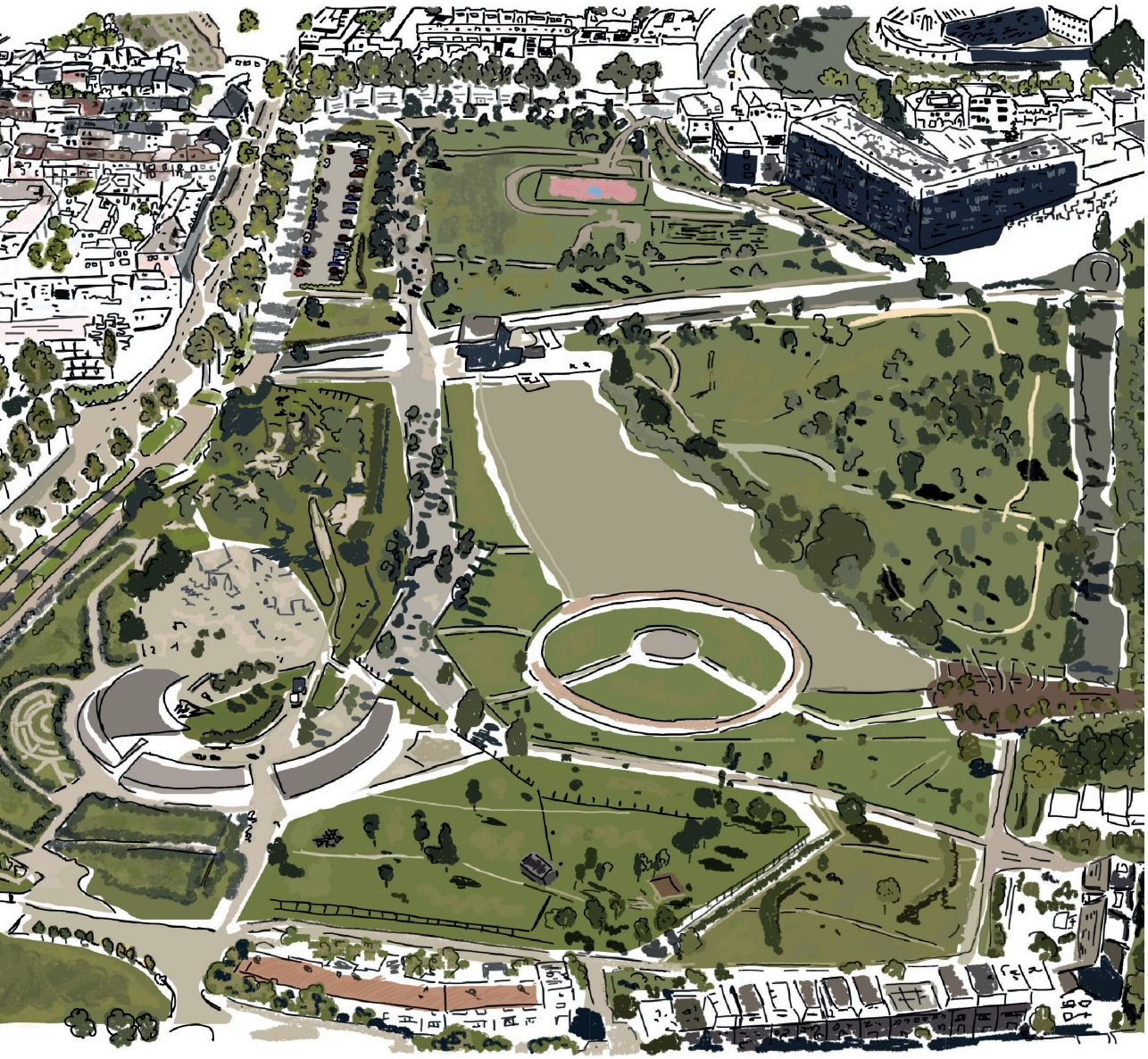
Biostimulation strategies

for in situ biodegradation of complex aromatic contaminant mixtures in groundwater



Dilan Camille Aydın

Propositions

1. A multi-treatment approach is essential for effective removal of mixtures of contaminants.

   (this thesis)

2. Claiming nitrate to be the best anaerobic electron acceptor is incorrect.

   (this thesis)

3. Nothing in science makes sense except in the light of chemistry.

4. Given the abundance of available alternatives, animal testing no longer serves as the default choice in scientific research.

5. Technological advancements designed for simplifying life often lead to increased workloads.

6. Freedom of speech is subject to limitations.

7. Many problems arise from the absence of empathy among individuals.

Propositions belonging to the thesis, entitled

Biostimulation strategies for in situ biodegradation of complex aromatic contaminant mixtures in groundwater

Dilan Camille Aydın, 19 January 2024

Biostimulation strategies for in situ biodegradation of complex aromatic contaminant mixtures in groundwater

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Biostimulation strategies for in situ biodegradation of complex aromatic contaminant mixtures in groundwater

Dilan Camille Aydın

Thesis

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“Everything is everywhere, but the environment selects”

Baas Becking

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Chapter 1

General Introduction

1.1. Former manufactured gas plant sites as source of soil and groundwater contamination

Manufacturing gas from coal and oil was a major worldwide industry from the middle of the 19th to the middle of the 20th century (Birak & Miller, 2009). Gas from manufactured gas plants (MGP) served as the primary lighting fuel. Following the availability of electricity, the use of manufactured gas shifted to heating and powering gas appliances until natural gas became available in mid-20th century. For decades, wastes from gas manufacturing processes were released to the environment by accidental spills and leaks, and even by intentional disposal practices (Murphy et al., 2005). These have led to extent contaminated soils and groundwater. Today, MGP sites that used to be located outside of the cities are now located in the heart of urban areas due to the expansion in city life (Birak & Miller, 2009). There are around 4500 former gasworks sites still existing in Europe and even more in the U.S (Murphy et al., 2005). Even though MGP sites were closed or abandoned decades ago, their environmental legacy can persist for a long time. For instance, they are major source of groundwater contamination and a concern for cities' drinking water sources. Therefore, measures need to be taken to prevent further migration of contaminants.

1.2. Aromatic hydrocarbons

Wastes generated from gas manufacturing depend on the type of gas produced; however they all contained tar. Tar is an opaque viscous liquid, enriched in high molecular weight compounds, mostly composed of various organic aromatic compounds (Birak & Miller, 2009; Sperfeld, Rauschenbach, et al., 2018). Aromatics are ringed hydrocarbon molecules and can be divided to two groups: monoaromatic hydrocarbons harboring one aromatic ring such as benzene, toluene, ethylbenzene and xylenes (BTEX) and polyaromatic hydrocarbons (PAHs) with two or more fused benzene rings (Chandra et al., 2013; Varjani, 2017) such as naphthalene (Figure 1.1).

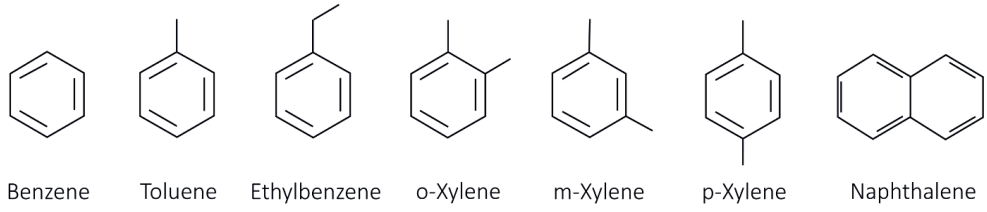


Figure 1.1 Chemical structure of monoaromatic hydrocarbons (BTEX), and naphthalene as a representative polyaromatic hydrocarbons.

Monoaromatic hydrocarbons are relatively stable due to the benzene ring structure and the lack of reactive functional groups. They are highly soluble and therefore relatively mobile in groundwater and reported to have acute toxicity as well as long-term potential carcinogenic effects (Foght, 2008; Lueders, 2017). For PAHs, low molecular weight (LMW) PAHs consist of up to 4 rings, while those with more than 4 rings are referred to as high molecular weight (HMW) PAHs (Bamforth & Singleton, 2005). PAHs are soluble in water however as the molecular weight increases, their solubility in water decreases (Haritash & Kaushik, 2009). HMW PAHs are known to be more persistent in the environment due to their molecular stability and hydrophobicity and can be more toxic than LMW PAHs. Given their properties mentioned above, majority of the aromatic hydrocarbons form a major threat to groundwater quality and aquifer ecosystem health (Lueders, 2017), therefore it is relevant to understand and predict their fate in groundwater systems.

The organic contaminants can be distributed differently in the environment based on their chemical properties. They can dissolve in water, be absorbed on solid organic particles, turn to soil gas and/or form a separate liquid layer commonly known as non-aqueous phase liquid (NAPL) (Logeshwaran et al., 2018). In the case of NAPLs, rather than rapidly dissolving away in the groundwater, it would primarily remain as a separate phase and tend to move downwards from unsaturated zone into the water saturated zone, reaching at the water table (Murphy et al., 2005). Light NAPLs (LNAPLs) float on the water table as they are less dense than water, forming a thin layer that spreads across the water table and capillary fringe. For instance, compounds such as BTEX known to be less dense than water, can be dissolved from LNAPLs. NAPLs denser than water (including coal tar residues with HMW aromatics) are referred as DNAPL and tend to migrate downward and act as long-term sources of dissolved phase contaminants (Hatipoğlu-Bağci & Motz, 2019; Murphy et al., 2005) (Figure 1.2). PAHs are relatively lightweight and behave as LNAPLs, however some HMW PAHs that are denser can act like DNAPLs and sink below the water table to form a separate phase. Due to their risk of dissolution and/or migration leading to contaminating drinking water supplies, control and treatment strategies to reduce the hazardous effects of the aromatic hydrocarbons are needed.

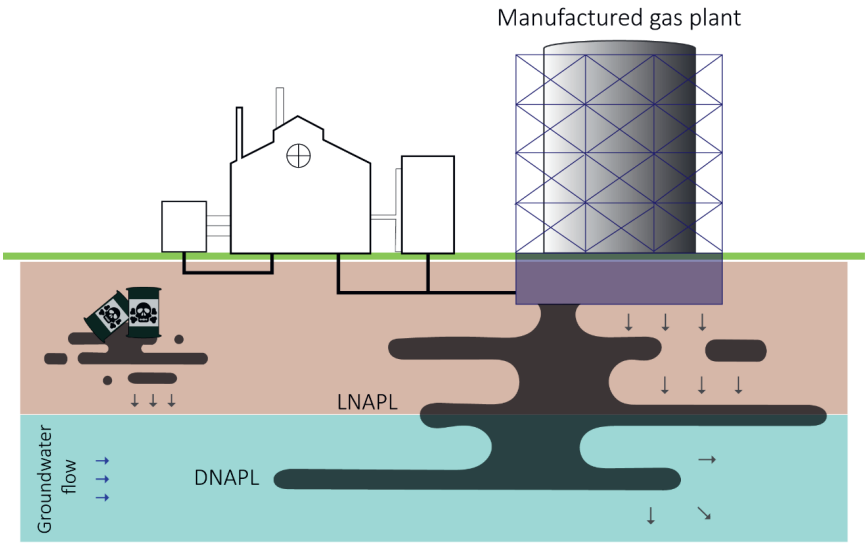


Figure 1.2 Representative figure of waste discharge at a manufactured gas plant site and its potential distribution in the subsurface.

1.3. Clean-up strategies for the removal of aromatic hydrocarbons from contaminated sites

There are different clean up methods for aromatic hydrocarbon removal from contaminated water, such as physical, chemical and biological treatment. These methods can be further sub-divided into ex situ and in situ remediation technologies (Farhadian, Duchez, et al., 2008). Ex situ remediation involves excavation of the contaminated material, which can then be treated, re-used, or re-injected using the pump and treat approach. The pump and treat approach is a common strategy applied in contaminated sites (Birak & Miller, 2009). Basically, the extracted material is pumped to the surface using a series of extraction wells and treated by physical-chemical techniques such as air stripping, activated carbon adsorption, precipitation, flocculation or application of chemical oxidation compounds such as O_3, O_2, H_2O_2 and Cl_2 (Majone et al., 2015). In some cases, to prevent the spreading of the contaminants, pump and treat strategy can be combined by implementation of steel barriers or slurry walls. Even though pump and treat is a widespread approach applied in contaminated sites, it may come with some limitations. It has a high energy demand leading to high treatment costs and may not be effective in treating contaminants that are strongly adsorbed onto the solid phase or present as a separate phase in the subsurface such as NAPLs. For such cases, the removal of contaminants depends to their slow dissolution kinetics meaning that long operational times will be needed, which will eventually increase the treatment cost (Majone et al., 2015).

Compared to ex situ treatment, in situ remediation comes with advantages such as low operating costs due to low energy request and less disturbance of the environment since the treatment occurs at the location, targeting the contaminant plume or the contamination source directly. As an example, among physical methods applied in situ, air stripping is the most common approach for removal of relatively shallow subsurface pollution with volatile organic compounds (VOC) removal including BTEX. But it is not an environmentally friendly technology as aromatics are transferred from liquid to gas phase, requiring intensive above ground treatment and risk education measures for the local living environment. As a chemical treatment, a common approach is injection of chemical oxidation compounds to the source of the pollution. This is an efficient technology to treat the contaminant in a short period of time however chemicals may lead to environmental consequences due to potential leaching or side reactions. Among in situ technologies, in most recent years biological treatment techniques has surpassed both physical and chemical treatment, becoming the common technology for groundwater remediation (Majone et al., 2015).

Bioremediation is a process that uses microorganisms' metabolic capabilities with the aim to transform toxic contaminants to less harmful products or non-toxic compounds such as CO_2, H_2O, CH_4 and biomass (El-Naas et al., 2014). Therefore, it is a sustainable and environmentally friendly approach, allying high efficiency and low costs (Chandra et al., 2013; Farhadian, Vachelard, et al., 2008). The microorganisms metabolize contaminants to obtain the energy and nutrients essential for cell growth. When natural conditions meet all the metabolic requirements for the biodegradation reactions, and no engineering intervention is needed, it is called intrinsic bioremediation or natural attenuation (Foght, 2008). This is an option when the rate of contaminant biodegradation is higher than the rate of contaminant transportation (Scow & Hicks, 2005). Intrinsic bioremediation can be combined with non-biological treatment methods such as soil excavation, vapor recovery system and pump and treat technologies. Such non-biological treatment approaches are more a temporary solution rather than permanent one as they are applied with intention to reduce the size of the contaminant plume in the site (Birak & Miller, 2009).

Even though intrinsic bioremediation is an option, contaminated sites may require further manipulation where environmental conditions can be optimized to stimulate microbial growth hence accelerate the microbial biodegradation process (Foght, 2008). Engineered bioremediation is often applied for a faster removal of the contaminants and to reduce the liability for costs required to maintain and monitor the site. This can be done by biostimulation, in which the growth of indigenous degraders is stimulated by the addition of nutrients, electron acceptors and/or other growth-stimulating materials. Another option is bioaugmentation, in which known degraders are added to supplement the existing microbial population (Das & Chandran, 2011). At sites with large pure product source zones where quick remediation is aimed, in situ chemical treatments (addition of oxidation compounds such as permanganate, persulfate, and Fenton's reagent) can be combined with bioremediation strategy and help to decrease DNAPL mass and reduce groundwater concentrations (Sutton et al., 2015). As it can be seen, there are many different approaches that can be applied to remediate a contaminated site. To choose the most effective method, it is essential to have a clear understanding of the degradation mechanisms of aromatic hydrocarbons and the factors that can influence the biodegradation process.

1.4. Biodegradation of aromatic hydrocarbons

Microorganisms have the capability to biodegrade almost all organic contaminants in order to use them for their own growth and reproduction. Contaminants serve as a source of carbon, essential for the formation of new cell constituents, and also provide electrons, which are necessary for obtaining energy (Hatipoğlu-Bağci & Motz, 2019). Briefly, microbes gain energy from contaminants by breaking chemical bonds and transferring the electrons from the contaminants to an electron acceptor. Contaminants like BTEX and aliphatic oil products are referred to as electron donors while electron recipients such as oxygen are known as electron acceptors. This energy gained from the electron transfer is further used for cell maintenance and growth. When oxygen is used as an electron acceptor by the microorganisms, it is referred to as aerobic biodegradation while in the absence of oxygen anaerobic biodegradation takes place by using alternative electron acceptors such as NO_3^-, Fe^{3+}, Mn^{4+}, SO_4^{2-}, HCO_3^- and highly halogenated organic compounds (El-Naas et al., 2014; Varjani, 2017). Because utilization of different electron acceptor leads to distinct metabolic pathways, aerobic and anaerobic degradation of aromatic hydrocarbons will be discussed separately.

1.4.1. Aerobic biodegradation

In aerobic zones, available oxygen is used by the microorganisms as a terminal electron acceptor and as a direct oxidant of the aromatic ring (Weelink et al., 2010). During aerobic degradation process, oxygen is first introduced to the molecule by progressive oxidation of the aromatic ring to produce carboxylic acids, or ring oxidation which produces substituted pyrocatechols. Then, carboxylic acids and pyrocatechols are transformed to substrates of the citrate cycle through cleavage of the aromatic ring ultimately leading to complete mineralization into CO_2 and H_2O (Hendrickx et al., 2006; Jindrova et al., 2002). Two bacterial multicomponent enzymatic systems, monooxygenases and dioxygenases, are responsible for the initial transformation of the monoaromatics and several aerobic metabolic pathways have been identified (Jindrová et al., 2002). Monooxygenases use one oxygen atom from the oxygen molecule to attack aromatic ring, whose products are subsequently transformed to pyrocatechols, while dioxygenases use two oxygen atoms to attack aromatic ring with the formation of 2-hydroxy-substituted compounds. Possible aerobic degradation routes of benzene, as a representative aromatic hydrocarbon compound, are summarized in Figure 1.3.

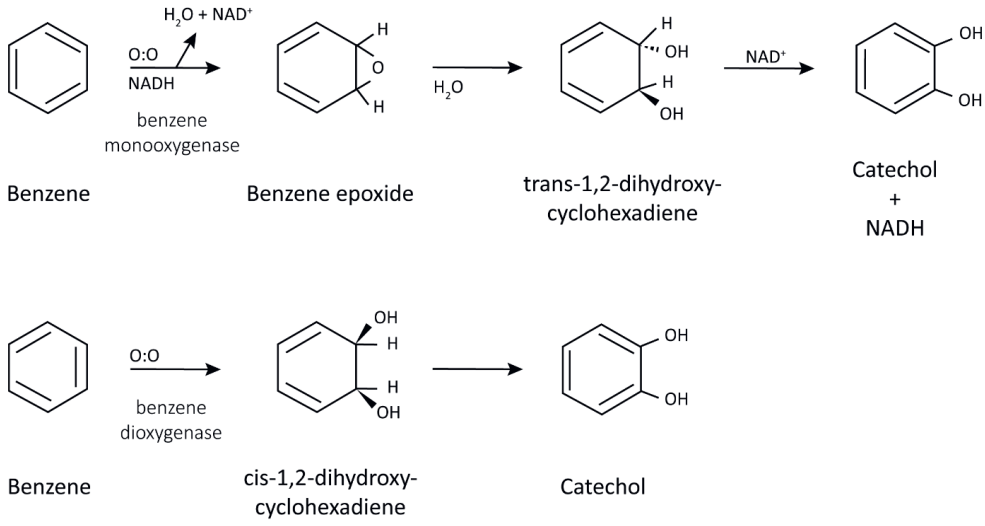


Figure 1.3 Reaction sequences for aerobic biodegradation of benzene via di and monooxygenase (adapted from Mancini et al. (2003)).

Oxygen is the most preferred electron acceptor by microorganisms due to its high energy yield which allows them to derive more energy during the degradation process (Christensen et al., 2000). Oxygen has also high redox potential which makes it an effective electron acceptor for aromatic compound degradation, as it can facilitate the transfer of electrons from the microbial catabolic pathways (Lovley, 1997). Under aerobic conditions, all monoaromatic compounds can be rapidly biodegraded (Weelink et al., 2010). Studies on aerobic degradation of monoaromatic hydrocarbons are broad and has been reviewed several times (Das & Chandran, 2011; El-Naas et al., 2014; Varjani, 2017) and various BTEX degraders have been isolated from different environments. Common aerobic BTEX degraders isolated from soil are *Pseudomonas*, *Burkholderia, Sphingomonas*, *Rhodococcus*, *Acinetobacter, Arthrobacter, Comamonas, Alcaligens*, *Acidovorax, Agrobacterium*, *Stenotrophomonas* and *Ralstonia* (El-Naas et al., 2014; Guzik et al., 2013; Hendrickx et al., 2006; Jiang et al., 2015; S. H. Lee et al., 2012). Even though complete removal of aromatics under aerobic conditions is promising, anaerobic conditions are prevalent in subsurface environments hence aerobic biodegradation is limited.

1.4.2. Anaerobic biodegradation

Under anaerobic conditions, oxygen is not available for the initial attack of the ring and therefore other pathways are used for the degradation process. Benzene biodegradation can occur via methylation to toluene, carboxylation to benzoate, or hydroxylation to phenol; and transformed to the central aromatic intermediate benzoyl-CoA which is further mineralized to CO_2 (Foght, 2008; Weelink et al., 2010) (Figure 1.4). The first step in the catabolism of toluene (Figure 1.4B) is the addition of fumarate to toluene to form benzylsuccinate by benzylsuccinate synthase (Bss) which is further converted to benzoyl-CoA which is known as the central intermediate in the anaerobic biodegradation of many aromatic hydrocarbons (Foght, 2008).

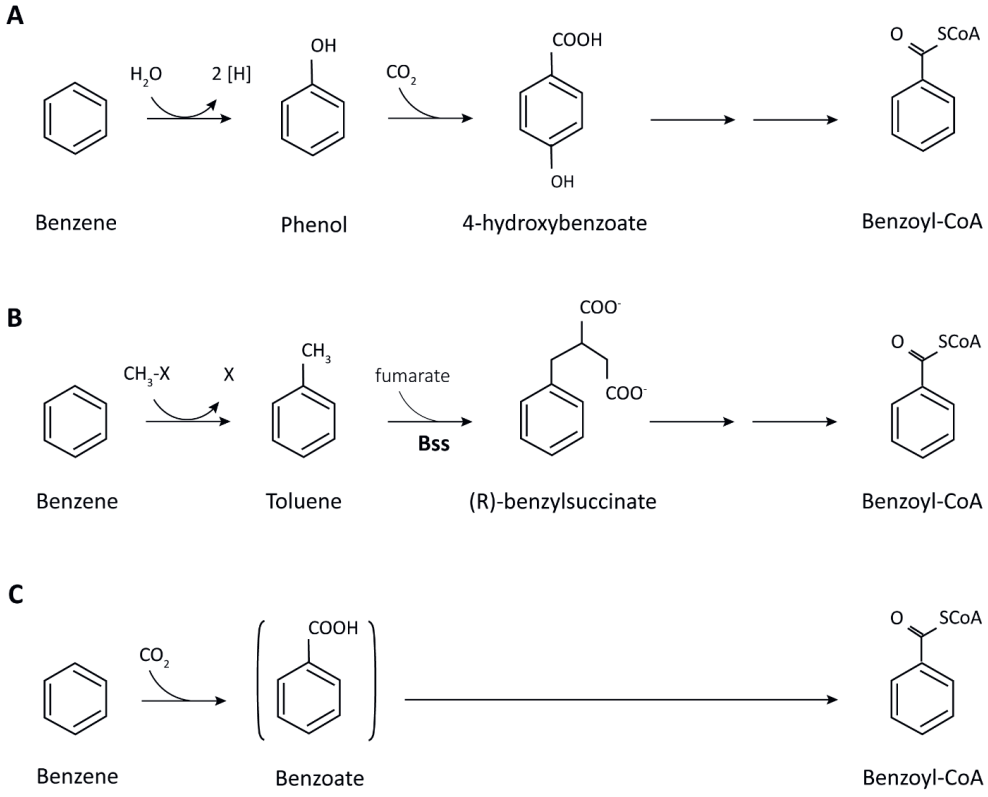


Figure 1.4 Possible anaerobic benzene activation steps and further transformation reactions to benzoyl-CoA as central metabolite (adapted from Foght (2008) and Weelink (2008)). **A**: hydroxylation, **B**: methylation, **C**: carboxylation of benzene, Bss: benzylsuccinate synthase.

In many polluted environments, oxygen is limited due oxygen being consumed faster than it can be replenished (Dou, Liu, Hu, et al., 2008). In the absence of oxygen, anaerobic degradation of aromatic hydrocarbons has been demonstrated under several different electron acceptors conditions (NO_3^-, Fe^{3+}, Mn^{4+}, SO_4^{2-}, HCO_3^-) with laboratory microcosms and enrichment cultures. However, hydrocarbon degradation under anaerobic conditions is often slower compared to aerobic degradation, due to less favorable reaction energetics with the alternate electron acceptors to oxygen (Christensen et al., 2000; Schreiber et al., 2004). Even though anaerobic degradation process is slower, diverse groups of anaerobic degraders of BTEX aromatics have been isolated from aromatic hydrocarbon contaminated aquifers. Members of the *Betaproteobacteria*, *Deltaproteobacteria* and *Clostridia* are the most common anaerobic hydrocarbon degraders (Lueders, 2017). Under nitrate-reducing conditions, *Azoarcus*, *Thauera Aromatoleum*'spp. and other members closely related *Rhodocyclaceae*, *Dechloromonas*, *Peptococcaceae* are known to degrade monoaromatic compounds (Chakraborty & Coates, 2004; van der Zaan et al., 2012; Weelink et al., 2010). *Geobacter* species are the most reported iron-reducing aromatic degraders (Chakraborty & Coates, 2004) while many sulphate-reducing BTEX degraders are related to *Desulfosarcina*, *Desulfobacula*, *Desulfotignum* spp. or to other *Desulfobacteraceae* (Lueders, 2017). In the absence of all other electron acceptors, methanogenic oxidation of BTEX hydrocarbons was reported for members of *Peptococcaceae*, *Deltaproteobacteria*

and different hydrogenotrophic (*Methanospirillum*, *Methanobacterium* spp.) or acetotrophic (*Methanosaeta* spp.) methanogens (Lueders, 2017; Ulrich & Edwards, 2003). As shown here, different microbial groups are playing a role in the biodegradation process dependent on the redox condition present. This issue will be extended in the following chapter together with many other factors that affects the biodegradation of aromatic hydrocarbons.

1.5. Factors affecting the suitability of bioremediation

The basic principles of microbial biodegradation of aromatic hydrocarbons are relatively straightforward. However, knowledge on microbial metabolisms are not fully understood, and successful use of microorganisms in bioremediation is complex. In order to identify and implement efficient remediation strategies, it is essential to thoroughly investigate and understand the complicating factors that significantly influence the fate of hydrocarbon removal in situ. These factors can be broadly classified as properties of the contaminant(s), environmental conditions of the site and the characteristics of the indigenous microbial community.

1.5.1. Properties of the contaminant(s)

Chemical structure: The chemical structure of the contaminant plays a significant role in its degradation. For aromatic hydrocarbons, the presence and position of functional groups (such as alkyl, halogen, nitro, or hydroxyl groups) can affect the reactivity and susceptibility of the compound to degradation processes. For instance, benzene is considered as the most difficult aromatic compound to be degraded. This is due to lack of additional functional group in benzene, limiting the number of microbial pathways available for its degradation. In contrast, toluene is relatively easy to be metabolized anaerobically due to the methyl group in addition to the benzene ring which facilitates the microbial attack. Hence, this structural feature allows for a greater variety of microbial degradation pathways and enzymes to be involved in its degradation (Weelink et al., 2010).

Substrate concentration: In addition to substrate properties and variation, the substrate concentration plays a significant role in biodegradation processes. High concentrations of the substrate can induce toxicity in microorganisms, thereby inhibiting biodegradation (El-Naas et al., 2014), while low concentration of contaminants may not be degraded or biodegraded very slowly. Too low concentration is often related to unavailability of the contaminants to microorganisms for instance if the contaminant is absorbed to soil or trapped to pores, and very little amount is dissolved in water. In some cases, uptake and metabolism of organic compounds stops at low concentration by the internal regulation mechanisms of the microbial cells (Hunt et al., 1998).

Substrate composition **(The mixture effect):** In contaminated sites, various type of pollutants are found together, and such mixtures present a complex and challenging problem for bioremediation (Thavamani et al., 2011). They can inhibit degradation by exerting toxicity and competitive inhibition or stimulate degradation by inducing the activity of required catabolic enzymes. Another type of substrate interaction occurs when one compound acts as a primary substrate, stimulating microbial growth, which in turn enhances the co-metabolism of another compound (Aburto & Peimbert, 2011; M. Chang et al., 1993; Foght, 2008). Consequently, it becomes crucial to explore substrate interactions to gain insights into the fate of contaminants and understand why certain compounds may persist in contaminated sites while others undergo degradation. In literature, various studies focus on single aromatic compound biodegradation as sole source of carbon however this is not a representation of in situ conditions. Studies where BTEX compounds have been investigated together are available

(Alvarez & Vogel, 1991; Deeb et al., 2001; Deeb & Alvarez-Cohen, 1999; S. K. Lee & Lee, 2002) however, in contaminated sites, BTEX often co-occurs alongside various pollutants which can significantly complicate the environmental remediation process (Deeb et al., 2001).

1.5.2. Environmental conditions

The suitability of bioremediation depends not only to pollutant properties but also on the site's geological and chemical characteristic (Phelps & Young, 1999). Various environmental factors can influence aromatic hydrocarbon degradation, including temperature, pH, soil type, moisture content, available nutrients and electron acceptors (Das & Chandran, 2011; Meckenstock et al., 2015; Schreiber et al., 2004; Varjani, 2017; Varjani & Upasani, 2017). In this context, emphasis will be placed on the availability of electron acceptors, as their rapid depletion within an aquifer is widely recognized as a significant constraint on bioremediation processes (Meckenstock et al., 2015).

The shape of the contaminant plume is defined by the groundwater flow which causes a lateral spreading of the leachate (Christensen et al., 2001). The reduced leachate creates a sequence of redox zones in the groundwater by methanogenic conditions being prevalent close to the contaminant source followed by sulfate, iron and manganese, nitrate-reducing conditions and finally aerobic processes toward the end of the plume (Christensen et al., 1994) (Figure 1.5). However, Meckenstock et al. (2015) propose that iron reduction, manganese reduction, and methanogenesis may occur simultaneously in the core of the contaminant plume, while nitrate, sulfate, and oxygen reduction take place at the fringes due to the rapid depletion of these dissolved electron acceptors in the plume core. Consequently, biodegradation with oxygen, nitrate, or sulfate reduction can only occur at the fringes of the plume, where electron acceptors are replenished from surrounding groundwater through dispersion and diffusion. Given the presence of different redox zones in the plume, it is important to investigate the biodegradation of the contaminants under various redox conditions as biodegradation is strongly dependent to the redox environment (Christensen et al., 1994).

In theory, microorganisms prioritize the use of electron acceptors with higher redox potentials, with the following order of $O_2 > NO_3^- > Fe^{3+} / Mn^{4+} > SO_4^{2-} > HCO_3^-$, due to the greater energy yield gained through their reduction (Christensen et al., 2000; Schreiber et al., 2004). To emphasize on the energy demands depending to the electron acceptor, the stoichiometric calculations of benzene, as a representative aromatic hydrocarbon, was given in the Table 1.1.

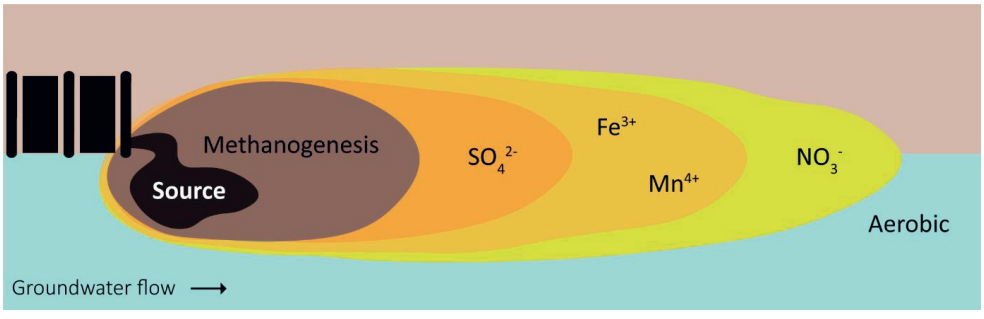


Figure 1.5 Longitudinal redox zonation concept in the contaminant plume (adapted from Christensen et al. (1994)).

Table 1.1 Stoichiometric equations and standard free energy changes for benzene oxidations with different electron acceptors (EA), without taken into account biomass formation (Weelink, 2008).

EA (ox/red)	Stoichiometric Equation (without biomass production)			$\Delta G^{o'}$ (kJ/mol)
O_2 / H_2O	$C_6H_6 + 7.5\ O_2 + 3\ H_2O$	$\rightarrow$	$6\ HCO_3^- + 6\ H^+$	-3173
Fe^{3+} / Fe^{2+}	$C_6H_6 + 30\ Fe^{3+} + 18\ H_2O^-$	$\rightarrow$	$6HCO_3^- + 30\ Fe^{2+} + 36\ H^+$	-3040
NO_3^- / N_2	$C_6H_6 + 6\ NO_3^-$	$\rightarrow$	$6\ HCO_3^- + 3\ N_2$	-2978
NO_3^- / NO_2^-	$C_6H_6 + 15\ NO_3^- + 3\ H_2O$	$\rightarrow$	$6\ HCO_3^- + 15\ NO_2^- + 6\ H^+$	-2061
SO_4^{2-} / H_2S	$C_6H_6 + 3.75\ SO_4^{2-} + 3\ H_2O$	$\rightarrow$	$6\ HCO_3^- + 1.875\ H_2S + 1.875\ HS^- + 0.375\ H^+$	-186
CO_2 / CH_4	$C_6H_6 + 6.75\ H_2O$	$\rightarrow$	$2.25\ HCO_3^- + 3.75\ CH_4 + 2.25\ H^+$	-124

As shown in Table 1.1, oxygen has the highest redox potential meaning that it would be preferred by the microorganisms as an electron acceptor for the oxidation reaction. Based on the information of Table 1.1, oxygen can be supplied to aquifers as a biostimulation strategy. However, this may not be as straight forward since addition of oxygen may come with certain disadvantages such as low solubility of oxygen in water and tendency to precipitate oxides which may decrease aquifer permeability (Wilson & Bouwer, 1997). Supplying oxygen to aquifers can also be very costly. Then, nitrate can be selected as an alternative electron acceptor since its energy yield is very close to oxygen. Iron is also an option as it also has similar energy changes as nitrate, but it is relatively insoluble and oxide precipitation may cause clogging hence it is often not preferred (Da Silva et al., 2005). As seen, relying only on theoretical knowledge may not always lead to the most effective decision-making hence the significance of practical studies becomes apparent. With an improved understanding of the effect of environmental conditions, we can gain valuable insights into the biodegradation process, helping to develop effective strategies to enhance the performance of the degraders. Since environmental conditions significantly influence the composition of microbial communities, gaining insight into microbial dynamics and their metabolic capacities is equally important.

1.5.3. Microbial community

Indigenous hydrocarbon degrading microorganisms play a significant role in bioremediation process (Varjani, 2017), especially bacteria which are known as primary degraders and most active agents for the removal of aromatic hydrocarbons (Meckenstock et al., 2015; Varjani & Upasani, 2017). Long-term exposure to hydrocarbons results in adaptation of the microbial community to use hydrocarbons as carbon and energy sources. Over time, certain microbial groups may develop specialized metabolic pathways and enzymes to efficiently degrade specific aromatic compounds. Through natural selection, these adapted microorganisms can become dominant within the community and contribute significantly to the biodegradation process (Leahy & Colwell, 1990; Varjani, 2017).

The rate of hydrocarbon biodegradation relies on the microorganisms hence to the microbial community structure, its diversity, and possible interactions. On one hand, the presence of diverse microbial species can enhance the overall biodegradation performance as different species possess distinct enzymatic pathways and metabolic capabilities that enable the degradation of various aromatic compounds. On the other hand, microbial communities can show antagonistic effects where the metabolic activities of distinct species inhibit the degradation process (El-Naas et al., 2014). The

identification of indigenous microorganisms and a comprehensive understanding of their functional capabilities in removing aromatic hydrocarbons is essential. Given the sensitivity of microorganisms to environmental changes, it becomes crucial to investigate the factors influencing microbial dynamics and identify stimulative or inhibitive conditions that can significantly impact the bioremediation process.

1.6. A case study: Griftpark

In this thesis, a specific case study was undertaken to explore the generic aspects of biodegradation of a complex aromatic hydrocarbon mixture by considering real-life situations and scenarios. A case study can provide evidence-based insights into specific issues and contribute to the design and optimization of pilot-scale and full-scale applications.

Griftpark is a recreational park located in the center of Utrecht, The Netherlands. It used to be a MGP site that produced gas from coal between 1859 to 1960, where also many other industrial activities were carried out. In 1980, extensive soil and groundwater pollution was discovered at the former MGP site, specifically contaminated with BTEX, PAHs, and other tar compounds. Today, to prevent the lateral spread of the historical pollutants, IBC method (isoleren: isolating, beheersen: constraining and controleren: monitoring) is used. For this, an underground bentonite wall was constructed around the park, which connected to a natural clay layer found 64 meters below ground level (Figure 1.6), with the goal of keeping the pollution in the 1st aquifer. However, the clay layer is not entirely impermeable and the hydraulic pressure in the 1st aquifer is higher than in the 2nd, meaning that contaminated groundwater may leak from the 1st down into the 2nd aquifer. Therefore, since 1999, groundwater has been continuously extracted from the park to maintain a low local water level and prevent the dissolved contaminants from infiltrating the surrounding drinking water reserves found in 2nd aquifer. The extracted water is transported to a nearby wastewater treatment plant (WWTP), where the pollutants are biologically treated in an aerated anti-bulking reactor.

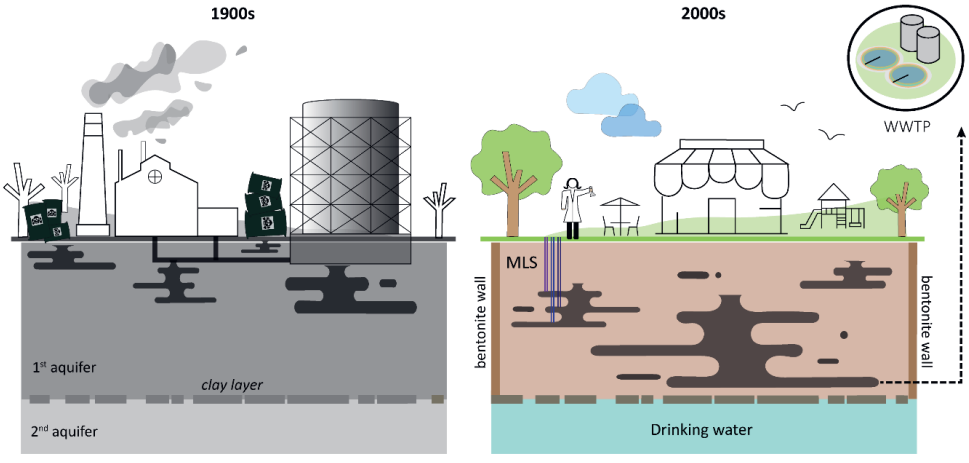
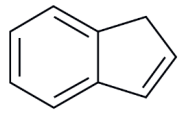


Figure 1.6 A representative overview of the Griftpark in the past (left), and present (right).

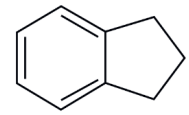
The pump and treat approach applied in Griftpark is only a temporary solution as it is very costly, and not sustainable. Through the installation of multi-level monitoring wells (MLS) and the collection of soil core samples within and around the park, various aromatic hydrocarbons were found to be prevalent in the sediment and groundwater of Griftpark, namely BTEX, indene, indane and naphthalene (BTEXIeIaN) (Hauptfeld et al., 2022; Oesterholt et al., 1997). Some non-organic compounds were present, such as cyanide, originating from the coal-gas factory, along with certain heavy metals as well as sulphate, potentially originating from the gas purifiers. The mobile PAH contamination is primarily attributed to LNAPL originating from tar components. Currently, the spread of LNAPL contamination is controlled through groundwater extraction however the spread of DNAPL contamination cannot be effectively managed using this method. Due to the high cost associated with the pump and treat process, the municipality of Utrecht is seeking alternative techniques for contaminant removal that do not pose additional risks to the surrounding area. Bioremediation is a promising solution as it is a cost-effective and environmentally friendly approach for addressing the contamination challenges in Griftpark. Nevertheless, before implementing a bioremediation approach, gaining insight into the present situation (contaminants, environmental limitations, composition and degradation capacity of the indigenous microorganisms) is essential to formulate effective bioremediation strategies for Griftpark.

1.6.1. Indene and Indane as emergent pollutants prevalent in the subsurface of Griftpark additional to BTEX and Naphthalene

BTEX and naphthalene are common contaminants found in MGP sites and have been studied and discussed by many researchers. However, indane and indene are not so well known. Indene contains a single benzene ring fused with a cyclopentene ring while indane is composed of one benzene ring fused to a cyclopentane ring (Figure 1.7). Despite being common tar oil contaminants, indene and indane received little attention. Most of the existing research has mainly focused on their metabolic conversions or biodegradation using pure cultures and under aerobic conditions (Allen et al., 1995; Buckland et al., 1999; Chartrain et al., 1998; Mundt et al., 2003; Wackett et al., 1988). Few studies were reported upon their anaerobic biodegradation (Kleemann & Meckenstock, 2011; Muhr et al., 2015). In recent years, these compounds have been relatively neglected, highlighting the need for further studies to understand the behavior and interactions of these compounds found alongside BTEX and naphthalene in order to develop effective remediation strategies for their removal in the context of Griftpark.



Indene



Indane

Figure 1.7 Chemical structure of indene and indane, tar oil compounds found in Griftpark additional to BTEX and naphthalene.

1.7. Knowledge gaps and research opportunities

The aforementioned review provides a broad summary of our current understanding of aromatic hydrocarbon degradation. However, various literature studies have revealed that the anaerobic biodegradation process of aromatic hydrocarbons is complex and highly site-specific. This is why further research is needed to come up with effective strategies for addressing the specific challenges posed by Griftpark but also for similar former MGP sites. Here, current research needs on aromatic hydrocarbon degradation will be addressed.

***Need for suitable analytical techniques*:** To investigate and monitor the biodegradation process, it is important to have a reliable measurement method for the routine quantification of the contaminant of interest. For aromatic hydrocarbons such as BTEX and naphthalene, various quantification methods are available however they may not suit to every researcher's need. Especially when dealing with complex mixtures, it is important to be able to measure all the compounds at once in order to save time. In the case of this thesis, BTEX, indene, indane and naphthalene needed to be quantified simultaneously, where low volume liquid samples were used to ensure minimal interference with the experimental conditions as routine measurements were planned. Even though BTEX and naphthalene quantification methods are broad, indene and indane quantification methods are limited. As might be expected, a method capable to quantify all the BTEXIeIaN compounds from liquid phase simultaneously, without the need of using high volumes of sample and which did not require complex sample preparation steps is not available. Therefore, an efficient and user-friendly method for quantifying BTEXIeIaN compounds is required for routine biodegradation investigations.

***Biodegradation of indene and indane as emergent contaminants*:** As previously mentioned, tar is a prevalent pollutant found in MGP sites. Indene and indane naturally occur in coal tar and crude oil, alongside with other aromatics such as BTEX and naphthalene, as in the case of Griftpark. These compounds also hold significant industrial importance. Despite their wide usage and unavoidable occurrence in many contaminated sites, the biodegradation of indene and indane remains poorly studied and largely unknown. Hence, further research is essential to explore the biodegradation of other tar compounds beyond BTEX and naphthalene such as indene and indane.

***Biodegradation of compounds present in complex aromatic mixtures*:** In literature, studies often focus on individual aromatic hydrocarbon degradation or consider monoaromatic compounds (BTEX) as a single pollutant. However, contaminated sites, such as Griftpark, commonly harbor a diverse mixture of hydrocarbons. As mentioned, the biodegradation of a compound in a mixture can differ from its degradation as an individual due to interactions among the contaminants and with the active microbial community in the subsurface. Relying on studies based on a single carbon source will often fail to accurately represent the real-site conditions, resulting in limited applicability and meaningful results when implementing in situ. Therefore, it is necessary to study complex mixtures to gain a comprehensive understanding of the biodegradation process working for these conditions.

***Investigation of multiple factors affecting biodegradation*:** In order to select efficient remediation strategies, it is essential to consider site-specific factors such as current redox conditions, pollution history, and the characteristics of the indigenous microbial community including their adaptation and biodegradation capacity. As these factors collectively influence the degradation of pollutants. While studies on anaerobic degradation of BTEX compounds exist, many of them tend to focus on one parameter, often on the use of different electron acceptors. However, it is crucial to conduct experiments that encompass multiple parameters simultaneously, in order to gain deeper insights into the biodegradation capabilities of indigenous microorganisms and their performance under various

conditions. By comprehensively exploring these factors, we can develop targeted approaches to efficiently remove complex mixtures containing diverse compounds with varying properties.

***Exploring the dynamics of microbial communities*:** Microbial analysis is a critical tool for gaining a fundamental understanding of the indigenous microbial community and their functional potential, including for organic pollutant biodegradation. Microbial communities are known to show variations in response to environmental conditions however current knowledge on interactions between the microbial community and environmental factors is limited and far from clear, despite their importance in exploring the ecological function of microorganisms. Therefore, further research exploring how the microbial community responds to co-varying environmental factors is necessary. Studies on microbial investigation on BTEX or naphthalene-degraders are broad but mostly focus on pure cultures or on environmental samples which are discussed in terms of their ecological significance. Microbial dynamics of indigenous microorganisms, harboring various microbial groups, can be investigated in detail with batch or column studies, where environmental conditions can be easily manipulated. By conducting more focused research in these controlled settings, we can enhance our comprehension of the relationships between microorganisms and their environments, leading to more effective strategies for pilot study designs of contaminated site remediation.

1.8. Aim and outline of this study

This thesis addresses the current research needs on aromatic hydrocarbon degradation, specifically focusing on the biodegradation of complex MGP site pollutants, by assessing multiple environmental factors, and their impact on the dynamics of microbial communities. The goal is to contribute to the development of innovative, effective, environmentally conscious, and cost-efficient strategies for the remediation of Griftpark subsurface but also to provide proof of principles so that obtained outcomes can be also applied to similar former MGP sites.

Chapter 1 is a review that covers topics such as MGP sites, their impact on the environment, the characteristics of aromatic hydrocarbons, their biodegradation process, and the use of bioremediation as a clean-up strategy. It also discusses some of the factors that relatively affect the biodegradation process and highlights areas where more research is needed. Finally, the case study Griftpark is presented. The provided information aims to serve as a helpful guide for understanding the more detailed research presented in the following chapters.

Chapter 2: Reduction in contaminant mass or concentration with time is a line of evidence of bioremediation. For routine monitoring of contaminant concentrations, a practical and accurate quantification method is therefore necessary. In Chapter 2, a HPLC method is presented which was specifically developed for the simultaneous detection and quantification of BTEXIeIaN compounds as well as another mixture harboring pollutants like monochlorobenzene, 1,2-dichlorobenzene and 1,4-dichlorobenzene. This HPLC method was used in all the experimental studies presented in this thesis (Chapter 3, 4, 5 and 6).

Chapter 3: The aerobic biodegradation potential of the BTEXIeIaN by the indigenous Griftpark subsurface microorganisms were investigated in batch experiments. Different substrate combinations (BTEX, BTEXIe, BTEXIa, BTEXN, BTEXIeIa, BTEXIeN, BTEXIaN) were additionally tested to gain generic knowledge about substrate interactions in complex mixtures and understand their effect on the microbial community. This study aimed to deliver a proof of principle for the biodegradation process of complex mixtures of aromatic compounds, contrary to single compound biodegradation studies, and provides insights into the dynamics of microbial communities involved.

Chapter 4: Aerobic biodegradation was proven to be highly effective in removing all the compounds of the BTEXIeIaN mixture (Chapter 3) however anaerobic processes may be the only feasible solution to remove pollutants in deep groundwater since oxygen is limited in subsurface, as in Grifpark. Injecting air would require a high energy cost, and technical complication. This is why, anaerobic biodegradation of the BTEXIeIaN mixture by indigenous Griftpark microorganisms was studied under nitrate and sulfate-reducing conditions through biostimulation and bioaugmentation experiments. For bioaugmentation, two different inoculums were tested. The first inoculum was obtained from a municipal wastewater treatment plant (WWTP) sludge which aimed to enhance the microbial diversity in the batch reactors by introducing the WWTP inoculum through bioaugmentation. The second inoculum consisted of indigenous microorganisms of Griftpark that were previously grown with toluene under nitrate-reducing conditions. In this case, the objective was to assess whether an indigenous microbial community, which had prior exposure to a simpler contaminant (toluene), could effectively facilitate the degradation of a more complex mixture (BTEXIeIaN).

Chapter 5: Anaerobic BTEXIeIaN mixture biodegradation showed long lag times and biostimulation/ bioaugmentation strategies were not effective in removing all compounds of the complex mixture (Chapter 4). Since complete removal of BTEXIeIaN compounds was shown under aerobic conditions in a relatively short time (Chapter 3), incorporating oxygen to remediation strategies became unavoidable. However, supply of oxygen can be very expensive and not efficient as it would be quickly consumed in the deep subsurface. Therefore, in Chapter 5, a technological perspective was adopted where lower oxygen concentrations could be utilized for BTEXIeIaN biodegradation along with nitrate. Considering this concept, BTEXIeIaN degradation was evaluated under microaerobic conditions in batch experiments and compared to fully aerobic and fully anaerobic conditions.

Chapter 6: Up until now, BTEXIeIaN biodegradation has been evaluated through batch experiments (Chapter 3, 4 and 5). However, batch studies may not fully represent real-site conditions due to their closed system nature. To address this limitation, anaerobic column experiments were conducted by introducing a controlled flow with the aim of flushing out non-adapted microorganisms, while also employing batch mode to enrich suitable degraders. In these experiments, substrate interactions were re-evaluated, as in Chapter 3, but this time focusing on benzene and toluene degradation as sole carbon sources, in dual mixes and complex mixture, under two different redox conditions (sulfate and nitrate) similar to Chapter 4. Furthermore, the impact of pollution history and alternating flow/batch mode were assessed. These combined factors were evaluated to understand their influence on microbial community dynamics. The aim was to identify the most influential factor that impacts the microbial community and, consequently, the biodegradation process.

In Chapter 7, the research findings from the experimental chapters are discussed in relation to their implications for potential bioremediation applications in Griftpark. The focus is placed on translating the obtained results as proof of principles into practical and innovative strategies that can be implemented in Griftpark and similar contaminated sites.

Chapter 2

Direct analysis of aromatic pollutants using HPLC-FLD/DAD for monitoring biodegradation processes

This chapter has been published as:

Aydin, D.C., Pineres, J.Z., Al-Manji, F., Rijnaarts, H. and Grotenhuis, T. (2021). Direct analysis of aromatic pollutants using a HPLC-FLD/DAD method for monitoring biodegradation processes. *Analytical Methods*, 13, 1635-1642.

Abstract

Industrial discharges resulting in contaminated groundwater is a global environmental problem. For such contaminated groundwater cases, bioremediation is a cost efficient and environmental friendly approach. Herewith, the determination and quantification of these pollutants has gained great importance and researchers are currently seeking to develop labor extensive, accurate and reliable methods for evaluating the biodegradation process. In this study, a HPLC method was developed and optimized for the quantification of 11 industrial pollutants studied as two different mixtures: Benzene, toluene, ethylbenzene, o, m/p-xylene, indane, indene, naphthalene (Mixture A) and benzene, monochlorobenzene, 1,2-dichlorobenzene, and 1,4-dichlorobenzene (Mixture B). The method harbors two different detectors: fluorescence detection and diode array. The fluorescence detector was used for mixture A to achieve lower quantification limits and to quantify separately o-xylene and indene due them showing similar wavelength behaviors. Limit of detection was found to be between 2 and 70 µg L^{-1} for mixture A and 290 µg L^{-1} for mixture B. Limit of quantitation was between 6 to 210 µg L^{-1} and 980 µg L^{-1} for mixture A and B, respectively. The novel part of this study is that aqueous samples can be directly measured with one-step sample preparation and comes with other advantages such as low volume of sampling from batch bottles and also avoids high cost, related with other analytical techniques. Therefore, this analytical method aims to facilitate the quantification of various aromatic hydrocarbons in laboratory batch samples and can be used as a routine monitoring tool for biological degradation processes of these 11 prevalent contaminants.

2.1. Introduction

Industrial wastes and accidental spills resulting in soil and groundwater contamination is becoming a global concern due to the toxicity and recalcitrance of many aromatic compounds (Birak & Miller, 2009). Common pollutants found in industrial or manufactured gasworks sites are known as benzene, toluene, ethylbenzene, xylene (BTEX), polyaromatic hydrocarbons (PAHs) and various chlorinated aromatic hydrocarbons (Jandera et al., 2001; Mundt et al., 2003). These compounds are specified as hazardous, mostly classified as carcinogenic, and posing risk to human health and to the environment (Birak & Miller, 2009; Jandera et al., 2001). Therefore, current research efforts are focused on the application of efficient remediation strategies for those compounds (Chakraborty & Coates, 2004).

Among all remediation technologies available, biodegradation is the preferred approach due to being economical, energy efficient and environmental friendly (Farhadian, Vachelard, et al., 2008). However, despite the wide range of biodegradation research reported, one of the main challenges is the lack of an adequate method for the analytical quantification of these pollutants. Developing a proper analytical method is a prerequisite in order to attain a reliable and accurate data during measurements (Kumar et al., 2014). Detailed information about different methods used for aromatic hydrocarbon determination and their quantification was reviewed by Farhadian, Vachelard, et al. (2008) and AlSalka et al. (2010). Each one of the discussed methods has its advantages and drawbacks, but main focus on method selection should be based on the aim of the experiment and also other factors such as sample preparation, cost and analysis time (Yamada et al., 2009). In aromatic hydrocarbon bioremediation research, one needs a single method that is comprehensive for all relevant compounds present in the pollution mixture often encountered in remediation projects. The method requires to be accurate, fast, cost-efficient (Yamada et al., 2009) and thereby budget attractive and easy to handle in laboratory and in situ pilot bioremediation technology development. For the measurement of monoaromatic compounds, Gas Chromatography Flame Ionization Detector (GC-FID), GC-Photo Ionization Detector (PID), GC-Mass Spectrometry (MS) (Y. Huang et al., 2013) and GC-Barrier Ionization Discharge Detector (BID) (Pascale et al., 2018) are employed which are useful for detection at low concentration range which is important to assess compliance with environmental thresholds (often in the $\mu g\ L^{-1}$ range). Additionally, GC technique and head-space (HS) sampling has been adopted by the Environmental Protection Agency (EPA) in many protocols. However these techniques come with disadvantages such as incompatibility to direct analyses in water samples and time consuming preparation steps (such as head-space extraction, purge and/or solid-phase microextraction) prior to analysis which may also require high cost equipment (Sarafraz-Yazdi et al., 2008), and laborious methods to identify intermediate products formed during bioconversion (Amy Tan et al., 2012). This renders these techniques less suitable to support lab and field technology development. The limitations of these GC-based methods can be overcome by using high performance liquid chromatography (HPLC), since it is more suited for direct aqueous sample analysis and can measure higher and wider ranges of concentrations for wide sets of aromatic pollutants and minimizing dilution or other pre-treatment steps (Amy Tan et al., 2012).

In this study, such a HPLC based method was developed with focus on the quantification of two different mixtures of compounds, namely one composed of benzene, toluene, ethylbenzene, o, m, p-xylene, indene, indane, naphthalene (which will be referred to as mixture A), and one with benzene, monochlorobenzene, 1,2-dichlorobenzene and 1,4-dichlorobenzene (referred to as mixture B) (Figure 2.1). These contaminants were selected upon their prominent presence in a former gasworks site and a chemical industrial area, respectively. The method aimed for a fast and reliable quantification of all compounds listed in Figure 2.1, by avoiding complex sample preparation steps. Additionally, this method was developed in order to facilitate the work of daily basis biodegradation experiments, where

reliable results can be obtained for aqueous samples with low sampling volumes and by avoiding high use of organic solvents in the monitoring of biodegradation processes.

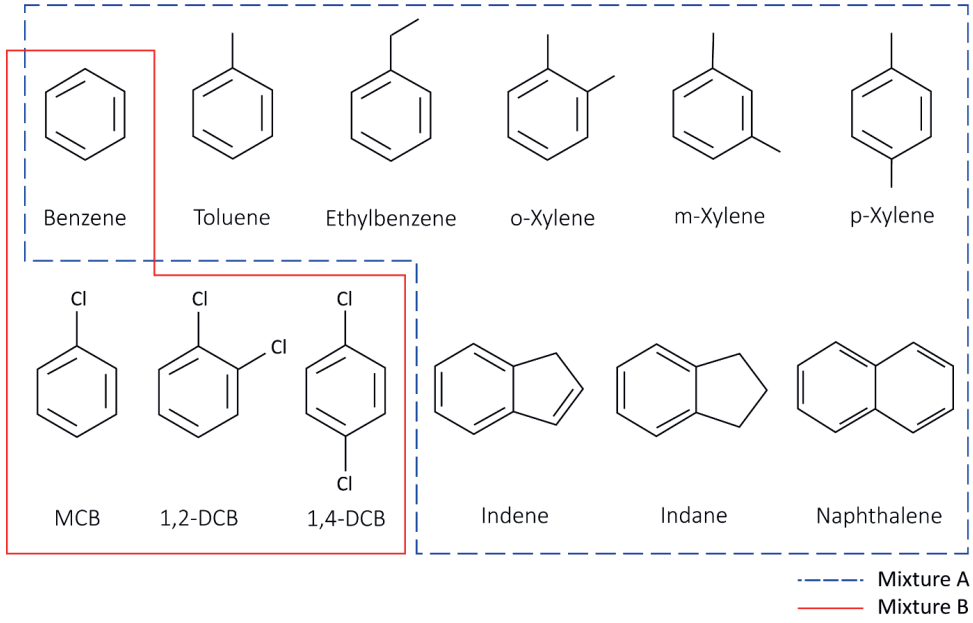


Figure 2.1 Chemical structure of the compounds used in this study. Mixture A (dashed line): benzene, toluene, ethylbenzene, o/m/p-xylene, indene, indane and naphthalene; Mixture B (line): benzene, monochlorobenzene (MCB), 1,2-dichlorobenzene (1,2-DCB) and 1,4-dichlorobenzene (1,4-DCB).

2.2. Materials and Methods

The proposed method was developed and optimized by use of HPLC with fluorescence detector (FLD) and Diode Array detector (DAD) validated in terms of specificity, matrix effect, linearity, precision, recovery and limits of detection (LOD) and quantitation (LOQ).

2.2.1. Chemicals

Chemicals used for standard solution and sample preparation are given in Table 2.1. ULC/MS grade absolute methanol was purchased from Biosolve (Netherlands).

2.2.2. Instrumentation

HPLC analyses were performed using a Thermo HPLC with Ultimate 3000 RS Diode Array detector and Ultimate 3000 RS fluorescence detector (Thermo Scientific Dionex, USA). For the separation of each analyte, a reversed phase chromatography was performed by using an Acclaim™ Phenyl-1 HPLC Column 150 x 4.6 mm, 3 µm (Thermo Scientific Dionex, USA). The mobile phase is an isocratic mixture of 60% methanol and 40% Milli-Q water.

Table 2.1 Information on the chemicals used in this study.

Chemicals	Purity (%)	Supplier
Benzene	> 99.7	VWR Chemicals (USA)
Toluene	≥ 99.9	Merck KGaA (Germany)
Ethylbenzene	99	Alfa Aesar (Germany)
o-Xylene	99	Alfa Aesar (Germany)
m-Xylene	99	Alfa Aesar (Germany)
p-Xylene	99	Acros Organics (Czechia)
Indane	95	Alfa Aesar (Germany)
Indene	≥ 99	Sigma-Aldrich (Germany).
Naphthalene	99	Sigma-Aldrich (Germany).
Monochlorobenzene	99	VWR Chemicals (France)
1,2-Dichlorobenzene	99	Acros Organics (Czechia)
1,4-Dichlorobenzene	99	Alfa Aesar (Germany)

2.2.3. Standard calibration solutions

Stock solutions for mixture A and B were prepared at concentrations of 1 g L^{-1} and 10 g L^{-1} in 100 mL volumetric flasks, respectively. Each analyte was dissolved individually in methanol and was stored at 4°C. Fresh working solutions were prepared daily by diluting the stock solutions to reach desired concentration values in different matrices (Milli-Q water and groundwater for mixture A; Milli-Q water and medium for mixture B).

2.2.4. Sample preservation

As the aromatics are volatile in water, tests demonstrated that all samples need to contain methanol as co-solvent in order to limit evaporation losses during HPLC autosampler procedures. Samples prepared only in Milli-Q water showed incoherent concentrations while with methanol, the concentration of the compounds were found to stay stable up to 12 hours (Table S2.1). Further, methanol addition showed higher resolution and efficiency on chromatographic results. Therefore, methanol was added to standard solutions and to the samples before auto-sampled injection to the HPLC.

