Anaerobic degradation of benzene and other aromatic hydrocarbons in a tar-derived plume: Nitrate versus iron reducing conditions

Johan A. van Leeuwen [a,c,*], Jan Gerritse [b], Niels Hartog [a,c], Siegmund Ertl [d], John R. Parsons [e], S. Majid Hassanizadeh [a]

[a] *Utrecht University, Department of Earth Sciences, Environmental Hydrogeology Group, Princetonplein 9, 3584 CC Utrecht, the Netherlands*
[b] *Deltares, Unit Subsurface and Groundwater Systems, Daltonlaan 600, 3584 BK Utrecht, the Netherlands*
[c] *KWR Water Research Institute, Groningenhaven 7, 3433 PE Nieuwegein, the Netherlands*
[d] *Hydroisotop GmbH, Woelkestrasse 9, Sweitenkirchen 85301, Germany*
[e] *University of Amsterdam, Institute for Biodiversity and Ecosystem Dynamics, Science Park 904, 1098 XH Amsterdam, the Netherlands*

ARTICLE INFO

ABSTRACT

The anaerobic degradation of aromatic hydrocarbons in a plume originating from a Pintsch gas tar-DNAPL zone was investigated using molecular, isotopic- and microbial analyses. Benzene concentrations diminished at the relatively small meter scale dimensions of the nitrate reducing plume fringe. The ratio of benzene to toluene, ethylbenzene, xylenes and naphthalene (BTEXN) in the fringe zone compared to the plume zone, indicated relatively more loss of benzene in the fringe zone than TEXN. This was substantiated by changes in relative concentrations of BTEXN, and multi-element compound specific isotope analysis for δ^2H and $\delta^{13}C$. This was supported by the presence of (*abcA*) genes, indicating the presumed benzene carboxylase enzyme in the nitrate-reducing plume fringe. Biodegradation of most hydrocarbon contaminants at iron reducing conditions in the plume core, appears to be quantitatively of greater significance due to the large volume of the plume core, rather than relatively faster biodegradation under nitrate reducing conditions at the smaller volume of the plume fringe. Contaminant concentration reductions by biodegradation processes were shown to vary distinctively between the source, plume (both iron-reducing) and fringe (nitrate-reducing) zones of the plume. High anaerobic microbial activity was detected in the plume zone as well as in the dense non aqueous phase liquid (DNAPL) containing source zone. Biodegradation of most, if not all, other water-soluble Pintsch gas tar aromatic hydrocarbon contaminants occur at the relatively large dimensions of the anoxic plume core. The highest diversity and concentrations of metabolites were detected in the iron-reducing plume core, where the sum of parent compounds of aromatic hydrocarbons was greater than 10 mg/L. The relatively high concentrations of metabolites suggest a hot spot for anaerobic degradation in the core of the plume downgradient but relatively close to the DNAPL containing source zone.

1. Introduction

Groundwater contamination by hydrocarbons is a global problem, mainly caused by spills and leaks related to the production and usage of petroleum products (Einarson and Mackay, 2001; Kueper et al., 2003; Röling and Van Verseveld, 2002). Typically, petroleum products are complex mixtures of hydrocarbons (McGregor et al., 2012; Sandercock and Du Pasquier, 2003; Sjögren et al., 1995). Once they invade the saturated subsurface as dense non-aqueous phase liquid (DNAPL), water

soluble compounds start partitioning from the DNAPL into the groundwater, forming contaminated groundwater plumes (Peters and Luthy, 1993). Processes that mainly reduce the actual removal of the dissolved contaminants in the groundwater plume are due to retardation through adsorption to the soil matrix, dilution through dispersion and biodegradation by microorganisms (Aronson and Howard, 1997; Micic et al., 2007; Oka et al., 2011; Rogers et al., 2002; Wiedemeier et al., 1999). At tar contaminated sites, besides the commonly reported benzene, toluene, ethylbenzene and xylenes (BTEX) in groundwater, other

---

* Corresponding author at: Utrecht University, Department of Earth Sciences, Environmental Hydrogeology Group, Princetonplein 9, 3584 CC Utrecht, the Netherlands.
E-mail address: j.a.vanleeuwen@uu.nl (J.A. van Leeuwen).

aromatic hydrocarbon compounds such as styrene, trimethylbenzenes, ethyltoluenes, indene, indane, naphthalene, methylnaphthalenes and methylindenes, are encountered (Micic et al., 2007; Schirmer et al., 2006; Zamfirescu and Grathwohl, 2001). For naphthalene, degradation rates are generally low compared to those of BTEX (Gerritse et al., 2004), while sorption dependent attenuation is a more prominent removal process due to a higher K_{oc} coefficient and relatively low water solubility (Rogers et al., 2002; Swartjes et al., 2011).

Biodegradation of one or multiple monocyclic aromatic hydrocarbons compounds has been shown to occur in laboratory and field studies under both oxic and anoxic conditions (da Silva and Corseuil, 2012; Gibson et al., 1968; Gieg et al., 2009; Grbic-Galic and Vogel, 1987) Biodegradation rates range widely and depend mostly on the molecular structure of the contaminant and the terminal electron-accepting processes (Foght, 2008; Meckenstock et al., 2016; Meckenstock et al., 2015). Complex mixtures of aromatic hydrocarbon compounds in groundwater systems often lead to sequential attenuation of the individual components (Suarez and Rifai, 1999). Biodegradation rates for some compounds might increase, in different redox zones, while they decrease for others (Suarez and Rifai, 1999; Swartjes et al., 2011). Moreover, in complex mixtures, co-metabolism can enhance biodegradation of certain recalcitrant compounds (Swartjes et al., 2011). In contrast, certain compounds can inhibit biodegradation due to toxicity (Swartjes et al., 2011). The complex interplay between multiple contaminant compounds that are present in groundwater plumes, abiotic and biotic attenuation processes, sequential redox conditions, and geohydrological setting makes it difficult to interpret biodegradation processes at contaminated sites.

While field studies have shown that under oxic conditions benzene biodegrades faster than toluene, ethylbenzene or xylenes (Alvarez et al., 1991; Suarez and Rifai, 1999), under anoxic conditions biodegradation of benzene is generally slower than that of other aromatic compounds (Weelink et al., 2010). Biodegradation of benzene under nitrate-reducing conditions has been observed (Atashgahi et al., 2018; Burland and Edwards, 1999; Coates et al., 2001; Dou et al., 2010), but the range of biodegradation rates is very wide. In laboratory experiments often benzene is the recalcitrant compound and toluene, ethylbenzene and xylenes (TEX) show biodegradation (Suarez and Rifai, 1999). However, at the field scale it was found that benzene potentially biodegrades. Nonetheless, biodegradation of TEX occurs at higher degradation rates than benzene (Aronson and Howard, 1997). It has been suggested that benzene biodegradation in shallow aquifers is actually enabled by the presence of low oxygen concentrations, along the fringe of the contaminant groundwater plumes (Aronson and Howard, 1997). Nonetheless, to date a variety of bacteria using nitrate as electron acceptor and capable of benzene degradation, have been isolated in a pure culture (Atashgahi et al., 2018; Coates et al., 2001; Dou et al., 2010; Vogt et al., 2011). More recently, laboratory studies showed strictly anaerobic degradation of benzene or naphthalene under iron or nitrate reducing conditions related to *Peptococcaceae* (members of the order *Clostridiales*). However, naphthalene degraders were not able to degrade benzene, indicating that benzene and naphthalene are biodegraded by different enzymes *abcA* and *nmsA* respectively. (Atashgahi et al., 2018; Cupples, 2016; Kleemann and Meckenstock, 2011; Meckenstock and Mouttaki, 2011; Musat et al., 2009; Toth et al., 2018; van der Waals et al., 2017; van der Zaan et al., 2012; Vogt et al., 2011). To our knowledge, no studies have been published on the relevance and presence of *Peptococcaceae* throughout contaminated plumes at the field scale.

Therefore, this field study focuses on the biodegradation of a wide range of hydrocarbons that derive from the dissolution of Pintsch gas tar, in a two-aquifer system dominated by denitrifying conditions in the surrounding native groundwater. This was performed to assess the differences on the biodegradation of individual hydrocarbon compounds and benzene in specific in the presence of different electron acceptors.

2. Materials and methods

2.1. Site description

The studied field site is located adjacent to the railway station in the city of Amersfoort, The Netherlands. On this site, a manufactured gas plant (MGP) was active from 1910 until 1958. The hydrogeological situation consists of a two-aquifer system. The shallow aquifer from 2 to 12 m below ground surface (mbgs) consists of medium to silty fine sands, and the deep aquifer from 12 to 85 mbgs of mainly coarse sands with gravel enclosures. The general direction of groundwater flow in the shallow aquifer is to the southeast and to the northwest in the deep aquifer (Fig. 1). Locally at the MGP site, groundwater flow shifts in the shallow aquifer, due to sheet piling containment measures and absence of the confining layer (Fig. 1). The hydraulic conductivity of the aquifers varies from k = 5 m/d in the shallow aquifer to k = 15 m/d in the deep aquifer. The two aquifers are separated by a clay/peat confining layer in the north (Fig. 2). The clay/peat layer thins out from more than 5 m thickness in the north, to about 50 cm or less at the MGP site. The aquitard fringe is situated some 50 m south of the DNAPL source zone (Fig. 2). In both aquifers, liquid phase tar is detected as free flowing DNAPL. More background on the site, partitioning behavior from the DNAPL towards the plume, and developed methods for detecting the dissolved contaminants and their metabolites were described earlier (Van Leeuwen et al., 2020).

The contaminated MGP site was divided into different zones, based on samples from groundwater sampling wells within each zone. The upgradient zone, represents non-impacted groundwater and is used as a reference. The source zone is the area where DNAPL is located, and the plume zone is representing the area of the groundwater dissolved contaminants. The fringe zone represents the area where contaminant concentrations in groundwater are diminishing and mixing with the surrounding groundwater occurs by dispersion. Downgradient is the area located in the direction of flow for groundwater and contaminant concentrations below 10 μg/L. In Table 1, the number of sampling wells in each zone is given.

2.2. Groundwater sampling

To create a two-dimensional, transect of the groundwater contamination in the two-aquifer system, groundwater samples were obtained from 34 sampling wells. The wells were selected along the groundwater flow direction (Fig. 1), as well as along a transversal cross section of the plume (Fig. 2). A comprehensive overview of the sampling wells is given in the supplementary information S3 to S9. To keep the groundwater samples anoxic while sampling, Marprene tubing (Rubber BV, Hilversum) in combination with a peristaltic pump (Watson-Marlow, type 604 U/R, MA, USA) was used. Some wells in the source zone contained DNAPL. To acquire clear groundwater samples, these wells were pumped at a flow rate of 10 L/h until groundwater breakthrough was established. Typically, two to sixty liters of tar was pumped before groundwater break through started (Table 2). Unaffected reference well 323 is located 145 m upgradient in the shallow aquifer.

