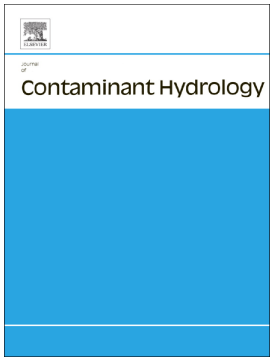

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BENZENE DEGRADATION IN CONTAMINATED AQUIFERS: ENHANCING NATURAL ATTENUATION BY INJECTING NITRATE

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Abstract

Natural attenuation processes depend on the availability of suitable electron acceptors. At the megasite Zeitz, concentrations of the main contaminant benzene were observed to increase constantly in the lower aquifer to levels of more than 2.5 mM. This was accompanied by decreasing concentrations of sulphate (SO_4^{2-}), which has been previously shown to be the main electron acceptor for benzene oxidation at this site, resulting in an electron acceptor-limited, sulphidic benzene plume. Therefore, a field experiment was conducted to stimulate benzene biodegradation by injecting nitrate (NO_3^-) into the sulfidic benzene plume aiming (i) to recycle sulphate by nitrate-dependent sulphide oxidation, and (ii) to serve as direct electron acceptor for benzene oxidation. Within 60 days, 6.74 tons sodium nitrate ($NaNO_3$) were injected into the lower aquifer, and the resulting biogeochemical effects within the benzene plume were monitored for more than one year by chemical and microbiological analyses of groundwater samples taken from various depths of ten monitoring wells located in three observation lines downstream of nitrate injection. Nitrate was microbiologically consumed, as shown by changes in $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ values, partial nitrite

accumulation, and changing ratios of Na^+/NO_3^-. Main electron donors for nitrate reduction were reduced sulfur compounds, verified by changing $\delta^{34}S\text{-}SO_4^{2-}$ and $\delta^{18}O\text{-}SO_4^{2-}$ values, partially increasing sulphate concentrations, and strongly increasing abundances of typical sulphur-oxidizing, nitrate-reducing bacterial taxa within the nitrate plume. The general absent hydrogen isotope fractionation of benzene, also in the sulphidic, nitrate-free part of the plume, indicates that benzene was not biodegraded by sulphate-reducing consortia. However, detected small carbon isotope fractionation of benzene points to *in situ* benzene biodegradation processes in the plume, probably supported by nitrate. In conclusion, nitrate injection resulted in changing redox conditions and recycling of sulphate in the sulphidic, sulphate-depleted benzene plume due to microbial oxidation of reduced sulphur species, leading to presumably favored conditions for *in situ* benzene biodegradation.

Keywords: benzene – compound specific stable isotope analysis – nitrate – sulphide oxidation, enhanced natural attenuation - bioremediation

1. Introduction

Besides chlorinated hydrocarbons, BTEX (benzene, toluene, ethylbenzene, xylenes) compounds are the most common groundwater pollutants that require remediation measures (Wiedemeier, Rifai et al. 1999). It is nowadays well understood that many monoaromatic pollutants can be degraded at oxic and anoxic conditions by several microorganisms (Farhadian, Vachelard et al. 2008, Weelink, van Eekert et al. 2010). Although aerobic biodegradation of BTEX is much faster than anaerobic biodegradation, the latter process is crucial for natural attenuation processes at BTEX-contaminated sites due to the low aqueous solubility and rapid consumption of oxygen. During anaerobic BTEX degradation, microorganisms transfer electrons from the target compounds to different terminal electron acceptors such as nitrate, ferric iron, sulphate, or carbonate, so that the plume is composed of different redox zones depending on the availability of electron acceptors (Meckenstock, Elsner et al. 2015). Benzene, which is a carcinogenic substance causing leukemia and further mutagenic diseases, is the worst degradable of the BTEX compounds at anoxic conditions (Vogt, Kleinsteuber et

al. 2011). Nevertheless, benzene mineralization at anoxic conditions has been observed with various electron acceptors such as carbonate, sulphate, ferric iron or nitrate, often at laboratory conditions (summarized by (Vogt, Kleinsteuber et al. 2011)). In previous studies, *in situ* benzene biodegradation in groundwater was shown to be stimulated by injecting nitrate and sulfate (Cunningham, Rahme et al. 2001) or nitrate, triethyl phosphate and persulfate (Xiong, Mathies et al. 2012). Pathways and extent of anaerobic benzene degradation in contaminated aquifers are mainly controlled by the prevailing biogeochemical conditions and the presence of specific benzene degrading microorganisms.

Concentrations of the inorganic sulphur compounds sulphide and sulphate are important markers for those biogeochemical conditions. The presence of free sulphide ($\Sigma(H_2S_{aq}, HS^-, S^{2-})$) in groundwater is always a clear indicator for sulphate-reducing conditions. Furthermore, the occurrence of free sulphide implies that any iron ions that had been present in the aquifer precipitated as insoluble iron-sulphur-compounds. Due to the very low solubility product of Fe-S-compounds, their precipitation reaction is very fast and usually complete. Fe-S-compounds represent an electron donor that is used up in the presence of suitable electron acceptors such as nitrate. The oxidation of Fe-S-compounds produces sulphate that can act as a further electron acceptor in the aquifer. Previous field and laboratory studies showed that sulphate is the preferential electron acceptor for anaerobic benzene degradation at the study site (Kästner, Fischer et al. 2006, Schirmer, Dahmke et al. 2006, Fischer, Theuerkorn et al. 2007, Vogt, Gödeke et al. 2007, Kleinsteuber, Schleinitz et al. 2008, Herrmann, Kleinsteuber et al. 2010, Rakoczy, Schleinitz et al. 2011, Taubert, Vogt et al. 2012). However, higher concentrations of free sulphide (> 50 mg L^{-1}) have been shown to inhibit benzene degradation under sulphate-reducing conditions in laboratory experiments with microbial communities from the test site (Taubert, Vogt et al. 2012), likely due to its known potential toxicity for aerobic and anaerobic organisms (Koschorreck 2008). In addition, benzene concentrations in the lower aquifer were observed to considerably increase so alongside with decreasing sulphate concentrations resulting in conditions in which benzene mineralization was limited by the available sulphate. However,

preliminary *on site* experiments showed that, in the presence of nitrate, free sulphide and presumably, Fe-S-compounds are quickly oxidized to sulphate by nitrate-reducing, sulphide oxidizing *Proteobacteria* (Heber 2013, Poser, Vogt et al. 2014). Furthermore, benzene mineralization at nitrate-reducing conditions by indigenous microbial communities from the Zeitz site has been verified in laboratory microcosm and *on site* column experiments (Keller et al. 2018), demonstrating the potential of the indigenous microbial community to switch between sulphate and nitrate as electron acceptors upon benzene mineralization.

Thus, a concept was developed for the lower aquifer based on addition of nitrate to recycle the natural electron acceptor sulphate by nitrate-dependent sulphide oxidation and to make an alternative electron acceptor available for anaerobic benzene oxidation; in the field experiment, we aimed to evaluate the biogeochemical processes in the benzene contaminated sulphidic aquifer triggered by nitrate injection. Nitrate is well soluble in water and provides a high oxidation potential, rising up the redox potential in the surrounding milieu. Compared to oxygen gas, which is a comparable strong electron acceptor, nitrate solutions can be easily injected into aquifers without forming gas phases interfering with the groundwater flow.

In the nitrate injection field experiment, reactive processes of the sulphur, nitrogen and carbon cycle were monitored by ^{34}S and ^{18}O isotope analysis of sulphate, ^{15}N and ^{18}O isotope analysis of nitrate, and ^{13}C and ^{2}H isotope analysis of benzene. Molecular biological methods were employed to characterize the impact of the nitrate injection on the groundwater microbial community within the benzene plume. The results are discussed with respect to the development of a strategy for monitored bioremediation to control BTEX plumes in sulphidic aquifers.

2. Materials and methods

2.1 Site description

2.1.1 History of the study site

The investigation area is located on the site of a former hydrogenation plant north of the city of Zeitz in Central Germany. That facility was installed in 1939 for the production of fuel, lubricants and paraffins out of lignite. During World War II, especially in the years 1944/45, the plant was partly destroyed by severe bomb strikes that resulted in a spilling of vast amounts of aromatic hydrocarbons into the soil. In 1963, a new plant for the production of benzene was established (Gödeke, Weiß et al. 2004). Between 1965 and 1977 at least nine documented production accidents contributed to the BTEX-contamination of the groundwater. Benzene entered the underground at several locations throughout the test site and contaminated groundwater down to 30 m (Gödeke, Richnow et al. 2006). After the German Reunification in 1990, all production facilities were shut down (Schirmer, Dahmke et al. 2006). Since then, numerous measures had been taken to apply active or passive remediation strategies in order to prevent a further spreading of the groundwater pollutants. The benzene-dominated BTEX plume was used as a field laboratory to study anaerobic BTEX degradation (Vieth, Kästner et al. 2005, Fischer, Bauer et al. 2006, Fischer, Theuerkorn et al. 2007, Fischer, Herklotz et al. 2008, Fischer, Gehre et al. 2009). Furthermore, the Helmholtz Center for Environmental Research installed a pilot plant for testing different groundwater remediation approaches based on enhancing the natural attenuation capability of the aquifer (Wachter, Dethlefsen et al. 2004, Gödeke, Richnow et al. 2006, Schirmer, Dahmke et al. 2006, Vogt, Gödeke et al. 2007). Especially, anaerobic benzene degradation at sulphate-reducing or nitrate-reducing conditions was investigated in different column systems filled with coarse sand and flushed with anoxic, benzene containing groundwater from the lower aquifer (GW 61) (Vogt, Gödeke et al. 2007, Knöller, Vogt et al. 2008, Taubert, Vogt et al. 2012, Poser, Vogt et al. 2014, Keller, Kleinsteuber et al. 2018).