The lowest methanol-to-sample ratio was determined for good reproducibility. In order to find optimal separation conditions and good stability in concentrations; different methanol:sample ratios (0:100, 10:90, 25:75, 50:50 v/v) were analyzed using mixture B (compounds in mixture B covers the same range of volatilization properties as for compounds in mixture A). For the analytical chromatographic method development, 25% of methanol was used in all samples unless otherwise specified.

2.2.5. Method Optimization

The operational parameters as sample injection volume, flow rate, mobile phase composition and the column temperature were optimized. The injection volume varied between 10 to 80 µL and two different flow rates of 0.7 and 1.2 mL min^{-1} were tested in line with Taleuzzaman et al. (2015). Methanol was selected as organic modifier in the mobile phase using the method developed by Amy Tan et al. (2012) and in consequence several methanol:water ratios were tested as eluents in HPLC.

The column temperature was varied for 25 and 35°C. All compounds of mixture A were measured with HPLC-FLD, while analytes from mixture B were determined with HPLC-DAD. The UV and excitation/emission wavelengths for each compound together with their retention times are presented in Table 2.2, and specific conditions of each detector is given in Table S2.2. The run time was 27 min for mixture A and 24 min for mixture B.

Table 2.2 The wavelengths and excitation\emission ranges of each compound applied to the HPLC measurements.

	Detector: FLD		Detector: DAD	
Compound	**FLD (Exc\Em)**	**Retention time (min)**	**DAD (nm)**	**Retention time (min)**
Benzene	210\260	9	210	7.76
Toluene	260\310	13.84		
Ethylbenzene	210\260	16.3		
Indene	280\330	17.1		
o-Xylene	210\260	17.4		
m/p-Xylene	260\310	18.1		
Indane	260\310	22.1		
Naphthalene	260\310	23.7		
Monochlorobenzene			210	12.48
1,2-Dichlorobenzene			210	19.83
1,4-Dichlorobenzene			210	20.65

DAD; Diode-array detector, FLD; Fluorescence detector, Exc\Em; Excitation\Emission

2.2.6. Specificity analysis

Each compound was first measured individually to evaluate any possible interfering peaks when samples are prepared as mixtures. A fluorescence spectrum was generated by defining the spectral excitation and emission wavelength and retention time for each compound was recorded. Samples prepared as in mixtures were run and peaks for each compound were compared with the spectrums of the individual compound samples.

Standard solutions for each compound individually at 1 g L^{-1} were prepared in methanol as initial solution and measured under optimized parameters presented in Table 2.2. Blank samples and individual compounds were analyzed in order to obtain a full UV spectrum between 210 to 340 nm. Then, mixture solutions of A and B (1 g L^{-1} and 10 g L^{-1}, respectively) dissolved in methanol, were tested and compared within the individual compound's results to confirm specificity.

2.2.7. Method development

This method was assessed in terms of matrix effect, linearity, intermediate precision, recovery and detection and quantitation limits as described by AlSalka et al. (2010) and Filho et al. (2016).

Matrix effect: The effect of different matrices was tested in order to observe any discrepancy between measurements. Therefore, for compounds in mixture A, two different matrices were selected: Milli-Q water and groundwater obtained from a former gasworks site. Stock solution of mixture A (1 g L^{-1}) was dissolved in six different 50 mL volumetric flask with methanol:given matrix (25:75 v/v) in order to reach final concentrations of 0.5, 1, 2.5, 5, 7.5 and 10 mg L^{-1}.

For mixture B, the matrix effect was tested in samples with Milli-Q water, and medium as prepared according to Lindeboom et al. (2011). Same procedure as in mixture A was applied, where 10 g L^{-1} of mixture B stock solution was dissolved in 50 mL of methanol:given matrix (25:75 v/v) in five different concentrations (1, 2.5, 5, 7.5 and 12 mg L^{-1}). Each peak for each compound was investigated for spectral information such as retention time, peak height and area. Pair t-test was done and p-values <0.05 were determined as an indicator of matrix effects.

Calibration curve: The calibration curves were generated in order to determine a linear relationship between concentration of the compound and the area in the chromatogram. Therefore, different concentrations between 0.5-10 mg L^{-1} for mixture A and 1-12 mg L^{-1} for mixture B were measured to assess the linearity of the method. Standard solution of each concentration was prepared by diluting methanol and given matrix (groundwater or medium) in 50 mL volumetric flasks as described previously. Measurements were performed in triplicate. The linearity and the correlation coefficient (R^2) for each compound was calculated with a linear regression model.

Accuracy and Precision: Accuracy of the method was tested in terms of intermediate precision and recovery. Intermediate precision was determined by calculating the percentage relative standard deviation (RSD) for each sample, at different concentration levels (0.5-10 mg L^{-1} for mixture A and 1-12 mg L^{-1} for mixture B) within different days. RSDs were determined with the Equation 2.1 (AlSalka et al., 2010):

$$Intermediate\ precision\ (\mathrm{RSD\%}) = (\mathrm{SD}/\mathrm{C_m})\ \mathrm{x}\ 100 \qquad (2.1)$$

Recovery experiments were performed at 10 mg L^{-1} concentration for both mixture A and B, in duplicate. The average percent recovery was calculated with the Equation 2.2 (Filho et al., 2016):

$$Recovery\ (\%) = (\mathrm{C}_{obs}/\mathrm{C}_r)\ \mathrm{x}\ 100 \qquad (2.2)$$

SD is the standard deviation, C_m is the mean value for the replicates, C_{obs} refers to the concentration observed in the samples and C_r is the initial spiked concentration of the reference sample.

LOQ and LOD: According to Eurochem Guidelines (Magnusson & Örnemark, 2014), the lowest concentration of the analyte that can be measured is referred as limit of detection (LOD), and the lowest level at which the performance is adequate is defined as limit of quantitation (LOQ). Within this method, the LOD and LOQ values were determined from the standard deviation of the results at low concentrations (SD) of the calibration curve and respective slope (S). Calculations were done by equations given below (Filho et al., 2016):

$$LOD = 3 \text{ x } (\text{SD}/\text{S}) \tag{2.3}$$

$$LOQ = 10 \text{ x } (\text{SD}/\text{S}) \tag{2.4}$$

2.3. Results

2.3.1. Sample preservation

Different methanol:sample ratios were tested with compounds of mixture B shown as in Table 2.3. As mentioned before, methanol was added to the samples in order to avoid evaporation of the volatiles during HPLC autosampler procedures. According to Eurochem Guidelines (Magnusson & Örnemark, 2014), the acceptance criteria to a relative standard deviation corresponds to 10% RSD response. Therefore, ratio of 25% methanol was selected as optimum by reason of showing minimum % RSD response values that is acceptable for all compounds. Additionally, it was preferred to 50% methanol by reasons of showing good reproducibility with the lowest LOD and LOQ possible for all the compounds, and also by having lower methanol-sample ratios. The same methanol:sample ratio was applied to mixture A, since compounds in the mixture A show similar structure and physical/chemical properties as the compounds in mixture B.

Table 2.3 Effect of the methanol content on the repeatability expressed in % RSD.

	RSD (%)			
Methanol (%)	**Benzene**	**MCB**	**1,2-DCB**	**1,4-DCB**
0	12	3	4.9	16.5
10	5.9	5.7	10.5	17
25	6.5	3.7	5.2	7.2
50	1.7	5.3	2.1	8.9

RSD; Relative standard deviation, MCB; Monochlorobenzene, DCB; Dichlorobenzene

2.3.2. Optimization

Optimal chromatographic conditions determined for this method are summarized in Table 2.4. Optimum conditions were determined according to better peak resolutions and smaller standard deviation outcomes.

Table 2.4 The final chromatographic conditions used for the detection of all compounds used in this study.

	Mixture	
Parameters	**A**	**B**
Volume	80 µL	50 µL
Flow rate	0.7 mL min^{-1}	0.7 mL min^{-1}
Mobile phase (isocratic mode)	60:40 (Methanol:Water) %	60:40 (Methanol:Water) %
Column temperature	35°C	35°C
Run time	27 min	24 min

Optimal range of injection volumes were determined as 80 µL for mixture A and 50 µL for mixture B. Amy Tan et al. (2012) reported that high injection volumes may lead to the presence of some residual peaks at the end of the analysis. In our study, where maximum injection volume was 80 µL, such phenomenon was not encountered.

To determine the optimum conditions for compound separation, different flow rates were tested upon their effect on the duration of the analysis and the chromatographic resolution (Amy Tan et al., 2012). For this study, 0.7 mL min^{-1} and 1.2 mL min^{-1} were tested. 0.7 mL min^{-1} showed higher efficiency and better analytical resolution.

2.3.3. Specificity analysis

To evaluate any possible interfering peaks between compounds, samples prepared as individual compounds were compared to samples prepared as mixtures within optimal conditions given in Table 2.4. For mixture B, overlapping peaks were not recorded and all compounds were well separated with use of DAD (Figure 2.2). For mixture A, all compounds could be quantified without interferences by use of FLD and the fluorescence properties of the compounds. Originally, o-xylene was detected with an excitation and emission wavelength of 210/260 nm with overlapping peaks between indene and o-xylene (Figure 2.3A). Changing indene detection to 280/330 nm (Figure 2.3B), differentiation and quantification of both compounds became possible.

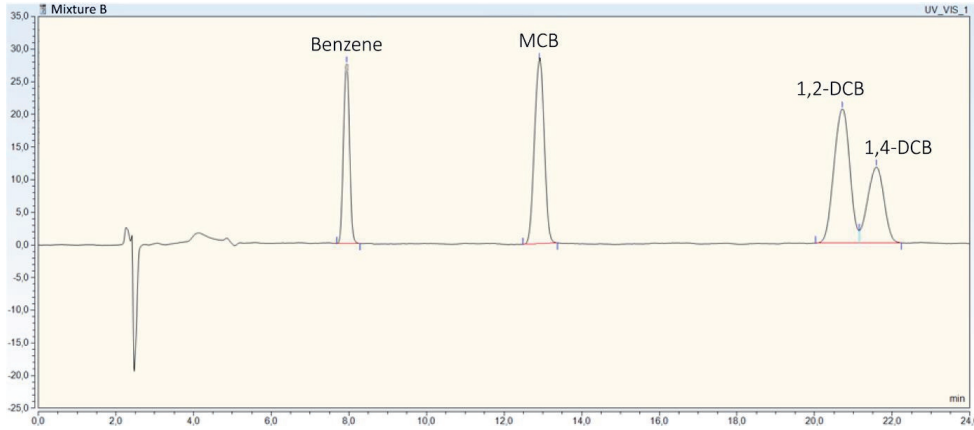


Figure 2.2 Chromatogram of all compounds in mixture B (12 mg L^{-1}), identified with a HPLC-DAD at 210 nm.

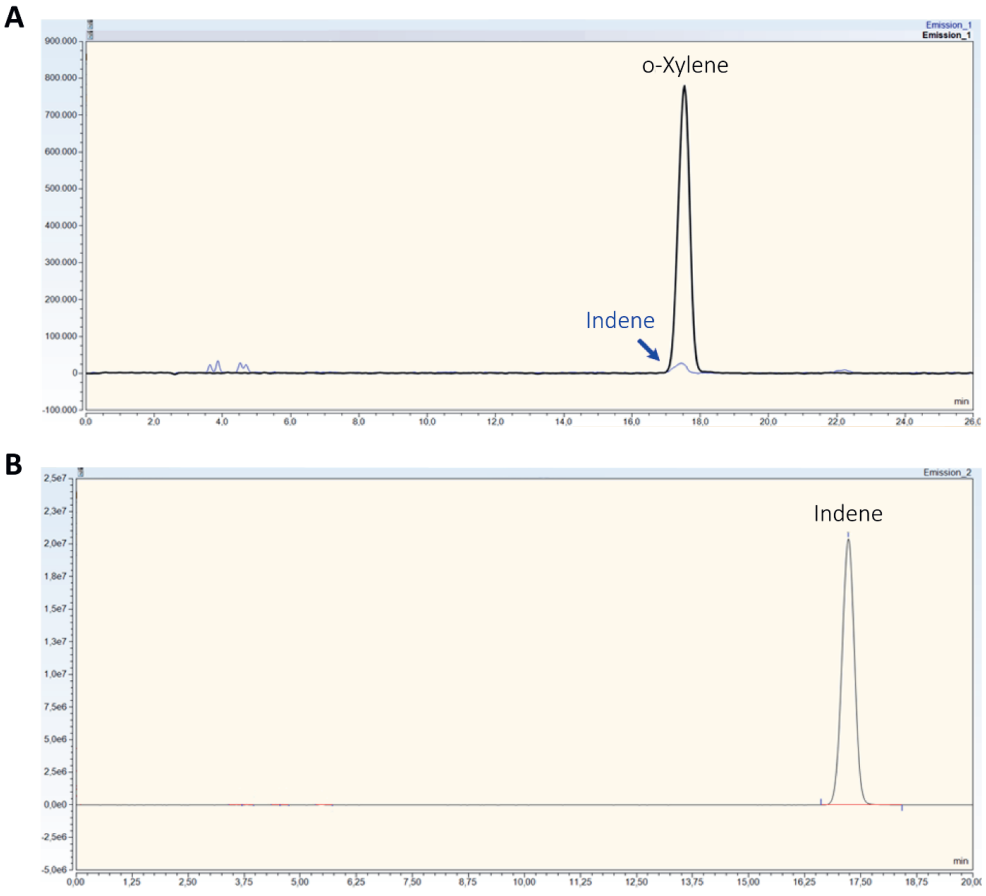


Figure 2.3 Chromatogram of o-xylene (10 mg L^{-1}) detected at 210/260 nm. An interference with the compound indene (20 mg L^{-1}) can be seen as a small peak (A) while chromatogram of indene alone at 280/330 nm (B) by the use of a HPLC-FLD.

2.3.4. Method development

Matrix effect: Mixture A samples, diluted in methanol and groundwater, were analyzed and probabilities (pair t-test values) at each concentration was calculated for both matrices. P-values smaller than 0.05 were accepted as matrix effect was detected, meaning that different matrices has an influence on the quantification. As seen in Table S2.3, a matrix effect was present for m/p-xylene at all concentrations and also for other compounds such as ethylbenzene, o-xylene, indane, indene and naphthalene at different concentrations. Mixture B prepared in methanol and medium was also compared and a matrix effect was observed at all concentrations for monochlorobenzene (Table S2.4) and at different concentrations for the other compounds. Therefore, it was concluded that standard solutions should always be prepared in the matrix of interest.

Calibration curve: Standard solutions of mixture A and B for calibration were prepared in defined matrices and analyzed within the estimated concentration range. A linear regression method was applied. The slope and the linear regression coefficient (R^2) for each analyte was calculated. Correlation coefficients calculated for each analyte were given in Table S2.5 and Table S2.6, respectively. The linearity of the current method was confirmed with correlation coefficient being higher than 0.99 for all the compounds.

Accuracy and Precision: Table S2.7 and Table S2.8 presents the results of intermediate precision of the proposed method for mixture A and B, respectively. For the precision, mixture A prepared at different concentrations (Table S2.7) in methanol:groundwater showed RSD values between 0.25 - 14.39%. RSD values varied between 0.68 - 4.01% for compounds of mixture B, prepared in methanol:medium at different concentrations (Table S2.8). Results were convenient due to RSD values for all compounds were below 20%, indicating a high level of precision as stated by Filho et al. (2016) and also meets the US EPA quality control criteria (AlSalka et al., 2010). Recovery values for both mixtures were found to be between 82 and 110% as given in Table S2.9 and Table S2.10. Obtained values were found to be within acceptable recovery limits determined as 80-120% according to US EPA quality control criteria (AlSalka et al., 2010).

LOD and LOQ: LOD and LOQ for each compound was defined and given in Table 2.5. LOD and LOQ is matrix dependent, therefore LOQ was determined for mixture A in groundwater and in medium for mixture B. The LOQ values found range between 6 µg L^{-1} (indene and naphthalene) and 210 µg L^{-1} (benzene) for mixture A and with 980 µg L^{-1} for all compounds in mixture B. These results were found to be convenient for researchers performing lab scale biodegradation monitoring experiments. When initial concentrations of 10 mg L^{-1} are applied, the proposed method allows observations in degradation of 90% (Mixture B) to over 98% (Mixture A) conversion of the studied compounds.

Table 2.5 LOD and LOQ values of the developed method for mixture A and B.

Compound	LOD (µg L^{-1})	LOQ (µg L^{-1})
Mixture A		
Benzene	70	210
Toluene	23	70
Ethylbenzene	36	110
o-Xylene	36	110
p/m-Xylene	5	15
Indane	12	36
Indene	2	6
Naphthalene	2	6
Mixture B		
Benzene	290	980
Monochlorobenzene	290	980
1,2-Dicholorobenzene	290	980
1,4-Dichlorobenzene	290	980

LOD; Limit of detection, LOQ; Limit of quantitation

2.4. Discussion

2.4.1. Use of phenyl column and FLD detector

This method was developed by following the study of Amy Tan et al. (2012) where they investigated BTEX and styrene with HPLC-DAD with use of a Phenyl-1 column. According to the manufacturer's information, the selected column has an unique selectivity of aromatic compounds and higher chromatographic performance. The bonded phase displays greater π-π interaction than other phenyl phases and provides enhanced aromatic selectivity and higher hydrophobic retention. Therefore, we used this column as recommended by Amy Tan et al. (2012) contrary to common use of C18 columns selected for highly volatile compounds. Unfortunately, the use of phenyl column was not sufficient for the detection and quantification of all compounds in mixture A in one single run. This issue was solved by supplementing the method of Amy Tan et al. (2012) with the use of FLD detector. This way, compounds showing peaks at similar retention times such as o-xylene and indene could be differentiated with use of fluorescence detector under different excitation/emission range. By use of HPLC-DAD; benzene, monochlorobenzene, 1,2-dichlorobenzene and 1,4-dichlorobenzene was quantified in a single run. Using the same method with FLD; BTEX, indane, indene and naphthalene could be also quantified in a single run.

2.4.2. Sample preparation steps and low LOD concentration

Farhadian, Vachelard, et al. (2008) reported that HPLC is often not suitable for accurate separation of ethylbenzene from xylene isomers however, in the study of AlSalka et al. (2010) they were able to quantify BTEX from environmental samples with use of HPLC-DAD without any interference between the compound of interests. Scarcely, in the study of Alsaka et al., an additional step prior to injection to HPLC is needed where BTEX compounds are transferred from water to an organic solvent (acetonitrile) with help of N_2 gas. In that step, an amount of 75 mL of sample is needed for BTEX to be transferred to acetonitrile. Since they are dealing with environmental samples, this amount may not be considered as high. Solely, in laboratory spiked samples, minimizing the sample volume is extremely important especially if frequent sampling is unavoidable. This is why, this study focused on developing a method without a time-consuming preparation step where high sample volumes might be wasted. Keeping in mind that, the sample preparation step developed by Alsaka et al. allows to reach low LOD values that are crucial for environmental sampling, i.e. showing compliance to or exceedance over legislative thresholds that are between 0.1-50 µg L^{-1} (BTEX) (Sethi & Di Molfetta, 2019).

Pascale et al. (2018) published a quantitative analytical GC-BID method for monitoring BTEX in low contaminated water samples and were able to determine BTEX in concentrations below legal limits (µg L^{-1}). The disadvantage of this method is that again, high sample volumes are needed (15 mL) and it is only applicable for quantification at very low BTEX concentrations. When high concentrations are applied (higher than trace BTEX), chromatographic peaks of all analytes could interfere with the adjacent peaks or with the baseline. Their method can be a good option for quality control purposes but not ideal for laboratory spiked samples where high concentrations are studied and routine monitoring/sampling is needed.

2.4.3. Quantification of indane and indene

This study reports for the first time the direct quantification of indane and indene in water samples using HPLC even though these compounds are commonly found at high concentrations in groundwater of many industrial sites but are often not further studied for analytical or remediation purposes. Mundt & Hollender (2005) inspected indane and indene in groundwater samples and quantified these with

the use of LC–DAD UV/–FLD/–MS but in their study, samples needed to be treated with solid phase extraction (SPE) material prior to the analysis. These authors also tested spiked non-contaminated groundwater samples, found low LOD values, namely 0.4 µg L^{-1} for indene and 1.6 µg L^{-1} for indane, but recovery rates were found to be below 25%. In the study reported here, only a one-step preparation (methanol addition) is needed before injection to HPLC, and recovery rates were between 82 - 110%, in the range of acceptable limits for recovery according to US EPA methods.

2.4.4. HPLC method to facilitate routine lab-scale biodegradation experiments

Taking all results into account, the HPLC method described above is reliable, practical and fits for analysis of the contaminants commonly found in manufactured gas plant sites and chemical industrial areas. The tested method showed to be specific as all compounds could be identified and quantified at high accuracy applied in biological degradation experiments often in the range of 0.1 to 10 mg L^{-1}. Benzene had a somewhat higher LOQ value of 210 µg L^{-1}, which wasn't hampering the experiment for this study. Further, no laborious pre-treatment of samples were needed and sample volumes required for quantification was rather minimal (>375 µL). Proposed injection volumes within the method will allow a serial sampling of experimental batch bottles without significant changes of the total liquid volume in such batch experiments. Therefore, this method can facilitate the work of biodegradation researchers, as reliable results can be obtained with low sampling volumes and a relatively simple analytical method avoiding use of organic solvents or complex extraction methods in the monitoring of in situ or laboratory based biodegradation processes.

2.5. Conclusions

A single step analysis method for the quantification of 11 different (aromatic and chlorinated) compounds in aqueous samples was developed and validated by use of HPLC-FLD/DAD. The optimum conditions for HPLC operation was determined as a mobile phase of 60% methanol, with 50 µL (mixture B) and 80 µL (mixture A) injection volume, at a flow rate of 0.7 mL min^{-1} and a detection of 210 nm wavelength and use of FLD for the separation of indene and xylene isomers at different excitation and emission wavelength pair. Under determined optimum conditions, chlorinated and non-chlorinated aromatic compounds could be quantified within 24 and 27 min, respectively. The method was also tested and confirmed in terms of intermediate precision and recovery, showing acceptable results fitting the quality control criteria of US EPA methods.

To the best of our knowledge, this is the first time that indane and indene, together with BTEX and naphthalene were measured in aqueous samples with an HPLC method. Additionally, LOD and LOQ values were defined for all the compounds, within the detection range of 0.1 to 10 mg L^{-1}, suitable for investigation of lab-scale biodegradation processes. The method showed to be simple and easy to use for daily basis biodegradation measurements.

Acknowledgements

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Supplementary information to Chapter 2

Table S2.1 Concentration and recovery percentages for Mixture A compounds (10 mg L^{-1}), injected at different times after preparation.

Concentration (mg L^{-1})								
Time (hours)	**B**	**T**	**E**	**o- X**	**Ie**	**m/p-X**	**Ia**	**N**
1	10.74	10.65	10.51	10.25	9,97	10.88	10.48	10.52
2	10.56	10.52	10.44	10.20	9.93	10.58	10.34	10.39
6	10.81	10.75	10.62	10.59	10.27	10.45	10.44	10.47
12	9.12	9.56	9.60	9.59	9.53	9.69	9.91	9.98
24	6.55	7.62	7.96	8.02	7.65	8.25	8.63	8.80
Recovery (%)								
1	107	107	105	103	100	109	105	105
2	106	105	104	102	99	106	103	104
6	108	107	106	106	103	104	104	105
12	91	96	96	96	95	97	99	100
24	66	76	80	80	77	83	86	88

B; benzene, T; toluene, E; ethylbenzene, X; xylene, Ia; indane, Ie; indene, N; naphthalene

Table S2.2 Specific conditions of each detector used in this study.

UV			
Wavelength (nm)	**Bandwidth (nm)**	**ref Wavelength (nm)**	**ref Bandwidth (nm)**
210	1	600	1
FLD			
Excitation (nm)	**Emission (nm)**	**Sensitivity**	**Filter wheel**
210	260	4	auto
260	330	2	auto
280	310	4	auto

For the UV detector, the data collection rate is 50 and the response time is 500 ms.

The FLD detector has a multichannel performance, where the collection data rate and response time can be defined itself. In this study, the standard option with a maximum of 4hz data collection rate and the response time less than 250 ms was used. When the filter wheel is auto, the detector automatically selects a filter wheel position.

Table S2.3 T-test P-values at each concentration tested for the compounds in mixture A, in groundwater. Highlighted columns represent p-values < 0.05, indicating a significant matrix effect.

	Mixture A							
Concentration (mg L^{-1})	**B**	**T**	**E**	**o-X**	**m/p-X**	**Ia**	**Ie**	**N**
0.5	0.429	0.868	0.678	0.669	< 0.001	0.609	0.477	0.124
1	0.076	0.220	0.009	0.038	0.002	0.189	0.189	0.110
2.5	0.116	0.238	0.159	0.150	0.002	0.017	0.078	0.380
5	0.676	0.208	0.205	0.359	0.001	0.141	0.009	0.004
7.5	0.850	0.423	0.390	0.278	0.002	0.986	0.070	0.001
10	0.762	0.530	0.432	0.278	0.001	0.158	0.097	0.003

B; benzene, T; toluene, E; ethylbenzene, X; xylene, Ia; indane, Ie; indene, N; naphthalene

Table S2.4 T-test P-values at each concentration tested for the compounds in mixture B, in medium. Highlighted columns represent p-values < 0.05, indicating a significant matrix effect.

	Mixture B			
Concentration (mg L^{-1})	**Benzene**	**Monochlorobenzene**	**1,2-DCB**	**1,4-DCB**
1	0.055	0.031	0.276	0.059
2.5	0.043	0.005	0.033	0.001
5	0.007	0.020	0.062	0.017
7.5	0.007	0.027	0.067	0.016
12	0.020	0.016	0.017	0.053

DCB; Dichlorobenzene

Table S2.5 Intercept, slope and R^2 values of the linear calibration line from the compounds of mixture A.

	Mixture A							
Compounds	**B**	**T**	**E**	**o-X**	**m/p-X**	**Ia**	**Ie**	**N**
Intercept (a)	12116	59006	72661	62736	639601	200006	231467	200006
Slope (b)	2194	8707	9974	4655	81357	142190	35704	106999
R^2	0.998	0.9984	0.9988	0.9968	0.9986	0.9993	0.9988	0.9994

B; benzene, T; toluene, E; ethylbenzene, X; xylene, Ia; indane, Ie; indene, N; naphthalene

Table S2.6 Intercept, slope and R^2 values of the linear calibration line from the compounds of mixture B.

	Mixture B			
Compounds	**Benzene**	**Monochlorobenzene**	**1,2-DCB**	**1,4-DCB**
Intercept (a)	0.8476	1.7032	1.4351	1.3978
Slope (b)	0.324	0.8452	0.9298	1.1396
R^2	0.9987	0.9971	0.9936	0.9916

DCB; Dichlorobenzene

Table S2.7 RSD values for each compound in mixture A, at different concentrations for the determination of intermediate precision of the method.

	Intermediate Precision (%)							
Concentration (mg L^{-1})	**B**	**T**	**E**	**o-X**	**m/p-X**	**Ia**	**Ie**	**N**
0.5	10.91	8.19	7.30	4.34	1.11	9.15	2.09	0.25
1	14.39	10.35	7.29	5.88	0.25	9.24	0.59	2.22
2.5	4.47	1.82	1.66	2.46	2.60	1.39	2.75	3.39
5	1.78	3.88	2.64	2.18	0.96	13.22	2.39	3.05
7.5	4.58	3.38	1.98	1.83	3.12	0.61	1.49	2.81
10	7.78	6.14	3.55	3.14	3.53	12.64	2.86	2.74

B; benzene, T; toluene, E; ethylbenzene, X; xylene, Ia; indane, Ie; indene, N; naphthalene

Table S2.8 RSD values for each compound in mixture B, at different concentrations for the determination of intermediate precision of the method.

	Intermediate Precision (%)			
Concentration (mg L^{-1})	**Benzene**	**Monochlorobenzene**	**1,2-DCB**	**1,4-DCB**
1	2.88	0.68	2.56	3.51
2.5	3.43	2.45	2.57	3.68
5	3.67	4.01	1.48	3.46
7.5	1.57	1.89	2.46	3.16
12	1.60	1.58	3.00	3.65

DCB; Dichlorobenzene

Table S2.9 Recovery values and mean recovery percentages obtained at 10 mg L^{-1} for mixture A.

	Recovery (10 mg L^{-1})							
	B	**T**	**E**	**o-X**	**m/p-X**	**Ia**	**Ie**	**N**
Sample 1	8.70	10.31	10.05	10.09	10.19	10.18	10.31	10.64
Sample 2	8.88	10.89	11.24	11.63	10.98	10.85	10.89	11.36
Recovery % (mean)	88	106	106	109	106	105	106	110

B; benzene, T; toluene, E; ethylbenzene, X; xylene, Ia; indane, Ie; indene, N; naphthalene

Table S2.10 Recovery values and mean recovery percentages obtained at 10 mg L^{-1} for mixture B.

	Recovery (10 mg L^{-1})			
	Benzene	**MCB**	**1,2-DCB**	**1,4-DCB**
Sample 1	8.33	10.62	9.49	9.35
Sample 2	8.01	10.23	9.32	9.50
Recovery % (mean)	82	104	94	94

MCB; Monochlorobenzene, DCB; Dichlorobenzene

Chapter 3

Indene, Indane and Naphthalene in a mixture with BTEX affect aerobic compound biodegradation kinetics and indigenous microbial community development

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Abstract

BTEX (benzene, toluene, ethylbenzene, xylene) are common pollutants often found in former gasworks sites together with some other contaminants like indene, indane and naphthalene (Ie, Ia, N). This study aimed to evaluate the inhibitory or stimulative substrate interactions between BTEX, and Ie, Ia, N during aerobic biodegradation. For this, batch bottles, containing originally anaerobic subsurface sediments, groundwater, and indigenous microorganisms from a contaminated former gasworks site, were spiked with various substrate combinations (BTEX, BTEXIe, BTEXIa, BTEXN, BTEXIeIa, BTEXIeN, BTEXIaN, BTEXIeIaN). Subsequently concentrations were monitored over time. For the BTEXIeIaN mixture, initial concentrations were between 1 to 5 mg L^{-1}, and all compounds were completely degraded by the microbial consortia within 39 days of incubation. The experimental data were fitted to a first order kinetic degradation model for interpretation of inhibition/stimulation effects between the compounds. Results showed that indene, indane, and naphthalene inhibited the degradation of benzene, toluene, ethylbenzene, o-xylene, with benzene being the most affected compound. M/p-xylene is the only compound whose biodegradation is stimulated by the presence of indene and indane (individually or mixed) but inhibited by the presence of naphthalene. 16S rRNA amplicon sequencing revealed differentiation in the microbial communities within the batches with different substrate mixtures, especially within the two microbial groups *Micrococcaceae* and *Commamonaceae.* Indene had more effect on the BTEX microbial community than indane or naphthalene and the presence of indene increased the relative abundance of *Micrococcaceae* family. In conclusion, co-presence of various pollutants leads to differentiation in degradation processes as well as in microbial community development. This sheds some light on the underlying reasons for that organic compounds present in mixtures in the subsurface of former gasworks sites are either recalcitrant or subjective towards biodegradation, and this understanding helps to further improve the bioremediation of such sites.

3.1. Introduction

Soil and groundwater contamination with petroleum hydrocarbons is a widespread environmental problem (Lueders, 2017). Aromatic hydrocarbons such as BTEX (benzene, toluene, ethylbenzene, xylene) are commonly used as industrial solvents and materials in fine chemical and petrochemical industries (Estévez et al., 2005). Such petroleum products often end up being released to the environment due to industrial activities related to refining, transportation, use, and disposal. They are also the waste product of the purification of the coal gas at gasworks sites (Murphy et al., 2005). This is of particular concern since BTEX compounds are defined as environmental priority pollutants by environmental agencies and classified as toxic and carcinogens (Lueders, 2017). Due to their high solubility in water, they can be easily transported over large distances by groundwater (Birak & Miller, 2009; Lueders, 2017). Therefore, contamination of aquifers, and consequently drinking water sources by such pollutants is a serious threat to the environment and human health.

An appealing solution to remove BTEX from the environment is bioremediation. Bioremediation relies on natural biodegradation, which is a sustainable, eco-friendly, and cost-effective process for contaminant breakdown, wherein microbes act as degraders with the goal of converting BTEX to less toxic or non-toxic compounds (H. Huang et al., 2021). Several factors are known to influence the biodegradation process such as pollutant characteristics (type, concentration, availability), environmental conditions (temperature, pH, availability of inorganic nutrients and electron acceptors) and microbial communities (adaptation, active biomass concentration, activity degradation potential) (Varjani, 2017). Substrate interactions are another factor that should be considered, as inhibitory or stimulative interactions between contaminants can greatly affect the degradation performance. Because contaminated sites typically involve various pollutant mixtures, these interactions should be studied carefully (Deeb & Alvarez-Cohen, 1999; Littlejohns & Daugulis, 2008; Zhou et al., 2011). Research on aerobic BTEX degradation and the interaction between BTEX compounds has been documented in earlier studies (Alvarez & Vogel, 1991; Deeb & Alvarez-Cohen, 1999; S. K. Lee & Lee, 2002). However, at contaminated sites, BTEX often co-occurs in combination with other pollutants. There are few studies investigating the multi-substrate effect of BTEX with pollutants such as naphthalene (Gülensoy & Alvarez, 1999), tetrahydrofuran (Zhou et al., 2011), methyl ter-butyl ether (Deeb et al., 2001) and ethanol (J. Cho et al., 2007; Da Silva et al., 2005). However, there is a knowledge gap regarding the interaction between BTEX and other potential co-occurring contaminants such as indene and indane.

Beside the multi-substrate effect, it is important to study the microbial community responsible for the biodegradation process and the effect of the presence of complex contaminant mixtures on the microbial community. Although the number of papers on the molecular analysis of microbiomes is increasing, the interaction between microbial community and changes of environmental factors are still far from clear. Jiao et al. (2016) investigated the succession patterns of the microbial community in response to various pollutants, while Huang et al. (2021) showed that BTEX played a key role in shifting the microbial community in groundwater. Because various pollutants often co-occur in polluted areas like former gasworks sites, understanding potential interactions is necessary for the design and optimization of engineered bioremediation processes to treat sites that have been contaminated for decades.

In this study, BTEX, indene, indane and naphthalene were selected as the contaminant mixture. This selection was made based on the contaminants found in abundance at a former gasworks site for over 100 years. Information about indene and indane degradation in literature is scarce, and substrate interaction between BTEX together with indene, indane and naphthalene is not available. The present study focuses on the effect of indene, indane and naphthalene on the aerobic biodegradation of BTEX

compounds under laboratory conditions by the indigenous microorganisms coming from a former gasworks site. Ultimately, outcomes of this study can help to obtain insights into the biodegradation process of various mixtures of contaminants and additionally contribute to implementing efficient bioremediation strategies towards protecting drinking water sources.

3.2. Materials and Methods

3.2.1. Sediment and groundwater sampling

Sediments and groundwater used in this study were collected from Griftpark, Utrecht (The Netherlands), a former gasworks site. From 1859 to 1960, several manufactured gas factories were located here, and many other industrial activities were carried out at this location. Notable contaminants in the subsurface and groundwater of the site were detected as BTEX, indene, indane and naphthalene (Hauptfeld et al., 2022). Subsurface sediments from Griftpark were collected from drillings (November 2018) at 38-38.5 m below ground level (bgl) and groundwater was pumped out from 8-10 m bgl through monitoring wells (October 2020). Sediments and groundwater samples were collected from the same well, at different depths as described before. All samples were immediately stored in glass containers at 4°C and in the dark until use.

3.2.2. Chemicals

The chemicals used in this study were reagent or analytical grade. Benzene (>99.7%) was purchased from VWR Chemicals (USA); toluene (>99.9%) from Merck KGaA (Germany); ethylbenzene, m-xylene, o-xylene (>99%) and indane (>95%) from Alfa Aesar (Germany); p-xylene (99%) from Acros Organics (Czechia), and indene and naphthalene (>99.9%) were obtained from Sigma-Aldrich (Germany).

3.2.3. Experimental set-up

Experiments were conducted in batch reactors of 250 mL autoclaved, clear glass bottles. Each reactor contained 20 g of sediment as the source of inoculum and 150 mL of groundwater as media. The pH of the samples (sediment + groundwater) was measured between 7-7.5. Air was present in the headspace (~100 mL) at 1 atm pressure in order to supply enough oxygen for the complete removal of contaminants. Bottles were capped with Butyl/PTFE-coated septa and aluminum crimp caps. Reactors were incubated in a rotary shaker at 120 rpm and 20 °C, in the dark between 13 to 39 days depending on the experimental set-up.

All bottles were spiked with the contaminant mixture of interest. A pure mix solution was prepared, and relevant compounds were purely mixed instead of being dissolved in a carrier solvent. For this, 100 mg of each compound was pipetted in a 1.5 mL clear glass vial and vortexed for one minute. The equivalent volume of 100 mg of the compound was calculated based on the density. For instance, the density of benzene is 0.8765 g mL^{-1}. For 100 mg of benzene, 0.114 mL was pipetted. This procedure was applied for all compounds except naphthalene as it is found in solid form. Once naphthalene crystals were completely dissolved, 10 µL pure mix was injected into the reactors with a 10 µL glass syringe. The aim was to have between 1-5 mg L^{-1} of each compound in the reactors. The bottles were incubated overnight to equilibrate the hydrocarbons between gas and liquid phases and then was sampled to determine the initial concentration.

To test the effect of indene, indane and naphthalene on BTEX biodegradation, eight different substrate combinations (set-up) were made as BTEX, BTEXIe, BTEXIa, BTEXN, BTEXIeIa, BTEXIeN, BTEXIaN and BTEXIeIaN (Table 3.1). Each set-up contained five bottles: three active bottles and two abiotic controls. Control bottles were sterilized by standard autoclaving procedure for 30 min at 121 °C. Sodium azide (NaN_3) and mercury chloride ($HgCl_2$) were added as biocides to prevent any potential microbial activity during the experiment. Abiotic controls were prepared for each set-up to discern volatilization and adsorption from biodegradation process. Experiments were performed in triplicate and experimental error was calculated as the standard deviation of triplicate samples.

Table 3.1 Eight substrate combinations (set-up) used in the batch experiments. (+); compound present in the bottle, (-); compound missing in the bottle.

	Substrate mixture							
Compounds	BTEX	BTEXIe	BTEXIa	BTEXN	BTEXIeIa	BTEXIeN	BTEXIaN	BTEXIeIaN
Benzene	+	+	+	+	+	+	+	+
Toluene	+	+	+	+	+	+	+	+
Ethylbenzene	+	+	+	+	+	+	+	+
o-Xylene	+	+	+	+	+	+	+	+
m/p-Xylene	+	+	+	+	+	+	+	+
Indene	-	+	-	-	+	+	-	+
Indane	-	-	+	-	+	-	+	+
Naphthalene	-	-	-	+	-	+	+	+

3.2.4. Analytical measurements

Each contaminant was measured and quantified by using HPLC-FLD equipped with an Acclaim™ Phenyl-1 HPLC column 150 x 4.6 mm, 3 µm (Thermo Scientific Dionex, USA). The operating parameters and flow rates have been described previously (Aydin et al., 2021). Compounds were analyzed by sampling the liquid phase from bottles using a 1 mL syringe equipped with a 0.4 mm needle. One mL liquid sample containing sediment and groundwater was centrifuged at 15000 rpm for 10 min, and 750 µL supernatant was transferred to HPLC vials. Finally, 250 µL methanol was added to the vial in order to limit evaporation losses during HPLC autosampler procedures. Chromeleon software (Thermo Fischer Scientific, USA) was used for analysis of the data. Sampling proceeded until one contaminant was left or until contaminant concentrations in the bottles dropped below their detection limits in the HPLC method (2-70 µg L^{-1}). After each measurement, the average amount of contaminant present in the samples from the active bottles were normalized to the average amount of contaminants present in the sample of the controls (Equation 3.1).

$$Normalized\ concentration = \frac{Mean\ active}{Mean\ control} \quad (3.1)$$

Gas chromatography (GC-2010, Shimadzu) was used for headspace gas measurements for monitoring O_2, CO_2, N_2 and CH_4 with the method of De Wilt et al. (2018). For this, 2 mL of gas was sampled from the headspace of the bottles and injected to the GC equipment.

3.2.5. Kinetic parameters

In order to examine the substrate interactions for all compounds in the various BTEXIeIaN mixtures, kinetic modeling was performed using the experimental data. The results of the batch data were fitted with the first-order kinetic model from Equation 3.2.

$$\frac{dS_i(t)}{dt} = -\lambda_i S_i(t) \quad (3.2)$$

$S_i(t)$ is the concentration of the i^{th} aromatic hydrocarbon and λ_i its first order degradation rate constant. The solution was fitted with a non-linear least squares curve fit in Python, with the rate constant λ_i and initial concentration $S_i(0)$ as fitting parameters using Equation 3.3.

$$S_i(t) = S_i(0) * e^{-\lambda_i t} \quad (3.3)$$

The initial concentration $S_i(0)$ was included as fitting parameter because measured initial concentrations were not reliable as the hydrocarbons dissolve into each other and may precipitate (i.e., naphthalene). The control batches showed some decrease in concentrations, indicating the occurrence of sorption and/or volatilization as complementary to biodegradation processes. Therefore, before fitting, the concentration data were normalized with the control batch data (Equation 3.1).

Comparisons of experimental and fitted degradation curves of each BTEXIeIaN compound in the various mixtures were used to assess the effect of indene, indane and naphthalene (individually or mixed) on the other compounds, as well as on each other. As all batches were performed in triplicates, degradation rate constants calculated for each of the batch tests were normalized to controls and averaged. To assess the quality of the model fits, the coefficient of determination (R^2) was used. Also, for R^2, the mean R^2 value of the three fits was used. The lag phase was taken out for the individual compounds of each batch series for optimal fitting and were separately reported (Figure S3.1). To study inhibitive/stimulative effects of Ie, Ia or/and N on the compound of interest more easily, the ratio of the rate constants was calculated for each compound. For example, the effect of indene on benzene degradation within BTEX mixture is calculated by using Equation 3.4:

$$\alpha_{B,Ie} = \lambda_B \frac{BTEXIe}{BTEX} \quad (3.4)$$

where α is the ratio of the rate constants (i.e., for benzene in the presence of indene compared to the absence of indene ($\alpha_{B,Ie}$)), obtained by dividing the degradation rate constant of the compound (λ_B) in the mixture of interest (BTEXIe) to the degradation rate constant in the mixture BTEX without indene. The equations used for the calculations of all the relevant ratios of rate constants can be found in Table S3.1. Values of α<1 indicate inhibition, and α>1 indicates stimulation of biodegradation through the presence of the added extra compound(s) to the BTEX mixture.

3.2.6. DNA extraction and sequencing

In order to understand the impact of different substrate mixtures on microorganisms, one batch from each set-up was sampled for microbial community analysis. The aim was to investigate the difference in microbial communities between the set-ups and the changes in the microbial community when exposed to different substrate mixtures.

For DNA analysis, nine different samples were collected. For the representation of the native microbial consortium, the sample T0 was collected before spiking the batch bottle with any contaminant. To compare the native microbial community to the final community of each set-up, eight different samples were taken at the end of the experiment (day 13-39 depending to the set-up).

An amount of 5 mL sediment-groundwater mix was collected from all set-ups and T0. Batch bottles were shaken vigorously before sampling in order to have a homogenous sample of sediment-groundwater mix. Then, to allow cell precipitation, samples were centrifuged for 10 min at 15000 rpm. After centrifugation, the supernatant was discarded, and the pellet was stored at -80°C until further use for DNA extraction. Microbial DNA was extracted from each sediment sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). After processing the samples in a bead beater to lyse the cells, DNA was extracted according to the manufacturer's instructions. The isolated DNA was used as template for amplifying the V3 and V4 region of 16S rRNA via Illumina sequencing using the primer sets described by Takahashi et al. (2014).

3.2.7. Processing and analysis from sequencing data

Sequence analysis of the raw data was performed in NG-Tax version 2.1.74 using default settings as described in Poncheewin et al. (2020). Taxonomy was assigned using the SILVA reference database version 138.1. Paired-end libraries were demultiplexed using read pairs with perfectly matching barcodes. Amplicon sequence variants (ASVs) were picked as follows: for each sample, sequences were ordered by abundance and a sequence was considered valid when its cumulative abundance was ≥ 0.1%. ASVs are defined as individual sequence variants rather than a cluster of sequence variants with a shared similarity above a pre-specified threshold, such as operational taxonomic units (OTUs). All analyses were performed in R version 4.2.0 (https://R-project.org/).

Raw sequences with barcode and primer removed and supporting metadata were deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number PRJEB58637.

3.3. Results and Discussion

3.3.1. Complete degradation of BTEXIeIaN under aerobic conditions

Figure 3.1 shows that all the compounds of the BTEXIeIaN mixture were degraded within 39 days, under aerobic conditions in the laboratory. Processes like volatilization and absorption were discarded as all data was normalized as described in Section 3.2.4. Complete removal of indene, indane and naphthalene was observed in the two out of the three active bottles. This occurred at day 34 for indene and indane, and at day 39 for o-xylene. Similarly, for the other substrate mixtures tested, all compounds were also completely degraded, although both the order in degradation and the degradation times differed (Figure S3.2).

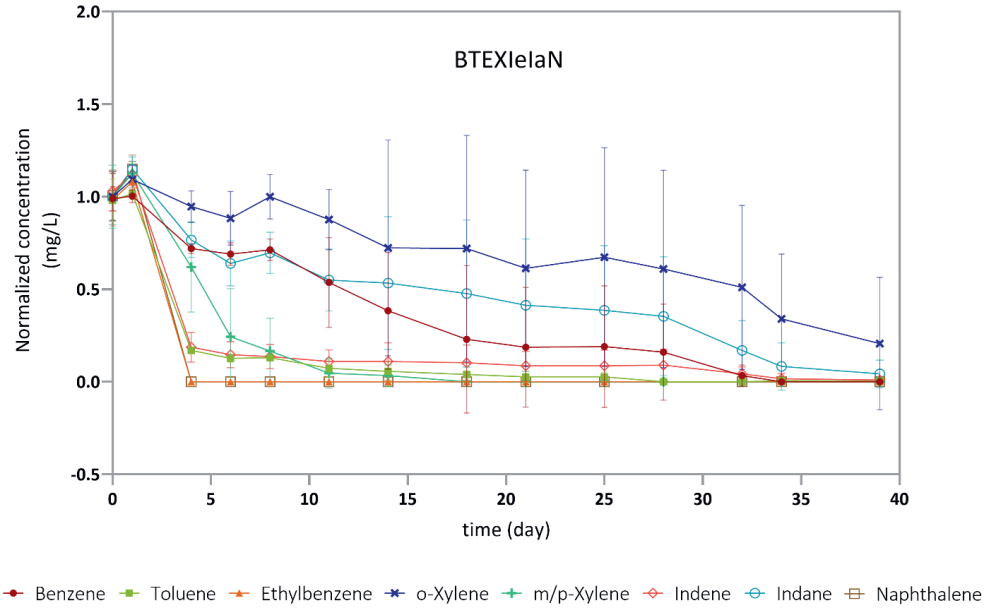

Figure 3.1 Biodegradation of BTEXIeIaN mixture under aerobic conditions in 39 days. The figure used the mean and standard deviations from experimental triplicates, normalized to the mean of abiotic controls.

Aromatic hydrocarbon degradation mostly occurs under aerobic conditions. Oxygen is needed for both ring activation and cleavage of the aromatic structure and also as the electron acceptor for its complete degradation by bacteria catalyzed by mono or dioxygenases (El-Naas et al., 2014). BTEX compounds often occur as a mixture in contaminated sites; therefore, an organism that could degrade all the different components of BTEX is more desirable (Singh & Celin, 2010). However, microorganisms capable of degrading different types of compounds such as the BTEX and polyaromatic hydrocarbons have very rarely been reported (Y. Lee et al., 2019). Various studies (Alvarez & Vogel, 1991; M. Chang et al., 1993; Oh et al., 1994) investigated single strains on BTEX degradation showing that single strains are not capable of removing all compounds simultaneously and efficiently. For BTEX degradation, microbial consortia were found to be more powerful than pure cultures (Mukherjee & Bordoloi, 2012). In this study, a variety of microorganisms were present, likely with different biodegradation capacities, enabling a complete degradation of the complex mixture harboring both monoaromatic and polyaromatic compounds. This means that in this subsurface sediment system none of the compounds are intrinsically recalcitrant in the presence of oxygen. These results showed the intrinsic aerobic degradation potential of deep surface microorganisms and suggests that oxygen could possibly be an alternative solution to remove deep subsurface BTEXIeIaN contaminations, instead of purely anaerobic strategies. Further studies about BTEXIeIaN biodegradation at different oxygen concentration could facilitate use of the aerobic degradation potential reported in this study.

3.3.2. Substrate degradation patterns during BTEXIeIaN biodegradation

Substrate degradation patterns of individual compounds in the BTEXIeIaN mixture vary depending on the substrate combinations used in each set-up (Table 3.2). The degradation time was defined as the moment the concentration of individual compound declined below the detection limit. In this way, the order of degradation could be established. Overall, fastest degradation was observed for ethylbenzene and/or naphthalene. For all cases where naphthalene was present in the mixture (i.e., BTEXN, BTEXIeN, BTEXIaN, BTEXIeIaN), it was the first compound to be fully degraded either with ethylbenzene (BTEXIaN and BTEXIeIaN) or ethylbenzene being the second compound degraded. After ethylbenzene, the order in degradation varied but was often observed as toluene, m/p-xylene, benzene, indene, indane and finally o-xylene. As shown in Table 3.2, o-xylene was found to be the slowest degrading compound in all set-ups except BTEXIaN.

Table 3.2 Degradation order of each compound for each set-up. The compounds were given in the order of degradability starting with easily degradable compound to the most difficult one. The colour is adjusted per column: light blue (1) represents the fastest to reach complete degradation and dark blue (5-7) represents the slowest to reach complete degradation.

	BTEX	BTEXIe	BTEXIa	BTEXN	BTEXIeIa	BTEXIeN	BTEXIaN	BTEXIeIaN
Naphthalene				1		1	1	1
Ethylbenzene	1	1	2	2	1	2	1	1
Toluene	2	2	3	4	2	3	2	3
m/p-Xylene	3	3	1	3	1	6	3	2
Benzene	4	4	4	5	3	4	5	4
Indene		5			4	5		5
Indane			5		5		5	6
o-Xylene	5	6	6	6	6	7	4	7

The degradation order of m/p-xylene was found to be inconsistent in the presence of different co-substrates (Table 3.2). M/p-xylene was degraded first in the set-up of BTEXIa and BTEXIeIa, and as the second compound in BTEXIeIaN mixture. In BTEXIeN, m/p-xylene was removed second to last, and it was removed after indene, making this the only set-up where indene is degraded before m/p-xylene. This was observed in all triplicate bottles. The IeN combination appeared to inhibit m/p-xylene degradation, whereas indane, and indane together with indene, contributed to a faster completion of m/p-xylene degradation.

Literature studies are in line with our findings of ethylbenzene being the most easily degraded compound when tested with BTEX mixtures. This was observed by Deeb & Alvarez-Cohen (1999) with different BTEX combinations but also in the presence of BTEX with other co-pollutants as n-propylbenzene or 1,2,4-trimethylbenzene. Results show that among BTEX compounds, ethylbenzene is always the first to be degraded no matter the co-pollutant. Interestingly, when BTEX compounds were tested individually, toluene is the first compound to be degraded (Deeb & Alvarez-Cohen, 1999). This was supported by El-Naas et al. (2014) as toluene being the most easily biodegradable among the

BEX compounds due to the presence of the substituent group on the ring that facilitates the attack on the side chain or oxidization of the aromatic ring. For isomers of xylene, they were reported to be degraded last among BTEX compounds (Alvarez & Vogel, 1991; Littlejohns & Daugulis, 2008) with o-xylene being more recalcitrant than the others (E. Cho et al., 2009). In this study, the degradation pattern varied among set-ups, mostly with m/p-xylene as mentioned before, which led to further investigations on substrate interactions. Differences in the findings show that an investigation of the multi-substrate effect is needed for a better understanding and for predicting the biodegradation behavior of BTEX compounds and/or other co-occurring contaminants.

3.3.3. Effect of indene, indane and naphthalene on biodegradation kinetics of BTEXIeIaN compounds

Biodegradation potential of individual compounds may be inhibited or stimulated by the presence of other compounds in the mixture. To understand the possible substrate interactions between each compound of the BTEXIeIaN mixture, the experimental data was fitted to a model. In this model, the first order reaction constants (1/day) of each compound in each set-up was calculated (Table S3.2). These results were qualitatively interpreted as low sampling frequency reduced the validity of rigorous quantitative analysis. Thus, Table S3.2 was used to make a relative comparison of the different degradation rate constants. For this, ratio of rate constants of interest (α) was calculated as in equations given in Table S3.1 and results were given in Table 3.3.

Benzene is the compound that is most strongly inhibited by the addition of indene, indane and naphthalene, individually or combined. This phenomenon raises environmental concerns about benzene because of its high mobility and because it tends to persist in the environment due to its low sorption and slow degradation rate compared to the other aromatic hydrocarbons (Huang & Li, 2014). Benzene is also the most dangerous compound among monoaromatics and possesses carcinogenetic risks (Lueders, 2017). Despite the above-mentioned concerns, it should be mentioned that although benzene is the most inhibited compound, it will not necessarily be degraded last. In Table 3.2 it can be observed that o-xylene is often the last compound to be removed from the batches.

Table 3.3 Substrate interactions calculated based on ratio of the rate constants. Darker blue indicates more strongly inhibited (α<1) and darker yellow more strongly stimulated (α>1) biodegradation. Gridlines are no value.

	BTEXIe	BTEXIa	BTEXN	BTEXIeIa	BTEXIeN	BTEXIaN	BTEXIeIaN
Benzene	0.32	0.23	0.19	0.15	0.19	0.12	0.12
Toluene	0.84	0.31	0.17	0.51	0.48	0.41	0.28
Ethylbenzene	0.93	0.54	1.18	0.81	0.77	0.82	0.85
o-Xylene	0.71	0.74	0.95	0.41	0.04	0.31	0.40
m/p-Xylene	3.74	3.25	0.86	3.89	0.22	0.38	0.68
Indene				0.11	0.77		0.99
Indane				0.21		0.51	0.34
Naphthalene					0.62	0.64	0.66

Among the BTEX compounds, m/p-xylene, in the absence of naphthalene, is the only compound stimulated by the presence of indene or/and indane (Table 3.3). Additionally to m/p-xylene, ethylbenzene had a slight stimulation effect by naphthalene. Gülensoy & Alvarez (1999) studied biodegradation capabilities of indigenous microorganisms exposed to different combinations of aromatic hydrocarbons of BTEXN. They reported enhanced degradation of naphthalene by ethylbenzene. In this study, a vice-versa effect was recorded (Table 3.3, BTEXN mixture). For all the other compounds, only inhibition patterns were observed in presence of indene, indane and/or naphthalene.

Our results suggest that substrate interactions among BTEXIeIaN differ from each other in various mixtures, despite the similarities in the chemical properties and structures of these compounds. One of the possible reasons for such inhibition/stimulation effects during BTEXIeIaN degradation can be the presence of broad-spectrum enzymes having different affinities for each compound, due to the repression or induction of genes involved in the degradation of one compound but not another (Aburto & Peimbert, 2011). Even though the focus of this study is far from an enzymatic approach, there was an effort made to study the microbial communities present in the different mixtures to provide an additional perspective to the BTEXIeIaN biodegradation patterns. Insights about the changes in the complex microbial community over time with different substrate combinations might be a first step to elucidate the complex interactions related to inhibition phenomena.

3.3.4. Microbial community is influenced by different substrate mixtures

To get an overview of the differences in microbial community between the set-ups, beta-diversity analyses were done, and the results are displayed in Figure 3.2. The Principal Coordinate Analysis (PCA) (Figure 3.2) gives a visualization of the microbial communities between the different set-up conditions by Weighted Unifrac index. In Figure 3.2, Axis 2 explains the variability of microbial communities by 11.1% and Axis 1 does it by 75%. It should be mentioned that the following results are descriptive, and the differences in the microbial community between samples could not be statistically proven due to the lack of replicates for DNA analyses in the different set-ups.

Differences in the substrate mixtures leads to differences in the microbial community. In this study, highest differences in microbial composition were found between BTEXIeIaN and BTEX. In Figure 3.2, the BTEX set-up and BTEXIeIaN are positioned in the two different sides of the Axis 1, and all other set-ups were found to be in-between. This can be expected: as more compounds are added to the mixture, more changes may occur in the community with an increase or decrease in microbial diversity. This can also be dependent on the contaminant type (i.e., chemical structure) and the microbial degradation potential rather than the variety of contaminants (i.e., five contaminant vs eight). Although there is no clear grouping in Figure 3.2 based on the presence of the different substrate mixtures, there is a distinct distribution when either indene (blue) or indane (yellow) are present. On one hand, mixtures that contain indene are positioned closely to BTEXIeIaN, gathered on the left side of the graph. On the other hand, samples containing indane are positioned more toward the BTEX mixture, and commonly on the right side of the graph. This paper focuses on the impact of indene, indane and naphthalene on BTEX; therefore, when comparing differences of microbial composition, it is observed that BTEXIe is the mixture differentiating from BTEX the most compared to BTEXIa and BTEXN. As a conclusion, the presence of indene shows to exert selective pressure on the BTEX microbial community rather than indane or naphthalene.

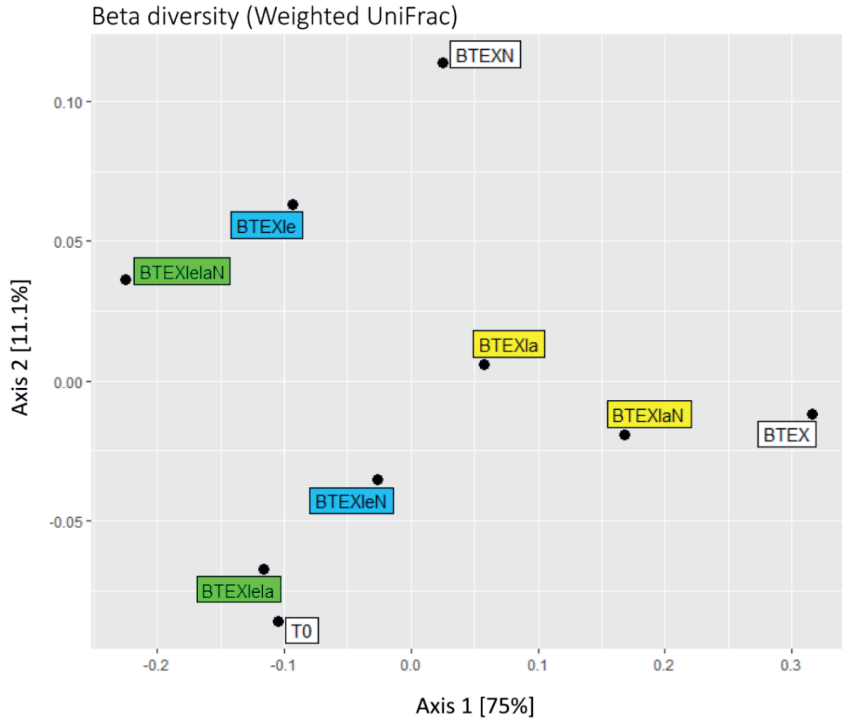

Figure 3.2 Beta-diversity analysis using the Weighted Unifrac distances to investigate the microbial community differences among each set-up. (T0: Data collected from sediment sample before addition of any contaminant). Samples containing indene were represented in blue, samples containing indane in yellow and samples with indene and indane in green.

The microbial communities in the batches with BTEXIeIaN and BTEXIeIa were rather similar meaning that naphthalene may not be playing a significant role in shaping the final microbial community. This is likely due to naphthalene being the first compound to be degraded (Table 3.2, Figure S3.2); therefore, due to its short-time presence, it may not have a major effect in the final microbial consortium. The sediment sample T0 was sampled as the representative starting point of the experiment. The position of T0 is very close to BTEXIeIa, meaning that the final microbial communities between T0 and BTEXIeIa are similar. Since the initial sample (T0) comes from a former gasworks site, mostly contaminated with an BTEXIeIaN mixture, it can be expected that T0 is closer to the position of BTEXIeIaN rather than BTEX. However, as mentioned before, original sediment samples were collected from 38 m bgl where anaerobic conditions are prevalent. This finding leads to the hypothesis that the indigenous microorganisms coming from deep surface were versatile and capable of quickly adapting to aerobic conditions thus, showing promising aerobic degradation capacities, which is important for development of cost effective in situ remediation strategies. Similar results were previously reported (Aburto et al., 2009; Fahy et al., 2006), where microorganisms enriched from a benzene-contaminated anaerobic groundwater were able to degrade benzene aerobically. The implications of these findings for treatment practices will be discussed in the section of Future Perspectives.

