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ENVIRONMENTAL BIOTECHNOLOGY

Microaerobic biodegradation of aromatic hydrocarbon mixtures: strategies for efficient nitrate and oxygen dosage

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Abstract

The biodegradation of organic aromatic compounds in subsurface environments is often hindered by limited dissolved oxygen. While oxygen supplementation can enhance in situ biodegradation, it poses financial and technical challenges. This study explores introducing low-oxygen concentrations in anaerobic environments for efficient contaminant removal, particularly in scenarios where coexisting pollutants are present. An innovative strategy of alternating nitrate-reducing and microaerobic conditions to stimulate biodegradation is proposed, utilizing nitrate initially to degrade easily-degradable compounds, and potentially reducing the need for additional oxygen. Batch experiments were conducted to assess the biodegradation of a BTEX, indene, indane, and naphthalene mixture using groundwater and sediments from an anaerobic contaminated aquifer. Two set-ups were incubated for 98 days to assess the redox transitions between microaerobic (oxygen concentrations < 0.5 mg O_2 L^{-1}) and nitrate-reducing conditions, aiming to minimize external electron acceptor usage while maximizing degradation. Comparative experiments under fully aerobic and fully anaerobic (nitrate-reducing) conditions were conducted, revealing that under microaerobic conditions, all compounds were completely degraded, achieving removal efficiencies comparable to fully aerobic conditions. A pre-treatment phase involving nitrate-reducing conditions followed by microaerobic conditions showed more effective utilization of oxygen specifically for contaminant degradation compared to fully aerobic conditions. Contrarily, under fully anaerobic conditions, without oxygen addition, partial degradation of ethylbenzene was observed after 400 days, while other compounds remained. The outcomes of this study can provide valuable insights for refining strategies involving oxygen and nitrate dosages, thereby enhancing the efficacy of in situ bioremediation approaches targeting complex hydrocarbon mixtures within anaerobic subsurface environments.

Key points

- *BTEX, indene, indane, and naphthalene mix biodegraded under microaerobic conditions*
- *Subsurface microorganisms swiftly adapt from nitrate to microaerobic conditions*
- *More oxygen directed to hydrocarbon biodegradation via a pre-anaerobic treatment*

Keywords BTEX · Microaerobic conditions · Nitrate-reducing conditions · Oxygen supplementation · Redox transitions · Bioremediation

Introduction

Before natural gas, cities and towns throughout the US 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) sites is coal tar which contains a mixture of various aromatic hydrocarbons (Sperfeld 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 because of their slow dissolution (Birak and Miller 2009). Benzene, toluene, ethylbenzene, and xylenes (BTEX) generate the most concern among hydrocarbon contaminants due to their high solubility and toxicity (Chakraborty and Coates 2004). Therefore, methods

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to remove contaminants like BTEX from groundwater and subsurface sediments of former MGP sites 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 and 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 and 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.

Adequate oxygen dosage is challenging due to the low solubility of oxygen in water (~ 10 mg L^{-1} at 15 °C), and the rapid consumption of oxygen in reduced aquifer environments. Furthermore, oxygen may lead to precipitation of oxidates, which can lead to less permeable aquifers, competing with aerobic conversion processes (Wilson and 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, is inexpensive, and non-toxic to microorganisms at concentrations below 500 mg NO_3^- L^{-1} (Hutchins 1991). Limitations for nitrate application are the maximum acceptable concentration 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 zonation can be observed as methanogenic conditions occur close to source of pollution, followed by sulfate, manganese, and iron, nitrate-reducing conditions, and finally aerobic conditions at the end of the plume. However, this process may not be as straightforward as described by Meckenstock et al. (2015), suggesting that oxygen, nitrate, or sulfate reduction can occur at the plume's edges, where electron acceptors are replenished by dispersion and diffusion from surrounding groundwater. Once all dissolved electron acceptors are depleted, methanogenesis and Fe(III)- or Mn(IV)-reduction may dominate in the plume core. Additionally, redox zones near sediment–water interfaces can exhibit 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 (Wilson and Bouwer 1997; Firmino et al. 2018). 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 (Wilson and Bouwer 1997; Firmino et al. 2018). 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 (Weelink et al. 2010; Firmino et al. 2018). Furthermore, oxygen-based conversion generally yields more microbial biomass, which can enhance the subsequent denitrification processes that lead to biodegradation of subsurface contaminants (Su and Kafkewitz 1994). There are several publications focusing on the microaerobic biodegradation of individual BTEX compounds (Su and Kafkewitz 1994; Yerushalmi et al. 2001, 2002; Aburto et al. 2009) and on BTEX as a mixture (Hutchins et al. 1992; Olsen et al. 1995; Da Silva et al. 2005; Firmino et al. 2018; Siqueira et al. 2018) with diverting outcomes on biodegradability of the individual compounds of the BTEX and other aromatic compounds. Nonetheless, for compounds such as indene and indane, the biodegradability in microaerobic conditions has not been reported prior to this study. Studies with much more complex mixtures of organic aromatic compounds are scarce, while mixtures—and not the individual compounds—are found in contaminated sites (Deeb et al. 2001; Gusmão et al. 2006; Zhou et al. 2011). In our previous study, where batch experiments were conducted under aerobic conditions, showed 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 was studied by investigating the potential use of oxygen, at lower concentrations, 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}). Furthermore, transitional redox scenarios were explored, involving shifts between microaerobic conditions and complete anaerobic (nitrate-reducing)

conditions, and vice versa. Both scenarios 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 sequential use of oxygen and nitrate (and vice versa), contributing to the development of efficient remediation strategies for aromatic and other hydrocarbons in the subsurface environments.

Materials and methods

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 were 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 to 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 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 2-L 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.

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 1). All set-ups consisted of three active bottles as experimental triplicates, and two abiotic duplicate controls to discriminate between biotic and abiotic processes in this study.

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 min 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 20 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 1. Oxygen dosage is explained in detail further in "*Adjustment of the oxygen concentrations*".

Table 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

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 S1, 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 (1:1:1:1:1:1:1:1:1, 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 analysing the compounds in liquid phase (Table S2).