2.1.2 Hydrogeology of the investigation area

Geologically, the test site consists of a sequence of two heterogeneous aquifers with a thickness between 12 and 20 m (Gödeke, Richnow et al. 2006) that are separated by a discontinuous sulphur-rich (2%) lignite-clay layer (Schirmer, Dahmke et al. 2006). The upper (Quaternary) aquifer is partly

unconfined and represents a sand deposit from the Pleistocene Elster glacial period. The lower (Tertiary) aquifer consists of gravel deposited by an Eocene river. A schematic cross section of the area is given in the supporting information (Figure 1b). Benzene contaminations occur in both aquifers (up to 1600 mg L^{-1} in the upper and up to 250 mg L^{-1} in the lower aquifer (Gödeke, Richnow et al. 2006)). The general groundwater flow direction is north-east (Figure 1a) (Gödeke, Weiß et al. 2004).

2.2 Nitrate injection and groundwater infiltration

6.74 tons of $NaNO_3$ were injected as 35 % solution (19.23 m^3, 4110 mM) into the groundwater flow over a period of 60 days (23rd July to 20th September 2015). The nitrate solution was stored in four stainless steel tanks, each having a volume of 1500 L. The tanks were flushed with dinitrogen (N_2) prior to storage of nitrate solution to remove oxygen. The nitrate solution was pumped into the infiltration well in a rate of 10.9 L h^{-1} via stainless steel connections. The groundwater was pumped from well GW EB2 (Figure 1a), enriched with NO_3^- in the injection well and pumped via injection well into the lower aquifer at 40 m below ground in a rate of 1500 L h^{-1} during the whole time of the field experiment. Groundwater from EB2 was taken from a depth of 22-30 m, contained high concentrations of benzene and was sulfidic (Figure 2), hence represented the original plume conditions of the lower aquifer.

2.3 Biogeochemical monitoring

Downstream of the injection well, water samples were collected in nine groundwater monitoring wells located in three observation lines on a weekly to biweekly basis (Figure 1).

The observation wells were equipped with a multi-level packer system (IMW, Germany) described in Schirmer, Jones et al. (1995) giving access to different depth levels of the lower aquifer (Table 1).

2.4 Chemical analyses

Electrical conductivity (EC) and pH were measured immediately after sampling using a multiparameter probe (WTW Germany). The concentrations of sulphide (S^{2-}), nitrite (NO_2^-), sulphate (SO_4^{2-}), nitrate (NO_3^-), sodium (Na^+) and benzene (C_6H_6) were analysed in the laboratory within 24 hours using methods as follows.

Benzene concentrations were determined by headspace gas chromatography as described elsewhere (Keller, Kleinsteuber et al. (2018). Na^+, NO_3^- and SO_4^{2-} were analysed by ion chromatography as described by Vogt, Gödeke et al. (2007). NO_2^- was photometrically analysed after reaction with sulfanilamide and N-(1-naphthyl)-ethylendiamine-dihydrochloride as described elsewhere (Keller, Kleinsteuber et al. (2018). Sulphide (H_2S, HS^-, S^{2-}) concentrations were determined photometrically according to Cline (1969) using modifications described by Herrmann, Kleinsteuber et al. (2008). Sulphide samples were immediately fixed in zinc acetate solution (3%) to prevent losses by chemical or biological oxidation reactions.

2.5 Isotope Analyses

2.5.1 Isotope analyses of ^{34}S-SO_4^{2-}, ^{18}O-SO_4^{2-}, ^{34}S-S^{2-}, ^{15}N-NO_3^- and ^{18}O-NO_3^-

Samples for sulphide concentrations and corresponding isotopic signature measurements were fixed immediately after sampling by mixing with 3 % Zn-acetate-solution leading to complete precipitation of ZnS. For sulphur isotope analyses in the laboratory, ZnS was subsequently converted to Ag_2S (Canfield, Raiswell et al. 1986, Fossing and Jørgensen 1989). Dissolved sulphate was precipitated as $BaSO_4$. Sulphur isotopic compositions were measured after conversion of $BaSO_4$ or AgS_2 to SO_2 using an elemental analyzer (continuous flow flash combustion technique) coupled with an isotope ratio mass spectrometer (Delta S, ThermoFinnigan, Bremen, Germany). Sulphur isotope measurements were performed with an analytical error of the measurement of better than 0.3‰, and results are reported in delta notation ($\delta^{34}S$) as part per thousand (‰) deviation relative to the Canõn Diablo Troilite (CDT) standard (according to general eq. 1):

$$\delta_{sample}\ [‰] = [(R_{sample} - R_{standard})/R_{standard}] \cdot 1000 \qquad (1)$$

Oxygen isotope analysis of prepared barium sulphate samples was carried out by high temperature pyrolysis at 1450°C in a TC/EA connected to a delta plus XL mass spectrometer (Thermo-Finnigan, Bremen, Germany) with an analytical error of better than +/-0.5 ‰. Results of oxygen isotope measurements are expressed in delta notation ($\delta^{18}O$) as part per thousand (‰) deviations relative to Vienna Standard Mean Ocean Water (VSMOW). The normalization of oxygen isotope data of sulphate was carried out using the reference material NBS 127 with an assigned $\delta^{18}O$ value of +8.7 ‰ (VSMOW).

Water samples for nitrate isotopic analysis were filtered through 0.22 µm cellulose acetate filters and stored at 4°C in the dark in high-density polyethylene bottles. To measure the isotopic composition of dissolved nitrate, the denitrifier method using whole cells of *Pseudomonas chlororaphis* strain ATCC #13985 was applied (Sigman, Casciotti et al. 2001, Casciotti, Sigman et al. 2002). A DELTA V Plus mass spectrometer in combination with a GasBench II from Thermo Scientific was used for nitrate isotope determination. Isotopic ratios are expressed in delta notation according to eq. 1 relative to atmospheric nitrogen for $\delta^{15}N$ and relative to Vienna Standard Mean Ocean Water (VSMOW) for $\delta^{18}O$.

The standard deviations for nitrogen and oxygen isotope measurements of nitrate were ±0.4 and ±1.6 ‰, respectively. Each sample was measured as duplicate and represents a mean value. For calibration of nitrogen and oxygen isotopic signatures, the following international standards were used: USGS32, USGS34, USGS35 and IAEA NO3.

2.5.2 Isotope analysis of ^{13}C-benzene and ^{2}H-benzene

Carbon isotope signatures of benzene were determined by carbon isotope ratio mass spectrometry (GC-C-IRMS) using a GC 7890A (Agilent Technologies, Palo Alto, CA, USA) coupled via a ConFlo IV interface (Thermo Finnigan, Bremen, Germany) with MAT 253 (Thermo Finnigan, Bremen, Germany). The combustion furnace was held at 1030°C on a Cu/Ni catalyst. Benzene in the GC effluent stream was oxidized to CO_2 and H_2O in the combustion furnace, and transferred online to the mass spectrometer to determine the carbon isotope ratios.

Hydrogen isotope signatures of benzene were determined by gas chromatography pyrolysis isotope ratio mass spectrometry (GC-P-IRMS). The hydrogen of benzene was quantitatively converted online into hydrogen gas. A GC 7890A (Agilent Technologies, Palo Alto, CA, USA) was coupled via a ConFlo IV interface to a MAT 253 system from the year 2007/08 (Thermo Finnigan, Bremen, Germany). Samples were thermally decomposed by online pyrolysis in non-porous alumina tube reactors at 1420°C

For both carbon and hydrogen isotope analyses, gaseous benzene (1 mL) was injected into the GC, previously separated from aqueous water samples (10 mL) by incubation at 70°C for 20 min in an agitator (rotation regime: 250 rpm for 5 s and no rotation for 2 s). Benzene was injected in a split/splitless injector held at 250°C (splits ranged from splitless to a split at 1:40) and separated on a capillary column (Zebron ZB1, 60 m x 0,32 mm x 1 µm, Phenomenex, Torrance, CA, USA) under a constant helium flow at 2 mL min^{-1}. The temperature program started at 40°C, was held for 5 min isothermally, and was then increased at a rate of 3 K min^{-1} to 90°C. The temperature was then increased by 20 K min^{-1} to 300°C and held for 5 min. All samples were measured in at least three replicates. The precision of the mass spectrometers were checked with the reference gas (CO_2, H_2) and a benzene standard of known isotope composition. The total analytical uncertainty incorporating both accuracy and reproducibility for $\delta^{13}C$ values was always better than ± 0.5 ‰ and for the $\delta^{2}H$ values always better than ± 10 ‰. Isotope ratios were expressed in the δ-notation ($\delta^{13}C$ and $\delta^{2}H$) relative to international isotope standards of the International Atomic Energy Agency using eq. 2 (Coplen 2011), Vienna Pee Dee Belemnite (V-PDB) and Vienna Standard Mean Ocean Water (V-SMOW) for stable carbon and hydrogen isotopes, respectively.

$$\delta^{13}C_{benzene} \text{ and } \delta^{2}H_{benzene} = \frac{Rsample}{Rstandard} - 1 \qquad (2)$$

2.6 Analysis of groundwater microbial communities

For the microbial community analysis, 1 L groundwater was sampled and filtered (Millipore polycarbonate filters with 0.22 µm pore size; Merck KGaA, Darmstadt, Germany) in the laboratory on the same day. Filters were stored at -20°C until further processing. DNA was extracted from the frozen filters using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA samples were analysed by terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of bacterial 16S rRNA genes as described by Sträuber, Lucas et al. (2016) using the restriction enzymes *Hae*III, *Rsa*I or *Bst*UI (New England Biolabs, Frankfurt (Main), Germany). Based on T-RFLP profiles, representative samples were selected for a more detailed analysis of the community composition by amplicon pyrosequencing of bacterial 16S rRNA genes on the GS Junior platform as described by Ziganshin, Liebetrau et al. (2013). Raw sequence data were processed with the QIIME 1.8.0 pipeline (Caporaso, Kuczynski et al. 2010) as described in detail by Sträuber, Lucas et al. (2016). De-multiplexed sequences of the analyzed samples were deposited at the ENA.