Similar to this study, investigation of different substrates and their effect on the microbial communities and diversity was studied by Banerjee et al. (2022) and Jiao et al. (2016). Banerjee et al. (2022) showed that different bacterial communities played a role in the degradation of the different xylene isomers. Jiao et al. (2016) studied the differences in microbial composition among different pollutants, namely phenanthrene (PHE), n-octadecane (C18), PHE and C18, and concluded that different pollutants and their combinations influenced the bacterial community by variations of the composition and relative abundance of the phylogenetic groups. Since changes in microbial community can be associated with functional capabilities (Strickland et al., 2009), this study shows the importance of microbial community characterization in response to the pollutant.

3.3.5. Microbial composition in different substrate mixtures

Members of *Proteobacteria* phylum might be related to BTEX degradation; as shown in the compositional plot (Figure 3.3), this phylum showed high relative abundance in all the set-ups. Contamination with hydrocarbons has been associated with an increase of members belonging to *Proteobacteria* (Alfreider & Vogt, 2007). Chen et al. (2022) reported some indigenous microorganisms coming from polluted groundwater or soil, such as *Actinobacteria*, *Proteobacteria* and some members of *Actinomycetota* such as *Rhodococcus and Arthrobacter* strains, to have high BTEX degradation activity. Outcomes of this study showed that the highest relative abundance of *Proteobacteria* was observed in the BTEX mixture and the lowest in BTEXIeIaN. This finding is in line with the differences discussed in Section 3.3.4, where the microbial communities from BTEXIeIaN differed the most from the communities in the presence of BTEX without other compounds.

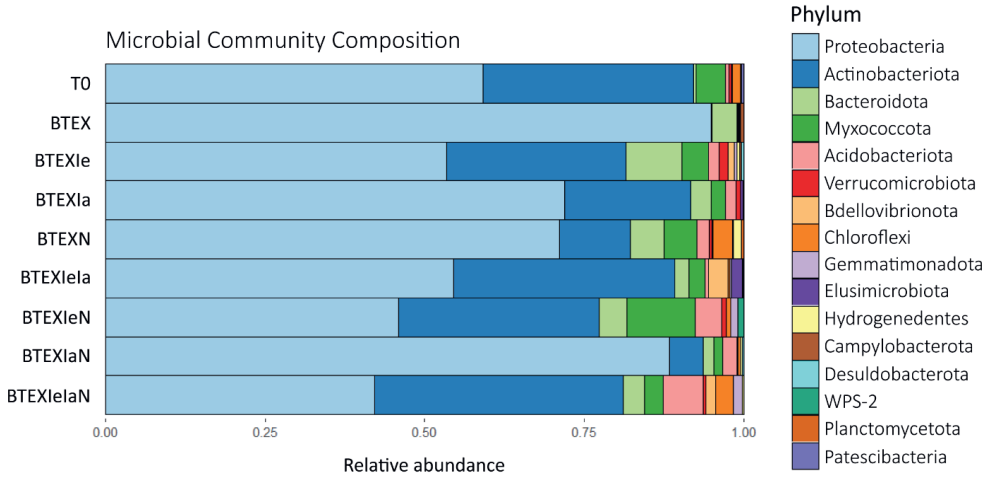

Figure 3.3 Relative abundance of the most abundant phyla for different substrate mixtures.

Presence of indene, indane and naphthalene promotes the abundance of *Actinobacteriota* phylum. As shown in Figure 3.3, higher relative abundance of *Actinobacteriota* was observed in the mixtures containing either indene, indane or naphthalene but not in the BTEX mixture. Set-ups including indene especially have higher relative abundance of *Actinobacteriota.* This was also the case for T0, which is the sample taken at the beginning of the experiment prior to spiking with any contaminant. It can be hinted that *Protobacteria* might be playing a role in BTEX degradation, while *Actinobacteriota* could be more related with degradation of co-substrates such as indene, indane and/or naphthalene. Further studies are needed to obtain insights about the degradation capacity of the afore mentioned microbial groups.

Different substrate mixtures showed different microbial compositions, with varying relative abundances. Figure 3.4 shows a more detailed display of microbial communities analyzed at genus level with compositional plots of the top 30 abundant genuses present in each set-up. As shown here, common hydrocarbon degraders reported in literature such as *Acinetobacter* and *Pseudomonas* (S. Jiao et al., 2016; S. H. Lee et al., 2012; Varjani, 2017), *Burkholderiales* (S. H. Lee et al., 2012), *Commamonadacea* (Táncsics et al., 2018), *Micrococcus* (Varjani, 2017), *Rhodocyclaceae* and *Sphingomonas* (S. H. Lee et al., 2012; Varjani, 2017), were also found in the samples. Since the sediment used as inoculum in this study comes from a hydrocarbon-contaminated site, and from a deep anaerobic subsurface sample, it is interesting that common aerobic BTEX degraders were detected. However, this is the first study where microbial investigations were performed with indene, indane and naphthalene together with BTEX. Here it is shown that common hydrocarbon degraders can also be detected in the presence of indene, indane and/or naphthalene. Whether these microbial groups play a role in their biodegradation and also in the biodegradation in deeper subsurface needs further investigation.

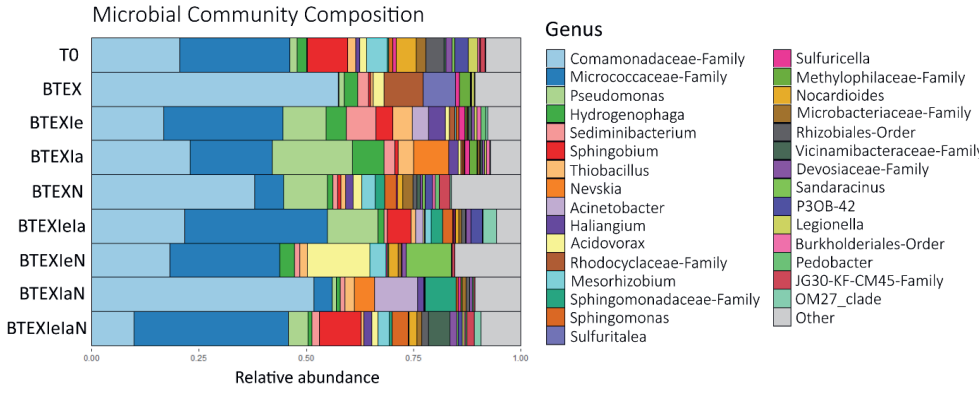

Figure 3.4 Relative abundance of the most abundant genus for different substrate mixtures.

In order to understand which genus showed high abundance and leads to a differentiation among the set-ups, microbial communities at genus level were presented as a heat map (Figure 3.5). It was observed that the main difference between set-ups concerned mainly two families: *Micrococcaceae* and *Comamonadaceae*. Our results show that the presence of indene increased the relative abundance of *Micrococcaceae* family, indicating that members of the *Micrococcaceae* family may play an important role in indene degradation. Also, *Comamonadaceae* showed high relative abundance in the BTEX set-up, suggesting that it might respond to BTEX biodegradation. As previously discussed for the beta-diversity analysis (Figure 3.2), the biggest differences in microbial communities were found to be between the set-ups of BTEX and BTEXIeIaN. In Figure 3.5, a high abundance of *Comamonadaceae* for BTEX was observed with no *Micrococcaceae*, while for BTEXIeIaN, the opposite effect was observed, showing higher relative abundance of *Micrococcaceae* than *Comamonadaceae*. From the heat map, set-ups showing higher *Micrococcaceae* relative abundance would always be mixtures containing indene. In order to combine the result of Figure 3.2 and Figure 3.5, the left side of the beta-diversity graph, harboring indene containing mixtures could be related to the abundance of members of *Micrococcaceae* family, while the right side to an abundance of *Comamonadaceae.* Within these findings, it can be concluded that the differences in the bacterial community structure observed in this study are influenced by different substrate mixtures, and indene had the strongest influence on the BTEX microbial community.

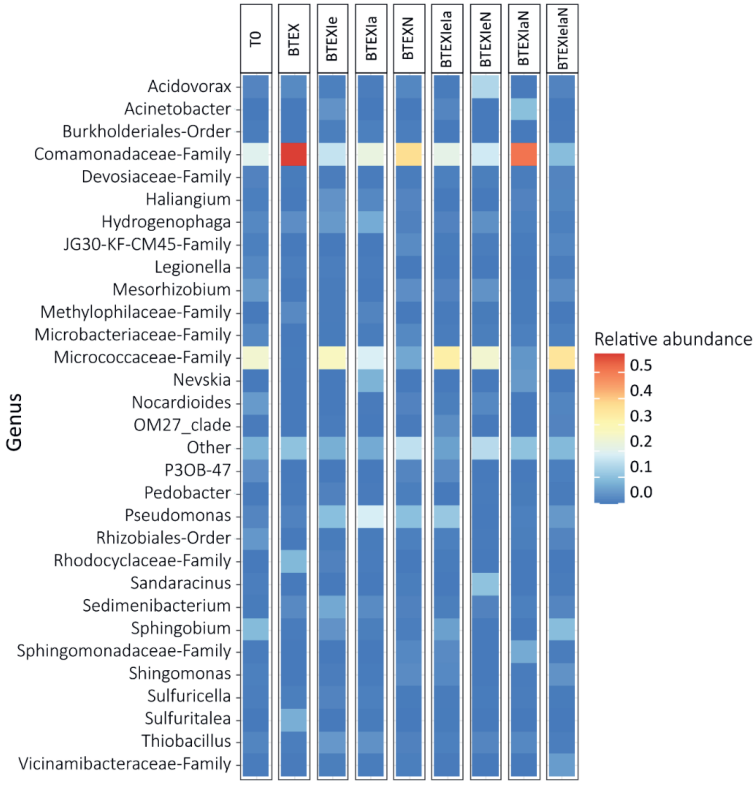

Figure 3.5 Microbial composition of each set-up at genus level, represented as a heat-map. Uncultured and unclassified species were given with the name of the last identified rank.

3.3.6. Future perspective

In this study, sediment samples coming from a previously contaminated site were used to test the biodegradation capacity of indigenous microbial communities towards BTEXIeIaN. The indigenous microbial community, which originated from an anoxic environment, showed aerobic degradation potential for BTEXIeIaN. Most anaerobic degraders of monoaromatic compounds reported under nitrate-reducing conditions are *Azoarcus*, *Thauera* and *Aromatoleum* spp., and other closely related *Rhodocyclaceae* are known to degrade toluene and/or ethylbenzene or xylenes (Lueders, 2017). Weelink et al. (2010) reported that *Thauera* and *Azoarcus* species are facultative anaerobes, capable of oxidizing toluene under nitrate- reducing conditions. In this study, members of *Rhodocylaceae* were found to be present only in the BTEX set-up but with a low relative abundance (Figure 3.5). Members of *Pseudomonas* (Fahy et al., 2006) and *Acidovorax* (Aburto et al., 2009) were reported to be capable of degrading benzene under aerobic, microaerobic and nitrate-reducing conditions. The mentioned microbial groups were not abundant in the set-ups of this study: however, Benedek et al. (2018) investigated BTEX-degrading and/or biofilm-forming communities under aerobic and oxygen-limited conditions and reported that at family level, both enrichments were dominated by *Comamonadaceae* (aerobic, 71%; oxygen-limited, 43%). Members of *Comamonadaceae* were found to be abundant for most of the set-ups in this study (Figure 3.5) which may explain the success of aerobic degradation of BTEXIeIaN compounds in the sediments originating from an anaerobic environment. Considering all this information, oxygen can be used as an electron acceptor for the removal of the BTEXIeIaN mixture present at a site that has been contaminated for over 100 years. The technological development regarding oxygen dosing in the ground and its potential risks need further investigations.

Knowing the multi-substrate effect in complex mixtures will help to understand why a particular compound may persist in a contaminated site while other compounds are degraded. Mechanisms leading to inhibition are unknown for such complex mixtures, but it can be hypothesized that the reason of persistent contaminants may not be related to a microbial degradation capacity limitation but rather due to the effect of mixture toxicity. For such cases, the focus should be toward strategies of having a less complex mixture and investigate on methods for the removal of potential inhibitors. For instance, indene showed to be exerting selective pressure in the microbial communities; therefore, focusing first on indene removal may prevent hindering the biodegradation of other substrates present.

In this study, it was demonstrated that variation in multi-substrates impacts the microbial community composition. Knowing which microbes can survive in the presence of contaminants, and which are active in biodegradation of those compounds, is relevant for the application of bioremediation technologies. In this study, we found that members of the *Micrococcaceae* family can survive in the presence of indene and indane; however, we cannot conclude yet that these types of microbes are involved in indene biodegradation. Studying this i.e., by microbial enrichment and molecular physiology studies, would further elucidate the potential for the use of these microbial communities for in situ bioaugmentation. More detailed studies on microbial dynamics during biodegradation of BTEXIeIaN are needed to understand biodegradation behavior of such complex mixtures and contribute to improved bioremediation strategies.

3.4. Conclusions

This study showed that microbial consortia originating from anaerobic subsurface could fully degrade BTEXIeIaN compounds under aerobic conditions. It was also shown that the co-occurrence of different pollutants affects degradation of individual compounds and is explained by the differences in microbial community structures. Indene, indane, and naphthalene inhibited the degradation of benzene, toluene, ethylbenzene, and o-xylene, with benzene being the most affected. In absence of naphthalene, m/p-xylene was the only compound whose biodegradation was stimulated by indene and indane (individually or mixed). Microbial analysis revealed differentiation in the microbial communities within different substrate mixtures, with indene having more effect on the BTEX microbial community than indane or naphthalene. It was hypothesized that indene degradation might be related to members of *Micrococcaceae* family. These outcomes, together with literature reports, lead to the statement that the multi-substrate effect on biodegradation is inevitable and is an important factor in shaping microbial communities. Investigation of such substrate interactions, together with microbial analyses, will help to design and optimize better remediation strategies, where laboratory observations can be better extrapolated to the field.

Acknowledgements

The authors would like to thank Alraune Zech and Tom Bastiaan for their help with Python and rate analysis and to Elizabeth Carlisle for proofreading the manuscript. This research was supported by the Municipality of Utrecht (the Netherlands) and is part of the project 'BestParc Utrecht' with the identification mark 4281188/170821/1002-gl.

Supplementary information to Chapter 3

Table S3.1 Equations for the calculation of the ratio of the rate constants for each compound used in the experiments. For instance in the equation $\alpha_{B,\,Ie} = \lambda_B(BTEXIe) / \lambda_B(BTEX)$, is $\alpha_{B,\,Ie}$ the ratio of two rate constants for benzene λ_B in the mixture (BTEX) in the presence (BTEXIe) and absence (BTEX) of Ie.

Benzene	**Toluene**
$\alpha_{B,\,Ie} = \lambda_B(BTEXIe) / \lambda_B(BTEX)$ $\alpha_{B,\,Ia} = \lambda_B(BTEXIa) / \lambda_B(BTEX)$ $\alpha_{B,\,N} = \lambda_B(BTEXN) / \lambda_B(BTEX)$ $\alpha_{B,\,IeIa} = \lambda_B(BTEXIeIa) / \lambda_B(BTEX)$ $\alpha_{B,\,IeN} = \lambda_B(BTEXIeN) / \lambda_B(BTEX)$ $\alpha_{B,\,IaN} = \lambda_B(BTEXIaN) / \lambda_B(BTEX)$ $\alpha_{B,\,IeIaN} = \lambda_B(BTEXIeIaN) / \lambda_B(BTEX)$	$\alpha_{T,\,Ie} = \lambda_T(BTEXIe) / \lambda_T(BTEX)$ $\alpha_{T,\,Ia} = \lambda_T(BTEXIa) / \lambda_T(BTEX)$ $\alpha_{T,\,N} = \lambda_T(BTEXN) / \lambda_T(BTEX)$ $\alpha_{T,\,IeIa} = \lambda_T(BTEXIeIa) / \lambda_T(BTEX)$ $\alpha_{T,\,IeN} = \lambda_T(BTEXIeN) / \lambda_T(BTEX)$ $\alpha_{T,\,IaN} = \lambda_T(BTEXIaN) / \lambda_T(BTEX)$ $\alpha_{T,\,IeIaN} = \lambda_T(BTEXIeIaN) / \lambda_T(BTEX)$
Ethylbenzene	**m/p-Xylene**
$\alpha_{E,\,Ie} = \lambda_E(BTEXIe) / \lambda_E(BTEX)$ $\alpha_{E,\,Ia} = \lambda_E(BTEXIa) / \lambda_E(BTEX)$ $\alpha_{E,\,N} = \lambda_E(BTEXN) / \lambda_E(BTEX)$ $\alpha_{E,\,IeIa} = \lambda_E(BTEXIeIa) / \lambda_E(BTEX)$ $\alpha_{E,\,IeN} = \lambda_E(BTEXIeN) / \lambda_E(BTEX)$ $\alpha_{E,\,IaN} = \lambda_E(BTEXIaN) / \lambda_E(BTEX)$ $\alpha_{E,\,IeIaN} = \lambda_E(BTEXIeIaN) / \lambda_E(BTEX)$	$\alpha_{m,p\text{-}X,\,Ie} = \lambda_{m,p\text{-}X}(BTEXIe) / \lambda_{m,p\text{-}X}(BTEX)$ $\alpha_{m,p\text{-}X,\,Ia} = \lambda_{m,p\text{-}X}(BTEXIa) / \lambda_{m,p\text{-}X}(BTEX)$ $\alpha_{m,p\text{-}X,\,N} = \lambda_{m,p\text{-}X}(BTEXN) / \lambda_{m,p\text{-}X}(BTEX)$ $\alpha_{m,p\text{-}X,\,IeIa} = \lambda_{m,p\text{-}X}(BTEXIeIa) / \lambda_{m,p\text{-}X}(BTEX)$ $\alpha_{m,p\text{-}X,\,IeN} = \lambda_{m,p\text{-}X}(BTEXIeN) / \lambda_{m,p\text{-}X}(BTEX)$ $\alpha_{m,p\text{-}X,\,IaN} = \lambda_{m,p\text{-}X}(BTEXIaN) / \lambda_{m,p\text{-}X}(BTEX)$ $\alpha_{m,p\text{-}X,\,IeIaN} = \lambda_{m,p\text{-}X}(BTEXIeIaN) / \lambda_{m,p\text{-}X}(BTEX)$
o-Xylene	**Indene**
$\alpha_{o\text{-}X,\,Ie} = \lambda_{o\text{-}X}(BTEXIe) / \lambda_{o\text{-}X}(BTEX)$ $\alpha_{o\text{-}X,\,Ia} = \lambda_{o\text{-}X}(BTEXIa) / \lambda_{o\text{-}X}(BTEX)$ $\alpha_{o\text{-}X,\,N} = \lambda_{o\text{-}X}(BTEXN) / \lambda_{o\text{-}X}(BTEX)$ $\alpha_{o\text{-}X,\,IeIa} = \lambda_{o\text{-}X}(BTEXIeIa) / \lambda_{o\text{-}X}(BTEX)$ $\alpha_{o\text{-}X,\,IeN} = \lambda_{o\text{-}X}(BTEXIeN) / \lambda_{o\text{-}X}(BTEX)$ $\alpha_{o\text{-}X,\,IaN} = \lambda_{o\text{-}X}(BTEXIaN) / \lambda_{o\text{-}X}(BTEX)$ $\alpha_{o\text{-}X,\,IeIaN} = \lambda_{o\text{-}X}(BTEXIeIaN) / \lambda_{o\text{-}X}(BTEX)$	$\alpha_{Ie,\,Ia} = \lambda_{Ie}(BTEXIeIa) / \lambda_{Ie}(BTEXIe)$ $\alpha_{Ie,\,N} = \lambda_{Ie}(BTEXIeN) / \lambda_{Ie}(BTEXIe)$ $\alpha_{Ie,\,IaN} = \lambda_{Ie}(BTEXIeIaN) / \lambda_{Ie}(BTEXIe)$
Indane	**Naphthalene**
$\alpha_{Ia,\,Ie} = \lambda_{Ia}(BTEXIeIa) / \lambda_{Ia}(BTEXIa)$ $\alpha_{Ia,\,N} = \lambda_{Ia}(BTEXIaN) / \lambda_{Ia}(BTEXIa)$ $\alpha_{Ia,\,IeN} = \lambda_{Ia}(BTEXIeIaN) / \lambda_{Ia}(BTEXIa)$	$\alpha_{N,\,Ie} = \lambda_N(BTEXIeN) / \lambda_N(BTEXN)$ $\alpha_{N,\,Ia} = \lambda_N(BTEXIaN) / \lambda_N(BTEXN)$ $\alpha_{N,\,IeIa}\ \lambda_N(BTEXIeIaN) / \lambda_N(BTEXN)$

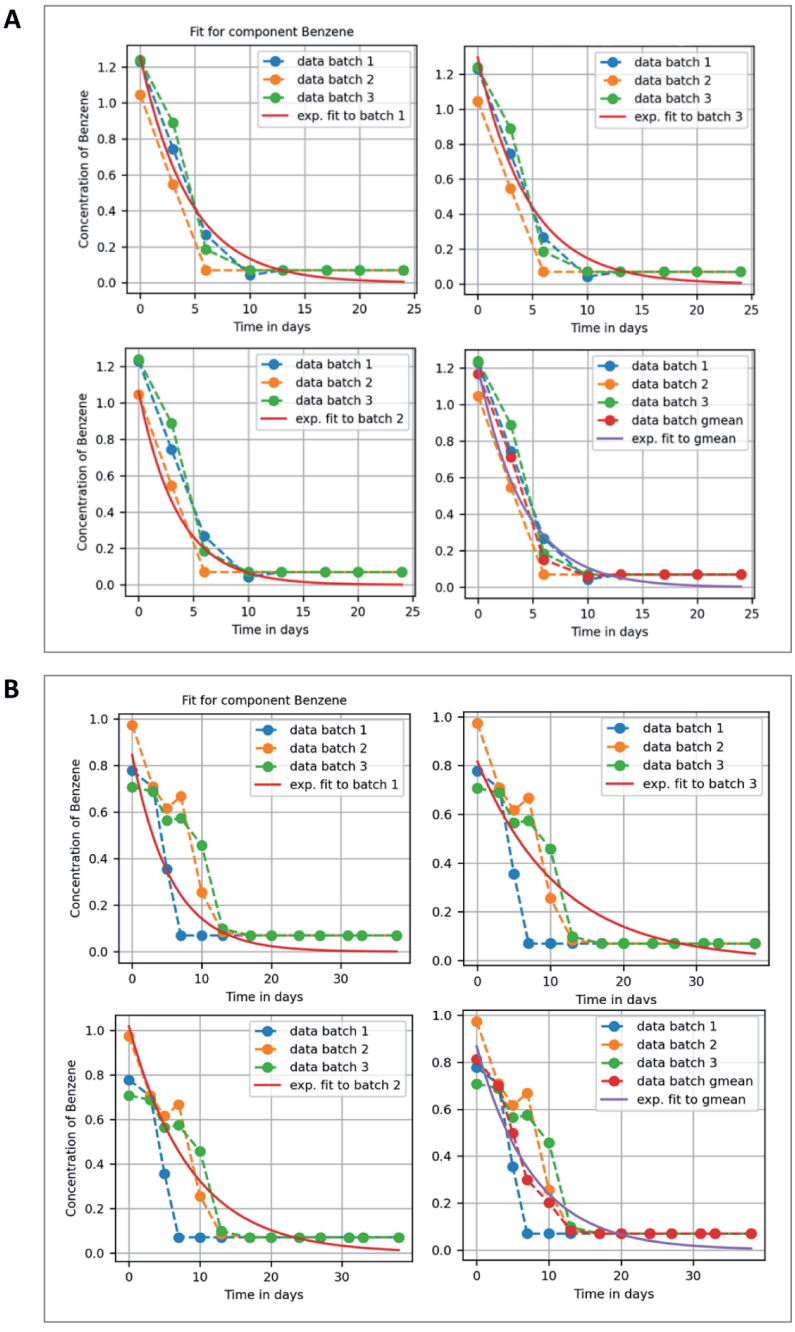

Figure S3.1 Plots of the different fits performed for benzene in the BTEXIa (A) and BTEXIeIaN (B) mixture. For rate calculations the mean of the fits to the three batches was used although they did not differ much from the fit of the geometric mean of the data. Concentrations were normalized with control batches.

Table S3 2-9 is given in excel format due to the data set being very large and can be accessed via this link: https://ars.els-cdn.com/content/image/1-s2.0-S0045653523020283-mmc2.xlsx. The excel file contains the raw HPLC data of the active batch bottles (triplicate) and control bottles (duplicate). Starting concentration of all compounds (mg L^{-1}) and for every sampling day is given for each set-up.

Table S3.2 Mean of the degradation rate constants (1/day) of the triplicate batches normalized to control batches. Batches with only one data point above detection limit (d.l.) are color coded. ±; standard deviations

	B	T	E	o-X	m/p-X	Ie	Ia	N
BTEX	1.03±0.05	1.76±0.06	1.27±0.04	0.23±0.02	0.44±0.08	-	-	-
BTEXIe	0.33±0.04	1.47±0.24	1.18±0.08	0.16±0.06	1.65±0.33	0.51±0.08	-	-
BTEXIa	0.24±0.03	0.55±0.23	0.69±0.26	0.17±0.02	1.44±0.19	-	0.21±0.08	-
BTEXN	0.20±0.01	0.31±0.01	1.49±0.35	0.22±0.06	0.38±0.04	-	-	3.22±0.01
BTEXIeIa	0.15±0.01	0.90±0.03	1.03±0.03	0.10±0.02	1.72±0.03	0.06±0.02	0.04±0.01	-
BTEXIeN	0.20±0.06	0.84±0.40	0.98±0.06	0.01±0.01	0.10±0.02	0.39±0.08	-	1.99±0.07
BTEXIaN	0.12±0.02	0.72±0.09	1.04±0.03	0.07±0.00	0.17±0.00	-	0.11±0.01	2.06±0.01
BTEXIeIaN	0.13±0.04	0.48±0.03	1.07±0.01	0.09±0.05	0.30±0.11	0.50±0.15	0.07±0.05	2.11±0.02

Only one data point above d.l in:	
	All batches
	2 batches
	1 batch
	Not applicable

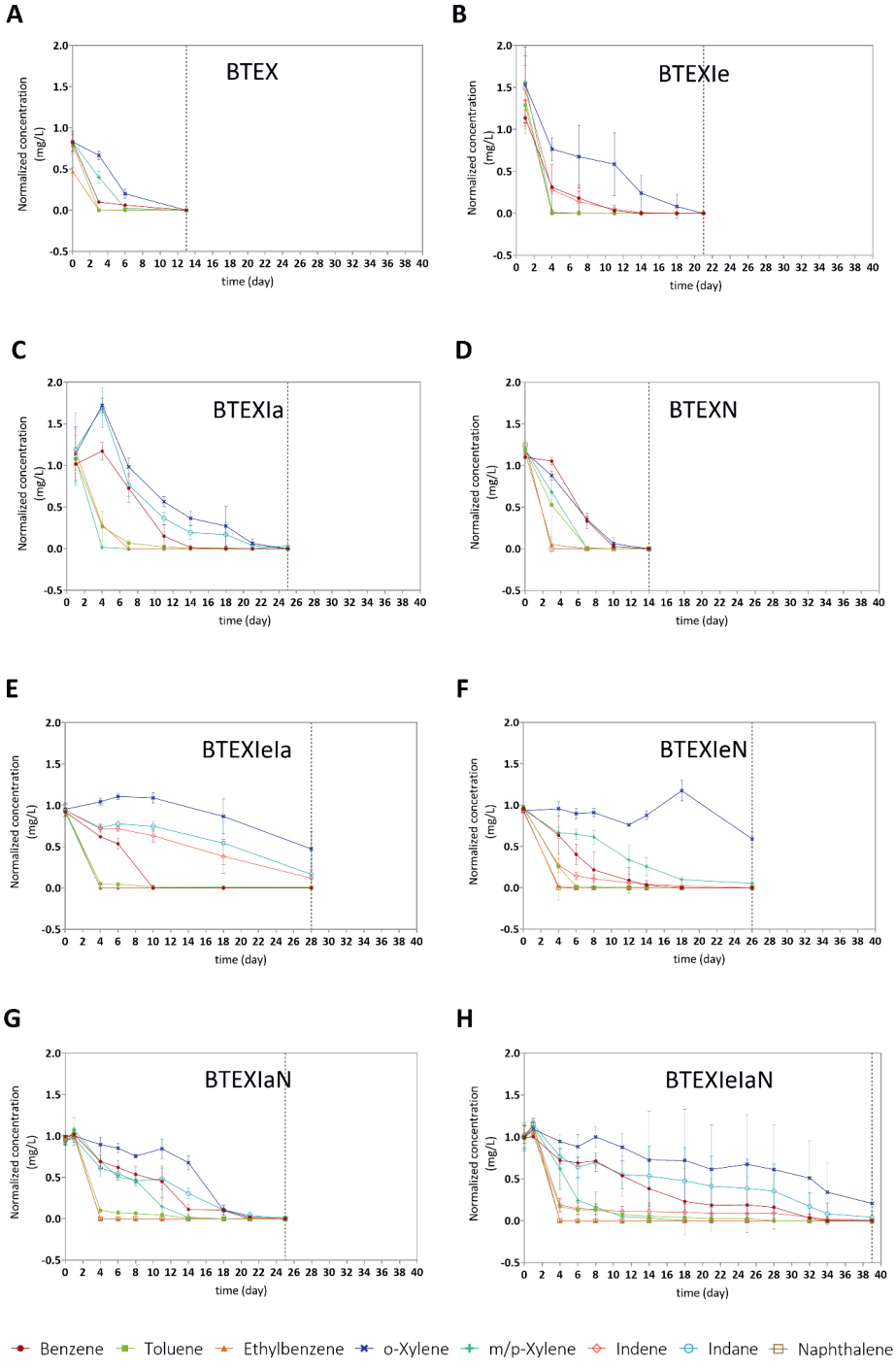

Figure S3.2 Biodegradation patterns for each set-up (with mean and standard deviations of the triplicate samples); A: BTEX, B: BTEXIe, C: BTEXIa, D: BTEXN, E: BTEXIeIa, F:BTEXIeN, G: BTEXIaN, H: BTEXIeIaN.

Chapter 4

Laboratory batch studies on biostimulation and bioaugmentation strategies for the BTEX, Indene, Indane, and Naphthalene mixture degradation under nitrate and sulfate-reducing conditions

Aydin, D.C., Jansen, B., Rijnaarts, H. and Grotenhuis, T.

Abstract

In this study, biostimulation and bioaugmentation strategies were tested in laboratory batch experiments to enhance the biodegradation of a complex aromatic hydrocarbon mixture composed of BTEX, indene (Ie), indane (Ia) and naphthalene (N) under nitrate and sulfate-reducing conditions. BTEXIeIaN mixture biodegradation was studied as it was found to be prevalent in the deep subsurface of a former gasworks site. Biostimulation was tested by supplying the batch bottles with nitrate or sulfate as electron acceptor. The results demonstrated that biostimulation with sulfate yielded the most favorable outcomes as complete removal of toluene, o-xylene, and m/p-xylene was achieved while biostimulation with nitrate resulted with only partial degradation of ethylbenzene. For the bioaugmentation approach, two different inoculums were evaluated: one derived from a wastewater treatment plant (WWTP), enriched with NO_3^- and SO_4^{2-}, and the other consisting of indigenous microorganisms originating from the MGP site, grown with toluene as the sole source of carbon under nitrate-reducing conditions. Bioaugmentation with the WWTP inoculum was not effective for the total removal of the mixture as only toluene biodegradation was observed under sulfate-reducing conditions and ethylbenzene was partially removed in the nitrate set-ups. The toluene-enriched inoculum performed better as ethylbenzene removal efficiencies were higher compared to other nitrate set-ups. Based on these findings, the recommended remediation strategy for anaerobic groundwater contaminated with BTEXIeIaN mixtures was by biostimulating with sulfate as electron acceptor, and be conservative in bioaugmentation.

4.1. Introduction

Monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene (BTEX) are common contaminants found in many environments worldwide (Foght, 2008). They are a great concern to groundwater quality and aquifer ecosystem health due to some of the compounds being toxic or even carcinogenic (Jahn et al., 2005). Indene (Ie) and indane (Ia) are tar oil compounds and can be found as contaminants in former manufactured gas plant (MGP) sites (Mundt & Hollender, 2005). Naphthalene is a common polyaromatic hydrocarbon pollutant, often detected in contaminated sites together with BTEX and harbors cancerogenic properties (Y. Huang & Li, 2014). The removal of such aromatic hydrocarbons often relies on microbial bioremediation (Foght, 2008) and because of limited oxygen concentrations in the contaminated aquifers, anaerobic degradation of such aromatic compounds plays a major role for their bioremediation processes (Lueders, 2017).

The main strategy for aromatic hydrocarbon degradation is natural bioremediation however natural attenuation of hydrocarbons is often limited by electron acceptor availability. In such cases, biostimulation strategy can be applied where suitable and sufficient electron acceptors are supplied into aquifers to enhance the biodegradation process (Foght, 2008). It is possible that indigenous microorganisms may not harbor the appropriate enzyme system hence lack proper metabolic capacities for the degradation of the contaminants or low microbial biomass may lead to low biodegradation rates. Therefore, bioaugmentation strategies can be applied which is the addition of proper degraders to the environment. This strategy can be useful for the removal of a complex molecule or a mixture of compounds that can only be broken down by a very specific combination of microorganisms (El Fantroussi & Agathos, 2005). No matter which strategy is applied, laboratory experiments are needed to optimize the biodegradation process so that effective bioremediation strategies can be further applied to sites polluted with various aromatic hydrocarbon compounds.

This study aimed to enhance the biodegradation of a complex mixture containing BTEXIeIaN compounds through anaerobic batch experiments by testing different biostimulation and bioaugmentation strategies. BTEXIeIaN mixture degradation was evaluated as it is a mixture found in the deeper subsurface (more than 30 m below groundwater level) of the Griftpark, a former gasworks site located in Utrecht, the Netherlands. For biostimulation, nitrate and sulfate were separately supplied to the different batch bottles as potential electron acceptors. Nitrate was selected as its energy yield in biodegradation processes is close to oxygen (Wilson & Bouwer, 1997) while sulfate was chosen due to the aquifer materials used in the experiments originated from sulfate-reducing conditions. Two different inoculums were tested for bioaugmentation. The first inoculum was obtained from a municipal wastewater treatment plant (WWTP) sludge. The sludge sample was enriched for a period of 6 months with both nitrate and sulfate to increase the population of nitrate and sulfate degraders in the reactor. The aim was to enhance the microbial diversity in the batch reactors by introducing especially organic denitrifying microbes from the WWTP inoculum. It was hypothesized that the addition of a diverse microbial population can contribute to the presence of various enzymatic systems, which in turn can facilitate the degradation of different compounds. The second inoculum consisted of indigenous microorganisms of Griftpark that were previously grown with toluene under nitrate-reducing conditions. In this case, the objective was to assess whether an indigenous microbial community, which had prior exposure to a simpler contaminant (toluene), could effectively facilitate the degradation of a more complex mixture (BTEXIeIaN) once bioaugmented. Bioaugmentation test with the WWTP inoculum was studied under nitrate and sulfate-reducing conditions while the toluene enriched Griftpark inoculum was only evaluated under nitrate-reducing conditions. The findings of this study can provide valuable insights for determining the most effective strategy for future

bioremediation approaches aimed at removing the complex BTEXIeIaN mixture found in the deep subsurface of the former gasworks site, Griftpark.

4.2. Materials and Methods

4.2.1. Batch reactor preparation

The batch reactors were prepared following the same procedure as described in the previous study (Aydin et al., 2023) with minor modifications. Batch bottles of 250 mL volume were filled with 20 g sediment, and 150 mL groundwater. The sediment, sourced from the former gasworks site at a depth of 38 m below groundwater level (bgl), served as the inoculum while the groundwater obtained from 10 m bgl acted as the matrix. To achieve anaerobic conditions in the bottles, both in the liquid phase and headspace, the groundwater was flushed with 100% nitrogen gas for 10 min followed by a gas exchange procedure with nitrogen by maintaining a pressure of 1.50 bar.

To assess nitrate and sulfate-reducing conditions, anaerobic batch bottles were supplied with the respective electron acceptor. The sediment and groundwater originally contained 2 mg L^{-1} NO_3^- and 50 mg L^{-1} SO_4^{2-}. To ensure that the electron acceptor was not limiting, an excess amount of the appropriate electron acceptor was added to the relevant bottles, aiming a total of 100 mg of the required electron acceptor.

To differentiate between biotic and abiotic processes, control bottles were prepared by sterilization with autoclaving procedure at 121°C for 30 minutes, repeated twice. In order to keep abiotic conditions throughout the experiments, control bottles were treated with biocides consisting of 1 mL of 260 g L^{-1} NaN_3 and 2.5 mL of 0.5 g L^{-1} $HgCl_2$.

Finally, all batch bottles were spiked with the BTEXIeIaN mixture. For this, a pure compound mixture was prepared consisting equal amounts (100 mg) of each compound into glass vial and vortexed for a minute, to allow to a dissolution in each other, including the naphthalene crystals. Next, 10 µL of the pure mixture was injected to the bottles using a glass syringe. To allow complete homogenization of the contaminants in the bottles, they were left overnight in the shakers at 120 rpm and 20°C, in dark.

4.2.2. Bioaugmentation inoculums

Two different inoculums were prepared for the bioaugmentation tests: the WWTP inoculum and the Griftpark inoculum. For the WWTP inoculum, sludge samples were obtained from the anaerobic part of the WWTP. Then, 20 g of WWTP sludge was transferred to 250 mL batch bottles and supplemented with 150 mL anaerobic basal mineral medium (Table S4.1). The bottles were made anaerobic within the same gas exchange procedure as presented above. To the medium, 1.5 g L^{-1} NO_3^- and 2.3 g L^{-1} SO_4^{2-} were added simultaneously and further contaminated with the BTEXIeIaN mixture (0.5 to 4 mg L^{-1}). The WWTP culture was operated for a period of 6 months. At the halfway point, which was after 3 months, the culture was transferred to a fresh medium with lower NO_3^- and SO_4^2 concentrations (< 120 mg L^{-1}). The transfer involved a final dilution of 15% (v/v) to ensure the introduction of fresh nutrients and maintain the culture's vitality and growth. For the Griftpark inoculum, 250 mL anaerobic batch bottles with 20 g sediment and 150 mL groundwater supplied with ~600 mg L^{-1} NO_3^- and ~130 mg L^{-1} SO_4^{2-} were contaminated with 8.8 mg L^{-1} toluene as sole source of carbon. Once toluene was fully biodegraded, 15% (v/v) of the inoculum was utilized for the bioaugmentation experiments. Both inoculums were introduced into their designated bottles 20 days after the start of the experiment for effective observation of the bioaugmentations' impact.

4.2.3. Experimental set-up

This study was divided into two sections as nitrate-reducing and sulfate-reducing conditions. For nitrate-reducing conditions, three experimental set-ups were designed where biostimulation was assessed by nitrate addition (N), and bioaugmentation was studied with the WWTP inoculum (NW) and Griftpark inoculum (NG). For sulfate-reducing conditions, biostimulation was tested with sulfate addition (S), and the bioaugmentation test was conducted with the WWTP inoculum (SW) (Table 4.1).

Table 4.1 Experimental set-ups for biostimulation (N, S) and bioaugmentation (NW, NG, SW) tests. **N**: **N**itrate-reducing conditions, **S**: **S**ulfate-reducing conditions, **W**: bioaugmentation with **W**WTP inoculum, **G**: bioaugmentation with indigenous **G**riftpark inoculum enriched with toluene. -: Not added.

Set-up	Electron Acceptor	Bioaugmented Inoculum
N	NO_3^-	-
NW	NO_3^-	WWTP
NG	NO_3^-	Griftpark (toluene enriched)
S	SO_4^{2-}	-
SW	SO_4^{2-}	WWTP

Each experimental set-up consisted of a total of five bottles, out of which three were active bottles and two control bottles. To account for any potential volatilization or absorption effects within the batch bottles, the active bottles were normalized to the control bottles by dividing the average of the BTEXIeIaN compound concentrations of the three active bottles to the average of the two control bottles.

4.2.4. Analytical methods

To assess the biodegradation process, BTEXIeIaN compounds were quantified using the HPLC-DAD method previously outlined by Aydin et al. (2021). In summary, a 1 mL of sample was extracted from liquid phase of the bottles by a syringe. Then, samples were transferred to an Eppendorf tube and centrifuged for 10 min at 15000 rpm to allow precipitation of the sediment. Following centrifugation, 750 µL supernatant was transferred to a HPLC glass vial. To ensure stability and prevent volatilization during the autosampler procedure, 250 µL of methanol was added to the vial containing the supernatant and vortex for a minute.

Nitrate (NO_3^-), nitrite (NO_2^-) and sulfate (SO_4^{2-}) concentrations were quantified by ion chromatography (IC) (Dionex ICS-2100, Thermo, USA) equipped with an AS17-Column. The left-over supernatant from the HPLC measurements were used for the IC analysis. All samples were diluted with Milli-Q water before measurements (70 µL supernatant, 630 µL Milli-Q water) in order to be within the limit of detection range of the equipment.

For headspace analysis, concentrations of O_2, CO_2, N_2 and CH_4 were monitored with gas chromatography (GC) (GC-2010, Shimadzu) with the method of De Wilt et al. (2018) by sampling 2 mL gas from the bottles. Parallel to GC, oxygen concentrations were also measured via a non-invasive oxygen sensor spot (Spot SP-PSt3 PreSens, Germany) before and after sampling to ensure no oxygen

was introduced during sampling procedures. Chromeleon software (Thermo Fischer Scientific, USA) was used for analysis of the data from both liquid and gas chromatography.

4.3. Results and Discussion

4.3.1. Enrichment cultures

The aim of collecting sludge samples from the anaerobic part of a municipal WWTP was to obtain a consortium with varied microbial groups that may harbor diverse metabolic properties. This inoculum was used for bioaugmentation purposes with aim to enhance the biodegradation of all the compounds of the BTEXIeIaN mixture. The enrichment culture was monitored for about 200 days. The HPLC analytical results showed some variations in the BTEXIeIaN concentrations due a matrix effect caused by WWTP organics interfering with the separation and quantification HPLC method (Supplementary data, Figure S4.1). Despite the HPLC data being highly variable, making it difficult to draw any conclusions regarding the biodegradation performance of the BTEXIeIaN compounds, the IC results showed a rapid decrease in the NO_3^- and SO_4^{2-} concentrations (Figure S4.2). Nitrate was fully consumed while sulfate was partially degraded on day 80, indicating the presence and activity of the nitrate and sulfate degraders in the WWTP culture. On day 113, the WWTP culture was re-supplemented with NO_3^- and SO_4^{2-}, and some consumption of both electron acceptors was observed on day 190 (Figure S4.2). As the culture showed evidence of nitrate and sulfate degraders, 15% (v/v) of the inoculum was transferred to a fresh medium, contaminated with the BTEXIeIaN mixture. Because the transfer inoculum was diluted by its transfer to the fresh medium (15% v/v), the matrix effect issue in the HPLC measurement was no longer a problem, and BTEXIeIaN concentrations could be accurately measured. The WWTP transfer culture did not show any biodegradation for the BTEXIeIaN compounds (Figure S4.3) however some consumption was observed in the electron acceptors (Figure S4.4) meaning that microorganisms were active and capable to utilize NO_3^- and SO_4^{2-} as substrate but couldn't biodegrade the BTEXIeIaN compounds during the given period of time of 90 days. Even though BTEXIeIaN biodegradation was not observed, because the WWTP transfer inoculum could utilize NO_3^- and SO_4^{2-}, a decision was made to utilize it as inoculum for bioaugmentation set-ups NW and SW to evaluate the biodegradation potential of a microbial group different from the indigenous microbiome.

The indigenous Griftpark inoculum was first grown with toluene as a sole source of carbon, under nitrate-reducing conditions. The HPLC results showed complete biodegradation of toluene within 50 days while this was not the case for the abiotic control bottle (Figure S4.5). The IC results showed that nitrate was partially consumed while sulfate concentrations were stable (Figure S4.6). For the removal of 8.8 mg L^{-1} toluene, an stochiometric amount of 42.68 mg L^{-1} NO_3^- was needed. In total, 132 mg L^{-1} NO_3^- was consumed in the batch bottle showing that nitrate was most likely utilized for both the biodegradation process and also for organic matter conversion. As the Griftpark inoculum was capable to remove toluene under nitrate-reducing conditions, its performance was further evaluated within the more complex BTEXIeIaN mixture by transferring 15% (v/v) of the inoculum to the bioaugmentation NG set-up.

4.3.2. Nitrate-reducing conditions

Biostimulation (set-up N) and bioaugmentation (set-ups NW and NG) approaches were tested under nitrate-reducing conditions for a period of 446 days (Figure 4.1). In the biostimulation set-up N, no biodegradation was observed for the BTEXIeIaN compounds during the first 300 days (Figure 4.1A). At the end of the experiment, 47% of the ethylbenzene was degraded, while the other compounds

removal efficiency was below 10% (Supplementary data, Table S4.2). A similar pattern was observed in the bioaugmentation set-up of NW (Figure 4.1B, Table S4.2), indicating that the WWTP inoculum did not have any positive effect on the biodegradation of the BTEXIeIaN mixture. Different than N and NW, in the NG set-up, a 87% removal of ethylbenzene occurred (Figure 4.1C, Table S4.2). This finding showed that prior exposure to toluene may have had a slightly positive effect on the removal of ethylbenzene, however its effectiveness fell short of expectations for the complete removal of the BTEXIeIaN mixture compounds. It is interesting that toluene was not fully biodegraded in the NG set-up, as it would be expected that some toluene degraders were supplemented to the bottles. Possibly, the toluene biodegradation was inhibited by the presence of the other aromatic compounds (BEXIeIaN) or microorganisms capable to remove toluene preferred to utilize ethylbenzene when both compounds are present in pollutant mixture. In our previous study, biodegradation of the BTEXIeIaN mixture was studied using the same aquifer material as in this study, and ethylbenzene biodegradation occurred prior to toluene however these experiments were performed under aerobic conditions (Aydin et al., 2023). For the IC results, nitrate was slightly consumed in set-up N, while sulfate was not utilized by the microorganisms (Figure S4.7A). Similar pattern was observed for the NG set-up (Figure S4.7C). In set-up NW, almost 500 mg L^{-1} NO_3^- was consumed while SO_4^{2-} concentrations were stable meaning that NO_3^- in NW set-up was utilized mostly for the organic matter conversion (Figure S4.7B). Very high NO_2^-concentrations was detected for the NW set-up (70 to 120 mg L^{-1}) due to high NO_3^- consumption. High NO_2^- concentrations may have a toxic effect on the microorganisms (Philips et al., 2002). However since partial ethylbenzene removal could still be observed starting from day 340 (Figure 4.1B) and the NW biodegradation performances were not that different than set-up N and NG (Table S4.2), where NO_2^- concentrations were below 5 mg L^{-1} throughout the experiment, lack of biodegradation of the total BTEXIeIaN mixture compounds in set-up NW may not be fully attributed to NO_2^- toxicity.

Results of nitrate set-ups showed that biostimulation with nitrate was not effective for the removal of the BTEXIeIaN compounds. For bioaugmentation strategies, the Griftpark inoculum (NG set-up) performed slightly better than the WWTP inoculum (NW set-up) (Table S4.2). In the NW set-up, the metabolic capacity of a microbial group different from the indigenous microbiome to degrade a complex aromatic pollutant mixture was evaluated however the results clearly indicated that the initial assessment of their potential was absent or very limited. Long-term exposure to pollution results in adaptation of the microbial community to use the contaminants as carbon and energy sources. Over time, certain microorganisms may develop distinct enzymes and special metabolic properties to efficiently degrade specific compounds (Varjani, 2017). Through natural selection, these adapted microbial groups can become dominant within the community and contribute significantly to the biodegradation process (Leahy & Colwell, 1990). Although the WWTP inoculum contained a diverse microbial community, including some potential nitrate reducers, it did not possess the ability to degrade any of the BTEXIeIaN compounds. As the WWTP inoculum was originating from municipal WWTP, it is unlikely that the microorganisms encountered BTEXIeIaN contamination hence the microbial community did not show any signs of evolving or developing an increased capacity for hydrocarbon biodegradation in the given experimental time. Therefore, it was concluded that the WWTP culture was unsuitable for bioaugmentation purposes to enhance the BTEXIeIaN mixture biodegradation under nitrate-reducing conditions despite its microbial diversity. Longer exposure time with BTEXIeIaN mixture may be needed for the biodegradation to occur, but it would be questionable then whether the newly introduced microbes would be the origin of the biodegrading bacteria, or that these would be the offspring of evolving indigenous microbes.

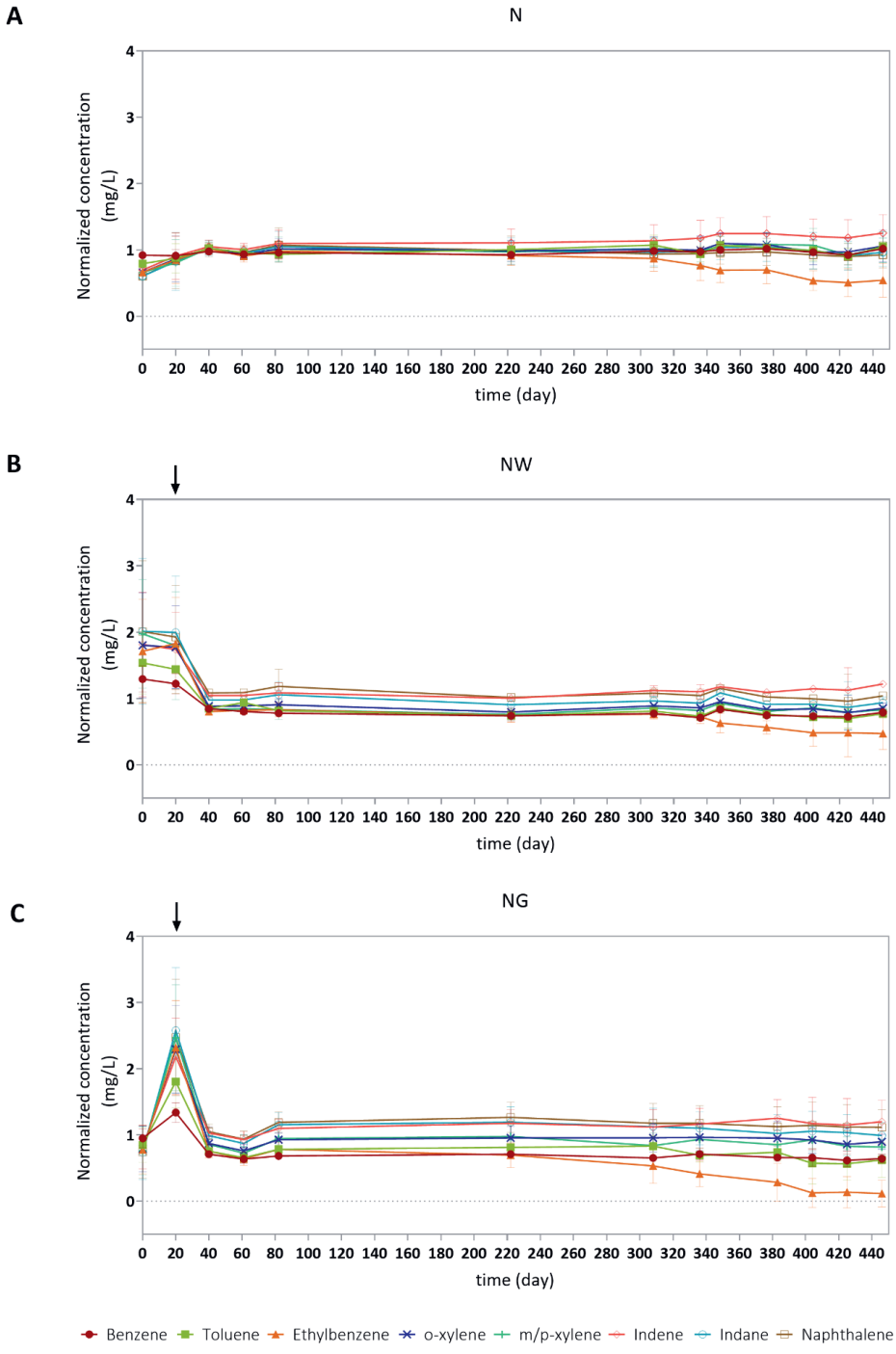

Figure 4.1 Normalized concentrations of the BTEXIeIaN compounds in the N(A)*, NW(B) and NG(C) set-ups. Experimental error was observed on day 20 as high standard deviations were found. Arrows represent bioaugmentation on day 20. **Figure 4.1A also represented as Figure 5S2 in Chapter 5, submitted for publication*.

4.3.3. Sulfate-reducing conditions

Under sulfate-reducing conditions, the biostimulation approach was investigated with set-up S, while the suitability of the WWTP inoculum for bioaugmentation was evaluated in set-up SW. It is important to note that the Griftpark inoculum enriched with toluene was not evaluated for any of the sulfate tests as it was grown only under nitrate-reducing conditions.

In the biostimulation set-up S, the initial 80-day period showed no removal of the BTEXIeIaN compounds (Figure 4.2A). On day 300, almost complete removal of toluene was observed and 82% of o-xylene was biodegraded (Figure 4.2A, Table S4.3). To confirm the process of biodegradation, the bottles were re-spiked with toluene and o-xylene on day 300 and a similar trend was observed, with toluene and o-xylene being almost fully biodegraded within 116 days (at day 446). Additional to toluene and o-xylene, m/p-xylene was fully biodegraded at day 446 (Figure 4.2A, Table S4.3). The IC results showed that some sulfate was consumed compared to the abiotic control bottles (Figure S4.8A). The consumed sulfate at the end of 446 days was 152 mg L^{-1} which is 6.9 times higher than the sulfate needed for the consumed compounds. This showed that sulfate was also used for natural organic matter conversion.

The set-up SW, bioaugmented with the WWTP inoculum, was not as effective as the biostimulation approach since partial removal of toluene was reported on day 300 (Figure 4.2A, Table S4.3). The bottles were re-spiked with toluene on day 300 and differently to S, o-xylene and m/p-xylene were not biodegraded in this set-up meaning that the bioaugmented inoculum had negative effect on the xylene degraders (Table S4.3). The lack of xylene biodegradation in SW may be attributed to the interaction between microbial communities (i.e., competitive inhibition) which potentially inhibited the biodegradation process. The IC measurements showed that 536 mg L^{-1} sulfate was consumed which is 3.5 times higher than what was consumed in the S set-up (Figure S4.8B) meaning that the WWTP inoculum continued to utilize sulfate however sulfate was mostly likely consumed for organic matter conversion rather than biodegradation as only toluene was removed from the bottles. Based on this finding, it was concluded that the WWTP inoculum was not a suitable candidate for enhancing the biodegradation of the BTEXIeIaN mixture under sulfate-reducing conditions.

Based on literature, among monoaromatic hydrocarbons, toluene and ethylbenzene are relatively easy to degrade (Akmirza et al., 2016; Weelink et al., 2010), therefore it can be expected that they degrade first. However, benzene and o-xylene are more difficult to degrade and often reported to be recalcitrant under anaerobic conditions (Akmirza et al., 2016). It was unexpected that o-xylene was one of the first compounds to be biodegraded by the indigenous microorganisms in set-up S. A similar outcome was observed in the study of Edwards & Grbić-Galić, (1994) where microbial consortium from a creosote-contaminated sediment was tested and toluene and o-xylene were degraded but no degradation was recorded for benzene, ethylbenzene, m/p-xylene or naphthalene under methanogenic conditions. In their study, also long lag times were recorded for toluene (100 to 120 days) and o-xylene (200 to 255 days) biodegradation. Another study reported that an enrichment of different iron-reducing cultures were able to oxidize all BTEX compounds where toluene and o-xylene degradation started immediately without any significant lag phase and were fully degraded within 39 days (Jahn et al., 2005). For the other compounds, longer lag times (16 to 61 days) were needed for their removal. It is important to emphasize that in their study single aromatic compounds were used as sole source of carbon which is not reflecting the in situ situation at most contaminated sites. This may explain the success of the complete removal of all the BTEX compounds since interactions such as substrate competition and metabolite inhibition can influence the degradation. This was previously shown and discussed for BTEXIeIaN compounds under aerobic conditions (Aydin et al., 2023). Edwards & Grbić-Galić (1994) discussed that despite the structural similarities among monoaromatics,

selectivity toward specific compounds can be attributed to the substrate specificity of the inoculum. Given the outcomes of this study, and above mentioned studies, it is possible that alternative mechanisms play a role in the successful degradation of toluene and o-xylene, rather than a substrate specificity. For m/p-xylene, a study reported that toluene at concentrations below 10 mg L^{-1} had a stimulatory effect on anaerobic biodegradation of m-xylene and p-xylene while toluene concentrations above 25 mg L^{-1} inhibited their degradation (Dou, Liu, & Hu, 2008a). However, in this study, even though toluene concentrations were relatively low, the biodegradation of m/p-xylene was negatively affected by toluene. This was evident as m/p-xylene was degraded once toluene was removed from the bottles (Figure 4.2A, day 400).

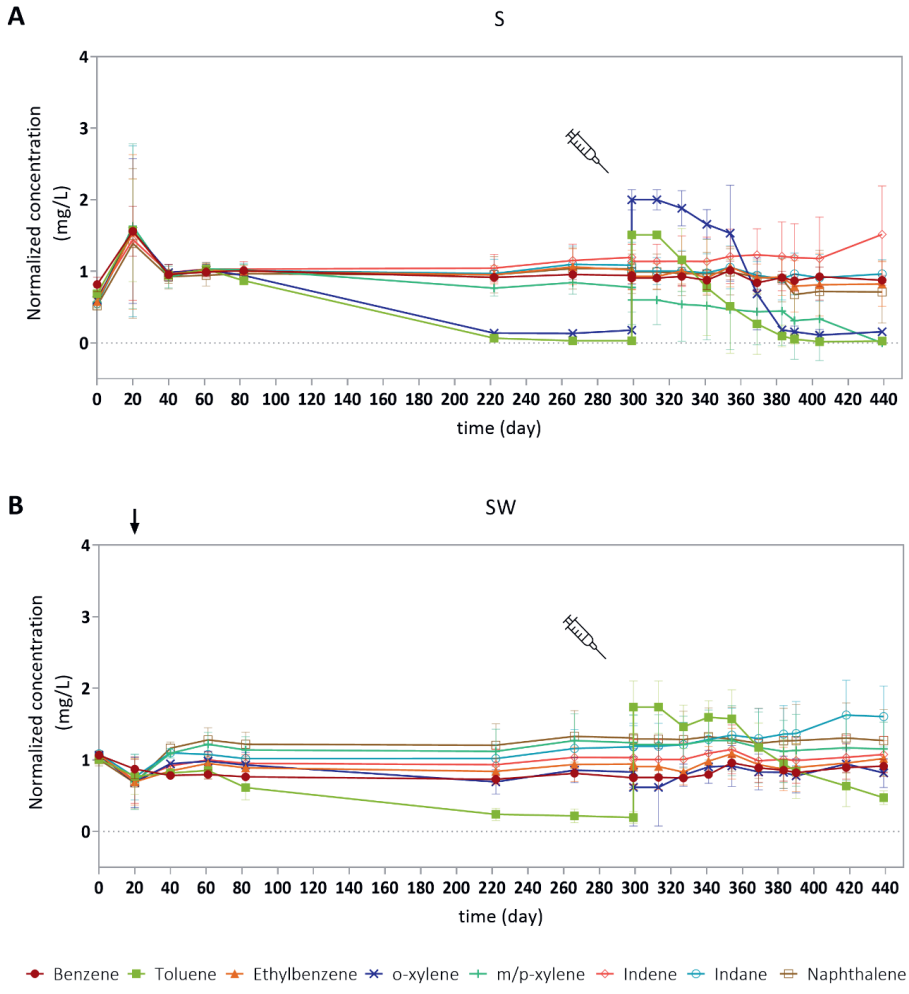

Figure 4.2 Normalized concentrations of the BTEXIeIaN compounds in the S (A) and SW (B) set-ups. Experimental error was observed on day 20 as high standard deviations were found. Arrows represent bioaugmentation on day 20 and syringes represent the re-addition of toluene and o-xylene to the bottles.

4.3.4. Summary

Outcomes of the biostimulation experiments showed that biodegradation of BTEXIeIaN compounds were electron acceptor specific. In nitrate-biostimulated set-up (N), only ethylbenzene was degraded while in the sulfate-biostimulated set-up (S), the biodegradation of toluene, o-xylene and m/p-xylene was observed. Meaning that different compounds were preferred as carbon source by the indigenous microorganisms of Griftpark, under different redox conditions. Similar observations were reported by Phelps & Young (1999), where toluene was degraded in various redox conditions (NO_3^-, SO_4^{2-}, Fe(III), CO_3^-) while benzene was only removed in sulfate-reducing condition. Another conclusion was that different redox conditions affect the degradation preference order of the compounds. For instance, ethylbenzene was the first compound to be utilized under nitrate-reducing conditions while for sulfate-reducing conditions, toluene was preferred. In our previous study, where BTEXIeIaN degradation was investigated under aerobic conditions, ethylbenzene and naphthalene were the first compounds to be degraded by the indigenous microorganisms (Aydin et al., 2023). Therefore, in the case of BTEXIeIaN compounds, the degradation preference order by the indigenous microbial community is determined by the prevailing redox conditions.