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 to discard soil. An amount of 750 µL supernatant was then transferred to HPLC-glass vials and 250 µL methanol was added 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 supernatant used for the HPLC measurements were also used for the IC analysis. For this, the supernatant was diluted tenfold with Milli-Q water before measurements to be within the limit of detection range of the equipment.

For headspace analysis, concentrations of O_2 and CO_2 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 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.

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 the atmospheric gas pressure (Table S3) and should be ~ 2.5 mg based on the ideal gas law (Equation S1 and Equation S2, Table S4). After oxygen addition to anaerobic bottles, the oxygen gas in the headspace was measured with GC at around 1.3% (Table S5) which matched to the theoretical calculations (~ 2.5 mg O_2) given in Table S4. Additional to headspace measurements, oxygen concentrations in liquid phase were measured via an oxygen sensor and was ~ 0.8 mg L^{-1} (Table S6) which corresponds to ~ 0.12 mg. To understand the distribution of the added oxygen in gas and liquid phase, calculations based on Henry's Law were done (Table S7). The distribution of oxygen in gas and liquid phase was found to be close to the theoretical Henry's coefficient (Table S7). 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 S8 and S9).

Experimental design

The experimental design for set-up I and II was summarized in Fig. 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

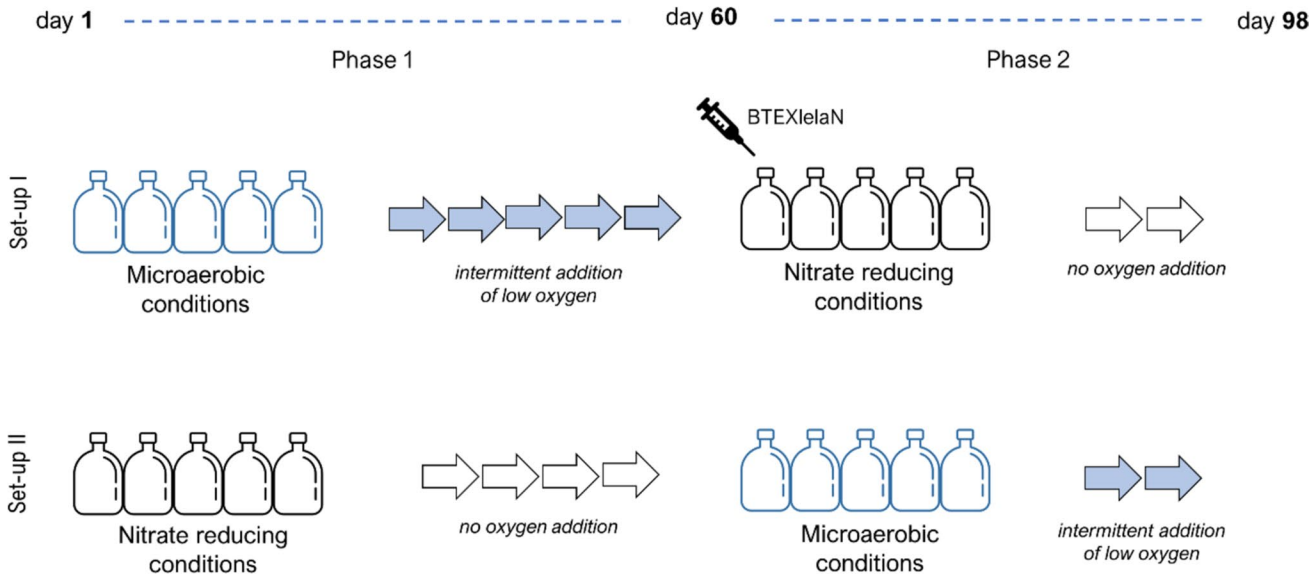

Fig. 1 Scheme for the experimental design of the set-up I and II

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. This condition served as a representation of the in situ conditions, involving prolonged exposure of microorganisms to the BTEXIeIaN mixture under anaerobic conditions. 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.

Results

Assessment of BTEXIeIaN mixture biodegradation under transitional redox 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 (Fig. 2A). Control bottles showed no biodegradation (Fig. 2B), confirming a biotic removal process in the active bottles. Oxygen was added intermittently on days 1, 7, 14, 21 and 42 with initial concentration of ~ 0.7 mg O_2 L^{-1}, reducing to 0.2 mg O_2 L^{-1} (Fig. 3). This concentration remained consistent until subsequent oxygen addition, suggesting the constant reading of 0.2 mg O_2 L^{-1} 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 after correction around 0.5 mg O_2 L^{-1}. Reduction in the oxygen concentrations (Fig. 3) were in line with the biodegradation data (Fig. 2), indicating BTEXIeIaN removal at oxygen 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, initiating a new phase to assess biodegradation under nitrate-reducing conditions (Phase 2). Notably, in the absence of oxygen, BTEXIeIaN biodegradation did not occur as indicated by negligible changes in compound concentrations, consistent with control bottle observations (Fig. 2). The findings from set-up I lead to the conclusion that oxygen is essential for the bioremediation of the BTEXIeIaN mixture. Additionally, it was shown that low oxygen concentrations (~ 0.5 mg O_2 L^{-1}) are sufficient for the complete degradation of the complex mixture comprising various aromatic hydrocarbons.

Set-up II: From nitrate-reducing conditions to microaerobic conditions

In set-up II, nitrate-reducing conditions were prevalent during the initial 60-day period, and throughout that time, the BTEXIeIaN mixture was not biodegraded (Fig. 4). The biodegradation of the compounds was observed starting from day 60, when set-up II bottles received low oxygen concentrations for the first time (Fig. 4A). In the second oxygen addition (day 67), biodegradation continued and almost all the compounds of the mixture were biodegraded. The DO concentrations measured for set-up II batch bottles (Fig. 5) exhibited a similar trend to set-up I, decreasing from approximately 0.8 to 0.3 mg O_2 L^{-1}. The reduction in oxygen concentrations was consistent with the biodegradation data (Fig. 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. These results suggest that microorganisms could adapt to microaerobic conditions after a pre-anaerobic period and biodegrade the BTEXIeIaN mixture within a short timeframe.