3. Results and discussion

3.1. Pre-experimental hydrochemical conditions in the aquifer and ratio of the nitrate injection experiment

In groundwater well EB2 located in the upstream of the injection well, benzene, sulphate and sulphide concentrations were monitored for more than 10 years from June 2006 until December 2016 (Figure 2). Beginning in March 2011 (t ~ 1700d), benzene concentrations continuously increased from below 0.5 mM to up to 2.5 mM in December 2012 (t = 2367d); afterwards, benzene concentrations dropped to levels in the range of 0.75 to 1 mM until the end of monitoring. Sulphate concentrations decreased from around 4 mM to less than 0.5 mM in December 2012. Until December 2016, sulphate concentrations gradually increased to maximal concentrations of around 2.5 mM. The groundwater was always sulphidic; sulphide concentrations ranged from less than 0.1 mM to more than 2 mM; highest concentrations were observed in the last years of monitoring. Notably, sulphide concentrations of around 1.5 mM were shown in laboratory experiments to strongly inhibit benzene mineralization at sulphate-reducing conditions (Taubert, Vogt et al. 2012). Furthermore, sulphate concentrations were far not sufficient for complete benzene mineralization from 2012 to 2016 (Figure 2), considering that the mineralization of 1 mol benzene is coupled to the reduction of 3.75 mol sulphate (Weelink, van Eekert et al. 2010). The data show that a plume partially depleted in sulphate and containing high benzene and sulphide loads, was flowing in direction of the test field, which motivated us to perform a nitrate injection field experiment to test an enhanced natural attenuation (ENA) approach based on nitrate reduction.

Previous investigations suggested that the prevailing hydrochemical conditions in the aquifer support the occurrence of bacterial sulphate reduction as the main process for anaerobic benzene degradation (Schirmer, Dahmke et al. 2006, Vogt, Gödeke et al. 2007, Knöller, Vogt et al. 2008). To verify the assumption of sulphate-reducing conditions, we conducted an initial screening especially focused on the correlation between sulphate concentrations and the isotopic composition of dissolved groundwater sulphate.

During the initial screening, sulphate concentrations and $\delta^{34}S$ values varied between 4.4 mM (422 mg L^{-1}) and 0.45 mM (43 mg L^{-1}) and between 8.8 and 51.3 ‰, respectively. The left plot in Figure 3 shows the relationship between sulphate concentrations (expressed as the fraction of reacted sulphate) and the sulphur isotopic signature of groundwater sulphate. The observed high and very high $\delta^{34}S$ values above 20 ‰ are a first indication for an isotopic enrichment associated with bacterial reduction of groundwater sulphate. Increasing $\delta^{34}S$ values in concert with decreasing sulphate concentrations provide further evidence for the occurrence of sulphate reduction. The observed correlation follows a logarithmic relationship that may be expressed by a general logarithmic equation (Rayleigh equation) ($\delta^{34}S\text{-observed} = \varepsilon Ln(C/C\text{-}0) + \delta^{34}S\text{-initial}$, where ε is the isotopic enrichment factor). According to Knöller, Vogt et al. (2006), an isotopic enrichment factor between -35 and -40 ‰ can be expected for straightforward bacterial sulphate reduction in a hydrochemical setting with similar conditions as present in the investigated aquifer. Fitting the Rayleigh equation to the observed field data yields an apparent enrichment factor of ca. -18 ‰. Compared to the expected values for ε of -35 and -40 ‰, the field based ε is relatively low. This indicates that bacterial sulphate reduction is not the sole process controlling sulphate concentrations and isotopic compositions. If concentration changes occur without changes of $\delta^{34}S$ values, the field based enrichment factor appears to be lower than the expected ε. Such fractionation patterns are associated with the process of dilution.

The right plot of Figure 3 shows the dual sulphate isotope space and the correlation between the sulphur and oxygen isotopic composition of groundwater sulphate during the initial screening (July 15, 2015). The relationship between $\delta^{18}O\text{-}SO_4$ and $\delta^{34}S\text{-}SO_4$ is characterized by a strong positive linear correlation with a slope close to a quarter. This slope can be considered as typical for isotope fractionation associated with bacterial sulphate reduction. Therefore, any further processes affecting the isotopic composition of sulphate do not seem to be likely. This implies that mixing of sulphate from isotopically different sources does not occur in the aquifer.

No correlation between sampling depth and sulphate isotopic composition is obvious. However, no isotopic enrichment was observed in the deepest sampling level suggesting that bacterial sulphate reduction is of minor importance below 45 m depth.

3.2 Nitrate injection and temporal development of sulphate, sodium ions, nitrite and nitrate concentrations

Nitrate was injected in a section of the lower confined tertiary sand aquifer (Figure 1) that is affected by the benzene contaminant plume. Figure 4 shows the temporal development of nitrate, nitrite, sulphate and sodium concentrations in those wells of each observation line that seemed to be most impacted by the nitrate injection (BB1 in the first line, 17A in the second line, 31A in the third line). The reason for the observed high nitrate impact seems to be the depth of the respective wells. BB1, 17A and 31A are the wells with the deepest drilling depth in each line. Therefore, morphology and groundwater flow direction determine the flow direction of the injected substance. The investigated aquifer showed a background concentration of 1.4 – 2.2 mM Na^+ (32 to 51 mg L^{-1} Na^+) with an average of 1.7 mM (39 mg L^{-1}). In contrast to the reactive behavior of nitrate, a biogeochemical turnover of sodium is not expected so that Na^+, the counter cation of the applied nitrate solution, was considered as a conservative tracer during the field experiment. Due to the longest relative travel time of the injected solution through the aquifer, we expected that the strongest biogeochemical impact of the nitrate injection should be visible in observation wells 31A/02 and 44A/02 in the third observation line.

Line 1: In the first observation line, only the lowermost sampling levels (P5) were affected by the nitrate injection. Nitrate concentrations started to increase after 42 days in the lowest sampling level (P5). The highest nitrate concentration of 57.8 mM was measured 56 days after the start of the injection. Simultaneously, sodium concentrations reached its maximum of 56.4 mM. Particularly in the deepest observation level, the concentration of nitrite rose from undetectable to 0.2 mM.

Line 2: A hydrochemical impact of the nitrate injection is visible in all measured sampling levels (P1, P2, P3, P4). Sodium and nitrate concentrations in the two upper observation levels showed a minor peak 119 days after the beginning of nitrate injection. At the same time, highest nitrate concentrations in the two lower levels (P3 = 45.4 mM, P4 = 47.3 mM) were observed. In the further course of the experiment until its end after 427 days, nitrate concentrations remained relatively constant on a plateau between 37 and 40 mM. Sodium concentrations showed similar behavior. Nitrite concentrations increased significantly in the two lowest sampling levels. Maximum nitrite concentrations were reached after 266 days (P3 = 0.9 mM, P4 = 1.8 mM). Sulphate concentrations increased continuously especially in the lowest sampling level. Highest sulphate concentrations of 8.2 mM were detected after 322 days.

Line 3: The increase of nitrate and sodium concentrations in the third observation line began, especially in the lowest level P4, 196 days after the start of the injection. The highest nitrate concentrations of 7.75 mM were measured after 210 days. The upper two sampling levels showed a minor effect of the nitrate injection. The increase of the nitrate concentration in the second lowest level (P3) was slightly more pronounced compared to levels P1 and P2 but still very small with 0.93 mM. Generally, sulphate concentrations varied considerably in all levels between 2 mM and 5.2 mM. While highest sulphate concentrations in level P3 (5.4 mM) were measured after 103 days, the maximum sulphate peak of 5.1 mM in the observation level P4 was observed after 196 days. An increase of nitrite concentrations was detected in the two lowermost observation levels only. Maximum nitrite concentrations of around 3 mM were reached in level P4 after 266 days.

3.3 Benzene concentrations and benzene carbon and hydrogen isotope signatures

Generally, benzene may be removed from the contaminated groundwater by microorganisms using the injected nitrate as electron acceptor, as shown in previous laboratory experiments with indigenous microbial communities from the site (Keller, Kleinsteuber et al. 2018). To assess potential benzene degradation processes, benzene concentrations and benzene carbon and hydrogen isotope signatures were monitored at all sampling locations throughout the duration of the experiment.

Benzene concentrations at the investigated site showed significant temporal and local variability. The highest benzene concentrations (> 1.9 mM) in the first observation line were determined in the uppermost horizons of BB1 and BB2. In BB3, benzene concentrations varied between 0.38 mM and 1.28 mM. Benzene concentrations in the plume might be affected by the mixing of the benzene-containing infiltration groundwater taken from well EB2 into the existing plume. Benzene concentrations below the detection limit were found in BB5, demonstrating that this well was located outside the contaminant plume. In the second observation line, benzene concentrations varied between 0 and 0.46 mM. These low concentrations suggest that the benzene plume passed the second line right between wells 17A/00 and 27A/02.