2.3. Aromatic hydrocarbon analysis

For the determination of concentrations of aromatic hydrocarbon compounds in the groundwater samples, a gas chromatographic headspace analyses method was used. Analyses were performed on a Shimadzu GC-2010 gas chromatograph equipped with a PAL auto sampler (Shimadzu Benelux, 's Hertogenbosch, The Netherlands) as previously described (Van Leeuwen et al., 2020). The detected hydrocarbon contaminant concentrations per sample are given in section S5 of the supplementary information.
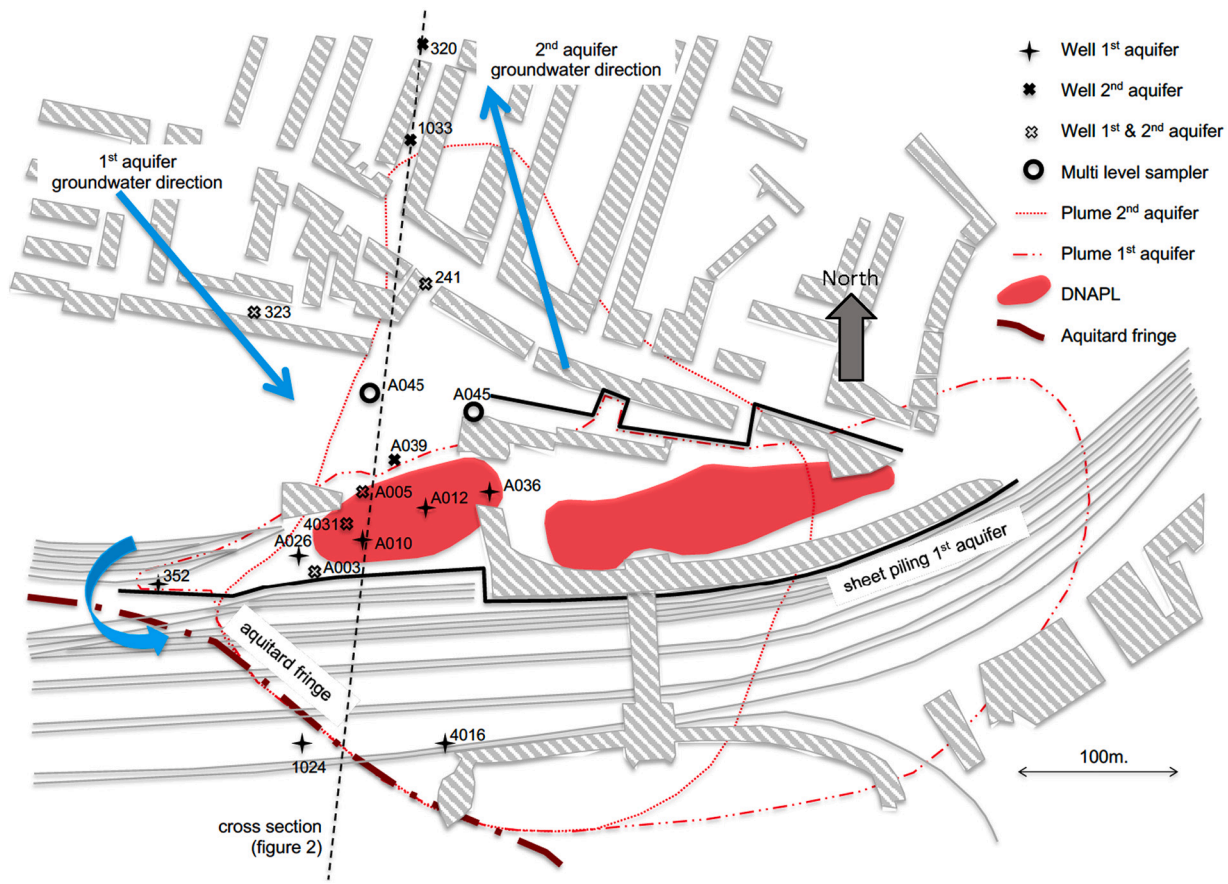
Fig. 1. Plan view of the investigated site. The four-pointed stars show the locations of groundwater monitoring wells in the shallow aquifer; x-shapes show monitoring well locations, both in shallow and deep aquifers; donut shapes represent multi-level samplers. Dashed line represents location of cross section shown in Fig. 2.
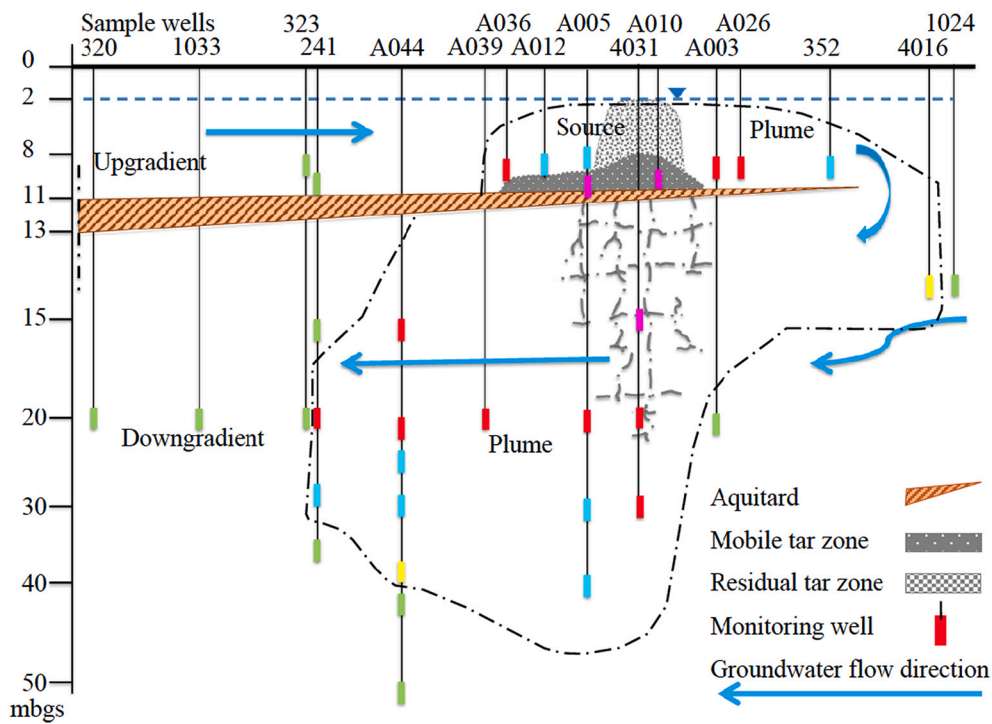

Fig. 2. Cross section of a site conceptual model (not to scale) showing the two aquifer systems and the thinning aquitard clay/peat layer. Concentrations indicated in colors are measured sum of benzene, toluene, ethyl-benzene, xylenes, and naphthalene. Green represents 0–100 µg/L, yellow 100–1000 µg/L, light blue 1000–10,000 µg/L, red 10,000–100,000 µg/L and magenta 100,000–1,000,000 µg/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Overview of contaminated site zonation and number of groundwater sampling wells per zone. Upgradient: ± 140-m upgradient from the source zone.

Well location	Upgradient	Source zone	Plume zone	Fringe zone	Downgradient
Number of wells	2 well	5 wells	13 wells	6 wells	8 wells

Plume zone: No DNAPL presence in sampling well and at least one contaminant >2 mg/L. Source zone: DNAPL presence within the sampling well. Fringe zone: No DNAPL present in sampling well and each contaminant <2 mg/L. Downgradient: sample wells are hydrogeological downgradient and each compound <10 μg/L.

Table 2

Wells sampled in the DNAPL-source zone, with well screen depth in meters below ground surface (mbgs), tar height measured from bottom of the well.

Sampling well	A010	A005	A012	A036A	4031	4031
Well screen (mbgs)	2–12	10–12	10–12	10–12	16–17	20–21
Tar height (m)	6	3.8	2.7	2.5	0.3	0.3
PBGB (L)	60	35	10	15	5	2

Pumped tar volume before groundwater breakthrough (PBGB) is given in liters (L).

2.4. Compound specific stable isotope analysis

To identify anaerobic biodegradation and the occurring biotransformation reactions CSIA ($\delta^{13}C$ and δ^2H) was performed. Samples from the contaminated groundwater plume were measured in duplicate for benzene, toluene ethylbenzene, xylenes, indene and naphthalene on a GC–MS-IRMS system consisting of a Thermo scientific Trace GC Ultra (GC), a Thermo scientific ISQ (qMS), a Thermo scientific GC Isolink (interface) and a Thermo scientific Delta V Advantage (IRMS). A comprehensive description of the used method is described in section S2 of the supplementary information. The $\delta13C$ and $\delta2H$ data per sample for the detected hydrocarbon contaminants are given in section S8 and S9 respectively of the supplementary information.

2.5. Semi-quantitative metabolite analysis

Putative metabolites of aromatic hydrocarbon degradation were analyzed by liquid chromatography, using a Shimadzu Nexera UHPLC system and a Bruker maXis 4G quadrupole orthogonal accelerated time-of-flight mass spectrometer as described (Van Leeuwen et al., 2020). Analytes were semi-quantified by using a linear relation between polarity and the molar response factor, where standards with known concentrations were used to define the unknown ones through correlation (Wang et al., 2010). The metabolite data per sample are given in section S6 of the supplementary information.

2.6. Analysis of anions, manganese and iron

Groundwater samples were analyzed for concentrations of anions (SO_4^{2-}, NO_3^- and NO_2^-) by liquid chromatography (LC) on a Dionex ICS-1500 equipped with an IONPAC AS14 anion exchange column and an A SRSs-Ultra 14 mm suppressor (Dionex Corporation, Sunnyvale CA, USA). Concentrations of dissolved manganese and iron in the groundwater samples were measured by a Perkin-Elmer Avio 500 inductively coupled plasma-optical emission spectrometer (ICP-OES). For both LC and ICP-OES analysis, groundwater was sampled in 1.5 mL vials with screw caps and silicon PTFE lined septa (Grace). The samples were sterilized in the field by addition of 0.6 mL of a 68% solution of nitric acid (Sigma-Aldrich Chemie N.V., Zwijndrecht, The Netherlands). An overview of environmental conditions such as pH, redox, electric conductivity (EC), dissolved organic carbon (DOC) and oxygen are given in section S3 of the supplementary information. Physico chemical data is given in section S4 of the supplementary information.

2.7. DNA extraction and qPCR analysis

For the extraction of DNA, 1-l groundwater samples were collected from monitoring wells in clean green glass bottles. To prevent the introduction of oxygen, the bottles were filled completely from the bottom up, and let to overflow for a few minutes before sealing with a polypropylene screw cap. The bottles were immediately put-on ice in a cool box and transported to the laboratory. The 1-l samples were vacuum filtered within 24 h. For filtration, 47-mm diameter, 0.22 μm pore size, hydrophilic filters were used (Millipore BV, Amsterdam, The Netherlands). DNA was subsequently extracted with the MoBio Powerlyzer kit according to the supplier's protocol (MoBio, CA, USA). DNA extracts were stored at −80 °C until analyzed.

Genes of general groups of micro-organisms were detected that signify redox conditions, such as nitrate reducers (*narG*), sulfite reducers (*drsA*), iron (III) reducers (16S rRNA *Geobacteraceae*), methanogens (*mcrA*), and *Archaea* (16S rRNA) Additionally, genes indicating bacteria and enzymes were targeted that are known to metabolize benzene, such as *Peptococcaceae* and benzene carboxylase (*abcA*). Furthermore, two kinds of benzylsuccinate synthase (*bssA*) genes, involved in toluene activation were analyzed: those associated with sulfate-, iron (*bssA*_SRB) reducing and nitrate (*bssA*) reducing micro-organisms. Additionally, total bacteria were quantified (16S rRNA). An overview of all analyzed genes encoding and primers, is given in the supplementary information S1. Assays for qPCR analysis were performed on a CFX96 Real-time PCR machine (Bio-Rad) as described (van der Waals et al., 2017). Detected gene copies for used essays are given per sample in section S7 of the supplementary information.
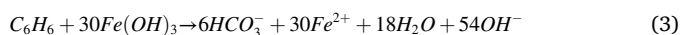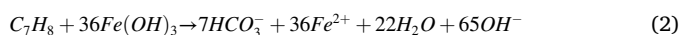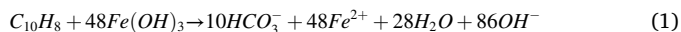
3. Results and discussion

3.1. Distribution of electron acceptors

Dissolved oxygen concentrations were below 0.5 mg/L, indicating anoxic conditions in all sampled groundwater wells. In groundwater wells surrounding the contaminated area, nitrate (24–33 mg/L) and sulfate (31–36 mg/L) were present. A comprehensive list of hydrogeochemical data can be found in the supplementary information section S3 and S4. Nitrate was depleted in the upgradient part of the source zone. The complete oxidation of 1 mol of benzene or toluene to CO_2, would require reduction of 6 or 7.2 mol of nitrate to N_2, respectively (Burland and Edwards, 1999), neglecting possible nitrate reduction to ammonium. The influx of groundwater containing 24–33 mg/L nitrate can therefore serve as an electron acceptor for the oxidation of only a relatively small fraction of the contaminant load present.