Set-up III and IV: BTEXIeIaN degradation under fully aerobic and fully anaerobic conditions

Additionally, to set-up I and II, separate experiments were conducted under fully aerobic conditions (set-up III) and fully anaerobic conditions (set-up IV), without assessment of any transitional redox conditions. On one hand, within set-up III, all compounds of the BTEXIeIaN mixture were fully biodegraded within 15 days (Supplementary Fig S1). On the other hand, in set-up IV, under prevailing nitrate-reducing conditions, only the degradation process of ethylbenzene started, taking a substantial duration of 308 days and continued until the end of the experiment by day 400 (Supplementary Fig S2). Even after this prolonged period of 400 days, all compounds, including ethylbenzene, remained in the bottles. These results indicate that fully anaerobic conditions were ineffective in completely removing the BTEXIeIaN compounds, thus confirming the essential role of oxygen in the rapid conversion of these contaminants.

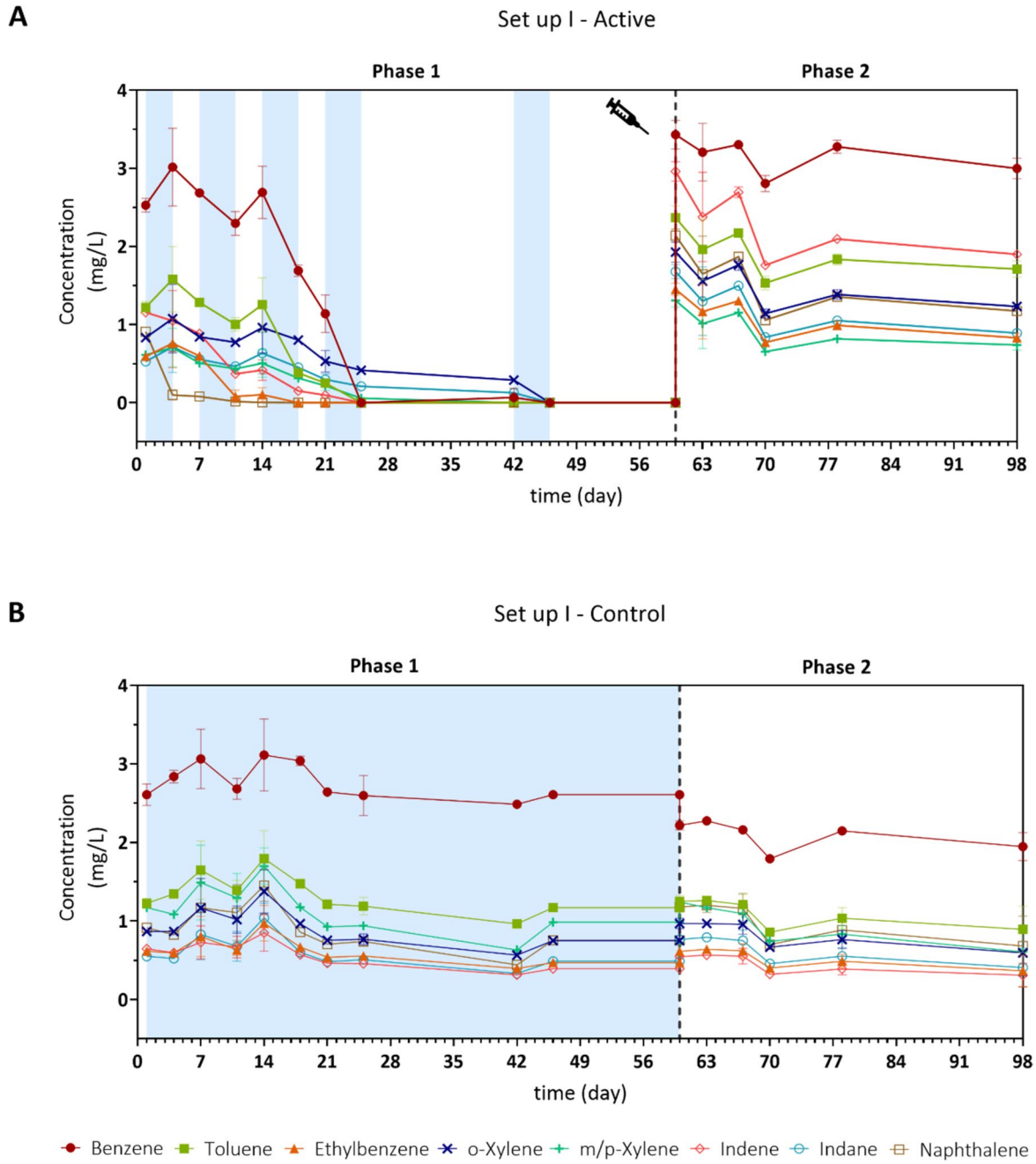

Fig. 2 Set-up I: BTEXIeIaN biodegradation with intermittent addition of low oxygen (**A**) in active bottles and (**B**) in controls. Microaerobic conditions shown in blue. Control bottles received low oxygen addition only on day 1, as oxygen was not consumed throughout phase 1. The figures represent the mean of the triplicate active bottles (**A**) and duplicate controls (**B**). On day 54, gas exchange procedure was performed, leading to some decrease in concentrations in control bottles (measured on day 60)

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, reaching a concentration around 0.5 mg O_2 L^{-1}. In contrast, set-up II required only two similar oxygen spikes to achieve almost complete conversion of the same amount of complex substrate. This indicated a variation in oxygen utilization between the different oxygen

