesel. The blended mixtures will be distinguished where relevant through designation of the percentage of FAME present in the material being studied.

Guidance on the safe use and handling of biodiesel has been developed both in Europe and the USA (CONCAWE 2009; NREL 2009). However, there is also interest in understanding the fate and behaviour of biodiesel in the event of a release to the subsurface as a

result of accidents, leakages or spills. Considerable work has already been undertaken to examine the fate of other biofuels such as ethanol (Morgan *et al.* 2014) but far less research appears to have been published on biodiesel. Although the aerobic and anaerobic biodegradation of biodiesel has been documented, uncertainty remains on how biodiesel partitions and degrades in the subsurface either alone or in influencing the fate of other hydrocarbons. The focus of this literature review is to summarize the characteristics and fate of FAME in the context of natural attenuation mechanisms that would be expected to control the fate of FAME in the subsurface.

Biodiesel composition

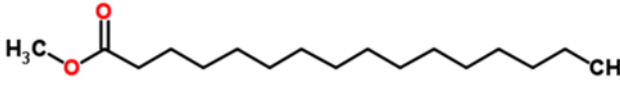
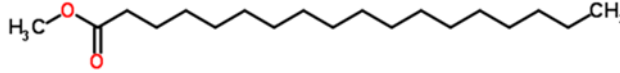
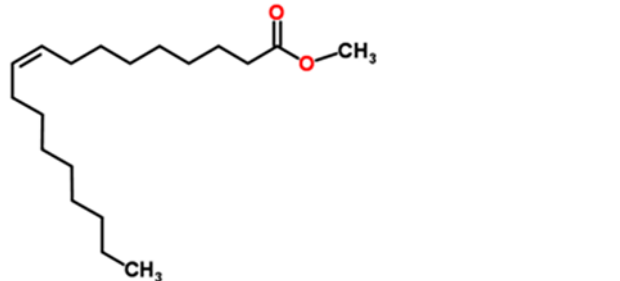
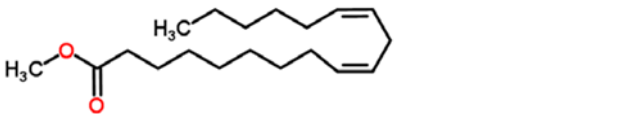
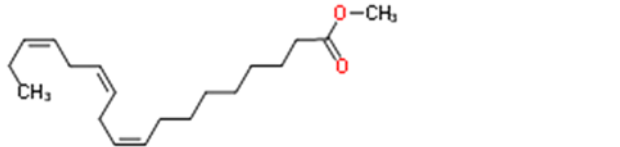
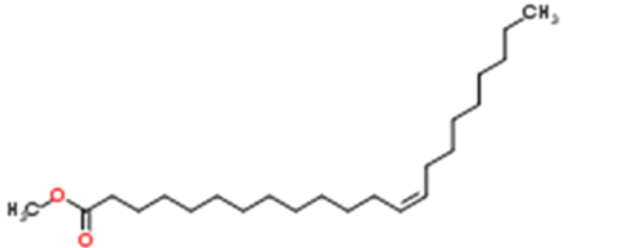
FAME composition in biodiesel

FAME have the general molecular structures $\text{CH}_3(\text{CH}_2)_n\text{COOCH}_3$ (saturated) and $\text{CH}_3(\text{CH}_2)_n(\text{CH})_x\text{COOCH}_3$ (unsaturated) and examples of FAME that are produced from the main vegetable oils used for biodiesel production are summarized in Table 1.

The composition of FAME within a given biodiesel (B100) will depend on a number of factors including the origin of the feedstock used and the manufacturing process (Energy Institute 2008). In addition, the genotype, growing seasons and growing conditions have all been found to affect oil content and fatty acid profiles (Hollebone *et al.* 2008). Figure 1 summarizes the relative proportions of fatty acids present in various common feedstocks.

In the USA, the three primary feedstocks are soybean (accounting for almost 70% of virgin plant-based feedstock production in 2014), canola and yellow grease (US EIA 2015). In Europe, CONCAWE (2009) noted that the FAME products sold for fuel blending are most commonly a mixture of different FAME products manufactured from different feedstocks. In a recent study of diesel composition being sold in 60 filling stations across Germany, the

Table 1. Examples of FAME

Palmitic acid methyl ester (C16:0)	
Stearic acid methyl ester (C18:0)	
Oleic acid methyl ester (C18:1)	
Linoleic acid methyl ester (C18:2)	
Linolenic acid methyl ester (C18:3)	
Erucic acid methyl ester (C22:1)	

Numbers describing each acid indicate the number of carbon atoms in the chain followed by the number of unsaturated carbon-carbon bonds in the chain. The 2D images are sourced from www.Chempider.com

source of the FAME in the biodiesel component included rapeseed (53%), palm (25%), soybean (11%) and coconut (11%) (UFOP 2013).

Other components of biodiesel

Contaminants of B100 can include methanol, water, catalyst, glycerol, free fatty acids, soaps, metals, and mono-, di- and triglycerides (Moser 2009). Additives can include antioxidants, biocides, cold flow enhancers, cetane enhancers, NO_x reducers, water dispersants and anti-foaming agents (Hodam 2008).

Physical properties

FAME physical properties

FAME have the appearance of a colourless to yellow or brown liquid with little odour. The physical properties of FAME relative to their primary use as a fuel have been extensively studied (Knothe 2005) and the composition of FAME within a particular feedstock have been shown to influence strongly the properties of biofuels (Sanford *et al.* 2009; Knothe 2012).

When predicting environmental behaviour it is important to bear in mind that biodiesel is a complex and variable mixture of both

FAME and other compounds, and that test results may be biased by trace components.

Alptekin & Canakci (2008) reported on the measurement of the density and viscosity of six biodiesels (sunflower, canola, soybean, cottonseed, corn oils and waste palm oil). Density measurements varied from 0.87 to 0.88 g cm⁻³, similar to typical diesel densities (0.86–0.89 g cm⁻³), and viscosity varied from 3.97 to 4.34 mm² s⁻¹ (diesel 2.71–3.43 mm² s⁻¹). These properties of FAME are, therefore, in or close to the range reported for those of conventional diesel.

Ginn *et al.* (2009) noted that no measurements of either the *K*_{ow} or vapour pressures for biodiesel fuels at ambient environmental temperatures have been reported, although some measurements for single components are available. A number of these properties are difficult to measure, although some experimental data exist for some compounds such as n-octanol–water partition coefficients, aqueous solubilities and Henry's Law constants (Krop *et al.* 1997) and volatility (Freitas *et al.* 2012).

For the purpose of this review, mirroring the approach undertaken in the review by Energy Institute (2008), a simple assessment of the physico-chemical properties of single FAME compounds has been undertaken using the US EPA Estimation Program Interface (EPI) Suite (US EPA 2014) and the results are presented in Table 2. Where available, the table also includes measured water solubility values reported by Krop *et al.* (1997).

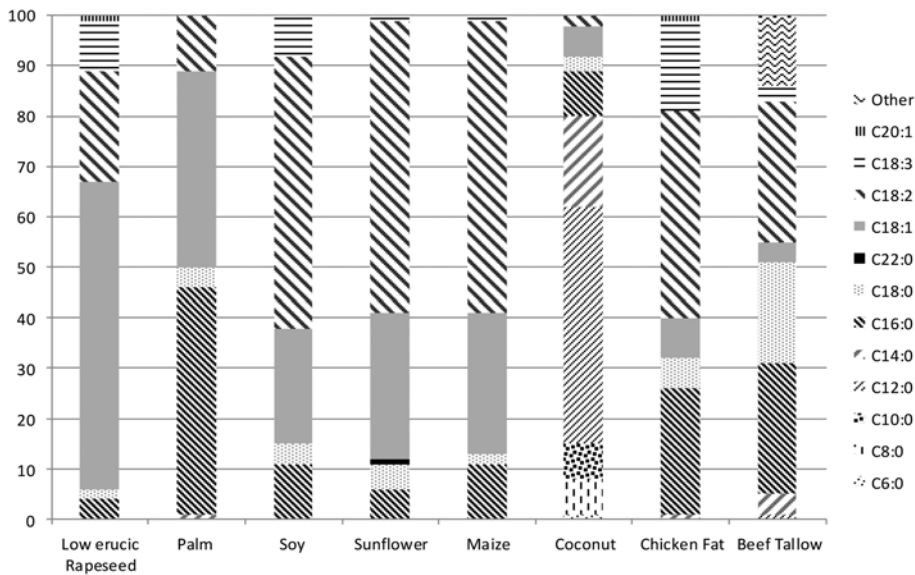


Fig. 1. Typical fatty acid composition of FAME feedstocks (adapted from Moser 2009).

Overall and consistent with an earlier review (Energy Institute 2008), the aqueous solubilities of single FAME compounds are low ($<0.1 \text{ mg l}^{-1}$) and FAME have very low volatility and a high affinity for organic matter with a minimum $\log K_{ow}$ of 6.29. FAME are, therefore, likely to be strongly sorbed to natural organic matter and their transport significantly retarded in soil and aquifer systems.

In its review of biofuels, the ITRC summarized and compared the key properties of biodiesel in relation to conventional diesel, gasoline and ethanol. It concluded that biodiesel was by far the least soluble in water and least mobile either in the aqueous or vapour phases (ITRC 2011).

The values of aqueous solubility reported for single FAME in Table 2 are in contrast to other values reported in literature. For example, Von Wedel (1999) reported that the saturation concentration of biodiesel in fresh water was 14 mg l^{-1} at a temperature of 17°C , though the biodiesel in question and analytical method used were not reported. Ginn *et al.* (2009) noted that biodiesel is on average 15–25 times more soluble than diesel. Using data derived from an earlier study, Chiaranda (2011) reported solubilities of single FAME in the range $2.9 - 7.2 \text{ mg l}^{-1}$ and effective solubilities (calculated using Raoult's Law) in a soy-based B100 biodiesel of between 0.036 and 0.5 mg l^{-1} . Toso (2010) reported the solubility of FAME in the range $10 - 20 \text{ mg l}^{-1}$. However, comparison of the reported solubilities with the derived values is difficult without understanding the biodiesel in question and the analytical methods

used. As previously above, biodiesel is a complex and variable mixture of organic compounds and may contain traces of compounds that have a higher solubility in water. During solubility testing these trace components may partition from the biodiesel into the aqueous phase, resulting in a test result that over-predicts the solubility of the major FAME components.

Ultimately the solubility of biodiesel or biodiesel-amended conventional diesel in water will be specific and will involve partitioning between multiple phases (Ginn *et al.* 2009). Given that some studies estimate FAME solubility at concentrations that would indicate a potential to cause contamination of water then FAME should be considered in a risk assessment and should be assessed on a site-by-site basis.

Other physical properties of FAME of relevance to natural attenuation

An important physical characteristic of FAME that has relevance to their fate and behaviour in the subsurface is their susceptibility to oxidation when exposed to oxygen in ambient air (Knothe 2007). FAME are more susceptible to autoxidation than conventional diesel. For example, Yassine *et al.* (2012) identified methanol, hexanal, *n*-butyl acetate, diethylene glycol monobutyl ether and diethylene glycol monobutyl ether acetate as autoxidation products of soybean FAME.

Table 2. Estimated and observed physicochemical properties of some single FAME

Name	CAS number	Molecular weight	Estimated water solubility from $\log K_{ow}$ (mg l^{-1}), 25°C	Measured water solubility (mg l^{-1}), 22°C	Henry's Law constant estimate ($\text{atm m}^3 \text{ mol}^{-1}$)	$\log K_{ow}$ estimate	Vapour pressure estimate (mmHg), 25°C
Stearic acid methyl ester	112-61-8	298.5	9.3×10^{-4}	3.0×10^{-4}	1.4×10^{-2}	8.4	3.3×10^{-5}
Erucic acid methyl ester	1120-34-9	352.6	6.6×10^{-5}	–	7.8×10^{-3}	9.3	1.1×10^{-6}
Linoleic acid methyl ester	112-63-0	294.5	1.9×10^{-2}	2.1×10^{-2}	1.9×10^{-3}	6.8	9.9×10^{-5}
Linolenic acid methyl ester	301-00-8	292.5	5.8×10^{-2}	9.2×10^{-2}	2.1×10^{-4}	6.3	3.1×10^{-5}
Oleic acid methyl ester	112-62-9	296.5	5.5×10^{-3}	4.4×10^{-3}	8.0×10^{-3}	7.5	1.6×10^{-4}
Palmitic acid methyl ester	112.39-0	270.5	9.1×10^{-3}	4.0×10^{-3}	1.9×10^{-2}	7.4	4.8×10^{-4}

Factors influencing autoxidation and the nature of reactions have been described in detail by Knothe (2007) & Salam *et al.* (2012) and include elevated temperature and the presence of light or extraneous materials such as metals or initiators. Oxidation, because it specifically influences biodiesel quality, has been the subject of considerable research and European biodiesel standards include an oxidative stability specification. Nevertheless, autoxidation may have an important role in natural attenuation.

