

EiCLaR bulletin

CL:AIRE's EiCLaR bulletins describe *in situ* bioremediation technology developments and tools created within the EiCLaR project. This bulletin describes the development of a new approach to the *in situ* bioremediation of chloroethenes.

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Monitored bioaugmentation and remediation

1. INTRODUCTION

Chloroethenes are used as a solvent/cleaning agent in the metal industry as well as service industry (e.g. dry cleaning). Due to improper handling and storage, these chemicals were released into the environment. Based on their higher density compared to water, once in the subsurface the chloroethenes will sink to the aquitard, potentially penetrating the complete thickness of the aquifer, where they can accumulate as dense non aqueous phase pools, acting as a long term source of the contaminants.

The monitored bioremediation approach developed in the EiCLaR project utilizes a specialized bacteria consortium in a bioaugmentation approach in order to facilitate the productive aerobic degradation of the chloroethenes in the subsurface. The key benefit in deploying an aerobic process compared to reductive dechlorination processes is the absence of stable toxic and carcinogenic intermediate products like cis-1,2-dichloroethene (cDCE) and vinyl chloride (VC) during contaminant degradation. Furthermore, reductive dechlorination requires auxiliary substrates and anaerobic by-products such as sulfite and methane are formed. During the co-metabolic, non-productive aerobic degradation, also an auxiliary substrate is used as growth substrate as well as energy source in the microbial metabolism. The chloroethenes are degraded fortuitously, resulting in a rather inefficient usage of the oxygen that needs to be provided in order to facilitate the aerobic microbial metabolism of auxiliary substrates. The productive aerobic microbial degradation process utilizes chloroethenes as growth substrate as well as energy source, therefore omitting the need of auxiliary substrates. Therefore, the oxygen usage efficiency is increased by up to 100 times.

In the scope of the EiCLaR project, successful bioaugmentation with an aerobic metabolic trichloroethene (TCE) degrading bacterial consortium was demonstrated with chloroethene contaminated groundwaters. As a field-monitoring tool-kit, a selective molecular biological quantitative polymerase chain reaction (qPCR) method to evaluate the degradation potential for aerobic metabolic TCE degradation has been established. Since oxygen is limited at most contaminated sites, with the usage of electrolysis and application of

oxygen releasing compounds, different approaches to deliver oxygen have been investigated.

The objectives of the bulletin are (i) to provide a literature review-based overview of the state of the technology as well as criteria to consider when choosing this new technology (section 2) and (ii) to report on further research undertaken within the scope of the EiCLaR project (section 3).

2. BACKGROUND TO THE TECHNOLOGY

Previously, the bioremediation of chlorinated ethenes such as TCE was considered to be only viable under anaerobic conditions through organohalide respiring bacteria or by co-metabolic aerobic processes (Figure 1). The reductive dechlorination uses the chloroethenes as electron acceptor and relies on hydrogen as electron donor. In engineered processes, the hydrogen is provided by fermentation of auxiliary substrates like acetate, lactate or glycerol. In addition to the need of the auxiliary substrates, only bacteria of the *Dehalococcoides* family can facilitate complete dechlorination of perchloroethene (PCE) and TCE requiring strongly reduced groundwater conditions. The degradation of the chloroethenes is a cascade of dechlorination steps exchanging a proton with a chlorine substituent, resulting in the appearance and possible accumulation of the intermediates cDCE and the carcinogenic VC (Dolinová *et al.*, 2017).

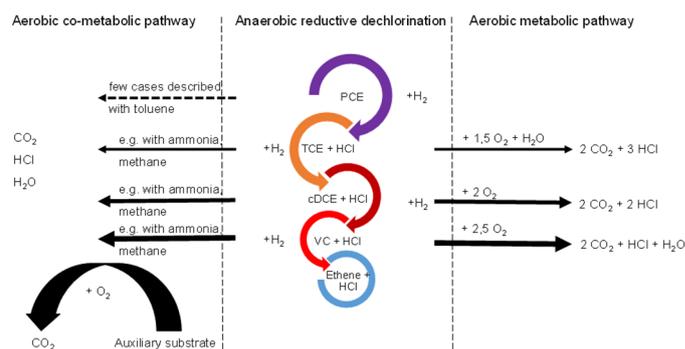


Figure 1. Possible pathways for the degradation of chloroethenes. The size of the arrows indicates the relative degradation rate compared to other processes of the same category.