Successful application of bioaugmentation techniques relies on the identification and isolation of suitable microbial strains as well as their ability to survive and remain active in the environment. Using a microbial consortium rather than a pure culture for bioaugmentation purposes may be more advantageous as it provides the metabolic diversity and robustness needed to remove a wide range of compounds. With this approach in mind, this study used a WWTP consortium for the removal of a complex hydrocarbon mixture as it would harbor a diverse range of microbial groups with broad metabolic capacities. Despite its metabolic diversity and the rather long exposure to BTEXIeIaN mixture (> 400 days), there was no evidence of compound biodegradation by the inoculum. This suggests that aromatic degraders were not present in the WWTP inoculum and that the mixture did not stimulate any aromatic hydrocarbon degradation capacity development. Adapted populations exhibit higher remediation rates than those with no contamination exposure history. This is why set-ups bioaugmented with the WWTP inoculum (NW and SW) did not perform better than the other set-ups (N, NG, S). The reason that the set-up S performed the best can be attributed to the above mentioned exposure history, as the Griftpark sediment originated from a sulfate-reducing environment, which in return facilitated the biodegradation of the compounds as the microorganisms were adapted to sulfate as an electron acceptor, and possibly to some of the BTEXIeIaN compounds as carbon source.

4.4. Conclusions

This study investigated the biodegradation of the BTEXIeIaN mixture under nitrate and sulfate-reducing conditions through biostimulation and bioaugmentation approaches. The biostimulation experiments revealed that utilization of sulfate as an electron acceptor was more effective for the removal of the BTEXIeIaN compounds. Results also showed electron acceptor specificity toward certain compounds, and that BTEXIeIaN compound degradation order is affected by different redox conditions. The bioaugmentation experiments demonstrated that an off-site diverse-microbial consortium do not guarantee an enhancement in BTEXIeIaN compound removal as the exposure history to contaminants plays a significant role. To facilitate biodegradation process, an enrichment culture containing indigenous microorganisms grown in a single substrate may be beneficial for the removal of other compounds when further exposed to a mixture. In the case of BTEXIeIaN mixture removal, bioremediation strategies in Griftpark focusing on biostimulation with sulfate would be more beneficial compared to bioaugmentation approaches.

Supplementary information to Chapter 4

Medium Composition

For the basal medium preparation, Sorensen's phosphate buffer (50 mL), macronutrient stock solution (6 mL L^{-1}) and trace elements stock solution (0.6 mL L^{-1}) were mix in demi-water and completed to 1 L (pH 7).

Table S4.1 Medium composition used in this study.

Macronutrient stock solution
$MgSO_4 \cdot 7H_2O$ (9 g L^{-1})
NH_4Cl (170 g L^{-1})
$CaCl_2 \cdot 2H_2O$ (8 g L^{-1})
Trace elements stock solution
$FeCl_2 \cdot 4H_2O$ (2 g L^{-1})
$MnCl_2 \cdot 4H_2O$ (0.5 g L^{-1})
$ZnCl_2$ (50 mg L^{-1})
$(NH_4)6Mo7O_24 \cdot 4H_2O$ (90 mg L^{-1})
$NiCl_2 \cdot 6H_2O$ (50 mg L^{-1})
$CoCl_2 \cdot 6H_2O$ (2 g L^{-1})
$CuCl_2 \cdot 2H_2O$ (30 mg L^{-1})
H_3BO_3 (50 mg L^{-1})
$Na_2SeO_3 \cdot 5H_2O$ (100 mg L^{-1})
EDTA – tripex 2 (1 g L^{-1})
HCl 36% (1 mL L^{-1})
Na-resazurin (0.5 g L^{-1})

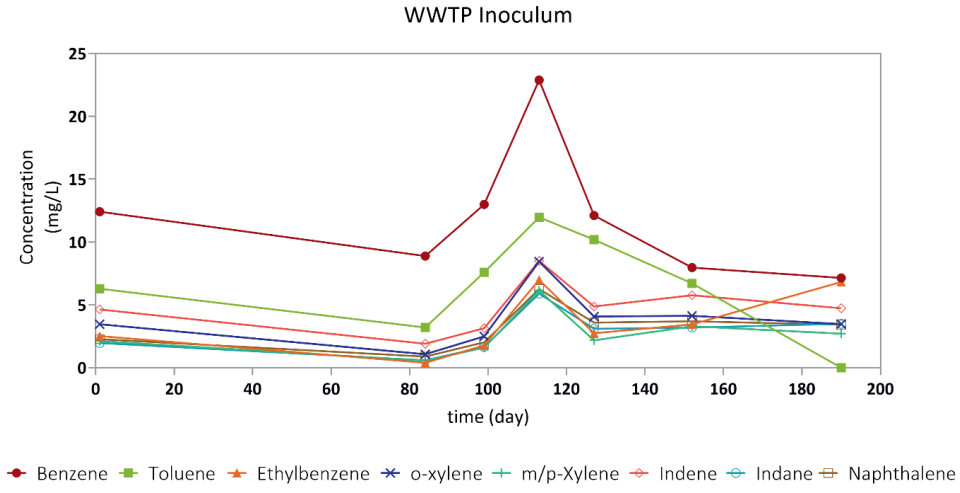

Figure S4.1 Concentrations of the BTEXIeIaN compounds (mg L^{-1}) in the WWTP culture. Variation in the data was due to the complex matrix affecting the HPLC quantification method.

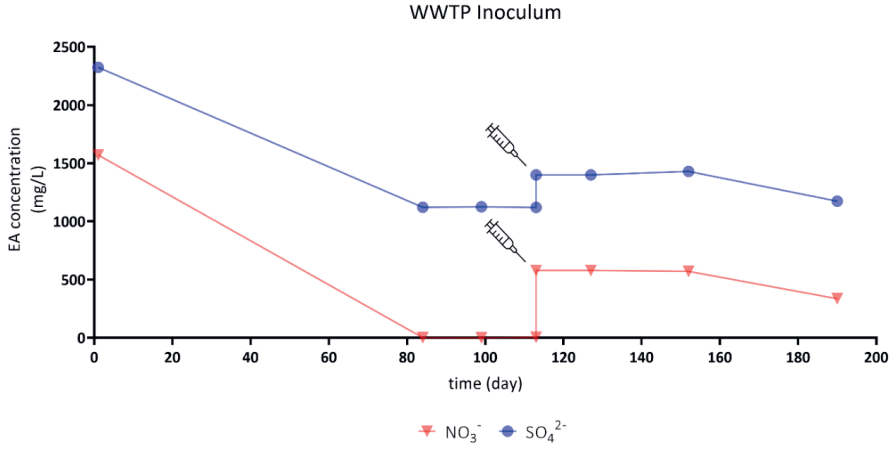

Figure S4.2 Nitrate (NO_3^-) and sulfate (SO_4^{2-}) concentrations in the WWTP culture. Syringes represent re-addition of electron acceptors (day 113). Nitrite (NO_2^-) was not detected throughout the experiment.

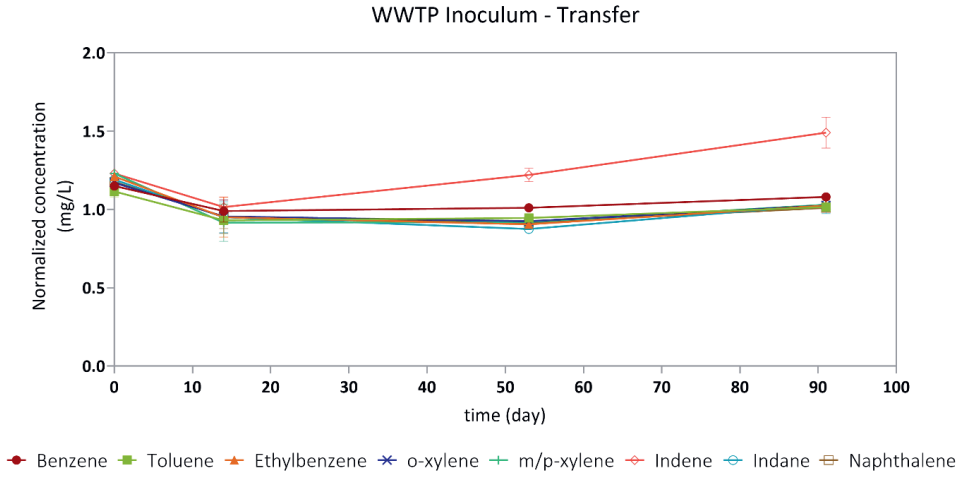

Figure S4.3 Normalized concentrations of the BTEXIeIaN compounds in the transferred culture from the WWTP inoculum (15% v/v).

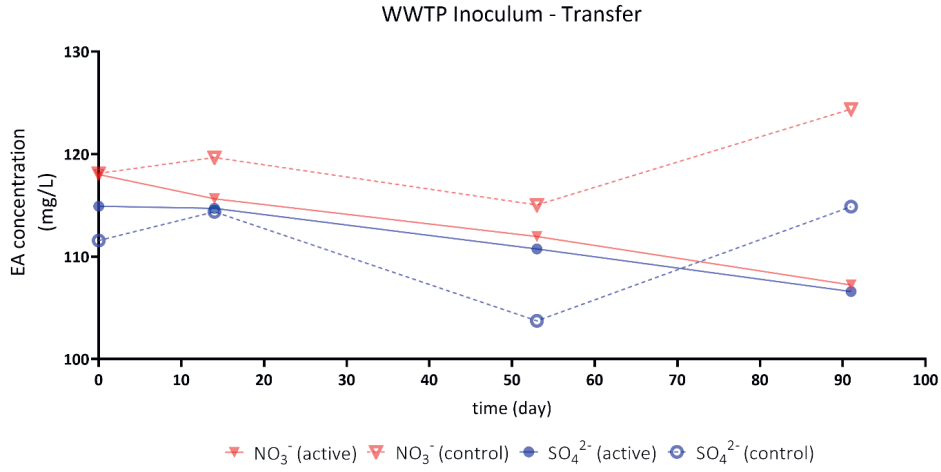

Figure S4.4 Nitrate (NO_3^-) and Sulfate (SO_4^{2-}) concentrations in the transferred culture from the WWTP inoculum (15% v/v) in the active and control bottles. Nitrite (NO_2^-) was not detected throughout the experiment.

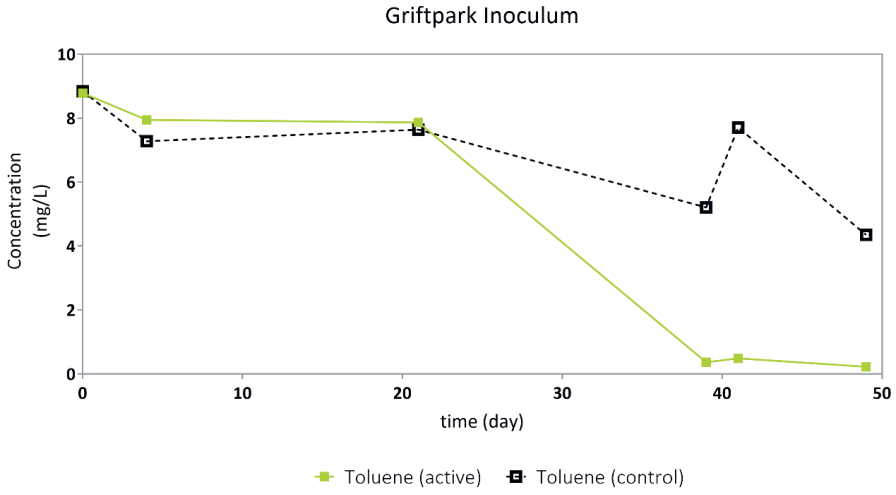

Figure S4.5 Toluene concentrations in the active and control bottles of the indigenous Griftpark inoculum.

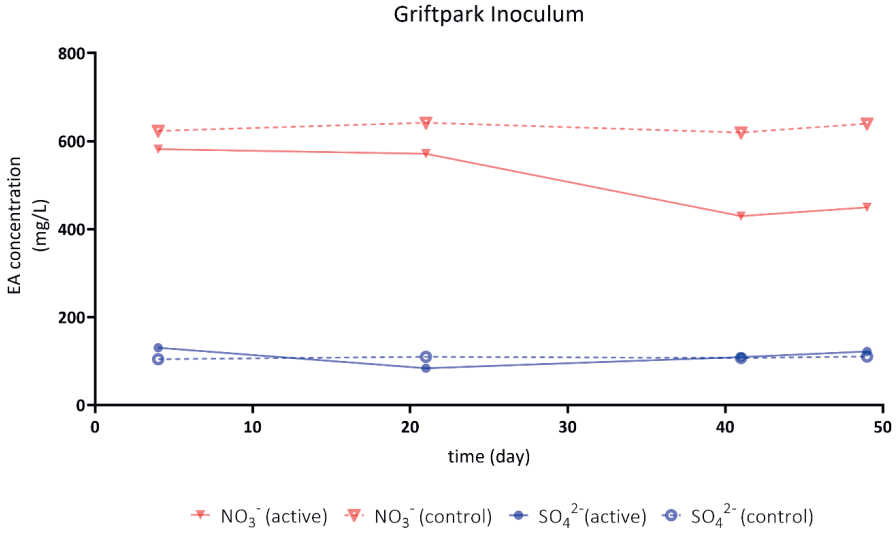

Figure S4.6 Nitrate (NO_3^-) and sulfate (SO_4^{2-}) concentrations in the active and control bottles of the indigenous Griftpark inoculum enriched with toluene. Nitrite (NO_2^-) was not detected through the experiment.

Table S4.2 Average of the removal efficiencies between day 40 to 446 in the nitrate set-ups, for each compound. Red columns indicate negative removal efficiency and blue columns represent biodegradation. Higher removal efficiency is represented with darker blue.

Removal efficiency (%)

	N		NW		NG	
Benzene	-3.63	± 3.67	7.05	± 4.53	9.98	± 1.81
Toluene	-3.21	± 4.36	10.10	± 2.94	18.71	± 24.64
Ethylbenzene	46.54	± 19.19	40.59	± 24.82	86.52	± 19.07
m/p-xylene	-0.66	± 11.51	0.66	± 2.15	4.53	± 6.24
o-xylene	-2.31	± 12.88	3.47	± 7.18	-2.82	± 1.11
Indane	6.93	± 10.21	4.01	± 7.59	0.10	± 5.71
Indene	-18.08	± 13.85	-16.48	± 2.30	-15.17	± 17.14
Naphthalene	9.42	± 10.05	4.19	± 4.52	-5.27	± 10.31

±: standard deviation

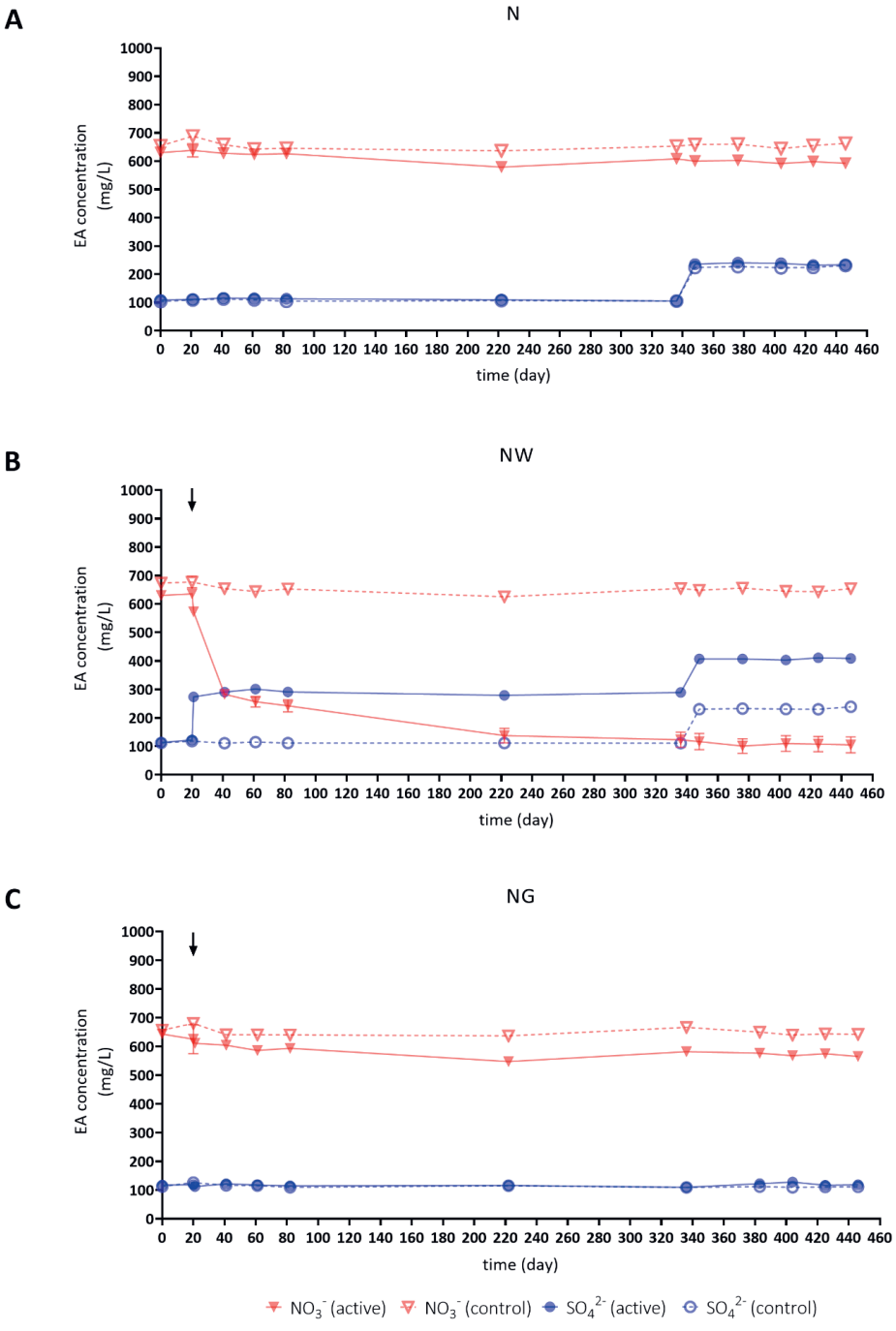

Figure S4.7 NO_3^- and SO_4^{2-} concentrations in the active and control bottles for the N (A), NW (B) and NG (C) set-ups. NO_2^- concentrations were found to be below 5 mg L^{-1} for set-up N and NG throughout the experiment, while high NO_2^- concentrations (70 to 120 mg L^{-1}) were measured for NW set-up.

Table S4.3 Average of the removal efficiencies of the bottles on day 300 and 446 in the sulfate set-ups, for each compound. Red columns indicate negative removal efficiency and blue columns represent biodegradation. Higher removal efficiency is represented with darker blue.

Removal Efficiency (%)

	S				SW			
	day 300		day 446		day 300		day 446	
Benzene	1.93	± 5.01	3.46	± 3.62	4.30	± 5.54	-20.53	± 6.27
Toluene	97.16	± 2.89	98.26	± 2.47	75.82	± 7.18	72.66	± 0.40
Ethylbenzene	-4.79	± 11.75	11.57	± 22.98	-10.71	± 22.31	-15.04	± 9.24
m/p-xylene	17.81	± 20.73	100.00	± 0.00	11.68	± 20.06	5.35	± 3.97
o-xylene	81.61	± 2.15	92.11	± 2.22	-11.79	± 19.57	-7.66	± 4.34
Indane	-11.11	± 14.65	3.89	± 3.38	-12.90	± 25.75	4.62	± 2.39
Indene	-20.14	± 14.53	-28.34	± 28.80	-6.97	± 20.01	-34.48	± 2.55
Naphthalene	-9.80	± 14.09	3.82	± 0.40	-11.90	± 24.46	2.26	± 1.45

±: standard deviation

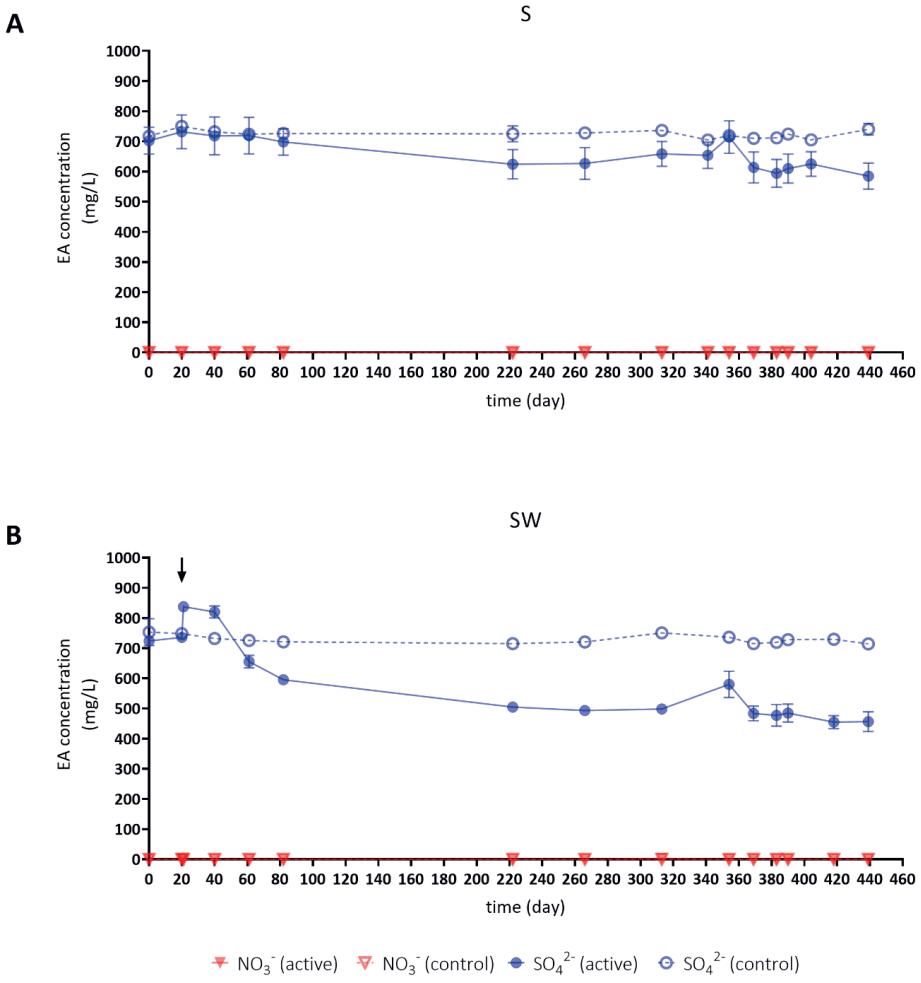

Figure S4.8 NO_3^- and SO_4^{2-} concentrations in the active and control bottles for the S (A) and SW (B) set-ups. NO_3^- was not added to the system, therefore it was measured as zero throughout the experiment. NO_2^- was not detected through the experiment.

Chapter 5

Microaerobic biodegradation of BTEX, Indene, Indane and Naphthalene pollution mixtures in groundwater: Defining strategies for efficient nitrate and oxygen dosage

This chapter has been submitted for publication:

Aydin, D.C., Aldas-Vargas, A., Grotenhuis, T. and Rijnaarts, H. (*under review*)

Abstract

Biodegradation of organic aromatic compounds in subsurface is often limited by dissolved oxygen. Providing low oxygen concentrations to stimulate in situ biodegradation of such compounds in anaerobic environments can help removing the contaminants in an efficient way. BTEX compounds often co-occur in combination with other pollutants at contaminated sites, affecting their mutual biodegradation. In this study, the biodegradation of BTEX, indene, indane and naphthalene pollution mixtures was assessed in batch experiments, with groundwater and sediments originating from an anaerobic contaminated aquifer, under fully aerobic, fully anaerobic and microaerobic conditions (<0.5 mg O_2 L^{-1}). Additionally, transitions between microaerobic to complete anaerobic (nitrate-reducing) conditions, and vice versa, were investigated to determine the most efficient approach where minimal external electron acceptor dosage is required while maximal degradation is achieved. Under microaerobic conditions, all compounds were fully degraded, and the removal efficiency was comparable to fully aerobic conditions. In contrast, batch experiments conducted under fully anaerobic-nitrate-reducing-conditions led to only partial degradation of ethylbenzene after an extended incubation period of 400 days. This is why, a pre-treatment period of nitrate-reducing conditions, followed by microaerobic conditions, was assessed. This approach resulted in complete biodegradation and additionally demonstrated a more effective utilization of oxygen for the degradation of the contaminants when compared to fully aerobic conditions. Outcomes of this study can help to develop oxygen and nitrate dosage strategies for the efficient in situ bioremediation of complex hydrocarbon mixtures in anaerobic subsurface.

5.1. Introduction

Before natural gas, cities and towns throughout the U.S. and Europe relied upon gas manufactured from coal and oil (Murphy et al., 2005). The manufacturing process and the waste from the industrial activities led to the contamination of the soil and groundwater over the years. Common contamination in manufactured gas plants (MGP) is coal tar which contains a mixture of various aromatic hydrocarbons (Sperfeld, Rauschenbach, et al., 2018). These contaminants can percolate to the subsurface as non-aqueous phase liquids (NAPL), and migrate downwards, ending up as a long-term source of contamination in groundwater as a result of their slow dissolution (Birak & Miller, 2009). Benzene, toluene, ethylbenzene and xylenes (BTEX) generate the most concern among hydrocarbon contaminants due to their high solubility and toxicity (Chakraborty & Coates, 2004). Therefore, methods to remove contaminants like BTEX from groundwater and subsurface sediments of former MGPs are necessary.

Biodegradation of aromatic hydrocarbons can be in many situations the most effective way to remove these contaminants from the environment (Meckenstock et al., 2015). Many studies have reported complete removal of such compounds under aerobic conditions and those studies were summarized in the review of Das and Chandran (2011). However, biodegradation in aquifers is often limited by either low concentrations of dissolved oxygen or a complete absence of oxygen leading to various types of anaerobic conditions. When oxygen is not available, the best electron acceptor for degradation process is nitrate (NO_3^-), followed by manganese (Mn^{4+}), ferric iron (Fe^{3+}), sulfate ($SO4^{2-}$) and carbon dioxide (CO_2) (Hatipoğlu-Bağci & Motz, 2019). Even though these alternatives to oxygen are often present in naturally anaerobic systems, aerobic biodegradation is often much faster than anaerobic contaminant transformation (Chakraborty & Coates, 2004; Varjani, 2017) and lead to complete removal of all the contaminants. Therefore, adequate oxygen dosage for enhancing biodegradation in oxygen limited environments can be a good strategy for the removal of recalcitrant compounds under anaerobic conditions.

Determining an adequate oxygen dosage is complex due to the low solubility of oxygen in water (~10 mg L^{-1} at 15°C), its rapid consumption in reduced aquifer environments, and the precipitation of oxidates, which leads to less permeable aquifers and competes with aerobic conversion processes (Wilson & Bouwer, 1997). Compared to oxygen, nitrate has the next level energy yield as electron acceptor for aromatic hydrocarbons degradation, and is favored by its high solubility in water, absence of precipitate formation, inexpensive and non-toxic to microorganisms at concentrations below 500 mg NO_3^- L^{-1} (S. R. Hutchins, 1991). Limitations for nitrate are the concentration regulation in drinking water (max 50 mg NO_3^- L^{-1} in the Netherlands (European Environment Agency, 2016)) and as mentioned, potentially longer time needed for biodegradation to take place. Therefore, balancing the advantages and disadvantages of these two different electron acceptors (oxygen and nitrate) in the subsurface can be helpful for development of successful remediation strategies.

In nature, redox zones in sediments close to the sediment-water interface display microaerobic conditions (oxygen concentration <2 mg O_2 L^{-1}) (Olsen et al., 1995; Yerushalmi et al., 2002). In those zones, facultative anaerobes such as denitrifying bacteria are abundant because of their ability to use both oxygen and nitrate (Firmino et al., 2018; Wilson & Bouwer, 1997). For organic contaminant biodegradation, facultative anaerobes have been shown to use oxygen to metabolize organics and once oxygen is depleted, degradation of the intermediates can occur under nitrate-reducing conditions (Firmino et al., 2018; Wilson & Bouwer, 1997). Thus, sequential electron acceptor usage can potentially contribute to the removal of aromatic organic compounds such as benzene where the most challenging step for microorganisms is the breakdown of the stabile ring structure (Firmino et al., 2018; Weelink et al., 2010). Furthermore, oxygen based conversion generally yields more microbial biomass and can

thereby enhance denitrification processes beneficial for the biodegradation of subsurface contaminants (Su & Kafkewitz, 1994). There are several publications focusing on the microaerobic biodegradation of individual BTEX compounds (Aburto et al., 2009; Su & Kafkewitz, 1994; Yerushalmi et al., 2001, 2002) and on BTEX as a mixture (Da Silva et al., 2005; Firmino et al., 2018; S.-R. Hutchins et al., 1992; Olsen et al., 1995; Siqueira et al., 2018) with diverting outcomes on biodegradability of the individual compounds of the BTEX and other aromatic compounds. Studies with much more complex mixtures of organic aromatic compounds are scarce, while mixtures -and not the individual compounds- are generally found in contaminated sites (Deeb et al., 2001; Gusmão et al., 2006; Zhou et al., 2011). Our previous study, where batch experiments were conducted under aerobic conditions, have shown that compounds in the mixture influence their mutual biodegradation (Aydin et al., 2023). With the aim of taking our previous work one step forward, the biodegradation of complex aromatic organic compound mixtures were studied by investigating the potential use of oxygen together with nitrate.

This study focused on the biodegradation of a mixture composed of BTEX, indene (Ie), indane (Ia) and naphthalene (N) which were found to be prevalent in the subsurface of a former gasworks site. The complete removal of the BTEXIeIaN mixture was previously reported under aerobic conditions (Aydin et al., 2023). In this study, biodegradation of BTEXIeIaN mixture was tested under microaerobic conditions (~0.5 mg O_2 L^{-1}) by investigating the changes from microaerobic to complete anaerobic (nitrate-reducing) conditions and vice versa. The degradation performances of the BTEXIeIaN mixture under various conditions were evaluated to determine the most efficient approach, aiming to minimize electron acceptor usage while achieving maximal degradation. The overall goal is to gain insight into the biodegradation of BTEXIeIaN through the combined use of oxygen and nitrate, and contributing to the development of efficient remediation strategies for aromatic and other hydrocarbons in the subsurface environments.

5.2. Materials and Methods

5.2.1. Sediment and Groundwater sampling

Sediment and groundwater samples were collected from the Griftpark, a former gasworks site located in Utrecht (The Netherlands). The subsurface and groundwater of the site was found to be contaminated with a hydrocarbon mixture composed of BTEXIeIaN compounds (Hauptfeld et al., 2022). For the study, sediment samples were taken from 38 – 38.5 m below ground level (bgl) and the groundwater was collected from the same well (8 m bgl). Both samples showed no contamination of BTEXIeIaN above the limit of detection by HPLC analysis (Aydin et al., 2021) prior to experiments but were most probably exposed to the contamination because above and below the sampled area, contamination was reported. The sediment samples were mixed in an anaerobic tent in order to have a homogenous composition, and was equally distributed to ambered glass jars, fully filled to limit the headspace, then closed with Teflon coated lids. Groundwater samples were stored separately in 2L glass bottles, fully filled without allowing any headspace. All bottles were stored at 4°C, in dark, upside down (inside of a water-filled bucket for glass jars) to keep anaerobic conditions and prevent any oxygen leak during storage period.

5.2.2. Batch reactor preparation

For the batch experiments, 250 mL serum glass bottles were used as reactors. In order to mimic site conditions, serum bottles were filled with 20 g sediment as the source of inoculum, and with 150 mL groundwater as the media. Different set-ups were prepared with different headspace and electron acceptor compositions (Table 5.1). All set-ups consisted of three active bottles as experimental triplicates, and two abiotic duplicate controls.

Table 5.1. Information on the chemical composition of the batch bottles for each set-up.

Set-up	Headspace	Nitrate	**Oxygen Addition** *(~2,5 mg O_2 per injection)*
I	100% N_2	~400 mg L^{-1}	Day 1, 7, 14, 21, 42
II	100% N_2	~400 mg L^{-1}	Day 60, 67
III	Air (21% O_2)	~50 mg L^{-1}	No addition
IV	100% N_2	~600 mg L^{-1}	No addition
V	100% N_2	~50 mg L^{-1}	Day 6, 18

All bottles were sealed with butyl/PTFE-coated stoppers and aluminum crimp caps prior to the headspace modification using the gas exchanger. Except for set-up III, the groundwater was flushed with 100% nitrogen gas for 10 minutes and a gas exchange procedure with nitrogen was applied and set to 1.50 bar to achieve anaerobic conditions both in liquid and the headspace. For fully aerobic conditions (set-up III), the groundwater was not flushed, and headspace consisted of air. Thus, no gas exchange procedure was performed. All the control bottles were autoclaved at 120°C, for 30 min. This procedure was repeated two times. After sterilization, headspace modification was made (except set-up III) with use of gas exchanger using filters (0.22 µm) to prevent any contamination from the added gas. Finally, biocides (1 mL of 260 g L^{-1} NaN_3 and 2.5 mL of 0.5 g L^{-1} $HgCl_2$) were added to all control bottles to ensure sterile conditions throughout the experiment. Once all bottles (experimental and controls) were prepared, the BTEXIeIaN mixture was added. To create microaerobic conditions (set-up I, II, V), anaerobic bottles received oxygen injection via a syringe on the days indicated in Table 5.1. Oxygen dosage is explained in detail further in section 5.2.5.

5.2.3. BTEXIeIaN mixture preparation

In order to have a mixture with equal mass ratio, 100 mg of each compound was mixed within each other without the use of any carrier solvent. The mass calculations were made as in Supplementary Table S5.1 and the calculated volumes were added to vials and vortexed for one minute until complete dissolution of all compounds was achieved. Then, 10 µL from the pure mix was added to the batch bottles via a glass syringe, with estimating an equal mass ratio (1:1:1:1:1:1:1:1, B:T:E:o-X:m/p-X:Ie:Ia:N) with an initial concentration of ~8.9 mg for each compound. It was aimed to create an equally amount of each compound of BTEXIeIaN per bottle. Due to redistribution of the mixture over the phases soil, liquid and gas, some variation occurred when analyzing the compounds in liquid phase (Table S5.2).

5.2.4. Analytical methods

To monitor biodegradation, quantification of BTEXIeIaN compounds was done by sampling the liquid phase and analyzed with HPLC-FLD equipped with a Phenyl-1 (Thermo) HPLC column as previously described (Aydin et al., 2021). Briefly, 1 mL sample was taken with a syringe from each bottle and centrifuged at 15000 rpm for 10 min. An amount of 750 µL supernatant was then transferred to HPLC-glass vials and 250 µL methanol was added in order to keep the volatile compounds stable during autosampler period.

Nitrate (NO_3^-), nitrite (NO_2^-) and sulfate (SO_4^{-2}) concentrations were quantified by an ion chromatography (IC) (Dionex ICS-2100, Thermo, USA) equipped with an AS17-Column. The left-over supernatant from the HPLC measurements were used for the IC analysis. All samples were diluted with Milli-Q water before measurements (70 µL supernatant, 630 µL Milli-Q water) in order to be within the limit of detection range of the equipment.

For headspace analysis, concentrations of O_2, CO_2, N_2 and CH_4 were monitored with gas chromatography (GC) (GC-2010, Shimadzu) with the method of De Wilt et al. (2018) by sampling 2 mL gas from the bottles. Parallel to GC, oxygen concentrations were also measured via a non-invasive oxygen sensor spot (Spot SP-PSt3 PreSens, Germany) before and after sampling to ensure no oxygen was introduced during sampling procedures. The oxygen sensor was attached at the bottom of the batch bottles in order to measure the dissolved oxygen present in the liquid phase. Chromeleon software (Thermo Fischer Scientific, USA) was used for analysis of the data from both liquid and gas chromatography.

5.2.5. Adjustment of the oxygen concentrations

In order to achieve microaerobic conditions in the desired batch bottles, 8.5 mL atmospheric air was injected to the anaerobic bottles via a syringe. The theoretical amount of oxygen in 8.5 mL air was calculated by considering of the atmospheric gas pressure (Table S5.3) and should be ~2.5 mg based on the ideal gas law (Equation S1 and Equation S2, Table S5.4). After oxygen addition to anaerobic bottles, the oxygen gas in the headspace was measured with GC at around 1.3% (Table S5.5) which matched to the theoretical calculations (~2.5 mg O_2) given in Table S5.4. Additional to headspace measurements, oxygen concentrations in liquid phase were measured via an oxygen sensor and was ~0.8 mg L^{-1} (Table S5.6) which corresponds to ~0.12 mg. In order to understand the distribution of the added oxygen in gas and liquid phase, calculations based on Henry's Law were done (Table S5.7). The distribution of oxygen in gas and liquid phase was found to be close to the theoretical Henry's coefficient. Oxygen measurements were performed periodically, before and after the sampling procedure, as well as after the addition of new oxygen. Dissolved oxygen concentrations measured in liquid phase were below 2 mg O_2 L^{-1} and therefore considered as microaerobic conditions (set-up I, II and V). As the added oxygen concentrations were insufficient for the complete removal of the total organic pollutant in the bottles, repeated additions of oxygen were needed over time to periodically re-install short term microaerobic conditions. For fully aerobic bottles (set-up III), the oxygen was measured at the start of the experiment as 19% in the headspace and the dissolved oxygen was ~8 mg L^{-1} in liquid (Table S5.8 and TableS5.9).

5.2.6. Experimental design

The experimental design for set-up I and II was summarized in Figure 5.1. To elaborate, set-up I started with microaerobic conditions, achieved by introducing low oxygen concentrations on day 1. Once the available oxygen was depleted, there was a brief 3-day interval without oxygen.

Following this, set-up I underwent a series of five cycles involving intermittent low-oxygen addition (Phase 1). Subsequently, after complete removal of all the compounds, the bottles were re-spiked with the BTEXIeIaN mixture (added on day 59 and measured on day 60). From that point onward, set-up I no longer received oxygen supply (Phase 2). Set-up II did not receive any oxygen at the beginning of the experiment, therefore nitrate-reducing conditions were prevailing in phase 1. At day 60, set-up II was switched to microaerobic conditions by receiving low-oxygen for the first time, followed by a second addition on day 67 (Phase 2). The experiment stopped for both set-ups at day 98. Set-ups III, IV and V were prepared at a later stage to allow for a comparison between the biodegradation capacity of BTEXIeIaN and the consumption of electron acceptors. This comparison was made in relation to the conditions observed in set-ups I and II.

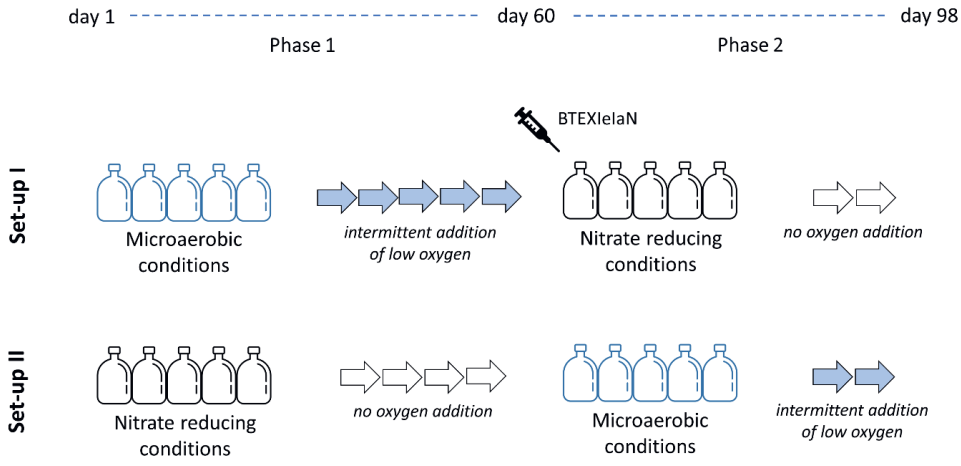

Figure 5.1 Scheme for the experimental design of the set-up I and II.

5.3. Results and Discussion

5.3.1. Biodegradation of BTEXIeIaN mixture under microaerobic conditions

Set-up I – From microaerobic conditions to nitrate-reducing conditions: In set-up I, complete biodegradation of BTEXIeIaN compounds was observed when low oxygen concentrations were added intermittently to the active bottles during phase 1 (Figure 5.2A). Biodegradation was not observed for the control bottles (Figure 5.2B) indicating that the removal of the compounds was a biotic process. Oxygen was added intermittently to the batches of set-up I, on days 1, 7, 14, 21 and 42. The added oxygen concentrations were ~0.7 mg O_2 L^{-1} and reduced to 0.2 mg O_2 L^{-1} (Figure 5.3). This concentration remained consistent until further oxygen addition, which seems that the constant reading of 0.2 mg O_2 L^{-1} on the oxygen sensors corresponds to an absence of oxygen. This could be related with either the sensitivity of the oxygen sensor to low concentrations, or a potential disturbance in the initial calibration. Therefore, the initial microaerobic conditions in this study were between, after correction, ~0.5 mg O_2 L^{-1}. Reduction in the oxygen concentrations (Figure 5.3) were in line with the biodegradation data (Figure 5.2), meaning that removal of the BTEXIeIaN compounds occurred in the presence of oxygen and at concentrations below 0.5 mg O_2 L^{-1}. After complete removal of the contaminants, active bottles of set-up I were re-spiked with the BTEXIeIaN mixture on day 60. This time BTEXIeIaN biodegradation was tested under nitrate-reducing conditions (Phase 2). When oxygen was not present in the bottles, BTEXIeIaN biodegradation did not occur, as the small decrease observed in the concentrations of the compounds was also noticed in the control bottles (Figure 5.2B). Therefore, for set-up I, it was concluded that oxygen is needed for a bio-removal of the BTEXIeIaN mixture, and that the low oxygen concentrations were sufficient for the complete degradation of the complex mixture containing various aromatic hydrocarbons.

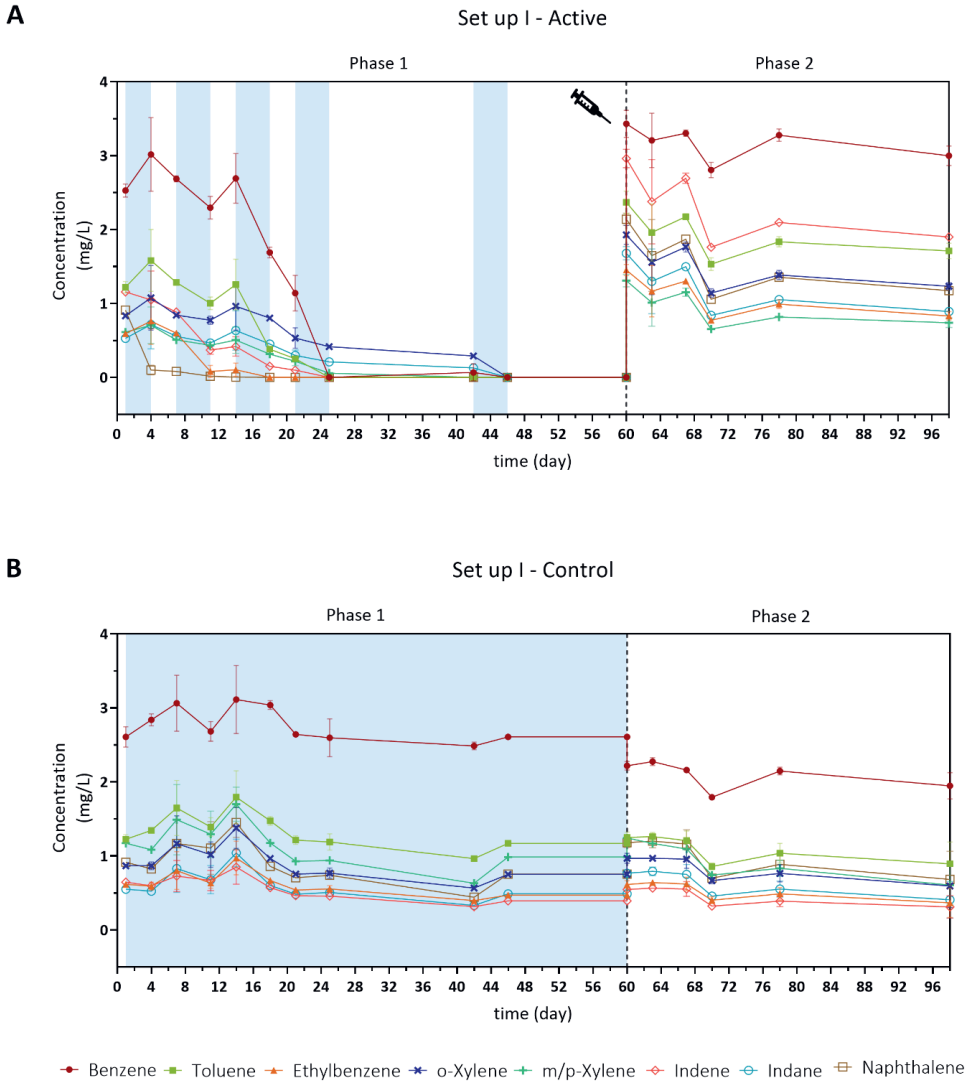

Figure 5.2 Set-up I: BTEXIeIaN biodegradation with intermittent addition of low oxygen (A) in active bottles and (B) in controls. Microaerobic conditions shown in blue. Control bottles received low oxygen addition only on day 1, as oxygen was not consumed throughout phase 1. The figures represent the mean of the triplicate active bottles (A) and duplicate controls (B).

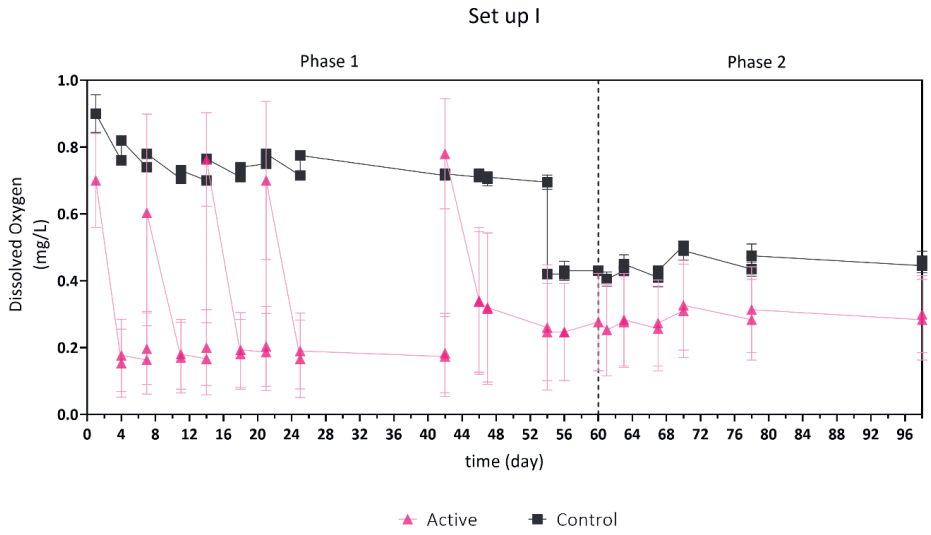

Figure 5.3 Mean of the dissolved oxygen concentrations of the active bottles and controls for set-up I, measured with oxygen sensor. At day 54, gas exchange procedure was applied with nitrogen gas (N_2, 100%).

Complete removal of the complex BTEXIeIaN mixture was achieved at concentrations below 0.5 mg O_2 L^{-1} in this study. In literature, microaerobic conditions were defined as below 2 mg O_2 L^{-1} (Olsen et al., 1995) however, a range of oxygen concentrations were also reported for the aerobic conversion of the BTEX compounds under microaerobic conditions. Su and Kafkewitz (1994) tested BTX degradation at 2% oxygen, and reported that xylenes and toluene could be degraded, but benzene was recalcitrant. Yerushalmi et al. (2001) tested benzene biodegradation under different DO concentrations (1 to 0.05 mg O_2 L^{-1}) and showed that at lower DO concentrations, the removal efficiency of benzene decreased (100% to 34%). Aburto et al. (2009) also investigated removal of benzene at low DO concentrations (2.57 to 0 mg O_2 L^{-1}) and concluded that very low concentrations of oxygen are sufficient to allow in situ biodegradation of benzene. As shown, individual BTEX compounds exhibit different performances under different oxygen concentrations. When BTEX was assessed as a mixture, in column experiments, 80% degradation was observed for TEX compounds and <50% for benzene (Firmino et al., 2018). These results were obtained with continuous injection of oxygen to the reactors with concentration of 0.18 L O_2 L^{-1}. Hutchins et al. (1992) also performed column experiments where the DO concentration was 1 mg O_2 L^{-1}, with oxygen being continuously flushed to the reactors. Some removal of benzene was reported under microaerobic conditions (without nitrate), and microaerobic conditions together with nitrate however this was below 50%, while TEX degradation almost did not occur for both conditions. In their study, nitrate-reducing conditions were required for the optimum TEX degradation. All the above references showed that there are no specific rules for BTEX degradation (individually or as mixture) under microaerobic conditions which was also discussed by Yerushalmi et al. (2002). This is explained by the fact that the oxygen concentration required to initiate aerobic conversion depends on various factors, such as bacterial population, substrate characteristics and environmental conditions. Therefore, investigating the biodegradation capacity of the indigenous microorganisms, as in this study, is crucial for each remediation case.

The experimental set-up I showed that indigenous microorganisms were capable to fully remove all the BTEXIeIaN compounds under low oxygen concentrations, however couldn't biodegrade the contaminants when further tested under fully anaerobic, nitrate-reducing conditions. The transition from microaerobic conditions to nitrate-reducing conditions was therefore unsuccessful, however complete removal of BTEXIeIaN mixture under microaerobic conditions is promising, especially for effective bioremediation purposes.

Set-up II – From nitrate-reducing conditions to microaerobic conditions: In set-up II, for the initial 60-day period, nitrate-reducing conditions were prevalent and during that time, the BTEXIeIaN mixture was not subjected to degradation (Figure 5.4). Biodegradation of the compounds was observed starting from day 60, when set-up II bottles received low oxygen concentrations for the first time (Figure 5.4A). In the second oxygen addition (day 67), biodegradation continued and almost all the compounds of the mixture were removed. If the experiment was extended over a longer duration and one more application of oxygen had been introduced, it is highly likely that complete removal of the compounds could have been achieved. The DO concentrations measured for set-up II batch bottles were given in Figure 5.5. Similar to the results of set-up I, the oxygen concentrations decreased from ~0.8 to 0.3 mg O_2 L^{-1} and were in line with the biodegradation data (Figure 5.4). Despite supplying oxygen to the control bottles on day 60, all compounds were still present on day 98, with concentrations remaining close to their initial values. Hence, the results indicated that following a pre-anaerobic period, microorganisms could swiftly adapt to microaerobic conditions and possess the capacity to fully biodegrade the BTEXIeIaN mixture in a short period of time.

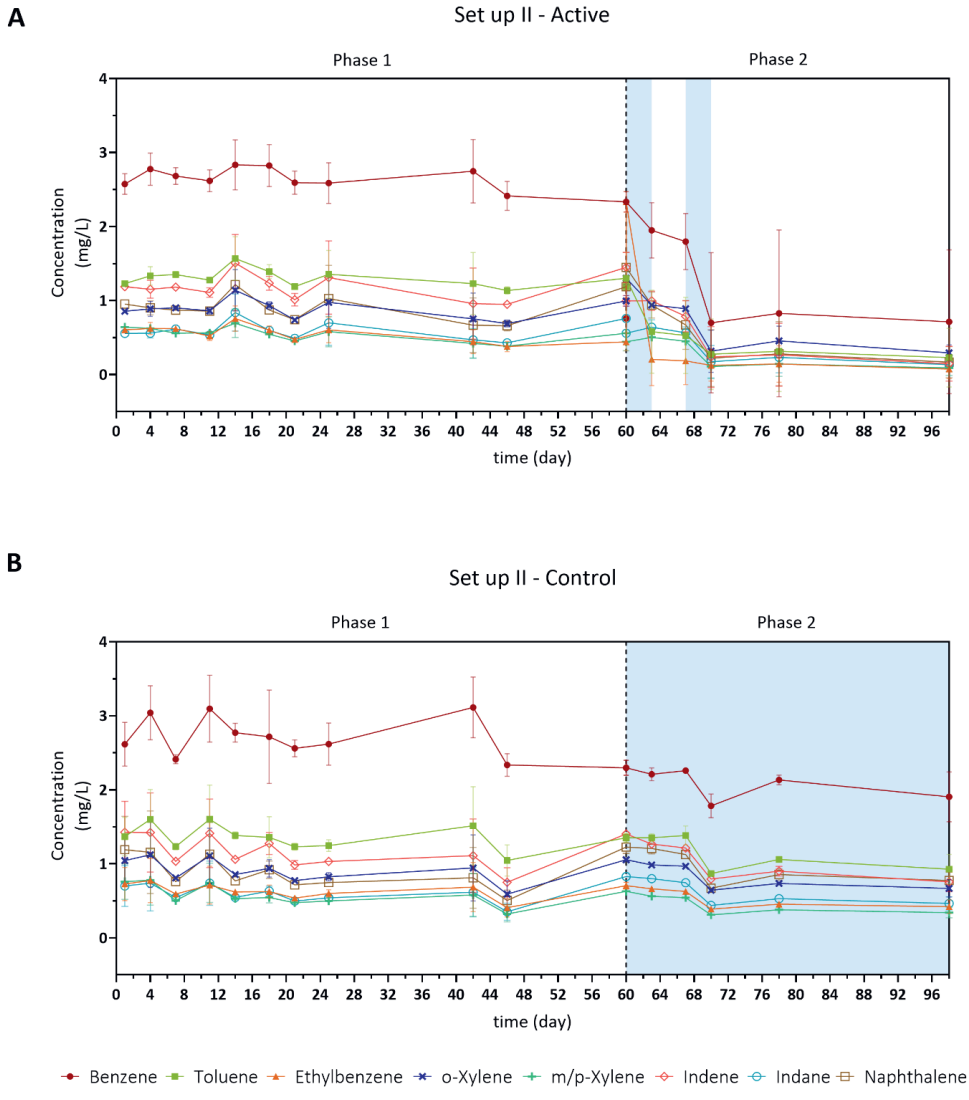

Figure 5.4 Set-up II: BTEXIeIaN degradation with intermittent addition of low oxygen (A) in active bottles and (B) in controls. Microaerobic conditions shown in blue. Control bottles received low oxygen addition only on day 60, as oxygen was not consumed throughout phase 2. The figures represent the mean of the triplicate active bottles (A), and duplicate controls (B).

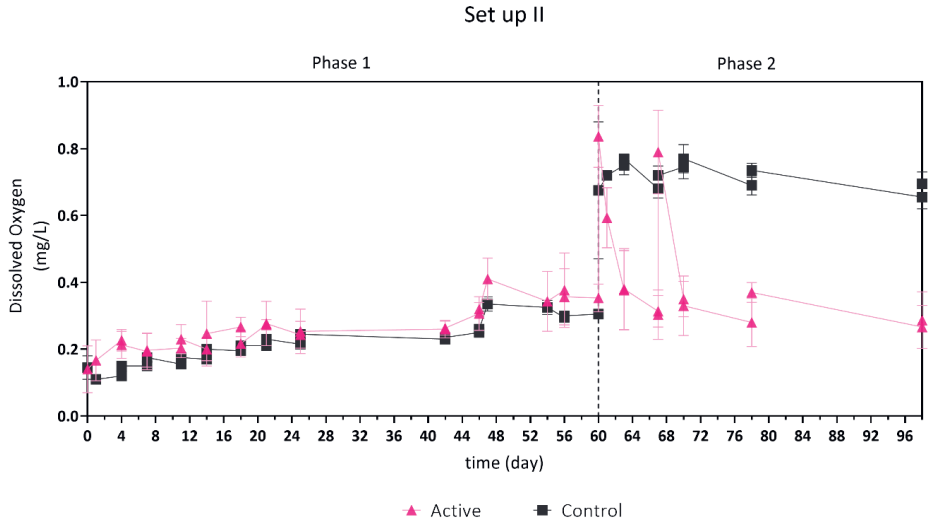

Figure 5.5 Mean of the dissolved oxygen concentrations of the active bottles and controls for set-up II, measured with oxygen sensor. At day 54, gas exchange procedure was applied with nitrogen gas (N_2, 100%).

The objective of the set-up II experiment was to mimic in situ bioremediation conditions, simulating the prolonged exposure of microorganisms to BTEXIeIaN mixture under anaerobic conditions. As shown, after a 60-day anaerobic period, the bottles were supplied with low oxygen concentrations as if oxygen was supplied to the subsurface of contaminated zones. This revealed that microorganisms were capable of transitioning from anaerobic conditions to microaerobic conditions and effectively biodegrading the BTEXIeIaN mixture. A similar approach was tested by Siqueira et al. (2018) where BTEX biodegradation was tested under anaerobic conditions first. In their case, high removal efficiencies were obtained for TEX compounds (70-80%) whereas benzene's removal efficiency was around 55%. After the anaerobic period of 50-75 days, synthetic air was introduced continuously to investigate BTEX removal under microaerobic conditions. Removal efficiencies then increased to > 85%, 89% and 67% for EX, T and B, respectively. Increase in the removal efficiencies with micro-aeration was also reported in the study of Firmino et al. (2018) where more than 80% removal of all BTEX compounds was achieved. In our study, benzene exhibited the least degradation among the BTEXIeIaN compounds under microaerobic conditions, similar to the finding of Siqueira et al., as mentioned above. Under anaerobic conditions, the BTEXIeIaN compounds were not degraded in the given experimental time contrary to the above studies mentioned. Therefore, oxygen seems to be the key reagent for the complete removal of the BTEXIeIaN mixture by the indigenous microorganisms.

In summary, in both set-ups, all compounds of BTEXIeIaN mixture were biodegraded where initial oxygen concentrations were ~0.5 O_2 L^{-1}. Oxygen was added intermittently; therefore, the aerobic conversion of the mixture most probably occurred at even lower concentrations than 0.5 O_2 L^{-1}. These findings are promising, as in set-up I, full removal of all compounds including benzene was achieved. Moreover, with an additional oxygen application to set-up II, complete removal of all compounds would likely have been achieved as well. Notably, this study exhibited complete removal of all compounds even in a complex mixture of BTEX and IeIaN, which sets it apart from previous reports on BTEX (Da Silva et al., 2005; Firmino et al., 2018; S.-R. Hutchins et al., 1992; Olsen et al., 1995; Siqueira

et al., 2018). Another strong point of this study was the demonstration of the transition between microaerobic and nitrate conditions. This highlighted the microorganisms' ability to adapt to microaerobic conditions after an extended period of anaerobic conditions (Set-up II). The reverse process did not occur, emphasizing the one-way nature of this adaptation.

Set-up III and IV – BTEXIeIaN degradation under fully aerobic and fully anaerobic conditions: Additionally, to set-up I and II, separate experiments were performed with fully aerobic (set-up III) and fully anaerobic conditions (set-up IV). On one hand, within set-up III, all compounds of the BTEXIeIaN mixture were fully biodegraded within 15 days (Supplementary Figure S5.1). On the other hand, in set-up IV, under prevailing nitrate-reducing conditions with no introduction of oxygen to the bottles, the degradation process of ethylbenzene started after 308 days (Supplementary Figure S5.2). By day 400, all compounds of the BTEXIeIaN mixture remained present, with ethylbenzene exhibiting partial degradation.

Outcomes of this study showed that fully anaerobic conditions are unsuccessful in complete removal of the BTEXIeIaN compounds and oxygen is essential for the rapid conversion of these contaminants. Additionally, it was shown that an excess supply of oxygen is unnecessary (as in set-up III), since complete biodegradation of the mixture was also shown at microaerobic conditions with set-up I and II. This is particularly crucial, especially when dealing with mixtures containing various aromatic hydrocarbons that exhibit varying susceptibilities to anaerobic biodegradation (Foght, 2008). For instance, ethylbenzene degradation is commonly observed under nitrate-reducing conditions and rarely with other electron acceptors. In contrast, xylene degradation was reported with nitrate, sulphate or iron but its occurrence mostly depends to the specific isomer (Weelink et al., 2010). The compound-dependent nature of biodegradation activity poses an additional challenge in bioremediation when dealing with mixtures containing various compounds with distinct properties, and this is even more challenging when anaerobic conditions prevail. In emergent remediation strategies, particularly in scenarios where toxic compounds like BTEX pose threats to the environment, the implementation of micro-aeration can offer a more guaranteed solution especially for remediation projects with a high risk reduction urgency.