Well 44A/02 in the third observation line showed the highest measured benzene concentrations at the test site (P1: ~2.2 mM, P2: ~1.3 mM, P3: ~1 mM). In well 31A/02, the maximum benzene concentrations were detected in P2 with 0.77 mM. The other levels showed lower concentrations with high variations. Due to the general local and temporal variability of benzene concentrations in the investigated plume, no clear evidence about ongoing benzene degradations could be derived from benzene concentrations alone, so that benzene carbon and hydrogen isotope signatures were determined to detect benzene biodegradation. Carbon and/or hydrogen isotope fractionation principally indicates biodegradation of hydrocarbons (Vogt, Dorer et al. 2016) as most microbial hydrocarbon degradation pathways start with a biochemical reaction in which a C-H bond within the target hydrocarbon is cleaved (Vogt, Musat et al. 2018); those reactions are generally associated with carbon and/or hydrogen isotope fractionation (Elsner, Zwank et al. 2005), leading to an enrichment of ^{13}C and/or ^{2}H in the remaining hydrocarbon molecules. Figure 5 shows correlations of benzene concentrations and hydrogen (Figure 5a) or carbon (Figure 5b, c, d) isotope signatures of all samples analysed during the field experiment. For hydrogen, no significant correlation of benzene concentrations and isotope signatures was observed; hence no hydrogen isotope fractionation could be verified in the course of the experiment. In contrast, a small but significant enrichment of ^{13}C-benzene was determined, resulting in a carbon enrichment factor (ε_C) of -0.35 ± 0.06. This indicates that a part of the benzene was biodegraded. The magnitude of benzene carbon isotope fractionation

is comparable to benzene ε_C values previously determined in the upper aquifer of the Zeitz site at prevalent sulphate-reducing conditions (Fischer et al. 2009). Notably, isotope fractionation was more pronounced in samples affected by nitrate (Figure 5c, d), indicating benzene biodegradation at nitrate-reducing conditions. In previous laboratory experiments, benzene biodegradation at nitrate-reducing conditions by microbial communities of the Zeitz site was shown to be associated with moderate carbon isotope fractionation (Keller et al. 2018), supporting this assumption. In the study of Keller, Kleinsteuber et al. (2018), only a small hydrogen isotope effect was determined upon benzene degradation, which corresponds to the data observed in the field experiment; due to the generally lower sensitivity of hydrogen isotope analysis compared to carbon isotope analysis, small hydrogen isotope effects may not be detected under field conditions. However, since benzene degradation at sulphate-reducing conditions is generally associated with strong hydrogen isotope fractionation (Fischer, Herklotz et al. 2008, Mancini, Devine et al. 2008, Fischer, Gehre et al. 2009) the absence of hydrogen isotope fractionation during the field experiment indicates that benzene was not biodegraded by microorganisms using sulphate as electron acceptor.

3.4. Isotopic variability of groundwater nitrate

The injected nitrate had a nitrogen isotopic signature ($\delta^{15}N\text{-}NO_3^-$) of 4.5 ‰ and an oxygen isotopic signature ($\delta^{18}O\text{-}NO_3^-$) of 23.5 ‰ (Figure 6a). Considering the entire nitrate isotope dataset for samples collected throughout the duration of the experiment, a positive correlation in the dual isotope space (Figure 6a) and a negative correlation between isotope signatures and nitrate concentrations is visible (Figure 6b). Both correlations may be indicative for the occurrence of bacterial nitrate reduction. The regression line in the dual isotope plot considering all samples showed a slope of 0.4. After removing two outliers showing unusual low $\delta^{18}O\text{-}NO_3^-$ values in concert with relatively high $\delta^{15}N\text{-}NO_3^-$ values, a slope of 0.9 resulted. Denitrification normally leads to an equal fractionation of $\delta^{18}O$ and $\delta^{15}N$ which means that isotopes change in parallel (Chen and MacQuarrie 2005, Granger, Sigman et al. 2008). Deviations from this ratio have been used to

untangle individual pathways of nitrogen cycling specifically to address nitrite reoxidation (Sigman, Granger et al. 2005, Rafter, DiFiore et al. 2013). Other experimental studies on denitrification in sediments identified ratios between 0.8 and 1 (Dähnke and Thamdrup 2013, Prokopenko, Hirst et al. 2013, Kessler, Bristow et al. 2014), with minimum values lower than 0.33 in tidal flat sediments (Wunderlich, Meckenstock et al. 2013) suggesting substantial variabilities of isotope fractionation ratios in natural environments. Furthermore, the composition of the microbial community can impact the isotope fractionation ratio of nitrogen and oxygen isotopes of nitrate during denitrification (Dähnke and Thamdrup 2016). Positive isotopic shifts of both species strongly indicate that nitrate is consumed by the anaerobic bacterial denitrification processes. Unusual negative shifts especially for oxygen isotopes, as observed for two samples (see Figure 6a) are an indicator for reverse reactions of intermediates (for example nitrite), which can influence the isotopic signal of the residual nitrate pool (Casciotti, Sigman et al. 2002, Casciotti, Buchwald et al. 2011). Negative shifts of δ^{18}O- NO_3 by oxygen isotope exchange between nitrate and the ambient water do not seem very likely as this process is extremely slow under environmental conditions typical for groundwater ecosystems (Knöller, Vogt et al. 2011).

Measured nitrate concentrations in the groundwater varied significantly between 0 and 57.8 mM depending on the impact of the injected nitrate as well as dispersion/dilution processes (Figure 6b). High δ^{15}N-NO_3^- values with low nitrate concentrations indicate bacterial NO_3^- consuming anaerobic denitrification reactions. In all observation wells affected by the nitrate plume, increased nitrite concentrations (>0.1 mM) were detected proving *in situ* denitrifying processes.

Despite the positive effect of providing sulphate as electron acceptor for benzene degradation, microbial nitrate reduction produces nitrite as one intermediate that has to be considered as a strong inhibitor for dissimilatory sulphate reduction. Therefore, minor amounts of nitrate and its subsequent degradation product nitrite may stop the process of dissimilatory sulphate reduction in the aquifer.

3.5 Sulphate isotopic signatures and changes in the microbial community composition as indicators for nitrate-driven oxidation of reduced sulphur compounds

The injected nitrate plume reached the first well gallery after 56 days (NO_3^- = 57.8 mM), the second after 119 days (NO_3^- = 47.3 mM) and the last after 210 days (NO_3^- = 7.75 mM) (Figure 4, Table 2).

In line 1, sulphate concentrations varied in a low range and showed no significant trend (Figure 4). Corresponding isotopic signatures behaved differently (Figure 7). $\delta^{34}S$ as well as $\delta^{18}O\text{-}SO_4^{2-}$ decreased with the arrival of the nitrate plume and increase again after the nitrate had passed the observation line ($\delta^{34}S\text{- }SO_4^{2-}$: 13 ‰ to 6 ‰, $\delta^{18}O\text{- }SO_4^{2-}$ 9 ‰ to 4 ‰).

Line 2 showed a different behaviour due to the specific morphology of the aquifer in that section. Nitrate rich water accumulated in a morphological depression of the aquifer layers, which resulted in longer residence times of the nitrate spiked water due to more stagnant flow conditions. As a consequence, oxidizing conditions were present over a longer time period. Sulphate concentrations increased slowly and reached a constant level after 196 days. Corresponding sulphate isotopic signatures decreased in that stage and reached values of -4.4 ‰ ($\delta^{34}S\text{- }SO_4^{2-}$) and 1.7 ‰ ($\delta^{18}O\text{- }SO_4^{2-}$), respectively, caused by sulphide oxidation. Sulphate showed relatively low isotopic signatures even at the end of the experiment, which is probably due to the fact that nitrate concentrations were still high.

In line 3, sulphate isotopic signatures decreased before nitrate concentrations increased (Figure 7). This means that the oxidation of sulphide to sulphate - indicated by lighter sulphate isotopic signatures (Figure 7) - already began 100 days after the start of the experiment. When the entire sulphide pool was oxidized, nitrate concentrations increased. Corresponding $\delta^{15}N\text{-}NO_3^-$ values increased to a maximum of 68 ‰ (vs. AIR) with a certain time delay indicating that nitrate was used as electron acceptor. *In situ* oxidation of sulphide was confirmed by lighter sulphate isotopic signatures. Both observation wells (31A and 44A) in the third line showed continuous sulphidic conditions with concentrations between 0.2 mM and 0.7 mM before the experiment. With the

breakthrough of injected nitrate, sulphide concentrations decreased in all horizons showing that nitrate was utilized for the oxidation of sulphide to sulphate. This reaction appeared in observation line 3 in the lowest horizon after 104 days indicated by decreasing sulphate isotopic signatures (line 1 in Figure 7c). When the entire sulphide pool was oxidized, nitrate concentrations started to increase which was the case 173 days after injection (line 2 in Figure 7c). The maximum nitrate concentration of 7.75 mM was reached after 210 days. Then, nitrate concentrations continuously dropped. After 364 days, no nitrate was detectable anymore. Microbial sulphide oxidation could also be verified by changes in the groundwater microbial community, as shown in Figure 8. Abundances of taxa known for oxidation of reduced inorganic sulphur compounds (Helicobacteraceae, Sulfurimonas, Sulfuritalea, Campylobacterales after Campbell, Engel et al. (2006)) strongly increased alongside with the arrival of the nitrate, comprising of around 90% of the microbial groundwater community.

The highly dynamic temporal development of nitrate concentrations does not seem to affect the temporal development of benzene concentrations. Benzene concentrations varied between 0.03 mM and 0.24 mM (well 31A/02-P4) and no significant benzene concentration changes indicating benzene biodegradation could be detected during the field study (Figure 9b).

During the entire field experiment, two general processes influenced sulphate isotopic signatures: (1) oxidation of sulphide which resulted in decreasing $\delta^{34}S\text{-}SO_4^{2-}$ signatures with increasing sulphate concentrations; (2) dilution effects which provoke in variable sulphate concentrations (Figure 9c/d). These processes occur in varying extent in each observation well depending on the position of the nitrate plume. Thus, groundwater observation well 44A/02-P3 shows a lower impact on injected nitrate (maximum NO_3^- with 298 mg L^{-1}, Figure 9a) compared to 31A/02-P4 (maximum NO_3^- with 481 mg L^{-1}, Figure 9b). While both processes affecting sulphate isotope signatures are visible in well 44A/02-P3, sulphide-oxidizing processes are dominating sulphate isotope signatures in well 31A/0P4 (Figure 9d).

3.6 Conceptual Model

The overall impact of the nitrate injection on the hydrochemical development in the contaminated aquifer is illustrated in the conceptual model in Figure 10. Generally, the field experiment showed that the injection of nitrate leads to more favourable hydrochemical conditions in the aquifer with respect to the degradation of organic contaminants. In the case of our experiment, nitrate does not seems to be utilized as electron acceptor for contaminant degradation directly to a large extent. Instead, a two-step process chain results in an indirect use of nitrate for benzene attenuation. In the first step, nitrate acts as an electron acceptor for the microbial oxidation of sulphide. This process most likely affects both dissolved sulphide and sulphide precipitated in the aquifer matrix. Sulphide oxidation provides sulphate that is may be utilized in a second step as an electron acceptor for benzene degradation by bacterial sulphate reduction, as previously shown at this site. Besides the provision of sulphate, the oxidation of dissolved sulphide reduces the toxicity level of the groundwater, which improves the hydrochemical prerequisites for microbial activity. Unfortunately, due to the highly temporally and locally variable benzene concentrations in the contaminant plume before the start of the experiment combined with the open system conditions, an exact balancing of the two-step process was not possible.