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While aerobic co-metabolic processes avoid the possible accumulation of the stable intermediate products by mineralizing the chloroethenes, auxiliary substrates are required as growth substrate and energy source. Therefore, oxygen consumed will primarily be used in the degradation of the auxiliary substrate while the chloroethene degradation happens fortuitously. The fortuitous degradation of the chloroethenes results in a low oxygen utilization efficiency and therefore a high cost-low benefit situation.

A beneficial process, avoiding the formation of these intermediates and omitting the need of auxiliary substrates, is the aerobic metabolic degradation where the contaminants are used as growth substrate as well as energy source by the microorganisms, resulting in a high oxygen utilization efficiency (see Figure 2).

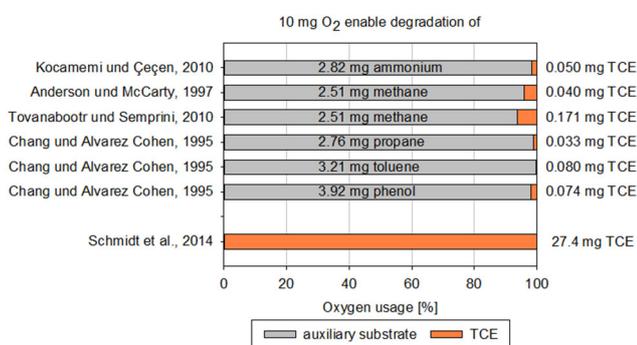


Figure 2. Oxygen utilization efficiency of aerobic co-metabolic TCE-degradation utilizing different auxiliary substrates compared to the aerobic metabolic TCE degradation.

The aerobic metabolic degradation of TCE was described for the first time in 2014 when investigating natural attenuation processes at a TCE/cDCE contaminated site in southern Germany (Schmidt *et al.*, 2014). The site potential for this process, which was a global novelty at this point in time, was assessed in a series of batch experiments. Cultivation of the site microbiota in a chloride free mineral salt medium and TCE as sole carbon source resulted in a stable process degrading several dosages of TCE (Figure 3), with contaminant concentrations of up to 46 mg/L (0.35 mM/L) (Schmidt *et al.*, 2014). The degradation of the TCE resulted in a stoichiometric release of chloride, which demonstrates the complete oxidative dechlorination of the TCE in this process. Due to the absence of other potential auxiliary substrates, proof is given that the process is indeed a metabolic TCE degradation, relying on the presence of the contaminant and the availability of dissolved oxygen.

Based on the rare observation of this degradation process, the potential of a bioaugmentation approach utilizing bacteria capable of aerobic metabolic TCE degradation will lead to a new, sustainable and efficient remediation option for sites contaminated with TCE and lower chlorinated ethenes, as shown in Figure 4.

The first proof of the viability of the bioaugmentation consortium has been accomplished in laboratory batch experiments with groundwater from a variety of chloroethene contaminated sites varying in physico-chemical composition of the groundwater and the contaminant profiles (Gaza *et al.*, 2019; Willmann *et al.*, 2023).

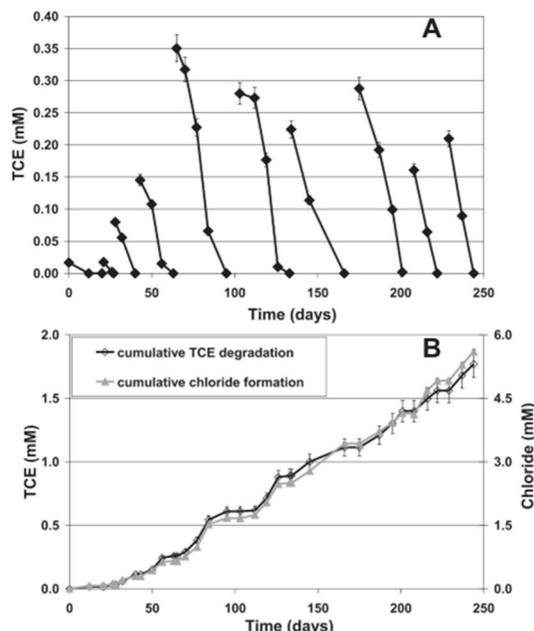


Figure 3. Aerobic TCE degradation in mineral salt medium (A): repeated TCE spiking to show repeatable, stable degradation. (B): Cumulative degradation of TCE and cumulative release of chloride ions caused by the dechlorination of the TCE (Schmidt *et al.*, 2014).