Besides oxidation caused by exposure to air (oxygen), biodiesel is also potentially subject to hydrolytic degradation owing to the presence of water (Knothe 2007). In absence of measured hydrolysis rates, DeMello *et al.* (2007) assumed that a base-catalysed pathway would control FAME hydrolysis and predicted base-catalysed hydrolysis half-lives of 7 years at pH 7 and 70 years at pH 6 (25°C) and 3 years and 19 weeks at pH 7.4 and 8.3, respectively. The latter results were considered upper bound estimates (DeMello *et al.* 2007) and may be significant in the context of natural attenuation where groundwater may typically be in the range pH 6–8.

Properties of additives

As indicated above, commercial biodiesel may be treated or supplemented with a variety of additives to improve storage and performance. The physical and chemical properties of these additives do not appear to have been systematically studied or reported in the context of potential influences on natural attenuation of FAME.

The Energy Institute (2008) reported the physical and chemical properties of a number of biodiesel antioxidants including tocopherol, *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and phenylenediamine. Tocopherol (naturally occurring vitamin E) and BHT were considered to be unlikely to have a significant migration potential as a result of low solubility and high affinity for organic matter but TBHQ and phenylenediamine were reported to be moderately soluble in water (748 and 40000 mg l⁻¹ respectively).

The active ingredients in the biocide Kathon FP 1.5 are a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one. Both substances are reportedly highly soluble in water and are considered mobile but have reported aerobic and anaerobic half-lives of less than 1.4 day and less than 0.2 day, respectively (Dow 2011).

Biodegradation of FAME

Because the fatty acid components of FAME are natural products and ubiquitous components of cellular membranes, a wide array of naturally occurring microorganisms in soil and groundwater environments are capable of their uptake and metabolism. Although methyl esters of fatty acids are not commonly found in nature, many fatty acids exist in organisms as glycerol esters (i.e. mono-, di- and triglycerides).

Numerous laboratory studies have been conducted to investigate the biodegradation of FAME from various feedstocks using different electron acceptors. When assessing these studies, it is important to note several aspects of the tests: the source of FAME, the microbial inoculum, whether inorganic nutrients have been added, and the analytical testing used to monitor disappearance or degradation of FAME.

General metabolism of fatty acids and their methyl esters

FAME molecules are similar in structure to their parent fatty acid esters, with the exception of the transesterification of the carboxylic group with methanol replacing glycerol to form a methyl ester. As discussed in a previous section, the ester functional group affects

physical properties and is also expected to have an effect on biodegradation. The biodegradation pathway for FAME molecules is a multistep process that is similar under both aerobic and anaerobic conditions. FAME are first de-esterified to form free fatty acids and methanol (Stolz *et al.* 1995; Sousa *et al.* 2007; Aktas *et al.* 2010). The free fatty acids then undergo sequential removal of two-carbon components through a process known as 'β-oxidation' as shown in Figure 2 (Stolz *et al.* 1995; Aktas *et al.* 2010). The methanol released is readily biodegraded under aerobic and anaerobic conditions.

This pathway has not been well studied for FAME, but generally follows the well-known biodegradation pathway for naturally occurring fatty acids and fatty acid esters (i.e. glycerides). Metabolism of glyceride esters starts with de-esterification to form free fatty acids and glycerol by esterase enzymes known as lipases, which have been found in all studied microorganisms (White *et al.* 1968). Lipases may also be involved in removal of the methyl ester group of FAME as the use of lipases as biocatalysts for the transesterification of oil feedstocks to produce FAME indicates that lipases can participate in esterification or de-esterification reactions involving fatty acid methyl esters (Ghaly *et al.* 2010). Stolz *et al.* (1995) found that bacterial strains that were capable of growth on FAME produced an esterase enzyme to cleave the methyl ester group (although the data were not shown). In addition, the de-esterification step for FAME may also occur through abiotic hydrolysis, as discussed above. More work is needed to identify the full enzymatic pathways for fatty acid degradation by these bacteria.

In a study looking at the degradation of FAME derived from soybean oil by several environmental inocula under anaerobic conditions (sulphate reduction and methanogenesis), Aktas *et al.* (2010) observed the formation of a series of lower carbon-chain length fatty acids as intermediates. The degradation of the soybean FAME, which are composed primarily of FAME molecules of even-number carbon chain lengths (including linoleic, oleic, stearic and palmitic methyl esters), produced a series of even-number carbon chain fatty acids that ranged from C24 (tetracosanoic acid) to C6 (hexanoic acid), as would be expected from the β-oxidation pathway. Lesser amounts of odd-numbered carbon chain fatty acids (C23 to C13) were also detected and were assumed to be due to the β-oxidation of the odd-number carbon chain FAME, although no specific data were presented to support this conclusion. The data from the study also did not provide information on whether unsaturated fatty acids were hydrogenated prior to undergoing β-oxidation or were degraded faster or slower than saturated fatty acids of the same chain length.

Given the diversity of microorganisms present in soil and groundwater, the β-oxidation pathway may not be the only one involved in FAME biodegradation. Alkanes, which have long hydrophobic alkyl chains similar to FAME but are lacking the more polar ester group, also undergo β-oxidation after conversion to fatty acids (Rojo 2009, 2010). Under aerobic conditions, alkanes are generally first oxidized by the addition of a terminal hydroxyl group, which is subsequently oxidized to an aldehyde and then to a carboxylic acid group to form a fatty acid. Hydroxylation can also occur at a subterminal carbon position. Under anaerobic conditions, another pathway involving the addition of fumarate to form an alkyl succinate has been postulated (Rojo 2010). Although not documented in the literature, reactions such as hydroxylation or carbon addition under either anaerobic or aerobic conditions are also possible for FAME given the structural similarities of the alkyl chain to alkanes, and may produce intermediates other than simple fatty acids. The EAWAG-BBD Pathway Prediction System (<http://eawag-bbd.ethz.ch/predict/>) provides a summary of available knowledge on these pathways.

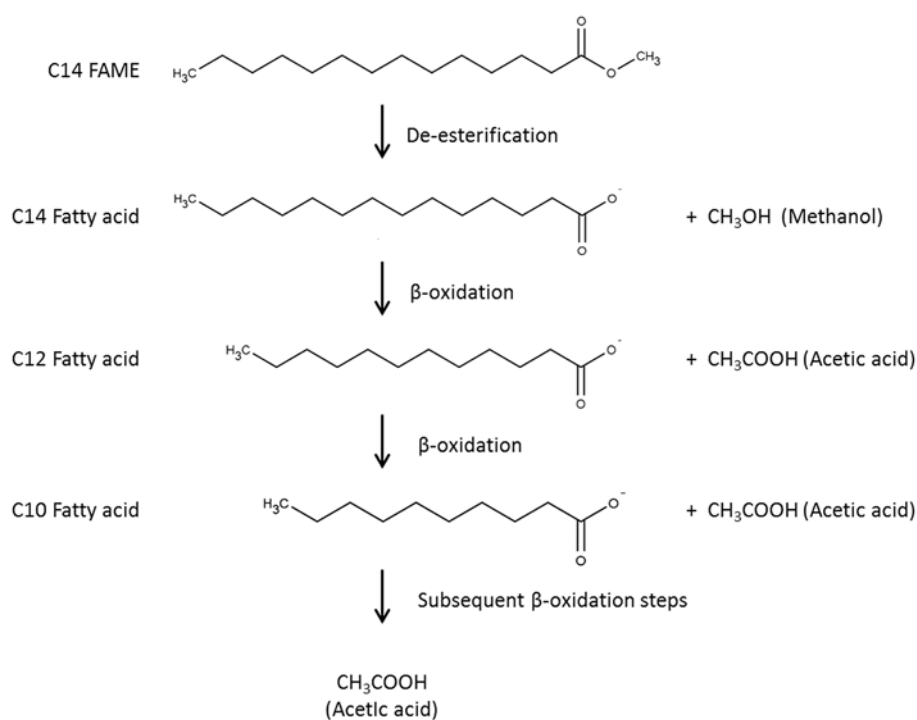


Fig. 2. General pathway for metabolism of FAME.

Laboratory studies of FAME degradation

The biodegradation of FAME has been investigated through laboratory studies under both aerobic and anaerobic conditions. As discussed above, these studies have been conducted using various FAME, inocula, test conditions and analytical methods.

Degradation under aerobic conditions

Laboratory testing has provided strong evidence for the biodegradation of FAME under aerobic conditions with mineralization to carbon dioxide. Over 20 studies were identified that evaluated the aerobic biodegradation of FAME, as summarized in Table 3.

The majority of the studies were conducted on either commercial or laboratory-produced FAME from rapeseed and/or soybean oils, with a few studies that used rapeseed or soybean ethyl esters. Isolated studies were also conducted on biodiesel produced from vegetable oil, animal fat, used cooking oil, castor oil and fish oil. Some researchers did not specify the feedstock or the alkyl ester of the biodiesel. Only one study (Ginn *et al.* 2012) considered the impact of additives on aerobic biodegradation. Studies were conducted on either pure fatty acid esters (B100) or blends with petroleum diesel (B2–B80).

Several of the aerobic studies were conducted using biological oxygen demand (BOD) procedures or modified procedures and used sewage sludge as the inoculum. These tests were monitored using oxygen uptake and/or carbon dioxide production. Because sewage sludge contains very high biomass concentrations, which have generally been acclimated to fats and other lipids, these tests do not simulate conditions of natural attenuation in the environment, but do provide evidence of aerobic biodegradability and mineralization by microorganisms. In most of these studies, 60–100% of soybean and rapeseed methyl or ethyl esters were reported to be mineralized to carbon dioxide within 21–28 days. One study that followed a standard BOD₅ protocol (USEPA Method 405.1) reported mean BOD₅ values ($n=6$) for soybean and rapeseed methyl and ethyl esters and neat oils ranging from 1.53 to 1.74 mg l⁻¹, as compared with 0.40 mg l⁻¹ for petroleum diesel as presented in Table 4 (Peterson & Moller 2005). The theoretical

BOD cannot be calculated for comparison because the concentrations of the esters and neat oils in the tests were not given; however, chemical oxygen demand (COD), which can be used as a surrogate, was tested in parallel. As shown in Table 4, the ratios of BOD/COD values indicate that under the short-term BOD₅ test conditions, the fatty esters were biodegraded to a larger extent than were the neat oils and petroleum diesel.

Other studies that were more representative of soil and aquifer conditions used either environmental media (soil, groundwater or surface water) or bacterial cultures isolated from environmental media as sources of the inocula. The rates and extent of degradation observed in these studies were generally lower than in studies using sewage sludges. For example, Vauhkonen *et al.* (2011) compared biodegradation of commercial rapeseed methyl esters and neat oil with either activated sludge or groundwater as the inoculum, as measured by oxygen uptake. Within 28 days, 60.8% RME and 65.8% neat rapeseed oil were biodegraded with the sludge inoculum, whereas only 19% rapeseed FAME and 9.9% neat rapeseed oil were biodegraded with groundwater as the inoculum. No further information was provided on the microorganisms present in the inocula.

To follow biodegradation of single biodiesel components, some studies monitored degradation using gas chromatography (GC) to follow loss of the fatty acid esters. However, in some studies, loss of the fatty acid esters was noted to occur rapidly in both the biotic and abiotic control microcosms (e.g. Yassine *et al.* 2013a,b), indicating that hydrolysis of the esters was possibly a non-biological process or that the abiotic microcosms had a biotic component. Therefore, data from studies that utilize only GC to follow fatty acid esters are not sufficient to confirm that biodegradation has occurred unless the studies can demonstrate a difference between the biotic microcosms and abiotic controls, or provide a supportive measurement such as oxygen uptake or carbon dioxide production. For example, although Yassine *et al.* (2013a,b) saw initial rapid loss in both the biotic microcosms and abiotic controls, the losses from the biotic microcosms were greater than those of the controls at later time points.

FAME from different feedstocks appear to be equally amenable to aerobic biodegradation based on the few studies that compared different FAME.