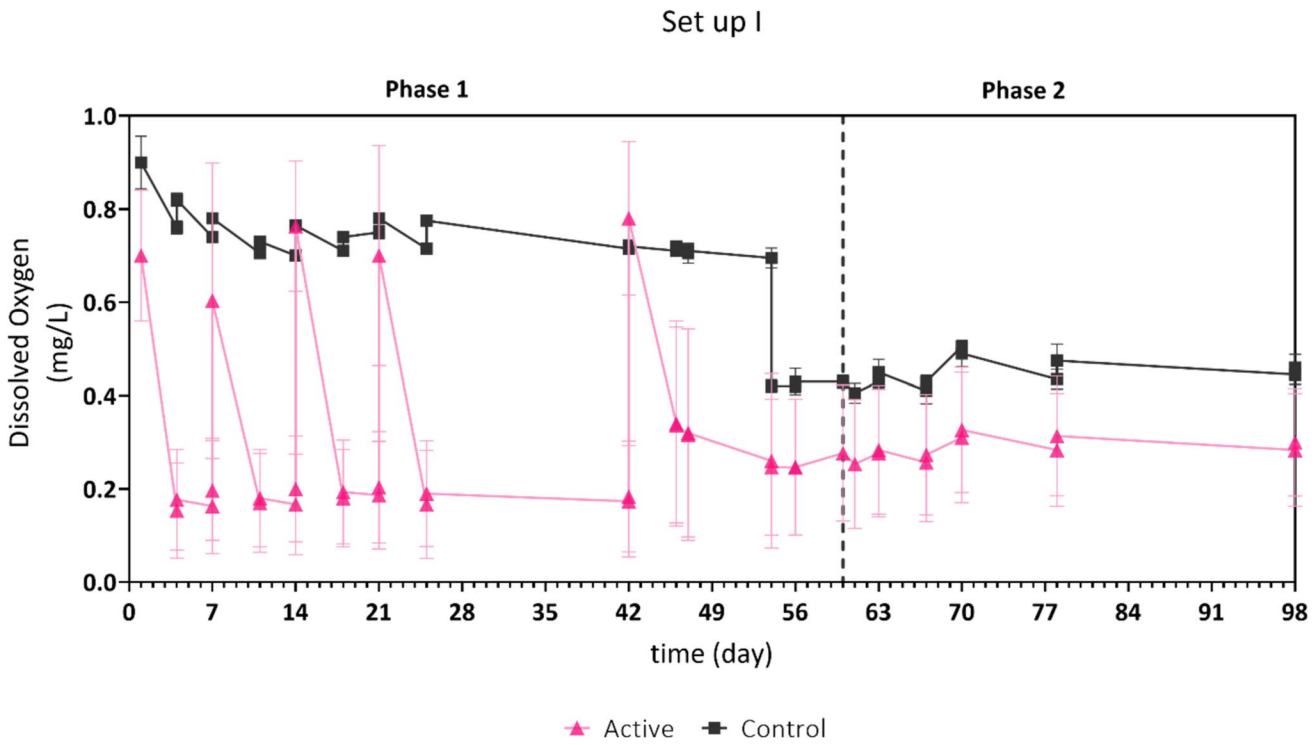

Fig. 3 Mean of the dissolved oxygen concentrations of the active bottles and controls for set-up I, measured with oxygen sensor. At day 54, gas exchange procedure was applied with nitrogen gas (N_2, 100%)

dosage set-ups. In order to investigate further, a comparison was made between the oxygen consumptions of set-up I and II (Table 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 S10. 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.

In set-up I, 22% of the consumed oxygen was utilized for BTEXIeIaN degradation (2.98 mg), leaving the 78% of the oxygen used for OM (10.59 mg) (Table 2). While in set-up II, 86% of the oxygen was consumed for biodegradation of the mixture (3.41 mg), with 14% for the OM (0.53 mg) (Table 2). This demonstrates that oxygen was more efficiently used for BTEXIeIaN degradation in set-up II compared to set-up I. This is likely due to the fact that in set-up II, most of the organic matter degradation occurred during the pre-anaerobic period (Phase 1), wherein nitrate was utilized as an electron acceptor. As a result, having a pre-anaerobic period before addition of low oxygen concentrations showed to be efficient in terms of directing most of the oxygen 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 S2); therefore, the theoretical O_2 values were higher and were calculated as 6.91 ($\pm$0.05) mg while the total oxygen consumption in the headspace and liquid phase was 19.48 ($\pm$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 oxygen 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%, indicating that additional oxygen would increase oxygen consumption in other processes, like OM degradation. Therefore, supplying an excessive amount of oxygen is unnecessary since, even under microaerobic conditions, all compounds can still be biodegraded.

Nitrate consumption under microaerobic and nitrate-reducing conditions

To assess the nitrate utilization by the indigenous microorganisms, nitrate consumption during phases 1 and 2 in set-ups I and II were monitored (Table 3). In set-up I, ~ 12 mg of nitrate was consumed in the active bottles during