5.3.2. Investigation on the microbial use of electron acceptors

In order to have an insight on the electron acceptor usage throughout the experiments, oxygen and nitrate consumptions were compared between set-ups. Additionally, the effect of nitrite on biodegradation as a by-product of denitrification process was investigated.

Specificity in oxygen consumption: BTEXIeIaN mixture was biodegraded in set-up I through the application of five oxygen spikes, while in set-up II, two similar spikes of oxygen were needed for almost complete conversion of the same amount of complex substrate. This indicated a variation in oxygen utilization between the different oxygen dosage set-ups. In order to investigate further, a comparison was made between the oxygen consumptions of set-up I and II (Table 5.2). First, the theoretical O_2 was determined by calculating the required oxygen per mg of consumed contaminant using the stochiometric values provided in Supplementary Table S5.10. Next, the consumed O_2 was calculated based on the total of consumed oxygen (mg) in the headspace and liquid phase. The oxygen used for organic matter (OM) degradation was derived by subtracting the theoretical oxygen values from the actual experimental oxygen measurements.

Table 5.2 Calculations of the actual oxygen consumption within the batch bottles (consumed O_2), the required oxygen for the biodegradation of the consumed compounds (theoretical O_2), and potential oxygen available for the organic matter (OM) in set-up I and II.

Set-up	**Consumed O_2 in the bottles** (mg)	**Theoretical O_2 for biodegradation** (mg)	**Potential O_2 available for OM** (mg)
I	13.57 ± 0.76	2.98 ± 1.32	10.59
II	3.94 ± 0.47	3.41 ± 0.20	0.53

In set-up I, the 78% of the consumed oxygen was utilized for OM (10.59 mg) leaving the 22% of the oxygen used for BTEXIeIaN degradation (2.98 mg) (Table 5.2). In set-up II, 14% of the oxygen was likely utilized for OM (0.53 mg) and 86% for biodegradation of the mixture (3.41 mg) (Table 5.2). This showed that oxygen was more efficiently used for BTEXIeIaN degradation in set-up II compared to set-up I. This is probably because in set-up II, most of the OM degradation occurred during the pre-anaerobic period (Phase 1), using nitrate as an electron acceptor. After oxygen addition at day 60 (Phase 2), oxygen was used mostly for the biodegradation process of the aromatic pollutants as some of the OM was probably consumed in the preceding days. During phase 1, limiting conditions were prevalent, allowing the microbes to use oxygen only for metabolization of the aromatic compounds rather than for OM. As a result, having a pre-anaerobic period before addition of low oxygen concentrations showed to be efficient in terms of oxygen usage towards BTEXIeIaN biodegradation.

Oxygen usage of set-up I and II was also compared to BTEXIeIaN degradation under fully aerobic conditions (set-up III). For set-up III, initial BTEXIeIaN concentrations were higher than set-up I and II (Table S5.2), therefore the theoretical O_2 values were higher and were calculated as 6.91 (±0.05) mg while the total oxygen consumption in the headspace and liquid phase was 19.48 (±0.44) mg. This signifies that 35% of the oxygen in set-up III was used for the biodegradation of BTEXIeIaN. While this percentage closely resembled the value in set-up I (22%), it is important to note that not all the oxygen introduced to the bottles in set-up III was utilized. This is because the O_2 measurements were stopped upon the complete removal of BTEXIeIaN. If the measurements were to continue, more oxygen would be consumed for the degradation of OM, as the BTEXIeIaN mixture was no longer present. This would likely result in a percentage lower than 35%. Firmino et al. (2018) investigated BTEX degradation under two different air flow rates (2 mL air min^{-1} vs 1 mL air min^{-1}). They demonstrated that, despite doubling the oxygen concentration, there were no significant differences in the removal efficiencies. This suggests that increasing oxygen concentrations in a system might not necessarily result in improved removal of a compound. Supply of low oxygen concentrations can even benefit in lowering the toxicity of oxygen to the anaerobic microorganisms (Krayzelova et al., 2015). In this study, the intermittent addition of low-oxygen proved to be as effective as maintaining fully aerobic conditions in terms of BTEXIeIaN removal (set-up I vs III) and exhibited even greater efficiency in oxygen utilization when combined with a pre-anaerobic period prior to low-oxygen addition (set-up II vs I-III).

Nitrate consumption under microaerobic and nitrate-reducing conditions: To understand the nitrate usage by the indigenous microorganisms, nitrate consumptions in phase 1 and 2 for set-up I and II were calculated (Table 5.3). For set-up I, during microaerobic conditions (Phase 1), ~12 mg of nitrate was consumed in the active bottles, while during nitrate-reducing conditions (Phase 2) only ~3 mg was utilized by the microorganisms. The reason for more nitrate consumption under microaerobic conditions can be explained by the presence of oxygen, leading to an increase in biomass growth of facultative anaerobes with OM as electron donor substrate, subsequently to higher nitrate

consumption. As mentioned before, oxygen may contribute by enhancing the growth rate of biomass and increasing the size of the indigenous microbial population. A larger and faster-growing population will lead to a higher rate of denitrification (Olsen et al., 1995). It is unknown from this data whether nitrate was consumed for BTEXIeIaN biodegradation or OM. Nitrate consumptions were higher in active bottles as opposed to controls, indicating that nitrate consumption was a biotic process. However, it is unlikely that this nitrate consumption was employed for the biodegradation of the mixture, given that oxygen is a more energetically favorable electron acceptor than nitrate (Lueders, 2017). Hence, microorganisms are likely to preferentially utilize oxygen. Furthermore, when nitrate was solely present during phase 2, the consumption of nitrate was lower than what was theoretically required for the compounds removed during that phase (4.44 ± 0.58 mg). This implies that nitrate was probably utilized for other processes, such as OM degradation rather than biodegradation of the aromatic compounds which are apparently more difficult to degrade compounds compared to OM, for the nitrate-reducing reducing bacteria.

In set-up II, ~6 mg nitrate was consumed during phase 1, under nitrate-reducing conditions. Once oxygen was added to the bottles during phase 2, nitrate consumption continued; however, it was not as high as in set-up I-phase 1 (microaerobic conditions). One potential explanation is that more OM was present at the beginning of the experiment leading to a higher consumption of nitrate in set-up I bottles. Additionally, set-up I bottles received five oxygen addition while set-up II bottles only received two additions, meaning that higher nitrate consumption in set-up I was related to more oxygen dosage, which could have enhanced the biomass growth of facultative anaerobes.

Table 5.3 Mean of the nitrate consumed in set-up I and II batch bottles.

Set-up I	**Phase 1** (*microaerobic*)	**Phase 2** (*nitrate-reducing*)	**Total**
Active (mg)	11.95 ± 0.95	3.09 ± 0.47	15.04 ± 0.50
Controls (mg)	4.59 ±0.70	-1.07 ± 1.35	3.52 ± 3.34
Set-up II	**Phase 1** (*nitrate-reducing*)	**Phase 2** (*microaerobic*)	**Total**
Active (mg)	5.75 ± 1.05	3.13 ± 0.35	8.89 ± 1.08
Controls (mg)	4.39 ± 0.41	-1.07 ± 0.42	3.32 ± 2.30

Overall, the oxygen and nitrate measurements (Table 5.2 and Table 5.3) showed that both electron acceptors were utilized under microaerobic conditions. According to literature, different scenarios are possible where oxygen and nitrate can be consumed simultaneously or sequentially. Simultaneous consumption of both electron acceptors is termed as aerobic denitrification where the denitrification process is carried out by aerobic denitrifiers in the presence of oxygen (Yang et al., 2020). Simultaneous consumption of oxygen and nitrate can also occur in the co-presence of aerobic microorganisms and dentrifiers at microaerobic zones where aerobic microorganisms consume oxygen by allowing denitrifiers to perform anaerobic processes. As an example, in situ microbial communities at different benzene contaminated groundwater sites were investigated by Aburto et al. (2009) for their potential in anaerobic as well as aerobic benzene degradation. Their results showed that, both aerobic and anaerobic microbes were present in the contaminated groundwater and concluded that even at low concentrations, oxygen seemed necessary to initiate benzene biodegradation, and that anaerobic microbes also contributed to the completion of the degradation. Some microorganisms can use oxygen to introduce hydroxyl groups into the aromatic ring as in aerobic pathways, followed by the cleavage

step occurring via anaerobic pathways. This was supported by Yerushalmi et al. (2001) where simultaneous presence of aerobic and anaerobic intermediates of benzene (catechol and benzoic acid, respectively) were detected under microaerobic conditions.

In this study, it is unknown whether oxygen and nitrate were consumed simultaneously or sequentially. Nevertheless, nitrate appeared not to be used for BTEXIeIaN biodegradation but mainly for OM degradation. However, when microaerobic conditions were applied, microbes could quickly adapt to these conditions and biodegrade the BTEXIeIaN compounds, without any lag phase (set-up II-phase 2). This activity could not be prolonged when transitioning from microaerobic conditions to nitrate-reducing conditions (set-up I-phase 2); hence the microorganisms were not capable of anaerobic BTEXIeIaN degradation during the given experimental time. The sediment samples used in this study were derived from an anaerobic environment that had remained devoid of oxygen. Despite this anaerobic origin, the BTEXIeIaN mixture exhibited degradation in the presence of oxygen suggesting the presence of facultative anaerobes, given the concurrent consumption of both oxygen and nitrate. In a prior study, the microbial consortium from the same sediment used in this experiment was analyzed by 16s rRNA amplicon, showing the presence of microbial groups with facultative anaerobic members (i.e., *Pseudomonas* and *Acidovorax*) (Aydin et al., 2023). Even though indigenous microorganisms could not biodegrade BTEXIeIaN mixture under nitrate-reducing conditions, they were tolerant to low oxygen concentrations as nitrate consumption continued after switching from microaerobic to nitrate-reducing conditions (set-up I-phase 2).

Monitoring nitrite production: High nitrate consumption can lead to high nitrite production, which can be toxic for the microorganisms (Chayabutra & Ju, 2000; Philips et al., 2002) and end up inhibiting the biodegradation process (Zhu et al., 2020). Therefore, nitrite concentrations in set-up I and II were monitored throughout the experiment. As shown in Figure 5.6, nitrite concentrations were almost three times higher in set-up I compared to set-up II. As mentioned in the previous chapter, more addition of oxygen in set-up I have led to more nitrate consumption, which ended up to higher accumulation of nitrite. To understand the cause behind the lack of biodegradation in set-up I, after switching to fully anaerobic conditions (Figure 5.2-phase 2), the potential connection to high nitrite production was investigated. For this, an additional experiment (Set-up V, Figure 5.7) was performed where BTEXIeIaN degradation was tested under microaerobic and nitrate-reducing conditions but with lower nitrate concentrations (~50 mg L^{-1}) than in set-up I and II (430-450 mg L^{-1}).

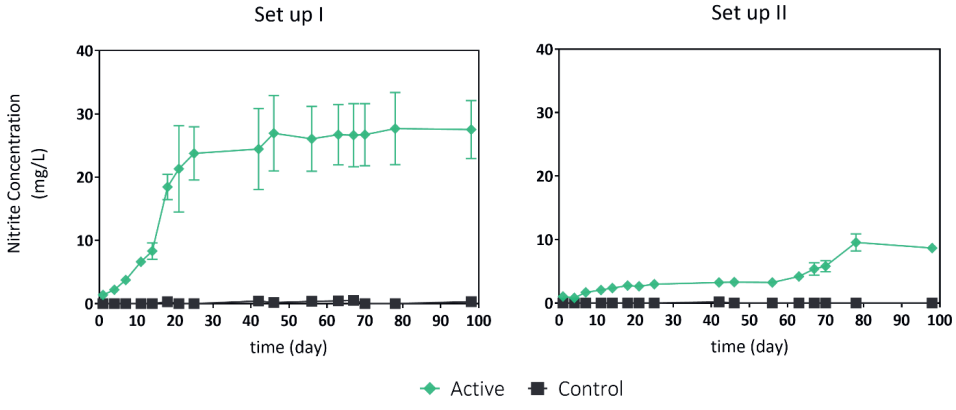

Figure 5.6 Mean of the produced nitrite concentrations in the active bottles and controls for set-up I and II.

Set-up V bottles were prepared fully anaerobic, with the nitrate concentrations of ~50 mg L^{-1} at the beginning of the experiment. First addition of low-oxygen (~2.08 mg) was on day 6 and the second (~2.25 mg) on day 18 (Figure 5.7). After day 18, no more oxygen was supplied to the batch bottles in order to observe if transition from aerobic to anaerobic degradation of BTEXIeIaN would occur. However, no removal of the contaminants was observed from day 30 to 116 (Figure 5.7). The nitrite concentrations in set-up V were found to be ~5.43 mg L^{-1} by the end of the experiment, which was one order of magnitude lower compared to set-up I (~27.53 mg L^{-1}) and comparable to set-up II (~8.66 mg L^{-1}). As shown, lower nitrate concentrations led to lower nitrite production, but the aromatic compound mixture was still not degraded. This indicated that nitrite was not the reason for the lack of degradation in set-up I during phase 2. This phenomena can also be confirmed in set-up II. On day 70, when transitioning from microaerobic conditions to nitrate-reducing conditions, despite having lower nitrite concentrations than those in set-up I, the residual compounds were still not biodegraded (Figure 5.4 and Figure 5.6). The fact that the compounds still remained in the absence of oxygen is unsurprising, given that it took 308 days for the removal of certain compounds to take place under fully anaerobic conditions in set-up IV (Figure S5.2). Thus, it can be concluded that, for the groundwater and sediment of this contaminated MGP site, the indigenous microorganisms cannot switch from aerobic to anaerobic -nitrate reducing- biodegradation within 60 days, and a longer period of time might be needed for the adaptation to anaerobic processes, or there are intrinsic (i.e. thermodynamic or enzyme mechanistic) reasons hampering these nitrate-reducing pollutant degradation processes.

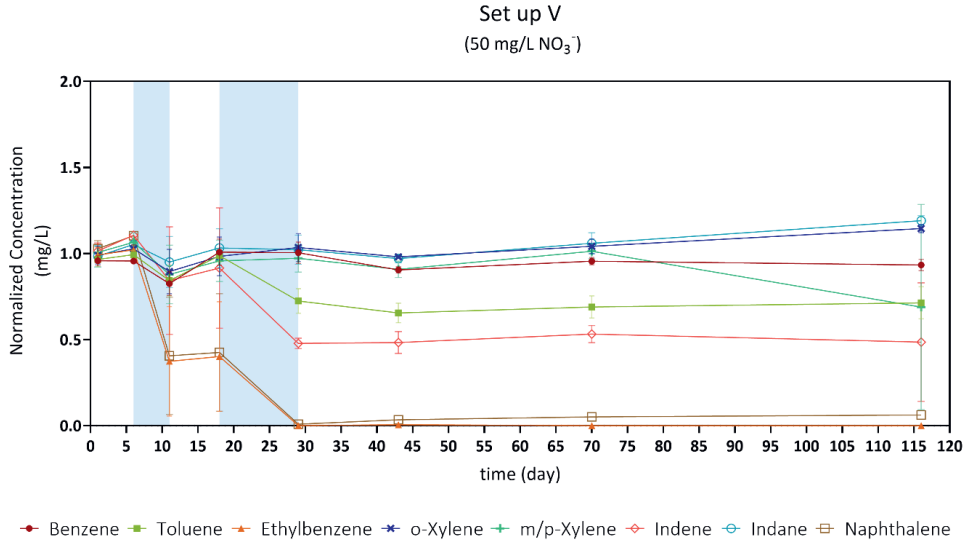

Figure 5.7 Biodegradation of BTEXIeIaN in set-up V, with lower nitrate concentrations (50 mg L^{-1}). Blue periods represent microaerobic conditions.

5.3.3. Implications for future remediation strategies

Pump and treat approach is the conventional solution commonly applied to contaminated sites with aromatic compounds with NAPL sources, preventing the spreading of contaminant plumes (Sakr et al., 2023). However, such treatment techniques have disadvantages such as transferring contaminants from one medium to another, high cost and maintenance requirements, and long duration of operation (Yerushalmi et al., 1999). Biological treatment can be a more efficient and economical strategy for aromatic hydrocarbon degradation in anaerobic aquifers, but oxygen limitation is one of the major problems affecting the performance of microorganism as most of contaminated sites suffer from lack of high energy yield electron acceptors (oxygen, nitrate) (Meckenstock et al., 2015). Although BTEX can be degraded under different redox conditions, anaerobic degradation is usually slower than aerobic bioconversion (El-Naas et al., 2014; Varjani, 2017), especially in complex mixtures where inter-compound inhibitions can occur (Aydin et al., 2023; Dou, Liu, & Hu, 2008b; Zhou et al., 2011). The limitation of anaerobic BTEX (plus other compounds) degradation can be overcome by adding oxygen into the system to favor the initial degradation of BTEX compounds (Firmino et al., 2018). Nevertheless, oxygen addition to naturally anaerobic aquifers can be a costly and inefficient process due to the low solubility of oxygen (Weelink et al., 2010), and potential occurrence of clogging effects by formation of particulate metal oxides. Therefore, efficient oxygen supply strategies to bioremediate deep anaerobic surfaces is needed. For instance, intermittent addition of low-oxygen can be applied by Micro-Nano Bubbles (MNBs). MNBs are gaining much attention in recent years and are used to enhance treatment effects in groundwater remediation. Due to their large specific surface area, long retention time and high oxygen transfer efficiency, MNBs filled with air or oxygen can improve the availability of dissolved oxygen in groundwater (Haris et al., 2020). As a result, these bubbles can stimulate the aerobic conversion of aromatic compounds that are recalcitrant under anaerobic conditions. Based on the observations of this study, in order to enhance the performance of MNBs, a pre-treatment method with nitrate, with consideration of drinking water legislation limits, can be applied where nitrate consumes a significant portion of the most reactive OM. Therefore, a-pre-treatment with nitrate can be beneficial before oxygen addition which can be supplied (via MNBs) intermittently and be principally used for biodegradation of contaminants. In this study, comparing the consumed oxygen of set-up II (microaerobic) and III (fully aerobic) showed that excess of oxygen is unnecessary for the BTEXIeIaN removal, when present with another electron acceptor like nitrate. It was also shown that, high dosages of oxygen increases the nitrate consumption, leading to high nitrite concentrations. Hence, an adequate dosage strategy for minimal amounts of oxygen and nitrate is needed to create a safe and effective bioremediation solution.

5.4. Conclusions

The results of this study have important implications for in situ bioremediation of aromatic compounds in naturally anaerobic aquifers. Providing low oxygen concentrations allowed for complete removal of a BTEXIeIaN mixture, while nitrate without oxygen did not lead to any biodegradation. Combining oxygen and nitrate showed an effective strategy to reduce the amount of oxygen dosed: a pre-treatment period under nitrate-reducing conditions followed by intermittent oxygen dosage creating microaerobic conditions (<0.5 mg O_2 L^{-1}) resulted in an efficient use of oxygen for the biodegradation of the contaminants. It appears that nitrate is used for conversion of reactive natural OM conversion in the anaerobic phase, while the oxygen is primarily used for pollutant biodegradation in the subsequent microaerobic phase. A careful dosage strategy for nitrate and oxygen is needed to create safe conditions for protecting groundwater against high nitrate and nitrite concentrations, while

effectively remediating the organic aromatic pollutants such as BTEXIeIaN mixtures, and this requires further optimization studies for each specific polluted aquifer considered.

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Supplementary information to Chapter 5

Supplementary data 1: BTEXIeIaN mixture

Table S5.1 Volumes added to achieve equal mass of 100 mg of each compound for the preparation of the pure BTEXIeIaN mixture.

Compound	Density ($g\ mL^{-1}$)	Amount (mg)	Volume added (µL)
Benzene	0.879	100	114
Toluene	0.862	100	116
Ethylbenzene	0.867	100	115
o-Xylene	0.880	100	114
m-Xylene	0.860	100	116
p-Xylene	0.860	100	116
Indene	0.965	100	104
Indane	0.996	100	100
Naphthalene	1.160	100	Solid (0.1 g)

Concentrations of the pure mixture of BTEXIeIaN measured in HPLC

Once that the pure mix was added to the batch bottles, the mass ratio was found to be not equally distributed (5:2:1:2:1:2:1:2, B:T:E:o-X:m/p-X:Ie:Ia:N). Initial concentrations for each compound at the beginning of the experiment is given in Table S5.2. It is hypothesized that the uneven distribution is due to headspace-liquid distribution in the batch bottles. As the analysis were done only by monitoring the liquid phase, it was assumed that compounds were also present in gas phase or absorbed to the sediment resulting to variable ratios of the compounds in the bottles. Since all bottles were prepared within the same procedure, and had similar initial BTEXIeIaN concentrations and ratios, this issue was disregarded as it would not affect the experimental design. This issue was by-passed by comparing the active bottles to the control bottles.

Table S5.2 Initial concentrations (mean of five bottles: 3 active and 2 control) for each compound at the beginning of the experiment for set-up I, II and III.

	Initial concentration ($mg\ L^{-1}$)		
	Set-up I	**Set-up II**	**Set-up III**
Benzene	2.56 ±0.09	2.54 ±0.16	3.58 ±0.09
Toluene	1.22 ±0.06	1.26 ±0.15	2.10 ±0.06
Ethylbenzene	0.60 ±0.02	0.64 ±0.13	1.19 ±0.05
o-Xylene	0.85 ±0.02	0.91 ±0.18	1.80 ±0.05
m/p-Xylene	0.63 ±0.03	0.68 ±0.13	1.15 ±0.07
Indene	1.16 ±0.02	1.27 ±0.23	2.41 ±0.10
Indane	0.54 ±0.02	0.60 ±0.15	1.38 ±0.05
Naphthalene	0.91 ±0.02	1.03 ±0.24	1.54 ±0.30

±; standard deviation

Supplementary data 2: Oxygen calculations

Absolute oxygen values added to the bottles

On day 1, 4, 7, 14, 21, 42 (set-up I) and 60, 67 (set-up II) oxygen was added to the bottles. For this 8.5 mL air was injected. First, the amount of 8.5 mL air was converted to mol based on the ideal gas law (Equation S5.1) and further to mg of oxygen (Equation S5.2). For this the atmospheric pressure of each day of was considered (Table S5.3).

Table S5.3 Pressure (P) of the atmospheric air on the days of oxygen addition.

	Date	day	Hg	mbar	atm
Set-up I	24-02-2022	1	29.7	1005.8	1.01
	03-03-2022	7	30.1	1019.3	1.02
	10-03-2022	14	30.2	1022.7	1.02
	17-03-2022	21	30.4	1029.5	1.03
	07-04-2022	42	29.0	982.10	0.98
Set-up II	25-04-2022	60	29.9	1012.53	1.01
	02-05-2022	67	30.2	1022.69	1.02

For the calculation of the added oxygen (n), Equation S5.1 and Equation S5.2 were used based on the values given in Table S5.4.

Calculation of the oxygen (in mol) based on the ideal gas law:

$$n = P*V / R*T \quad \text{(S5.1)}$$

Calculation of oxygen (mol to mg):

$$n*32\ (\text{oxygen in g}) * 1000\ (\text{to mg}) *21/100\ (21\%\ \text{oxygen in air}) \quad \text{(S5.2)}$$

Table S5.4 Theoretical calculation of oxygen (in mg) added to the bottles for set-up I and II.

Set-up	day	P (atm)	V (L)	R (L atm K^{-1} mol^{-1})	T (°K)	n (mol)	n (g)	n (mg)	*21 (21% oxygen)
I	1	1.01	0.0085	0.082	293.15	0.00	0.01	11.38	2.39
	7	1.02	0.0085	0.082	293.15	0.00	0.01	11.53	2.42
	14	1.02	0.0085	0.082	293.15	0.00	0.01	11.57	2.43
	21	1.03	0.0085	0.082	293.15	0.00	0.01	11.65	2.45
	42	0.98	0.0085	0.082	293.15	0.00	0.01	11.11	2.33
									Total: 12.02 mg
II	60	1.01	0.0085	0.082	293.15	0.00	0.01	11.46	2.39
	67	1.02	0.0085	0.082	293.15	0.00	0.01	11.57	2.42
									Total: 4.82 mg

Table S5.5 Oxygen percentage measured in GC (in blue) and their calculations to mg (in orange) for each bottle of set-up I (SI) and set-up II (SII). C1 and C2 are duplicates of the control bottles.

day 1	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.14	100	0.1	0.0821	293	0.00	0.15	151.65	1.14		1.73	
SI-2	1.26	100	0.1	0.0821	293	0.01	0.17	167.61	1.26		2.11	
SI-3	1.17	100	0.1	0.0821	293	0.00	0.16	155.64	1.17		1.82	
SI-C1	1.51	100	0.1	0.0821	293	0.01	0.20	200.87	1.51		3.03	
SI-C2	1.31	100	0.1	0.0821	293	0.01	0.17	174.27	1.31		2.28	
Active (mean)									*1.19*	*±0.05*	*1.89*	*±0.16*
Control (mean)									*1.41*	*±0.10*	*2.66*	*±0.38*
day 7	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.39	102	0.102	0.0821	293	0.01	0.19	188.61	1.39		2.62	
SI-2	1.52	102	0.102	0.0821	293	0.01	0.21	206.24	1.52		3.13	
SI-3	1.46	102	0.102	0.0821	293	0.01	0.20	198.10	1.46		2.89	
SI-C1	1.49	102	0.102	0.0821	293	0.01	0.20	202.17	1.49		3.01	
SI-C2	1.24	102	0.102	0.0821	293	0.01	0.17	168.25	1.24		2.09	
Active (mean)									*1.46*	*±0.05*	*2.88*	*±0.21*
Control (mean)									*1.37*	*±0.13*	*2.55*	*±0.46*
day 14	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.42	104	0.104	0.0821	293	0.01	0.20	196.45	1.42		2.79	
SI-2	1.55	104	0.104	0.0821	293	0.01	0.21	214.44	1.55		3.32	
SI-3	1.50	104	0.104	0.0821	293	0.01	0.21	207.52	1.50		3.11	
SI-C1	1.40	104	0.104	0.0821	293	0.01	0.19	193.69	1.40		2.71	
SI-C2	1.22	104	0.104	0.0821	293	0.01	0.17	168.78	1.22		2.06	
Active (mean)									*1.49*	*±0.05*	*3.08*	*±0.22*
Control (mean)									*1.31*	*±0.09*	*2.39*	*±0.33*
day 21	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.79	106	0.106	0.0821	293	0.01	0.25	252.41	1.79		4.52	
SI-2	1.89	106	0.106	0.0821	293	0.01	0.27	266.51	1.89		5.04	
SI-3	1.72	106	0.106	0.0821	293	0.01	0.24	242.53	1.72		4.17	
SI-C1	1.59	106	0.106	0.0821	293	0.01	0.22	224.20	1.59		3.56	
SI-C2	1.30	106	0.106	0.0821	293	0.01	0.18	183.31	1.30		2.38	

Table S5.5 continued...

Active (mean)								*1.80*	*±0.07*	*4.58*	*±0.36*
Control (mean)								*1.45*	*±0.15*	*2.97*	*±0.59*
day 42	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.47	108	0.108	0.0821	293	0.01	0.21	211.19	1.79		3.78	
SI-2	1.68	108	0.108	0.0821	293	0.01	0.24	241.36	1.89		4.56	
SI-3	1.64	108	0.108	0.0821	293	0.01	0.24	235.62	1.72		4.05	
SI-C1	1.26	108	0.108	0.0821	293	0.01	0.18	181.02	1.59		2.88	
SI-C2	1.07	108	0.108	0.0821	293	0.00	0.15	153.73	1.3		2.00	
Active (mean)									*1.80*	*±0.07*	*4.13*	*±0.32*
Control (mean)									*1.45*	*±0.15*	*2.44*	*±0.44*
day 60	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SII-1	1.12	112	0.112	0.0821	293	0.01	0.17	166.87	1.12		1.87	
SII-2	1.19	112	0.112	0.0821	293	0.01	0.18	177.30	1.19		2.11	
SII-3	1.12	112	0.112	0.0821	293	0.01	0.17	166.87	1.12		1.87	
SII-C1	1.28	112	0.112	0.0821	293	0.01	0.19	190.71	1.28		2.44	
SII-C2	1.34	112	0.112	0.0821	293	0.01	0.20	199.65	1.34		2.68	
Active (mean)									*1.14*	*±0.03*	*1.95*	*±0.11*
Control (mean)									*1.31*	*±0.03*	*2.56*	*±0.12*
day 67	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SII-1	1.42	114	0.114	0.0821	293	0.01	0.22	215.19	1.31		2.82	
SII-2	1.41	114	0.114	0.0821	293	0.01	0.21	214.43	1.34		2.87	
SII-3	1.36	114	0.114	0.0821	293	0.01	0.21	205.49	1.28		2.63	
SII-C1	1.40	114	0.114	0.0821	293	0.01	0.21	211.70	1.24		2.63	
SII-C2	1.40	114	0.114	0.0821	293	0.01	0.21	211.70	1.37		2.90	
Active (mean)									*1.31*	*±0.02*	*2.77*	*±0.10*
Control (mean)									*1.31*	*±0.07*	*2.76*	*±0.14*

Table S5.6 Dissolved oxygen (DO) concentrations measured with oxygen sensor for each sampling day. Mean of DO concentrations of active bottles (triplicate) and controls (duplicate) are given with the standard deviations (±) for set-up I and II. Oxygen additions are shown in blue.

	Set-up I – DO (mg L^{-1})				Set-up II – DO (mg L^{-1})			
Day	Active		Control		Active		Control	
1	**0.70**	±0.11	**0.90**	±0.04	**0.17**	±0.05	**0.11**	±0.01
4B	**0.15**	±0.08	**0.76**	±0.01	**0.23**	±0.03	**0.12**	±0.01
4A	**0.18**	±0.09	**0.82**	±0.01	**0.21**	±0.03	**0.15**	±0.01
7B	**0.16**	±0.08	**0.74**	±0.00	**0.19**	±0.04	**0.15**	±0.01
7A	**0.20**	±0.09	**0.78**	±0.00	**0.20**	±0.04	**0.18**	±0.00
7O2	**0.60**	±0.24	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
11B	**0.17**	±0.09	**0.71**	±0.01	**0.20**	±0.02	**0.16**	±0.01
11A	**0.18**	±0.09	**0.73**	±0.00	**0.23**	±0.04	**0.18**	±0.00
14B	**0.17**	±0.09	**0.70**	±0.00	**0.20**	±0.03	**0.17**	±0.01
14A	**0.20**	±0.09	**0.77**	±0.01	**0.25**	±0.08	**0.20**	±0.00
14O2	**0.76**	±0.11	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
18B	**0.18**	±0.09	**0.71**	±0.00	**0.27**	±0.02	**0.20**	±0.01
18A	**0.19**	±0.09	**0.74**	±0.00	**0.22**	±0.03	**0.210**	±0.00
21B	**0.24**	±0.12	**0.83**	±0.01	**0.35**	±0.01	**0.29**	±0.00
21A	**0.28**	±0.10	**0.86**	±0.00	**0.36**	±0.05	**0.31**	±0.00
21O2	**0.78**	±0.19	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
25B	**0.25**	±0.09	**0.80**	±0.01	**0.32**	±0.03	**0.30**	±0.01
25A	**0.27**	±0.09	**0.86**	±0.01	**0.33**	±0.05	**0.33**	±0.01
42B	**0.25**	±0.10	**0.80**	±0.01	**0.34**	±0.02	**0.31**	±0.00
42A	**0.26**	±0.10	**0.80**	±0.01	**0.34**	±0.02	**0.32**	±0.01
42O2	**0.71**	±0.30	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
46B	**0.42**	±0.18	**0.79**	±0.00	**0.39**	±0.04	**0.33**	±0.01
46A	**0.42**	±0.17	**0.80**	±0.00	**0.40**	±0.02	**0.34**	±0.01
54*	**0.34**	±0.15	**0.78**	±0.02	**0.34**	±0.07	**0.33**	±0.02
56B	**0.33**	±0.12	N.A.	N.A.	**0.38**	±0.09	**0.3**	±0.01
56A	**0.33**	±0.12	**0.51**	±0.02	**0.36**	±0.07	**0.3**	±0.01
60	**0.36**	±0.12	**0.51**	±0.01	**0.35**	±0.03	**0.31**	±0.01
60O2	N.A.	N.A.	N.A.	N.A.	**0.84**	±0.08	**0.68**	±0.15
61	**0.33**	±0.11	**0.49**	±0.02	**0.59**	±0.07	**0.72**	±0.00
63B	**0.36**	±0.11	**0.51**	±0.01	**0.38**	±0.10	**0.75**	±0.02
63A	**0.36**	±0.11	N.A.	N.A.	**0.38**	±0.10	**0.77**	±0.01
67B	**0.34**	±0.10	**0.49**	±0.02	**0.31**	±0.04	**0.68**	±0.02
67A	**0.35**	±0.10	**0.51**	±0.01	**0.3**	±0.06	**0.72**	±0.02
67O2	N.A.	N.A.	N.A.	N.A.	**0.79**	±0.10	N.A.	N.A.
70B	**0.39**	±0.11	**0.59**	±0.01	**0.35**	±0.04	**0.75**	±0.03
70A	**0.41**	±0.11	**0.57**	±0.02	**0.33**	±0.07	**0.77**	±0.03
78B	**0.36**	±0.10	N.A.	N.A.	**0.28**	±0.06	**0.69**	±0.02
78A	**0.39**	±0.10	**0.56**	±0.03	**0.37**	±0.02	**0.74**	±0.02
98B	**0.36**	±0.10	**0.53**	±0.02	**0.27**	±0.05	**0.66**	±0.03
98A	**0.38**	±0.09	**0.54**	±0.02	**0.29**	±0.07	**0.7**	±0.03

B: before sampling, A: after sampling, O2: After oxygen addition, N.A. No addition, or no measurement, *;gas exchange

Supplementary data 3: Distribution of oxygen in gas and liquid phase based on Henry's Law

The relation between the concentration in the gas phase and the concentration in the liquid phase can be described by the dimension less Henry coefficient (K_H) by using Equation S5.3.

$$K_H = m = C_g / C_l \quad \text{(S5.3)}$$

K_H = dimensionless Henry constant (-)

m = distribution coefficient (-)

C_g = concentration contamination in the gas phase (mol/m^3)

C_l = concentration contamination in the liquid phase (mol/m^3)

The dimensionless Henry coefficient (K_H) can be derived from the ideal gas law as shown in Equation S5.4.

$$K_H = m = H / R*T \quad \text{(S5.4)}$$

H = Henry coefficient (Pa m^3/mol)

R = gas constant (J/mol/K)

T = temperature (K)

Table S5.7 Mean of the Henry's coefficients calculated for set-up I (day 1) and set-up II (day 60) bottles based on Equation S5.3. and Equation S5.4.

Set-up	C_g	C_l	K_H	H*
I	21.96 ± 4.64	0.78 ± 0.13	28.13 ± 2.57	688.16 ± 62.82
II	21.93 ± 3.20	0.77 ± 0.13	29.79 ± 8.98	728.91 ± 219.82

*For water in equilibrium with the atmosphere, the concentration is governed by Henry's Law, and KH for O_2 is 769.23 L atm mol^{-1} at 298 K.

Supplementary data 4: Oxygen concentrations in set-up III

Table S5.8 Oxygen percentage measured in GC (in blue) and their calculations to mg (in orange) for each bottle of set-up III (SIII).

Set-up III - Oxygen												
day 1	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SIII-1	1.14	100	0.100	0.0821	293	0.00	0.15	151.65	16.97		25.74	
SIII-2	1.26	100	0.100	0.0821	293	0.01	0.17	167.61	17.19		28.81	
SIII-3	1.17	100	0.100	0.0821	293	0.00	0.16	155.64	16.88		26.27	
SIII-C1	1.51	100	0.100	0.0821	293	0.01	0.20	200.87	16.86		33.87	
SIII-C2	1.31	100	0.100	0.0821	293	0.01	0.17	174.27	16.83		29.33	
Active (mean)									*17.01*	*±0.13*	*26.94*	*±1.34*
Control (mean)									*16.85*	*±0.02*	*31.60*	*±2.27*
day 3	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SIII-1	1.14	101	0.101	0.0821	293	0.00	0.15	153.17	16.01		24.52	
SIII-2	1.26	101	0.101	0.0821	293	0.01	0.17	169.29	15.59		26.39	
SIII-3	1.17	101	0.101	0.0821	293	0.00	0.16	157.20	15.30		24.05	
SIII-C1	1.51	101	0.101	0.0821	293	0.01	0.20	202.88	19.10		38.75	
SIII-C2	1.31	101	0.101	0.0821	293	0.01	0.18	176.01	19.07		33.56	
Active (mean)									*15.63*	*±0.29*	*24.99*	*±1.01*
Control (mean)									*19.09*	*±0.02*	*36.16*	*±2.59*
day 8	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SIII-1	1.14	102	0.102	0.0821	293	0.00	0.15	154.68	11.78		18.22	
SIII-2	1.26	102	0.102	0.0821	293	0.01	0.17	170.97	11.27		19.27	
SIII-3	1.17	102	0.102	0.0821	293	0.00	0.16	158.75	10.55		16.75	
SIII-C1	1.51	102	0.102	0.0821	293	0.01	0.20	204.89	19.19		39.32	
SIII-C2	1.31	102	0.102	0.0821	293	0.01	0.18	177.75	19.09		33.93	
Active (mean)									*11.20*	*±0.50*	*18.08*	*±1.03*
Control (mean)									*19.14*	*±0.05*	*36.63*	*±2.69*
day 15	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SIII-1	1.14	103	0.103	0.0821	293	0.00	0.16	156.20	7.30		11.40	
SIII-2	1.26	103	0.103	0.0821	293	0.01	0.17	172.64	7.33		12.65	
SIII-3	1.17	103	0.103	0.0821	293	0.01	0.16	160.31	6.91		11.08	
SIII-C1	1.51	103	0.103	0.0821	293	0.01	0.21	206.90	18.36		37.99	
SIII-C2	1.31	103	0.103	0.0821	293	0.01	0.18	179.49	18.35		32.94	
Active (mean)									*7.18*	*±0.19*	*11.71*	*±0.68*
Control (mean)									*18.36*	*±0.00*	*35.46*	*±2.52*

Table S5.9 Dissolved oxygen (DO) concentrations measured with oxygen sensor for each sampling day. Mean of DO concentrations of active bottles (triplicate) and controls (duplicate) are given with the standard deviations (±) for set-up III.

Set-up III – DO (mg L^{-1})								
Bottle \ Day	0	1B	3B	3A	8B	8A	15B	15A
Active (mean)	**8.43**	**6.36**	**4.32**	**4.42**	**2.85**	**2.92**	**2.10**	**2.18**
	±0.74	±0.29	±0.19	±0.19	±0.04	±0.04	±0.10	±0.08
Control (mean)	**10.90**	**7.51**	**6.18**	**6.26**	**5.33**	**5.44**	**4.91**	**5.27**
	±0.91	±0.42	±0.32	±0.31	±0.32	±0.28	±0.31	±0.27

B: before sampling, A: after sampling

Supplementary data 5: BTEXIeIaN biodegradation under fully aerobic and fully anaerobic conditions

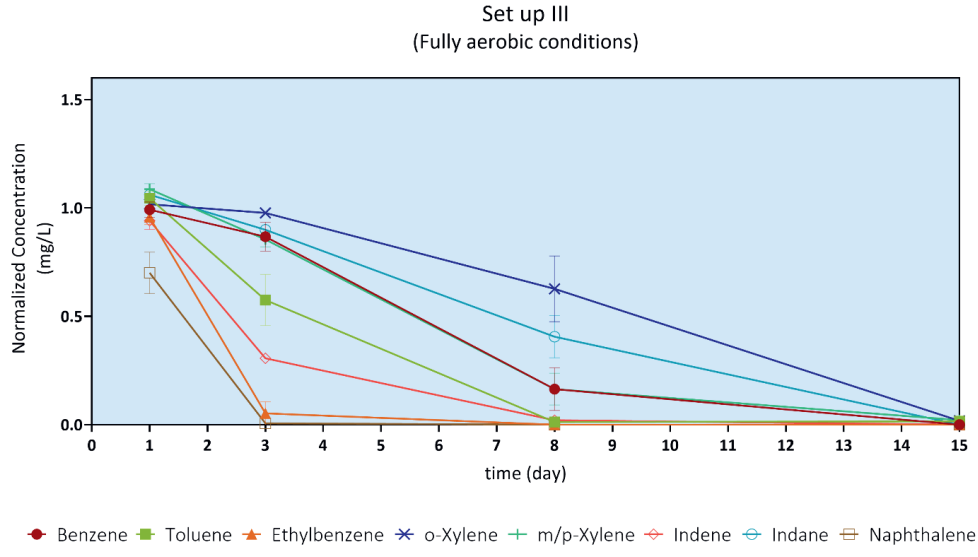

Figure S5.1 BTEXIeIaN concentrations in set-up III, under fully aerobic conditions.

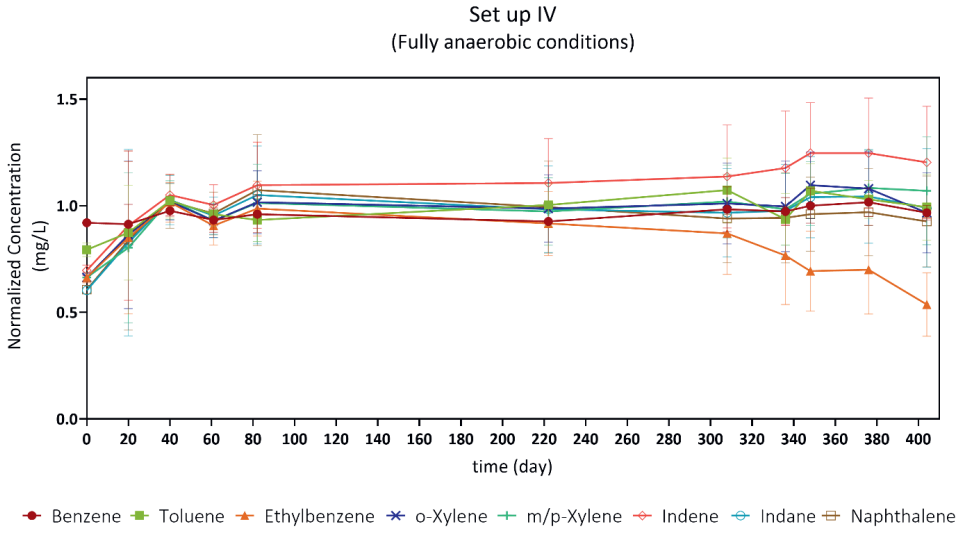

Figure S5.2 BTEXIeIaN concentrations in set-up IV*, under nitrate-reducing conditions without any oxygen addition. *Also represented as Figure 4.1A in Chapter 4.

Supplementary data 6: Stochiometric calculations

Table S5.10 Theoretical calculations for electron acceptor (EA) needed per mg of compound.

	mg compound / mg EA			
	mol		**mg**	
	oxygen	**nitrate**	**oxygen**	**nitrate**
Benzene	7.5	6.0	3.07	4.76
Toluene	9.0	7.2	3.13	4.85
Ethylbenzene	10.5	8.4	3.16	4.91
o-xylene	10.5	8.4	3.17	4.91
m/p-xylene	10.5	8.4	3.17	4.91
Indene	11.0	8.8	3.03	4.70
Indane	11.5	9.2	3.11	4.83
Naphthalene	12.0	9.6	3.00	4.64
Total (BTEXIeIaN)	82.5	66	24.83	38.49

Chapter 6

Impact of redox condition and co-contaminant exposure history on anaerobic microbial communities during Toluene and Benzene biodegradation in laboratory soil columns

A modified version of this chapter has been submitted for publication:

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Abstract

Aromatic compounds are present as highly toxic and persistent contaminants in both aquatic and terrestrial environments, demanding effective remediation of contaminated areas. In this study, laboratory column experiments were performed with aquifer sediments from a contaminated former gasworks site. Toluene and benzene biodegradation as a sole source of carbon was assessed under different redox conditions. Results showed that toluene biodegradation was more efficient under sulfate-reducing conditions (80%) compared to nitrate-reducing conditions (55%) during initial exposure. Subsequently to toluene biodegradation, benzene showed an opposite effect where removal efficiencies were 44.5% and 59.5% under sulfate and nitrate-reducing conditions, respectively. These findings led to the conclusion of compound-specific redox-condition efficiency. The relatively rapid removal of the compounds under anaerobic conditions (within one to two months) was attributed to the alternating flow-batch approach applied to the columns where selective enrichment of specific microorganisms was stimulated by alternating biomass washout at high flow rate and growth at batch mode. Additionally to single compound experiments, different substrate combinations were tested, within different exposure order to investigate the effect of substrate composition and pollution history on the biodegradation of the aromatic hydrocarbons. Toluene biodegradation was not affected by the presence of benzene, but slightly inhibited by preincubation with a complex mixture of various aromatic contaminants (BTEX, indene, indane and naphthalene). On the contrary, benzene removal efficiencies were reduced to 8% by the presence of toluene, but not affected (40-50% removal) by the complex mixture. When the columns were pre-exposed to either toluene or benzene, their biodegradation continued unaffectedly when subsequently fed with a more complex mixture. Meaning that once the microbial community had been adapted to biodegrade toluene or benzene as single compound, co-contaminants appear not to interfere the biodegrading community. Beta-diversity analysis revealed distinct microbial community clustering between sulfate and nitrate-reducing columns, emphasizing the greater influence of redox conditions on microbial community composition compared to substrate composition or pollution history. Despite certain columns receiving the same substrate mixture at specific times, differences among the microbial consortia were observed in each column, implying the influence of the substrate exposure order (pollution history) on microbial community dynamics. It is essential to consider all the findings derived from this study when developing effective strategies for the bioremediation of groundwater contaminated with complex mixtures containing various aromatic hydrocarbons.

6.1. Introduction

Manufactured gas plants (MGP) were the main energy source for lighting, heating, and cooking between 1820 and 1950 (Thavamani et al., 2011). Waste generated during the manufacturing process ended up contaminating soils, groundwater and sediments (Murphy et al., 2005). Such wastes comprise mixtures of organic substances such as monoaromatic hydrocarbons like benzene, toluene, ethylbenzene, and xylenes (BTEX) and polycyclic aromatic hydrocarbons (PAH) (Thavamani et al., 2011). Hydrocarbon pollutants are one of the most persistent organic substances with toxic and cancerogenic properties (Varjani, 2017). Due to their relatively high water solubility and mobility, these pollutants can travel far distances, migrate into groundwater, and consequently contaminate drinking water supplies (Dou et al., 2008). Due to fast urbanization, many former MGP sites are now located in city centers and posing a risk to human health and the environment (Birak & Miller, 2009). Therefore, there is an urgent need for sustainable clean-up solutions to remove historical pollutants from contaminated sites.

Bioremediation is an economical, energy efficient and environmentally friendly approach for the removal of BTEX from soil and groundwater (Dou, Liu, & Hu, 2008b). The primary mechanism in bioremediation is biodegradation, in which microorganisms use diverse metabolic capabilities for the detoxification process of contaminants (Das & Chandran, 2011). Biodegradation of BTEX compounds strongly depends on the terminal electron acceptor processes (Schreiber et al., 2004). The best electron acceptor for degradation process is oxygen (O_2), followed by nitrate (NO_3^-), manganese (Mn_4^+), ferric iron (Fe^{3+}), sulfate (SO_4^{2-}) and carbon dioxide (CO_2) (Hatipoğlu-Bağci & Motz, 2019). Even though BTEX compounds can be degraded under both aerobic and anaerobic conditions, anerobic biodegradation is much slower (Varjani, 2017). This needs attention as BTEX-contamination is often found in subsurface where conditions are anaerobic due to the rapid consumption of oxygen (Wu et al., 2022).

For the selection of efficient remediation strategies, site characteristics such as available electron acceptors, indigenous microbial community and their biodegradation capacity together with pollution history needs to be investigated in depth as all the above would have an effect on the biodegradation of the current pollutants (Phelps & Young, 1999). Natural systems are complex and testing the diverse factors influencing biodegradation processes on field scale might be challenging due to environmental conditions not being optimal and hence extending the duration of the experiments. Fortunately, the fate of contaminants can be studied in laboratory within column experiments as an ex situ representation of a small section of the natural system and allow for the convenient testing and control of various parameters. Numerous studies on BTEX biodegradation have been conducted in column experiments (Anid et al., 1993; Da Silva & Alvarez, 2004; S.-R. Hutchins et al., 1992; Langenhoff et al., 1996; Meckenstock et al., 2015; Patterson et al., 1993; Tan et al., 2006; Zhao et al., 2015), most of them focusing on one parameter, often on the use of different electron acceptors. However, investigating multiple parameters within the same experiment will lead to high quality insight on the biodegradation performance of the indigenous microorganisms. Implementing a diverse range of specific experimental conditions that take into account the site-characteristics can help to design effective remediation strategies and enhance the efficiency in terms of time and cost.

In this study, column experiments were performed to obtain insights into the underlying mechanisms of stimulating biodegradation of subsurface aromatic contaminants, often found in groundwater systems in contact with former gasworks sites. In this research, the Griftpark site, located in the center of Utrecht (The Netherlands) was used as example case study. The site exhibited the presence of various aromatic

contaminants, namely BTEX, indene (Ie), indane (Ia), and naphthalene (N). Lab-scale-soil-column studies were conducted to simulate Griftpark-like in situ conditions. The columns were operated with an alternating flow regime thus aiming to select for specific microorganisms. During the flow mode, slow growing and non-adhering microorganism were washed out, while at batch mode, the compound-degrading biomass was allowed to degrade the contaminants, grow and retain in the column. First, biodegradation of toluene and benzene were investigated as single compound, within a duplex mix (T+B) and complex mixture (BTEXIeIaN) under nitrate and sulfate-reducing conditions. Toluene was selected as, under anaerobic conditions, it is a relatively easy degrading compound while benzene biodegradation can be rather challenging (Weelink et al., 2010). Each column was fed with distinct substrate composition and exposure orders, which allowed to study the effect of pollution history on the developed biodegradation capacity of the column biomass. The influence of environmental variables in the columns were further investigated with microbial community analysis. Beta-diversity analysis was employed to assess differences in microbial communities based on various parameters such as redox conditions, substrate composition, and pollution history. Additionally, abundant microbial groups were identified in each column. This study aimed to gain insight on the biodegradation capabilities of indigenous microorganisms across multiple parameters and use this knowledge to contribute to the development of more efficient designs for in situ treatment of complex dissolved aromatic mixtures found in the subsurface, for instance, at former gasworks sites.

6.2. Materials and Methods

6.2.1. Sediment samples and medium

Sediment samples used in this study were collected from Griftpark, a former gasworks site, located in Utrecht (The Netherlands). Samples were taken from 29.5-30 m below ground level where sulfate-reducing conditions were found to be prevalent. Sediment samples were extracted using a coring device and stored in closed soil liners to prevent any oxygen leakage. Samples were stored in dark, at 4°C until use.

Basal mineral medium was used as a matrix for the experiments. The composition of the medium was given as supporting information (Supplementary data 1, Table S6.1). To create nitrate-reducing conditions in certain columns, 45-50 mg L^{-1} $NaNO_3$ was added to the medium while sulfate was not added as it was naturally present in the sediment samples (20-25 mg L^{-1}).

6.2.2. Column set up and operation

Four up-flow columns filled with subsurface sediment and medium were used to simulate the subsurface of Griftpark (Figure 6.1). The glass columns were 6 cm internal diameter and 32 cm long, equipped with four sampling ports (SP1, SP2, SP3, SP4) which were positioned at 5, 10, 15 and 25 cm from the inlet, respectively. All columns were filled with the Griftpark sediment under continuous flushing of N_2 to prevent oxygen leak. Sediment and anaerobic medium were added simultaneously to ensure homogenous distribution and prevent preferential pathways. Once filled, columns were placed in a cabinet, at room temperature (18-22°C) covered with light-blocking Plexiglas to prevent algae growth and photodegradation.

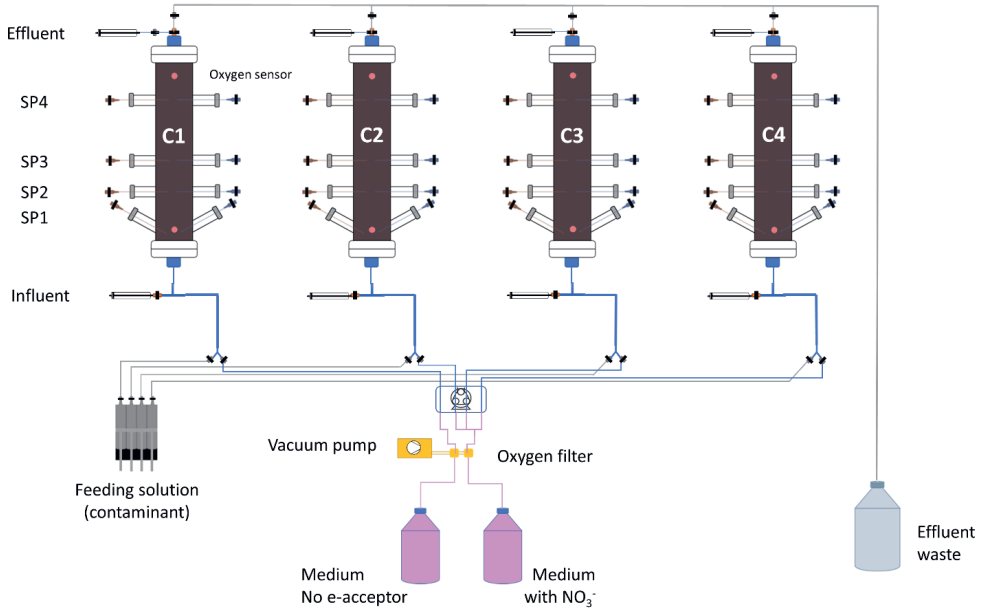


Figure 6.1 Scheme of the column set up. Soil samples were sampled from the left side, and liquid samples from the right side of the columns. **SP:** Sampling port

Columns were operated in medium saturated up-flow mode. Column 1 (C1) received medium (pH 7) without additional electron acceptor representing the sulfate-reducing conditions as in Griftpark while the other columns (C2, C3 and C4) were fed with medium supplemented with nitrate. The medium was refreshed weekly and was pumped to the column by a peristaltic pump with a flow rate of ~10 mL h^{-1}. Before entering to the columns, the medium had to pass through a membrane contactor (3M™ Liqui-Cel™ MM Series Membrane Contactor) connected to a vacuum pump to de-gas the liquid and obtain an oxygen free medium. Oxygen levels were regularly monitored using non-invasive oxygen sensors (Spot SP-PSt3, PreSens) attached at the bottom and top of the columns. In order to contaminate the columns, a contaminant feeding solution was prepared by filling 100 mL sterile Milli-Q water to sterile batch bottles and then made anaerobic by a gas exchange procedure, and pressurized to 1.5 atm. Next, the contaminant of interest was injected to the capped batch bottle via a 10 µL glass syringe as previously described by Aydin et al. (2023) and allowed to mix in a shaker overnight (120 rpm, 20°C), in dark. Once the contaminants were dissolved, the feeding solution was transferred to gas-tight glass syringes and injected to columns via a syringe pump with a flow rate of 1 mL h^{-1}. The total flow rate in the columns were around 11 mL h^{-1} (Supplementary data 2, Table S6.2). To monitor the biodegradation process throughout the column, liquid samples were collected from the right side of the column via a syringe and 0.5 mm x 16 mm needles. One mL liquid sample was taken from each sampling point with the order of effluent, SP4, SP3, SP2, SP1 and the influent. This procedure was done once a week.

6.2.3. Analytical measurements

To monitor biodegradation, quantification of the contaminants was done to liquid samples with HPLC-FLD equipped with a Phenyl-1 (Thermo) HPLC column as previously described (Aydin et al., 2021). Briefly, 1 mL liquid sample was taken from each sampling point and centrifuged at 15000 rpm for 10 min. An amount of 750 µL supernatant was then transferred to HPLC-glass vials and 250 µL methanol was added in order to keep the volatile compounds stable during autosampler period. Samples were analyzed on the same day of sampling (<12 h).

Nitrate and sulfate concentrations were quantified by an ion chromatography (IC) (Dionex ICS-2100, Thermo, USA) equipped with an AS17-Column. The left-over supernatant from the HPLC measurements were used for the IC analysis. All samples were diluted with Milli-Q water before measurements (70 µL supernatant, 630 µL Milli-Q water) in order to be within the limit of detection range of the equipment. Chromeleon software (Thermo Fischer Scientific, USA) was used for analysis of the data from both liquid and gas chromatography.

6.2.4. DNA extraction and sequencing

All columns were sampled at different time periods to investigate the microbial communities. An amount of 5 mL sediment-medium mix was collected via a syringe from SP1 and SP4 (left side of the column) in triplicate. For this, a thicker needle (1.2 x 40 mm) was used. Collected samples were then transferred to an Eppendorf tube and were centrifuged for 10 min at 15000 rpm to allow cell precipitation. After centrifugation, the supernatant was discarded, and the pellet was stored at -80°C until further use for DNA extraction. Microbial DNA was extracted from each sediment sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). After processing the samples in a bead beater to lyse the cells, DNA was extracted according to the manufacturer's instructions. DNA concentration in each sample was quantified using Qubit®(Life Technologies, Carlsbad, CA, USA). In case of sufficient DNA concentration (> 0.2 ng μL^{-1}), the samples were purified with DNA Clean & Concentrator-5 Kit (Zymo). The isolated and purified DNA was used as template for amplifying the V3 and V4 region of 16S rRNA via Illumina sequencing using the primer sets described by Takahashi et al. (2014).

6.2.5. Processing and analysis from sequencing data

For the processing and analysis from the sequencing data, the default pipeline from USEARCH was used (https://www.drive5.com/usearch/manual/uparse_pipeline.html). First, the quality assessment of the raw sequencing data was performed using fastQC and reported using multiQC. Paired-end reads were merged using USEARCH -fastq_mergepairs with an expected product size of 464 base pairs (Edgar, 2010). Forward and reverse primers were stripped, and the merged reads were quality filtered to retain high-quality reads (Edgar & Flyvbjerg, 2015). Denoising was accomplished through ASV (amplicon sequence variant) prediction and chimeric sequence filtering (Edgar, 2016b). The resulting denoised ASVs were used to create an operational ASV table by mapping them to the original sample sequences. Taxonomic classification was performed using the SINTAX algorithm in USEARCH with the Silva database V138 (Edgar, 2016a). ASV classifications with a confidence level below 80% were excluded from specific taxonomy ranks. Raw sequences with barcode and primer removed and supporting metadata were deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number PRJEB66348.

6.2.6. Biodegradation experiments

To investigate different parameters affecting biodegradation of benzene and toluene, soil columns were operated for 36 weeks. The experiment was divided to three phases (Phase 1, 2, and 3) based on the contaminant injected to the columns (Figure 6.2). Each phase was 12 weeks long and were operated in two modes. Flow mode and batch mode (shown in Figure 6.2 as grey and darker gray) were run for 3 weeks, repeated two times, sequentially.

In flow mode, the columns were flushed with medium and fed with the contaminants continuously with a total flow rate of 11 mL h^{-1}, resulting in an up flow velocity of 23.41 cm d^{-1} (Supplementary data 2, Table S6.2) with an HRT of approximately 1.36 day. This flow served two purposes: firstly, to create a selection pressure in the columns by flushing the non-adhered microorganisms out of the system if their growth rate upon the substrate was below the flow rate. Secondly, it allowed to flush out inhibitory by-products such as nitrite or toxic intermediates. The modelled groundwater speed at Griftpark is estimated to be 0.1 cm d^{-1}. When applied to the soil column, it would have resulted in an HRT of 320 days. Therefore, to enhance the washout of non-specific BTEXIeIaN degrading non-adhering microorganisms from the soil columns, the flow rate was set about 235 times higher as the natural conditions. After 3 weeks of flow mode, the medium and feeding solution were no longer pumped to the columns transitioning to a batch mode for the following 3 weeks. The HRT in flow mode was measured as 45 h (1.8 day) with fluorescein as a tracer (Supplementary data 2, Figure S6.1). When considering the duration of the subsequent batch mode of 3 weeks, it can be viewed as a virtual HRT of 21 days (504 h). Therefore, the duration of the batch mode can be seen as comparable to about 14 times higher flowrate compared to field site of Griftpark.

The contaminants and electron acceptors used in each phase of the experiment was given in Figure 6.2. Column C1 did not receive any additional electron acceptors, and natural attenuation under sulfate-reducing conditions were investigated since sulfate was already present in the sediment. Nitrate-reducing conditions were investigated for columns C2, C3 and C4, hence sulfate was also present in the columns as second electron acceptor. Under both redox conditions, toluene and benzene biodegradation were tested as single compound, in duplex mixture (T+B) and within complex mixture (BTEXIeIaN). Differently to C3 and C4, C1 and C2 had a 4 week of preparation time (Phase 0), where the experimental conditions and column set-up were tested with toluene prior to the actual experiment (36 weeks).