Sulfate concentrations decreased along the groundwater path of flow and remained low (0.1–7.5 mg/L) throughout the plume zone, along the groundwater path of flow. In only 4 out of 34 sampling wells was sulfate below 1 mg/L.

Dissolved iron and manganese were not detected in the uncontaminated upstream reference well. In contrast, dissolved iron and manganese were detected in relatively high concentrations up to 14.6 mg/L, within the contaminated plume and fringe zones. This is an indication of iron(III) and manganese(IV) reduction by bacteria such as *Geobacteraceae*, which are known to use a wide range of aromatic compounds (Nealson, 1994). Dissolved iron and manganese were detected at similar concentrations, which is remarkable, since generally iron is five to ten times more abundant in soil systems than manganese (Nealson, 1994). This observation may be explained by precipitation of ferrous iron with sulfide produced through sulfate reduction. The solubility in water of FeS is negligible, whereas that of MnS is 47 mg/L at 18 °C (Nealson, 1994). It was reported that about 60% of the iron (III) had been reduced to iron (II) in the source zone, and 35% of the ferric iron in

the plume zone due to biodegradation of aromatic hydrocarbons from a tar contaminated site (Wege, 2005).This suggests that at least 60% of iron (III) in an aquifer was available to microorganisms for biodegradation of hydrocarbons over several decades. Moreover, based on this outcome calculating the potential biodegraded contaminant load through iron(II) reduction, provides an insight, in present and future groundwater plume stability. Stoichiometric relationships for the oxidation of naphthalene, toluene and benzene are as follows:

$$C_{10}H_8 + 48Fe(OH)_3 \rightarrow 10HCO_3^- + 48Fe^{2+} + 28H_2O + 86OH^- \quad (1)$$

$$C_7H_8 + 36Fe(OH)_3 \rightarrow 7HCO_3^- + 36Fe^{2+} + 22H_2O + 65OH^- \quad (2)$$

$$C_6H_6 + 30Fe(OH)_3 \rightarrow 6HCO_3^- + 30Fe^{2+} + 18H_2O + 54OH^- \quad (3)$$

Stoichiometric relations imply that 2,7 kg of ferric iron are needed to biodegrade 1 mol (128 g) of naphthalene. For biodegradation of 1 mol of benzene (78 g) or toluene (92 g) 1.7 and 2 kg of ferric iron are needed, respectively (Eq. (2) and (3)). In the Netherlands, generally 1.5–30 g/dm^3 Fe(OH)$_3$ is present in sandy soils (De Vries, 1999). This implies that for every kilogram of naphthalene a soil volume of on average 1.3 m^3 is potentially sufficient as electron acceptor. The total dimension of the contaminant plume zone at the investigated site is approximately 3.3 million m^3. The contaminated soil volume of the plume at the former MGP site in Amersfoort would potentially hold sufficient Fe(III) as electron acceptor for the biodegradation of 2.5 million kg naphthalene. Moreover, acceptors such as nitrate and sulfate flow into the groundwater contaminated plume, over an inflow area of 12,000 m^2 (300 m^1 wide and down to 40mbgs). The aquifer flows at a constant rate of ±25 m/y, which implicates 300,000m^3/y of water inflow into the contaminated area. This amount of groundwater contained of 0,024 kg/m^3 and 0,035 kg/m^3 of nitrate and sulfate respectively in the upgradient reference well. This would roughly contribute an additional 7200 kg/y of nitrate and 10,500 kg/y of sulfate as potential terminal electron acceptor to the contaminated area. Ferric iron is not replenished like nitrate and sulphate and might potentially deplete. Moreover, various chemical reactions between iron and sulfur can occur in the fringe zone. Due to a relatively large iron reducing volume of the plume compared to a small nitrate reducing volume at the fringe zone, reduction of ferric iron coupled to relatively slower biodegradation of most hydrocarbon contaminants within the plume zone, appears quantitatively to be of greater significance than coupled to nitrate reduction in the fringe zone.

3.2. Distribution of aromatic hydrocarbons

In the source zone, the relatively light hydrocarbon compounds partition from the tar into the surrounding groundwater. The liquid DNAPL in the source zone, is considered to be an instantaneous solute source. The composition of the initial solute mixture (C_0) is determined by averaging the concentrations of partitioned tar compounds to water and is shown in (Fig. 3) and described by (Van Leeuwen et al., 2020). The nine most abundant compounds that have partitioned into the groundwater are: naphthalene (29%), followed by toluene (22%) and benzene (15%). Other compounds such as 2-methylnaphthalene (2-MN), styrene, indene, xylenes and ethylbenzene were detected varying from 8% down to 2%. The other 15 detected aromatic compounds at lower concentrations than ethylbenzene are not considered here (not shown in Fig. 3). An overview of all detected contaminant compounds per sampling well is given in the supplementary information S5.

Propagation of the contaminant mixture by groundwater transport phenomena, such as dispersion/dilution and retardation have an prominent effect on the concentration profile along the groundwater path of flow. Composition of the aromatic hydrocarbon mix is besides dispersion, affected by retardation. For each compound. K_{oc} values and retardation values for the nine most abundant aromatic hydrocarbons detected in the contaminant groundwater are given in Table 3 (Abraham
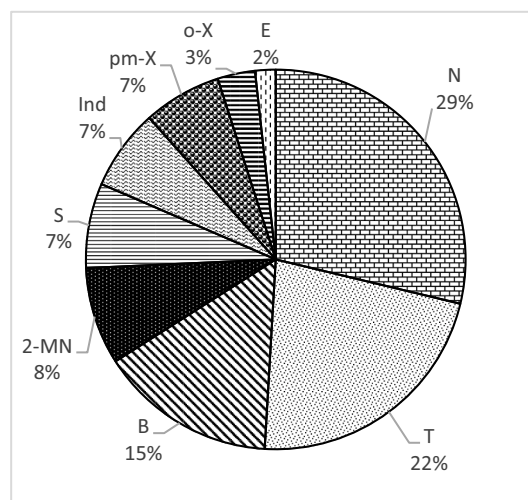
Fig. 3. Nine most abundant hydrocarbon compounds in the aqueous phase within the DNAPL source zone. Concentrations represent the average of four samples taken from the pool zone. N = naphthalene, T = toluene, B = benzene, 2-MN – 2-methylnaphthalene, S = styrene, Ind = indene, pm-X = para- and metaxylene, o-X = orthoxylene, E = ethylbenzene.

Table 3

Log Koc of the nine most abundant aromatic hydrocarbons compounds partitioned into the groundwater in the source zone.

	Log K_{oc}	Retardation factor
		R
N	2.91	2.14
2-MN	2.83	1.95
pm-X	2.48	1.42
E	2.48	1.42
o-X	2.46	1.41
S	2.33	1.3
Ind	2.92	2.17
T	2.17	1.21
B	1.73	1.08

2-methylnaphthalene (2-MN), naphthalene (N), para- and metaxylene (pm-X), ethylbenzene (E), orthoxylene (o-X), styrene (S), Indene (Ind), toluene (T), benzene (B). organic carbon content (foc) = 1%, bulk density 1.8, porosity 0.32.

et al., 1994; Freeze and Cherry, 1979). The K_{oc} values used are obtained from chemical database (gsi-net.com).

In the plume zone, the composition of the contaminant mixture has changed, compared to the source zone. In the source zone naphthalene and toluene were the most abundant compounds (Fig. 3), whereas in the plume zone, the most abundant compounds were ethylbenzene and benzene, with values of 0.56 and 0.71C/C$_0$, respectively (Fig. 4). This may be attributed to the retardation effect, as the K_{oc} value for ethylbenzene, benzene and other aromatic hydrocarbons is lower than that of naphthalene (Fetter, 1999) (Table 3). Another compound from the most abundant hydrocarbon compounds, 2-methylnaphthalene (2-MN), has a relatively high K_{oc}, and is expected to decrease less than naphthalene, based on retardation. However, 2-MN decreased relatively the most of all compounds from the source zone to the plume zone (Fig. 4). Naphthalene is expected to decrease the most if only retardation played a role. This is indicative for biodegradation of 2-MN within or relatively close (± 10 m^1) to the source zone.

Further downgradient (± 50–150 m^1), from the plume zone to the fringe zone, the highest relative loss was not observed for 2-MN, but for styrene and toluene. This suggests, further down the groundwater path of flow in the contaminated groundwater plume, biodegradation is different for 2-MN than close to the source zone and relatively less than for styrene and toluene.
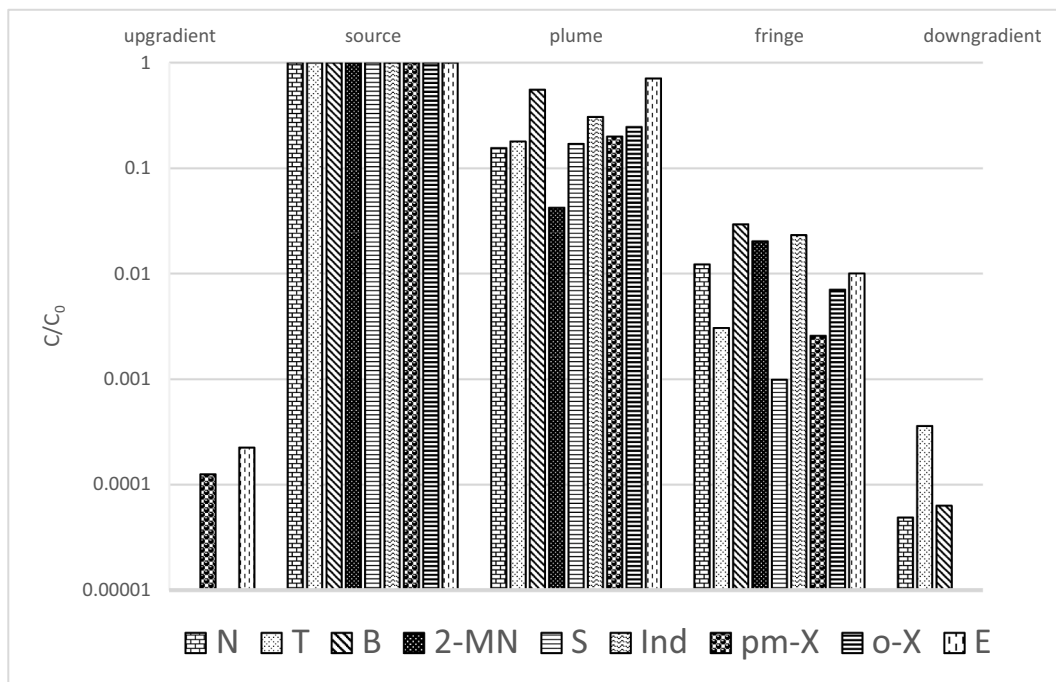
Fig. 4. Aromatic compounds per contamination zone. From left to right average concentrations sampled in each zone. Sample wells per zone are given in Table 1. Abbreviations on x-axis represent: N = naphthalene, T = toluene, B = benzene, 2-MN = 2-methylnaphthalene, S = styrene, Ind = indene, pm-X = pm-xylene, o-X = o-xylene, E = ethylbenzene.