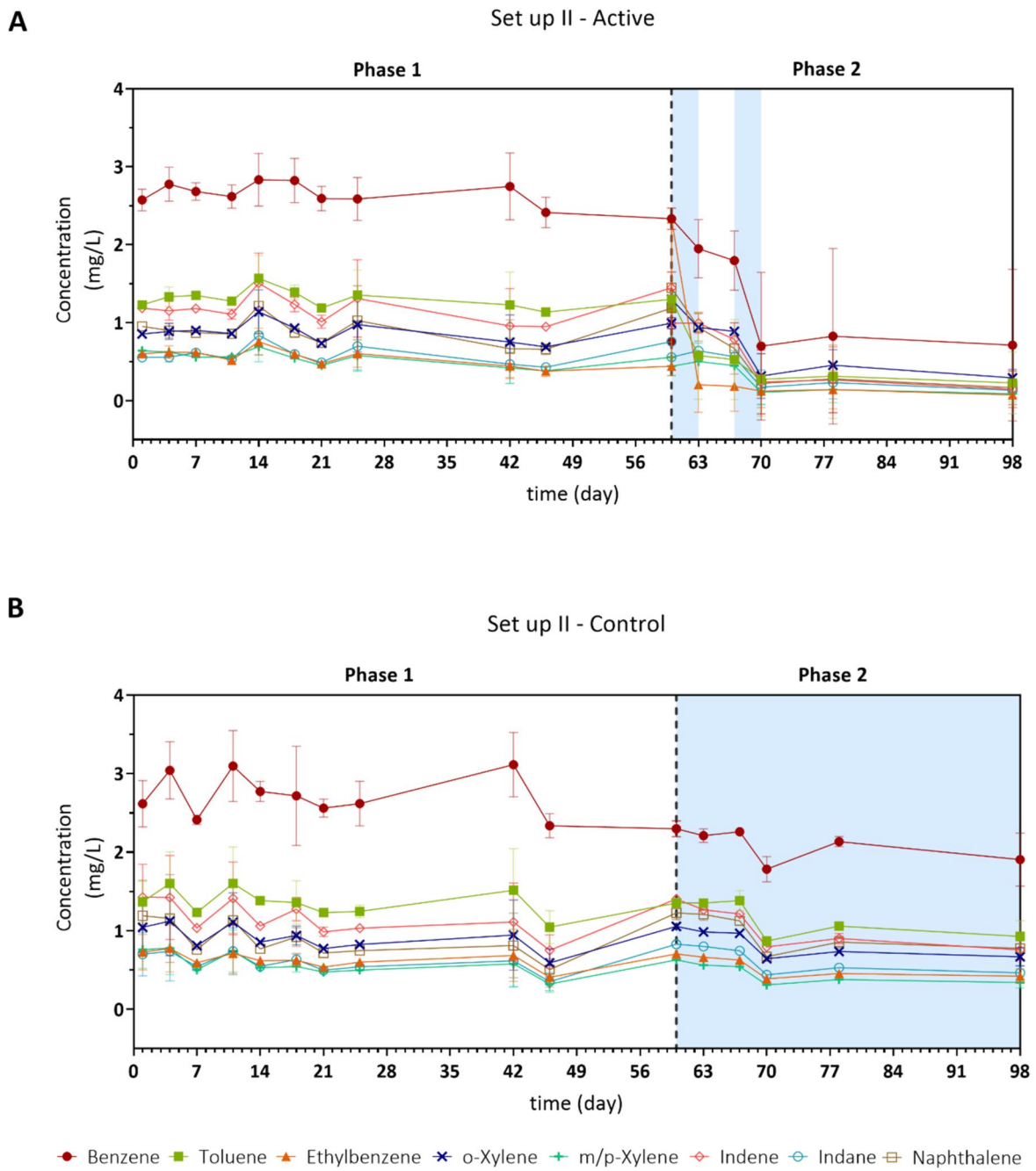

Fig. 4 Set-up II: BTEXIeIaN degradation with intermittent addition of low oxygen (**A**) in active bottles and (**B**) in controls. Microaerobic conditions shown in blue. Control bottles received low oxygen addition only on day 60, as oxygen was not consumed throughout phase 2. The figures represent the mean of the triplicate active bottles (**A**), and duplicate controls (**B**)

microaerobic conditions (Phase 1), while only ~ 3 mg was utilized during nitrate-reducing conditions (Phase 2). Nitrate consumptions were higher in active bottles as opposed to controls, indicating that nitrate consumption was a biotic process. It is unknown from our data whether nitrate was consumed for BTEXIeIaN biodegradation or OM. However, it is unlikely that this nitrate consumption was employed for the biodegradation of the contaminant mixture since when nitrate was solely present in set-up I 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.

In set-up II, ~ 6 mg nitrate was consumed during phase 1, under nitrate-reducing conditions. Once oxygen was added

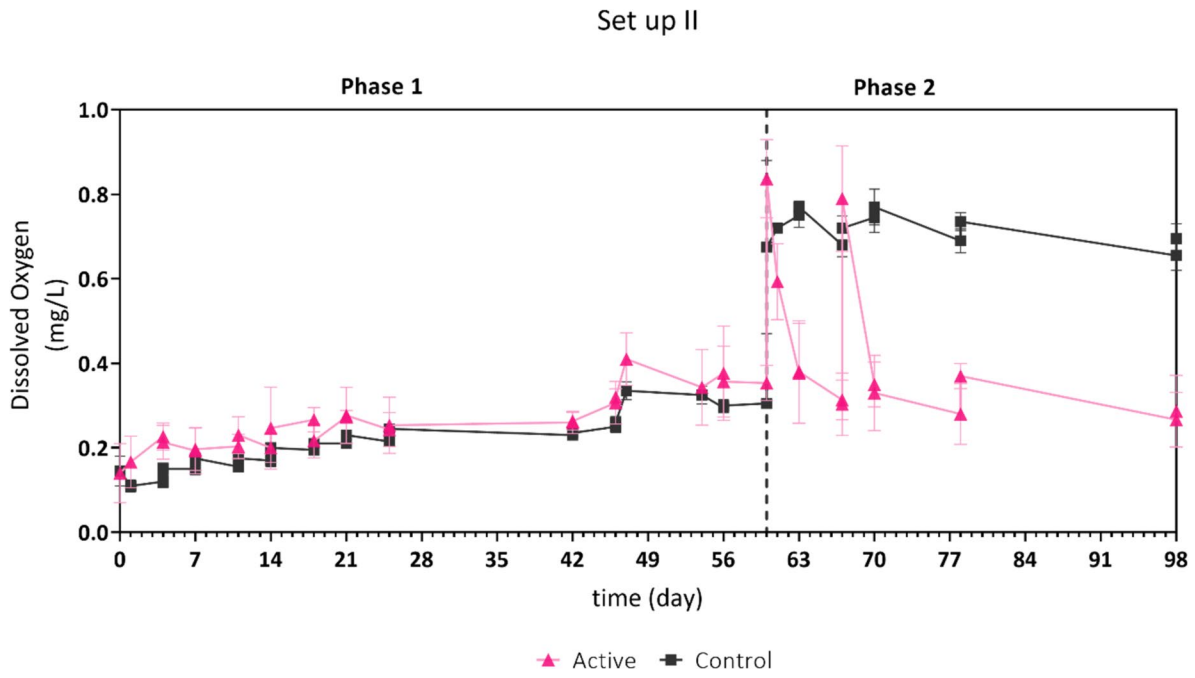

Fig. 5 Mean of the dissolved oxygen concentrations of the active bottles and controls for set-up II, measured with oxygen sensor. At day 54, gas exchange procedure was applied with nitrogen gas (N_2, 100%)

Table 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

Table 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

to the bottles during phase 2, nitrate consumption continued; however, it was not as high as in set-up I-phase 1 (microaerobic conditions). It is possible that more OM was present at the beginning of the experiment leading to a higher consumption of nitrate in set-up I bottles. Furthermore, bottles in set-up I underwent five oxygen injections, whereas those in set-up II received only two, indicating a correlation between higher nitrate consumption in set-up I and increased oxygen dosage. This correlation is also supported by the data from set-up I, where more nitrate was consumed in phase I in the presence of oxygen compared to phase 2, where nitrate-reducing conditions prevail.