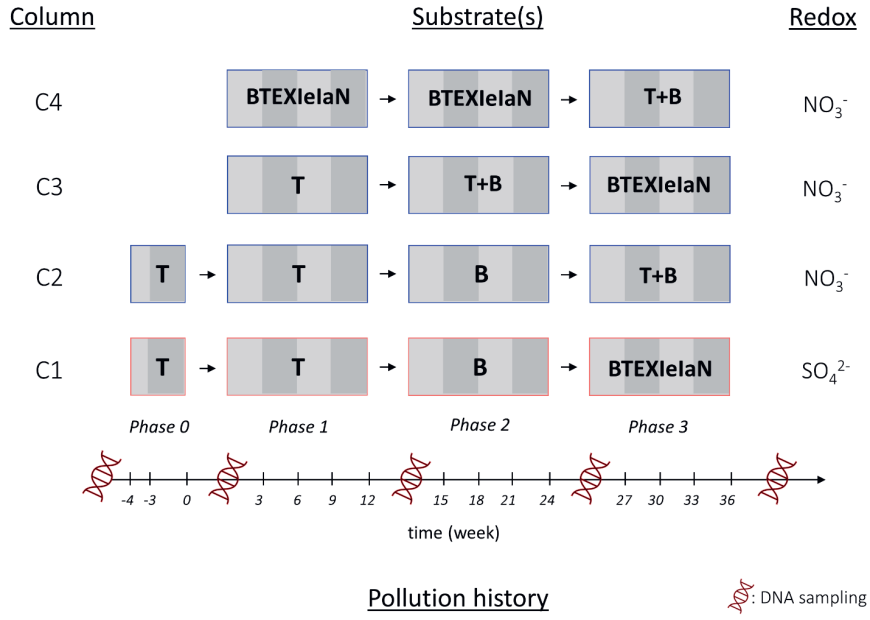


Figure 6.2 Experimental scheme of this study. Flow mode is represented in light gray and batch mode in darker gray. Phase 0 indicates preparation time and was only done in C1 and C2 with one week of flow mode, and 3 weeks of batch mode. All the columns were run with flow mode followed by a batch mode of 3 weeks, repeated two times sequentially (12 weeks in total for each phase). Compounds fed to the columns during each phase was indicated as **T:** toluene, **B:** benzene, **B + T:** duplex mix and **BTEXIelaN:** complex mixture. Factors investigated throughout the experiment (36 weeks) were shown as the substrate(s), redox and changes in time. DNA samples were taken between each phase, represented with red DNA symbols in the timeline.

6.2.7. Data interpretation

Samples were taken from the columns on a weekly basis during both flow and batch mode. The interpretation of the collected data was summarized and presented in Supplementary data 3, Figure S6.2. Briefly, during flow mode, the concentrations of the contaminants were analyzed and compared throughout the column, at various sampling points. To evaluate the efficiency of contaminant removal three calculation options were employed as (i) influent to effluent, (ii) SP1 to effluent and (iii) SP1 to SP4 (Supplementary data 3, Table S6.3-6).

As there was no flow present during the batch mode, the concentrations of the compound(s) from each sampling port was monitored over the three weeks and the removal efficiencies were calculated by comparing each week to the following week. Next, the average of the removal efficiencies over the three weeks were calculated for each port. Finally, as a representation of what was happening throughout the column, the average of the removal efficiencies of the four ports were calculated. A visual summary of the data interpretation process was given in Supplementary data 3, Figure S6.2.

6.3. Results and Discussion

Biodegradation couldn't be observed during the flow modes (Supplementary data 3, Table S6.3-6) despite the different calculation options used to evaluate removal efficiencies mentioned in section 6.2.7. The reason why biodegradation couldn't be observed during flow mode could be attributed to (i) high inflow rate of the substrate, which most likely exceeded the biodegradation rate or (ii) the rate of biomass growth being slower than the rate at which biomass is being washed out from the columns. This showed that the flow rate used in the experiment, which was approximately 235 times higher than the vertical groundwater velocity in Griftpark, could not be balanced with the selection and growth of biomass within the duration of the experiment. Therefore, for the biodegradation related data presented in the following sections, only the batch mode data was discussed.

6.3.1. Redox conditions: Toluene and benzene biodegradation under sulfate and nitrate-reducing conditions during batch mode

In order to understand the effect of the electron acceptor on aromatic hydrocarbon degradation, the easiest degradable aromatic hydrocarbon (toluene) and the most persistent one (benzene) were assessed individually under two different redox conditions (SO_4^{2-} and NO_3^-). For this, sulfate-reducing column C1 and nitrate-reducing column C2 were investigated. The batch modes during P0, P1 and P2 of C1 and C2 were compared to each other as these columns received the same substrate composition with different redox conditions (Figure 6.3).

Toluene biodegradation: Nearly complete biodegradation of toluene was recorded under both redox conditions within 16 weeks (Figure 6.3, P1-BM2). In greater detail, as outlined in Supplementary data 4, it was observed that toluene removal gradually increased with time from BM0 to the end of BM2 (Supplementary data 4, Figure S6.3). During preparation phase (P0) toluene was partially degraded even though it was introduced to the columns for the first time. Under sulfate-reducing conditions, 80% removal of toluene was observed and under nitrate-reducing conditions 55% toluene was biodegraded (Figure 6.3, BM0). Therefore, toluene degradation was more efficient under sulfate-reducing condition in this part of the experiment. From an energy gain perspective, nitrate-reducing conditions should theoretically be more favorable than sulfate-reducing conditions for toluene biodegradation. One explanation could be that the sediment used in the experiments originated from a sulfate rich subsurface environment without nitrate. Thus, in the first phase, a relative abundance of adapted sulphate-reducing microorganisms over nitrate-reducing bacteria would be expected. Further, in P1, removal efficiencies of toluene under sulfate and nitrate-reducing conditions were found to be close (Figure 6.3, BM1 and BM2) indicating that a microbiome exposed to sulfate-reducing conditions for decades can adapt to nitrate-reducing conditions within 70 days (end of BM1). This demonstrates the subsurface microbiome's high adaptive capacity to changing anaerobic redox conditions, especially when comparing this time to the natural HRT of 320 days.

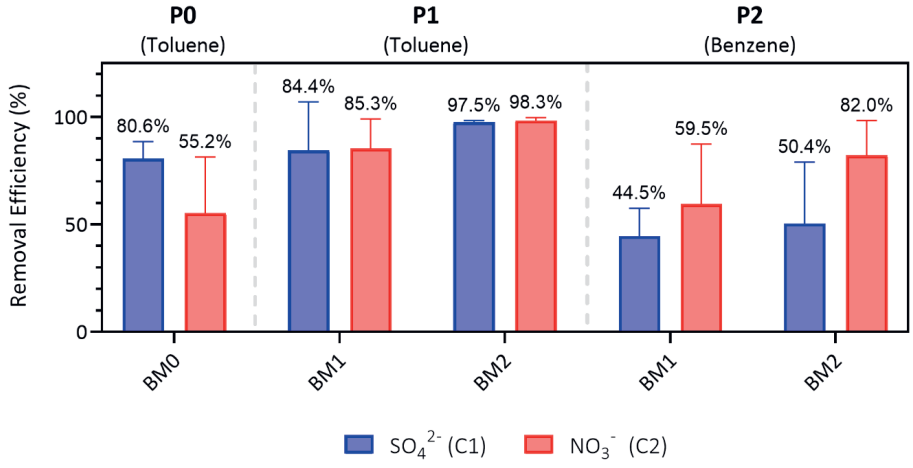


Figure 6.3 Comparison of toluene and benzene biodegradation (individually) under sulfate-reducing conditions (C1, in blue) and nitrate-reducing conditions (C2, in red) in columns operated in batch mode. **P:** Phase (0,1,2), **BM:** Batch mode, **BM0:** Preparation phase (batch mode).

Benzene biodegradation: After complete removal of toluene, both columns were spiked with benzene as a single substrate. Unlike toluene, benzene was not fully biodegraded (Figure 6.3, P2). In BM1, 44.5% benzene conversion was observed under sulfate-reducing conditions and 59.5% at nitrate-reducing conditions. Although complete removal was not achieved, a relatively brief adaptation period of 42 days was observed for benzene degradation to start. Another column study showed that toluene was readily degraded within 1–2 months under various redox conditions (nitrate, iron, manganese, sulfate, methanogenic) while benzene was recalcitrant up to 525 days (Langenhoff et al., 1996). A shorter adaptation time observed for benzene, in this study, could be explained by the applied selection procedure by high flow rate in flow mode. Toluene is relatively easy to degrade anaerobically (Weelink et al., 2010). This is attributed to the broader phylogenetic variety of organisms with the capacity for toluene degradation (Boll et al., 2020) and that toluene degraders are more likely to have genes/operons capable of synthesizing the benzyl succinate synthase enzyme (Bss) (Wartell et al., 2021). Benzene biodegradation is often reported to be slow, incomplete, and subject to long lag times (Foght, 2008). This is due to the stable chemical structure of benzene and the absence of any methyl or other side groups, which would have facilitated enzymatic attacks (Weelink et al., 2010). In the current study, benzene was not recalcitrant but, as suggested by others in the literature, its degradation was much slower compared to toluene. This could also be due to both columns being exposed to toluene for 16 weeks before the introduction of benzene (Figure 6.3, P0 and P1). It is highly likely that the columns already harbored a substantial population of toluene degraders when benzene was introduced. More time may be required to allow the biomass of benzene degraders to increase sufficiently for complete benzene removal. In any case, the results are promising as in both columns, the microbial consortium was capable of degrading toluene and subsequently benzene (P0 to P2). Several microorganisms have been isolated and identified to be capable of degrading the individual BTEX compounds; however, these microorganisms typically show limited versatility in their ability to utilize more than one aromatic hydrocarbon as a substrate (Nagarajan & Loh,

2015). Hence, the biodegradation of both toluene and benzene as found in the present study can most likely be attributed to the presence of more than one microorganism within the microbial community, delivering a wider variety of metabolic enzymes needed for BTEX biodegradation.

For benzene biodegradation, nitrate-reducing conditions were found to be more suitable than sulfate-reducing conditions when introduced to the column for the first time (Figure 6.3, P2) which contrasts with what was observed for toluene (Figure 6.3, P0). This phenomenon was also reported by Keller et al., where benzene degradation coupled to nitrate reduction was more favorable energetically, than sulfate reduction alone (Keller et al., 2018). IC results showed that in C2, where nitrate and sulfate are present simultaneously, microorganisms consumed nitrate as an electron acceptor prior to sulfate during the batch mode (Supplementary data 5, Figure S6.7). This was recorded during both toluene and benzene degradation. Even though nitrate was consumed prior to sulfate in C2, toluene biodegradation was higher under sulfate-reducing conditions in C1. Therefore, sulfate-reducing conditions appear to be more favorable for toluene biodegradation while nitrate-reducing conditions was likely more efficient for benzene removal. This showed that, under distinct redox conditions, toluene and benzene exhibited diverse degradation performances and that electron acceptors with higher redox potentials (NO_3^- > Fe^{3+}> SO_4^{2-} > HCO_3^-) may not always result in higher contaminant removal (Daghio et al., 2017; Lueders, 2017). Here, biodegradation was affected by the redox condition present, but was also dependent on the chemical properties of the contaminant. Additionally, prior exposure to one of the contaminants (toluene) may have influenced the biodegradation of the other compound (benzene). Findings of this experiment highlighted the challenges in predicting the biodegradation of compounds, revealing that environmental fluctuations exert compound-specific impacts on biodegradation efficiency.

6.3.2. Toluene and benzene biodegradation in presence of different substrate mixtures

In the presence of multiple substrates, the substrate interaction can enhance, have no effect or inhibit the degradation of the compounds (Littlejohns & Daugulis, 2008). In this study, toluene and benzene were investigated as single compound (T or B), in a duplex mix of substrates (T+B) and in a complex mixture (BTEXIeIaN), under different redox conditions (SO_4^{2-} or NO_3^--reducing conditions). All phases (P0, P1,P2 and P3) of each column were investigated and their removal efficiencies during their batch modes (BM) were summarized (Figure 6.4). The aim was to unravel and understand the multiple substrate effect on biodegradation of toluene and benzene with focus on the higher conversion rates.

Column 1 ($SO4^{2-}$, T ➔ B ➔ BTEXIeIaN): For the sulfate-reducing column (C1), toluene biodegradation was slightly affected by the presence of the BTEXIeIaN mixture. As shown in Figure 6.4-C1, toluene was fully degraded in P1-BM2 (toluene alone) and P3-BM2 (complex mixture). However, in P3, toluene removal was 71% in BM1, being lower compared to other BMs in P1 and P3. This is probably due to the addition of toluene after 12 weeks of benzene spiking (P2), which affected the subsequent toluene biodegradation (P3) as the microbial community was adapting to benzene as substrate for the degradation (P2). Alternatively, the presence of other aromatic hydrocarbons could have negatively affected the initial toluene degradation. For benzene, during P2 (benzene alone) and P3 (complex mixture), similar removal efficiencies were observed. The fact that benzene biodegradation was not inhibited by the presence of other compounds (P3) can be explained by the 12 weeks of benzene exposure during P2 prior to P3. In conclusion, in the sulfate-reducing C1 column, no major substrate inhibition effects were recorded for both toluene and benzene when exposed to a complex mixture.

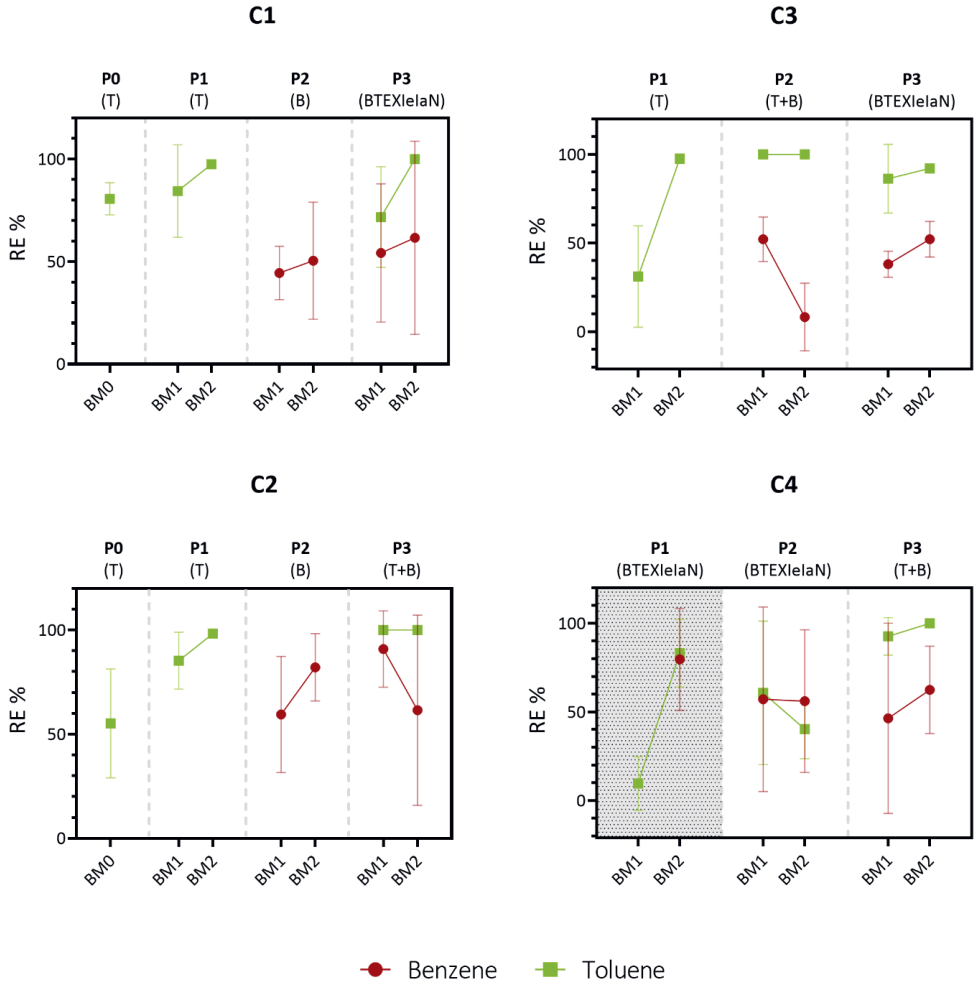


Figure 6.4 Removal efficiencies of toluene and benzene during batch modes of all phases, for all columns. C4-P1 was discarded due to some operational issues leading to inconsistent concentrations during injection procedure. **RE%**: Removal efficiency in percentage. **C1:** Sulfate-reducing column, **C2-4:** Nitrate-reducing columns.

Column 2 (NO_3^-, T ➔ B ➔ T+B): Under nitrate-reducing conditions (C2), toluene biodegradation was not affected by the presence of benzene, as at the end of P1 (toluene alone) and P3 (duplex mixture) similar removal efficiencies were recorded (Figure 6.4-C2). This finding was in contrast with the study of Dou, Liu, & Hu (2008) which reported that the presence of benzene inhibited the degradation of toluene under nitrate-reducing conditions. The variation in outcomes in this study can be directly attributed to the composition of the microbial community, and the interactions among the microbial groups.

For benzene, its biodegradation was affected by the presence of toluene, as lower removal efficiencies were recorded in P3-BM2 (duplex mixture) than in P2-BM2 (benzene alone). A study reported that the simultaneous presence of benzene and toluene had a slightly mutual inhibitory effect on biodegradation under aerobic conditions (Chang et al., 2001). In the study of Alvarez & Vogel, (1991), no difference was observed in benzene degradation whether benzene was present alone or in combination with toluene in aquifer materials. For a pure culture of *Pseudomonas sp.*, it was found that traces of toluene enhanced benzene removal while with pure cultures of *Arthrobacter sp*. toluene was not degraded in the absence of benzene. In this study, a mixed culture was investigated which makes it even more challenging to predict the biodegradation performances of the compounds. Based on our findings, as well as the variations in the existing literature, making broad generalizations about the interactions between benzene and toluene is not feasible. This limitation is primarily due to the fact that the degradation of these compounds relies heavily on the involvement of specific microorganisms.

Column 3 (NO_3^-, T ➔ T+B ➔ BTEXIeIaN): The biodegradation of toluene and benzene was also investigated in C3, under nitrate-reducing conditions but with different substrate mixture combinations than in C2. In P1, when toluene was present alone, 31% removal was observed in the first batch mode (BM1) and increased to 100% removal in the second batch mode (BM2) (Figure 6.4-C3). Since toluene was added to the column for the first time (no preparation phase), low removal efficiency during BM1 was probably related to low biomass of toluene degraders. In P2, when toluene and benzene were present as a duplex mix, toluene continued to be fully degraded (Figure 6.4-C3). This showed that toluene degradation was not affected by the presence of benzene similar to the result obtained in C2-P3. In P3, toluene degradation was slightly inhibited by the presence of other compounds, as complete removal of toluene did not occur. Compared to the other phases, P3 was the only phase where complete degradation of toluene was not observed however if the experimental time would prolong, most probably toluene would be fully removed after adaption to a mixture as observed in C1-P3. It is important to mention that toluene concentrations were 10 times lower (0.09 mg L^{-1}) in P3, than P1 and P2. This choice was made in order to have the same overall hydrocarbon concentration throughout the experiment. At low concentrations, biodegradation can be inhibited due to the scarcity of carbon and energy source for bacterial growth (Dou, Liu, Hu, et al., 2008). The concentrations at P3 were probably not as low as to inhibit the biodegradation process since in C1-P3, also low concentrations were present, and complete degradation of toluene could still be obtained. Inhibition of toluene degradation in C3-P3 was most probably due to the simultaneous presence of the additional hydrocarbons (BEXIeIaN) .

For benzene removal, P2 and P3 were investigated, as benzene was never spiked as single compound in C3. When spiked as a duplex mixture (T+B) during P2, 52% of benzene was removed in the first batch mode. As the column was exposed to benzene for the first time, it was not surprising that benzene was not fully removed. Interestingly, in the second batch mode (BM2), removal of benzene lowered to 8%. The lack of degradation of benzene can possibly be explained by the preferential utilization of other compounds, as found by others (Phelps & Young, 1999). It is possible that microorganism consumed toluene, prior to benzene, due to toluene's relatively easy degradability. This finding was in line with results of C2-P3, where benzene was partially degraded, and toluene fully removed.

Dou, Liu, & Hu (2008) reported that addition of toluene enhanced benzene degradation under nitrate-reducing conditions. Contrary to their study, Da Silva & Alvarez (2004) reported that benzene removal was inhibited by the presence of toluene under methanogenic conditions. In our study, it seems that the presence of toluene had a negative effect on the biodegradation of benzene under nitrate-reducing conditions. Especially because the microbial community was exposed to toluene for 12 weeks prior to benzene addition. In contrast, during P3, benzene removal was higher than in P2, when present within a complex mixture (BTEXIeIaN). The removal efficiencies of benzene were 38% (BM1) and 52% (BM2) in the P3. Some substrates could enhance the degradation of others through the induction of enzymes or could act as a primary substrate stimulating microbial growth, thereby favoring the co-metabolism of another (but similar) compound (Alvarez & Vogel, 1991). In this case, the presence of other components may have enhanced benzene degradation. On one hand, Dou, Liu, & Hu (2008) reported that low concentration of m-xylene enhanced benzene degradation. On the other hand, Chang et al. (2001) reported that ethylbenzene is the most potent inhibitor of BTEX degradation, and p-xylene hindered benzene degradation. Based on the results of this study, benzene was not fully degraded in the presence of the complex mixture, however removal efficiencies were higher than in duplex mixture (T+B). It is possible that longer exposure to benzene (P2 and P3) lead to a better adaptation to benzene bioconversion, than during a phase with toluene as single substrate.

Column 4 (NO_3^-, BTEXIeIaN ➔ BTEXIeIaN ➔ T+B): In C4, a contrasting strategy was employed compared to the other columns. Instead of injecting a simple-to-complex mixture, the approach was reversed, involving the injection of a complex-to-simple mixture. However, it is important to mention that during P1, mixing issues were encountered in the inlet of the column, as some of the compounds couldn't be detected in the inlet due concentrations below detection limits of HPLC (Aydin et al., 2021). This issue was later on fixed in P2. Therefore, P1 was excluded from the discussion and only P2 and P3 were compared (Figure 6.4-C4).

When toluene was introduced to the column within a complex mixture, the toluene removal efficiencies were found to be low (<50%) compared to columns which have been exposed to more simple substrate combinations prior to mixture addition (C1-P3 and C3-P3). Therefore, it is clear that an adaptation period as a single compound helps for the removal of the toluene when further exposed to a more complex mixture. In P3, C4 was spiked with the duplex mixture and higher removal was recorded for toluene compared to toluene present in complex mixture. This confirmed that the simultaneous presence of the other hydrocarbons (BEXIeIaN) had a negative effect on toluene degradation and inhibited its degradation by 50%. For benzene, when present in a complex mixture (C4-P2), the removal efficiencies were low however they were not that different than what was reported in C1-P3 and C3-P3. Furthermore, when C4-P3 was compared to C2-P3, the removal efficiency was higher in C4-P3, showing that longer exposure time (24 weeks) to benzene (C4-P1 and P2) have helped to remove benzene when present in duplex mixture (C4-P3). While in C2, benzene exposure was only for 12 weeks (C2-P2) and therefore, lower removal efficiencies were observed. From these findings, it was concluded that, exposure time is an important factor influencing the biodegradation performance of the microorganisms additional to redox conditions, and multi-substrate effect. Additionally, this experiment showed that simplifying the mixture from complex to simpler components led to an improvement in the biodegradation of toluene and benzene. This phenomenon of lowering number of compounds in a mixture can occur for instance at the fringe area of a contaminated site. The physical and chemical properties of aromatic compounds (e.g., solubility, vapor pressure, K_{ow}, K_{oc}) are important characteristics which can affect their mobility hence their distribution in

the subsurface (Odermatt, 1994). Due to the distinct transportation rates of the aromatic hydrocarbons, a more pronounced separation of compounds will occur in the plume fringe area, leading to less complex mixtures.

In this study, additional to substrate interactions, it was demonstrated that the 'pollution history', defined here as the addition order of compounds to the columns and their exposure time, influenced the individual biodegradation of these compounds. For instance, when C3-P3 and C4-P2 were compared, where both columns received BTEXIeIaN mixture but with different contamination history, it was observed that toluene removal efficiencies were much lower in C4-P2, because this column was never exposed to toluene as single compound prior to BTEXIeIaN exposure. Therefore, an adaptation phase with toluene alone helped for its removal when it was further present within a complex mixture. Another example can be given for C2-P3, C3-P2 and C4-P3 when the mentioned columns received dual mixtures (T+B) with different pollution history. Toluene biodegradation was not different among the investigated period, as toluene was not affected by the presence of benzene. For benzene, a variation in removal efficiencies were observed: in C2-P3 and C4-P3 (BM2), ~60% benzene biodegradation was reported while in C3-P2 (BM2), this was ~10%. Apparently, benzene was more sensitive to the presence or absence of other aromatic substrates than toluene in this column. Because the C3 column was never fed with benzene alone, removal efficiencies were found to be low when exposed together with toluene for the first time. While in C2, higher removal of benzene was recorded as a 12 week period of benzene as sole source of carbon may have helped to develop benzene degrading biomass before exposed to the duplex mixture (T+B) later. Finally, in C4, even though benzene was never present as single compound as in C3, a 36 week period of benzene feeding to the column may have helped microorganisms with adaptation to benzene therefore compared to C3-P2, which may have resulted in higher removal efficiencies in C4-P2.

In conclusion, findings obtained from all the columns showed that, (i) redox conditions played a crucial role in the biodegradation efficiency of toluene and benzene, with sulfate-reducing conditions favoring toluene and nitrate-reducing conditions favoring benzene biodegradation. Benzene, often reported as recalcitrant under anaerobic conditions, was partially degraded within 42 days, and its relatively rapid biodegradation within both redox conditions was attributed to high flow rates. (ii) Substrate interactions have distinct effects on the biodegradation of individual compounds. Toluene biodegradation was not affected by the presence of benzene but was slightly influenced by other hydrocarbons, while benzene's biodegradation was affected by toluene but not by other hydrocarbons. Moreover, an improvement in compounds biodegradation was recorded when transitioning from complex to simpler mixtures. (iii) It is crucial to consider previous exposure to specific compounds when studying biodegradation processes, as the findings showed that an adaptation period as single compound can facilitate the biodegradation of the compounds when exposed further to a complex mixture. At the end, the tested factors had compound-specific impact on the biodegradation efficiency of toluene and benzene, which confirms the complexity of predicting the biodegradation of these compounds. This study emphasizes the need to investigate various environmental factors affecting biodegradation process, particularly in complex mixtures.

6.3.3. Investigating the electron acceptor consumption

Additional to HPLC data, IC measurements were performed to monitor the electron acceptor utilization throughout the experiment (Supplementary data 5, Figure S6.7). For C2, C3 and C4, more nitrate was consumed than what was required for total amount of contaminant biodegraded meaning that nitrate was used for other processes than biodegradation of contaminants such as organic matter degradation. The same phenomena was reported for sulfate consumption in C1 but only during P1 (BM1 and BM2) (Supplementary data 5, Figure S6.7). In P2 and P3, a slight increase in sulfate concentrations were observed especially during P3, but this can be due to high standard deviations, and that actually some sulfate might be consumed, but couldn't be detected. A more distinct increase in sulfate concentrations was reported for C2, C3 and C4 throughout the experiment, compared to C1. The increase in sulfate concentrations was around 0.97 to 4.82 mg L^{-1} in C1 while for the other columns, it was up to 27 mg L^{-1}. The higher increase in sulfate concentrations for C2, C3 and C4 could be explained by the sulfide oxidization. For instance, Eckford & Fedorak (2002) showed that the addition of nitrate stimulated bacterial oxidation of sulfide, where nitrate can be used as an electron acceptor for sulfate production. This was also discussed by Keller et al., that by adding nitrate, sulfides could be oxidized back to sulfate using nitrate as the electron acceptor, thereby replenishing sulfate in the aquifer (Keller et al., 2018). This could explain the higher nitrate consumption than what was theoretically required for the removal of the aromatic compounds. What is certain is that a correlation between nitrate reduction and sulfate production was observed, especially during batch modes.

In the presence of both sulfate and nitrate, either sulfate or nitrate may be the preferred electron acceptor, or both electron acceptors can be reduced concomitantly (Eckford & Fedorak, 2002). In our study, nitrate was used preferentially over sulfate (C2,C3,C4) and sulfate was utilized only when nitrate was not present (Figure S6.7, C1-P1). It is possible that microorganisms would switch to sulfate, in the absence of nitrate as reported in the study of Cunningham et al. (2001). For instance, in C3-P2 (BM2), nitrate was fully consumed (Figure S6.7), and increase in sulfate concentrations was the lowest (Table S6.7). This can be an indication of sulfate utilization, as there was no increase contrary to other BMs and phases, but it's important to note that this is a preliminary assumption, and additional investigations are required to confirm it.

6.3.4. Microbiological investigations

Differences in microbial community composition based on the tested factors

The effect of redox conditions, substrate composition, pollution history, flow mode and location upon columns on the microbial communities were studied within all the columns. For this, the columns were sampled at different time intervals, as illustrated in Figure 6.1. Samples were taken from the beginning, between each phase and at the end of the experiment however, after the DNA extraction procedure, some samples, especially from the start of the experiment (P1), exhibited low DNA concentrations which prevented these samples to proceed with the sequencing (Supplementary data 6, Table S6.8). Despite this limitation, it was clear that DNA concentrations were initially lower at the start of the experiment; however, over the course of the study, the microbial biomass increased, especially during P2. This trend highlighted the microorganisms' ability to adapt to the prevailing environmental conditions applied to the columns.

The impact of redox conditions and subjected substrate(s) on the microbial community composition is presented in Figure 6.5, by beta-diversity analysis of the sequenced samples. The Principal Coordinate Analysis (PCA) gives a visualization of the microbial communities between the different columns where PC1 explains the variability of microbial communities by 34% and PC2 does it by 12.8%. The results clearly demonstrated a distinct separation between sulfate-reducing column (C1) and nitrate-reducing column inoculums (C2,C3 and C4). Despite their common sediment origin, addition of nitrate in C2, C3 and C4 induced a shift in the microbial community composition, that became distinct from the C1 microbiome. This finding supports the idea that microorganisms are highly responsive to changes in redox conditions. Subsequently, it explains the variations in biodegradation performance for different compounds based on the availability of different electron acceptors, as observed in the biodegradation results with toluene and benzene (Section 6.3.1).

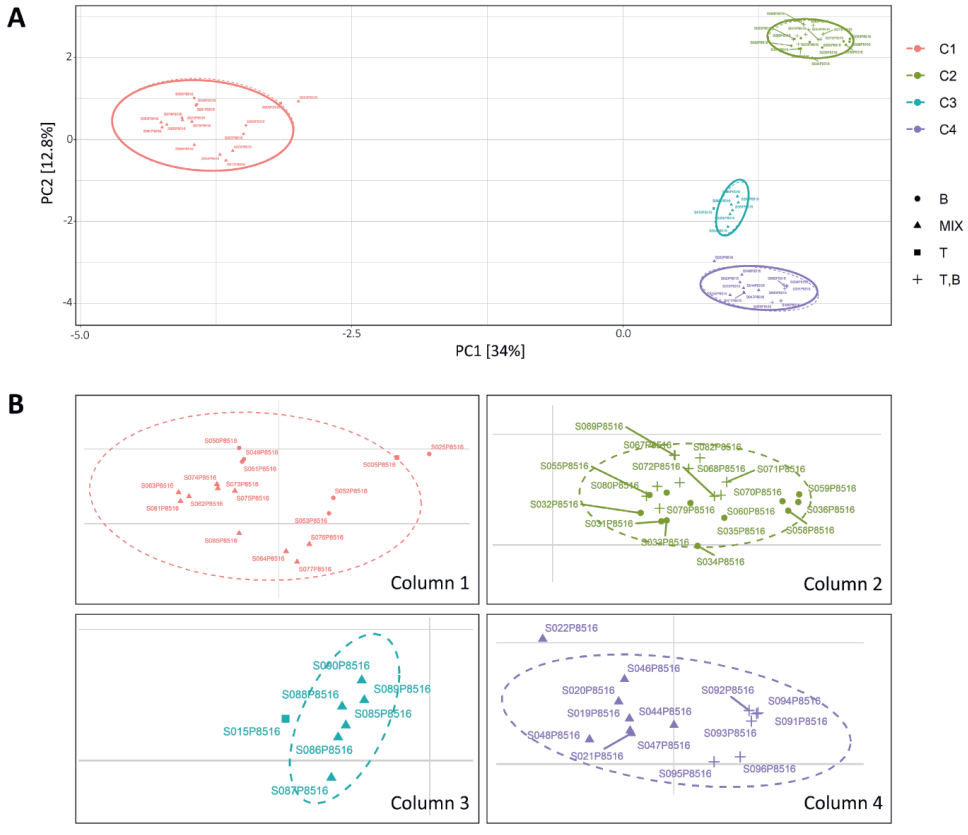


Figure 6.5 Beta-diversity analysis to investigate the microbial community differences among samples. The impact of redox conditions shown with sulfate-reducing column C1 being distant from nitrate-reducing columns C2, C3 and C4 (A). Zoomed in version of figure A, representing the distribution of the samples in each column based on the substrate, shown in different shapes (B).

Figure 6.5 also allowed to investigate the effect of the substrate on the microbial community composition. As shown (Figure 6.5B), no clear clustering was reported based on the substrate fed to the columns. For instance, C1, C3 and C4 were all sampled when fed with the BTEXIeIaN and these samples did not show similarities as they were positioned distant from each other (Figure 6.5A). Similar comparison can be done with C2 and C4, when both columns were fed with dual mix of T+B. Again, when subjected to the same substrate mix, samples from C2 and C4 displayed dissimilarities (Figure 6.5A), suggesting that substrate's influence on the microbial community composition was comparatively less affecting than redox conditions.

The variations in microbial communities, even though the columns received the same substrate, but in different orders, can possibly be attributed to the variation in pollution history. During C2-P3 period and C4-P3 period, despite supplying the same substrate mix (T+B) and maintaining identical redox conditions (NO_3^-) to both columns parallelly, the dissimilarity could only be explained with their pollution history, as it is the only distinguishable factor that differs between these columns. Therefore, it can be concluded that pollution history plays an important role in defining the microbial communities under similar redox conditions. This conclusion can also be supported by the evident clustering of each sample within its respective column as shown in Figure 6.5.

In this study, two other factors that may have influenced the microbial community composition are alternating flow/batch mode and sampling location in the column. For the alternating flow/batch approach, samples were taken only from C1 and C2, during both flow and batch mode, while all columns were sampled from SP1 and SP4. The results did not reveal distinct clusters based on these factors (Supplementary data 6, Figure S6.8). This can be explained by the influence of the batch mode, which aids in achieving a more uniform microbial community distribution throughout the entire column. If the experiment had been conducted solely under continuous flow conditions, this homogenization effect might not have been as evident. Furthermore, it's important to highlight that the relatively small number of samples collected could potentially limit the extent of our investigation regarding these parameters. Therefore, conducting additional analyses may be required to achieve a more comprehensive understanding about the impact of these factors on the microbial community dynamics.

Beta-diversity analysis helped to visualize the impact of the tested factors on microbial community and showed that redox conditions had the most impact which were in line with various studies. In the study of Wu et al. (2022), BTEX biodegradation was tested under various redox conditions and showed that microbial communities were influenced by different electron acceptors. In another study (Fahy et al., 2005), the relationship between bacterial community structures and geochemistry of BTEX polluted groundwater was investigated and found that structure of microbial communities was greatly affected by a reduction in redox potential rather than the substrate (benzene). For the pollution history, Phelps & Young (1999) studied BTEX biodegradation in four different sediments, originating from pristine or polluted sites, and showed that the removal efficiency of the different BTEX components in anoxic sediments was dependent on the prevailing redox conditions as well as on the characteristics and pollution history of the sediment. Based on the existing literature, and outcomes of this study, redox conditions plays a major role in the microbial community dynamics. Therefore, for optimizing remediation strategies in sites contaminated with complex mixtures, as in Griftpark, investigation into redox conditions demonstrated to be an efficient initial approach for biostimulation.

Key microbial groups in response to electron acceptor supplementation

As redox conditions showed to have more impact than the others studied parameters on shaping the microbial community, abundant microbial groups in sulfate (C1) and nitrate-reducing column (C2, C3, C4) were investigated. For this, the top 30 abundant microbial groups were defined (Figure 6.6).

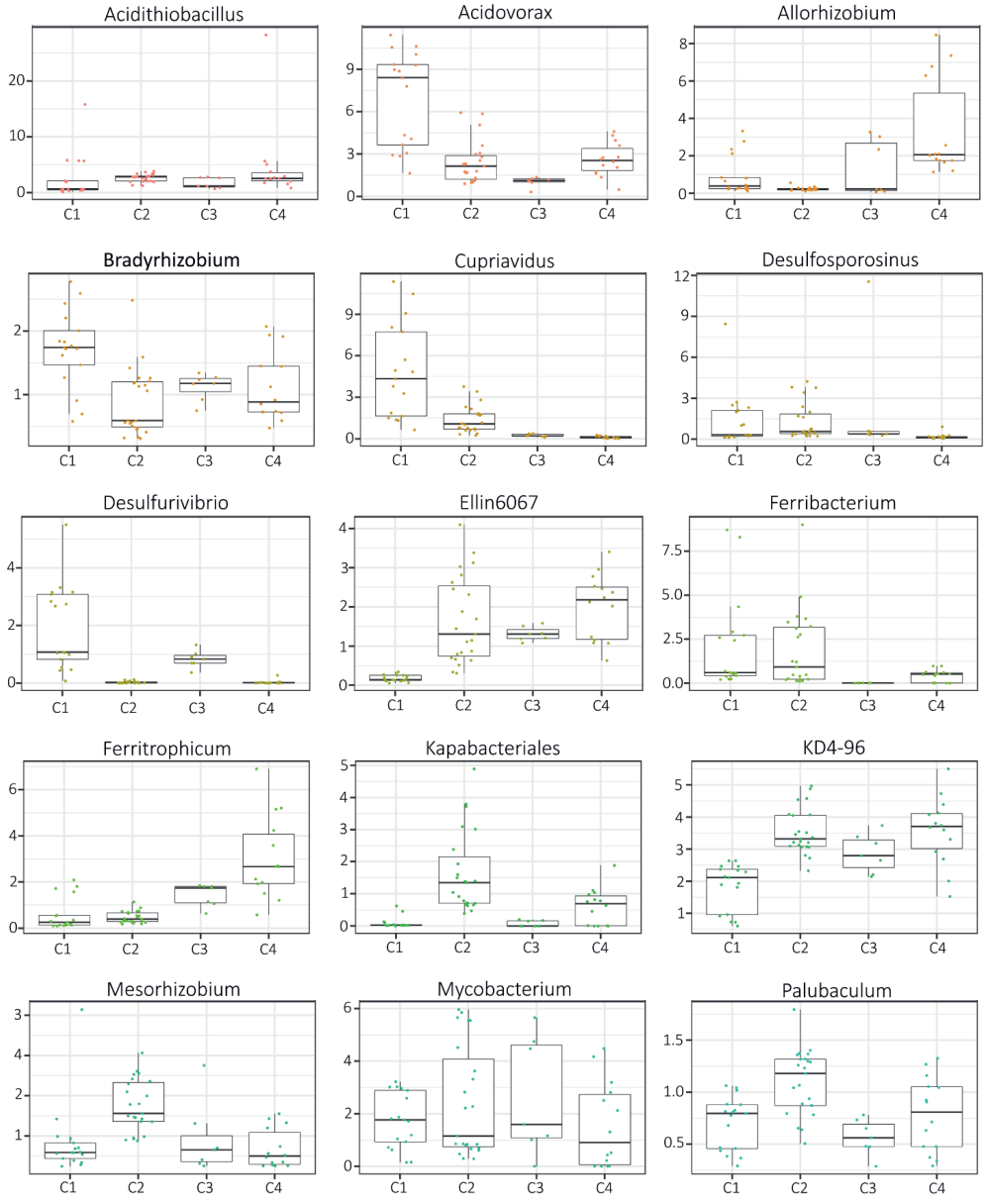
In the sulfate-reducing column C1, members of *Acidovorax*, *Cupriavidus*, *Desulfurivibrio* and *Reyranella* showed higher abundance compared to nitrate-reducing columns. The mentioned members were found to be dominant during C1-P2 and P3 except for *Acidovorax*, showing abundance only during C1-P3. It is likely that members of *Acidovorax* are stimulated with the presence of complex BTEXIeIaN mix under sulfate-reducing conditions. Microorganisms that were abundant in the columns were also identified in other studies as hydrocarbon-degrading species. *Acidovorax* is a common BTEX-degrader (Aburto & Peimbert, 2011; Fahy et al., 2006) and was also detected in abundance at oxygen-limited hydrocarbon-contaminated environments (Aburto & Peimbert, 2011; Benedek et al., 2018; Y. Huang & Li, 2014). *Desulfurivibrio*, a sulfate and sulfur-reducing bacteria was reported for its ability to degrade naphthalene under anaerobic conditions (Campbell et al., 2021). *Cupriavidus* is associated with aerobic monoaromatic hydrocarbon degradation however its capacity to degrade toluene under nitrate-reducing conditions was also demonstrated (Tofalos et al., 2018). Based on our findings, *Cupriavidus* might be capable to degrade aromatic hydrocarbons also under sulfate-reducing conditions as its high abundance (~9%) was recorded in C1-P3 samples. *Reyranella* was previously reported as toluene oxidizing-denitrifying microorganism (H. Jiao et al., 2022).

For nitrate-reducing columns, Ellin6067 (member of *Nitrosomonadaceae*), KD4-96 (belonging to *Chloroflexi* phylum), *Pseudolabrys*, *Rhodanobacter*, SFR-FBR-L83 and *Thermincola* showed higher abundance compared to the sulfate-reducing column, C1. Additionally, *Kapabacteriales*, *Rhodanobacter* and *Thermincola* showed relatively high abundancy in C2 and C4 while *Rhodococcus* species showed high abundance in C3 and C4. For C2, high abundance in *Mesorhizobium and Thiobacillus* was shown, while for C4, higher *Ferritrophicum* was detected.

Members that were found to be abundant in all the columns, regarding their redox condition, were *Mycobacterium*, *Sulfuritalea*, *Sulfuricella* and *Thiobacillus*. *Mycobacterium* was reported in aerobic enrichment cultures of toluene and benzene (Yoshikawa et al., 2016). Even though its biodegradation was not reported, *Sulfuritalea*-related microorganisms are commonly detected at hydrocarbon-contaminated sites (Sperfeld, Diekert, et al., 2018). Interestingly, while all columns showed high abundance of *Pseudomonas*, C2 exhibited the opposite. *Pseudomonas*, known as facultative anaerobe, is recognized as important hydrocarbon degrader (Aburto & Peimbert, 2011; Fahy et al., 2006). As C2 was the only column that did not receive BTEXIeIaN, it is possible that members of *Pseudomonas* are stimulated by the complex mix. This observation underscores the impact of the second important parameter, the pollution history.

The prevalent microorganisms commonly associated with sulfate and nitrate-reduction, along with hydrocarbon degradation, as outlined by Lueders (2017) and Weelink et al. (2010), were not identified as predominant within our columns. However, a shared characteristic among the abundant microorganisms in our columns is their facultative anaerobic nature. In our previous study, aquifer material obtained from the similar location was used to assess the aerobic biodegradation of the different BTEXIeIaN mixtures by investigating the prevalent microbial communities (Aydin et al., 2023). The prevalent microorganisms in the previous study differed from those in the current one, reaffirming the significant role of redox conditions in shaping microbial communities. Given the demonstrated aerobic degradation potential of

the deep-surface sediment in the previous study, the presence of facultative anaerobes in this context becomes rather logic. This finding carries implications, suggesting that introducing oxygen could prove to be effective in cases where certain compounds exhibit recalcitrance under anaerobic conditions.



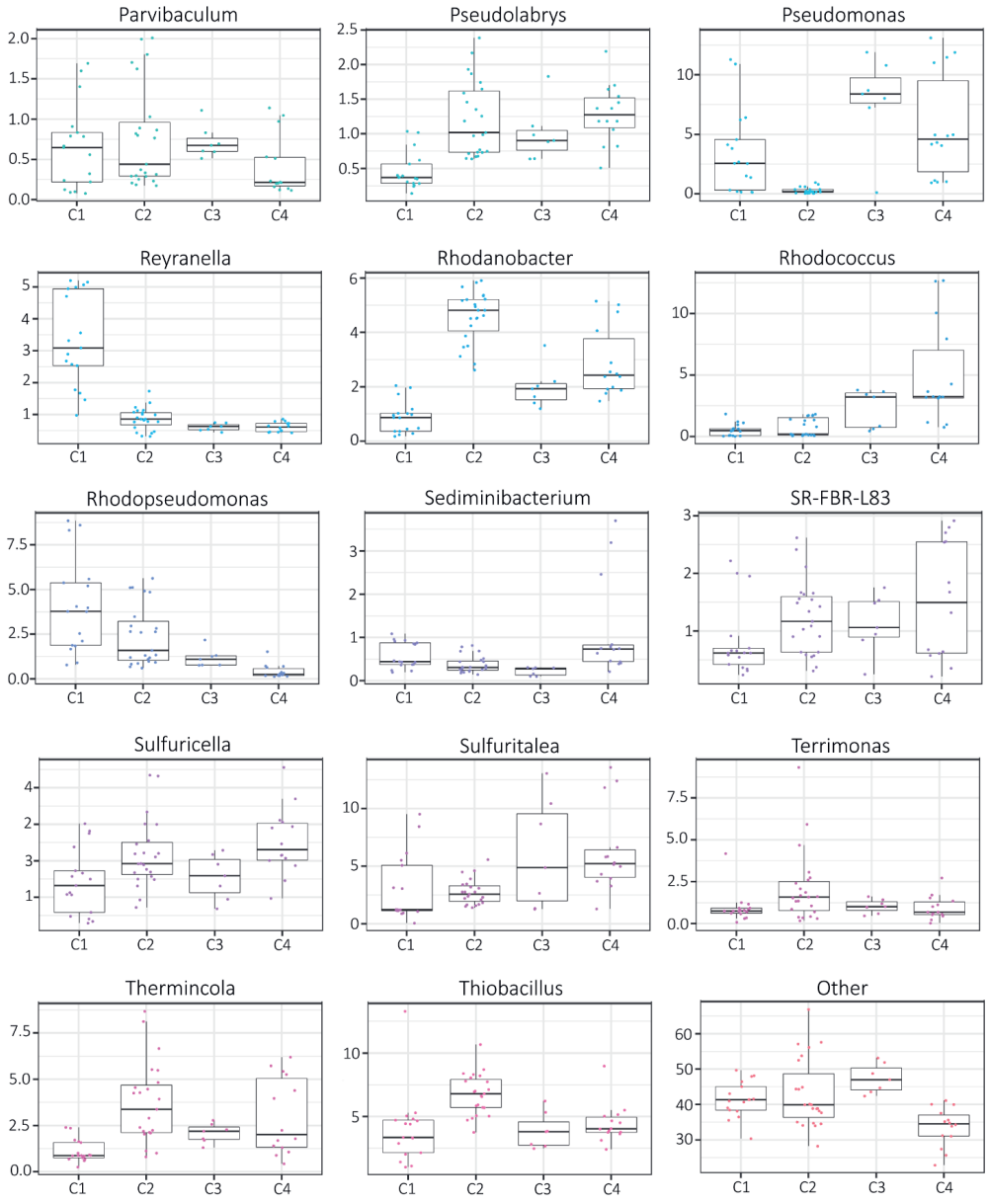


Figure 6.6 Box-plot figures of the top 30 abundant genuses identified in sulfate-reducing column (C1) and nitrate-reducing columns (C2, C3 and C4). The abundance is given in percentage (%).

6.4. Conclusions

In this study, anaerobic biodegradation of toluene and benzene was studied in laboratory soil-columns, to obtain more realistic insights on the impact of various environmental factors, including redox conditions and co-contaminant exposure history, on the microbial community dynamics hence biodegradation. Toluene was more efficiently biodegraded under sulfate-reducing conditions while benzene removal efficiency was higher under nitrate-reducing conditions. Furthermore, substrate interactions showed that toluene biodegradation was relatively unaffected by benzene but influenced by the complex mixture, whereas benzene biodegradation was mostly inhibited by toluene but not by the complex mix. These findings demonstrated the distinct impacts of redox conditions and substrate interactions on the biodegradation of these compounds. The high adaptive capacity of indigenous microorganisms in biodegrading subsequently toluene and benzene, in relatively short time was attributed to the alternating flow/batch mode approach, where high flow rates were applied to the columns. An initial adaptation period, where a single compound was supplied, enhanced the biodegradation of these compounds when subsequently exposed to complex mixtures. Additionally, improved biodegradation efficiency was observed for both compounds when transitioning from complex to simplified mixtures. This finding is relevant for subsurface contaminant plumes, where varying transportation rates can lead to scenarios involving complex to simplified mixtures. In conclusion, our research highlighted the compound-specific nature of aromatic hydrocarbon biodegradation, within different environmental conditions tested. Outcomes of this study can help to design effective bioremediation strategies in contaminated sites harboring various aromatic hydrocarbons. It is proposed to prioritize pilot studies with focus on enhanced redox conditions, as it was shown that the microbial community dynamics were affected by it the most, by still considering factors such as co-contaminant exposure history.

Acknowledgments

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Supplementary Information to Chapter 6

Supplementary data 1: Medium Composition

For the basal medium preparation, Sorensen's phosphate buffer (50 mL), macronutrient stock solution (6 mL L^{-1}) and trace elements stock solution (0.6 mL L^{-1}) were mix in demi-water and completed to 1L (pH 7).

Table S6.1 Medium composition used in this study.

Macronutrient stock solution
$MgSO_4 \cdot 7H_2O$ (9 g L^{-1})
NH_4Cl (170 g L^{-1})
$CaCl_2 \cdot 2H_2O$ (8 g L^{-1})
Trace elements stock solution
$FeCl_2 \cdot 4H_2O$ (2 g L^{-1})
$MnCl_2 \cdot 4H_2O$ (0.5 g L^{-1})
$ZnCl_2$ (50 mg L^{-1})
$(NH_4)6Mo7O_24 \cdot 4H_2O$ (90 mg L^{-1})
$NiCl_2 \cdot 6H_2O$ (50 mg L^{-1})
$CoCl_2 \cdot 6H_2O$ (2 g L^{-1})
$CuCl_2 \cdot 2H_2O$ (30 mg L^{-1})
H_3BO_3 (50 mg L^{-1})
$Na_2SeO_3 \cdot 5H_2O$ (100 mg L^{-1})
EDTA – tripex 2 (1 g L^{-1})
HCl 36% (1 mL L^{-1})
Na-resazurin (0.5 g L^{-1})

Supplementary data 2: HRT test

For the hydraulic retention time (HRT) test, fluorescein was used as a tracer. For this, 100 mg L^{-1} stock solution was prepared and connected to the syringe pump, with a flow rate of 0,55 mL h^{-1}. Based on the information given in below, the final fluorescein concentration in the column was calculated as 5 mg L^{-1}.

Table S6.2 Column information.

Column info		
Height	32	cm
Diameter	6	cm
Column Volume (V_c)	904.8	cm^3
Porosity (ε)	0.4	
Pore Volume (V_c* ε)	361.9	cm^3
Liquid Volume (V_{liq}=V*ε)	542.9	cm^3
Surface A	28.27	cm^2
Flow rate (ϕ)	10.5	mL h^{-1}
Syringe pump flow rate	0.55	mL h^{-1}
Total flow (ϕ_{total})	11	mL h^{-1}
Total flow (day)	264	mL d^{-1}
Upflow velocity liquid (v = ϕ_{total}/(A*ε)	23.41	cm d^{-1}

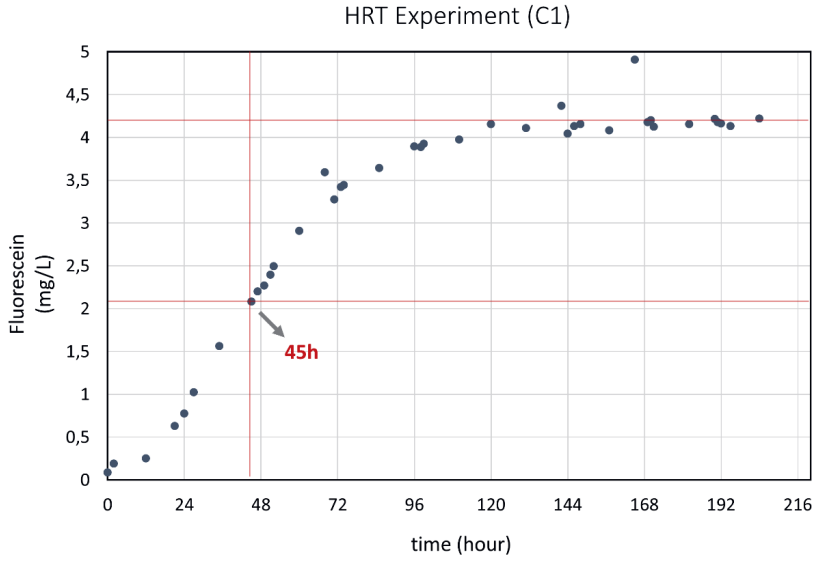

Figure S6.1 HRT measurements performed with fluorescein as a tracer in C1. As a maximum concentration, average concentration of the data points between 120 to 204h was taken and found as 4.2 mg L^{-1}. The half concentration was then 2.1 mg L^{-1} corresponding to 45h as the HRT.

Supplementary data 3: Data Interpretation

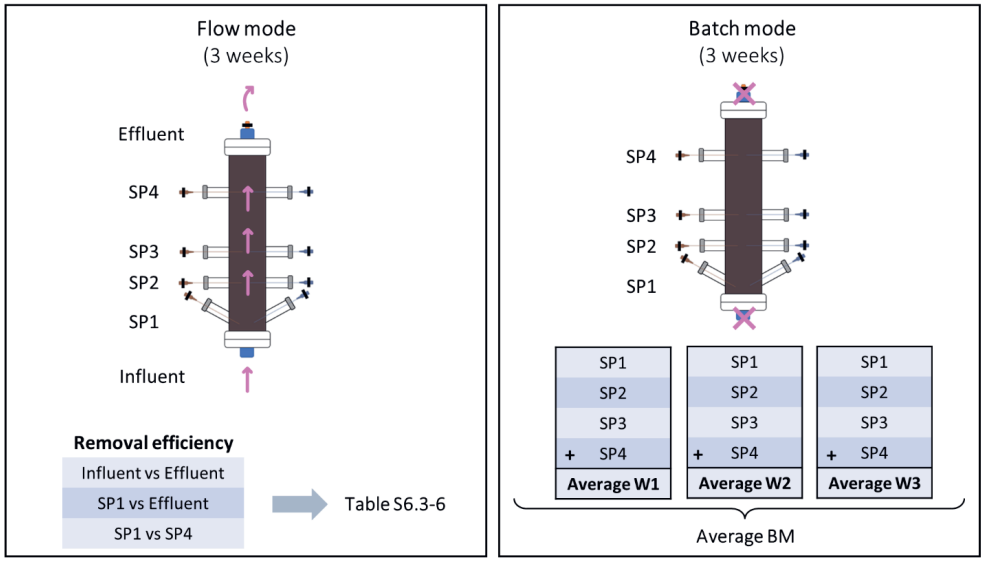

Figure S6.2 Overview of the data interpretation process during flow and batch mode. SP: Sampling Port, BM: Batch Mode, W: week

Removal efficiencies of toluene and benzene during flow and batch mode

Table S6.3 Removal efficiency of toluene and benzene given in % for Column 1 (C1). Darker Blue: High removal efficiency, Darker Red: Negative removal (inefficient).

Column 1		day	Toluene			Benzene		
			In-Ef	SP1-Ef	SP1-4	In-Ef	SP1-Ef	SP1-4
Phase 0	FM0	-21	93.6	94.64	45.85			
	BM0	-14			45.86			
		7			62.6			
		0			33.08			
Phase 1	FM1	7	47.46	-1131.73	-758.88			
		14	73.73	-523.32	-65.95			
		21	22.05	-1603.89	-1596.11			
	BM1	28			NA			
		35			100			
		42			-6.32			
	FM2	49	56.01	25.36	51.49			
		56	-11.22	-57.99	4.21			
		63	98.82	60.75	58.49			
	BM2	70			94.36			
		77			8.07			
		84			61.88			
Phase 2	FM1	91				71.92	-13.89	-9.99
		98				60.67	-19.57	29.4
		105				38.31	-1.13	5.19
	BM1	112						-9.88
		119						-9.11
		126						21.6
	FM2	133				30.75	-20.73	4.48
		140				74.23	-14.44	-2.49
		147				79.01	-16.8	-3.52
	BM2	154						16.01
		161						42.99
		168						53.48
Phase 3	FM1	175	79.44	-9.61	9.34	64.91	-28.45	-11.79
		182	68.68	-31.88	-43.51	58.05	-7.33	-12.62
		189	84.51	-1.43	21.99	73.67	-28.14	-13.57
	BM1	196			22.6			-61.98
		203			29.32			27
		210			100			32.03
	FM2	217	53.25	-51.31	-1.82	46.7	-10.64	-21.64
		224	44.51	16.39	-1	16.52	28.66	3.37
		231	84.31	-44.65	4.21	79.54	-52.21	7.13
	BM2	238			100			2.34
		245			100			22
		252			100			34

Table S6.4 Removal efficiency of toluene and benzene given in % for Column 2 (C2). Darker Blue: High removal efficiency, Darker Red: Negative removal (inefficient).

Column 2		day	Toluene			Benzene		
			In-Ef	SP1-Ef	SP1-4	In-Ef	SP1-Ef	SP1-4
Phase 0	FM0	-21	85,4	68,95	35,01			
	BM0	-14			9,7			
		7			31,98			
		0			61,26			
Phase 1	FM1	7	69,78	-50,61	-12,02			
		14	13,96	-60,94	-72,41			
		21	72,73	-127,64	-6,55			
	BM1	28			NA			
		35			-122,11			
		42			54,53			
	FM2	49	88,3	15,46	12,52			
		56	76,44	14,25	19,9			
		63	74,48	23,15	32,14			
	BM2	70			100			
		77			100			
		84			100			
Phase 2	FM1	91				88,21	-52,26	-32,16
		98				61,9	-1,14	11,02
		105				-103,23	4,06	45,2
	BM1	112						-4,92
		119						17,91
		126						50,98
	FM2	133				76,7	-23,42	24,23
		140				81,99	26,33	64,91
		147				67,7	-12,84	2,42
	BM2	154						40,43
		161						28,77
		168						33,43
Phase 3	FM1	175	14,91	-14,61	9,26	15,5	-1,27	7,31
		182	77,91	17,42	-23,02	64,67	0,71	-19,1
		189	95,21	-21,3	18,96	92,18	-31,26	7,04
	BM1	196			100			100
		203			100			100
		210			100			100
	FM2	217	94,08	-38,03	-62,68	93,25	-37,03	-63,41
		224	97,66	46,69	-3,96	92,51	-28,8	-8,77
		231	99,22	83,32	71,88	98,13	67,19	39,79
	BM2	238			100			100
		245			100			100
		252			100			100

Table S6.5 Removal efficiency of toluene and benzene given in % for Column 3 (C3). Darker Blue: High removal efficiency, Darker Red: Negative removal (inefficient).

Column 3		day	Toluene			Benzene		
			In-Ef	SP1-Ef	SP1-4	In-Ef	SP1-Ef	SP1-4
Phase 1	FM1	7	98,94	88,12	84,36			
		14	3,12	-27	55,52			
		21	93,89	91,86	70,02			
	BM1	28			57,58			
		35			71,78			
		42			76			
	FM2	49	NA	NA	NA			
		56	NA	8,34	37,66			
		63	99,49	98,14	97			
	BM2	70			87,05			
		77			77,2			
		84			-1274,48			
Phase 2	FM1	91	80,86	59,21	75,88	82,25	67,15	34,15
		98	-614,47	20,99	82,28	-784,82	-8,02	16,19
		105	99,36	96,31	100	75,76	-18,97	18,57
	BM1	112			100			33,71
		119			100			100
		126			100			100
	FM2	133	48,22	-40,97	13,1	42,91	-41,85	-0,9
		140	59,27	4,13	49,24	51,08	-6,93	12,51
		147	76,48	25,11	79,69	61,97	-8,73	18,24
	BM2	154			75,94			16,01
		161			100			-2,49
		168			100			-11,75
Phase 3	FM1	175	53,49	42,58	58,36	35,18	17,59	3,07
		182	100	100	100	75,77	5,46	2,83
		189	100	100	100	86,44	6,71	14,23
	BM1	196			100			-49,03
		203			100			48,84
		210			100			30,61
	FM2	217	-29,42	-89,78	50,02	-62,6	-110,73	24,2
		224	100	100	100	69,85	-6,56	22,76
		231	100	100	100	75,12	-2,64	39,83
	BM2	238			100			39,46
		245			100			30,02
		252			100			36,26

Table S6.6 Removal efficiency of toluene and benzene given in % for Column 4 (C4). Darker Blue: High removal efficiency, Darker Red: Negative removal (inefficient).