Further downgradient at the boundary of the groundwater plume, where the plume zone transitions into the fringe, and nitrate reducing conditions prevail, the concentration of ethylbenzene is observed to decrease 2.5 times more than the upgradient decrease from source- to plume zone. Remarkably, the ratio of benzene to toluene changes at these downgradient nitrate reducing conditions at the boundaries of the contaminated groundwater plume. Upgradient from source zone to

plume zone and from plume zone to fringe zone toluene concentrations are lower than benzene, moreover, further downgradient this is reversed, and toluene concentrations are higher than benzene (Fig. 4).

Overall, the relative decrease of contaminant compound differences from source zone to plume zone compared to fringe zone to downgradient in other ratios than cannot be explained by retardation. This suggests that biodegradation occurs for most contaminant compounds.
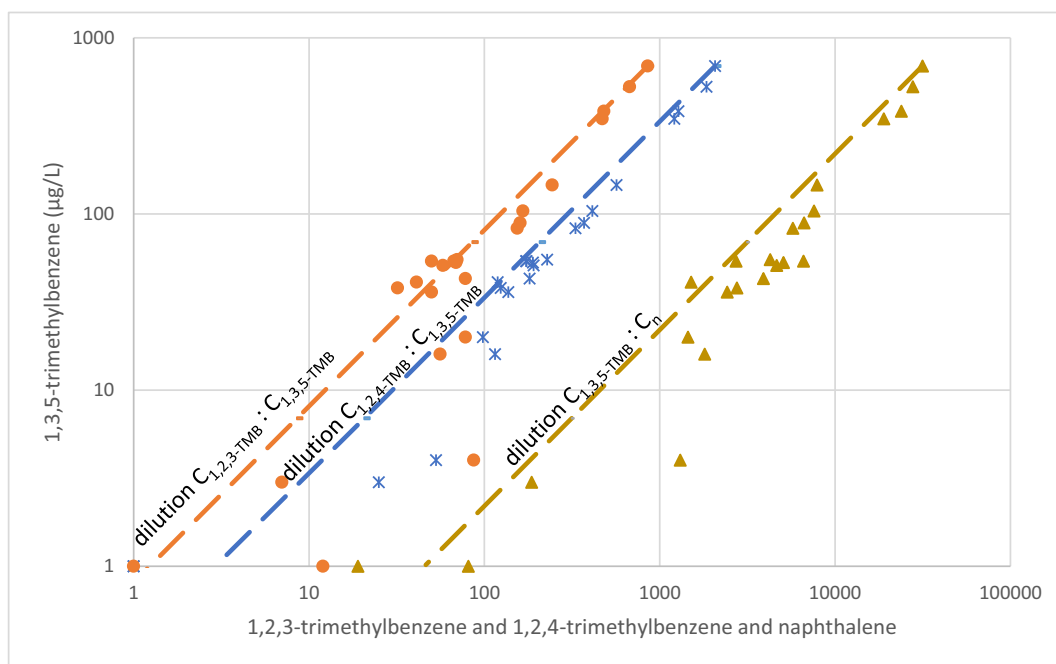

Fig. 5. Concentrations of 1,3,5-trimethylbenzene ($C_{1,3,5-TMB}$) versus 1,2,3,-trimethylbenzene in orange ($C_{1,2,3-TMB}$) 1,2,4-trimethylbenzene in blue ($C_{1,2,4-TMB}$) and naphthalene in ochre (C_n). The dotted line represents 1-D dilution. All three contaminants show a relatively good linear fit considering soil heterogeneity and retardation over the ±300-m sampled trajectory of the groundwater plume. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Moreover, decreases of individual contaminant compounds within the mix, differ along the groundwater path of flow in different zones within the contaminant plume (Fig. 4).

To interpret the composition changes of compounds in the complex contaminant mixture along the path of groundwater flow, two-dimensional plots were produced, where the concentration of 1,3,5-trimethylbenzene (1,3,5-TMB) was plotted versus contaminant compounds as presented in Fig. 4 ($C_{1,3,5\text{-}tmb}$: C_x) using all obtained groundwater samples (Figs. 5 and 6). Where $C_{1,3,5\text{-}TMB}$ is the concentration is of 1,3,5TMB and C_x the concentration of a specific aromatic hydrocarbon from the contaminant mix.

Naphthalene, 1,2,3-trimethylbenzene (1,2,3-TMB) and 1,2,4-trimethylbenzene (1,2,4-TMB) show a comparative fit to linear dilution or dispersion only (Fig. 5). Benzene and toluene show the least fit to linear dilution and benzene as well as toluene concentration decrease relatively more than 1,3,5-TMB at relatively lower concentrations (Fig. 6). Although soil heterogeneity and retardation effects play a role in groundwater transport of contaminants, relatively for the contaminant mix benzene shows the most deviation from dilution and naphthalene together with trimethylbenzenes the least (Fig. 5). This indicates that naphthalene and trimethylbenzenes are the most conservative compounds in the contaminant mix. Tri-substituted alkylbenzenes are less vulnerable to microbial degradation than monosubstituted alkylbenzenes (Peng et al., 2021). Moreover 1,3,5-TMB is recognized as the most conservative degrading aromatic hydrocarbon compound (Aamand et al., 1989; Aronson and Howard, 1997; Baedecker et al., 2011; Peng et al., 2021; Richnow et al., 2003). Other contaminant compounds such as xylenes, ethylbenzene, styrene and 2-methylnaphthalene show, however less distinct, decreased relative concentrations corresponding to benzene and toluene versus 1,3,5-TMB.

Naphthalene and benzene both non-substituted cyclic aromatic hydrocarbons are also known as anaerobically slow degrading compounds relative to other water soluble tar constituents (Aronson and Howard, 1997; Flanagan et al., 2014; Wiedemeier et al., 1999). The data obtained from this contaminated groundwater plume show that naphthalene is behaving in accordance, however benzene is not. Benzene concentrations decrease in the same order as toluene concentrations when plotted to the most persistent compound in the mix 1,3,5-TMB (Fig. 6).

Considering various redox conditions along the path of flow, concentration ratios for benzene (C_B) and toluene (C_T) could be occurring at different conditions and are given in (C_B:C_T), Fig. 7. In the source zone, which is considered an instantaneous point source, concentrations are relatively high for benzene (30,000 μg/L) and toluene (32,000 μg/L). The majority of samples containing dissolved iron and manganese, yet no nitrate, show a ratio of greater than one for benzene versus toluene (Fig. 7). This could be explained by relatively more adsorption of toluene to the soil matrix since benzene has a lower retardation factor. However, some of the samples show a ratio smaller than one for benzene versus toluene (Fig. 7). These samples relate to the fringe zone and its nitrate reducing conditions. The surrounding groundwater contains nitrate, which mixes in with the contaminated groundwater, due to dispersion. The relatively high toluene versus low benzene concentrations, indicate more loss of benzene than of toluene under the nitrate reducing conditions at the fringe of the contaminant plume. This composition change was also observed in sample locations above the source zone, where an inflow of nitrate containing groundwater from upgradient occurs. The lower ratio of benzene versus toluene in the up- and downgradient locations cannot be explained by retardation effects or dilution through dispersion and therefore indicates greater biodegradation of benzene than toluene at nitrate reducing conditions in the plume fringe.

3.3. Multi-element compound-specific isotope fractionation

Fractionation of stable isotopes of hydrogen ($^2H/^1H$) and carbon ($^{13}C/^{12}C$) was determined for BTEX, indene, and naphthalene in contaminated groundwater samples. To determine direct insight in the reaction mechanisms for biodegradation, hydrogen (δ^2H) and carbon ($\delta^{13}C$) enrichments were combined, into two-dimensional compound specific isotope analysis plots. The slope of the linear regression, leads to the apparent reaction mechanism and is expressed as the lambda value (Λ) (Kümmel et al., 2016) (Fig. 8). Lambda values indicate putative reaction mechanisms of aromatic hydrocarbon degradation pathways corresponding to carboxylation, fumarate addition, and hydroxylation (Vogt et al., 2016). The Λ-value found for toluene ($\Lambda = 25$) and o,m-xylene ($\Lambda = 20$) both corresponded to anaerobic degradation via
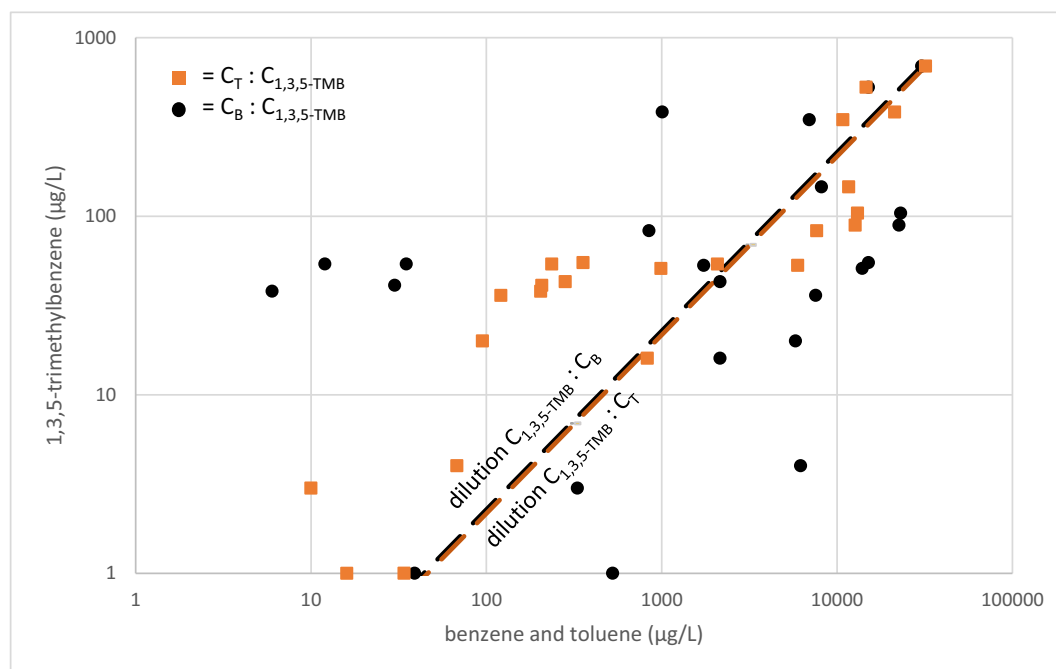