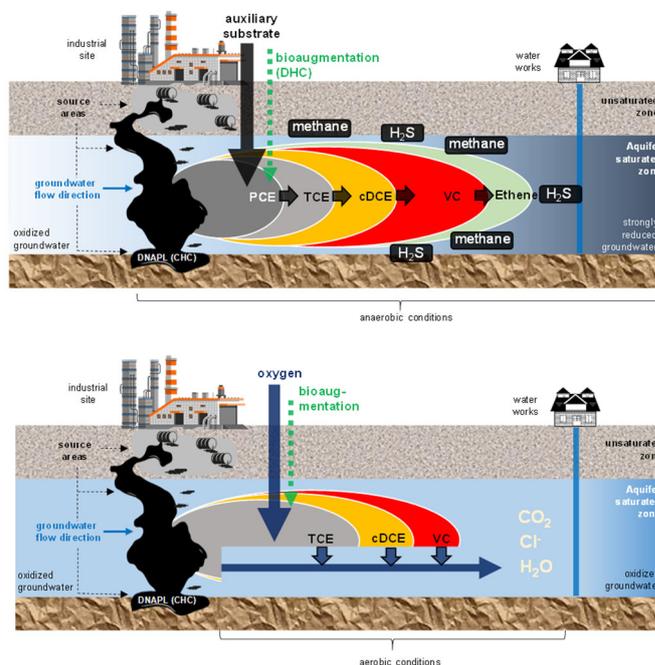


Figure 4. Process schematic for biodegradation of chloroethenes via reductive dechlorination resulting in critical metabolites (e.g. VC) and anaerobic side product (e.g. H₂S) formation (top) and the aerobic approach through bioaugmentation with aerobic metabolic chloroethene degraders and oxygen infiltration (bottom).

3. DEVELOPMENT OF THE TECHNOLOGY AT LAB-SCALE

In laboratory batch experiments, a variety of chloroethene contaminated groundwaters from approximately 10 different sites with different physico/chemical composition of the groundwater and aquifer properties have been investigated to assess the suitability of the enriched microbial consortium that is capable of the aerobic

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metabolic TCE degradation to a wide variety of environmental conditions. These investigations not only demonstrated the viability of the bioaugmentation consortium, but also led to the identification of additional sites that possess intrinsic potential for the aerobic metabolic TCE degradation.

The bioaugmentation approach was successful with low contaminant concentrations (~600 µg/L) (see Figure 5) as well as high contaminant concentrations (~30 mg/L), demonstrating resilience against the toxicity of high concentrations as well as the suitability as a polishing step in the aftercare of previous treatments that resulted in a residual contamination.

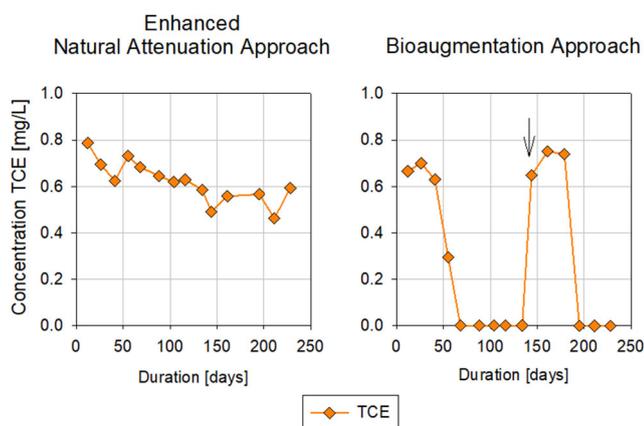


Figure 5. Comparison of the enhanced natural attenuation approach supplying oxygen as terminal electron acceptor with the bioaugmentation approach. The arrow indicates TCE-spiking of the batch experiment. Studies were done with contaminated groundwater of EiCLaR field sites.