Column 4		day	Toluene			Benzene		
			In-Ef	SP1-Ef	SP1-4	In-Ef	SP1-Ef	SP1-4
Phase 1	FM1	7	87,33	4,79	15,53			
		14	24,93	3,19	-6,01			
		21	75,04	-9,35	-0,59			
	BM1	28			0,66			
		35			16,6			
		42			28,52			
	FM2	49	NA	NA	NA			
		56	67,55	4,47	1,93			
		63	19,09	-16,12	-50,57			
	BM2	70			-223,55			
		77			37,77			
		84			-1260			
Phase 2	FM1	91	13,04	53,32	-4,43	57,99	45,84	10,58
		98	-119,29	-128,77	-31,41	-57,26	-93,51	-20,36
		105	35,05	-1,89	-32,73	24,75	-31,63	-42,35
	BM1	112			22,59			32,06
		119			66,67			100
		126			100			100
	FM2	133	65,51	-7,26	8,42	45,21	-30,17	-24,26
		140	35,66	-106,73	-12,29	39,21	-21,87	3,09
		147	80,85	82,56	7,85	100	100	-30,22
	BM2	154			12,75			8,74
		161			36,71			56,76
		168			45,47			45,61
Phase 3	FM1	175	-54,95	-18,69	5,45	-29,22	4,02	9,23
		182	-16,15	-63,8	-20,41	-55,32	-72,17	-26,18
		189	96,84	45,14	-21,89	94,29	22,3	-20,5
	BM1	196			-19,81			-8,92
		203			4,8			47,06
		210			100			91,19
	FM2	217	-47,12	2,11	-11,16	-55,27	-12,75	-20,12
		224	58,37	-27,59	-20,6	50,32	-12,85	-12,14
		231	97,85	100	100	92,86	92,13	13,93
	BM2	238			100			67,91
		245			100			71,55
		252			100			73,56

Supplementary data 4: Toluene and Benzene Concentrations

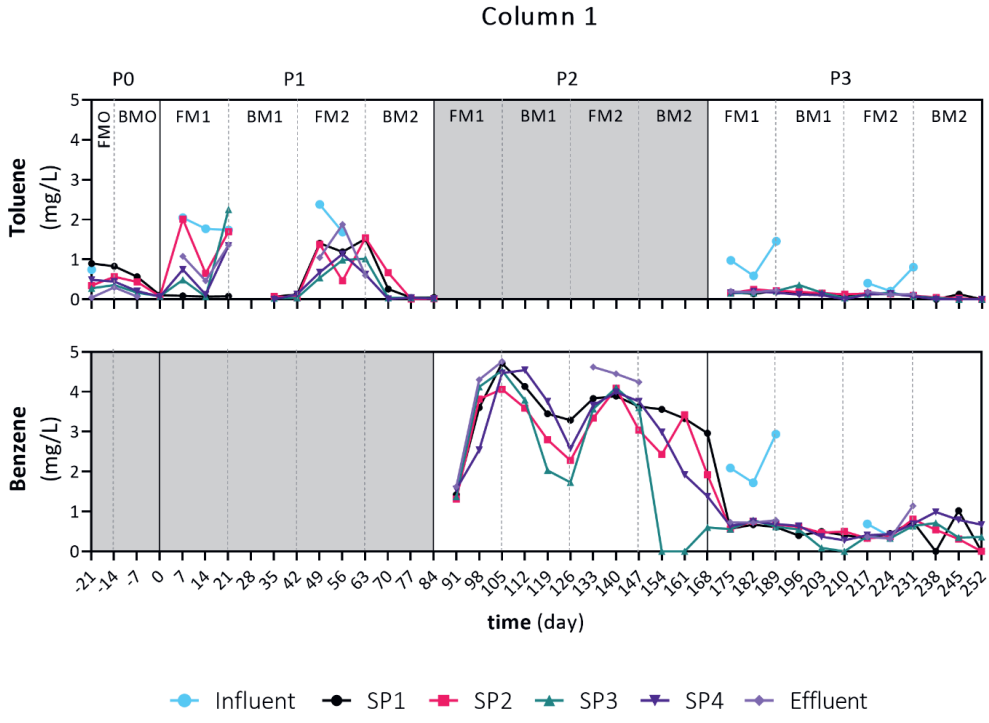

Figure S6.3 Toluene and benzene concentrations measured throughout the column C1. Shadowed area represent no addition of the contaminant, and therefore was not detected in the HPLC.

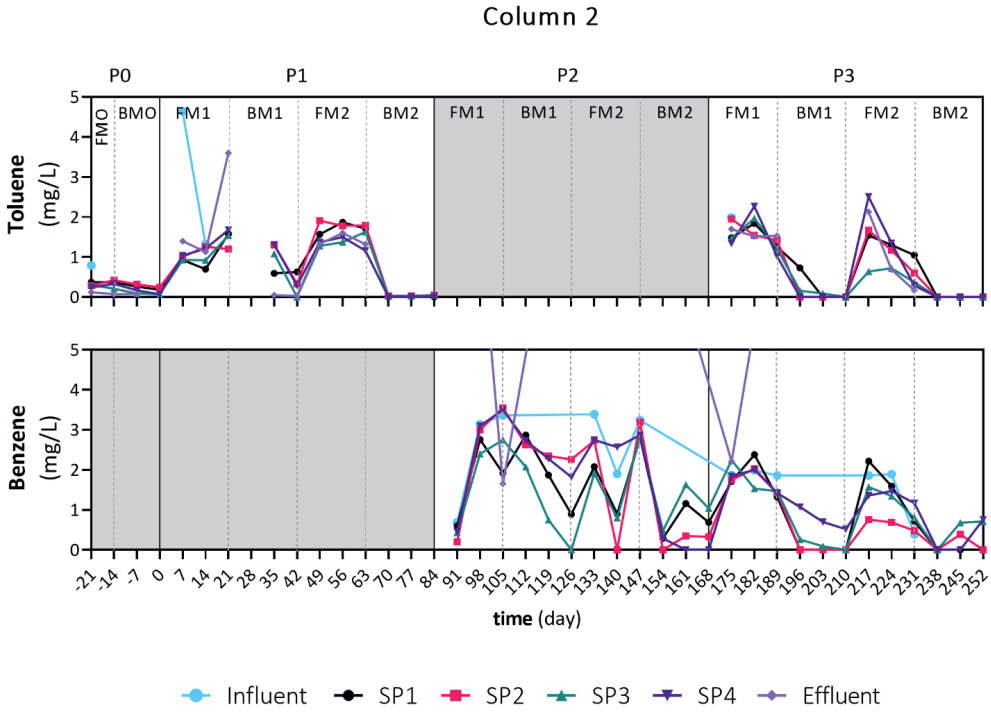

Figure S6.4 Toluene and benzene concentrations measured throughout the column C2. Shadowed area represent no addition of the contaminant, and therefore was not detected in the HPLC.

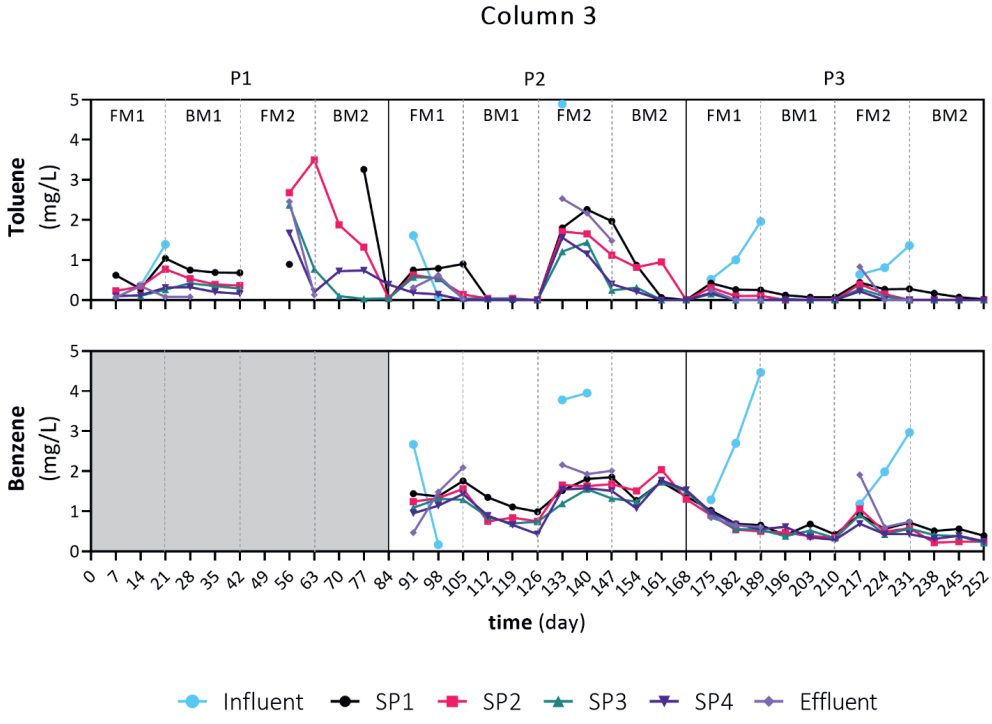

Figure S6.5 Toluene and benzene concentrations measured throughout the column C3. Shadowed area represent no addition of the contaminant, and therefore was not detected in the HPLC.

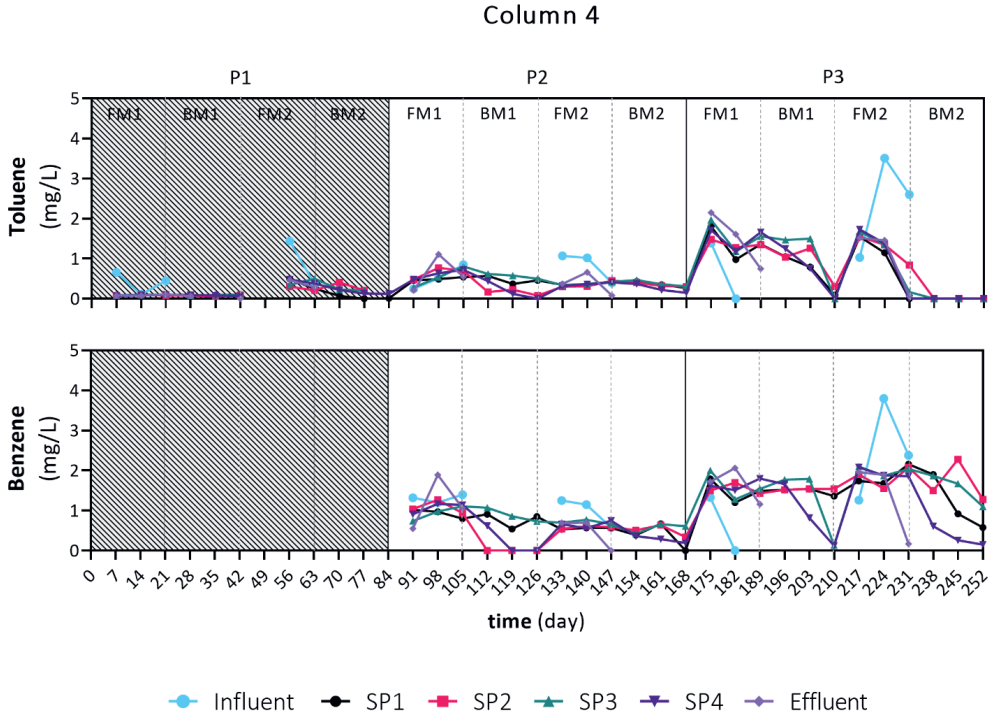

Figure S6.6 Toluene and benzene concentrations measured throughout the column C4. Data from P1 (shadowed area with diagonal lines) was not discussed due to mixing issue that lead to low and inconsistent concentrations, which couldn't be accurately measured.

Supplementary data 5: Sulfate and Nitrate Concentrations

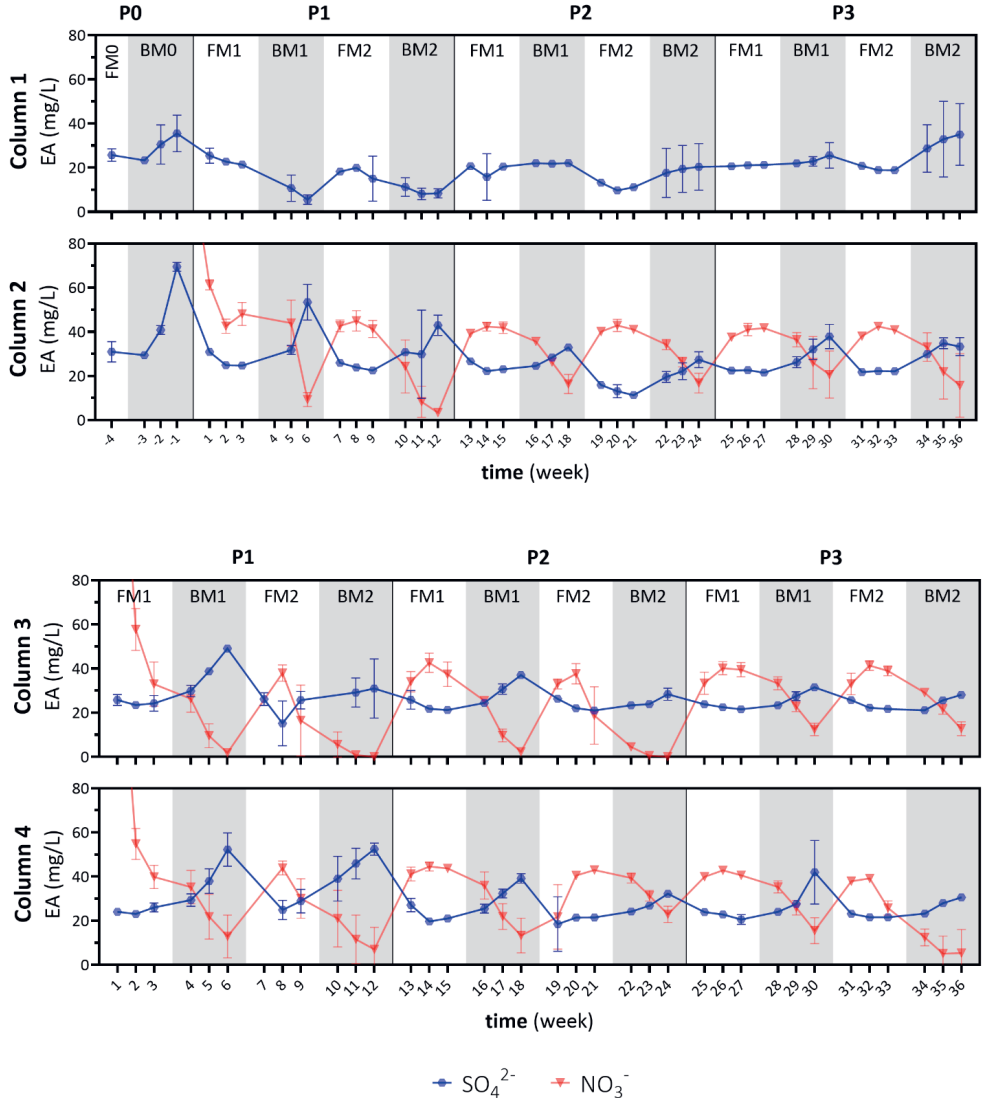

Figure S6.7 Nitrate and sulfate concentrations (mg L^{-1}) measured in each column throughout the experiment. EA; electron acceptor.

Stoichiometric Calculations

Table S6.7 Stochiometric calculations for all the columns, during the batch mode of all phases. All results are given in mg L^{-1}.

	C1						C2					
	SO_4^{2-}			NO_3^-			SO_4^{2-}			NO_3^-		
	N	C	C/N	N	C	C/N	N	C	C/N	N	C	C/N
P0-BM0	1.89	-4.82	-2.55				0.78	-9.87	-12.71	0.80	24.55	30.62
P1-BM1	5.31	15.86	2.99				3.44	-28.74	-8.34	3.56	38.83	10.91
P1-BM2	5.40	8.72	1.62				7.24	-20.43	-2.82	7.48	32.99	4.41
P2-BM1	9.11	-0.34	-0.04				8.04	-9.86	-1.23	8.30	25.6	3.08
P2-BM2	8.17	-0.97	-0.12				11.05	-16.1	-1.46	11.41	24.29	2.13
P3-BM1	4.30	-2.71	-0.63				11.68	-16.45	-1.41	12.06	21.08	1.75
P3-BM2	4.33	-2.12	-0.49				4.96	-11.17	-2.25	5.12	25.14	4.91

	C3						C4					
	SO_4^{2-}			NO_3^-			SO_4^{2-}			NO_3^-		
	N	C	C/N	N	C	C/N	N	C	C/N	N	C	C/N
P0-BM0												
P1-BM1	0.87	-24.87	-28.45	0.90	31.18	34.53	0.14	-26.18	-187.77	0.14	26.92	186.96
P1-BM2	12.90	-15.81	-1.23	13.33	16.53	1.24	6.16	-25.1	-4.08	6.36	22.47	3.53
P2-BM1	4.90	-6.27	-1.28	5.06	31.64	6.25	10.32	18.99	1.84	10.66	30.43	2.85
P2-BM2	4.97	-1.12	-0.23	5.13	14.98	2.92	4.07	-10.68	-2.62	4.21	20.02	4.76
P3-BM1	2.65	-9.95	-3.75	2.74	27.04	9.86	9.80	-21.43	-2.19	10.12	25.05	2.48
P3-BM2	5.57	-6.39	-1.15	5.75	26.17	4.55	7.02	-8.99	-1.28	7.25	20.43	2.82

Negative values represents production, while positive values are consumption. **N**: Electron acceptor needed, **C**: Electron acceptor consumed. C/N is used to measure the variance between required (needed) and actual consumption values (consumed). Gray area: not applicable.

Supplementary data 6: DNA quantification

Table S6.8 Qubit results were used to determine DNA concentrations (µg/µL) of the samples collected during the experiment. The figure employs a color-coded scheme representing these concentrations. Darker shades of green indicate higher DNA concentrations, while red represents low DNA concentrations. Samples with DNA concentrations below 0.2 µg/µL were not suitable for sequencing.

	Column 1		Column 2		Column 3		Column 4	
	Sample Info	µg/µL	Sample Info	µg/µL	Sample Info	µg/µL	Sample Info	µg/µL
Phase 1	C1,P1, SP1, FM, rep1	0.158	C2,P1, SP1, FM, rep1	0.081	C3,P1, SP1, BM, rep1	0.127	C4,P1, SP1, BM, rep1	0.242
	C1,P1, SP1, FM, rep2	0.193	C2,P1, SP1, FM, rep2	low	C3,P1, SP1, BM, rep2	0.272	C4,P1, SP1, BM, rep2	0.182
	C1,P1, SP1, FM, rep3	0.167	C2,P1, SP1, FM, rep3	low	C3,P1, SP1, BM, rep3	0.158	C4,P1, SP1, BM, rep3	0.147
	C1,P1, SP4, FM, rep1	0.073	C2,P1, SP4, FM, rep1	0.054	C3,P1, SP4, BM, rep1	low	C4,P1, SP4, BM, rep1	0.256
	C1,P1, SP4, FM, rep2	0.282	C2,P1, SP4, FM, rep2	0.086	C3,P1, SP4, BM, rep2	low	C4,P1, SP4, BM, rep2	0.165
	C1,P1, SP4, FM, rep3	0.404	C2,P1, SP4, FM, rep3	0.228	C3,P1, SP4, BM, rep3	low	C4,P1, SP4, BM, rep3	low
Phase 2	C1,P2, SP1, FM, rep1	0.414	C2,P2, SP1, FM, rep1	3.64				
	C1,P2, SP1, FM, rep2	0.193	C2,P2, SP1, FM, rep2	5.51				
	C1,P2, SP1, FM, rep3	0.203	C2,P2, SP1, FM, rep3	3.07				
	C1,P2, SP4, FM, rep1	low	C2,P2, SP4, FM, rep1	4.72				
	C1,P2, SP4, FM, rep2	low	C2,P2, SP4, FM, rep2	1.07				
	C1,P2, SP4, FM, rep3	low	C2,P2, SP4, FM, rep3	0.87				
	C1,P2, SP1, BM, rep1	2.51	C2,P2, SP1, BM, rep1	2.6	C3,P2, SP1, BM, rep1	0.36	C4,P2, SP1, BM, rep1	0.678
	C1,P2, SP1, BM, rep2	2.06	C2,P2, SP1, BM, rep2	3.04	C3,P2, SP1, BM, rep2	1.07	C4,P2, SP1, BM, rep2	1
	C1,P2, SP1, BM, rep3	2.59	C2,P2, SP1, BM, rep3	2.25	C3,P2, SP1, BM, rep3	0.607	C4,P2, SP1, BM, rep3	0.737
	C1,P2, SP4, BM, rep1	0.086	C2,P2, SP4, BM, rep1	0.71	C3,P2, SP4, BM, rep1	0.728	C4,P2, SP4, BM, rep1	0.476
	C1,P2, SP4, BM, rep2	0.062	C2,P2, SP4, BM, rep2	1.32	C3,P2, SP4, BM, rep2	0.537	C4,P2, SP4, BM, rep2	0.251
	C1,P2, SP4, BM, rep3	0	C2,P2, SP4, BM, rep3	0.689	C3,P2, SP4, BM, rep3	0.211	C4,P2, SP4, BM, rep3	0.158
Phase 3	C1,P3, SP1, FM, rep1	0.6	C2,P3, SP1, FM, rep1	0.129				
	C1,P3, SP1, FM, rep2	0.413	C2,P3, SP1, FM, rep2	0.149				
	C1,P3, SP1, FM, rep3	0.28	C2,P3, SP1, FM, rep3	low				
	C1,P3, SP4, FM, rep1	0.098	C2,P3, SP4, FM, rep1	0.289				
	C1,P3, SP4, FM, rep2	0.064	C2,P3, SP4, FM, rep2	0.431				
	C1,P3, SP4, FM, rep3	low	C2,P3, SP4, FM, rep3	0.285				
	C1,P3, SP1, BM, rep1	0.547	C2,P3, SP1, BM, rep1	0.614	C3,P3, SP1, BM, rep1	0.422	C4,P3, SP1, BM, rep1	0.398
	C1,P3, SP1, BM, rep2	0.669	C2,P3, SP1, BM, rep2	1.04	C3,P3, SP1, BM, rep2	0.743	C4,P3, SP1, BM, rep2	0.356
	C1,P3, SP1, BM, rep3	0.694	C2,P3, SP1, BM, rep3	0.475	C3,P3, SP1, BM, rep3	0.472	C4,P3, SP1, BM, rep3	0.248
	C1,P3, SP4, BM, rep1	0.05	C2,P3, SP4, BM, rep1	0.219	C3,P3, SP4, BM, rep1	0.539	C4,P3, SP4, BM, rep1	0.306
	C1,P3, SP4, BM, rep2	0.093	C2,P3, SP4, BM, rep2	0.268	C3,P3, SP4, BM, rep2	0.349	C4,P3, SP4, BM, rep2	0.28
	C1,P3, SP4, BM, rep3	low	C2,P3, SP4, BM, rep3	0.127	C3,P3, SP4, BM, rep3	0.249	C4,P3, SP4, BM, rep3	0.226

C: Column, P: Phase, SP: Sampling port, FM: Flow mode, BM: Batch mode, rep: replicate, low: below detection limit

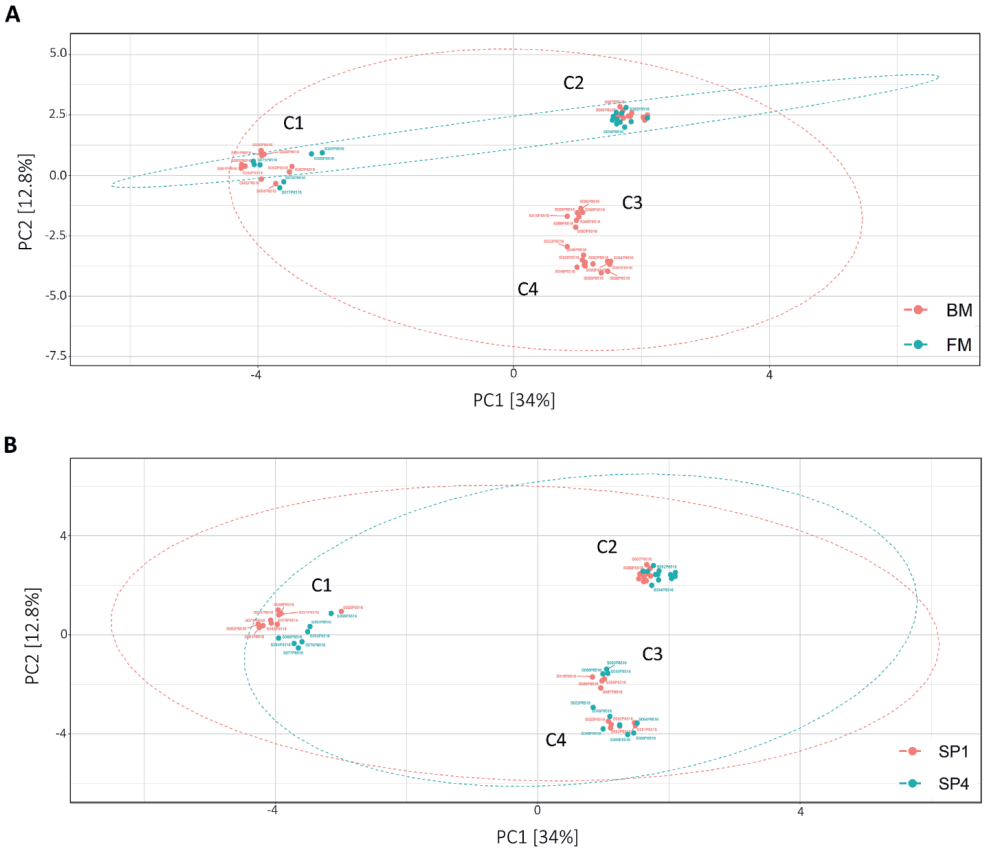

Figure S6.8 Beta-diversity analysis for differences in microbial communities depending on (A) alternating flow (FM) and batch (BM) mode in the samples of C1 and C2, (B) sampling location in the columns as SP1 (entrance) and SP4 (exit). Clear sample clusters were not apparent in the dataset.

Chapter 7

General Discussion

7.1. Introduction

Intensive anthropogenic activities, extreme use of chemicals and disposal of large volumes of wastes during past decades have resulted in numerous contaminated sites around the world (Panagos et al., 2013). Such sites are an important source of pollution, causing serious environmental problems on the terrestrial and aquatic ecosystems. In this thesis, former Manufactured Gas Plant (MGP) sites, their historical wastes and resulting groundwater pollution with aromatic hydrocarbons were the main focus (Chapter 1). However, many other hazardous substances are released into the environment from different sources, for instance heavy metals from industrial or commercial sites, radioactive contamination from thermal power plants, chlorinated hydrocarbons from manufacturing of synthetic solvents, and point source and diffuse pollution by insecticides and pesticides and many other (Badawi et al., 2000; Guvenç et al., 2003; Järup, 2003; Papp et al., 2002). Depending on the properties of the contaminants, they can cause different effects on ecosystems and human health. Epidemiological studies point that the increase in health incidents such as lymphoma, leukemia, and liver and breast cancers, might be attributed to continuous exposure of humans to low concentrations of pollutants (Badawi et al., 2000). This is why management of contaminated sites is crucial to ensure that the quality of the environment is maintained to meet environmental standards as well as health and legal requirements to prevent or reduce the significant impacts of contaminants on human health and ecosystems.

Even though implementation measures are taken today, many sites exist with historical contamination and require intensive management strategies. In the United States, it is estimated that there are currently between 3,000 to 5,000 former MGP sites distributed across the country (Environmental Protection Agency, 1999). In Europe, approximately 4,500 former MGP sites exist (Thomas, 2014) and around 217 gasworks sites have been reported in the Netherlands (Stichtingbehoudvanoud.nl, 2023). While many of these MGP sites were originally located outside of the cities, with the expansion of urban areas, they are now situated in the inner-city areas (Environmental Protection Agency, 1999). When redevelopment is planned for the locations of these former MGP sites, there is a risk of potential liabilities. To address and minimize these risks, it is crucial to conduct historical and geological waste assessments (Hatheway, 1997). This will help in making informed decisions about site remediation and in ensuring that any environmental hazards are properly managed or removed.

This PhD study addressed the pollution problems of former MGP sites, using the Griftpark in Utrecht, the Netherlands, as an example, with a focus on groundwater pollution and its remediation. The most common approach for groundwater remediation is the so called pump and treat approach. Despite the advantage of easy immediate implementation, this technique has several downsides: it requires high energy, needs constant monitoring, can be very expensive and often does not perform as efficiently as expected (Majone et al., 2015). At best, it prevents the spreading of contamination rather than providing a permanent clean-up solution (Birak & Miller, 2009). In Griftpark, the contaminants are pumped to a WWTP and biologically treated in an aerated anti-bulking reactor. However, this costs approximately 200.000 – 300.000 euros annually. Furthermore, according to calculations, the pump and treat strategy may need to continue for at least 100 years to reduce the flux of contaminant from the pollution sources in the Griftpark subsurface, so that acceptable concentrations will be reached in the groundwater downstream of the Griftpark. This is why, the municipality of Utrecht (owner of the site) is seeking for an alternative strategy capable of reducing costs by at least 90% while still meeting environmental and legal standards.

In situ bioremediation is an environmentally friendly and cost-effective alternative to physical and chemical methods and highly promising technology for remediation of contaminated soil, groundwater and sediments. Despite its advantages, in situ bioremediation is less standardized and requires high multi-disciplinary knowledge of the geochemistry, hydrogeology, microbiology and ecology (Majone et al., 2015). This is why laboratory optimization experiments are needed before any in site applications, where site conditions are preserved and all potential impacts on biodegradation mechanisms are evaluated. Once preliminary information is obtained by laboratory-scale studies, pilot-scale field tests can be conducted. Even though these steps require intense research and long monitoring time, it will help for a better design, a more reliable operation and permanent solution.

To facilitate the planning of remediation activities at contaminated sites, three-dimensional numerical groundwater transport models can be employed. Such models are able to both simulate and predict behavior of contaminants over reasonably long time scales and under a variety of circumstances and conditions (Prommer et al., 2000). To achieve realistic results, comprehensive site characterization is essential. Site characterization includes parameters such as flow velocity, hydraulic conductivity, and dispersity, which can be obtained through drilling, sampling, pumping tests, and tracer tests. Reactive transport models can integrate all available hydrological, hydrogeological, and hydrogeochemical information to establish a quantitative framework for evaluating the long-term groundwater risks posed by contamination (Steefel et al., 2005). In the case of Griftpark, the presence of a large pond lined with cement-bentonite and presumed rubble and pipelines in the shallow subsurface made these techniques impractical and cost-prohibitive. Furthermore, due to the inherent subsurface heterogeneity, deterministic hydrogeological models always carry a degree of uncertainty. However, despite the challenges arising from data scarcity, a PhD from Utrecht University, parallel to this thesis, focused on the qualitative understanding of biodegradation of tar aromatic hydrocarbons and the subsequent geochemical response at Griftpark through the implementation of a reactive transport model. Combining laboratory research (this thesis), field studies and numerical modelling (Utrecht University) can provide valuable insights into the understanding of complex sites, such as Griftpark, predict the fate of contaminants in the subsurface, and contribute to risk assessments and the identification of potential new control and remediation strategies.

This thesis focused on understanding the biodegradation mechanisms of the BTEXIeIaN mixture, which are prevalent groundwater contaminants found in the subsurface of Griftpark. Additionally, it aimed to propose strategies for enhancing the biodegradation process of these contaminants. First, a practical analytical method was developed for the accurate and simultaneous quantification of all the contaminants (Chapter 2). Next, biostimulation strategies were assessed with addition of oxygen (Chapter 3 and 5), nitrate and sulfate (Chapter 4 and 6) while also assessing different bioaugmentation approaches (Chapter 4). Throughout the thesis, factors that could potentially affect the biodegradation process were investigated, including substrate composition and interactions (Chapter 3 and 6), flow hydrodynamics and the pollution history (Chapter 6). In addition to the biodegradation investigation, the influence of the aforementioned factors on microbial dynamics were studied (Chapter 3 and 6). The findings presented in this thesis aimed to provide insights for guiding future decisions on pilot studies in the subsurface of Griftpark and also to contribute to the development of effective clean up strategies for other heavily polluted sites.

In previous chapters, scientific discussions were addressed upon the research presented. In this chapter, the generic scientific knowledge derived from the laboratory studies (Chapter 2, 3, 4, 5 and 6) will be discussed upon their relevance to the case study, the Griftpark.

7.2. Thesis Outcomes

Before starting discussing the outcomes of this thesis, it is important to emphasize on how biodegradation was evaluated throughout this study. There are different lines of evidence to confirm the biodegradation in laboratory experiments. The first one is the decrease in substrate concentration which indicates that microorganisms are utilizing and degrading the compound as a carbon and energy source. This information can be supported by changes in chemical data such as: consumption of growth stimulating materials (i.e. electron acceptors), production of intermediate compounds or final metabolic by-products such as CO_2. Another evidence is microbial growth, which can be investigated by monitoring biomass by cell counting, biomarker analysis or by studying the microbial population dynamics with use of molecular techniques (Wittebol & Dinkla, 2015). In this thesis, contaminant concentrations were monitored by HPLC analysis (Chapter 2) while chemical data was evaluated by use of ion chromatography (NO_3^-, NO_2^-, SO_4^{2-}) or gas chromatography (O_2, CO_2, CH_4) (Chapter 3, 4, 5 and 6). Increase in biomass was assessed indirectly during molecular analyses, where DNA concentrations were quantified with Qubit fluorometer (Chapter 3 and 6). As a decrease in contaminant concentration is one of the most significant indicator of biodegradation, Chapter 2 needs attention since an analytical method was specifically developed for the simultaneous detection and quantification of all compounds in the BTEXIeIaN mixture.

7.2.1. Significance of the developed HPLC method for routine biodegradation monitoring

In the context of this thesis, a method capable to detect and quantify all the BTEXIeIaN compounds simultaneously was required. Since the focus was studying the biodegradation of BTEXIeIaN, routine sampling was necessary. However, a high sampling frequency had the potential to disturb the experiment. This is why, using low volumes of samples for quantifying these compounds was crucial. Additionally, the interest was on monitoring the biodegradation process in liquid phase, necessitating the analysis of aqueous samples. Therefore, HPLC was selected as the measurement equipment rather than to common known GC measurement techniques. Routine measurements meant also serious time investment, therefore the method needed to be fast and practical. When dealing with low concentrations or volatile compounds such as BTEX, sample preparation steps are often required. This can be time consuming, costly and may require high volumes of samples (Yamada et al., 2009). The limit of detection (LOD) and limit of quantification (LOQ) values for each compound were aligned with the experimental design. For example, for benzene, which exhibited the highest LOD and LOQ values, precise quantification of an 80% degradation rate was attainable at an initial concentration of 1 mg L^{-1}. Moreover, it was possible to detect a degradation level as high as 93%. Consequently, when designing the experiments, concentrations above 1 mg L^{-1} were chosen for benzene to ensure reliable quantification. In summary, Chapter 2 demonstrated the importance of considering the researcher's needs when designing and developing a method. By carefully addressing factors such as simultaneous detection and quantification, routine sampling requirements, low sample volumes, analysis of aqueous samples, practicality, and cost considerations, the method successfully enabled the accurate quantification of BTEXIeIaN compounds within 27 minutes (per sample) without the need for complex sample preparation steps.

The HPLC method for BTEXIeIaN mixture quantification was preferred to be shared in details so that other researchers could replicate the work or can build upon existing methods, refine them, or adapt them for their own research purposes. Despite the wide range of biodegradation research studies, adequate methodology publications are limited. One of the reasons can be due to the word limitations in the research papers, leading to summarize the quantification method and limiting the transparency and reproducibility of the method. Another reason can be that, research papers which present novel findings tend to receive more attention and recognition. Consequently, researchers may prioritize publishing such type of papers over methodology papers, which are often considered less ground-breaking. Writing methodology papers requires the collective expertise and input of various individuals involved in the method development process where more time and effort to document and publish is needed. However, knowledge and experience needs to be shared to facilitate the work of researchers in similar field but also to contribute to the overall advancement of scientific knowledge.

7.2.2. Experimental considerations

Laboratory optimization experiments are needed before any in situ bioremediation applications where site conditions are preserved as much as possible and all potential impacts on biodegradation mechanisms are evaluated. In this thesis, experiments were designed with intention to be close to Griftpark's subsurface conditions. For batch experiments (Chapter 3, 4 and 5) sediment from the subsurface of Griftpark (35-38.5 m bgl) was used as a source of inoculum, and solid matrix in batch and column experiments. This way, the biodegradation performance of the indigenous microorganisms were tested and insight on their metabolic potential were obtained. Additionally, as a liquid matrix, groundwater from Griftpark, sampled from 8 to 10 m bgl, was used. This aimed to preserve the redox conditions dominant in Griftpark's subsurface. Intentionally, we did not add any supplements, such as vitamins and nutritional yeast, to the bottles as we wanted to test the 'minimalist conditions' where biodegradation could still occur, which can also be viewed as the 'worst case scenario'. For column experiments (Chapter 6), again sediment from Griftpark (29.5 -30 m bgl) was utilized and a flow was provided with continuous pumping of a medium, imitating a groundwater flow. Groundwater from Griftpark was not selected as the matrix for the column study. This decision was made as it would be impractical and inconvenient to continuously sample groundwater from the site to supply to the columns. It would result in extensive sampling of liters of groundwater each week, which would come with storage challenges, such as preserving the anaerobic conditions. Therefore, a basal medium was prepared similar to the properties of the Griftpark groundwater, and still possess the 'minimalist conditions'. Since batch experiments were closed systems, and not fully representing the in site conditions, we advanced the laboratory experiments by performing column studies where the effect of a flow (in our case, higher than Griftpark's groundwater flow) and microbial selection pressure can be further evaluated.

Another challenging experimental decision was the temperature used for the batch and column studies. All experiments were performed at room temperature (18-22°C) which was higher than the average groundwater temperature known as 10-15°C (Brown et al., 2020). This is important, as temperature influences the microbial growth and hence the biodegradation process (Farhadian, Vachelard, et al., 2008; Varjani, 2017). Different microbial species have distinct temperature ranges at which they can effectively degrade the aromatic hydrocarbons however it is often reported that higher temperatures (30-40°C in soil environments) enhance microbial activity and accelerate biodegradation processes (Das & Chandran, 2011). As anaerobic biodegradation of aromatic hydrocarbons is a slow process and the time of this PhD

project was limited to 4 years, it was decided to employ ambient room temperature conditions for the experiments. This selection was made with the intention of obtaining faster results. To compensate this decision, the Arrhenius equation can be applied, enabling the estimation or prediction of the degradation rate constant at any environmental temperature (El-Naas et al., 2014). It is generally assumed that biodegradation rates are reduced at lower temperatures, as represented by the Q10 factor which describes the change in the degradation rate over a 10 °C temperature range (Davenport et al., 2022). To assess the applicability of the Arrhenius correction for biodegradation rates under environmentally relevant temperatures, Brown et al. (2020) studied the biodegradation of hydrocarbons within various temperatures. Their results showed that temperature-dependence was still evident but appeared to be lower than predicted by the Q10 factor proposed. For the findings of this thesis, Arrhenius equations can still be applied to extrapolate laboratory biodegradation rates to field conditions however, based on the findings of Brown et al., for more accurate predictions, it is recommended that any degradation simulation study for a given chemical considers test systems adapted to the intended test temperature.

7.2.3. Biodegradation experiments

As in situ bioremediation mostly relies on the metabolic capacities of microorganisms, it is important to gain insight on their biodegradation potential (Cunningham et al., 2001; Phelps & Young, 1999) before implementation of any time-consuming and expensive field applications. Natural attenuation is an effective and common method for removing contaminants like aromatic hydrocarbons from contaminated sites. However, it has limitations as it is a slow process that requires continuous monitoring of a contaminant plume, which can result in high long-term costs (Lovley, 1997). In the case of Griftpark, the slow biodegradation of the BTEXIeIaN compounds in the subsurface leads to a situation where the contaminants are transported more quickly than they can be broken down meaning that they risk to migrate from the first aquifer to the second aquifer and contaminate the drinking water supply of the city of Utrecht. Hence, strategies to enhance the biodegradation activity of the indigenous microorganisms are necessary. To achieve this, it is important to gain insights on the factors that may have an affect on the biodegradation process. In this thesis, factors that may influence the biodegradation performance of the Griftpark microorganisms were evaluated. These factors included the substrate composition and interaction (Chapter 3 and 6), redox conditions (Chapter 3, 4, 5 and 6), historical pollution/adaptation (Chapter 6) and flow (Chapter 6). Hence their impact on the microbial communities were studied (Chapter 3 and 6).

Substrate interactions in complex mixtures: Tar oil is a complex mixture containing a large number of hydrophobic compounds (Murphy et al., 2005) with different susceptibilities for microbial degradation (Mundt et al., 2003). When chemical mixtures such as tar oil compounds enter the environment, substrate interactions can take place and influence the microbial biodegradation. Positive substrate interactions include inducing the required catabolic enzymes, increased biomass growth and co-metabolism while negative interactions can cause to inhibition by toxicity, catabolite repression, competitive inhibition for enzymes, or depletion of electron acceptors (Alvarez & Vogel, 1991). The influence of multiple substrates on biodegradation kinetics depend on (i) the complexity of the mixture, for instance the chemical properties of the compounds and the number of components, (ii) the concentration of the compounds or overall chemical concentration and (iii) the microbial biomass composition whether a pure or mixed bacterial culture is used (El-Naas et al., 2014).

Numerous studies have been published on substrate interactions with BTEX compounds, conducted in both aerobic conditions (Abuhamed et al., 2004; Alvarez & Vogel, 1991; S. W. Chang et al., 2001; Deeb & Alvarez-Cohen, 1999; Reardon et al., 2000) and anaerobic conditions (Da Silva & Alvarez, 2004; Dou, Liu, & Hu, 2008b; Meckenstock et al., 2004; Phelps & Young, 1999). For instance, Abuhamed et al. observed that toluene had a stronger inhibitory effect on benzene degradation compared to the effect of benzene on toluene degradation. A similar observation was reported by Da Silva and Alvarez under nitrate-reducing conditions. These findings align with the column experiments where toluene biodegradation was unaffected by benzene, but benzene removal efficiency decreased in the presence of toluene (Chapter 6). Despite these consistent observations in Chapter 6 and the references mentioned, the literature reports inconsistent findings regarding substrate interactions among BTEX compounds. Consequently, there is no general rule for predicting the substrate interactions for BTEX mixtures (Dou, Liu, & Hu, 2008b). This variation can be attributed to differences in these studies upon substrate concentration and composition, and the use of either pure or mixed cultures. Therefore, these interactions are case-specific which underscores the importance of conducting investigations into substrate interactions within complex mixtures present in the contaminated sites and their indigenous microbial communities. Such studies can provide valuable insights into why certain compounds may persist in contaminated sites while others are degraded.

In Griftpark, a variety of chemicals are present as contaminants across the park, distributed at varying depths. This thesis specifically focused on BTEXIeIaN compounds (Benzene, Toluene, Ethylbenzene, Xylenes, Indene, Indane and Naphthalene) due to their significant prevalence in the park's subsurface. However, the distribution of the compounds varied throughout the park (Figure 7.1). For example, in Well B2, 74% of the contamination was attributed to naphthalene while in Well B, 50% of the contamination consisted of benzene, and naphthalene made up 20%. Figure 7.1 was the inspirational figure for the study presented in Chapter 3. The aim was to understand: (i) whether Griftpark microorganisms were capable of aerobically degrading the BTEXIeIaN compounds and (ii) if the slow biodegradation in the subsurface was due to the metabolic capacities of indigenous microorganisms or a consequence of substrate interactions within the mixture. As it was impossible to test all the mixture combinations present in and around Griftpark, a proof-of-principle approach was considered where the effect of indene, indane and naphthalene on BTEX biodegradation was investigated. Results showed that all compounds could be fully biodegraded aerobically, no matter the substrate combination tested, however degradation rates of the compounds differed among the set-ups indicating substrate interactions in the mixture.

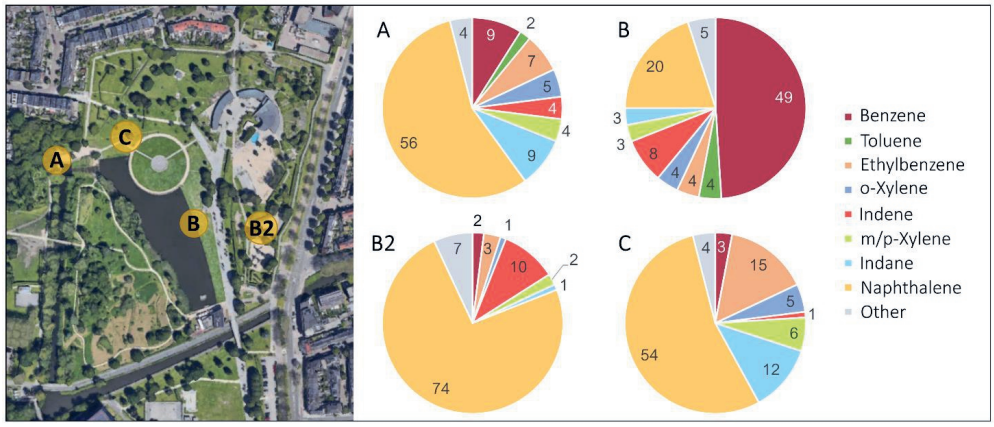

Figure 7.1 Various combinations of substrates with different percentages, identified from soil samples taken around the Griftpark through soil drillings. Very low concentrations were found in Well A, as it is located outside the park. The data was obtained from Deltares, a project partner that explored the natural attenuation potential of Griftpark.

In Chapter 3, eight different substrate mixtures were evaluated with increasing substrate complexity (BTEX to BTEXIeIaN). The fastest degradation was obtained within the less complex mixture of BTEX within 13 days while the most complex BTEXIeIaN mixture was degraded in 39 days, even though the overall hydrocarbon concentrations were similar (~22 mg L^{-1}). Results were fitted to first-order kinetics modelling which confirmed the substrate interaction in mixtures by showing that all compounds except m/p-xylene were affected by the presence of indene, indane and or naphthalene. As this was the first study to investigate the effect of indene and/or indane on m/p-xylene, it was not possible to compare our findings with other studies. However, this study showed clearly that chemical properties were an important factor in the substrate interactions as o-xylene was inhibited by the presence of indene and indane contrary to m/p-xylene even though they have very similar chemical structures. Furthermore, the number of components clearly influenced the biodegradation performance. Knightes & Peters (2006) tested substrate interactions in systems up to nine hydrocarbons, and suggested that as the number of compounds increases, the interactions become more complex. This was also confirmed within our column study (Chapter 6), where toluene biodegradation was not affected by the presence of benzene (one compound) but inhibited in the presence of eight additional compounds.

The order of biodegradation of the compounds was also evaluated within the different mixtures. In all the set-ups tested in Chapter 3, biodegradation started with ethylbenzene and/or naphthalene (if present), and o-xylene consistently was the last compound to be fully biodegraded. This information is valuable for the case of Griftpark, as in Figure 7.1, most of the contamination in the presented wells was attributed to naphthalene, meaning that if oxygen can be supplied to the subsurface of Griftpark effectively, the first compound expected to be removed would be naphthalene. It can also be assumed that once naphthalene is degraded, it can stimulate the biodegradation of the other compounds, as the mixture will become less complex, there would likely be fewer substrate interactions taking place. At the end, the subsurface scenario of Griftpark upon substrate composition was evaluated through batch experiments, in the

presence of oxygen, by employing a proof-of-principle approach that underscored the importance of working with complex mixtures.

Biodegradation of BTEXIeIaN compounds under various redox conditions: In the case of aromatic hydrocarbons, biodegradation is strongly dependent on the terminal electron-acceptor (Schreiber et al., 2004). The biodegradation rates of aromatic hydrocarbons are often reported to be higher under aerobic conditions compared to anaerobic environments (Weelink et al., 2010). For a long time it was thought that anaerobic BTEX degradation was not possible (Chakraborty & Coates, 2004). However, in recent decades, various studies have reported successful BTEX biodegradation under anaerobic conditions (Bin et al., 2002; Varjani, 2017) including sulfate-reducing (Dou, Liu, Hu, et al., 2008), nitrate-reducing (Meckenstock et al., 2016) iron-reducing (Botton & Parson, 2006), manganese-reducing (Meckenstock et al., 2016) and methanogenic conditions (Ramos et al., 2013). Naphthalene degradation has also been reported under sulfate, manganese and nitrate-reducing conditions (Langenhoff et al., 1996). A detailed review on recent studies on anaerobic hydrocarbon biodegradation was published by Wartell et al. (2021).

Despite aromatic hydrocarbons could be anaerobically biodegraded, the specificity and rates of biodegradation for each compound is redox-dependent (Schreiber et al., 2004). For example, some studies reported higher naphthalene degradation rates under sulfate-reducing conditions compared to nitrate (B. V. Chang et al., 2003; Maillacheruvu & Pathan, 2009) while Lu et al. (2012) reported the opposite, that two-ringed PAHs could be degraded faster with nitrate compare to sulfate-reducing conditions. The variations in the biodegradation performance of aromatic compounds is related to the composition of the microbial community, including the types of species present, their enzymatic capabilities, and the distinct environmental conditions they encounter (Wartell et al., 2021). Despite having a lower redox potential and Gibbs Energy value than denitrification or iron reduction, sulfate reduction appears to be more successful in hydrocarbon biodegradation, largely due to the diverse enzymatic capabilities associated with sulfate-reducing bacteria (Maillacheruvu & Pathan, 2009). It has also been reported that nitrate-reducers are more sensitive to environmental and chemical changes, which can lead to inhibition in the biodegradation process (Wartell et al., 2021).

To determine the most effective electron acceptor for the biodegradation of the BTEXIeIaN mixture, a series of experiments under different redox conditions were conducted throughout this thesis. All compounds of the BTEXIeIaN mixture could be fully removed under aerobic conditions, in a period of 39 days which is relatively rapid (Chapter 3). The sediment used in the aerobic experiments originated from a depth of 30-40 meters below ground level, indicating that the microorganisms within the subsurface of Griftpark possess aerobic degradation capabilities. In Chapter 4, anaerobic conditions were assessed for the biodegradation of the BTEXIeIaN mixture with nitrate or sulfate as electron acceptors. The sediment samples used in anaerobic experiments were originating from the same location as those employed in the aerobic experiments. Despite originating from an anaerobic sediment source, indigenous microorganisms displayed a notable difference in biodegradation performance between aerobic and anaerobic conditions. For nitrate-reducing conditions, in a period of 400 days, only ethylbenzene was partially degraded while all the other compounds were recalcitrant (Chapter 4). For sulfate-reducing conditions, toluene, o-xylene were fully removed at day 300, and m/p-xylene was completely degraded in 440 days. Hence, all the other compounds were not fully degraded (Chapter 4). The biodegradation of the BTEXIeIaN compounds under various redox conditions, studied in this thesis, was summarized in Table 7.1.

Table 7.1 Summary of the compounds that were biodegraded under specific redox conditions in this thesis.

	Chapter 3	Chapter 4		Chapter 5
		Redox conditions		
Compound	**O_2**	**NO_3^-**	**SO_4^{2-}**	**Low O_2**
Benzene	+	-	-	+
Toluene	+	-	+	+
Ethylbenzene	+	±	-	+
o-Xylene	+	-	+	+
Indene	+	-	-	+
m/p-Xylene	+	-	+	+
Indane	+	-	-	+
Naphthalene	+	-	-	+

+: fully degraded, ±: partially degraded, -: not degraded

As demonstrated, biodegradation of BTEXIeIaN compounds was very slow or incomplete under anaerobic conditions (Table 7.1). When comparing the biodegradation performances of indigenous microorganisms under aerobic and anaerobic conditions, it was observed that for certain compounds, the removal process took nearly ten times longer in the absence of oxygen. On one hand, this finding is logical, considering that oxygen is a very strong and energetically favorable electron acceptor (Schreiber et al., 2004). On the other hand, it might be expected that microorganisms adapted to anaerobic conditions for decades would perform better under anaerobic conditions. When comparing nitrate to sulfate-reducing conditions, despite nitrate being a more oxidative electron acceptor (Christensen et al., 2000; Lovley, 1997), the performance of indigenous microorganisms with sulfate was faster (Table 7.1). The sediment, sourced as an inoculum for both experiments, originated from sulfate-reducing conditions hinting that the origin of the sediment might be an important factor affecting the biodegradation process. Since microorganisms were adapted to sulfate-reducing conditions, it is likely that they performed better with sulfate contrary to nitrate. This observation was an inspiration for the column experiments (Chapter 6), as the pollution history, in this case previous exposure to sulfate, clearly influenced the biodegradation process of certain compounds. However aerobic conditions demonstrated greater effectiveness compared to sulfate-reducing conditions (Table 7.1). This controversy might hint that, redox conditions might impose a stronger selection pressure than pollution history, which was further evaluated in Chapter 6.

Another interesting finding was that based on the electron acceptor, different compounds were biodegraded with different order. For instance, under aerobic conditions, ethylbenzene or naphthalene (if present) were the first compounds to be biodegraded, while o-xylene was the last (Chapter 3). Under nitrate-reducing conditions, ethylbenzene maintained its status as the primary compound to be degraded however, contrary to aerobic conditions, naphthalene did not take the lead in biodegradation; instead, it was recalcitrant. Surprisingly, o-xylene, less favored for aerobic biodegradation (Chapter 3), was among the first compounds to degrade alongside toluene under sulfate-reducing conditions (Chapter 4). Notably, ethylbenzene displayed recalcitrance under sulfate-reducing conditions (Chapter 4). This implies that the biodegradation sequence of compounds is influenced by the type of electron acceptor present, and various compounds display varying levels of biodegradation activity under distinct redox conditions. This finding aligns with the study by Phelps & Young (1999) where, under nitrate-reducing conditions, the investigated microbial consortium initially degraded toluene, followed by ethylbenzene and m-xylene. In

contrast, under sulfate-reducing conditions, the degradation order was reported as toluene, followed by o-xylene and m-xylene. These results demonstrate that certain compounds are degraded in distinct orders within different redox conditions even though the microbial consortium had the same origin.

In conclusion, results of Chapter 3 and 4 showed that for a rapid removal of the BTEXIeIaN mixture, oxygen is needed. However, supplementing oxygen to the subsurface can be challenging and costly. Therefore, a decision was made to incorporate oxygen from a technological standpoint by combining the utilization of low oxygen levels with nitrate (Chapter 5). The strategy of pre-exposure to nitrate-reducing conditions, followed by the addition of low oxygen concentrations, efficiently directed oxygen toward BTEXIeIaN biodegradation. By supplying nitrate first, the denitrifying microorganisms consumed the organic matter through denitrification process, leaving BTEXIeIaN as the only carbon source for subsequent aerobic biodegradation. A similar approach was discussed by Da Silva et al. (2005), where they suggested to provide anaerobic electron acceptors to enhance the biodegradation of BTEX compounds in the absence of oxygen but also to reduce the biological oxygen demand in the system, allowing the available subsequent oxygen used for BTEX removal. In our study (Chapter 5), BTEXIeIaN was not biodegraded under nitrate-reducing conditions within the given time frame. However, nitrate was used for organic matter removal, which led to a more efficient oxygen utilization when low oxygen concentrations was later introduced into the system for hydrocarbon removal. This strategy can help to design future in situ bioremediation applications and help to reduce the cost, as less oxygen will be required. Findings of Chapter 5 also showed that indigenous microorganisms could quickly adapt from fully anaerobic conditions to microaerobic conditions. This was not surprising, as in Chapter 3 and 4, the biodegradation capabilities of microorganisms under both aerobic and anaerobic conditions were demonstrated.

Biodegradation of BTEXIeIaN compounds with flow conditions: In this thesis, both batch and column experiments were performed to understand certain environmental factors influencing the biodegradation process of the BTEXIeIaN compounds. Batch experiments are closed systems, where homogeneous conditions are present. The constant mixing within the bottles, as the bottles were kept on shakers throughout the experiment, allows for consistent concentration of contaminants within the system. Whereas, column experiments are lacking from this consistent mixing, resulting in a heterogeneous environment. The flow provided, can cause a concentration gradient in the liquid phase along the column length, leading to the formation of layers with differing concentrations unlike the well-mixed system observed in batch experiments. While this might appear disadvantageous, column experiments are more representative of the in situ environment, as the groundwater flow naturally introduces heterogeneity and intricate patterns in the subsurface, rendering predictions about chemical transport and fate challenging (Hatipoğlu-Bağci & Motz, 2019). Hence, in this thesis, column experiments allowed to simulate conditions closer to those present in Griftpark's subsurface. While it is impossible to replicate the exact subsurface conditions of the site in the laboratory, attempts were made to establish boundary conditions that closely resemble the in situ environment. Batch experiments were important for assessing preliminary steps to gain insights into factors affecting biodegradation process while column studies allowed to evaluate hydrodynamic conditions from a more representative perspective to real-life situations. Therefore, the concepts explored in previous chapters within batch experiments (Chapter 3 and 4) were further tested through column experiments (Chapter 6).

Effect of the flow dynamics: In literature, several studies have been published on BTEX compounds and/or naphthalene using continuous systems (Da Silva et al., 2005; Da Silva & Alvarez, 2004; S. R. Hutchins, 1991; Langenhoff et al., 1996; Meckenstock et al., 2004). In the study by Da Silva & Alvarez (2004), anaerobic columns were operated for three years. Toluene was biodegraded within two years, while benzene remained recalcitrant. In the study of Langenhoff et al. (1996), toluene was biodegraded within one to two months under various electron acceptors (nitrate, sulfate, iron, manganese, and bicarbonate). Naphthalene was partially biodegraded with nitrate and manganese and completely removed with sulfate. However, benzene remained recalcitrant over the experimental period of 375 to 525 days. These results suggest that certain compounds may require extended timeframes for complete removal. In the case of benzene, it was often reported as recalcitrant despite extended experimental durations.

In this thesis, both toluene and benzene could be biodegraded in column experiments, under both nitrate and sulfate-reducing conditions. Toluene biodegradation was reported above 90% while benzene biodegradation was mostly above 50% under all substrate combinations tested. Differently, in Chapter 4, where biostimulation was assessed in batch experiments by addition of nitrate or sulfate, it was observed that toluene was biodegraded under sulfate-reducing conditions, and not nitrate, while benzene was recalcitrant regardless of the redox situation over the period of 440 days (Table 7.1). The success in toluene and benzene biodegradation in the columns, under all the given substrate combinations and redox conditions, can be attributed to the alternating flow/batch approached implemented into the system. In the flow mode, continuous replenishment of nutrients and contaminants eliminated any limitations imposed by factors such as insufficient nutrient availability and low contaminant concentrations. Additionally, flow mode prevented the accumulation of inhibitory by-products, such as nitrite or sulfide, which can be toxic to nitrate and sulfate-reducing microorganisms, respectively (Wartell et al., 2021). Furthermore, the flowing medium exerts selective pressure, resulting in the removal of non-adaptive microorganisms from the system. In subsequent batch mode, the selected and succeeding microbial groups are allowed to biomass growth, and therefore biodegradation is stimulated. Due to this selection pressure, along with continuous nutrient and carbon source replenishment and removal of inhibitory products, compounds were biodegraded in a relatively shorter time compared to the experiments performed in batch bottles (Chapter 4), and the studies mentioned earlier.

Microbial adaptation: The success of anaerobic toluene and benzene biodegradation in the columns under both redox conditions can also be attributed to factors related to historical pollution and adaptation, in addition to the influence of flow. The pollution history factor was mentioned by Phelps & Young (1999) where anaerobic BTEX biodegradation was studied under various redox conditions within four different sediment originating from pristine or polluted sites. They observed that BTEX removal rates were higher in sediments with a long history of pollution. In general, microorganisms do not degrade contaminants upon initial exposure but they can develop the capability to degrade contaminants after prolonged exposure by several mechanisms such as metabolic adaptation including enzyme induction, biomass growth of degraders, and horizontal gene transfer (H. Huang et al., 2021; Lueders, 2017).

In this thesis, batch experiments presented in Chapter 4, gave a hint of the fact that pollution history can have an influence on biodegradation of the BTEXIeIaN compounds. This was shown with the bioaugmentation experiments under nitrate-reducing conditions. Bottles that received only nitrate (biostimulation with nitrate addition) were compared to the bottles that were bioaugmented with the Griftpark inoculum enriched with only toluene (biostimulation with nitrate + bioaugmentation). Findings showed that the set-up bioaugmented with toluene-enriched Griftpark inoculum degraded toluene more

efficiently compared to the ones that were not bioaugmented. Then, it was hypothesized that microorganisms could be 'trained' for the biodegradation process. This is why, in the column experiments, the indigenous microorganisms were 'trained' by feeding the columns subsequently with different substrates of increasing complexity (Chapter 6). For instance, Column 3 was first fed with toluene (T), followed by duplex mixture (T+B) and finally the complex mixture (BTEXIeIaN) while almost the opposite was tested with Column 4, where the column was first fed with the complex mixture (BTEXIeIaN) and then with a simplified substrate of duplex mix (T+B). Microorganisms, initially introduced to a single source of carbon, exhibited enhanced performance when they were further exposed to a mixture, particularly in comparison to those encountering the mixture without the benefit of a prior exposure period. Therefore, bioaugmentation strategies using indigenous microorganisms, where they are exposed to various substrates with increasing complexity, can be successful in achieving complete removal of all the compounds within the mixture, simultaneously.