Fig. 6. Concentrations of 1,3,5-trimethylbenzene ($C_{1,3,5\text{-}TMB}$) versus benzene (C_B) and toluene (C_T). The dotted line represents 1-D dilution. Benzene and toluene show a relatively bad linear fit considering soil heterogeneity and retardation over the ±300-m sampled trajectory of the groundwater plume.
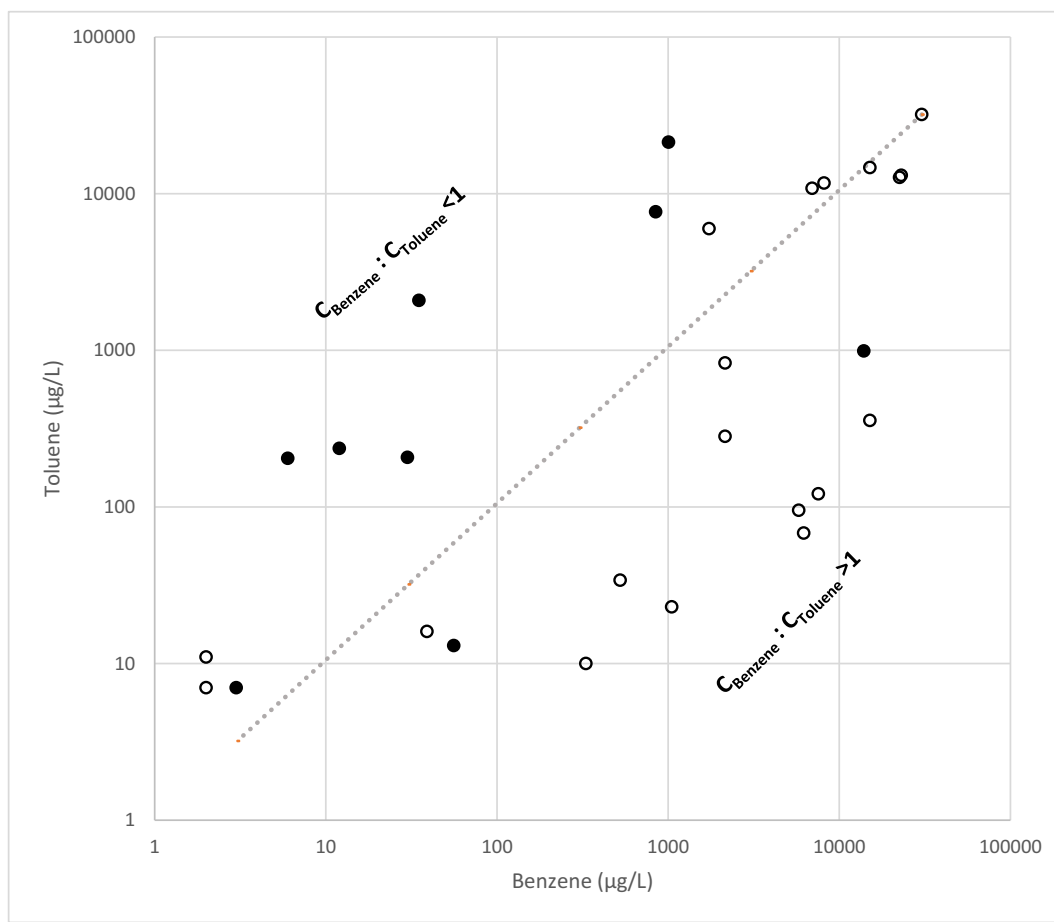
Fig. 7. Concentration of benzene versus toluene for all sampled wells, donut shapes represent samples containing no nitrate presence and black dots represents nitrate containing samples. The dotted line represents dilution only (C_B: $C_T = 1$). Ratio of benzene to toluene is smaller when nitrate is present, indicating more loss of benzene than toluene when nitrate is present. No replicate samples have been analyzed.

succinylation by fumarate addition (Fig. 8). Benzene isotope fractionation ($\Lambda = 19$), corresponds to degradation via the putative carboxylation reaction mechanism, in which benzene is carboxylated yielding benzoate (Abu Laban et al., 2010; Luo et al., 2014; Vogt et al., 2016).

While the generated lambda value for ethylbenzene suggests hydroxylation, this is based mainly on one datapoint representing high hydrogen fractionation. The latter suggest a different reaction mechanism. However, no lambda values are published that represent other reaction mechanisms for ethylbenzene such as fumarate addition. Furthermore, benzoylacetate was the only signature metabolite detected for ethylbenzene degradation, which relates to the hydroxylation mechanism. This metabolite was detected in 8 groundwater samples originating throughout the contaminated groundwater plume. An overview of all detected metabolites per monitoring well are given in supplementary information S6.

The Λ-value for naphthalene has a poor correlation ($R^2 = 0.51$) and does not correspond with a known carboxylation mechanism (Fig. 8). This can be explained by masking of kinetic isotope fractionation because of the relatively low solubility of naphthalene, resulting in diffusion controlled degradation (Vogt et al., 2018). The Λ-value for indene has the least correlation of the presented 2D plots and no reference data could be acquired to compare the results with (Fig. 8).

The lowest values for fractionation of hydrogen ($\delta^2 H$) and carbon ($\delta^{13} C$) isotopes were generally found in samples from the source zone and its proximity. This indicates that a relatively large fraction of the total amount of contaminants in the source zone has not been biodegraded. Small fractionation values of carbon and hydrogen might not be reliably quantified and could be underestimated due to ongoing

partitioning of aromatic hydrocarbons from the DNAPL into the bypassing groundwater (Van Leeuwen et al., 2020). Moreover, the partitioning effect induced by groundwater flow, could be increased by higher mass transfer rates caused by biodegradation (Langevoort and Hassanizadeh, 2009). Simultaneous biodegradation and source partitioning could underestimate the biodegradation effect in the source zone (Vogt et al., 2016). Moreover, ongoing partitioning of contaminant compounds from the instantaneous source to the groundwater, potentially could create a double masking effect due to the fact that the partitioning takes place to an already biodegrading hydrocarbon mixture in the water phase and simultaneously is diluted by inflow of less contaminated groundwater from upgradient. (Van Leeuwen et al., 2020).

3.4. Metabolites of aromatic hydrocarbon biodegradation

The highest total concentration of metabolites in a single well (545 µg/L) was found at the start of the plume (well A026). This well is located approximately 35 m downgradient of the source zone (Fig. 1, Fig. 2). This suggests that the start of the plume, governed by metal reducing conditions corresponds to a relatively high bio-active zone (Abu Laban et al., 2010; Beller et al., 2008; Jobelius et al., 2011a, 2011b; Kleemann and Meckenstock, 2011; Nealson, 1994), or metabolites accumulate due to conditions preventing complete contaminant mineralization. In the source zone the second highest concentration of metabolites in a single well (321 µg/L) was detected. In the fringe zone, upgradient and downgradient highest total metabolite concentrations for a single well were measured at 40 µg/L, 37 µg/L and 50 µg/L,
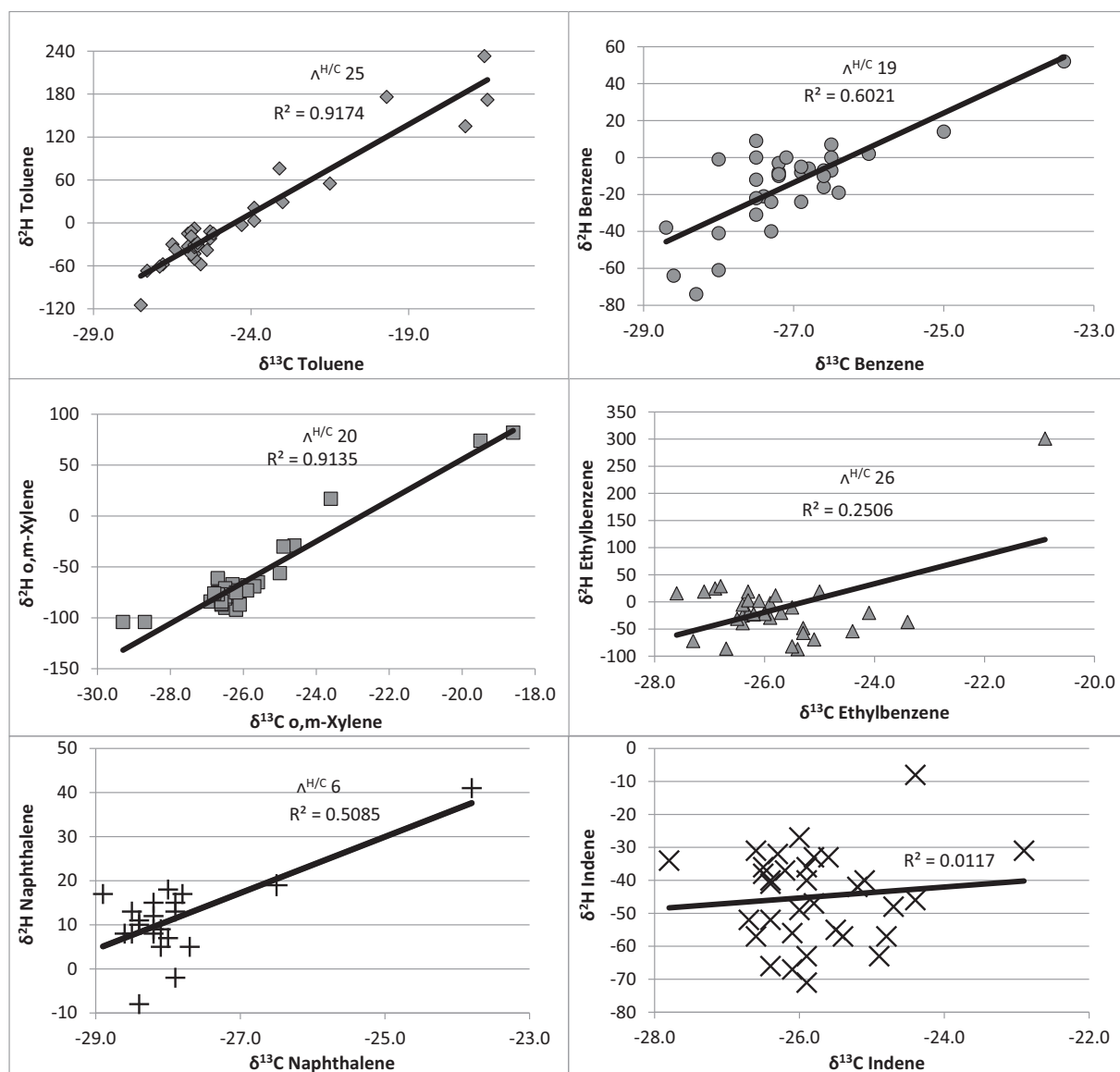
Fig. 8. Two-dimensional plots for δ^2H and $\delta^{13}C$. Lambda values (Λ) are shown for toluene, benzene, o,m-xylene, ethylbenzene and naphthalene. $\Lambda \cong \varepsilon$ bulk H/ε bulk C. Analytical standard deviation varies from ± 0.1–0.5 mUr for $\delta^{13}C$ and ± 2–8 mUr for δ^2H.

respectively. A comprehensive overview of all detected metabolites per groundwater sample is given in section S6 of the supplementary information.

These findings indicate that biodegradation occurs in the plume zone and in the source zone. The upgradient reference sample, fringe zone and downgradient samples, all show some metabolite presence, however at relatively low concentrations.

It has been reported that the highest concentrations of metabolites occurred at plume fringes (Abu Laban et al., 2010; Elsner, 2010), while at this MGP site in Amersfoort, The Netherlands, the highest concentrations were detected within the source zone and at the start of the plume. Limited electron acceptor availability at this site may have resulted in metabolite accumulation (Jobelius et al., 2011a). Another explanation for relatively high concentrations of metabolite presence is that although the source zone and the start of the plume zone have redox conditions such as iron reducing or methanogenic at which biodegradation is relatively slow, these conditions may be considered high bioactive zones, due to an abundance of both hydrocarbon compounds and their degrading bacteria. Absence or lower concentrations of metabolites in the fringe zone compared to the plume- or source zone, can

be explained by the relatively fast degradation of both parent compounds and their metabolites, due to nitrate availability in fringe zone.

Over 40%, of the metabolite concentration detected in the plume zone, were signature metabolites related to toluene degradation. The highest concentration was found for benzylsuccinic acid (211.8 μg/L), a signature metabolite for toluene (Callaghan, 2013; Griebler et al., 2004). While in the source zone benzylsuccinic acid was absent.

Although naphthalene showed little scatter when plotted versus o-xylene and is suggested to biodegrade less than benzene and toluene, approximately 25% of the metabolite concentration detected in the plume zone were related to the anaerobic naphthalene degradation. The highest concentration of suspect metabolite was found for naphthaleneacetic acid (67.3 μg/L), a so far metabolite earlier hypothesized, yet unverified, in the naphthalene methylation pathway (Safinowski and Meckenstock, 2006). Moreover, nine specific metabolites for naphthalene carboxylation were detected in multiple samples including dimethyl benzoic acid (36.8 μg/L), and 2-naphtoic acid (38.1 μg/L) (Callaghan, 2013). Naphthalene metabolite concentrations were generally the highest in the plume zone, except for 2-naphtoic acid (61 μg/L), which was detected as the highest concentration in the source

zone. The other naphthalene metabolites related to carboxylation were also present in the source zone, however at lower concentrations than in the plume zone. This either suggests that the first step in the carboxylation pathway is more apparent in the vicinity of the DNAPL than in the groundwater downgradient, or biodegradation is more limited and more accumulation occurs (Callaghan, 2013).