Table 3. Summary of aerobic laboratory studies of biodegradation of FAME and FAME–diesel blends

Reference	Source of inoculum	Media	Measurement	FAME and other substrates	Reported results
Peterson & Reece (1994)	Not specified; however, BOD tests generally use sewage sludge and shake flask test generally uses a weak bacterial inoculum	Aqueous solution (not further described); no abiotic control reported	BOD; measurement for shake flask test not described, but is generally DOC	Rapeseed methyl esters (RME) and rapeseed ethyl esters (REE) (probably synthesized at the university); diesel; dextrose	High BOD for RME and REE; in the shake flask test, 95% biodegradation of RME and REE within 28 days, compared with 40% of diesel; rate of degradation of RME similar to that of REE, but faster than that of dextrose and diesel
Stolz <i>et al.</i> (1995)	Pond or lake water; topsoil; culture grown on soybean FAME	Mineral salt medium	GC; methanol (by Draeger tubes)	Soybean FAME	100% FAME disappeared within 7 days with biomass and methanol production; nine aerobic FAME-degrading bacteria were isolated; detected esterase activity
Zhang <i>et al.</i> (1998)	Organic-rich soil and aerated activated sewage sludge (unacclimated to FAME)	Yeast extract, amino acids, minerals	CO ₂ production; GC	Soybean and rapeseed FAME; soybean and rapeseed ethyl esters (SEE and REE) synthesized at the university using transesterification; neat rapeseed oil, neat soybean oil; Phillips 2-D reference diesel; blends of REE and diesel	In 28 days, 85.5% soybean FAME and 88.5% rapeseed FAME were mineralized to CO ₂ compared with 87.8% for dextrose, 76.0–78.5% for the neat oils and 26.2% for diesel; as measured by GC, 100% of rapeseed ethyl ester disappeared within 3 days
Pasqualino <i>et al.</i> (2006)	Two different activated sludge samples	Water with mineral media	CO ₂ production	FAME from waste cooking oil (from BIONET Europa); diesel and gasoline from fuel station; blends of waste cooking oil FAME with diesel and gasoline	In 28 days, almost 100% of the FAME was mineralized to CO ₂ , whereas diesel and gasoline were mineralized at 50 and 56%
Makareviciene & Janulis (2003)	Unspecified bacterial culture	Natural water	Infrared absorbance	Rapeseed methyl ester; rapeseed ethyl ester; diesel	In 21 days, about 98% of RME and REE had degraded, compared with 61% of diesel
Schleicher <i>et al.</i> (2009)	Culture isolated from uncontaminated soil	Water with nutrients	Acid number by titration; microbial enumeration	Rapeseed methyl ester (commercial); diesel; B5, B10, B20 blends of the above	Increase in free fatty acid content when rapeseed methyl ester was incubated with a microbial culture; higher microbial numbers in B100 incubated under anaerobic than aerobic conditions
Russell <i>et al.</i> (2005)	Potting soil	Potting soil	GC	Soybean alkyl esters (ester not specified)	In 28 days, 80–90% of the soybean alkyl ester had disappeared
Peterson & Moller (2005)	Sewage sludge	Aqueous	BOD by EPA Method 405.1	Soybean and rapeseed methyl esters; rapeseed ethyl esters; neat soybean oil and rapeseed oil; Phillips 2-D reference diesel	BOD for methyl and ethyl esters and neat oils range from 1.5 to 1.7 × 10 ⁶ mg l ⁻¹ , compared with 0.4 × 10 ⁶ mg l ⁻¹ for petroleum diesel
DeMello <i>et al.</i> (2007)	Seawater (unacclimated to FAME)	Seawater	GC	FAME (parent oil not specified, but containing C13 to C18); commercial diesel; B20 blend of the above	90% degradation of FAME within 21 days, compared with no loss in abiotic control
Mariano <i>et al.</i> (2008)	River water, or soil from a petrol station with low fuel concentration	River water or soil from petrol station	Carbon dioxide (CO ₂) production	Laboratory-prepared castor oil alkyl ester blended with diesel to B5 and B20; commercial diesel and B2 (biodiesel component not specified)	CO ₂ production from castor oil alkyl ester was 1.6-fold higher than for diesel
Owsianiak <i>et al.</i> (2009)	Microbial consortium isolated from a crude oil site; identified by 16SrRNA to contain <i>Pseudomonas alcaligenes</i> , <i>Ochrobactrum intermedium</i> , <i>Sphingobacterium</i> sp., <i>Pseudomonas putida</i> , <i>Klebsiella oxytoca</i> , <i>Chryseobacterium</i> sp. and <i>Stenotrophomonas maltophilia</i> ; maintained in laboratory	Mineral medium at pH 7	GC	Commercial rapeseed methyl esters; diesel (EN 590:2004); B5, B10, B20, etc. blends of the above prepared in the laboratory	Within 7 days, 76% disappearance of RME, compared with 42% of diesel
Cyplik <i>et al.</i> (2011)	Microbial consortium isolated from crude oil site under aerobic conditions containing <i>Achromobacter</i> sp., <i>Alcaligenes</i> sp., <i>Citrobacter</i> sp., Comamonadaceae, <i>Sphingobacterium</i> sp., <i>Pseudomonas</i> sp. and <i>Variovorax</i> sp.	Mineral medium at pH 7	GC	Commercial rapeseed methyl esters; diesel (EN 590:2004); B20 blends of the above prepared in the laboratory	Within 5 days, complete disappearance of RME, compared with 10% disappearance of RME in the abiotic control and 20% diesel

Prince <i>et al.</i> (2008)	Rainwater from detention pond (unacclimated to FAME)	Pond water plus mineral salt medium	GC	Commercial soybean methyl esters; B20 (soybean FAME)	Half-lives for single FAME (palmitate, stearate, oleate and linoleate methyl esters) ranged between 2.1 and 2.6 days
Ginn <i>et al.</i> (2012)	Silty-loam soil	Soil slurry with mineral salts medium	GC with mass spectrometry detection; CO ₂ production	Soybean methyl esters; animal fat methyl esters; ultralow-sulphur diesel, B20 blends, with and without antioxidant (BioExtend containing TBHQ) and biocide additive	CO ₂ production was observed for a duration of 28–30 days, with no CO ₂ production in the absence of the inoculum (soil); no significant effect of additives on aerobic biodegradation
Vauhkonen <i>et al.</i> (2011)	Activated sludge, or groundwater	Mineral media, or groundwater	Oxygen uptake (OECD 301F test)	Rapeseed methyl esters (commercial); rapeseed oil	In 28 days, 60.8% RME and 65.8% rapeseed oil were biodegraded with a sewage sludge inoculum; only 19% RME and 9.9% rapeseed oil biodegraded with groundwater as the inoculum
Elazhari-Ali <i>et al.</i> (2012)	Sandy soil	Wet sandy soil, with and without inorganic nutrients (N, P); oxygen	GC of hydrocarbons	B20 made from commercial rapeseed FAME and 12 hydrocarbons blended in the laboratory to resemble gasoline or kerosene	Toluene was removed at essentially the same rate from the hydrocarbon-only mixture and B20 in the presence of added nutrients, but more rapidly from the hydrocarbon-only mixture (v. B20) in the absence of added inorganic nutrients
Meneghetti <i>et al.</i> (2012)	Two soils: clayey soil and sandy soil	Soil with and without fertilizer	CO ₂ production; GC	Vegetable oil methyl esters (source not specified)	Reductions of 16.8% in clayey soil and 58.5% in sandy soil were observed by GC in the absence of fertilizer after 110 days; higher percentage reductions were observed in the presence of fertilizer (59.8% in clayey soil and 90.4% in sandy soil; production of CO ₂ was reported, but appeared to be only a small proportion of the FAME
Yassine <i>et al.</i> (2013a)	Culture isolated from aeration tank of wastewater treatment plant and gasoline and triglyceride degrading cultures; acclimated to diesel and FAME	Aqueous minimal mineral medium containing vitamin	GC/MS	Commercial soybean methyl esters; low-sulphur diesel; blends of the above (B20, B40, B60 and B8)	Rapid loss initially of FAME from both biotic and abiotic microcosms; biological utilization rates for FAME increased with increasing carbon chain length and decreasing number of double bonds
Yassine <i>et al.</i> (2013b)	Culture derived from wastewater activated sludge and gasoline- and triglyceride degrading cultures (acclimated to FAME and FAME–diesel blends)	Aqueous minimal mineral medium containing vitamin	GC/MS; CO ₂ evolution; biomass production	Commercial soybean methyl esters; low-sulphur diesel; blends of the above (B20, B40, B60 and B80)	Rapid loss initially of FAME from both biotic and abiotic microcosm; 64% FAME mineralized within 7 days and 75% within 42 days; C10–C12 alkanes metabolized faster in the presence of FAME
Fuller <i>et al.</i> (2013)	Creek water	Creek water; oxygen	GC of polyaromatic hydrocarbons (PAH)	Tallow and canola oil FAME (10:90 w/w); commercial diesel; B20 blend of the above	After 28 days, a higher proportion of the mass of PAH pyrene, fluoranthene, methylpyrenes, and C2-alkyl fluoranthenes and pyrenes were removed in the B20 blend than in diesel alone
Horel & Schiewer (2014)	Cultures isolated from soil and adapted to (1) fish oil alkyl ester (2) Syntroleum, or (3) diesel	Unsaturated sandy soil with NPK fertilizer added	CO ₂ production; microbial enumeration; GC/MS	Fish oil alkyl ester (ester not specified); diesel; Syntroleum (synthetic oil)	Production of CO ₂ from fish oil alkyl ester lagged behind that from diesel and the synthetic oil even using the inoculum adapted to fish oil alkyl ester; however, maximum CO ₂ production in 28 days was similar for both diesel and fish oil alkyl ester (<20%)

(continued)

Table 3. (Continued)

Reference	Source of inoculum	Media	Measurement	FAME and other substrates	Reported results
Lisiecki <i>et al.</i> (2014)	Microbial consortium isolated from a crude oil site and enriched on diesel as soil source of carbon; contained <i>Achromobacter</i> , <i>Alcaligenes</i> , <i>Citrobacter</i> , Comamonadaceae, <i>Sphingobacterium</i> , <i>Pseudomonas</i> and <i>Variovorax</i>	Mineral salts medium; oxygen, but microcosms were saturated, undisturbed and sealed	CO ₂ production; GC of hydrocarbon fractions	Commercial rapeseed FAME; diesel (EN 590:2004); B10, B20, B30, B40, B50, B60, B70, B80, B90 blends of the above prepared in the laboratory	In the FAME and diesel microcosms, c. 58 and 30% of FAME and diesel were mineralized to CO ₂ within 578 days, respectively; the presence of FAME did not appear to enhance the rate or extent of diesel degradation; rates of CO ₂ production were as predicted by the ratio of FAME to diesel; residual fractions of aromatic and aliphatic hydrocarbons were generally the same in the B10 to B80 microcosms
Thomé <i>et al.</i> (2014)	Clayey soil	Unsaturated soil with intermittent air flow; no amendments	Weight per cent of residual hexane-extractable material	B20 blend of commercial soybean alkyl ester (ester not specified) with petroleum diesel	After 60 days, B20 reductions of up to 85% were observed for the aerated soils and 64% for the unaerated (possibly anoxic) control

Degradation under anaerobic conditions

In the absence of molecular oxygen, other terminal electron acceptors can support anaerobic biodegradation processes. In soil and groundwater environments, these processes are typically reduction of nitrate, sulphate and/or metals (ferric iron [Fe⁺³] and manganese [Mn⁺⁴]), as well as methanogenesis, which depends on biogenic carbon dioxide, acetic acid and certain other low molecular weight carbon compounds, as terminal electron acceptors for respiration. As summarized in Table 5, a number of studies in the literature provide evidence that biodegradation of FAME can be supported by nitrate reduction, sulphate reduction and methanogenesis; however, no laboratory studies on metal reduction were identified.

Adair & Wilson (2009, 2010a, b) have reported rates of methane production of 3 mg l⁻¹ methane per day from the biodegradation of biodiesel (source oil not specified) at 1000 mg l⁻¹, in microcosms constructed with aquifer sediments from a jet fuel release site and a commercial soybean FAME release site (which is discussed in more detail below). Although the longevity of methane production in biodiesel degradation is not known, methane generation may, like ethanol-release sites, be a significant risk driver at large-volume biodiesel release sites where the available electron acceptor capacity of the ground is greatly exceeded (Adair & Wilson 2010a).

No laboratory studies investigating FAME degradation through metal reduction (iron or manganese) have been identified; however, as discussed below, field evidence for iron reduction linked to the biodegradation of FAME has been observed (Toso 2010, 2012).

Although fewer laboratory studies have been conducted under anaerobic conditions than aerobic conditions, the studies all confirm that FAME biodegradation can occur through nitrate reduction, sulphate reduction and methanogenesis.

Effect of additives on biodegradation

Very few studies have reported on the presence of additives in the biodiesel used in biodegradation testing and even fewer have reported on whether the presence of an additive had an effect on biodegradation. As discussed in more detail below, Toso (2010, 2012) reported on a large-scale release from an above-ground tank in Minnesota, USA, of FAME containing the antioxidant *tert*-butylhydroquinone (TBHQ) at a concentration of 250 mg l⁻¹ in the source biodiesel. Evidence of both aerobic and anaerobic biodegradation of the released FAME was observed, indicating that the presence of TBHQ did not prevent biodegradation. However, whether TBHQ enhanced, inhibited or had no effect on biodegradation could not be determined from available information.

Butylated hydroxytoluene (BHT) is another antioxidant that is sometimes added to biodiesel. Salam *et al.* (2012) investigated the effect of BHT on the aerobic biodegradation of linoleate triglyceride using respirometry testing (carbon dioxide production and oxygen depletion). The tests used a canola oil-degrading microorganism inoculum that had been isolated from activated sludge. After 19 weeks, 61% of the linoleate triglyceride was mineralized to carbon dioxide in microcosms containing BHT, as compared with only 41% in microcosms without BHT. Significant polymerization was observed in the microcosms without BHT and the rigid polymers appeared to resist biodegradation. However, the biodegradation rate constants were similar in microcosms with and without BHT and indicated that BHT did not directly affect biodegradation but rather inhibited polymer formation and allowed the triglyceride to remain bioavailable.