Testing the 'training process' of the indigenous microorganisms through column experiments can be more meaningful compared to batch experiments, as in columns, the non-degrading and not selected microorganism would be flushed out of the system while the biomass of the active degraders would increase. In a scenario where all the components of the BTEXIeIaN mixture are sequentially introduced to the column, with increasing complexity, can allow to the development of a microbial consortium capable of simultaneously degrading all the BTEXIeIaN compounds. Moreover, this process can help microorganisms in gradually developing tolerance to the inhibitory effects of the hydrocarbons on each other, discussed previously as the substrate interactions (Chapter 3).

Testing single compound vs complex mixtures: In Chapter 3, the findings demonstrated that as the mixture becomes less complex, the inhibitory impact resulting from substrate interactions also decreases. To validate this concept under anaerobic conditions, columns experiments were performed by an increasing substrate complexity (from single compound to a complex BTEXIeIaN mixture). This approach helped to gain insight into the anaerobic biodegradation potential of individual compounds by the Griftpark microorganisms and allowed for a comparison with the biodegradation occurring within a mixture. When present as single compound, both toluene and benzene were biodegraded relatively rapidly under anaerobic conditions. However toluene's biodegradation was slightly hindered within a mixture compared to its standalone or together with benzene, while benzene biodegradation was inhibited by toluene, and not as much from the complex mixture. These findings demonstrated that, even though Griftpark microorganisms are capable to degrade both compounds individually, their biodegradation was inhibited due to having multiple substrates simultaneously, where interactions between hydrocarbons most likely hindered their biodegradation process. Therefore, substrate interactions observed within aerobic conditions (Chapter 3), were also validated under anaerobic scenarios (Chapter 6). It was concluded that, the limiting factor of the biodegradation process in Griftpark's subsurface was not attributed to indigenous microorganisms' degradation capacity, since they could degrade the compounds individually or within simpler mixtures, but more about the fact of having a complex mixture composed of nine aromatic hydrocarbons.

In contaminated sites, such as Griftpark, not only one contaminant is present but due to intense industrial activities, many different contaminants can be present simultaneously, as a mixture. Then, what is the purpose of performing single compound experiments, knowing that they could be biodegraded and why is it relevant for in situ applications dealing with complex mixtures? When considering the physical and chemical properties of aromatic compounds, such as solubility in water, octanol-water coefficient, organic

carbon partition coefficient, they are important characteristics which determine the distribution of the compounds in the subsurface (Lyman et al., 2020). For instance, based upon these characteristics, the relative mobility of the BTEX compounds in groundwater aquifers was predicted to be as xylene < ethylbenzene < toluene < benzene (Odermatt, 1994). When considering the distinct transportation rate of the BTEXIeIaN compounds, a natural chromatographic separation of the compounds can be observed within the subsurface of Griftpark (Figure 7.2). This separation might lead to plume segments containing single components and it is possible that all compounds could biodegrade before the groundwater reaches the second aquifer. For example, given benzene's high mobility, its plausible that at some point, only benzene might be present at the plume's fridge, meaning that at in situ conditions, it's conceivable that benzene biodegradation could occur. Additionally, in Chapter 6 biodegradation of aromatic hydrocarbons was assessed as a transition from a complex mixture (BTEXIeIaN) to a simpler substrate (T+B) under nitrate-reducing conditions (Column 4). The results demonstrated that after an extensive exposure to the complex mixture, when the column was subsequently supplied with the simpler substrate mix, the biodegradation of toluene and benzene notably improved. This phenomenon can be perceived as a representation of the earlier mentioned chromatographic effect. However, further investigations will be necessary to validate the occurrence of such a phenomenon in the subsurface, as other physical, chemical, and biological factors may also affect the distribution of the BTEXIeIaN compounds, including site heterogeneity, dissolution, sorption and natural attenuation (Valsala & Govindarajan, 2018).

In conclusion, the column experiments presented in Chapter 6 allowed for more intricate environmental manipulations compared to batch studies, as they were conducted over a longer term with frequent sampling. Frequent sampling was not an issue in column experiments due to the system's rapid self-recovery capabilities. This phenomenon is much more complex to control with batch experiments, as close systems often restrict the routine sampling procedure. The act of frequent sampling can progressively disrupt the experiment itself, as each sampling procedure introduces disturbances to the system. Notably, actions undertaken in batch experiments are difficult to reverse unless significant manipulations are implemented, consequently hindering the experiment. Thus, in this context, we highlight another noteworthy advantage of column studies, where long term experiments can be performed and multiple factors can be simultaneously tested, resulting in achieving more realistic outcomes.

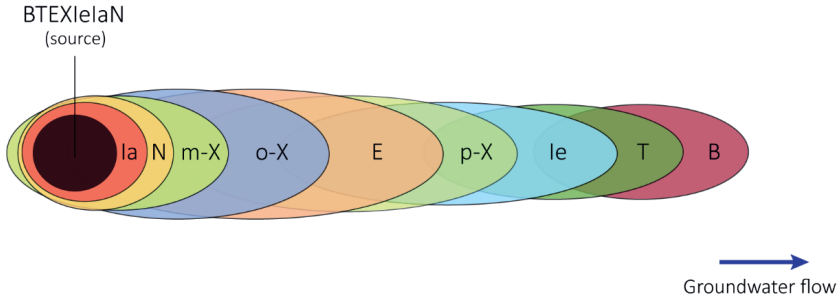

Figure 7.2 Illustration of the distribution of the BTEXIeIaN compounds in the subsurface based on their retardation factors calculated by their physical and chemical properties (K_{ow}, K_{oc} and K_d).

7.2.4. Microbial community analysis

Microorganisms play a vital role in the breaking down process of the contaminants, and the success of this process relies on their distinct and diverse metabolic pathways (Kebede et al., 2021). Therefore, understanding the community structure, its diversity, and the interactions among microorganisms is crucial in defining their aromatic hydrocarbon degradation potential and their specificity towards those compounds.

A consortium rather than a single species is more effective in the degradation of aromatic hydrocarbon mixtures (Farhadian, Duchez, et al., 2008) because most of the hydrocarbon degrading bacteria are unable to catabolize various types of hydrocarbon compounds at once due to the fact that they have limited catabolic enzymes (Kebede et al., 2021). Anaerobic BTEX biodegradation is a complicated process, and various microbes coexist and interact with each other to form intricate networks (Wu et al., 2022). For instance, benzene was found to be degraded via syntrophic interactions between *Peptococcaceae* and *Desulfobulbaceae*. While *Peptococcaceae* breakdown benzene into H_2 under iron-reducing conditions, *Desulfobulbaceae* benefits from this breakdown by using the hydrogen gas as an energy source (Kunapuli et al., 2007). It was also shown that H_2/acetate produced from the benzene breakdown by *Peptococcaceae*, under nitrate-reducing conditions, were further utilized by *Betaproteobacteria* (van der Zaan et al., 2012). In another study, microbial cocktails were prepared containing two species: *P. putida* F1 and *P. stutzeri* OX1. These species have shown the capability to biodegrade certain BTEX compounds and not all of them. The study involving a mixed culture and mixed substrate revealed that the composition of these species in the formulated microcosms significantly contributed to the efficient and rapid removal of all the BTEX compounds (Nagarajan & Loh, 2015). Therefore, when dealing with complex mixtures such as BTEXIeIaN, a microbial consortium would be more advantageous than pure cultures as they would harbor diverse species with distinct metabolic pathways and enzymatic capabilities. This diversity can allow to the degradation of a broader range of compounds, increasing the efficiency of contaminant removal. In the case of this thesis, a microbial consortium was studied which can explain the success in the complete biodegradation of all the compounds in all the mixture combinations, under aerobic and microaerobic conditions (Chapter 3 and 5). For anaerobic conditions, employing a mixed-electron approach can be advantageous as it could promote the growth of diverse microbial groups and may contribute to the simultaneous biodegradation of a wider range of compounds.

Organic pollutants exert influence on various ecological processes within the ecosystem. This influence, in turn, contributes to the selection of microbial degraders hence the biodegradation process (S. Jiao et al., 2016). In Chapter 3, BTEXIeIaN compounds and their combinations were investigated to understand the influence of the various substrate mixtures on the microbial community. Microbial analyses showed that distinct substrate mixtures led to differences in the microbial community composition. This was studied by Jiao et al. where the presence of different pollutants (phenanthrene, n-octadecane and $CdCl_2$), and their combinations influenced the bacterial community by variations of the composition and relative abundance of the phylogenetic groups (S. Jiao et al., 2016). In this thesis, indene had the strongest influence on the BTEX microbial community as set-ups harboring indene showed high relative abundance of *Micrococcaceae* family, hinting that members of the *Micrococcaceae* family may play an important role in indene degradation (Chapter 3). Additionally, it was proposed that *Comamonadaceae* might be responsible for BTEX biodegradation as it displayed the highest abundance within the BTEX set-up (Chapter 3). Similar investigations were performed in Chapter 6, within column experiments. When investigating the abundance of each microbial group within the samples, it was observed that all the samples collected

from Column 2 resulted in low abundance of *Pseudomonas*, which could be attributed to the fact that Column 2 was never exposed to the complex mixture of BTEXIeIaN. This observation was further validated by the fact that samples exhibiting a significant abundance of *Pseudomonas* were consistently associated with samples collected from the other columns (C1, C3 and C4) when fed with the BTEXIeIaN mixture. Therefore, findings observed in both Chapter 3 and 6 demonstrated that substrate composition have an influence on the microbial community dynamics.

Redox conditions play an important role in determining the microbial community structure and function (Wu et al., 2022). In Chapter 6, beta-diversity analysis was performed to discern variations in microbial communities across diverse parameters (redox conditions, substrate composition and its exposure order, flow/batch mode, location in the column). Notably, the most distinct clustering was observed within different redox conditions; with Column 1, the sulfate-reducing column, differentiating the most from the other nitrate-reducing columns. Microbial abundance analysis showed that, different microbial groups were abundant under sulfate and nitrate-reducing conditions. Moreover, the abundant groups under anaerobic conditions (Chapter 6) differentiated from the ones reported under aerobic conditions (Chapter 3). Nevertheless, it is important to mention that certain members were found in abundance in both aerobic and anaerobic experiments, such as *Pseudomonas, Acidovorax, Mesorhizobioum, Sulfuritalea, Sedimenibacterium* and *Sulfuricella* (Chapter 3 and 6). This could be attributed to the shared origins of sediment samples, sourced from proximate locations for all experiments. However, species like *Pseudomonas* and *Acidovorax* were reported in aromatic hydrocarbon biodegradation studies under both aerobic and anaerobic conditions (Aburto & Peimbert, 2011; Benedek et al., 2018; Fahy et al., 2006; Y. Huang & Li, 2014). Due to their versatile biodegradation capacity, they can be proposed as potential key candidates for in situ aromatic hydrocarbon bioremediation. To confirm this statement, enzyme activity assays or gene expression analysis should be performed to better understand the biodegradation capabilities of these microbial groups under various redox conditions. This is important because their abundance does not necessarily indicate their effectiveness in breaking down aromatic hydrocarbons.

A study investigated microorganisms responsible for the biodegradation of aromatic compounds in the Griftpark groundwater treatment pipeline (Hauptfeld et al., 2022). The pipeline microbiome showed two distinct groups of microorganisms, shifting from mainly anaerobic organisms in the park groundwater to a highly specialized aerobic community in the WWTP. The diversity of the microbial communities in the park-side was higher than in the WWTP, which contained many known aromatic hydrocarbon degradation genes. It was suggested that oxygen availability, rather than mixture of communities, drove the increase of aromatic hydrocarbon degradation pathways, leading to selection of effective aromatic hydrocarbon-degrading microbial groups. Their hypothesis can be supported by the findings within this thesis, where all the BTEXIeIaN compounds were fully removed in the presence of oxygen (Chapter 3 and 5) and not under anaerobic conditions (Chapter 4). Due to the thorough sampling of the treatment process from beginning to end, their results helped to understand how the microbial community changed at each step in bioremediation process. In parallel to their study, this thesis also showed the microbial community dynamics from fully anaerobic conditions (Chapter 6) to fully aerobic conditions (Chapter 3) and defined some key members that were in abundant under both conditions. Additionally, this thesis demonstrated the aerobic and anaerobic degradation potential of the deep-subsurface microorganisms of Griftpark.

At the end, the adaptability of the deep-subsurface microorganisms of Griftpark was shown by both biodegradation data (Chapter 3, 4, 5 and 6), and microbial analysis (Chapter 3 and 6). These findings can contribute for the design of pilot studies, where the versatile capacity of the indigenous microorganisms can be utilized to optimize and enhance bioremediation processes in the subsurface of Griftpark.

7.3. Pilot studies at Griftpark

The current application of pump and treat technology is not feasible for Griftpark, as it could potentially take several decades to clean-up the highly contaminated subsurface. In contrast, bioremediation offers a more a sustainable solution. While the operating cost of bioremediation might initially appear higher than those of pump and treat, its shorter operating timeframe usually results in overall reduction in total cost. Furthermore, bioremediation brings about additional benefits beyond time and cost considerations, such as decreased requirements for maintenance, labor, and supplies (National Research Council, 1993).

In highly contaminated sites, the excess electron donors (hydrocarbons) compared to the oxidation potential of electron acceptors can limit the biodegradation process due to the low redox potential and depletion of electron acceptors (Meckenstock et al., 2015). Lower redox potential results in anoxic conditions and reduces the rate of biodegradation, which is evident in the subsurface environment of Griftpark. This thesis has demonstrated that the indigenous microorganisms of Griftpark could biodegrade all compounds of the BTEXIeIaN mixture in the presence of oxygen (Chapter 3 and 5) and certain compounds under anaerobic conditions (Chapter 4 and 6). This indicated that the metabolic capacity required for the degradation of BTEXIeIaN compounds exists within the indigenous microorganisms, however inhibiting factors have been identified as: i) complexity of the contaminant mixture and ii) the absence of suitable electron acceptors. Building on these findings, four pilot projects are being developed as remediation approaches in Griftpark. These projects involve in situ biostimulation strategies by addition of oxygen, nitrate, sulfate and ex situ treatment of contaminated groundwater in a constructed wetlands in combination of microbubbles and activated carbon (Figure 7.3). In the following section, the decisions upon the pilot studies were discussed.

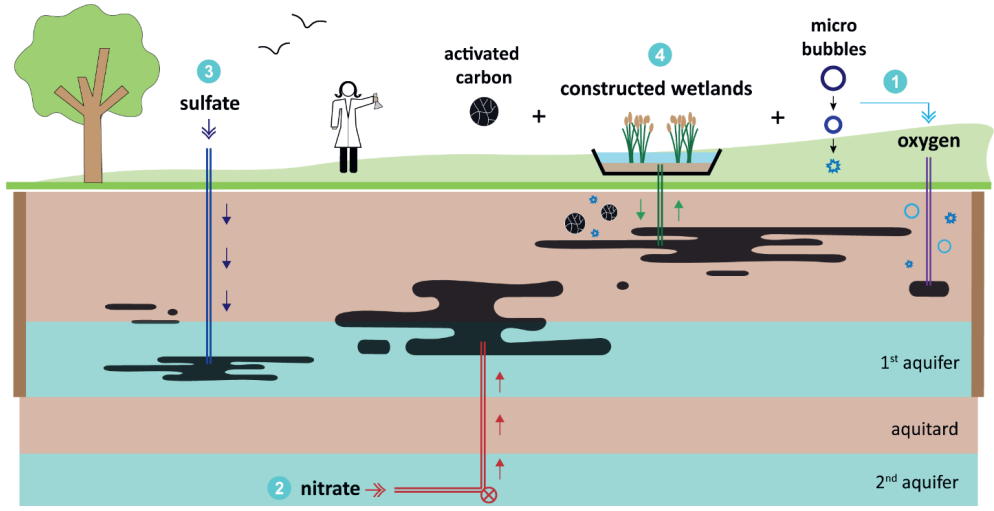

Figure 7.3 Pilots studies planned at the subsurface of Griftpark. **1**: In situ biostimulation with oxygen via microbubbles, **2**: In situ biostimulation with nitrate, **3**: In situ biostimulation with sulfate, **4**: Ex situ treatment in constructed wetlands combined with activated carbon and microbubbles.

7.3.1. In situ biostimulation with oxygen addition via microbubbles

To enhance the biodegradation process of the contaminants, oxygen is planned to be supplied to the subsurface of Griftpark by use of microbubbles. The findings presented in Chapters 3 and 5 demonstrated that, in the presence of oxygen, all the compounds of BTEXIeIaN mixture could be fully and rapidly biodegraded. Therefore, when dealing with urgent removal of these contaminants at a site like Griftpark, ensuring the subsurface receives a sufficient oxygen supply emerges as the most favorable approach. However, introduction of oxygen to the subsurface is often challenging. For instance, oxygen gas has limited water solubility and other oxygen carriers, such as H_2O_2 and liquid O_2, can be very costly and not always effective (National Research Council, 1993). In Chapter 5, we proposed a strategy of adding lower oxygen concentrations, at intermittent intervals. This created microaerobic conditions, and promoted microbial growth and enabled aerobic biodegradation, which may contribute to a potential cost reduction. The low transfer rate of oxygen from air to the water phase and rapid depletion of oxygen by the microbes makes the air sparging technology less attractive. While H_2O_2 can help to increase the biological activity, its rapid enzymatic decomposition offsets the benefits (National Research Council, 1993). The difficulties associated with oxygen delivery have led to the proposal of an alternative approach: microbubbles. Microbubbles are minute bubbles with diameters on the micrometer and can even be on nanometer scale (Nanobubbles). Their advantage over the other proposed oxygen injection methods is that they maintain their structural integrity for extended periods of time, facilitating gradual oxygen transfer as groundwater passes by, thus enhancing oxygen transfer efficiency (Haris et al., 2020). By considering its advantages, use of microbubbles can be an effective remediation strategy for the removal of BTEXIeIaN mixture from the subsurface of Griftpark.

7.3.2. In situ biostimulation with nitrate or sulfate injection

Two different biostimulation strategies have been planned to enhance the anaerobic biodegradation of contaminants within Griftpark. The first strategy involves introducing nitrate into the contaminated area (1st aquifer) by pumping nitrate-rich groundwater from the 2nd aquifer. The second strategy entails supply of sulfate to the contaminated zone through the use of monitoring wells. Based on the finding of this thesis, under nitrate-reducing conditions, ethylbenzene was the only compound to be (partially) biodegraded by the indigenous microorganisms while under sulfate-reducing conditions, toluene and all the xylene isomers could be fully removed (Table 7.1). Results from column studies showed that, under both redox conditions, toluene and benzene could be biodegraded despite to differences in their removal efficiencies (Chapter 4). These findings showed that indigenous microorganisms exhibit varying biodegradation patterns for specific BTEX compounds depending on the prevailing redox conditions. Thus, an effective strategy for enhancing the biodegradation process at Griftpark's subsurface, the supplementation of nitrate or sulfate to the contaminated zones could be useful. However, addition of a single electron acceptor might selectively stimulate the degradation of only certain compounds. MGP sites like Griftpark, often harbor mixtures of pollutants and for such cases, hence a mix-electron approach can provide a greater total electron acceptor capacity than a single electron acceptor and may allow to a simultaneous removal of all the aromatic hydrocarbons by the indigenous microbial consortium. For instance, the addition of a sulfate-nitrate mixture might concurrently enhance the removal of BTEX compounds. However, it's worth noting that indene, indane, and naphthalene were not biodegraded in any of the tested anaerobic scenarios, suggesting that they might necessitate oxygen as a strict electron acceptor. In such cases, sequential treatment schemes need to be planned. This concept was introduced in Chapter 5, where a pre-nitrate-reducing condition was established in batch bottles before the addition of a low oxygen concentration. Even though the BTEXIeIaN compounds were not biodegraded under nitrate-reducing conditions, within the tested timeframe, this sequential approach of introducing electron acceptors led to a more efficient utilization of oxygen by the indigenous microorganisms for the biodegradation process.

7.3.3. Ex situ treatment of contaminated groundwater in a constructed wetland in combination with microbubbles and activated carbon

In addition to in situ strategies, an ex situ strategy is planned for the Griftpark site, which involves setting up constructed wetlands with a combination of microbubbles and activated carbon. Constructed wetlands are a natural-based treatment technology, consisting of a support matrix, plants, and microorganisms (Imfeld et al., 2009). Use of constructed wetlands for removal of industrial effluent is becoming more and more popular due to their recognized advantages like being sustainable and environmentally friendly approach that do not require regular monitoring, high operation costs (Imfeld et al., 2009). Although this technology has the potential to be a suitable remediation strategy, it comes with some challenges such as phytotoxicity, slow biodegradation rates at lower temperatures, and routine monitoring requirements, including media porosity and clogging issues. Another concern is that, in case of phytovolatilization, contaminants are transferred from the groundwater to the atmosphere instead of being biodegraded, therefore it should be avoided in the populated areas (Jain et al., 2020). The combined use of microbubbles and activated carbon in a constructed wetland system may allow a synergistic effect that maximizes the removal of contaminants from the water, and eliminate the factors that would cause a disruption to the surrounding environment.

The outcomes of these pilot studies will determine whether the laboratory findings (presented in this thesis) can be effectively applied in practical field conditions (during pilot studies). Before considering large-scale remediation methods, it's essential to conduct smaller-scale pilot studies in specific areas of the site. These preliminary tests will provide insights into the most suitable approach for addressing the Griftpark contaminants. The goal is to identify the strategy that can achieve the most removal of the pollutants in a more sustainable and cost-efficient manner.

7.4. Recommendations

7.4.1. Further molecular-scale investigations

For cost effective site remediation strategies, information on site's chemistry (contaminants, intermediate products), geochemistry (redox conditions, available electron acceptors or electron donors), and microbiology (genera/genes responsible for biodegradation) is needed (National Research Council, 1993). In this thesis, attempts were made to replicate experiments under conditions similar to those of Griftpark with the aim to investigate both natural attenuation and biostimulation strategies within laboratory conditions. Our findings showed that Griftpark microorganisms can biodegrade the BTEXIeIaN compounds under different redox conditions. However, microorganisms' biodegradation activity were not confirmed at molecular level, where an assessment of enzymatic activity could have provided further insights. For instance, in both batch and column experiments, it would have been valuable to examine key catabolic genes associated with, for instance, BTEX biodegradation such as benzyl succinate synthase (Bss) or anaerobic benzene carboxylase (ABC), to validate the BTEX biodegradation activity of the indigenous microorganisms. It's important to note that the presence of the gene does not necessarily indicate its expression or the activity of the microorganisms. Therefore, it is proposed to monitor the gene expression over time, which involves comparing the quantification of the genes at the beginning and end of the experiment, by use of qPCR, and validate with decreasing contaminant concentrations and biochemical data. Another approach could be conducting RT-qPCR analysis, which quantifies the RNA transcribed from the genes, and provide direct information upon the catabolic enzymes activity.

In the case where the genera or genes responsible for the biodegradation are unknown, metagenomic analysis via Next Generation Sequencing (NGS) could be employed. By performing hierarchical clustering, samples with similar microbial composition can be grouped and correlated with specific contaminants. Alternatively, changes in microbial diversity can be monitored overtime by Principle Coordinate Analysis, similar to what has been performed in this thesis (Chapter 3 and 6). Given that this thesis investigated the biodegradation of a complex mixture of BTEXIeIaN, investigating multiple genes could indeed pose an extra challenge. However, when conducting experiments with single substrate, in the case of toluene which was the sole source of carbon at the start of column experiments (Chapter 6), toluene dioxygenase (TDO) could have been investigated throughout the experiment for a direct evidence of the biodegradation activity. In both batch and column experiments, an additional suggestion could have been to detect key intermediate products of aerobic and anaerobic biodegradation of aromatic hydrocarbons. This could have provided supplementary evidence of biodegradation activity by the indigenous microorganisms.

7.4.2. Use of in situ microcosms

Following preliminary laboratory investigations, prior to advancing to pilot studies, the utilization of in situ microcosms (ISMs) could be a time and cost-effective approach. ISMs serve as sampling units deployed within existing monitoring wells to evaluate natural attenuation and engineered bioremediation under in situ conditions (Taggart & Clark, 2021). ISM studies offer notable advantages over laboratory microcosms, which struggle to replicate exact in situ conditions, and pilot field studies, which are often expensive. After incubating the ISM units within monitoring wells, for a relatively short period of 30 to 60 days, the samplers can be analyzed for microbial, chemical, and geochemical parameters. For the case of Griftpark, ISMs could be employed, for instance, to compare natural attenuation with sulfate-based anaerobic biostimulation within the site conditions. At the end of a 60 day period, samples retrieved from both units, the un-amended unit and the sulfate-amended unit, could be subjected to qPCR analysis to detect BTEX-degrading genes, enabling a comparison between the two units. For example, if the un-amended unit shows lower BTEX-degrading genes compared to the amended unit, it will mean that sulfate addition stimulated the growth of bacteria capable of anaerobically degrading BTEX. By having multiple ISMs across various monitoring wells throughout the site, concise conclusions can be drawn within a relatively short timeframe. This approach eliminates the necessity of engaging in costly and time-consuming pilot studies. However, the feasibility of implementing these measures at Griftpark would require further communication and collaboration with the stakeholders.

7.4.3. Bioelectrochemical systems as a trending topic in aromatic hydrocarbon biodegradation studies

Bioelectrochemical systems (BES) have emerged as an innovative technology that has gained significant attention in recent years, particularly within the field of aromatic hydrocarbon biodegradation (Yang et al., 2020). Microbial BES are electrochemical devices consisting of one or two compartments, in which an anode and a cathode facilitates redox reactions catalyzed by microorganisms. Electrons are produced by microorganisms through the oxidation of organic matter and transferred to the anode. Then, they flow via a conductive material to the cathode where they can be utilized for biotic or abiotic reduction reactions (Kronenberg et al., 2017). By providing continuous electrons, BES offers an attractive solution to the challenges posed by conventional in situ biostimulation methods, which require periodic replenishment of proper electron acceptors (Yang et al., 2020). By providing a permanent supply and withdrawal of electrons for microorganisms, BES offers a cost-effective and low-maintenance approach.

Most BES studies, focusing on the removal of organic pollutants, are limited to laboratory or pilot-scale experiments. Furthermore, the majority of the studies published so far have addressed the treatment of groundwater containing single contaminants and limited attention has been directed towards complex mixtures of contaminants. While BES offers notable advantages, there are still several areas that require improvement for its utilization for in situ bioremediation purposes. Future studies should focus on the (i) progressive scale-up of the technology, (ii) enhancing electricity output, and (iii) improving the efficiency of aromatic hydrocarbon degradation within mixed contaminant scenarios.

7.5. Conclusion

Various bioremediation approaches are being tested and applied to a wide range of contaminants, including chlorinated solvents, pesticides and polychlorinated biphenyls. It is aimed that, the principles of practice outlined in this thesis can be extended to a much broader spectrum of contaminants. Hence, this thesis did not approach Griftpark simply as a case study but as a source of inspiration where a proof-of-principle approach was adopted with the goal of providing insights for the design and optimization of remediation projects that applies not only to MGP sites but also at any chemically contaminated area.

As we continually advance methods for remediating environmental issues, equal attention should be given to preventing contamination in the first place. MGP sites were once the pride of the industrialized world, but today they only represent environmental liabilities (Hatheway, 1997). Similarly, we are repeating past mistakes with the massive plastic production and inadequate waste management. By focusing on responsible waste management today, future generations won't be burdened with the task of conducting extensive research on remediating century-old contaminants. Instead, the priority can be placed on adopting a lifestyle that respects and conserves nature.

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Summary

Gas manufactured from coal and oil was a major worldwide industry from the middle of the 19th to the middle of the 20th century. For decades, wastes from gas manufacturing processes were released to the environment by accidental spills and leaks, and even by intentional disposal practices. These have led to extent contaminated soils and groundwater systems. Even though Manufactured Gas Plant (MGP) sites were closed or abandoned decades ago, their environmental legacy still persists today.

Griftpark, a former MGP site located in the center of Utrecht, the Netherlands, is one of the many sites that contain such historical contaminants. Tar oil, waste generated from the gas manufacturing process, is a common source of contaminants found in the subsurface of such MGP sites, which contains mixtures of various hydrocarbons. The prevalent contaminants found in the subsurface and groundwater systems of Griftpark includes benzene, toluene, ethylbenzene, xylenes (BTEX), indene (Ie), indane (Ia), and naphthalene (N). These aromatic hydrocarbons are classified as toxic, mostly as carcinogenic, and thus pose risks to human health and the environment. Due to their relatively high water solubility and mobility, these pollutants can travel far distances, and cause environmental concerns such as contaminating drinking water supplies. Therefore, there is an urgent need for sustainable clean up solutions to remove contaminants like BTEXIeIaN from groundwater and subsurface sediments, at the Griftpark, but also at many other MGP sites worldwide.

Among all remediation technologies available, bioremediation is an appealing strategy due to its sustainable, environmentally friendly, and cost-effective approach. Bioremediation relies on natural biodegradation, which is a process for the breakdown of contaminants, wherein microorganisms act as degraders with the goal of converting toxic contaminants into less or non-toxic compounds. However, the biodegradation process can be affected by various factors, including environmental conditions such as pH, temperature, and the available electron acceptor. Additionally, biodegradation depends on the type of contaminant, the concentration, and the number of contaminants present. Finally, as biodegradation is a process performed by microorganisms, the characteristics of the microorganisms, such as metabolic capacities, metabolic diversity, and microbial interactions, are also crucial. All these factors lead to site-specific investigations, which are important when designing effective biological clean-up strategies for a contaminated site.

This thesis investigated the biodegradation of BTEXIeIaN compounds by subsurface microorganisms in Griftpark under different redox conditions, ranging from aerobic to strictly anaerobic conditions. Furthermore, other factors that might affect the biodegradation process, such as substrate interactions within mixtures, pollution history, adaptation, and the flow effect, were evaluated. In parallel, microbial community analyses were performed to understand how variations in environmental conditions would impact microbial community dynamics. The goal was to identify the limiting factors for the biodegradation of deep subsurface contaminants and develop strategies to enhance the breakdown process carried out by the indigenous microorganisms.

Before investigating the biodegradation process of the BTEXIeIaN mixture under various conditions, it was essential to establish an accurate and practical quantification method for routine biodegradation experiments in the laboratory. In Chapter 2, a HPLC method was developed and optimized for the simultaneous quantification of BTEXIeIaN compounds. This method allowed for direct measurements from

aqueous samples, requiring only a one-step preparation process. This simplicity came with advantages, such as a low volume of sampling, allowing for long-term investigations without disrupting the experimental conditions. At the end, the developed method facilitated the quantification of various aromatic hydrocarbons in laboratory batch samples and served as a routine monitoring tool for biological degradation processes involving prevalent contaminants.

In Chapter 3, the aerobic biodegradation potential of indigenous microorganisms was assessed through batch experiments using anaerobic aquifer material from the Griftpark. All compounds from the BTEXIeIaN mixture were completely removed under aerobic conditions, highlighting the high aerobic degradation capacity of the subsurface microorganisms. Furthermore, substrate interactions within the mixture were evaluated by conducting experiments with different substrate combinations of increasing complexity. The simple BTEX mixture was fully biodegraded within 15 days, whereas the removal of the complex mixture took 39 days, indicating the occurrence of substrate interactions. To confirm this, experimental data were fitted to a first-order kinetic degradation model for interpretation of inhibition/stimulation effects between the compounds. The results revealed that indene, indane, and naphthalene inhibited the biodegradation of benzene, toluene, ethylbenzene, and o-xylene, with benzene being the compound most affected. In contrast to other monoaromatic hydrocarbons, m/p-xylene biodegradation was stimulated by the presence of indene and/or indane but inhibited by the presence of naphthalene. Microbial analysis showed that different substrate mixtures led to variations in microbial community composition. Indene had a greater effect on the BTEX microbial community than indane and naphthalene as samples containing indene showed a higher relative abundance of *Micrococcaceae*, while *Commamonaceae* had a higher relative abundance in BTEX samples. In conclusion, the co-occurrence of different pollutants affected the biodegradation of individual compounds, which can be explained by differences in microbial community structures. This finding is crucial when dealing with complex mixtures, as it helps to understand why a particular compound may persist in a contaminated site while others are degraded.

Although aerobic conditions were successful in removing all compounds from the BTEXIeIaN mixture, biodegradation in contaminated aquifers is often limited by the absence of oxygen, leading to various types of anaerobic conditions. When oxygen is not available, microorganisms can utilize other electron acceptors such as nitrate, manganese, ferric iron, sulfate, and carbon dioxide. In Chapter 4, the anaerobic biodegradation capacity of subsurface microorganisms in Griftpark was tested under nitrate and sulfate-reducing conditions. Anaerobic aquifer material from the same location as in the aerobic experiments was used for the batch experiments in this study. Under nitrate-reducing conditions, only ethylbenzene was partially biodegraded, but not until 300 days had passed. Under sulfate-reducing conditions, toluene, o-xylene, and m/p-xylene were biodegraded, but a period of 100 to 200 days for toluene and o-xylene, and 400 days for m/p-xylene were needed for their complete removal. The findings of this study demonstrated that under anaerobic conditions, only specific compounds from the BTEXIeIaN mixture could be biodegraded, and this process required a significant amount of time compared to aerobic conditions.

Slow or no biodegradation can be due to the low microbial biomass of indigenous microorganisms, or they may lack proper metabolic capacities. In this case, a bioaugmentation approach can be applied, involving the addition of suitable degraders to the environment. In Chapter 4, additional experiments were performed to test bioaugmentation strategies using two different inoculums. The first inoculum was enriched from a wastewater treatment plant (WWTP) and added to batch bottles containing subsurface aquifer material, with the goal of increasing microbial diversity in the system. The diverse range of microbial groups with broad metabolic capacities could facilitate the biodegradation of a greater number

of compounds within the BTEXIeIaN mixture. Bioaugmentation tests with the WWTP inoculum under nitrate-reducing conditions had no impact on the biodegradation process compared to the non-bioaugmented bottles. Under sulfate-reducing conditions, only toluene was biodegraded, demonstrating that bioaugmentation with the WWTP had a negative impact, as toluene, o-xylene, and m/p-xylene were biodegraded in the non-bioaugmented bottles. The second inoculum consisted of indigenous Griftpark microorganisms enriched with toluene as the sole source of carbon and nitrate as the electron acceptor. Toluene-enriched bottles performed better under nitrate-reducing conditions, as ethylbenzene removal efficiencies were higher compared to the non-bioaugmented and WWTP-bioaugmented set-ups. This led to the hypothesis that the indigenous microorganisms could be trained: once enriched with a single substrate, their degradation could be improved upon further exposure within a complex mixture.

The results from Chapter 3 and 4 revealed that rapid and complete removal of all compounds within the BTEXIeIaN mixture requires oxygen as an electron acceptor. However, adding oxygen to the subsurface is challenging, not always effective, and often costly. For this reason, in Chapter 5, BTEXIeIaN biodegradation was tested under microaerobic conditions, where lower oxygen concentrations were intermittently supplied to the system. The results showed that under microaerobic conditions, the BTEXIeIaN mixture could be fully removed. However, when the system switched to fully anaerobic conditions, BTEXIeIaN biodegradation ceased. Another strategy was tested where the batch bottles were first exposed to strictly anaerobic conditions, with nitrate supplied as the electron acceptor. During this period, BTEXIeIaN biodegradation did not occur; however, nitrate was consumed, most likely by other electron donors available in the system, such as organic matter. After pre-exposure to nitrate-reducing conditions, the bottles were supplied with low oxygen concentrations to create microaerobic conditions within the system. Once oxygen was present, all the compounds were biodegraded. The results showed that the bottles pre-exposed to nitrate-reducing conditions prior to microaerobic conditions required less oxygen for the complete removal of the mixture compared to the bottles exposed to microaerobic conditions directly. This is likely due to organic matter consumption in the pre-anaerobic phase, directing the subsequent available oxygen mainly towards BTEXIeIaN biodegradation. This approach demonstrated that the combined use of oxygen and nitrate can reduce the amount of oxygen needed in the subsurface, making it useful in designing cost-effective remediation strategies where oxygen use is limited due to its expense.

In Chapter 3, 4, and 5, batch experiments were conducted using aquifer material from Griftpark to replicate conditions similar to those in the contaminated site. However, batch experiments represent closed and homogeneous systems, which may not fully reflect real-life situations. In Chapter 6, column experiments were conducted to introduce a flow, which can create heterogeneity and intricate patterns in the system, similar to the groundwater flow in the subsurface. For this, four up-flow columns filled with sediment from the Griftpark subsurface were built and run in parallel to test various environmental conditions simultaneously and evaluate their impact on the biodegradation process. First, toluene and benzene biodegradation as sole source of carbon was studied within two redox conditions: nitrate and sulfate. Results showed a compound-specific redox condition efficiency where toluene removal efficiencies were higher under sulfate-reducing conditions, while benzene removal was more effective with nitrate as the electron acceptor. In addition to single compound experiments, columns were also fed with duplex (T+B) and complex mixtures (BTEXIeIaN) in different exposure orders. This allowed the investigation of substrate interactions as well as the study of pollution history and adaptation factors on the biodegradation process. Toluene biodegradation was not affected by the presence of benzene but was

slightly hindered by the presence of the complex mixture. In contrast, benzene biodegradation was hindered by the presence of toluene but not as much when present within the complex mixture. Different substrate interactions were observed, indicating that when dealing with complex mixtures containing contaminants with distinct properties, the biodegradation performance may vary under the given environmental conditions. Columns that were initially treated with a single substrate and then exposed to a more complex mixture were more successful in biodegrading the compound when it was part of the mixture. This suggests that indigenous microorganisms require an adaptation period with a simpler mixture before being capable of degrading multiple contaminants simultaneously. Beta-diversity analysis revealed distinct microbial community clustering between sulfate and nitrate-reducing columns, demonstrating the high impact of redox conditions on microbial community composition. Despite columns receiving different substrate(s), no distinct clustering was observed based on the provided substrate. However, each column exhibited distinct microbial community clustering, highlighting their uniqueness in microbial composition. This variation is likely influenced by different contaminant exposure sequences, referred to in this study as the pollution history factor. All the outcomes of Chapter 6 need to be considered when making decisions for an effective remediation approach, especially for sites harboring various types of contaminants.

The findings from the experimental chapters (Chapter 3, 4, 5, and 6) were discussed in Chapter 7 in the context of the Griftpark case study. Various pilot studies planned for removing contaminants from the subsurface of Griftpark were evaluated, and recommendations for future investigations into aromatic hydrocarbon biodegradation were proposed. This thesis contributed to a better understanding of the mechanisms behind the biodegradation process of various aromatic hydrocarbons. Moreover, the results indicated that the choice of remediation strategies (natural attenuation, biostimulation, or bioaugmentation) depends on the type of contaminants, redox conditions, and characteristics of the indigenous microbial communities. The outcomes of this thesis aim not only to assist in decision-making for the remediation process in the Griftpark case but also for similar sites contaminated with complex hydrocarbon mixtures such as BTEXIeIaN.

Özet

Kömür ve petrol ürünlerinden üretilen gaz, 19. yüzyılın ortasından 20. yüzyılın ortasına kadar dünya çapında önemli bir endüstriydi. Gaz üretim süreçlerinden kaynaklanan atıklar, uzun yıllar boyunca çevreye kazara veya kasıtlı bertaraf uygulamaları ile salınarak, toprak ve yeraltı su sistemlerinin kirlenmesine neden olmuştur. Günümüzde, gaz üretim tesisleri kapatılmış veya terkedilmiş olsalar da, yıllar önce çevreye vermiş oldukları zarar halen devam etmektedir.

Hollanda'nın Utrecht şehrinin merkezinde bulunan, eski bir gaz üretim tesis bölgesi olan Griftpark, bu tür tarihi kirleticileri barındıran dünyadaki birçok kontamine alandan sadece birisidir. Gaz üretim süreci sonucunda ortaya çıkan ve yapısında birçok karışık hidrokarbon içeren katran yağı, gaz üretim tesis bölgelerinde, özellikle yer altında sıklıkla görülen önemli bir kirletici kaynağıdır. Griftpark'ın yer altı ve yeraltı su sistemlerinde bulunan yaygın kirleticiler arasında benzen, toluen, etilbenzen, ksilen (BTEX), inden (Ie), indan (Ia) ve naftalin (N) bulunmaktadır. Bu aromatik hidrokarbonlar genellikle toksik olarak sınıflandırılır ve kanserojen olarak kabul edilir. Bu sebeple, insan sağlığı ve çevre için ciddi bir risk oluşturmaktadırlar. Sudaki yüksek çözünürlükleri ve hareketlilikleri nedeniyle bu kirleticiler uzun mesafeler katederek, içme suyu kaynaklarının kirlenmesi gibi çevresel endişelere sebep olabilmektedir. Bu nedenle, Griftpark'ta olduğu gibi, dünya genelinde birçok tarihi gaz üretim tesis bölgesinde görülen BTEXIeIaN benzeri kirleticilerin yeraltı suyu ve sedimanlardan arındırılmasına yönelik sürdürülebilir çözüm stratejilerine ihtiyaç duyulmaktadır.

Mevcut tüm remediasyon teknolojileri arasında biyoremediasyon sürdürülebilir, çevre dostu ve ekonomik olması nedeniyle öne çıkmaktadır. Doğal biyodegradasyon mekanizmasına dayanan biyoremediasyon, mikroorganizmalar kullanılarak toksik kirleticilerin daha az toksik veya toksik olmayan bileşiklere dönüştürülme sürecidir. Ancak, biyodegradasyon süreci çeşitli faktörlerden etkilenebilir. Bunlar arasında çevresel koşullar (pH, sıcaklık ve mevcut elektron alıcısı gibi), kirleticinin türü, konsantrasyonu ve çeşitliliği bulunmaktadır. Ek olarak, biyodegradasyon mikroorganizmalar tarafından gerçekleştirilen bir süreç olduğundan, mikroorganizmaların özellikleri, metabolik kapasiteleri, metabolik çeşitliliği ve mikrobiyal etkileşimleri gibi özellikler de önem taşımaktadır. Tüm bu faktörler, kontamine bir bölge için etkili biyolojik remediasyon stratejileri tasarlanırken değerlendirilmelidir.

Bu tez, Griftpark yeraltı mikroorganizmalarını kullanarak, aerobik ve anaerobik dahil olmak üzere, farklı redoks koşulları altında BTEXIeIaN karışımının biyodegradasyon sürecini araştırmıştır. Ayrıca, biyodegradasyon sürecini etkileyebilecek olan; bileşikler arası etkileşimler, kirlilik geçmişi, adaptasyon ve su akış etkisi gibi faktörler de değerlendirilmiştir. Aynı zamanda, çevresel koşullardaki değişikliklerin mikrobiyal topluluk dinamiklerini nasıl etkileyeceğini anlamaya yönelik mikrobiyal topluluk analizleri gerçekleştirilmiştir. Amaç, yerli mikroorganizmaların aromatik hidrokarbon biyodegradasyon potansiyellerini ölçmek, bu süreçte etkili olabilecek kısıtlayıcı faktörleri belirlemek ve yerli mikroorganizmaların biyodegradasyon aktivitesini artırmaya yönelik yeni stratejiler geliştirmektir.

BTEXIeIaN karışımının biyodegradasyon sürecini çeşitli koşullar altında incelemeye başlamadan önce, laboratuvar ortamında gerçekleştirilecek olan rutin biyodegradasyon deneyleri için doğru ve pratik bir ölçüm metodu geliştirmek önemliydi. Bu amaçla, 2. Bölüm'de, BTEXIeIaN karışımında bulunan her bir bileşiğin aynı anda ölçülebilmesi için bir HPLC yöntemi geliştirilmiş ve optimize edilmiştir. Bu yöntem, tek bir adımdan oluşan numune hazırlık süreci içererek, sıvı örneklerden doğrudan ölçümleri mümkün

kılmıştır. Bu yaklaşım aynı zamanda düşük hacimli örnek alımına olanak tanıdığı için deneysel koşullara fazlasıyla müdahale etmeden, uzun vadeli incelemelere imkan sağlamıştır. Sonunda, geliştirilen HPLC yöntemi ile laboratuvar örneklerinde çeşitli aromatik hidrokarbonların nicelendirilmesi kolaylaştırılmış ve bu tip yaygın kontaminantlar için rutin izleme aracı olarak da hizmet sağlayabilmiştir.

3. Bölüm'de, yerli mikroorganizmaların aerobik koşullar altında biyodegradasyon potansiyeli laboratuvar şişelerinde incelenmiş ve bunun için Griftpark'tan elde edilen anaerobik yeraltı su ve sediman örnekleri kullanılmıştır. BTEXIeIaN karışımındaki tüm bileşikler, aerobik koşullar altında tamamen degrede edilebilmiştir. Bu sonuç, yeraltı mikroorganizmalarının sahip olduğu yüksek aerobik degradasyon kapasitesinin varlığını kanıtlamıştır. Ayrıca, karışım içindeki bileşiklerin degradasyon sırasında birbirleri ile olan etkileşimleri, farklı karışım kombinasyonları ele alınarak değerlendirilmiştir. Örneğin, basit BTEX karışımı 15 günde tamamen degrede olurken, daha karmaşık BTEXIeIaN karışımının tamamen degrede edilmesi 39 gün sürmüştür. Bu da karışımlardaki bileşiklerin kendi arasındaki etkileşiminin degradasyon sürecine olan etkisini göstermiştir. Bu durumu doğrulamak için, elde edilen deneysel veriler, bileşikler arasındaki inhibisyon/stimülasyon etkilerini yorumlamak amacıyla birinci derece kinetik degradasyon modeline uydurulmuştur. Sonuçlar, indenin, indanın ve naftalinin benzen, toluen, etilbenzen ve o-ksen biyodegradasyonunu inhibe ettiğini, benzenin ise en çok etkilenen bileşik olduğunu göstermiştir. Diğer monoaromatik hidrokarbonlardan farklı olarak, m/p-ksen biyodegradasyonun inden ve/veya indanın varlığı tarafından stimüle edildiği, ancak naftalin varlığında inhibe edildiği görülmüştür. Gerçekleştirilen mikrobiyal analizler sonucunda, farklı içerikte olan karışımların, mikrobiyal topluluk kompozisyonunda varyasyonlara yol açtığını gözlemlenmiştir. İnden içeren örneklerde Micrococcaceae'nin, BTEX örneklerinde ise Commamonaceae'nin daha yüksek göreceli baskınlığa sahip olduğunu tespit edilmiştir. Sonuç olarak, farklı bileşiklerin bir arada bulunmasının, bu bileşiklerin bireysel biyodegradasyon sürecini etkilediği ve bu durumun, mikrobiyal topluluk yapılarındaki farklılıklardan dolayı olduğu açıklanmıştır. Bu bulgu, özellikle birçok farklı yapıda bileşik içeren kompleks karışımlarla kontamine olmuş alanlarda, belirli bir bileşiğin biyodegrede olurken, başka bileşiklerin herhangi bir değişikliğe uğramadan, uzun süre var olmasının nedenlerini anlamaya yardımcı olacaktır.

Aerobik koşullar altında, BTEXIeIaN karışımındaki tüm bileşikler tamemen degrede olabilmiştir ancak yeraltı koşullarında oksijen konsantrasyonu sınırlıdır ve bu durum, test edilen bileşiklerin biyodegradasyon sürecini kısıtlayabilir. Oksijenin mevcut olmadığı durumlarda mikroorganizmalar nitrat, manganez, demir, sülfat ve karbon dioksit gibi diğer elektron alıcılarını kullanabilir. Bu sebeple, 4. Bölüm'de, Griftpark yer altı mikroorganizmalarının anaerobik biyodegradasyon kapasitesi nitrat ve sülfat indirgeme koşulları altında test edilmiştir. Bu çalışmada, aerobik deneylerde kullanıldığı gibi yine Griftpark'tan elde edilen yer altı su ve sediman örnekleri kullanılmıştır. Nitrat indirgeme koşulları altında sadece etilbenzen degrede olurken, etilbenzenin kısmi degradasyonu için neredeyse 300 gün süre gerekmiştir. Sülfat indirgeme koşulları altında toluen, o-ksen ve m/p-ksen degrede olurken, toluen ve o-ksenin tamamen degrede edilebilmesi için 100-200 gün, m/p-ksen için ise 400 gün süre gerekmiştir. Bu çalışmanın bulguları, anaerobik koşullar altında yerel mikroorganizmalar tarafından yalnızca belirli bileşiklerin biyodegrede edilebileceğini ve bu sürecin aerobik koşullara kıyasla oldukça uzun sürdüğünü göstermiştir.

Yerel mikroorganizmaların düşük mikrobiyal biyomasına sahip olmaları veya uygun metabolik kapasitelere sahip olmamaları biyodegradayon sürecinin yavaşlamasına veya hiç gerçekleşmemesine sebep olabilir. Böyle bir durumda, degradasyon yeteneğine sahip mikroorganizmaların kontaminasyon bölgesine eklenmesini kapsayan biyolojik büyütme (biyo-büyütme) olarak adlandırılan yaklaşım uygulanabilir. Biyo-büyütme yöntemini incelemek amacıyla, 4. Bölüm'de iki farklı inokulum test edilmiştir. Test edilen ilk

inokulum, bir atıksu arıtma tesisinden elde edilmiş ve yeraltı örnekleri içeren şişelere eklenerek sistemdeki mikrobiyal çeşitliliğini artırmak amaçlanmıştır. Geniş metabolik kapasitelere sahip çeşitli mikrobiyal gruplar, BTEXIeIaN karışımındaki bir dizi bileşiğin degradasyonunu kolaylaştırabilir. Atıksu arıtım tesisi inokulumuyla yapılan, nitrat indirgeyen koşulda gerçekleştirilen biyo-büyüme testleri, biyo-büyüme içermeyen-nitrat indirgeyen deneylerle karşılaştırıldığında, test edilen inokulumun BTEXIeIaN bileşiklerinin degradasyon sürecine herhangi bir etkisi olmadığı görülmüştür. Sülfat indirgeyen koşullarda ise sadece toluen biyodegrede edilmiştir. Oysaki biyo-büyüme yapılmamış sülfat indirgeyen koşulların hakim olduğu şişelerde toluen, o-ksilen ve m/p-ksilen degrede edilmişti. Bu durum, atıksu inokulumu kullanılarak test edilen biyo-büyüme stratejisinin başarısızlığını ve hatta degradasyon sürecine inhibe edici bir etkisi olduğunu göstermiştir. Test edilen ikinci inokulum, toluenin tek karbon kaynağı ve nitratın elektron alıcı olarak kullanıldığı koşullarda büyütülmüş yerel Griftpark mikroorganizmalarını içermektedir. İkinci inokulum ile biyo-büyütülmüş şişelerde, nitrat indirgeyen koşullarda etilbenzen degradasyon yüzdeleri, biyo-büyütme stratejisi uygulanmamış veya atıksu inokulumu içeren biyo-büyütme deneyleri ile karşılaştırıldığında daha yüksek bulunmuştur. Sonuçlar, yerel mikroorganizmaların eğitilebileceği hipotezine yol açarak, tek bir karbon kaynağı ile büyütülen bir inokulumun, sonrasında kompleks bir karışıma maruz kaldığında daha kolay bir degradasyon süreci gerçekleştirilebileceğini öne sürmüştür.

3. ve 4. bölümlerin sonuçları, BTEXIeIaN karışımındaki tüm bileşiklerin hızlı ve tam degradasyonu için oksijenin kesin gerekliliğini ortaya koymuştur. Ancak oksijenin yeraltına eklenmesi zahmetli, her zaman etkili sonuç vermeyen ve genellikle maliyetli bir işlemdir. Bu nedenle, 5. Bölüm'de BTEXIeIaN bileşiklerinin degradasyonu, düşük oksijen konsantrasyonlarının sisteme aralıklı olarak sağlandığı mikroaerobik koşullarda test edilmiştir. Sonuçlar, mikroaerobik koşullar altında BTEXIeIaN karışımının tamamen degrede edilebileceğini göstermiştir. Ancak BTEXIeIaN bileşikleri şişelere tekrar eklendiğinde ve sistem tamamen anaerobik koşullara geçtiğinde, BTEXIeIaN degradasyonu devam etmemiştir. Test edilen başka bir stratejide ise şişeler ilk olarak elektron alıcının nitrat olduğu, tamamen anaerobik koşullara maruz bırakılmıştır. Bu dönemde BTEXIeIaN degradasyonu gerçekleşmemiş; ancak nitrat konsantrasyonlarındaki düşüş, organik madde gibi sistemdeki diğer elektron donörleri tarafından nitratın kullanıldığına işaret etmiştir. Nitrat indirgeyen koşullara önceden maruz kalmış bu şişelerde, bu kez düşük oksijen konsantrasyonları sağlanarak mikroaerobik koşullar yaratıldığında, tüm bileşikler degrede edilebilmiştir. Sonuçlar, mikroaerobik koşullara doğrudan maruz kalan şişelere göre nitrat indirgeyen koşullara önceden maruz kalan şişelerin, karışımın tamamen degrede edilebilmesi için daha az oksijene ihtiyaç duyduğunu göstermiştir. Bunun sebebi, muhtemelen, ön-anaerobik fazdaki organik madde tüketimi nedeniyle sonradan sağlanan oksijenin çoğunlukla BTEXIeIaN degradasyonuna yönlendirmesinden kaynaklanmaktadır. Bu bulgular, özellikle oksijen kullanımının maliyeti sebebiyle tercih edilmediği kontamine bölgeler için tasarlanacak remediasyon stratejileri açısından daha ekonomik bir yaklaşım sunabilir.

3, 4 ve 5. bölümlerde, Griftpark koşullarını taklit etmek için bölgeden elde edilen yeraltı su ve sediman örnekleri kullanılarak laboratuvar şişe deneyleri gerçekleştirilmiştir. Ancak şişelerde gerçekleştirilen deneyler kapalı ve homojen sistemleri temsil ettiğinden Griftpark yeraltı koşullarını tam olarak yansıtmayabilir. Bu nedenle, 6. Bölüm'de, heterojenlik yaratabilecek bir su akışı tanıtmak amacıyla kolon deneyleri gerçekleştirilmiştir. Bunun için, yeraltından alınan sediman ile doldurulmuş dört kolon tasarlanmış ve açık sistem düzeneği kurulmuştur. Aynı anda çeşitli çevresel koşulları test etmek ve bunların degradasyon sürecine etkisini değerlendirmek için kolonlar paralel olarak çalıştırılıp, kolonlardan eş zamanlı rutin örnekler alınmıştır. Öncelikle, tek karbon kaynağı olarak toluen ve sonrasında benzen

degradasyonu nitrat veya sülfat indirgeyen koşullarda incelenmiştir. Sonuçlar, toluen degradasyonunun sülfat, benzen degradasyonunun ise nitrat indirgeyen koşullarda daha yüksek olduğunu göstermiştir. Bu durum karbon kaynağına bağlı, redoks koşullarına spesifik bir degradasyon süreci olduğunu kanıtlamıştır. Tek karbon kaynaklı deneylerinin yanı sıra, kolonlar çift (T+B) ve karmaşık karışımlarla (BTEXIeIaN) beslenmiş ve her bir kolon bu karışımlara farklı beslenme sırasıyla maruz bırakılmıştır. Böylece, degradasyon süresince gerçekleşen bileşikler arası etkileşimler ve kontaminasyon geçmişi faktörü incelenebilmiştir. Toluen degradasyonu, benzenin varlığından etkilenmezken, BTEXIeIaN karışımında kısmen inhibe olduğu gözlemlenmiştir. Benzen degradasyonu, toluen varlığı tarafından inhibe edilirken, BTEXIeIaN karışımından etkilenmemiştir. Bileşikler arası gözlemlenen bu etkileşimler, farklı özelliklere sahip bileşenler içeren kompleks karışımlarla çalışılırken degradasyon performansının var olan çevresel koşullar altında değişebileceğini göstermektedir. İlk olarak tek bir substratla beslenen ve ardından daha karmaşık bir karışıma maruz bırakılan kolonların, bileşen sonrasında karışımın bir parçası olduğunda daha yüksek degradasyon performansı gösterdiği gözlemlenmiştir. Bu durum, yerel mikroorganizmaların birden fazla bileşiği aynı anda degrede etmeden önce, daha basit bir karışıma maruz bırakılacak bir adaptasyon süresine ihtiyaç duyduğunu kanıtlamıştır. Yapılan beta-çeşitlilik analizleri ile sülfat ve nitrat indirgeyen kolonlar arasında belirgin bir mikrobiyal topluluk kümelenmesi olduğu gözlemlenmiş ve redoks koşullarının mikrobiyal topluluk kompozisyonu üzerindeki yüksek etkisi doğrulanmıştır. Kolonlar farklı substrat(lar) ile beslenmelerine rağmen, sağlanan substrata dayalı belirgin bir mikrobiyal kümelenme gözlenmemiştir. Analizler, her kolona ait spesifik bir mikrobiyal kompozisyon göstermiştir. Kolonlar arası gözlemlenen mikrobiyal kompozisyonlardaki bu varyasyonun, kirlilik geçmişi faktörü olarak adlandırılan farklı kirleticilere maruz kalma sırasından dolayı olduğu öne sürülmüştür. Sonuç olarak, 6. Bölüm'de sunulan tüm bulgular, özellikle çok sayıda ve farklı çeşitlilikte bileşik içeren kontamine bölgeler için etkili bir remediasyon yaklaşımı belirlenmesi sırasında karar alınırken önem taşıyacaktır.

Bu tezde sunulan deneysel bölümlerin (Bölüm 3, 4, 5 ve 6) bulguları, Griftpark vakası kapsamında 7. Bölüm 'de tartışılmıştır. Griftpark'ın yeraltındaki kontaminasyonuna çözüm olarak sunulan ve planlanan çeşitli pilot çalışmalar değerlendirilmiş ve aromatik hidrokarbon degradasyonuna odaklı ileriye yönelik araştırmalar için önerilerde bulunulmuştur. Bu tez, çeşitli aromatik hidrokarbonların degradasyon sürecinin arkasındaki mekanizmaların daha iyi anlaşılmasına katkı sağlamıştır. Ayrıca, uygulanacak olan remediasyon stratejisi (doğal seyrelme, biyostimülasyon veya biyo-büyüme) seçim sürecinde, kirleticilerin türü, sunulan redoks koşulları ve yerel mikrobiyal topluluklarının özelliklerinin değerlendirilmemisinin ne kadar önem taşıdığı vurgulanmıştır. Bu tezin sonuçları, sadece Griftpark vakasına uygun bir remediasyon yöntemi seçiminde değil, aynı zamanda BTEXIeIaN gibi karmaşık hidrokarbon karışımlarıyla kontamine olan benzer bölgeler için tasarlanan remediasyon stratejilerine de katkı sağlayabilir.

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About the Author

Dilan Camille Aydın was born on September 26th, 1993, in Gaziantep, and was raised in the city of İzmir, Turkey. Motivated by her profound concern for nature and the environment, Dilan pursued a degree in biology, obtaining a Bachelor of Science from Hacettepe University (Ankara). Following her graduation in 2015, she volunteered for EKAD (Ecological Research Society), engaging in wildlife conservation work for endangered marine turtles in Belek-Antalya. Dilan furthered her studies by earning a Master's degree in Biochemistry from Middle East Technical University (Ankara) in 2018. For her MSc thesis, she studied indigenous hydrocarbon degraders, evaluated their kerosene degradation and biosurfactant production potentials. Amazed by the remarkable capabilities of microorganisms, Dilan realized her passion for continuing her studies. In 2019, she moved to the Netherlands, and started her PhD at the Environmental Technology (ETE) group. Her research focused on exploring biostimulation strategies to enhance the biodegradation process at Griftpark, a former gasworks site, with the goal to remediate the subsurface from historical contaminants, predominantly composed of aromatic hydrocarbons, by use of indigenous microorganisms. After completing her PhD, Dilan aims to continue working on environmental issues, focusing on the prevention and/or clean up of any type of pollution to ensure that future generations do not bear the environmental burdens caused by the excesses of earlier eras.

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Aydin, D.C., Faber, S.C., Attiani, V., Eskes, J., Aldas-Vargas, A., Grotenhuis, T., Rijnaarts, H. (2023). Indene, Indane and Naphthalene in a mixture with BTEX affect aerobic compound biodegradation kinetics and indigenous microbial community development. *Chemosphere*, 340, 139761.

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- Career Orientation, Wageningen Graduate Schools (2023)

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Oral Presentations

- *Towards finite aftercare: Bioremediation and 3D modelling at a former manufactured gas plant in Utrecht, the Netherlands*. AquaConSoil 2019. 20-24 May 2019, Antwerp, Belgium
- *Biodegradation of benzene, toluene and BTEXIeIaN under nitrate and sulfate-reducing conditions in a continuous system*. AquaConSoil 2023, 11-15 September 2023, Prague, Czechia

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