4. Conclusion

We conducted a field experiment to investigate biogeochemical processes associated to nitrate injection in a sulphidic aquifer contaminated mainly with benzene. To improve the biogeochemical conditions for benzene degradation, we injected 6.74 tons of sodium nitrate solution into the confined aquifer. The necessary amount of nitrate was calculated based on lab experiments and detailed information about the contaminated site from past studies (Gödeke, Weiß et al. 2004, Gödeke, Richnow et al. 2006). A monitoring programme was conducted documenting benzene, nitrate, sulphide and sulphate concentration and the respective isotopic compositions.

The field experiment proved that the prevailing impact of the nitrate injection was the regeneration of sulphate by microbial re-oxidation of reduced, potentially toxic sulphur compounds (Knöller, Vogt

et al. 2008, Poser, Vogt et al. 2014, Keller, Kleinsteuber et al. 2018). This leads to environmental-friendly conditions for the establishment of microorganisms which can use sulphate to degrade organic compounds (Herrmann, Kleinsteuber et al. 2010). No clear prove was obtained from concentration and isotope data that benzene was oxidized by indigenous microbial communities, although previous on-site and laboratory experiments have demonstrated the potential for microbial benzene oxidation with sulfate or nitrate as electron acceptor; the detected low but significant benzene carbon isotope fractionation give some hint for benzene oxidation by denitrifying consortia. It might be that the monitoring time period (around 15 months) was not long enough to verify *in situ* benzene oxidation.

Our study significantly contributes to the research field of modern, *in situ*, large scale remediation strategies for contaminated aquifers based on the concept of enhanced natural attenuation and bioremediation, respectively. A new approach of enhancing contaminant degradation on field scale was tested and documented. Our results indicate that nitrate injection results in a promising, accelerated improvement of the hydrochemical conditions in the previously sulphidic, highly contaminated aquifer leading to a more favourable environmental milieu for microorganisms in the aquifer with respect to the degradation of hydrocarbons.

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Figure 1. (a) Investigation area: injection well, three lines of observation wells and groundwater flow direction; (b) Cross-section of the investigated aquifer system close to Zeitz, Saxonia-Anhalt, Germany.

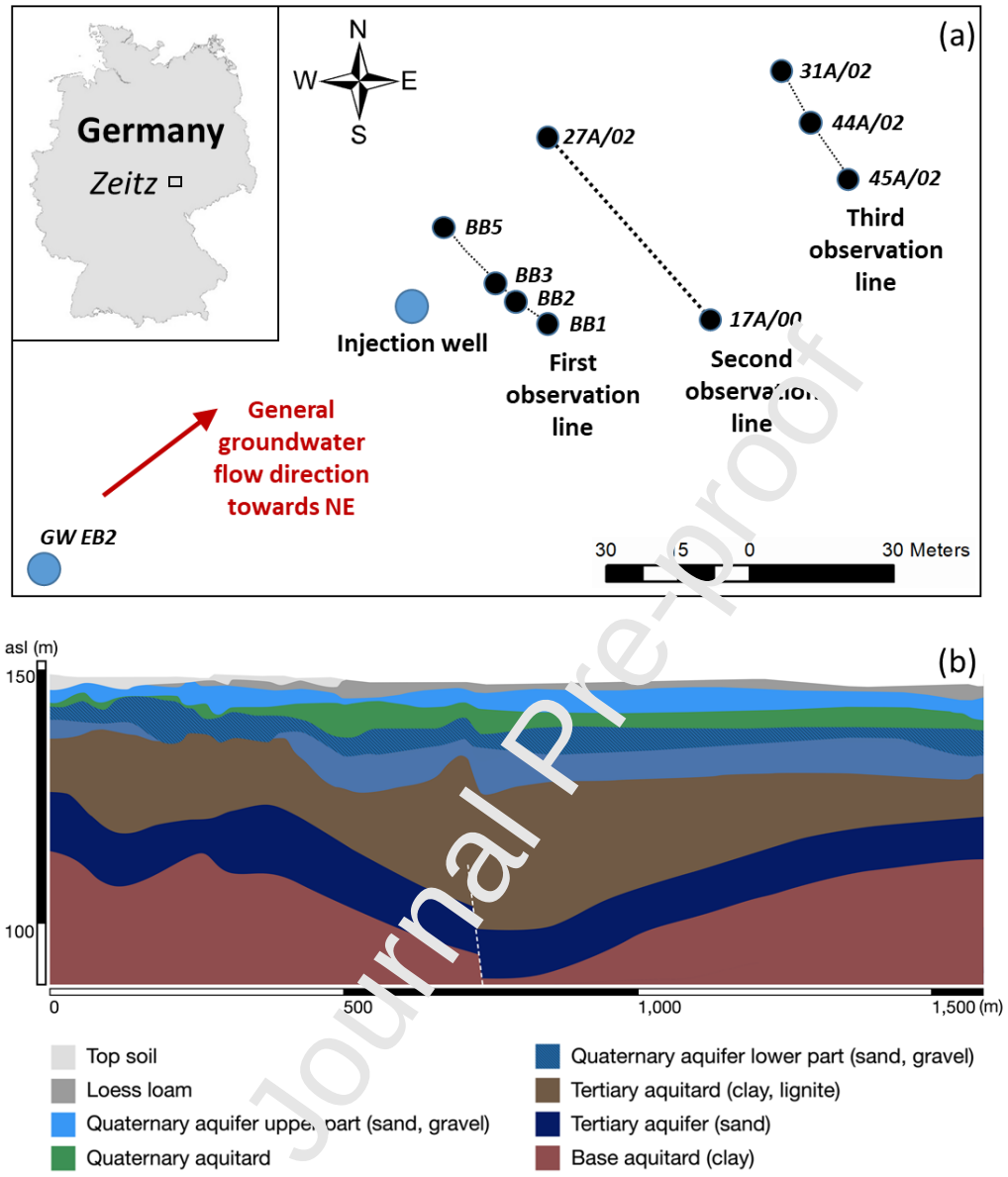


Figure 2: Concentrations of benzene (a), sulphate (b) and sulphide (c) in groundwater monitoring well EB2 from June 2006 (day 0) to December 2016.

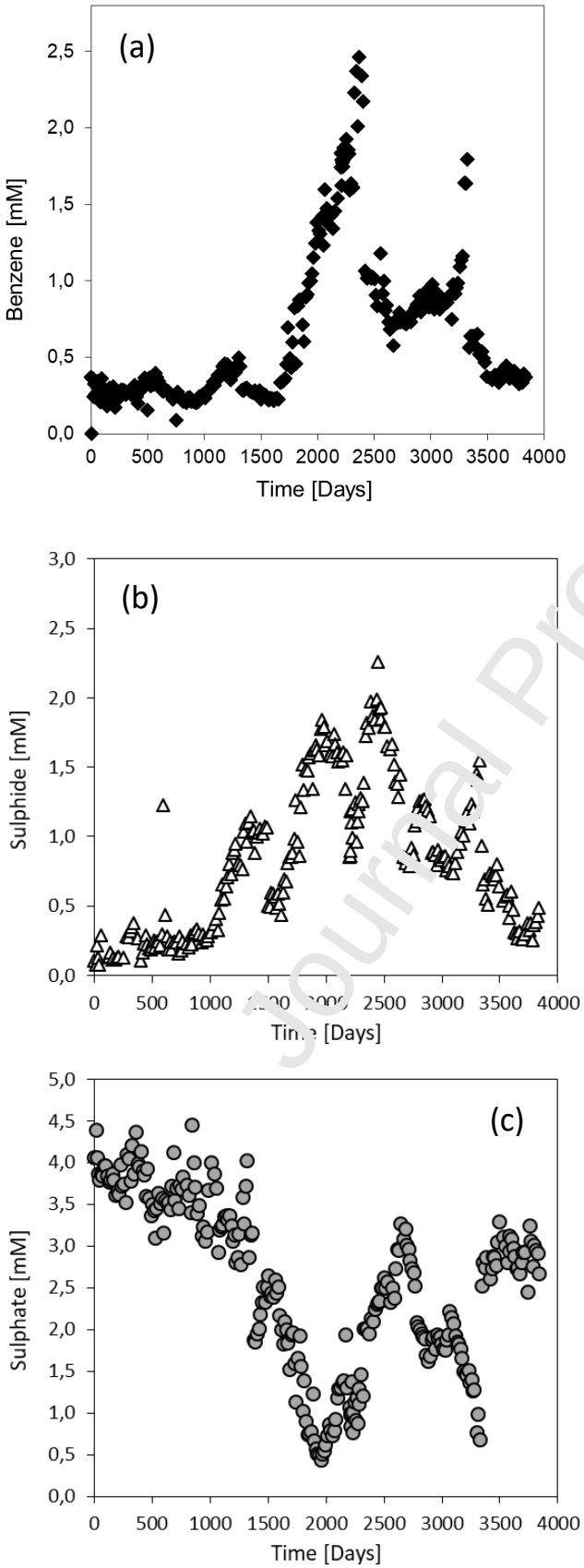


Figure 3. Correlation (a) between the fraction of reacted sulphate and the sulphur isotopic composition and (b) between the sulphur and oxygen isotopic composition of groundwater sulphate during the initial screening (July 15, 2015)