Ginn *et al.* (2012) investigated the effect of an antioxidant additive (BioExtend) and an unspecified biocide on the aerobic degradation of FAME from soybean oil and animal fat. The composition of BioExtend was not reported in the paper; however,

Table 4. Experimental BOD and COD values

Test material	Mean BOD (mg l ⁻¹)	Mean COD (mg l ⁻¹)	BOD/COD ratio
Rapeseed ethyl ester	1.67	2.64	0.63
Rapeseed methyl ester	1.53	2.43	0.63
Neat rapeseed oil	1.74	3.31	0.53
Soybean methyl ester	1.68	2.63	0.64
Neat soybean oil	1.57	2.70	0.58
Petroleum diesel	0.397	2.35	0.17

Data taken from Peterson & Moller (2005). BOD, mean biological oxygen demand ($n = 6$) for each test material; COD, mean chemical oxygen demand ($n = 3$) for each test material.

BioExtend is an antioxidant product from Eastman that contains TBHQ and a metal chelating agent. The microcosms were constructed with a soil inoculum and either without additives, with the antioxidant additive (BioExtend), or with the antioxidant and an unspecified biocide. No significant impacts on carbon dioxide production over 28–30 days from FAME (or parallel microcosms constructed with diesel or a B20 blend of either soybean or animal fat) were observed in the presence of either additive.

Partitioning behaviour of FAME in the subsurface

The Energy Institute (2008) anticipated that the likely fate and behaviour of FAME-containing biodiesel would be comparable with that of conventional diesel. With similar viscosity, density and hydrophobicity (see Table 2), B100 would form a light non-aqueous phase liquid (LNAPL) that would behave in a similar manner to diesel LNAPL (Ginn *et al.* 2012), and, given the solubility and partition coefficients, FAME would be strongly sorbed to soil organic matter and relatively immobile in groundwater (Energy Institute 2008). Ramos *et al.* (2013) summarized that biodiesel blend releases are not readily miscible in groundwater and behave as a fixed, decaying yet long-lived source with a relatively small region of influence compared with soluble biofuels such as ethanol.

The fate and behaviour of petroleum hydrocarbons in the subsurface is well understood. Detailed guidance on the processes of concern and the development of LNAPL conceptual site models is available from a number of sources (ITRC 2009; CL:AIRE 2014).

Elements of an LNAPL conceptual site model (Fig. 3) include an understanding of contaminant fate and transport in the unsaturated zone, the interaction of LNAPL with groundwater at the saturated zone interface and the subsequent migration and fate of either LNAPL or the dissolved phase in groundwater according to the prevailing hydrogeological regime.

FAME behaviour in the unsaturated zone

As part of the 2015 California EPA Multimedia Evaluation of Biodiesel, Ginn *et al.* (2012) conducted a series of small-scale experiments in 2D sandboxes to visualize the rates of biodiesel infiltration, redistribution and lens formation on the water table relative to that of ultralow-sulphur diesel (ULSD). The experiments found that Soy B100, Soy B20 and animal fat B20 did not exhibit any significant differences relative to ULSD. Animal fat B100 showed noticeable differences in the amount of residual material that occurred in the unsaturated zone and in the lens geometry. This was attributed to the physical properties including higher viscosity and interfacial tension of animal fat biodiesel relative to ULSD. The report concluded that these differences may become more pronounced at temperatures below 20°C.

Field studies that validate the above have not been published. Toso (2010) reported on the investigation of the release of $c. 110 \text{ m}^3$ of soy-based B100 (with TBHQ antioxidant (250 mg l⁻¹)) through corrosion holes in the base of an above-ground storage tank. The

release was adjacent to a river in an area of generally sandy soils and fill. Depth to groundwater was $c. 2\text{--}3 \text{ m}$ below ground surface. LNAPL was recovered from trenches around the release and 0.57 m of LNAPL was observed in a monitoring well near the tank, although this LNAPL was transient and did not reappear following initial removal via bailing. It was noted by Toso that the behaviour of B100 as an LNAPL seemed to match that reported in the California Multimedia Evaluation of Biodiesel sandbox study.

Behaviour of FAME in LNAPL

Fuller *et al.* (2013) designed a series of experiments in which a stable oil layer was maintained over an aqueous phase in microcosms and trends in compositional changes in FAME, sterols and hydrocarbon profiles were observed as a means of investigation alternatives for forensic assessment of biodiesel spills. The biodiesel used was produced from a commercial mixture of tallow and canola oil (10:90 w/w) and B20, B100 and conventional diesel only (B0) mixtures (the presence or absence of antioxidants or other additives in the biodiesel was not reported). The experiment was designed to mimic a surface water body. Analysis focused only on the LNAPL phase and not on water composition. In terms of overall mass changes, a gradual reduction in the B20 and B0 mass was observed so that only between 33 and 38% of the mass was remaining after 84 days, whereas 90% of the mass of B100 remained. For single FAME compounds, the results indicated that for both the B100 and B20 microcosms, major changes were evident after 7 days, and that the unsaturated FAME were the first to decrease in concentration and the polyunsaturated FAME decreased before monounsaturated FAME (Fig. 4). The concentration of saturated FAME did not change appreciably during the experiment. The removal of single FAME compounds generally occurred faster for B20 than for B100. A number of oxidation products were observed including 9-oxononanoic acid methyl ester and *cis*-3-octyl-oxiraneoctanoic acid methyl ester. Both are associated with oxidation of unsaturated C18 FAME. Photo-oxidation and biodegradation were considered the main weathering mechanisms for biodiesel and biodiesel blends.

Yang *et al.* (2013) studied the changes in mass and chemical composition in biodiesel blends stored in the dark at ambient temperatures ($c. 22^\circ\text{C}$) with air exposure for 190 days. Two commercial biodiesel samples manufactured from a soybean and canola oil (with added antioxidants) were tested, and also diesel and blended biodiesels (B5 soy, B20 soy and B20 canola). The mass loss curves indicated losses for pure diesel (B0) and the B5 and B20 blends but no losses in the B100. The loss was attributed to evaporation of the petroleum diesel components. Yang *et al.* (2013) noted that the mass loss by evaporation is significantly lower for biodiesel than for conventional petroleum diesel because of the higher boiling point of the biodiesel components. Losses owing to microbial activity and chemical degradation were negligible and Yang *et al.* implied that this was possibly due to unidentified antioxidants in the commercial biodiesel used.

Table 5. Summary of anaerobic laboratory studies of biodegradation of FAME and FAME–diesel blends

Reference	Source of inoculum	Media	Measurement	FAME and other substrates	Reported results
Stolz <i>et al.</i> (1995)	Pond or lake water; topsoil; culture grown on soybean FAME	Mineral salt medium; nitrate as electron acceptor	GC; methanol (by Draeger tubes); nitrite	Soybean FAME	100% removal of FAME (as measured by GC) was observed in 14 days in anaerobic microcosms compared with about 50% in the uninoculated controls; 100% removal was observed in 7 days in parallel aerobic microcosms; the denitrifying bacterium H1 (<i>B. solanocearum</i>) was isolated using soybean FAME as the soil source of carbon
Cyplik <i>et al.</i> (2010)	Microbial consortium isolated from crude oil site under aerobic conditions containing <i>Achromobacter</i> , <i>Alcaligenes</i> , <i>Citrobacter</i> , Comamonadaceae, <i>Sphingobacterium</i> , <i>Pseudomonas</i> and <i>Variovorax</i>	Mineral salt medium, pH 7; nitrate as electron acceptor	Nitrate and nitrite; GC	Commercial rapeseed FAME; commercial diesel	20% removal of FAME v. 5% removal of diesel within 5 days, with 100% removal of FAME over the same period in parallel aerobic microcosms; nitrate depletion and nitrite production was observed; the microbial community structure was similar when grown on FAME or diesel
Corseuil <i>et al.</i> (2011)	Groundwater and sediments (unacclimated to FAME)	Groundwater and sediments; nitrate and sulphate as electron acceptors	GC; nitrate and sulphate	FAME from soybean oil and castor oil (from Paraná Institute of Technology)	Rate and extent of soybean oil FAME removal was greater than for castor oil FAME; stearate (C18:0) disappeared more slowly from soybean FAME than the other C16 and C16 FAME; sequential depletion of nitrate followed by sulphate was observed with soybean oil FAME; however, only nitrate was depleted with castor oil FAME
Adair & Wilson (2009, 2010a,b)	Sediments from jet fuel release site; sediments from a biodiesel release site	Unspecified; with or without sulphate as electron acceptor	Methane	Biodiesel (unspecified)	Conversion of 3 mg biodiesel l ⁻¹ to methane was observed per day; the rate was similar in the presence and absence of sulphate
Aktas <i>et al.</i> (2010)	Anaerobic cultures: (1) hydrocarbon degradation under sulphate and methanogenic conditions from contaminated aquifer sediments at natural gas field; (2) alkane-degrading methanogenic culture from contaminated aquifer sediments at natural gas field; (3) marine oil degrading sulphate-reducing culture from sunken ship; (4) culture from seawater ballast tank; none, except possibly (4), were acclimated to FAME	Seawater or freshwater with CO ₂ /N ₂ headspace; sulphate as electron acceptor followed by methanogenesis after sulphate depletion	Sulphate reduction; methane production; GC	Soybean FAME	In the presence of FAME, sulphate was consumed by three of the inocula within 1 month with methane produced by two of the inocula; FAME was converted to free fatty acids ranging in chain length from C6 to 24 in the inoculated microcosms, whereas in the sterile controls only C16–C24 FAME were observed; the detection of the shorter chain length fatty acids was consistent with β -oxidation; sulphate reduction was observed
Borges <i>et al.</i> (2014)	Lagoon sediments; (unacclimated to FAME)	Mineral salt medium; sulphate as electron acceptor	Methane production; benzene by GC	Laboratory-produced FAME from soybean oil, castor oil and pork lard	Methane production was observed within about 40 days in all live microcosms and reached maximum methane production within 150–200 days; rates of methane production were similar among all three FAME (soybean, pork and castor); short-chain acids (acetate, propionate, formate, pyruvate and malonate) were detected with malonate the predominant acid in all three FAME microcosms
Sørensen <i>et al.</i> (2011)	Water obtained in summer or winter from a light diesel storage tank diluted to <i>c.</i> 10 ⁶ cells ml ⁻¹	Water from diesel tank; limited nitrate and sulphate	Methane production; biomass production	Commercial animal fat (pork lard) FAME; commercial fossil diesel	When water obtained during the summer was used as inocula, methane production was observed to occur for 13 days in the presence of FAME and blends of FAME with diesel (B5, B10, B20, B50), but not with diesel; a five-fold increase in biomass was also measured; no methane production was observed when water obtained during the winter was used as inocula

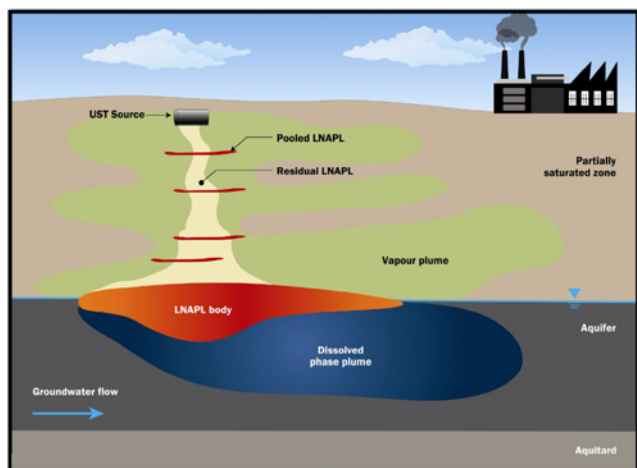


Fig. 3. Schematic diagram of LNAPL conceptual model (UST - Underground Storage Tank) (after CL:AIRE 2014).

Of the above, the latter study by Yang *et al.* (2013) and the experiment carried out in the dark by Khoury *et al.* (2011) may most closely represent conditions that would be expected in the subsurface, although these studies involved only one- or two-phase systems and interactions with soils were been considered.

Dissolution of FAME in groundwater

Detailed studies relating to the dissolution of FAME in groundwater were not identified during this review. However, a number of studies did examine the partitioning behaviour in aqueous solutions and these are summarized below.

The aqueous solubility of biodiesel, ultralow-sulphur diesel and biodiesel-ULSD mixtures was examined by Hollebhone *et al.* (2008) as part of a wider study into aquatic toxicity. The equilibrium concentrations of a range of biodiesels from different feedstocks were measured in freshwater for B100, B20 and B5 blends with petroleum diesel. The equilibrium water-accommodated fraction (WAF) concentration was considered to represent the aqueous solubility of the biodiesels and the WAF was drawn from the aqueous phase of a mixture in which a 1 l to 10 g ratio of water to test oil had been stirred at a constant rate for 16 h and rested for 30 min. The results indicated that for the ULSD the WAF analysed as total extractable materials via gas chromatography with flame ionization detection (GC-FID) varied from between 23 and 45 mg l⁻¹ with the soluble fraction consisting of BTEX and alkylated benzenes. For the biodiesels, WAFs were variable and ranged from 13 mg l⁻¹ for a

B100 soy to 101 mg l⁻¹ and 105 mg l⁻¹ for a fish and soy B100, respectively. Hollebhone *et al.* concluded that the solubility of biodiesels is highly variable even for the same feedstock type. The WAF of B5 and B20 mixtures of various biodiesels varied from 21 to 29 mg l⁻¹ and Hollebhone *et al.* noted that no measurable co-solvent effects were observed between biodiesel and diesel mixtures. The components in the WAF were not quantified but were considered heavier than those seen in the diesel and appeared to be ‘oxygenated hydrocarbons’.