Evidenced by the laboratory bioaugmentation studies, minor co-contaminations of cDCE are degraded by the TCE degrading consortium in a co-metabolic process.

While chloride-concentrations can be used as a suitable line of evidence for the mineralization of the chloroethenes in the lab, most field sites have a considerable chloride background in conjunction with low concentrations of chloroethenes invalidating this line of evidence. Alternative lines of evidence include molecular biological analysis via qPCR as well as isotopic fractionation.

Selective qPCR-analysis can verify the presence of competent bacteria and required functional genes, in order to determine the potential and growth of a site microbiota to facilitate different degradation pathways. For the monitored bioaugmentation and remediation (MBR)-approach, specific qPCR-primers have been established for the detection of the *Rhodocyclaceae* family (*Rho*), which is the key player in the aerobic metabolic TCE degradation as well as functional genes (*moABC*) partaking in the degradation pathway. With the *Rho*-primer bacteria of the *Rhodocyclaceae* family can be detected, however, not all species of the family are capable of the aerobic metabolic TCE degradation. Therefore, a more selective method targeting functional genes (*moABC*) involved in the degradation pathway was established to give evidence for the degradation potential. Figure 6 shows the detection of the specific bacteria and the functional genes during TCE degradation in a microcosm experiment. A qualitative summary of the primer selectivity is given in Table 1 in a range of laboratory cultures and environmental samples.

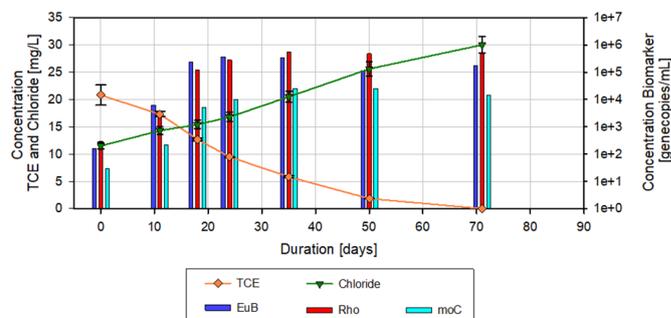


Figure 6. Development of TCE and chloride concentration compared to the amount of bacteria (*EuB*); the amount of *Rhodocyclaceae* (*Rho*) and the functional gene *moC*.

The comparison of general bacterial DNA (*EuB*) and specific TCE degrader DNA (*Rho*) indicates, that through enrichment of bacteria with TCE as sole carbon source in the microcosm, most bacteria present in the batch bottles belong to the specified bacterial family of *Rhodocyclaceae*.

The results indicate that the designed PCR-primers for *Rho* and *moC* can specifically detect metabolic TCE degrading bacteria only present at the SF site and in the bioaugmentation sample. Vice versa, this indicates that the relevant bacteria are not widespread in the environment and therefore bioaugmentation may be more important for TCE remediation than for some other contaminants (e.g. BTEX).

Table 1: Qualitative summary of the primer selectivity in a range of laboratory cultures and environmental samples.

Primer	Primer-functionality							
	Aerobic VC-degrading culture	Aerobic cDCE-degrading culture	Communal wastewater	BTEX contaminated site	PAH / NSO-HET / CE contaminated site	PAH / NSO-HET contaminated site	SF-site ¹⁾	Bioaugmentation lab experiment
<i>EuB</i>	✓	✓	✓	✓	✓	✓	✓	✓
<i>Rho</i>	X	X	X	X	X	X	✓	✓
<i>moC</i>	X	X	X	X	X	X	✓	✓
1)	Field site with aerobic metabolic TCE degradation			BTEX - benzene, toluene, ethylbenzene and xylene				
X	Primer had negative results			PAH - polycyclic aromatic hydrocarbons; CE - chloroethenes				
✓	Primer had positive results			NSO-HET - heterocyclic aromatic hydrocarbons containing nitrogen, sulfur, or oxygen				

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During the scale up experiments from the laboratory batches to column systems and technical scale setups (Figure 7), the usage of an immobilized bioaugmentation culture resulted in the shortest lag period before TCE-degradation was observed after inoculation. However, the injection of a culture grown in mineral media yielded positive results as well, allowing the choice of different inoculation approaches to apply the MBR-technology dependent on site specific properties.