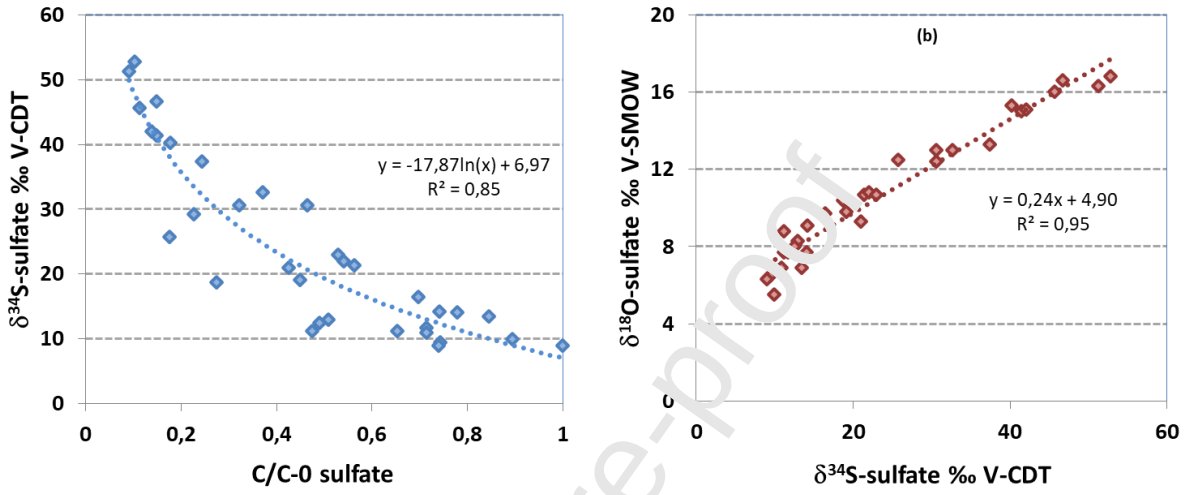


Figure 4. Temporal development of nitrate, nitrite, sulphate and sodium concentrations in representative observations wells of each observation line (BB1, 17A/00 and 31A/02)

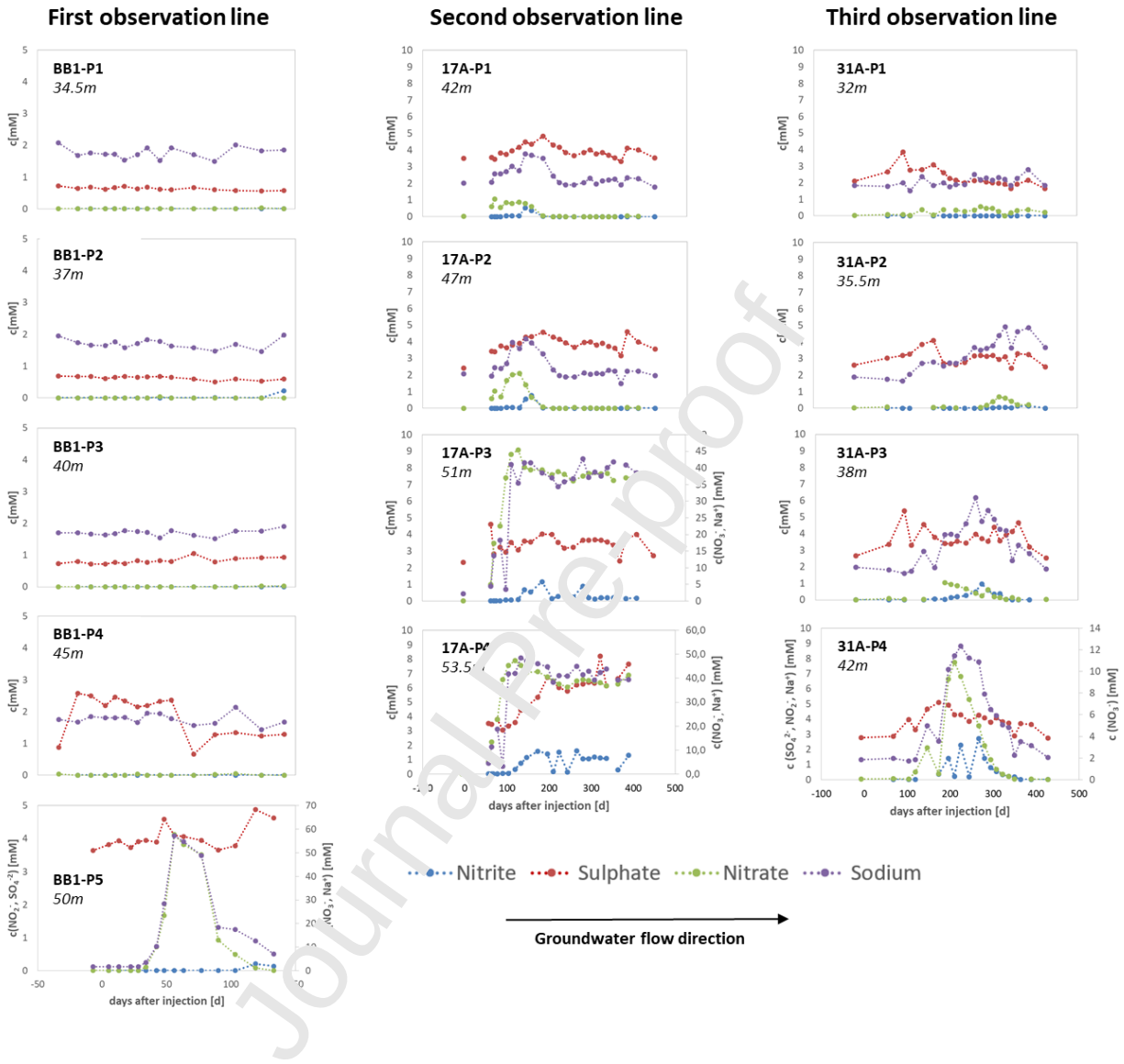
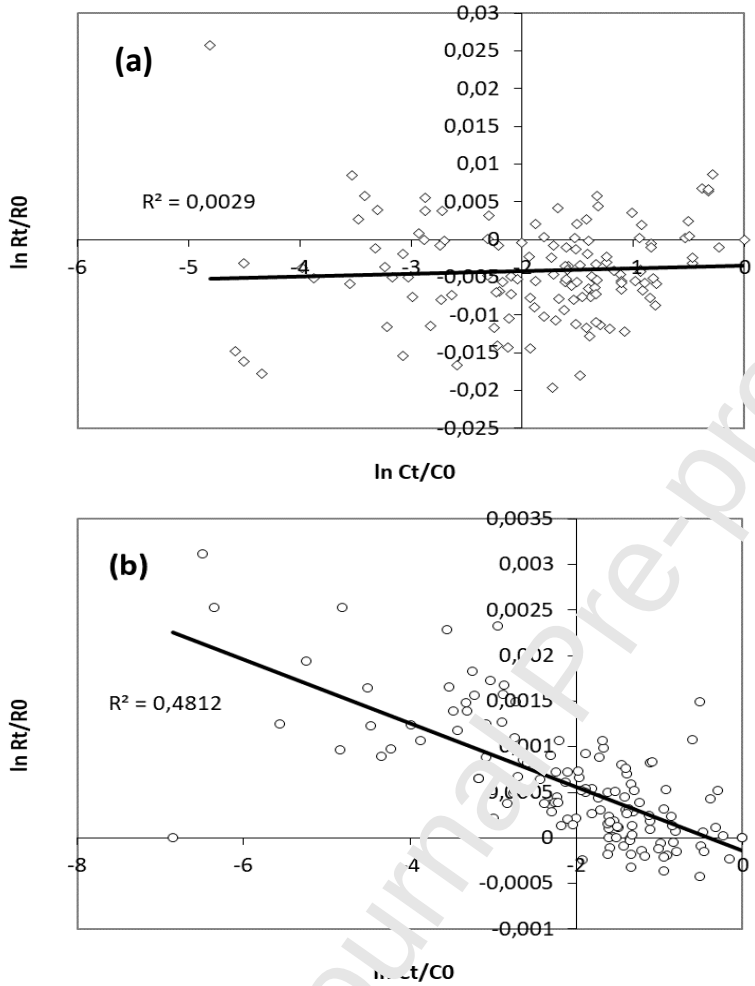
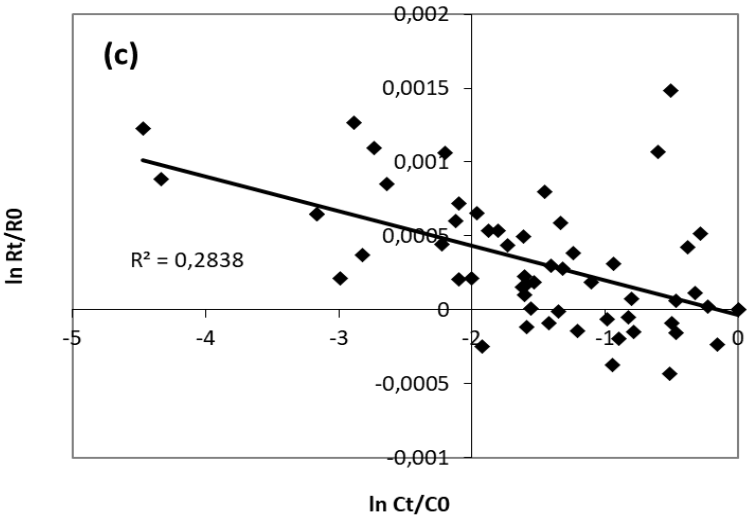


Figure 5: Rayleigh plots of (a) benzene concentrations versus benzene δ^2H values (all samples) and benzene concentrations versus benzene $\delta^{13}C$ values ((b): all samples; (c): samples without nitrate; (d): samples with nitrate). The resulting enrichment factors were ε_C = -0.35 ± 0.06 ‰ for carbon ((a), all values), ε_H = -0.35 ± 1.12 ‰ for hydrogen ((b), all values), ε_C = 0.24 ± 0.1 ((c), values of samples without nitrate) and ε_C = -0.37 ± 0.09 ((d), values of samples with nitrate).