Yassine *et al.* (2012) studied the partitioning behaviour of diesel (B0), soybean biodiesel (B100) and diesel-soybean biodiesel blends (B20, B40, B60 and B80) with water at a variety of oil loads. In the B20 blend, it was notable that the predicted water solubilities for each of the FAME varied in the range 10⁻² – 10⁻⁵ mg l⁻¹ and the measured WAF were in the range 10⁻⁰ – 10⁻⁴ mg l⁻¹. It was concluded that solubility of FAME was neither enhanced nor suppressed in the presence of diesel.

Analysis of the WAFs by gas chromatography with detection by mass spectrometry (GC-MS) indicated the presence of free-phase methanol, *n*-hexanal, *n*-butyl acetate, diethylene glycol monobutyl ether and diethylene glycol monobutyl ether acetate and other peaks that were reportedly not present in the parent oils. These products were attributed to the hydrolysis of FAME into free fatty acids and methanol. Further analysis indicated the loss of 5–10% of the FAME profile after the 24 h equilibration period, and in addition insoluble fine deposits assumed to be biodiesel oxidative polymerization products were also noted within the vessel. The findings suggested rapid autoxidation of biodiesel under the conditions studied.

No field-based measurements of FAME solubility in groundwater were identified for this review.

Field studies of biodegradation of FAME in the subsurface

The laboratory studies discussed in the previous sections provide evidence that biodegradation can occur under a range of redox conditions. However, field data are required to understand the significance of these processes for FAME biodegradation in soil and groundwater. A controlled release of FAME (B100) and biodiesel (B20) was implemented in Brazil in June 2008 and continues to be monitored. A large-scale accidental release of soybean FAME (B100) from an AST occurred in 2007 in the USA in Minnesota. The findings from these field studies are discussed below.

Controlled release of FAME (B100) and biodiesel (B20)

Chiaranda (2011) and Ramos *et al.* (2013) have reported on the results of field releases of B100 and B20 soybean oil FAME at a controlled

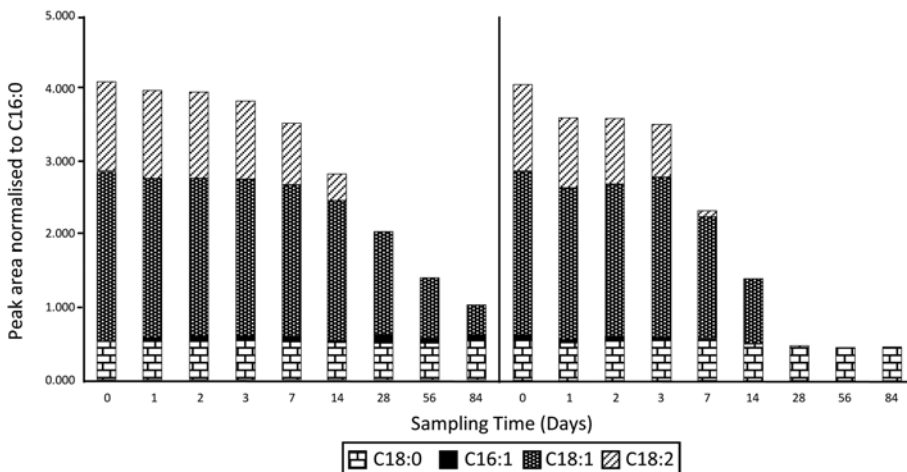


Fig. 4. Fatty acid methyl esters (FAME) profile changes of B100 and B20 over the course of the weathering experiment. The peak areas of the FAME are normalized to C16:0 (C14:0, C16:0 and C17:0 not shown) (adapted from Fuller *et al.* 2013).

field site at the Experimental Farm of Ressacada in Florianópolis, Brazil. The experiments commenced in June 2008 initially as natural attenuation studies (Chiaranda 2011) but some of the plots were subsequently amended with ammonium acetate to stimulate biodegradation (Ramos *et al.* 2011, 2013). At the commencement of studies, c. 100 l of each (B100 and B20) was released into each of two 1 m square plots that had been excavated 1.6 m down to the water table (Chiaranda & Corseuil 2010; Chiaranda 2011). Both plots were covered to limit rainfall into the excavations. The geology is generally a fine sand with some silt and clay. Groundwater flow velocity is slow and has been estimated at 6 m a^{-1} . For the study site reported groundwater temperatures range from 22°C in the winter to 26°C in the summer. The groundwater pH was 4; although not noted in the report, very acidic pH is common in Brazil.

Groundwater quality was measured at the source and c. 2 m downgradient over a period of 29 months. Each of the plots slowly changed from aerobic to anaerobic conditions with oxidation–reduction potentials in groundwater slowly changing from +430 to -20 mV . In the B100 plot, acetate concentrations peaked at about 120 mg l^{-1} between 1.5 and 2 years after the release. Changes indicative of a range of redox processes were apparent at the source and downgradient with alkalinity increasing from 50 to c. 250 mg l^{-1} , iron(II) peaking at about 100 mg l^{-1} between 1.5 and 2 years after the release, sulphate reducing from 25 mg l^{-1} to below detection over 12 months and methane concentrations in groundwater increasing from below the detection limit to a maximum of 30 mg l^{-1} , which is in excess of the solubility limit for methane in water ($25 - 28 \text{ mg l}^{-1}$ at the reported groundwater temperatures).

Analysis of the dominant terminal electron accepting processes (TEAP) over time indicated a transition from iron-reducing to methanogenic conditions. In the B20 plot, changes in TEAP were similar although less consistent than those in the B100 plot: dissolved oxygen was already depleted; nitrate was present at initial concentrations varying from c. 40 to 80 mg l^{-1} but was rapidly reduced to below detection in 3 months. Iron reduction was less marked, with dissolved concentrations peaking at c. 20 mg l^{-1} and sulphate increasing or stable. Methane peaked at about 30 mg l^{-1} between 1.5 and 2 years after the release. Concentrations of acetate remained below or close to detection limits for the whole experiment. For the B20 release, chemical analysis was also undertaken of benzene, toluene, ethylbenzene and xylenes (BTEX) and total hydrocarbons. BTEX concentrations were either stable, or more typically increased over the duration of the project. It was, however, not possible to determine whether biodegradation rates of BTEX were reduced in the presence of FAME as there was no diesel-only control. A follow-up paper by Ramos *et al.* (2014) focused solely on results from the plot amended with ammonium acetate, specifically examining the role of microbial community structure on fermentative–methanogenic biodegradation of aromatic hydrocarbons.

Large-scale environmental release of FAME

Although FAME and biodiesel blends have been in use since the late 1990s, only one accidental release of FAME into the environment has been reported in the literature Toso (2010, 2012). This release occurred in Minnesota, USA, in August 2007. Although single FAME compounds were not detected in groundwater (possibly owing to analytical detection limits above the aqueous solubilities), elevated concentrations of total organic carbon (TOC) and dissolved organic carbon (DOC) were detected at concentrations up to 2390 and 2340 mg l^{-1} , respectively. Short chain fatty acids including acetate and butyrate, which are products of biological beta-oxidation of FAME, were detected at a high concentration 3094 mg l^{-1} in one well only and accounted for 85% of DOC at that location. This indirect evidence for biodegradation

was further supported by the detection of high dissolved inorganic carbon (indicating the likely production of carbon dioxide), high dissolved methane and high iron(II) concentrations in groundwater. Soil gas measurements also detected high methane (up to 67% v/v) and carbon dioxide (up to 34% v/v) with complete depletion of oxygen at these same locations. Although no data on groundwater oxidation reduction potential (ORP) or dissolved oxygen were presented, the high concentrations of methane and iron(II), both of which form under strongly reducing conditions, provide good evidence that anaerobic conditions developed.

Taken together, these data provide strong evidence that biodegradation of FAME can occur under both aerobic and anaerobic conditions in the subsurface. The naturally occurring microbial population, which probably had no previous exposure to FAME, was capable of metabolizing FAME through multiple TEAP including aerobic, iron reduction and methanogenesis.

Ultimately, an understanding of the behaviour and natural attenuation of FAME in soil and groundwater will come only from surveying observations of FAME at multiple release sites, as was done in the past for gasoline and other petroleum products (Newell *et al.* 1989; Rice *et al.* 1995; Mace *et al.* 1997) and more recently for ethanol (Ruiz-Aguilar *et al.* 2003; Mackay *et al.* 2006; Morgan *et al.* 2014). The fates of FAME and biodiesel blends in different geologies, hydrogeological regimes and climates will provide a better view of how FAME will behave in the environment and influence the behaviour of petroleum diesel constituents.

Effect of FAME on the subsurface fate and transport of petroleum hydrocarbons

Potential mobilization of hydrocarbons

The potential use of FAME to mobilize hydrocarbons in the subsurface has been the subject of research, and previous reviews have highlighted a number of papers where FAME have been studied in the context of mobilizing crude oil or polycyclic aromatic hydrocarbons (PAH) from soils (Energy Institute 2008; ITRC 2011).

These works included a study by Miller & Mudge (1997) that evaluated the effect of application of a rapeseed-derived biodiesel on the weathering and movement of crude oil within artificial sand columns. The addition of biodiesel led to greater recovery of oil from the sediment when applied to relatively unweathered crude oil. Pereria & Mudge (2004) evaluated the use of B100 from rapeseed, soybean and cooking oil at the laboratory scale using trays and larger containers containing cobbles, gravel, and coarse and fine sand collected from nearby shores. Crude oil removal rates of up to 96% were reported for the rapeseed-derived biodiesel compared with a value of 15% in a control using water only. In the mesocosm experiments a ratio 1:1 of soybean biodiesel to crude oil removed 80% of the oil from cobbles and fine sands, 50% from coarse sand and 30% from gravel. Taylor & Jones (2001) examined the addition of a slow-release fertilizer (N:P:K 14:14:14), diesel and biodiesel on the biodegradation of coal tar in field and laboratory experiments. Increased degradation of naphthalene following application of biodiesel was attributed to tar solubilization and dispersion leading to an increase in PAH bioavailability.

More recently, Gong *et al.* (2010) studied the use of FAME in the solubilization of PAH from soils from a former manufactured gas plant site and two soil samples artificially spiked with PAH. The study undertook a comparison between the use of a commercial biodiesel (manufactured from waste oil), a laboratory-synthesized soybean oil biodiesel, soybean oil, methanol and a range of other surfactants. Total PAH removal of between 35% (commercial biodiesel) and 46% (soybean biodiesel) was reported, and this removal was greater than that observed for the parent compounds or commercial surfactants.

Each of the above case studies illustrates the potential for FAME at high concentrations (B100) to mobilize previously spilled and residual hydrocarbons in the subsurface, which could be relevant if a FAME (B100) spillage was to occur at a historically affected site. However, to date no field or case study examples of such behaviour appear to have been reported. Because of the probably site-specific nature of any such effects, further research in this area is recommended.

Effect of FAME on stability and behaviour of diesel LNAPL

Most of the work on the effect of FAME on the behaviour of diesel LNAPL has been undertaken in the context of the fate of biodiesel in the marine surface water environment.

DeMello *et al.* (2007) observed that in dispersion experiments simulating turbulent mixing of FAME blends of B20 or greater, FAME (unspecified source oil) appeared to stabilize oil droplets in the water phase by decreasing the oil water surface tension and, therefore, reducing oil droplet aggregation. DeMello *et al.* concluded that FAME may influence the transport, weathering rate and ecological impact of spilled biodiesel, and suggested that FAME may enhance dissolution rates of conventional hydrocarbons, and by extension increase their potential for biodegradation.

This initial observation was replicated by Hollebone *et al.* (2008), who reported that under high-energy conditions (rotating flask), B100 and B20 blends using canola, soy and waste frying oil were dispersed much more readily than the petroleum diesel fuel tested under the same conditions.

Both the above studies were undertaken in turbulent conditions unlikely to be representative of conditions in groundwater. Hollebone *et al.* (2008) noted that under low-energy conditions the biodiesels tested had very similar behaviour to the petroleum diesel.

In microcosms designed to mimic a free phase oil layer over surface water (Fuller *et al.* 2013), the overall total petroleum hydrocarbon (TPH) degradation rate and extent was similar but the PAH pyrene, fluoranthene and alkyl derivatives were found to degrade faster in a B20 biodiesel blend (tallow and canola oil) than in conventional diesel. The samples were incubated in a glass greenhouse and were exposed to day and night variations in light, temperature and humidity. Fuller *et al.* indicated that the glass filtered the sunlight and reduced the effects of UV-B radiation to 'negligible'. The study concluded that the presence of biodiesel had a significant effect on the weathering process resulting in accelerated loss of hydrocarbon components, and reported that the mechanism for this loss was probably photo-oxidation or aerobic biodegradation.

Yang *et al.* (2015) studied the photolysis behaviour of biodiesel and blends in surface water using biodiesel prepared from soybean, canola and animal fat. In blends of biodiesel and diesel photolysis of alkanes was reduced and it was suggested that this was due to biodiesel acting as a surfactant and stabilizing small oil droplets formed by agitation.