In the source and plume zone, other individual metabolites for anaerobic biodegradation were detected related to parent compounds ethylbenzene, indene, naphthalene, acenaphthene, phenanthrene and fluorene (Annweiler et al., 2000; Beller and Spormann, 1998; Callaghan, 2013; Chee-Sanford et al., 1996; Cozzarelli et al., 1990; Gieg and Suflita, 2002; Morasch et al., 2004; Safinowski, 2005; Tischler and Kaschabek, 2012). A few metabolites were found at highest concentrations within the source zone, such as dimethylbenzoic acid, methylbenzoic acid and benzoic acid. Another compound that was detected in groundwater samples, 2-methylindene, could possibly be a compound originating from the tar, or a metabolite formed through biodegradation of indene in the aquifer, similar to methylnaphthalenes (Callaghan, 2013). Furthermore 2-ethylhexanol, a metabolite for anaerobic biodegradation of styrene (Tischler, 2015) and 4-amino benzoic acid a metabolite from aniline (Schnell and Schink, 1991) degradation were detected.

Some metabolites, such as 3-o-toluoylpropionic acid and benzoic acid, were not only detected in the contaminated area, but also up- and downgradient, though in relatively low concentrations. These metabolites could originate from the contaminated zone upgradient; however, it is likely that benzoic acid is also formed during degradation of aromatic compounds in natural organic matter present in the aquifer.

High concentrations of metabolites in the source and plume zone, instead of at the fringe zone, might suggest that they were discharged from the DNAPL. However, since they are thermally relatively unstable, they would have disintegrated at the high temperatures during the Pintsch gas fabrication process (Van Leeuwen et al., 2020). The presence of carboxylated and succinylated metabolites therefore indicate active anaerobic degradation in the DNAPL containing source zone and the plume (Aitken et al., 2004; Bian et al., 2015; Callaghan, 2013; Elshahed et al., 2001; Head et al., 2003).

3.5. Distribution of microorganisms and enzymes

Gene copies of generic microorganisms, that use specific electron acceptors indicating redox conditions, were analyzed in groundwater samples. Gene copies indicating *Geobacteraceae*, nitrate and sulfate reducing micro-organisms, and methanogenic *Archaea* were found throughout the investigated area at concentrations from 10^3 per mL for methanogens up to 10^7 per mL for total bacteria (Fig. 9). This suggests that these micro-organisms co-exist, or that different microbial niches exist in a smaller domain than the one-meter filter length of the groundwater sampling wells (Rivett et al., 2008). Another possibility is that Archaea that perform pathways in the N-cycle are present, such as denitrification, although much less is known in Archaea than in bacteria (Cabello et al., 2004).

Gene copy concentrations of sulfite reducers (*drsA)* and iron reducing *Geobacteraceae* 16S rRNA were one order of magnitude higher in the source zone than observed anywhere else in the aquifer. Total 16S rRNA bacterial and *Archaea* genes in the source zone were two orders of magnitude higher than in the contaminant plume area (Fig. 9).

In samples from the source zone(s), gene copies of *nirS* and *narG* were detected in thousands or tens of thousands per mL of sampled groundwater, although no nitrate or nitrite was detected in those samples (Fig. 9).

In addition to detected genes related to use of specific electron acceptors, DNA was targeted for bacteria and enzymes known capable of anaerobic biodegradation of mono- and bicyclic aromatic hydrocarbons, such as *Peptococcaceae* 16S rRNA genes, benzene carboxylase *(abcA* genes)* and benzylsuccinate synthase (*bssA* genes)* (Fig. 10). The functional *bssA* genes for anaerobic toluene degradation via succinylation were found throughout the aquifer in relatively high concentrations, suggesting toluene degradation all over the investigated area. The highest gene copy numbers of *bssA* genes for iron and sulfate reducers were detected in the source zone, while the highest concentration of *bssA* genes of nitrate-reducers was detected in the plume zone (Fig. 10). These results indicate presence of microorganisms capable of toluene degradation by succinylation over the whole contaminated area, at iron, sulfate and nitrate reducing conditions.

The highest concentrations of *Peptococcaceae* 16S rRNA genes were detected in the source zone, but they were also present in the plume
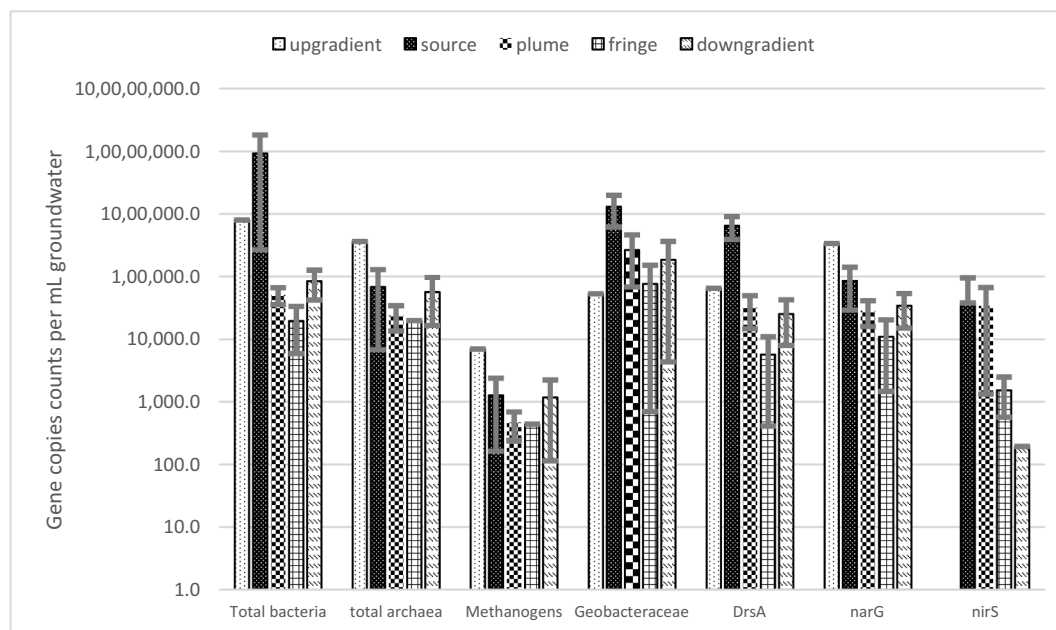

Fig. 9. Gene copy concentrations in distinct contamination areas, upgradient, source zone, plume area, fringe, downstream for total-, sulfate- and nitrate reducing, metal reducing Geobacteraceae, methanogens and archaea. The data was averaged over the number of wells in each contamination area as given in Table 1.
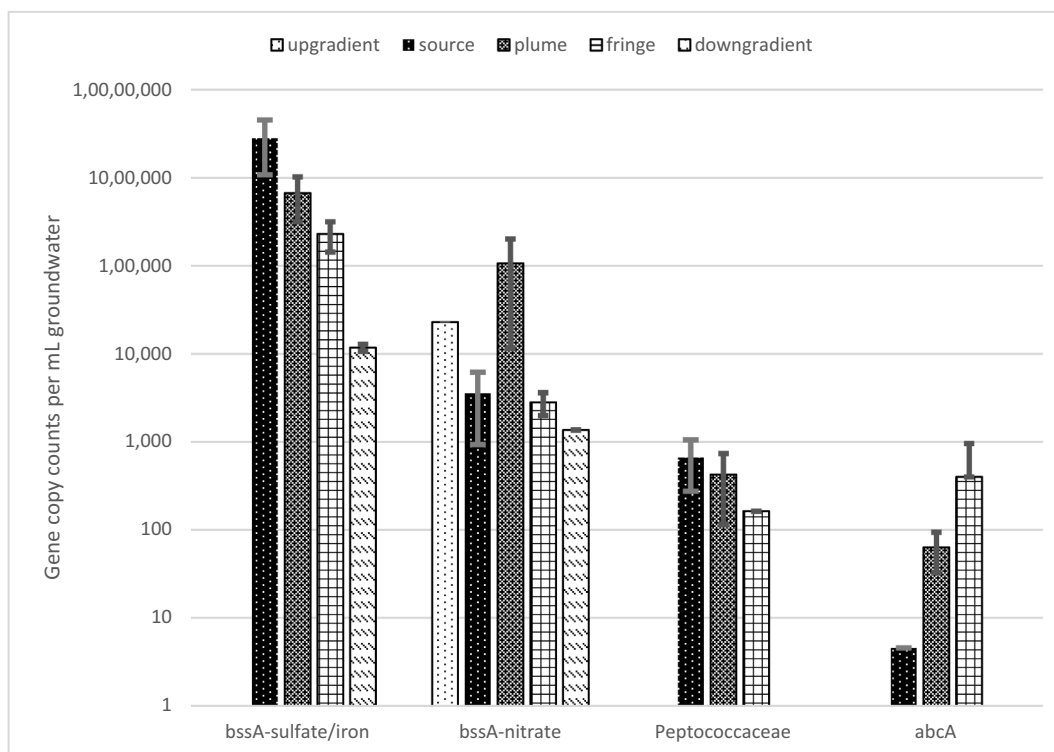
Fig. 10. Genes indicating microorganisms and enzymes capable of anaerobic toluene and benzene degradation. Gene copies are given for contaminant zone upgradient, source zone, plume area, plume fringe, downstream. For *Peptococcaceae*, benzene carboxylase (*abcA*)and benzylsuccinate synthase (*bssA*) at sulfate and nitrate reducing conditions.

zone, and at the fringe of the contaminated area (Fig. 9). *Peptococcaceae* 16S rRNA genes were detected in relatively low concentrations from hundreds up to thousands of gene copies per mL groundwater, compared to *bssA* genes. This suggests that *Peptococcaceae,* known to be involved in anaerobic benzene and naphthalene degradation, may have contributed to the anaerobic degradation of benzene and naphthalene at this site (Abu Laban et al., 2010; Kleemann and Meckenstock, 2011; Luo et al., 2014; van der Waals et al., 2017; van der Zaan et al., 2012).

Genes encoding for the benzene carboxylase enzyme (*abcA*) in nitrate reducing *Peptococcaceae* (Toth et al., 2021),were detected in relatively low concentrations in the source-, and plume zones. The highest concentrations of *abcA* genes were detected at the plume fringe (Fig. 10). This indicates that the carboxylation reaction mechanism may be involved in benzene degradation in the vicinity of the nitrate containing groundwater at the fringes of the plume. Copy numbers of *Peptococcaceae* were lowest, in fringe samples, and numbers of *abcA* genes were highest. Considering copy numbers of *Peptococcaceae* were highest and numbers of *abcA* genes were lowest in the source zone samples. This indicates that several *Peptococcaceae* were not involved in benzene degradation.

The lowest concentration of bacterial 16S rRNA gene copy numbers was found at the plume fringe, and downgradient outside the contaminant plume. Although concentrations of gene copies for bacteria and *Archaea* are relatively high in the upgradient reference well, genes indicating aromatic hydrocarbon degrading microorganisms such as benzylsuccinate synthase by sulfate and iron reducers, *Peptococcaceae* and benzene carboxylase (*abcA*) were below detection limit of the identification method. The amount of biodegraded contaminants is highest in the source zone. This most likely explains why the source zone is teaming with microbial life. In the fringe zone biodegradation is potentially the fastest under nitrate reducing conditions, however concentrations have decreased heavily before arriving that far downgradient, and thus are merely a polishing step influencing the length of the contaminant plume.