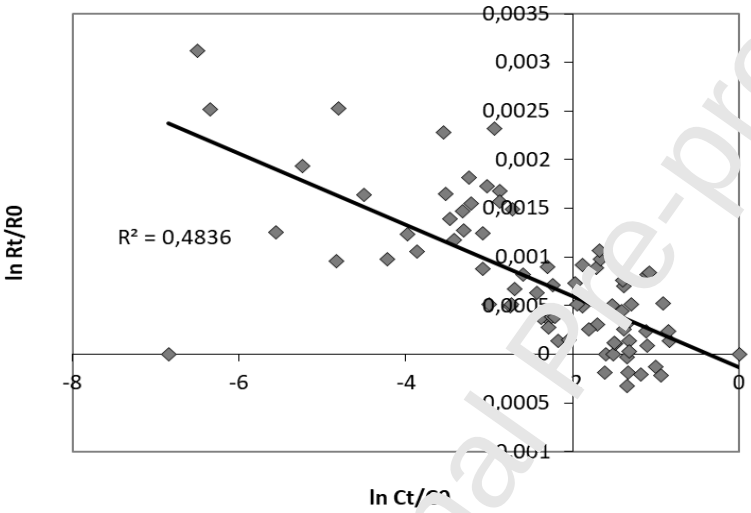


Figure 6. (a): Dual nitrate isotope plot for groundwater samples taken during the entire study period, isotopic signals are classified according to nitrate concentrations; (b): Relation between $\delta^{15}N\text{-}NO_3^-$ and nitrate concentrations for all sampling locations and horizons 1: Increasing $\delta^{15}N\text{-}NO_3^-$ with low nitrate concentrations indicate bacterial denitrification processes; 2: Dilution or contaminant plume outside of the sampling range.

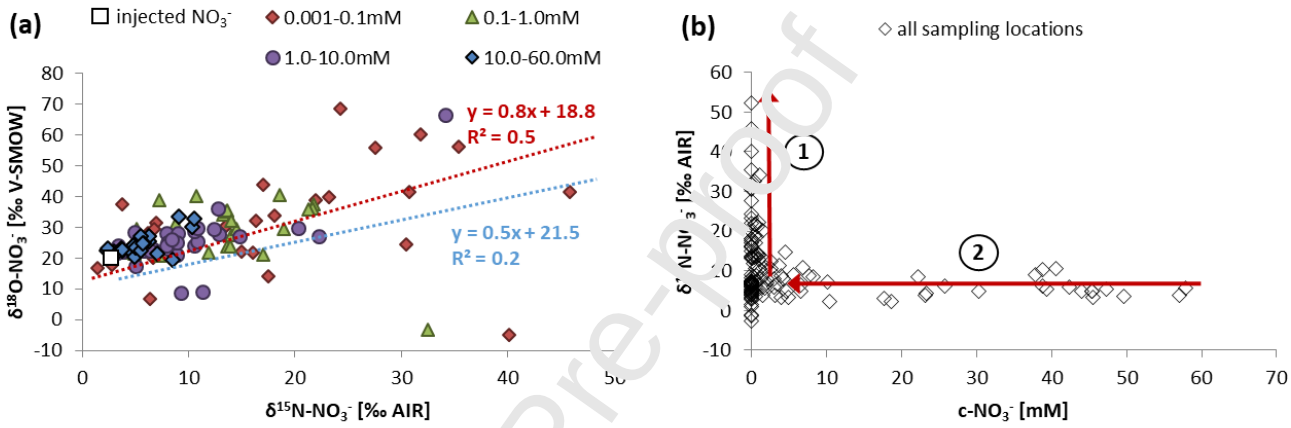


Figure 7: Temporal development of sulphate isotopic signatures as well as sulphate and nitrate concentrations in all observation lines (wells: BB1, 17A/00, 31A/02) and deepest horizons. In the last observation line, it can be differentiated between the start of sulphide oxidation to sulphate (1) and the oxidation of the entire sulphide pool, afterwards increasing nitrate concentrations (2)

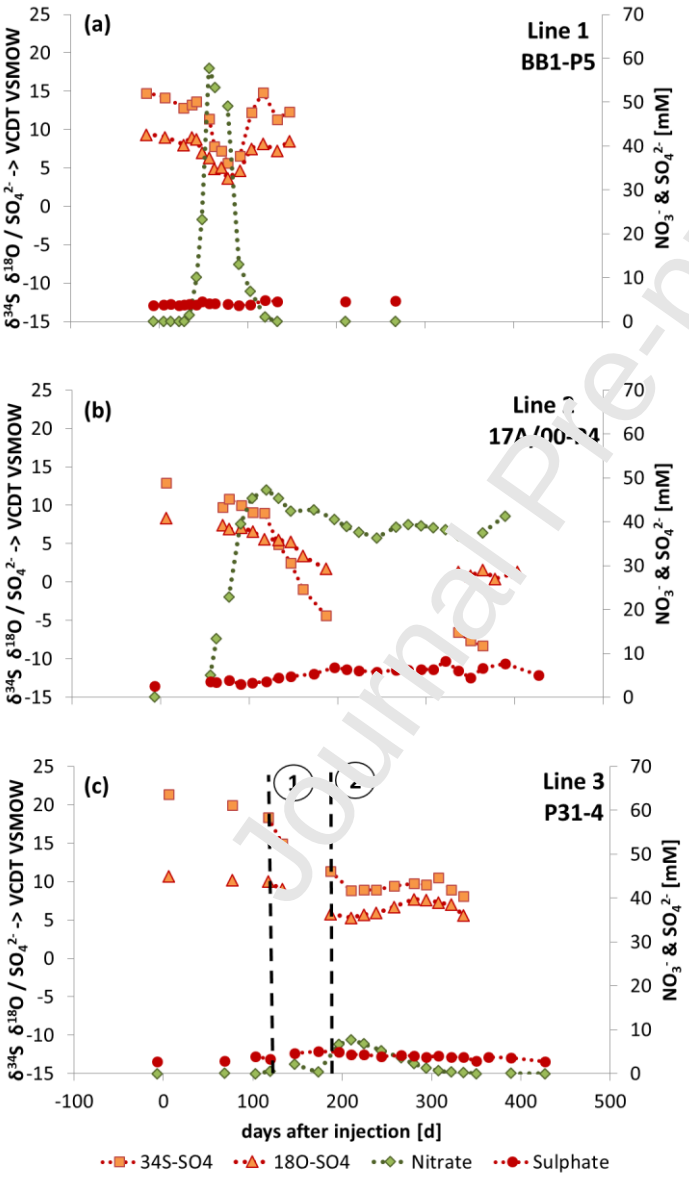


Figure 8. Relative abundances of bacterial taxa in groundwater samples from monitoring well 44/02-P3 before breakthrough of nitrate (day 63) and influenced by the nitrate plume (days 173, 224, 294 and 322). Abundances of taxa known for oxidation of reduced inorganic sulphur compounds (Helicobacteraceae, Sulfurimonas, Sulfuritalea) are indicated by symbols.

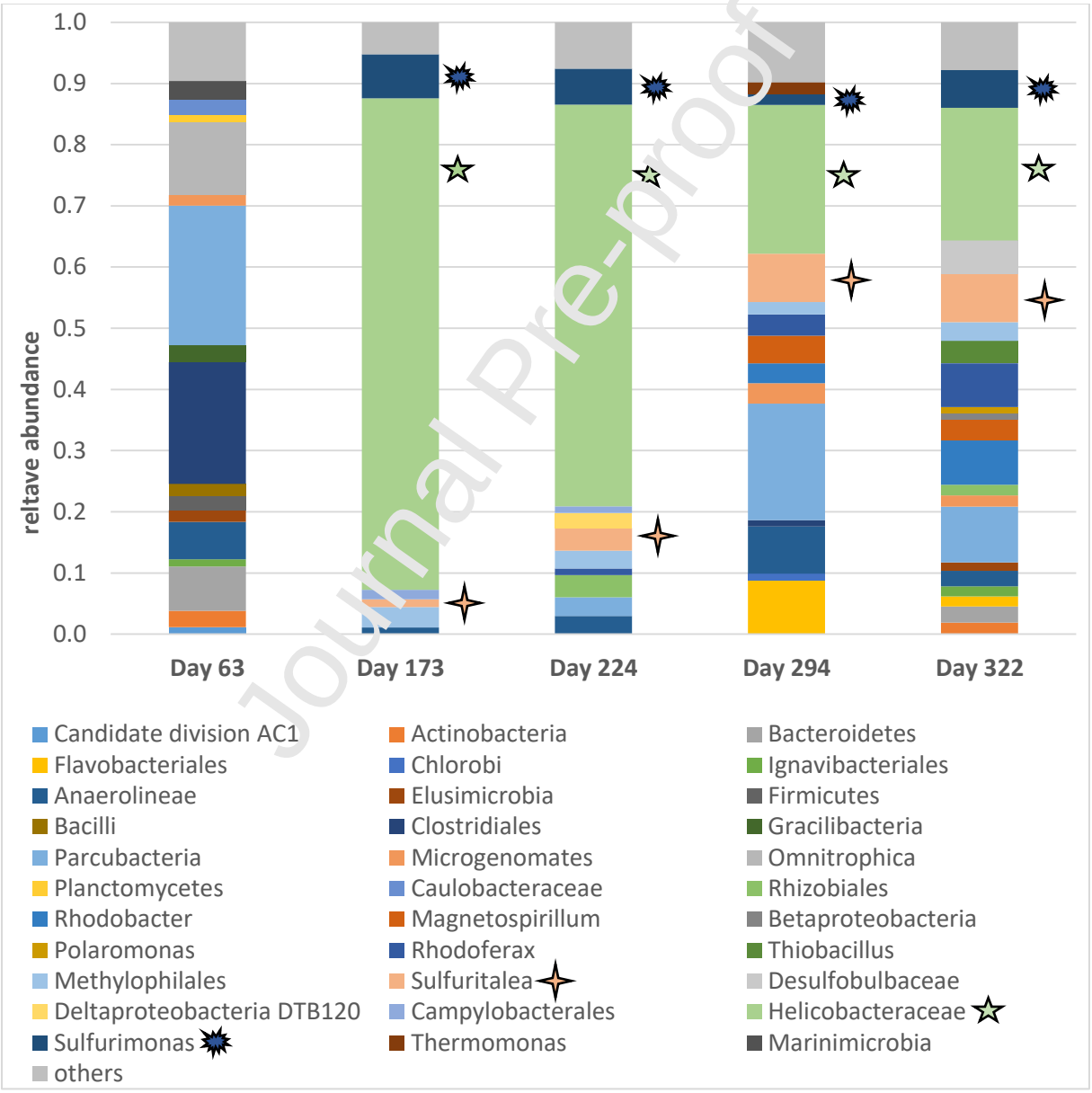


Figure 9. Temporal variation of nitrate and benzene concentrations in the lower horizons of well 44A/02-P3 (a) and 31A/02-P4 (b) in the third observation line. Corresponding spatial variations of sulphide oxidizing processes in the third line depending on the influence of the injected nitrate plume (well 44A/02-P3 (c) and 31A/02-P4 (d)), 1: Admixture of sulphate from sulphide oxidation, 2: Dilution and background variations.