Yassine *et al.* (2012) studied the batch partitioning of C10–C20 *n*-alkanes and four monoaromatic compounds in petrodiesel–soybean biodiesel mixtures (B20, B40, B60 and B80). The aqueous solubility of *n*-alkanes was found to increase with biodiesel content, this being attributed to the formation of stable colloids of *n*-alkanes. Overall, Yassine *et al.* concluded that a spill of biodiesel would be easier to bioremediate than a similar petroleum diesel spill owing to the enhanced dissolution of aliphatic compounds and enhanced oil–water micro-emulsion stability resulting in a larger oil–water interface area. They noted, however, that the partitioning behaviour of diesel and biodiesel blends will be particular to the mixture under study owing to inherent variability in the

physicochemical properties, chemical composition, feedstock source, oxidative stability, additives and methods of manufacture.

Overall FAME appears to have some stimulatory effect on diesel hydrocarbon biodegradation at concentrations of B20 and higher, but potential for changes at lower concentration blends has not been reported. However, to date no field or case study examples of such behaviour appear to have been reported.

Effect of FAME on partitioning of hydrocarbons

Coupled with (and difficult to separate from) the physical effects on the stability of LNAPL is the influence of FAME on the partitioning of particular hydrocarbons from LNAPL to either the vapour or aqueous phase. A number of studies have reported on such aspects.

In respect of the vapour phase transfer, DeMello *et al.* (2007) observed that diesel hydrocarbons evaporated at a similar rate from B0 (pure diesel) and biodiesel mixtures. This behaviour was corroborated by additional calculations of activity coefficients in simulated diesel and biodiesel mixtures, and it was concluded that FAME will not affect the rate of volatilization of diesel components.

This observation was later confirmed by Yang *et al.* (2013), who studied the weathering of diesel and biodiesel (B5 and B20 blends) in open air under ambient conditions (in the dark at 22°C) over a period of 190 days. In terms of partitioning and mass transfer to the aqueous phase, Hollebone *et al.* (2008) measured the WAF of B5 and B20 blends in comparison with that of the B0 diesel product. The WAFs of the blends were not significantly different from that of the diesel product and it was concluded that there were no measurable solvent effects between biodiesel and petrodiesel in the blends.

Chen *et al.* (2008) studied the partitioning of eight indicator PAH between water and the oil phase in a range of biodiesel formulations derived from waste edible oil (B100, B1, B5, B20) and concluded that the equilibrium partitioning depended on many factors, including sorption onto the aquifer matrix and the presence of potential surfactants, emulsifiers or cosolvents.

In the study of partitioning undertaken by Yassine *et al.* (2012) noted above, dissolved concentrations of aromatic compounds were found to closely obey Raoult's Law at all oil loads, with measured concentrations in aqueous solution not appreciably affected by filtration. On this basis, those researchers concluded that the dissolution of aromatics is not significantly modified in the presence of FAME.

Effect of FAME on biodegradation of petroleum hydrocarbons

The literature on the effects of FAME on diesel hydrocarbon biodegradation is summarized in Tables 3 and 5 for aerobic and anaerobic conditions, respectively. Key papers are discussed below.

Zhang *et al.* (1998) compared the rates of biodegradation of diesel (Phillips 2-D reference diesel) under aerobic conditions in the presence and absence of rapeseed ethyl ester using both mineralization to carbon dioxide and gas chromatography (GC). This paper was one of the first to report that fatty acid esters enhance the degradation of diesel. The tests were conducted on mixtures of rapeseed ethyl ester and diesel (B100, B80, B60, B20 and B0) in nutrient media inoculated with organic-rich soil, aerated activated sewage mixed liquor and raw domestic sewage water. The mixtures were then monitored for carbon dioxide evolution. The percentage of the added carbon that was mineralized to carbon dioxide over 28 days increased in proportion to the amount of rapeseed ethyl ester in the mixture. In a second experiment using a 50:50 diesel–rapeseed ethyl ester mixture (B50) and monitoring by GC, 94.8% of the diesel hydrocarbons was degraded within 4 days when present in the B50 mixture, compared with 53.5% of the pure diesel. Zhang *et al.*

concluded, based on the disappearance of diesel from the chromatograms, that the alkanes of diesel and the fatty acid ethyl esters were degraded at similar rates and estimated that the maximum removal rates as determined by CO₂ evolution were 25 mg l⁻¹ day⁻¹ for rapeseed ethyl ester and 12.5 mg l⁻¹ day⁻¹ for diesel.

Taylor & Jones (2001) examined the effect of the addition of nutrients, diesel and biodiesel on the biodegradation of coal tar contaminated soils in field and laboratory experiments. The extent of degradation of naphthalene in coal tar relative to controls was shown to increase by 85 and 52% respectively in the 55 day experimental period and when diesel was applied the increases were 85 and 96% respectively. Other PAH containing up to four rings were depleted to a lesser extent. The increases were attributed to tar solubilization and dispersion increasing PAH bioavailability.

In mixtures of waste cooking oil with gasoline and diesel, Pasqualino *et al.* (2006) reported that waste cooking oil biodiesel increased both the rate and extent (between a 10 and 40% enhancement) of mineralization of diesel and gasoline when measured through quantification of carbon dioxide evolution relative to control flasks.

DeMello *et al.* (2007) investigated the effect of FAME on the biodegradation of diesel in aerobic seawater microcosms. Diesel and FAME (B100, source oil not specified) were obtained from commercial sources and mixtures of diesel and FAME were prepared (B8 and B25). Several commercially available B20 mixtures were also obtained for comparison but the researchers noted that the proportion of diesel in these B20 commercial mixtures exhibited 'extreme variability', with one sample having almost no diesel present. Microcosms were constructed using seawater amended with nitrogen and phosphorus and either B8, B25, B100 or B0 (to *c.* 0.1125 mg l⁻¹). Abiotic controls were constructed in parallel. The microcosms were incubated under aerobic conditions. The microcosms were analysed by GC for *n*-alkanes, branched-chain alkanes, single FAME, bulk FAME and bulk diesel hydrocarbons. Biodegradation of B0 followed a typical pattern of loss of *n*-alkanes with relative increased proportions of branched-chain alkanes (farnasene, norpristane, pristane and phytane) and the unresolved compound mixture (UCM). A similar pattern of losses was observed in the B8 and B25 mixtures, along with loss of FAME. The *n*-alkanes were observed to disappear slightly more slowly initially in B8 and B25 than in the pure diesel; however, this effect was small. Overall, the conclusions from this study were that FAME has little effect on the biodegradation of straight-chain and branched-chain alkyl components of diesel under aerobic conditions.

Mariano *et al.* (2008) conducted experiments on the biodegradability of blends of castor oil FAME and diesel under aerobic conditions using respirometry testing. Laboratory-produced castor oil biodiesel (ester type unspecified), commercial diesel, blends of the castor oil FAME with diesel (B5, B10, B20), and a commercial B2 (source oil not specified) were incubated under aerobic conditions with either contaminated river water or fuel-contaminated soil from a petrol station. Carbon dioxide productions from castor oil FAME and the castor oil FAME–diesel blends were much higher than from diesel, whereas the carbon dioxide production from diesel was similar to the production from the commercial B2 blend. The carbon dioxide production from the B20 blend was essentially the same as predicted from the production observed from pure diesel (B100) and pure FAME (B0). Thus, the blending of FAME and diesel did not show enhancement of carbon dioxide production from the petroleum hydrocarbons.

Owsianiak *et al.* (2009) presented data from a 7 day flask experiment indicating that, under aerobic conditions, the addition of 10% commercial rapeseed FAME to diesel inhibited the extent of biodegradation of the diesel fraction by *c.* 10%, but that higher proportions (30% or more) slightly enhanced biodegradation of diesel (by *c.* 5%).

While investigating FAME biodegradation under anoxic or anaerobic conditions, Corseuil *et al.* (2011) also looked into the effect of FAME on the biodegradation of benzene and toluene. Microcosms were constructed using groundwater and aquifer sediment, purged with nitrogen to remove oxygen, and amended with either benzene and toluene (2.9 and 0.8 mg l⁻¹, respectively) or benzene, toluene and soy B100 (2.8, 0.8 and 54.8 mg l⁻¹, respectively), which formed both dissolved and organic phases. Abiotic controls poisoned with mercuric chloride were constructed in parallel. Biodegradation of toluene and benzene were both slower in the microcosms containing FAME than when incubated alone. Toluene was completely removed within 34 days in the presence and absence of FAME, whereas only 45% of benzene was removed in the presence of FAME and 90% in the absence of FAME. Corseuil *et al.* postulated that at least some if not all of the benzene removal might be due to aerobic degradation, as the set-up of the microcosms allowed for some oxygen leakage into the test containers. Overall, the results indicate that the presence of FAME can inhibit or at least slow the rate of biodegradation of toluene and benzene under anoxic or hypoxic conditions.

Elazhari-Ali *et al.* (2012) measured aerobic biodegradation rates of hydrocarbons in microcosms inoculated with wet sandy soil, with and without the addition of inorganic nutrients (N and P). A synthetic blend of single hydrocarbons was tested, and also the same blend in a B20 biodiesel (blended with commercial rapeseed FAME). The progress of biodegradation was measured by mass analysis of residuals at the end of the experiment (22 days) and by headspace analysis during the incubation. The addition of nutrients resulted in significantly greater hydrocarbon removal from both the hydrocarbon blend and B20 microcosms. In the microcosms with no added nutrients rates of toluene removal were greater in the hydrocarbon-only mixture than in the B20 microcosms, whereas the rate of removal of toluene was similar in microcosms with added nutrients. Although not discussed by those researchers, this observation is likely to be the result of nutrient limitation, with FAME degraders outcompeting hydrocarbon degraders for the available nutrients.

While conducting studies on the weathering of biodiesel and biodiesel–diesel blends, Fuller *et al.* (2013) also reported data on the effect of biodiesel on the degradation of diesel hydrocarbons. The tests were performed with creek water amended with diesel, biodiesel or a blend of biodiesel–diesel (B20) and aerated during incubation. The biodiesel component comprised a 10:90 w/w blend of tallow and canola oil FAME. After 28 days, a higher proportion of the mass of the PAH components pyrene, fluoranthene, methylpyrenes, and C2-alkyl fluoranthenes and pyrenes were removed from the B20 blend than from diesel alone. Because these PAH are not expected to significantly weather as a result of volatilization, Fuller *et al.* concluded that this impact of biodiesel might be due to photo-oxidation or aerobic degradation as the samples were exposed to sunlight in a greenhouse.

Yassine *et al.* (2013b) conducted aerobic degradation studies on blends (B20, B40, B60 and B80) of a commercial soybean FAME (B100) with low-sulphur petroleum diesel (B0) prepared in the laboratory. Microcosms were inoculated with a bacterial culture acclimated to FAME, diesel and biodiesel. The degradation rates of the *n*-alkanes were enhanced in the FAME blends although this appeared unlikely to be due to increased biomass production from FAME. Based on co-solubilization experiments, it appeared that the presence of FAME resulted in higher aqueous concentrations and bioavailability of *n*-alkanes. The effect of FAME on aromatic compounds during these experiments was mixed. Toluene, *o*-xylene and tetralin, which were not degraded in pure diesel or B20 during the 7 day incubation period, were completely degraded in B40 and B80 to below the detection limit over the same time. In B60, reportedly only ethylbenzene was degraded in multiple replicate experiments. The mineralization rate of diesel was

significantly enhanced in the blends with FAME ($p < 0.05$). Yassine *et al.* did not provide an explanation for the lack of expected biodegradation of toluene and other compounds in pure diesel and B20. Supporting data presented in graphical format indicate that some degradation of toluene may have occurred in the pure diesel and B20 microcosms.

Borges *et al.* (2014) conducted tests to determine whether the presence of FAME from soy, pork and castor oil would affect the biodegradation of benzene under anaerobic conditions. The microcosms were constructed with synthetic groundwater containing 22 mg l^{-1} sulphate and sediment, with either benzene alone or benzene ($3\text{--}4 \text{ mg l}^{-1}$) plus soybean, pork or castor FAME, or one of the three FAME alone (60 mg l^{-1} of B100 of each FAME). The microcosms were initially under oxidizing conditions, which changed to anaerobic conditions within 1 month. Only 17.7% of benzene was removed within 150 days in the benzene-only microcosms. Benzene removals in the FAME-containing microcosms were slightly less at 10.8, 14.4 and 6.7% for soy, pork and castor FAME, respectively, and it is not clear whether these are statistically different from the benzene-only case. Because no data from abiotic controls containing benzene were presented, benzene loss through volatilization cannot be quantified but may be contributing to these losses. Organic acid production can be an indicator of potential methane production and was found to be 34–56% less in FAME + benzene microcosms than in the FAME-only microcosms. Methane production data were not presented for the benzene-containing microcosms. Because other data presented in the paper indicate that these microcosms might have been nutrient-limited and that variability in the added sediment might contribute to observed differences, the observed effect of FAME on benzene degradation and benzene on FAME degradation cannot be assessed.