Monitoring nitrite production

High nitrate consumption can lead to high nitrite production which can be toxic for the microorganisms (Chayabutra and Ju 2000; Philips et al. 2002) and end up inhibiting the biodegradation (Zhu et al. 2020). As shown in Fig. 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 investigate the potential correlation between the lack of biodegradation observed in set-up I after transitioning to fully anaerobic conditions (Fig. 2-phase 2) and the presence of high nitrite concentrations, an additional experiment (Set-up V, Fig. 7) was performed where BTEXIeIaN degradation was tested under microaerobic and nitrate-reducing conditions, utilizing lower initial nitrate concentrations (~50 mg L^{-1}) than in set-up I and II (430–450 mg L^{-1}).

In Set-up V bottles, the first addition of low-oxygen (~2.08 mg O_2 L^{-1}) was on day 6, followed by the second addition (~2.25 mg O_2 L^{-1}) on day 18 (Fig. 7). After day 18,

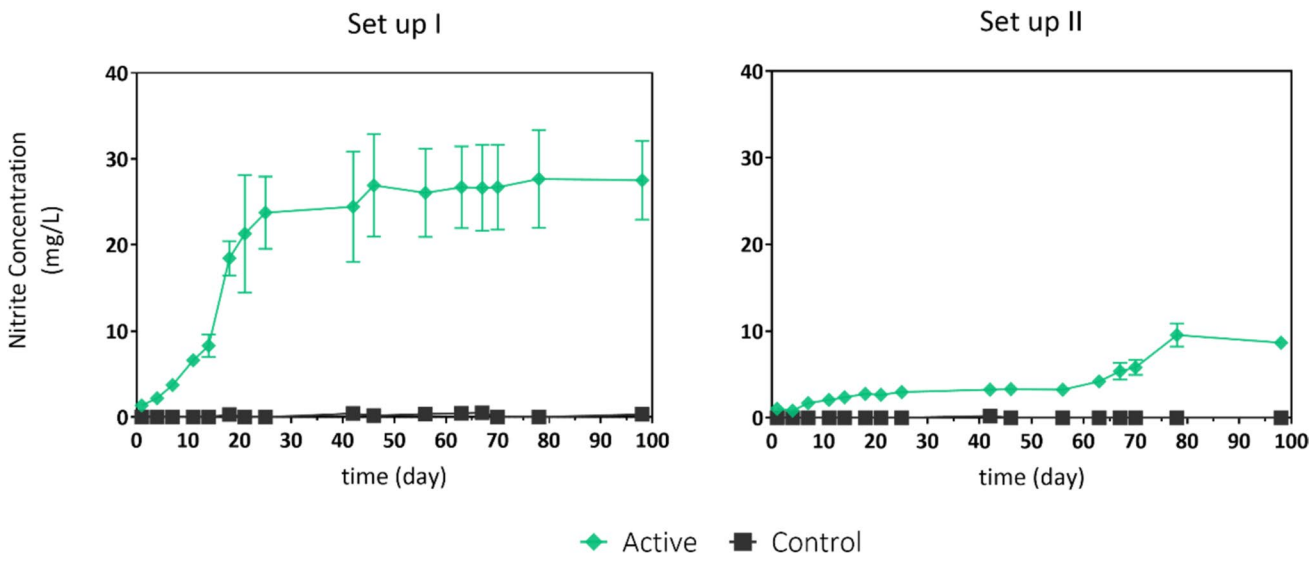

Fig. 6 Mean of the produced nitrite concentrations in the active bottles and controls for set-up I and set-up II

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 (Fig. 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.

Discussion

Complete biodegradation of all compounds in the BTEXIeIaN mixture under microaerobic conditions

The primary objective of this study was to assess whether indigenous microorganisms could degrade the BTEXIeIaN mixture under microaerobic conditions. In this study, all compounds within the mixture were successfully biodegraded at initial oxygen concentrations of ~ 0.5 mg O_2 L^{-1},