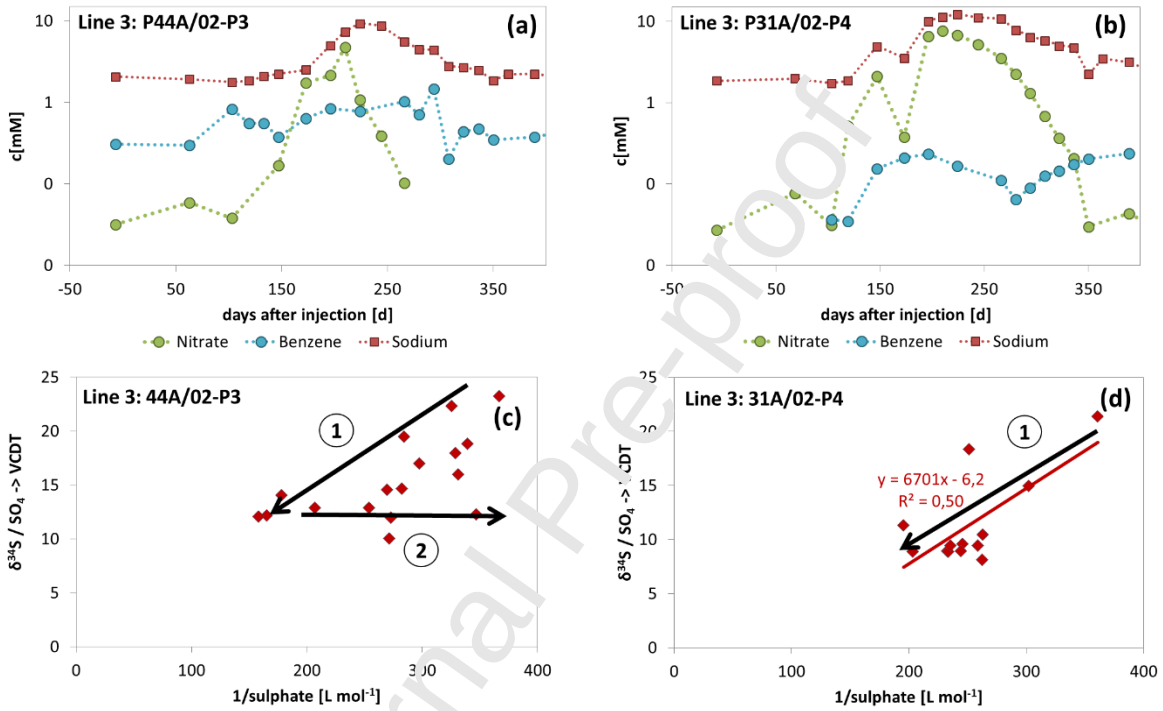


Figure 10: Conceptual model of ongoing processes in the lower aquifer after nitrate injection into the benzene contaminant plume. Sulphate reduction (*) as the last step for benzene degradation, could not be proved within this study.

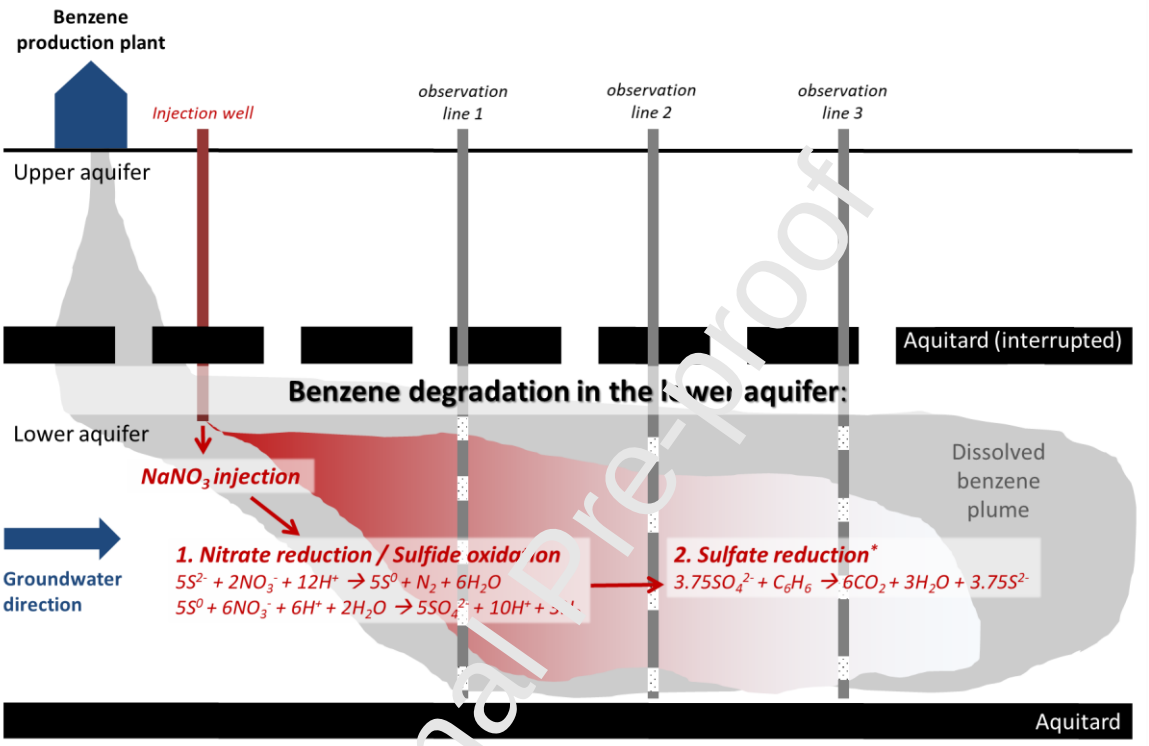


Table 1. Monitoring wells with depth, filter line and position of each horizon

Multi-level monitoring well	Maximum depth [m]	Filter line [m]	depth of installed pressure pumps
BB1	52,0	32,0 – 51,0	BB1-P1: 34,5 m BB1-P2: 37 m BB1-P3: 40 m BB1-P4: 45 m BB1-P5: 50 m
BB2	51,0	33,0 – 50,0	BB2-P1: 34 m BB2-P2: 37 m BB2-P3: 41 m BB2-P4: 45 m BB2-P5: 49 m
BB3	49,0	33,0 – 48,0	BB3-P1: 34 m BB3-P2: 39 m BB3-P3: 42 m BB3-P4: 47 m
BB5	44,5	29,6 – 42,5	BB5-P1: 30 m BB5-P2: 33,5 m BB5-P3: 37,5 m BB5-P4: 42 m
17A/00	54,0	40,0 – 54,0	17A/00-P1: 42 m 17A/00-P2: 47 m 17A/00-P3: 51 m 17A/00-P4: 53,5 m
27A/02	48,3	31,3 – 47,3	27A/02-P1: 32,5 m 27A/02-P2: 37,5 m 27A/02-P3: 43 m 27A/02-P4: 46,5 m
31A/02	44,0	31,0 – 43,0	31A/02-P1: 32 m 31A/02-P2: 35,5 m 31A/02-P3: 38 m 31A/02-P4: 42 m
44A/02	42,7	29,7 – 41,7	44A/02-P1: 31 m 44A/02-P2: 36,5 m 44A/02-P3: 41 m
45A/02	43,0	32,0 – 42,0	45A/02-P1: 33 m 45A/02-P2: 36 m 45A/02-P3: 38,5 m 45A/02-P4: 41 m

Table 2. Maximal nitrate concentrations in each observation line after start of injection of sodium nitrate solution (t=0)

Line of observation wells	**Maximum measured NO_3^- concentration**		**Time after the injection [d]**
	[mM]	**[mg L^{-1}]**	
First line	58	3585	56
Second line	47	2932	119
Third line	8	481	210

Author statement

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Term	Definition
Conceptualization	kk, hw, hr, cv
Methodology	chm, kk, cv, hw, rs, rl
Validation	all
Formal analysis	chm, cv, kk, rl
Investigation	chm, rl, kk, cv, rt
Resources	sk, cv, kk, hw, hr
Data Curation	chm, rl, cv
Writing - Original Draft	chm, cv
Writing - Review & Editing	kk, cv and others
Visualization	chm, cv
Supervision	kk, cv
Project administration	cv, kk, hw, hr
Funding acquisition	cv, kk, hw, hr

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- nitrate can be an efficient electron donor for benzene degradation in contaminated aquifers
- more than 7 tons of nitrate were injected into a contaminated aquifer to accelerate biodegradation
- injection of nitrate triggered sulfide oxidation and the produced sulphate was then utilized by sulphate-reducing bacteria for benzene degradation