Lisiecki *et al.* (2014) conducted studies on blends of rapeseed FAME with petroleum diesel (B0, B10, B20, B30, B40, B50, B60, B70, B80, B90 and B100) in sealed undisturbed saturated sand microcosms that were inoculated with a diesel-degrading consortium that had been isolated from a crude oil release site. These microcosms were designed to simulate an aquifer environment close to the water table with limited oxygen transfer from the gas phase into the water phase. The microcosms were incubated for 578 days. Production of carbon dioxide was measured throughout the incubation period. At the end of incubation, the residual fractions of aromatic hydrocarbons, aliphatic hydrocarbons, total petroleum hydrocarbons and FAME were determined by GC-FID. A shift in the bacterial consortium (measured using real-time polymerase chain reaction analyses) was observed, with the abundance of *Citrobacter* increasing with biodiesel content whereas *Achromobacter*, Comamonadaceae and, to some extent, *Pseudomonas* and *Variovorax* were suppressed. In the B100 and B0 microcosms, *c.* 58 and 30% of FAME and diesel were mineralized to carbon dioxide respectively. Overall, the presence of FAME did not appear to affect the rate or extent of diesel degradation. The rates of carbon dioxide production from the blends were generally predictable from the ratio of FAME to diesel and the rates of carbon dioxide production from pure diesel and pure FAME. The residual fractions of aromatic and aliphatic hydrocarbons were generally the same in the B10 to B80 microcosms. Approximately 35–40% of all carbon in all microcosms was not accounted for as carbon dioxide or residual hydrocarbons or FAME, and might have been converted into biomass or intermediate products, or, in the case of diesel, lost through volatilization.

Potential parallels between the impact of ethanol on benzene and BTEX plumes can be drawn for biodiesel. Comparison of BTEX plumes in the presence and absence of ethanol has shown that ethanol-containing BTEX plumes are significantly longer than BTEX-only plumes (Deeb *et al.* 2002; Ruiz-Aguilar *et al.* 2003; Mackay *et al.* 2006). The weight of evidence indicates that this may

be due to either or both of the following: (1) rapid biodegradation of ethanol depleting oxygen, nitrate and sulphate, and thus limiting hydrocarbon biodegradation to slower anaerobic processes such as fermentation; (2) production of high concentrations of hydrogen and acetate from fermentation of ethanol having an inhibitory effect on benzene degradation (Rakoczy *et al.* 2011). These observations open up the possibility that fermentation of the organic acids produced during biodegradation of FAME may have the same effects. However, to date, no data have been reported in the literature to support this theory; specific studies are needed to investigate such potential effects.

Conclusions and recommendations

The primary objective of this review was to provide insight into the natural attenuation of FAME in the subsurface. The key conclusions from the review are as follows.

The composition and properties of FAME within a given biodiesel vary according to the original FAME feedstock, the presence of additives and the blend of feedstock used in a commercial product. B100 (100% FAME) may be present at fuel production and storage sites, but the most commonly used and distributed forms of FAME-containing fuels are 5–20% (B5–B20) blends of FAME with petroleum diesel.

Single FAME compounds are of low aqueous solubility, low volatility and low mobility. In this context, a B5 or B20 FAME–petroleum diesel blend may be expected to behave similarly to petroleum diesel in the subsurface. The mixtures of FAME that have been studied in the peer-reviewed literature do not appear to enhance the solubility of hydrocarbons as a whole or of single components such as PAH or monoaromatic hydrocarbons.

FAME have been reported to undergo relatively rapid autoxidation and hydrolysis in aqueous solution, with 5–10% conversion to free fatty acids and methanol over a 24 h period. These more soluble, but equally biodegradable substances could increase the concentration of dissolved organic carbon in groundwater beyond that expected for the parent FAME. Confirmation of complete FAME biodegradation requires more than disappearance as detected by GC because intermediates produced through autoxidation and hydrolysis are not detectable by standard GC methods for FAME and diesel. With the exception of methanol, these intermediates are not known to be toxic, but could continue to affect water quality. Additional work is needed to explore these effects.

Numerous laboratory studies have been conducted to investigate the biodegradation of FAME (both bulk FAME and single compounds) from various feedstocks. The studies have utilized different test conditions, microbial inocula, amendments and analytical measurements.

Multiple laboratory studies have demonstrated that biological mineralization of FAME to carbon dioxide occurs under aerobic conditions. Oxygen depletion in response to FAME has also been observed at the two field sites.

Multiple laboratory studies have demonstrated biodegradation of FAME under methanogenic conditions. Methane production in response to FAME has also been observed at the two field sites.

A more limited number of laboratory studies have provided evidence that biodegradation of FAME occurs through nitrate and sulphate reduction. Nitrate and sulphate depletion in response to FAME biodegradation has also been observed at one field site.

Although no laboratory studies were identified that evaluated biodegradation of FAME through iron or manganese reduction, increases in dissolved iron in response to FAME have been observed at the two field sites. No data have been reported on manganese reduction.

Although studies support the overall conclusion that FAME is readily biodegradable under both aerobic and anaerobic conditions,

the specific details (rate, observation of a lag period, extent of degradation, preferential degradation of specific FAME) varied from study to study. Site-specific assessment of natural attenuation processes, in accordance with lines-of-evidence based good practice on Monitored Natural Attenuation (MNA), remain necessary to demonstrate MNA on a site-by-site basis.

FAME appears to enhance the biodegradability of diesel at concentrations of B20 and higher, but this effect has not been demonstrated at field scale in the context of a subsurface release of a FAME–diesel mixture. At sites with limited electron acceptors and macronutrients (nitrogen and phosphate), microorganisms that degrade FAME have the potential to deplete available electron acceptors and nutrients, resulting in an extended time for diesel degradation.

Under subsurface conditions labile biofuels, including ethanol and FAME, have a greater potential to biodegrade to methane compared with petroleum hydrocarbons. Accordingly, the potential for methane production is greater for a FAME biodiesel spill than for an equivalent diesel release. Studies conducted at ethanol spill sites indicate that, in addition to FAME content, the rate of methane formation will probably be dependent on factors such as geology, depth to water, ground temperature and soil moisture content. Off-gassing of methane and carbon dioxide can enhance volatilization of BTEX and other volatile compounds from groundwater and thereby increase the risk of vapour intrusion into buildings. It may be appropriate, therefore, to take the potential for methane production into account in the risk assessment of biodiesel release sites. Additional observations at biodiesel spill sites are needed to better understand the extent to which methane production may be an issue.

Overall, natural attenuation would appear to be significant in controlling the fate, behaviour and potential risks posed by biodiesel. Significant attenuation mechanisms are likely to include sorption, autoxidation and biodegradation via a variety of redox processes; the exact role and contribution of each will depend on the nature of the release, the characteristics of the particular FAME, the FAME mixture and the environmental setting. Such attenuation may have secondary impacts on the degradation of other components of the biodiesel and may in itself generate undesirable effects such as excessive methane.

The literature contains relatively few studies of the fate and behaviour of FAME at the field scale and few published case studies. Additional examples of either controlled or accidental releases of FAME or FAME–diesel mixtures would enhance understanding of the biodegradation processes discussed above and the behaviour of these processes in different geologies. Of particular interest would be additional information on the impact of FAME on the fate and transport of petroleum diesel and the production of methane.

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References

- Adair, C. & Wilson, J.T. 2009. Anaerobic biodegradation of biofuels (ethanol and biodiesel) and proposed biofuels (*n*-propanol, *iso*-propanol, *n*-butanol, and 2,5-dimethylfuran) in aquifer sediments. Presentation at National Tanks Conference, 30 March, Sacramento, CA.
- Adair, C. & Wilson, J.T. 2010a. Anaerobic biodegradation of biofuels (ethanol and biodiesel) and proposed biofuels (*n*-propanol, *iso*-propanol, *n*-butanol). Presented at Seventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds, 24–27 May 2010, Monterey, CA.
- Adair, C. & Wilson, J.T. 2010b. Anaerobic biodegradation of biofuels (ethanol, biodiesel, *n*-propanol, *n*-butanol, and *iso*-butanol) in aquifer sediment. Presented at the 22nd National Tanks Conference, 20–22 September, Boston, MA.
- Aktas, D.F., Lee, J.S., Little, B.J., Ray, R.I., Davidova, I.A., Lyles, C.N. & Sufliya, J.M. 2010. Anaerobic metabolism of biodiesel and its impact on metal corrosion. *Energy Fuels*, **24**, 2924–2928.
- Alptekin, E. & Canakci, M. 2008. Determination of the density and the viscosities of biodiesel–diesel fuel blends. *Renewable Energy*, **33**, 2623–2630.
- ASTM 2009. *Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels, ASTM D6751-09*. American Society for Testing and Materials International, West Conshohocken, PA, <http://www.astm.org>
- Borges, J.M., Dias, J.M. & Danko, A.S. 2014. Influence of the anaerobic biodegradation of different types of biodiesel on the natural attenuation of benzene. *Water, Air and Soil Pollution*, **225**, 2146.
- BSI 2013. *Liquid petroleum products. Fatty acid methyl esters (FAME) for use in diesel engines and heating applications, BS EN 14214:2012+A1:2014*. Requirements and test methods. British Standards Institution London UK.
- Chen, C.S., Lai, Y.-W. & Tien, C.-J. 2008. Partitioning of polynuclear aromatic hydrocarbons into water from biodiesel fuel mixtures. *Environmental Chemistry*, **5**, 435–444.
- Chiaranda, H.S. 2011. *Alterações biogeomórficas em águas subterrâneas impactadas por biodiesel de soja e misturas de diesel/biodiesel (B20)*. PhD thesis, Universidade Federal de Santa Catarina, Florianópolis, SC.
- Chiaranda, H.S. & Corseuil, H.X. 2010. Assessing natural attenuation of diesel/biodiesel mixtures spills in groundwater. In: *IWA World Water Congress and Exhibition, 2010, Montreal. Proceedings of IWA World Water Congress and Exhibition*, IWA Publishing, London.
- CL:AIRE 2014. *An illustrated handbook of LNAPL transport and fate in the subsurface*. CL:AIRE, London, ISBN 978-1-905046-24-9.
- CONCAWE 2009. *Guidelines for handling and blending FAME. Report 9/09*. Conservation of Clean Air and Water in Europe, Brussels.
- Corseuil, H.X., Monier, A.L., Gomes, A.P.N., Chiaranda, H.S., do Rosario, M. & Alvarez, P.J.J. 2011. Biodegradation of soybean and castor oil biodiesel: implications on the natural attenuation of monoaromatic hydrocarbons in groundwater. *Ground Water Monitoring & Remediation*, **31**, 111–118.
- Cyplik, P., Schmidt, M. *et al.* 2011. Relative quantitative PCR to assess bacterial community dynamics during biodegradation of diesel and biodiesel fuels under various aeration conditions. *Bioresource Technology*, **102**, 4347–4352.
- Deeb, R.A., Sharp, J.O. *et al.* 2002. Impact of ethanol on benzene plume lengths: microbial and modeling studies. *Journal of Environmental Engineering*, **128**, 868–875.
- DeMello, J.A., Carmichael, C.A., Peacock, E.E., Nelson, R.K., Arey, J.S. & Reddy, C.M. 2007. Biodegradation and environmental behaviour of biodiesel mixtures in the sea: an initial study. *Marine Pollution Bulletin*, **54**, 894–904.
- Dow 2011. *Kathon™ FP 1.5 Biocide. Safety Data Sheet*. Dow Chemical Company, Midland, MI.
- Elazhari-Ali, A., Singh, A.K., Davenport, R.J., Head, I.M. & Werner, D. 2012. Biofuel components change the ecology of bacterial volatile petroleum hydrocarbon degradation in aerobic sandy soil. *Environmental Pollution*, **173**, 125–132, <http://doi.org/10.1016/j.envpol.2012.10.010>
- Energy Institute 2008. *Biofuels – Literature Review of Potential Risks to UK Water Resources. Final Report*. Energy Institute, London.
- Freitas, S.V.D., Oliveria, M.B., Lima, Á.S. & Coutinho, J.A.P. 2012. Measurement and prediction of biodiesel volatility. *Energy Fuels*, **26**, 3048–3053.
- Fuller, S., Spikmans, V., Vaughan, G. & Guo, C. 2013. Effects of weathering on sterol, fatty acid methyl ester (FAME), and hydrocarbon profiles of biodiesel and biodiesel/diesel blends. *Environmental Forensics*, **14**, 42–49.
- Ghaly, A.E., Dave, D., Brooks, M.S. & Budge, S. 2010. Production of biodiesel by enzymatic transesterification: review. *American Journal of Biochemistry and Biotechnology*, **6**, 54–76.
- Ginn, T.R., Hatch, T.J., McKone, T.E. & Rice, D.W. 2009. *California Biodiesel Multimedia Evaluation Tier I Report*. University of California, Davis and University of California, Berkeley.
- Ginn, T.R., Hatch, T.J., McKone, T.E. & Rice, D.W. 2012. *California Biodiesel Multimedia Evaluation Tier II Report on aquatic toxicity, biodegradation and subsurface transport experiments*. University of California, Davis and University of California, Berkeley.
- Gong, Z., Wang, X., Tu, Y., Wu, J., Sun, Y. & Li, P. 2010. Polycyclic aromatic hydrocarbon removal from contaminated soils using fatty acid methyl esters. *Chemosphere*, **79**, 138–143.
- Hodam, R. 2008. *Biodiesel A Technical Report*. UST Leak Prevention Unit, State Water Resources Control Board, CA.
- Hollebone, B.P., Fieldhouse, B., Landriault, M., Doe, K. & Jackman, P. 2008. Aqueous solubility, dispersibility and toxicity of biodiesels. In: *Proceedings of International Oil Spill Conference, 2008, 1*, 929–936, International Oil Spill Conference, Washington, DC, <http://ioscproceedings.org/doi/pdf/10.7901/2169-3358-2008-1-929>
- Horel, A. & Schiewer, S. 2014. Influence of inocula with prior hydrocarbon exposure on biodegradation rates of diesel, synthetic diesel, and fish-biodiesel in soil. *Chemosphere*, **109**, 150–156.
- ITRC 2009. *Evaluating LNAPL Remedial Technologies for Achieving Project Goals*. Interstate Technology & Regulatory Council, Washington, DC.
- ITRC 2011. *Biofuels: Release Prevention, Environmental Behaviour, and Remediation*. Interstate Technology & Regulatory Council, Washington, DC.
- Khouri, R.R., Ebrahimi, D., Hejazi, L., Bucknall, M.P., Pickford, R. & Hibbert, D.B. 2011. Degradation of fatty acid methyl esters in biodiesels exposed to sunlight and seawater. *Fuel*, **90**, 2677–2683.