4. Conclusions

The results of this study show that benzene concentrations decrease relatively slow compared to other monoaromatic hydrocarbons at the iron reducing core of the groundwater plume but is the most decreasing component in the contaminant mix at the nitrate reducing plume fringe. Biodegradation of benzene was confirmed in the source zone and all along the groundwater path of flow by detection of signature and suspect metabolites, relevant micro-organisms, and fractionation of stable isotopes. Changes in stable isotopic ratios for $\delta^2 H$ and $\delta^{13}C$, along with carboxylated metabolites and micro-organisms responsible for anaerobic degradation of aromatic hydrocarbons, indicated biodegradation for benzene via the carboxylation mechanism. The concentration ratio of benzene versus toluene, ethylbenzene, xylenes and naphthalene shifted from greater than one at iron reducing conditions to smaller than one at nitrate reducing conditions. Moreover, benzene carboxylase (*abcA*) genes, were in accordance most abundant at the nitrate reducing plume fringe.

Overall composition of the contaminant compounds changes along the groundwater path of flow in other ratios than can be explained by dispersion nor adsorption. In addition, metabolites of xylenes and trimethylbenzenes were detected in the source zone at their highest concentrations. The highest diversity and concentrations of most metabolites were detected in the upgradient part of the plume zone, near the source zone.

This indicates active biodegradation in the vicinity of the DNAPL pool zone. The highest numbers of 16S rRNA gene copies for total bacteria, *bssA* of sulfate, iron-reducing *Geobacteraceae*, and *Peptococcaceae*, were detected within the source zone indicating a relatively high bio-active zone for biodegradation compared to the total groundwater contaminated zone.

Most if not all water-soluble aromatic hydrocarbon contaminants biodegrade at the relatively large dimensions of the anoxic plume core. However, relatively slow biodegradation of most hydrocarbon

contaminants at iron reducing conditions within the relatively large dimensions of the plume zone appears quantitatively to be of greater significance than relatively more biodegradation in the relatively small dimensions of the nitrate reducing fringe zone.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconhyd.2022.104006.

References

Aamand, J., Jørgensen, C., Arvin, E., Jensen, B.K., 1989. Microbial adaptation to degradation of hydrocarbons in polluted and unpolluted groundwater. J. Contam. Hydrol. 4, 299–312. https://doi.org/10.1016/0169-7722(89)90030-2.

Abraham, M.H., Chadha, H.S., Whiting, G.S., Mitchell, R.C., 1994. Hydrogen bonding. 32. An analysis of water-octanol and water-alkane partitioning and the Δlog p parameter of seiler. J. Pharm. Sci. 83, 1085–1100. https://doi.org/10.1002/jps.2600830806.

Abu Laban, N., Selesi, D., Rattei, T., Tischler, P., Meckenstock, R.U., 2010. Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. Environ. Microbiol. 12, 2783–2796. https://doi.org/10.1111/j.1462-2920.2010.02248.x.

Aitken, C.M., Jones, D.M., Larter, S.R., 2004. Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. Nature 431, 291–294. https://doi.org/10.1038/nature02922.

Alvarez, P.J.J., Anid, P.J., Vogel, T.M., 1991. Kinetics of aerobic biodegradation of benzene and toluene in sandy aquifer material. Biodegradation 2, 43–51. https://doi.org/10.1007/BF00122424.

Annweiler, E., Richnow, H.H., Antranikian, G., Hebenbrock, S., Garms, C., Franke, S., Francke, W., Michaelis, W., 2000. Naphthalene degradation and incorporation of naphthalene-derived carbon into biomass by the thermophile *Bacillus thermoleovorans*. Appl. Environ. Microbiol. 66, 518–523. https://doi.org/10.1128/AEM.66.2.518-523.2000.

Aronson, D., Howard, P.H., 1997. Anaerobic biodegradation of organic chemicals in groundwater: a summary of field and laboratory studies. Tech. Rep. 268.

Atashgahi, S., Hornung, B., Van Der Waals, M.J., Da Rocha, U.N., Hugenholtz, F., Nijsse, B., Molenaar, D., Van Spanning, R., Stams, A.J.M., Gerritse, J., Smidt, H., 2018. A benzene-degrading nitrate-reducing microbial consortium displays aerobic and anaerobic benzene degradation pathways. Sci. Rep. 8, 1–12. https://doi.org/10.1038/s41598-018-22617-x.

Baedecker, M.J., Eganhouse, R.P., Bekins, B.A., Delin, G.N., 2011. Loss of volatile hydrocarbons from an LNAPL oil source. J. Contam. Hydrol. 126, 140–152. https://doi.org/10.1016/j.jconhyd.2011.06.006.

Beller, H.R., Spormann, a.M., 1998. Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. J. Bacteriol. 180, 5454–5457.

Beller, H.R., Kane, S.R., Legler, T.C., Mckelvie, J.R., Lollar, B.S., Pearson, F., Balser, L., Mackay, D.M., 2008. Comparative assessments of benzene, toluene, and xylene natural attenuation by quantitative polymerase chain reaction analysis of a catabolic gene, signature metabolites, and compound-specific isotope analysis. Environ. Sci. Technol. 42, 6065–6072. https://doi.org/10.1021/es8009666.

Bian, X.-Y., Mbadinga, S.M., Liu, Y.-F., Yang, S.-Z., Liu, J.-F., Ye, R.-Q., Gu, J.-D., Mu, B.-Z., 2015. Insights into the anaerobic biodegradation pathway of n-alkanes in oil reservoirs by detection of signature metabolites. Nat. Rev. Microbiol. 5, 9801. https://doi.org/10.1038/srep09801.

Burland, S.M., Edwards, E.A., 1999. Anaerobic benzene biodegradation linked to nitrate reduction. Appl. Environ. Microbiol. 65, 529–533.

Cabello, P., Roldán, M.D., Moreno-Vivián, C., 2004. Nitrate reduction and the nitrogen cycle in archaea. Microbiology 150, 3527–3546. https://doi.org/10.1099/mic.0.27303-0.

Callaghan, A.V., 2013. Metabolomic investigations of anaerobic hydrocarbon-impacted environments. Curr. Opin. Biotechnol. 24, 506–515. https://doi.org/10.1016/j.copbio.2012.08.012.

Chee-Sanford, J.C., Frost, J.W., Fries, M.R., Zhou, J., Tiedje, J.M., 1996. Evidence for acetyl coenzyme A and cinnamoyl coenzyme A in the anaerobic toluene mineralization pathway in Azoarcus tolulyticus Tol-4. Appl. Environ. Microbiol. 62, 964–973.

Coates, J.B., Chakraborty, R., Lack, J.G., O'Connor, S.M., Cole, K.A., Bender, K.S., Achenbach, L.A., 2001. Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of Dechloromonas. Nature 411, 1039–1043. https://doi.org/10.1038/35082545.

Cozzarelli, I.M., Eganhouse, R.P., Baedecker, M.J., 1990. Transformation of Monoaromatic hydrocarbons to organic acids in anoxic groundwater environment. Environ. Geol. Water Sci. 16, 135–141. https://doi.org/10.1007/BF01890379.

Cupples, A.M., 2016. Contaminant-degrading microorganisms identified using stable isotope probing. Chem. Eng. Technol. 39, 1593–1603. https://doi.org/10.1002/ceat.201500479.

da Silva, M.L.B., Corseuil, H.X., 2012. Groundwater microbial analysis to assess enhanced BTEX biodegradation by nitrate injection at a gasohol-contaminated site. Int. Biodeterior. Biodegrad. 67, 21–27. https://doi.org/10.1016/j.ibiod.2011.11.005.

De Vries, F., 1999. Karakterisering van Nederlandse gronden naar fysisch-chemische kenmerken.

Dou, J., Ding, A., Liu, X., Du, Y., Deng, D., Wang, J., 2010. Anaerobic benzene biodegradation by a pure bacterial culture of *Bacillus cereus* under nitrate reducing conditions. J. Environ. Sci. 22, 709–715. https://doi.org/10.1016/S1001-0742(09)60167-4.

Einarson, M.D., Mackay, D.M., 2001. Peer reviewed: predicting impacts of groundwater contamination. Environ. Sci. Technol. 35, 66A–73A. https://doi.org/10.1021/es0122647.

Elshahed, M.S., Gieg, L.M., McInerney, M.J., Suflita, J.M., 2001. Signature metabolites attesting to the in situ attenuation of alkylbenzenes in anaerobic environments. Environ. Sci. Technol. 35, 682–689. https://doi.org/10.1021/es001571u.

Elsner, M., 2010. Stable isotope fractionation to investigate natural transformation mechanisms of organic contaminants: principles, prospects and limitations. J. Environ. Monit. 12, 2005–2031. https://doi.org/10.1039/c0em00277a.

Fetter, C.W., 1999. Contaminant Hydrogeology, 2nd ed. Waveland Press, Inc.

Flanagan, P.V., Kelleher, B.P., Allen, C.C.R., 2014. Assessment of anaerobic biodegradation of aromatic hydrocarbons: the impact of molecular biology approaches. Geomicrobiol J. 31, 276–284. https://doi.org/10.1080/01490451.2013.820237.

Foght, J., 2008. Anaerobic biodegradation of aromatic hydrocarbons: pathways and prospects. J. Mol. Microbiol. Biotechnol. 15, 93–120. https://doi.org/10.1159/000121324.

Freeze, R.A., Cherry, J.A., 1979. Groundwater, Vol. 604. Prentice-Hall, Englewood Cliffs, N.J.

Gerritse, J., van der Grift, B., Langenhoff, A., 2004. Contaminant Behaviour of Micro-Organics in Groundwater. John Wiley & Sons inc. https://doi.org/10.1036/0071425799

Gibson, D.T., Koch, J.R., Kallio, R.E., 1968. Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymatic formation of catechol from benzene. Biochemistry 7, 2653–2662. https://doi.org/10.1021/bi00847a031.

Gieg, L.M., Suflita, J.M., 2002. Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. Environ. Sci. Technol. 36, 3755–3762. https://doi.org/10.1021/es0205333.

Gieg, L.M., Alumbaugh, R.E., Field, J., Jones, J., Istok, J.D., Suflita, J.M., 2009. Assessing in situ rates of anaerobic hydrocarbon bioremediation. Microb. Biotechnol. 2, 222–233. https://doi.org/10.1111/j.1751-7915.2008.00081.x.

Grbic-Galic, D., Vogel, T.M., 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. 53, 254–260.

Griebler, C., Safinowski, M., Vieth, A., Richnow, H.H., Meckenstock, R.U., 2004. Combined application of stable carbon isotope analysis and specific metabolites determination for assessing in situ degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer. Environ. Sci. Technol. 38, 617–631. https://doi.org/10.1021/es0344516.

Head, I.M., Jones, D.M., Larter, S.R., 2003. Biological activity in the deep subsurface and the origin of heavy oil. Nature 426, 344–352. https://doi.org/10.1038/nature02134.

Jobelius, C., Anneser, B., Griebler, C., Meckenstock, R.U., Reineke, A., Frimmel, F.H., Zwiener, C., 2011a. Supporting Information to the manuscript Metabolites indicate hot spots of biodegradation and biogeochemical monitoring well gradients in a high-resolution monitoring well. Environ. Sci. Technol. 45, 1–12.

Jobelius, C., Ruth, B., Griebler, C., Meckenstock, R.U., Hollender, J., Reineke, A., Frimmel, F.H., Zwiener, C., 2011b. Metabolites indicate hot spots of biodegradation and biogeochemical gradients in a high-resolution monitoring well. Environ. Sci. Technol. 45, 474–481. https://doi.org/10.1021/es1030867.

Kleemann, R., Meckenstock, R.U., 2011. Anaerobic naphthalene degradation by Gram-positive, iron-reducing bacteria. FEMS Microbiol. Ecol. 78, 488–496. https://doi.org/10.1111/j.1574-6941.2011.01193.x.