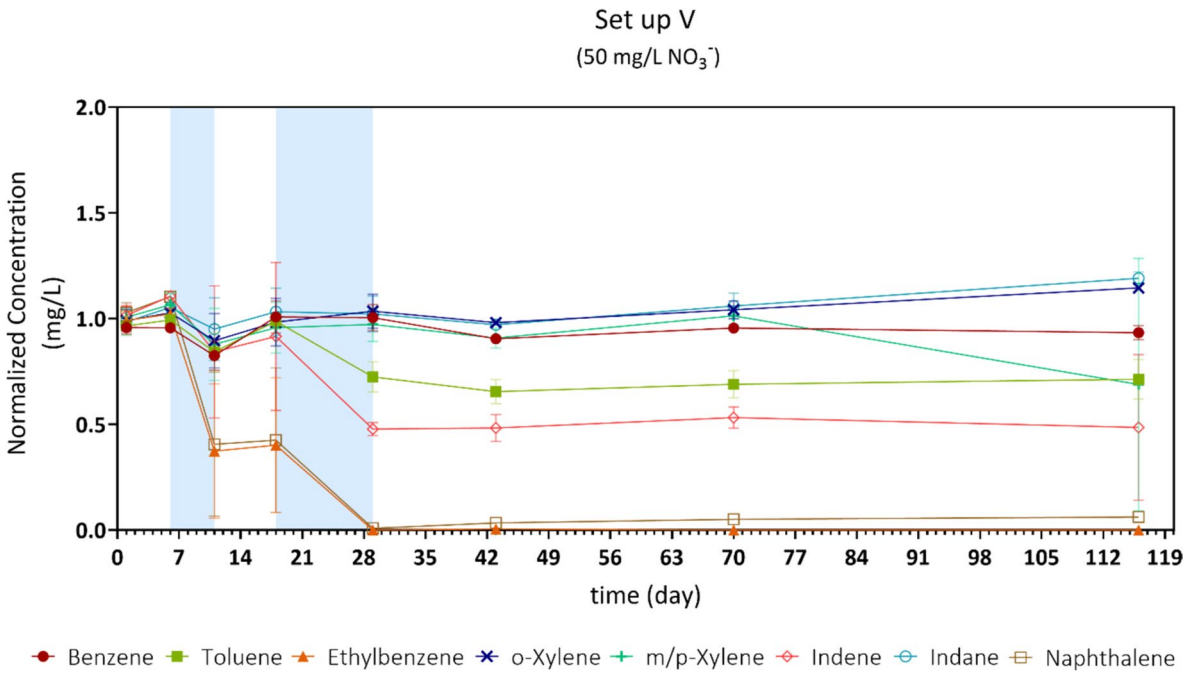

Fig. 7 Biodegradation of BTEXIeIaN in set-up V, with lower nitrate concentrations (50 mg L^{-1}). Blue periods represent microaerobic conditions

including recalcitrant compounds such as benzene. This sets apart our study from previous microaerobic studies on BTEX. Moreover, this study is the first to demonstrate the biodegradation of indene and indane under microaerobic conditions.

In literature, a range of oxygen concentrations to initiate BTEX compounds biodegradation under microaerobic conditions were reported. Su and Kafkewitz (1994) reported that while xylenes and toluene could be degraded at 2% oxygen, benzene remained recalcitrant. Yerushalmi et al. (2001) tested benzene biodegradation under different DO concentrations (1 to 0.05 mg O_2 L^{-1}) and demonstrated a decrease in removal efficiency at lower DO levels (100 to 34%). Similarly, Aburto et al. (2009) showed that very low concentrations of oxygen (2.57 to 0 mg O_2 L^{-1}) are sufficient for in situ biodegradation of benzene. When BTEX was assessed as a mixture, Firmino et al. (2018) reported 80% degradation was for TEX compounds and < 50% for benzene with continuous oxygen injection at 0.18 L O_2 L^{-1}. Hutchins et al. (1992) tested at DO concentration of 1 mg O_2 L^{-1}, continuously supplying oxygen to the reactors. Under microaerobic conditions (with and without nitrate), less than 50% benzene removal was observed while TEX degradation was minimal under both conditions.

All the references cited above demonstrate the variation in oxygen concentrations required to initiate the biodegradation of specific aromatic hydrocarbons. As mentioned by Yerushalmi et al (2002), oxygen concentration required to initiate aerobic conversion depends on several factors, such as bacterial population, substrate characteristics, and environmental conditions. Furthermore, oxygen levels can influence the expression of catabolic genes. For instance, a study (MartÍnez-Lavanchy et al. 2010) demonstrated that varying oxygen availability affected the expression of toluene catabolic genes. While the presence or absence of the substrate and its intermediates typically regulates these systems, it was found that below a certain oxygen threshold, bacteria significantly reduced the expression of catabolic genes, even when the inducer (toluene) was present at high concentrations. The same study also revealed that *Pseudomonas putida* efficiently responded to temporal fluctuations in oxygen levels. This finding is relevant to real-world scenarios, where seasonal or diurnal variations in oxygen levels may impact degradation performances. Additionally, oxygen concentrations can influence the selection of metabolic pathways during compound degradation. This was demonstrated (Duetz et al. 1994) by testing four different *Pseudomonas* strains capable of degrading toluene through distinct pathway. Results showed that under oxygen-limited conditions, the strain that hydroxylates toluene at the ortho position exhibited the highest growth rate compared to other strains.

Studies discussed in this section highlight the importance of investigating multiple factors—such as microbial community composition and metabolic capacity, substrate characteristics, and environmental conditions—that may simultaneously affect the biodegradation activity of contaminants. In our study, we focused on the effect of transitional redox conditions (aerobic/anaerobic) and oxygen concentrations (fully aerobic/microaerobic) on the biodegradation potential of the indigenous microbial community. Examining the degradation capacities of microorganisms for a variety of compounds across different oxygen levels can provide valuable insights for developing effective bioremediation strategies. This is especially critical in emerging remediation scenarios involving mixtures of diverse toxic compounds, such as BTEXIeIaN, which pose significant environmental threats. In such cases, micro-aeration emerges as a promising approach, particularly for projects requiring urgent risk reduction measures.

Microbial adaptation to transitional redox conditions

This study also evaluated the biodegradation capacity of indigenous microorganisms during transitional redox conditions. In set-up I, we tested whether the indigenous microorganisms could sustain the biodegradation of the BTEXIeIaN mixture using nitrate as an electron acceptor (Phase 2) following exposure to low oxygen levels (Phase 1). Results showed that microorganisms were unable to biodegrade the contaminants when tested under nitrate-reducing conditions. It is plausible that within the experimental timeframe, microorganisms failed to adapt to nitrate-reducing conditions after exposure to microaerobic conditions. For set-up II, the objective was to mimic in situ bioremediation conditions, simulating the prolonged exposure of microorganisms to BTEXIeIaN mixture under anaerobic conditions. For this, after a 60-day anaerobic period, the set-up II bottles were supplied with low oxygen concentrations as if oxygen were supplied to the subsurface of contaminated zones. This time, indigenous microorganisms were capable of transitioning from anaerobic conditions to microaerobic conditions and effectively biodegrading the BTEXIeIaN mixture. This highlighted the microorganisms' ability to adapt to microaerobic conditions after an extended period of anaerobic conditions. The reverse process (set-up I) did not occur, emphasizing the one-way nature of this adaptation. Siqueira et al. (2018) and Firmino et al. (2018) employed a similar approach, initially testing BTEX biodegradation under anaerobic conditions and subsequently under microaerobic conditions, where removal efficiencies increased with micro-aeration. Unlike findings in the literature, BTEXIeIaN could not be biodegraded under fully anaerobic conditions within the experimental timeframe of this study.