- Knothe, G. 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Processing Technology*, **86**, 1059–1070.
- Knothe, G. 2007. Some aspects of biodiesel oxidative stability. *Fuel Processing Technology*, **88**, 669–677.
- Knothe, G. 2012. Biodiesel composition and fuel properties. Powerpoint presentation. US Department of Agriculture/Agricultural Research Service/National Center for Agricultural Utilization Research (USDA/ARS/NCAUR), Peoria, IL.
- Krop, H.B., Van Velzen, M.J.M., Parsons, J.R. & Govers, H.A.J. 1997. *n*-Octanol–water partition coefficients, aqueous solubilities and Henry's law constants of fatty acid esters. *Chemosphere*, **34**, 107–119.
- Lisiecki, P., Chrzanowski, L. *et al.* 2014. Biodegradation of diesel/biodiesel blends in saturated sand microcosms. *Fuel*, **116**, 321–327.
- Mace, R.E., Fisher, R.S., Welch, D.M. & Parra, S.P. 1997. *Extent, Mass, and Duration of Hydrocarbon Plumes from Leaking Petroleum Storage Tank Sites in Texas*. Geologic Circular, **97-1**. Bureau of Economic Geology, University of Texas at Austin.
- Mackay, D.M., de Sieyes, N.R. *et al.* 2006. Impact of ethanol on the natural attenuation of benzene, toluene, and *o*-xylene in a normally sulphate reducing aquifer. *Environmental Science & Technology*, **40**, 6123–6130.
- Makarevicene, V. & Janulis, P. 2003. Environmental effect of rapeseed oil ethyl ester. *Renewable Energy*, **28**, 2395–2403.
- Mariano, A.P., Tomasella, R.C., de Oliveira, L.M., Contiero, J. & de Franceschi de Angelis, D. 2008. Biodegradability of diesel and biodiesel blends. *African Journal of Biotechnology*, **7**, 1323–1328.
- Meneghetti, L.R.R., Thomé, A., Schnaid, F., Prietto, P.D.M. & Cavellhao, G. 2012. Natural attenuation and biostimulation of biodiesel contaminated soils from southern Brazil with different particle sizes. *Geotecnia Ambiental*, artigo 3.
- Miller, N.J. & Mudge, S.M. 1997. The effect of biodiesel on the rate of removal and weathering characteristics of crude oil within artificial sand columns. *Spill Science & Technology Bulletin*, **4**, 17–33.
- Morgan, P., Firth, S. & Hildenbrand, B. 2014. Behaviour and effects of alcohol-blended petrol in the subsurface. *Quarterly Journal of Engineering Geology and Hydrogeology*, **47**, 267–279. <https://doi.org/10.1144/qjegh2014-047>
- Moser, B.R. 2009. Biodiesel production, properties and feedstocks. *In Vitro Cellular Development and Biotechnology—Plant*, **45**, 229–266.
- Newell, C.J., Hopkins, L.P. & Bedient, P.B. 1989. *A hydrogeologic database for ground-water modeling*. API Publication, **4476**. American Petroleum Institute, Washington, DC.
- NREL 2009. *Biodiesel Handling and Use Guide*, 4th edn. National Renewable Energy Laboratory, Golden, CO, NREL/TP-540-43672.
- Owsianiak, M., Chrzanowski, L., Sulc, A., Staniewski, J., Olszanowski, A., Olejnik-Schmidt, A.K. & Heipieper, H.J. 2009. Biodegradation of diesel/biodiesel blends by a consortium of hydrocarbon degraders: Effect of the type of blend and the addition of biosurfactants. *Biorescience Technology*, **100**, 1497–1500.
- Pasqualino, J.C., Montané, D. & Salvadó, J. 2006. Synergic effects of biodiesel in the biodegradability of fossil-derived fuels. *Biomass and Bioenergy*, **30**, 874–879.
- Pereria, M.G. & Mudge, S.M. 2004. Cleaning oiled shores: laboratory experiments testing the potential use of vegetable oil biodiesels. *Chemosphere*, **54**, 297–304.
- Peterson, C.L. & Moller, G. 2005. Biodiesel fuels: biodegradability, biological and chemical oxygen demand, and toxicity. *In*: Knothe, G., Van Gerpen, J. & Krahl, J. (eds) *The Biodiesel Handbook*. AOCSS Press, Champaign, IL, <https://www.scribd.com/doc/54171412/The-Biodiesel-Handbook-Knothe-Van-Gerpen-and-Krahl>
- Peterson, C.L. & Reece, D. 1994. *Toxicology, Biodegradability and Environmental Benefits of Biodiesel*, Biodiesel'94, Sioux Falls, SD, http://www.biodiesel.org/reports/19940101_mar-002.pdf
- Prince, R.C., Haitmanek, C. & Lee, C.C. 2008. The primary aerobic biodegradation of biodiesel B20. *Chemosphere*, **71**, 1446–1451.
- Rakoczy, J., Schleinitz, K.M., Müller, N., Richnow, H.H. & Vogt, C. 2011. Effects of hydrogen and acetate on benzene mineralisation under sulphate-reducing conditions. *FEMS Microbiology*, **77**, 238–247.
- Ramos, D.T., Chiaranda, H.S. & Corseuil, H.X. 2011. Assessment of stimulatory fermentative processes to enhance natural attenuation of groundwater contaminated with biodiesel (B20). International Symposium on Bioremediation and Sustainable Environmental Technologies, Reno, NV, 27–30 June 2011, Battelle Memorial Institute, Columbus, OH.
- Ramos, D.T., Busi da Silva, M.L., Chiaranda, H.S., Alvarez, P.J.J. & Corseuil, H.X. 2013. Biostimulation of anaerobic BTEX biodegradation under fermentative methanogenic conditions at source-zone groundwater contaminated with a biodiesel blend (B20). *Biodegradation*, **24**, 333–341.
- Ramos, D.T., Busi da Silva, M.L., Nossa, C.W., Alvarez, P.J.J. & Corseuil, H.X. 2014. Assessment of microbial communities associated with fermentative-methanogenic biodegradation of aromatic hydrocarbons in groundwater contaminated with a biodiesel blend (B20). *Biodegradation*, **25**, 681–691, <https://doi.org/10.1007/s10532-014-9691-4>
- Rice, D.W., Grose, R.D. *et al.* 1995. *California Leaking Underground Fuel Tank (LUFT) Historical Case Analysis*. Lawrence Livermore National Laboratory (LLNL) Study. UCRL-AR-122207, Lawrence Livermore National Laboratory, Livermore, CA.
- Rojo, F. 2009. Degradation of alkanes by bacteria – Minireview. *Environmental Microbiology*, **11**, 2477–2490.
- Rojo, F. 2010. Enzymes for aerobic degradation of alkanes. *In*: Timmis, K.N. (ed.) *Handbook of Hydrocarbon and Lipid Microbiology*. Springer, Berlin, https://doi.org/10.1007/978-3-540-77587-4_79
- Ruiz-Aguilar, G.M.L., O'Reilly, K. & Alvarez, P.J. 2003. A comparison of benzene and toluene plume lengths for sites contaminated with regular vs. ethanol-amended gasoline. *Ground Water Monitoring & Remediation*, **23**, 48–53.
- Russell, S., Rhykerd, R.L., Smiciklas, K.D. & Hamaker, C. 2005. Phytoremediation of petroleum diesel and biodiesel contaminated soil. Poster presented at The ASA–CSSA–SSSA International Annual Meetings, November 6–10, Salt Lake City, UT.
- Salam, D.A., Suidan, M.T. & Venosa, A.D. 2012. Effect of butylated hydroxytoluene (BHT) on the aerobic biodegradation of a model vegetable oil in aquatic media. *Environmental Science & Technology*, **46**, 6798–6805.
- Sanford, S.D., White, J.M., Shah, P.S., Wee, C., Valverde, M.A. & Meier, G.R. 2009. *Feedstock and Biodiesel Characteristics Report*. Renewable Energy Group, Ames, IA, www.regfuel.com.
- Schleicher, T., Werkmeister, R., Russ, W. & Meyer-Pittroff, R. 2009. Microbiological stability of biodiesel–diesel mixtures. *Biorescience Technology*, **100**, 724–730.
- Sørensen, G., Pedersen, D.V., Nørgaard, A.K., Sørensen, K.B. & Nygaard, S.D. 2011. Microbial growth studies in biodiesel blends. *Biorescience Technology*, **102**, 5259–5264.
- Sousa, D.Z., Pereira, M.A., Stams, A.J.M., Alves, M.M. & Smid, H. 2007. Microbial communities involved in anaerobic degradation of unsaturated or saturated long-chain fatty acids. *Applied and Environmental Microbiology*, **73**, 1054.
- Stolz, J., Follis, P., Floro, G., Donofrio, R., Buzzelli, J. & Griffin, W. 1995. Aerobic and anaerobic biodegradation of the methyl esterified fatty acids of soy diesel in freshwater and soil environments, http://www.biodiesel.org/reports/19950101_gen-273.pdf
- Taylor, L.T. & Jones, D.M. 2001. Bioremediation of coal tar PAH in soils using biodiesel. *Chemosphere*, **44**, 1131–1136.
- Thomé, A., Reginatto, C., Cecchin, I. & Colla, L.M. 2014. Bioventing in a residual clayey soil contaminated with a blend of biodiesel and diesel oil. *Journal of Environmental Engineering*, **140**, 06014005.
- Toso, M.A. 2010. Investigation of a large-scale subsurface biodiesel release. *In*: Fields, K.A. & Wickramanayake, G.B. (Chairs) *Remediation of Chlorinated and Recalcitrant Compounds*. Seventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA.
- Toso, M.A. 2012. The impact of biofuel releases on Minnesota's water resources. Presentation to BNSF Railway Hazardous Materials Team, <http://www.bnsfhazmat.com/refdocs/1326423967.pdf>
- UFOP 2013. *Raw Material Basis for Biodiesel Components in Diesel Fuels*. Union zur Förderung von Oel- und Proteinpflanzen, Berlin.
- US EIA 2015. *Monthly Biodiesel Production Report with Data for April 2015*. US Energy Information Administration (US EIA), Washington, DC, <http://www.eia.gov/biofuels/biodiesel/production/biodiesel.pdf>
- US EPA 2014. *Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.11*. US Environmental Protection Agency, Washington, DC.
- Vauhkonen, V., Lauhanen, R. *et al.* 2011. The phytotoxic effects and biodegradability of stored rapeseed oil and rapeseed oil methyl esters. *Agricultural and Food Science*, **20**, 131–142.
- Von Wedel, R. 1999. *Technical Handbook for Marine Biodiesel in Recreational Boats*. CytoCulture International, Pont Richmond, CA.
- White, A., Handler, P. & Smith, E.L. 1968. *Principles of Biochemistry*, 4th edn. McGraw–Hill, New York.
- Yang, Z., Hollebone, B.P., Wang, Z., Yang, C. & Landriault, M. 2013. Effect of storage period on the dominant weathering processes of biodiesel and its blends with diesel in ambient conditions. *Fuel*, **104**, 342–350.
- Yang, Z., Hollebone, B.P. *et al.* 2015. A preliminary study for the photolysis behavior of biodiesel and its blends with petroleum oil in simulated freshwater. *Fuel*, **139**, 248–256.
- Yassine, M.H., Wu, S., Suidan, M.T. & Venosa, A.D. 2012. Partitioning behaviour of petrodiesel/biodiesel blends in water. *Environmental Science & Technology*, **46**, 7487–7494.
- Yassine, M.H., Suidan, M.T. & Venosa, A.D. 2013a. Microbial kinetic model for the degradation of poorly soluble organic materials. *Water Research*, **47**, 1585–1595.
- Yassine, M.H., Wu, S., Suidan, M.T. & Venosa, A.D. 2013b. Aerobic biodegradation kinetics and mineralisation of six petrodiesel/soybean-biodiesel blends. *Environmental Science & Technology*, **47**, 4619–4627.
- Zhang, X., Peterson, C.L., Reece, D., Moller, G. & Haws, R. 1998. Biodegradability of biodiesel in the aquatic environment. *Transactions of the American Society of Agricultural Engineers*, **41**, 1423–1430.