Kueper, B., Wealthhall, G., Smith, J., Leharne, S., Lerner, D., 2003. An Illustrated Handbook of Dense Non-Aqueous Phase Liquids (DNAPL) Transport and Fate in the Subsurface.

Kümmel, S., Starke, R., Chen, G., Musat, F., Richnow, H.H., Vogt, C., 2016. Hydrogen isotope fractionation as a tool to identify aerobic and anaerobic PAH biodegradation. Environ. Sci. Technol. 50, 3091–3100. https://doi.org/10.1021/acs.est.5b04819.

Langevoort, M., Hassanizadeh, S.M., et al., 2009. A comprehensive review on biodegradation of DNAPLs in the vicinity of a source zone. PhD thesis No. 303 Geosciences, Utrecht university 7–59. ISBN/EAN: 978-90-5744-165-3, Submitted for publication.

Luo, F., Gitiafroz, R., Devine, C.E., Gong, Y., Hug, L.A., Raskin, L., Edwards, E.A., 2014. Metatranscriptome of an anaerobic benzene-degrading, nitrate-reducing enrichment culture reveals involvement of carboxylation in benzene ring activation. Appl. Environ. Microbiol. 80, 4095–4107. https://doi.org/10.1128/AEM.00717-14.

McGregor, L.A., Gauchotte-Lindsay, C., Nic Daéid, N., Thomas, R., Kalin, R.M., Nic, N., Thomas, R., Kalin, R.M., 2012. Multivariate statistical methods for the environmental forensic classification of coal tars from former manufactured gas plants. Environ. Sci. Technol. 46, 3744–3752. https://doi.org/10.1021/es203708w.

Meckenstock, R.U., Mouttaki, H., 2011. Anaerobic degradation of non-substituted aromatic hydrocarbons. Curr. Opin. Biotechnol. 22, 406–414. https://doi.org/10.1016/j.copbio.2011.02.009.

Meckenstock, R.U., Elsner, M., Griebler, C., Lueders, T., Stumpp, C., Aamand, J., Agathos, S.N., Albrechtsen, H.-J.J., Bastiaens, L., Bjerg, P.L., Boon, N., Dejonghe, W., Huang, W.E., Schmidt, S.I., Smolders, E., Sørensen, S.R., Springael, D., Van Breukelen, B.M., 2015. Biodegradation: updating the concepts of control for microbial cleanup in contaminated aquifers. Environ. Sci. Technol. 49, 7073–7081. https://doi.org/10.1021/acs.est.5b00715.

Meckenstock, R.U., Boll, M., Mouttaki, H., Koelschbach, J.S., Cunha Tarouco, P., Weyrauch, P., Dong, X., Himmelberg, A.M., 2016. Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons. J. Mol. Microbiol. Biotechnol. 26, 92–118. https://doi.org/10.1159/000441358.

Micic, V., Straub, K., Blum, P., Kappler, A., 2007. Natural attenuation of naphthalene and benzene at a former gasworks site. Water Sci. Technol. Water Supply 7, 145–153. https://doi.org/10.2166/ws.2007.077.

Morasch, B., Schink, B., Tebbe, C.C., Meckenstock, R.U., 2004. Degradation of o-xylene and m-xylene by a novel sulfate-reducer belonging to the genus *Desulfotomaculum*. Arch. Microbiol. 181, 407–417. https://doi.org/10.1007/s00203-004-0672-6.

Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R., Wilkes, H., Schink, B., Rabus, R., 2009. Anaerobic degradation of naphthalene and 2-methyl-naphthalene by strains of marine sulfate-reducing bacteria. Environ. Microbiol. 11, 209–219. https://doi.org/10.1111/j.1462-2920.2008.01756.x.

Nealson, K.H., 1994. Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation. Annu. Rev. Microbiol. 48, 311–343. https://doi.org/10.1146/annurev.micro.48.1.311.

Oka, A.R., Phelps, C.D., Zhu, X., Saber, D.L., Young, L.Y., 2011. Dual biomarkers of anaerobic hydrocarbon degradation in historically contaminated groundwater. Environ. Sci. Technol. 45, 3407–3414. https://doi.org/10.1021/es103859t.

Peng, L., Lin, Y., Meng, F., Wu, J., Zheng, Y., Sun, T., Wang, G., 2021. Environmental fate and aquatic effects of propylbenzenes and trimethylbenzenes: a review. Chemosphere 264, 128533. https://doi.org/10.1016/j.chemosphere.2020.128533.

Peters, C. a, Luthy, R.G., 1993. Coal-tar dissolution in water-miscible solvents – experimental evaluation. Environ. Sci. Technol. 27, 2831–2843. https://doi.org/10.1021/es00049a025.

Richnow, H.H., Meckenstock, R.U., Reitzel, L.A., Baun, A., Ledin, A., Christensen, T.H., 2003. In situ biodegradation determined by carbon isotope fractionation of aromatic hydrocarbons in an anaerobic landfill leachate plume (Vejen, Denmark). J. Contam. Hydrol. 64, 59–72. https://doi.org/10.1016/S0169-7722(02)00104-3.

Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W.N., Bemment, C.D., 2008. Nitrate attenuation in groundwater: a review of biogeochemical controlling processes. Water Res. 42, 4215–4232. https://doi.org/10.1016/j.watres.2008.07.020.

Rogers, S.W., Ong, S.K., Kjartanson, B.H., Golchin, J., Stenback, G.A., 2002. Natural attenuation of polycyclic aromatic hydrocarbon-contaminated sites: review. In: Pract. Period. Hazardous, Toxic, Radioact. Waste Manag., Vol. 6, pp. 141–155. https://doi.org/10.1061/(ASCE)1090-025X(2002)6:3(141).

Röling, W.F.M., Van Verseveld, H.W., 2002. Natural attenuation: what does the subsurface have in store? Biodegradation 13, 53–64. https://doi.org/10.1023/A:1016310519957.

Safinowski, M., 2005. Anaerobic Biodegradation of Polycyclic Aromatic Hydrocarbons.

Safinowski, M., Meckenstock, R.U., 2006. Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Environ. Microbiol. 8, 347–352. https://doi.org/10.1111/j.1462-2920.2005.00900.x.

Sandercock, P.M.L., Du Pasquier, E., 2003. Chemical fingerprinting of unevaporated automotive gasoline samples. Forensic Sci. Int. 134, 1–10. https://doi.org/10.1016/S0379-0738(03)00081-1.

Schirmer, M., Dahmke, A., Dietrich, P., Dietze, M., Gödeke, S., Richnow, H.H., Schirmer, K., Weiß, H., Teutsch, G., 2006. Natural attenuation research at the contaminated megasite Zeitz. J. Hydrol. 328, 393–407. https://doi.org/10.1016/j.jhydrol.2005.12.019.

Schnell, S., Schink, B., 1991. Anaerobic aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*. Arch. Microbiol. 155, 183–190. https://doi.org/10.1007/BF00248615.

Sjögren, M., Li, H., Rannug, U., Westerholm, R., 1995. A multivariate statistical analysis of chemical composition and physical characteristics of ten diesel fuels. Fuel 74, 983–989. https://doi.org/10.1016/0016-2361(95)00056-B.

Suarez, M.P., Rifai, H.S., 1999. Biodegradation rates for fuel hydrocarbons and chlorinated solvents in groundwater. Bioremediat. J. 3, 337–362. https://doi.org/10.1080/10889869991219433.

Swartjes, F.A., et al., 2011. Dealing with contaminated soils. In: Soil Use and Management. Springer (Kluwer Academic Publishers). https://doi.org/10.1111/j.1475-2743.1991.tb00867.x.

Tischler, D., 2015. Microbial Styrene Degradation. SpringerBriefs Microbiol. https://doi.org/10.1007/978-3-319-24862-2.

Tischler, D., Kaschabek, S.R., 2012. Microbial styrene degradation: from basics to biotechnology. Microb. Degrad. Xenobiotics 67–99.

Toth, C.R.A., Berdugo-Clavijo, C., O'farrell, C.M., Jones, G.M., Sheremet, A., Dunfield, P.F., Gieg, L.M., 2018. Stable isotope and metagenomic profiling of a methanogenic naphthalene-degrading enrichment culture. Microorganisms 6, 1–17. https://doi.org/10.3390/microorganisms6030065.

Toth, C.R.A., Luo, F., Bawa, N., Webb, J., Guo, S., Dworatzek, S., Edwards, E.A., 2021. Anaerobic benzene biodegradation linked to the growth of highly specific bacterial clades. Environ. Sci. Technol. 55, 7970–7980. https://doi.org/10.1021/acs.est.1c00508.

van der Waals, M.J., Atashgahi, S., da Rocha, U.N., van der Zaan, B.M., Smidt, H., Gerritse, J., 2017. Benzene degradation in a denitrifying biofilm reactor: activity and microbial community composition. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-017-8214-8.

van der Zaan, B.M., Saia, F.T., Stams, A.J.M.M., Plugge, C.M., de Vos, W.M., Smidt, H., Langenhoff, A.A.M.M., Gerritse, J., 2012. Anaerobic benzene degradation under denitrifying conditions: Peptococcaceae as dominant benzene degraders and evidence for a syntrophic process. Environ. Microbiol. 14, 1171–1181. https://doi.org/10.1111/j.1462-2920.2012.02697.x.

Van Leeuwen, J.A., Hartog, N., Gerritse, J., Gallacher, C., Helmus, R., Brock, O., Parsons, J.R., Hassanizadeh, S.M., 2020. The dissolution and microbial degradation of mobile aromatic hydrocarbons from a Pintsch gas tar DNAPL source zone. Sci. Total Environ. 722, 137797 https://doi.org/10.1016/j.scitotenv.2020.137797.

Vogt, C., Kleinsteuber, S., Richnow, H.H., 2011. Anaerobic benzene degradation by bacteria. Microb. Biotechnol. 4, 710–724. https://doi.org/10.1111/j.1751-7915.2011.00260.x.

Vogt, C., Dorer, C., Musat, F., Richnow, H.-H.H., 2016. Multi-element isotope fractionation concepts to characterize the biodegradation of hydrocarbons – from enzymes to the environment. Curr. Opin. Biotechnol. 41, 90–98. https://doi.org/10.1016/j.copbio.2016.04.027.

Vogt, C., Musat, F., Richnow, H.-H., 2018. Compound-Specific Isotope Analysis for Studying the Biological Degradation of Hydrocarbons, Anaerobic Utilization of Hydrocarbons, Oils, and Lipids. https://doi.org/10.1007/978-3-319-33598-8_18-1.

Wang, M., Guo, B., Huang, Z., Duan, J., Chen, Z., Chen, B., Yao, S., 2010. Improved compatibility of liquid chromatography with electrospray tandem mass spectrometry for tracing occurrence of barbital homologous residues in animal tissues. J. Chromatogr. A 1217, 2821–2831. https://doi.org/10.1016/j.chroma.2010.02.042.

Weelink, S.A.B., van Eekert, M.H.A., Stams, A.J.M., 2010. Degradation of BTEX by anaerobic bacteria: physiology and application. Rev. Environ. Sci. Biotechnol. 9, 359–385. https://doi.org/10.1007/s11157-010-9219-2.

Wege, R., 2005. Heft 143 Untersuchungs- und Überwachungsmethoden für die Beurteilung natürlicher Selbstreinigungsprozesse im. Grundwasser von Ralf Wege.

Wiedemeier, T., et al., 1999. Natural Attenuation of Fuels and Chlorinated Solvents in the Subsurface. John Wiley & Sons Inc.

Zamfirescu, D., Grathwohl, P., 2001. Occurrence and attenuation of specific organic compounds in the groundwater plume at a former gasworks site. J. Contam. Hydrol. 53, 407–427. https://doi.org/10.1016/S0169-7722(01)00176-0.