Compound-specific biodegradation activity represents a challenge when dealing with mixtures containing various pollutants with distinct properties. This is even more challenging when anaerobic conditions prevail (Foght 2008). For

instance, ethylbenzene degradation is commonly observed under nitrate-reducing conditions but rarely with other anaerobic electron acceptors. Conversely, xylene degradation has been reported with nitrate, sulfate, or iron, but its occurrence depends on the specific isomer (Weelink et al. 2010). Moreover, some compounds, such as indene and indane, have not been reported to biodegrade under anaerobic conditions. Consequently, for complex mixtures, assessing transitional redox conditions is particularly crucial, as it allows us to leverage the advantages of different redox conditions. For instance, easily anaerobically biodegradable compounds like ethylbenzene can be removed during a pre-anaerobic phase, thereby reducing the mixture's complexity, and potentially benefiting from the inhibition of substrate interactions among compounds (Aydin et al. 2023). After removing easily-degradable compounds, recalcitrant compounds under anaerobic conditions can be biodegraded with low oxygen injections, enabling microaerobic conditions to occur. This approach minimizes the presence of compounds requiring oxygen for their removal, thereby reducing oxygen dosage, which could be beneficial in terms of cost and mitigating side effects such as toxicity.

Microbial use of electron acceptors

Efficiency in oxygen utilization and its role in nitrate consumption

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). 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. Their study 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, based on the oxygen results, it was concluded that an excess of oxygen is unnecessary for the biodegradation of the aromatic hydrocarbons as they could already be biodegraded under microaerobic conditions, with intermittent oxygen supply. Additionally, introducing a pre-anaerobic period before supplying low oxygen concentrations has demonstrated to prioritize oxygen usage for BTEXIeIaN biodegradation over other biotic processes. This is explained by the fact that during this pre-anaerobic phase, nitrate was utilized for OM degradation, thereby conserving more oxygen to facilitate the biodegradation of aromatic hydrocarbons.

In set-up I, higher nitrate consumption was observed under microaerobic conditions compared to nitrate-reducing conditions. This could be attributed to the presence of oxygen, which enhances the growth rate of biomass and increases the size of the indigenous microbial population. A larger and faster-growing population is known to lead to a higher rate of denitrification (Olsen et al. 1995). Similarly, higher nitrate consumption was observed in set-up I compared to set-up II during microaerobic conditions as set-up I bottles received five oxygen addition while set-up II bottles only received two, confirming that higher nitrate consumption is attributed to more oxygen dosage.

Overall, the oxygen and nitrate measurements (Tables 2 and 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 conducted 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 (Aburto et al. 2009). 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 microorganisms were present in the contaminated groundwater. At low concentrations, oxygen seemed necessary to initiate benzene biodegradation, and that anaerobic microorganisms 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 also supported by Yerushalmi et al. (2001) where simultaneous presence of aerobic and anaerobic intermediates of benzene (catechol and benzoic acid, respectively) was 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 for OM degradation. However, when microaerobic conditions were applied, microorganisms could quickly adapt to these conditions and biodegrade the BTEXIeIaN compounds, without any lag phase (set-up II-phase 2). This demonstrated the tolerance of microorganisms to low oxygen concentrations and their ability to swiftly adapt from anaerobic to microaerobic conditions. 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. This inference is further supported by our previous study, where we examined the microbial consortium extracted from sediment collected from the same location as the sediment used in this experiment. Through 16S rRNA amplicon analysis, we identified microbial groups, such as *Pseudomonas* and *Acidovorax* (Aydin et al. 2023) known to include facultative anaerobes among their members. In conclusion, understanding the pivotal role of facultative anaerobes in transitioning between redox conditions could be essential for optimizing remediation strategies, underscoring the necessity for further research in this area.

Implications for future remediation strategies

This study is the first, to our knowledge, to explore the removal efficiency of an aromatic hydrocarbon mixture under alternating nitrate-reducing and microaerobic conditions in a vice-versa manner, comparing the outcomes not only between these conditions but also with fully aerobic and fully anaerobic environments. Unlike previous research focused on single compounds or simpler mixtures (such as BTEX), this study addresses the biodegradation of a more complex contaminant mixture (BTEXIeIaN), which reflects real-world situations where multiple contaminants are present simultaneously. Notably, this is also the first microaerobic study conducted with indene and indane. By using sediment and groundwater samples originated from a contaminated subsurface, our experimental design includes indigenous microbial communities closely mimicking real-site conditions, adding further relevance to the findings. By evaluating various transitional redox conditions and their impact on the biodegradation of different aromatic compounds, this study provides new insights for the field and practical guidance for the application of nitrate-oxygen strategies in site bioremediation.

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 (Dou et al. 2008; Zhou et al. 2011; Aydin et al. 2023). The biodegradation requirements can differ for each compound in a mixture. For instance, while some compounds can be biodegraded under anaerobic conditions, others may remain recalcitrant without the presence of oxygen. In such cases, using both oxygen and nitrate can be beneficial. In our case, the limitation of anaerobic BTEX degradation (as well as other compounds) can be overcome by adding oxygen to the system, promoting the initial degradation of aromatic 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 are needed.

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 our findings, 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-pretreatment with nitrate can be beneficial before oxygen addition which can be supplied (via MNBs) intermittently and be principally used for biodegradation of contaminants.

In conclusion, 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 biodegradation of all the compounds within the BTEXIeIaN mixture. 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. For in situ applications, 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 complex mixtures such as BTEXIeIaN harboring various aromatic pollutants. While these findings provide a basis for remediation strategies, further site-specific pilot tests are necessary for addressing similar contamination in aquifers.

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Data availability The raw data that support the findings of this study are available on request from the corresponding author.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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