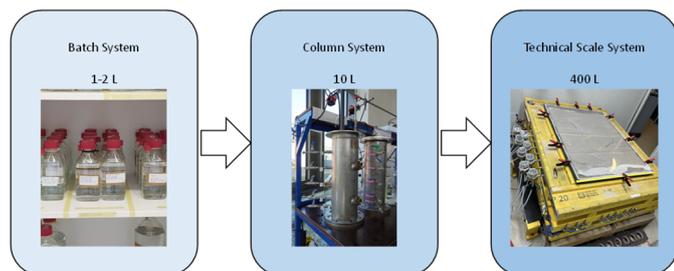


Figure 7. Overview of the experiment setups used for the different scales. Batch system: mineral medium with TCE as sole carbon source and inoculation with laboratory liquid enrichment culture. Column system: Flow-through columns (total volume ~10 L; pore volume ~4 L volume flow of 1 L/d, resulting in a retention time of ~4 days) with silica sand as carrier material and inoculation by integration of layer with immobilized bacteria on sand. Technical scale system: dimension LxWxH 115 cm x 80 cm x 60 cm, with Dorsilit 8 as carrier material as porous soil medium, inflow and outflow area has been filled with gravel to achieve a higher hydraulic conductivity, inoculated with ~20 L of silica sand (52.6 kg) with biomass.

Due to the aerobic nature of the degradation process, oxygen needs to be supplied in order to enhance the remediation process. In the scope of the EiCLaR project, oxygen supplementation was successfully performed in a bio-electro approach utilizing electrolysis to produce oxygen *in situ* (Hertle *et al.*, 2023).

Electrolysis can not only support the aerobic metabolic degradation of TCE, but, as shown previously in small scale (Lohner *et al.*, 2011; Lohner and Tiehm, 2009) and further developed by the Chinese project partners, lead to a shift in the redox conditions and stimulating the degradation of chloroethenes as well (Cai *et al.*, 2023; Cao *et al.*, 2024; Shi *et al.*, 2023). It was also demonstrated that sequential anaerobic/aerobic treatment is promising for PCE contaminated sites.

4. DEVELOPMENT OF THE TECHNOLOGY AT FIELD-SCALE

The MBR-technology had its first application at the SF-site (south Germany as described in Schmidt *et al.* (2014)) in a semi-bioaugmentation approach established through a groundwater circulation setup. The SF-site has a fractured aquifer, resulting in a high hydraulic conductivity of the subsurface. While the bacteria were already present on the site itself, the treatment area was expanded by bioaugmenting the proximity with oxidized and bacteria enriched groundwater from the primary treatment area. By doing so, the lag period noticed in the enhanced degradation of the pilot treatment area (Figure 8) is omitted. Oxygenation of the groundwater has been performed in a pressure vessel with technical oxygen before being redistributed into the subsurface.

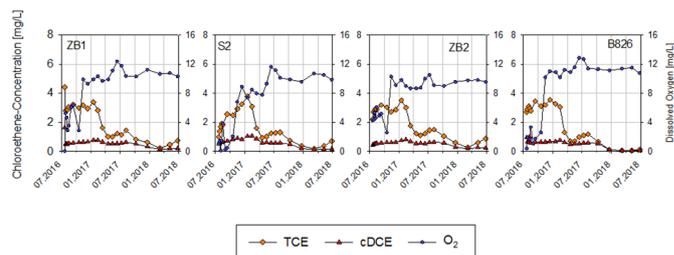


Figure 8. Development of TCE, cDCE and Dissolved Oxygen during the first 24 months of groundwater circulation treatment in the wells included in the pilot plant setup.

The decrease in TCE and cDCE is accompanied by an increase of the compound specific isotopic signature ($\delta^{13}\text{C}$). Unlike non-destructive processes (e.g. dilution), biodegradation favours the degradation of chloroethenes with the lighter carbon isotope ^{12}C . The favoured degradation of the lighter carbon isotopes leads to an increase in ^{13}C in the residual contamination, therefore resulting in a heavier isotope composition of the remaining chloroethenes (the $\delta^{13}\text{C}$ -isotope signature becomes less negative) (Figure 9).

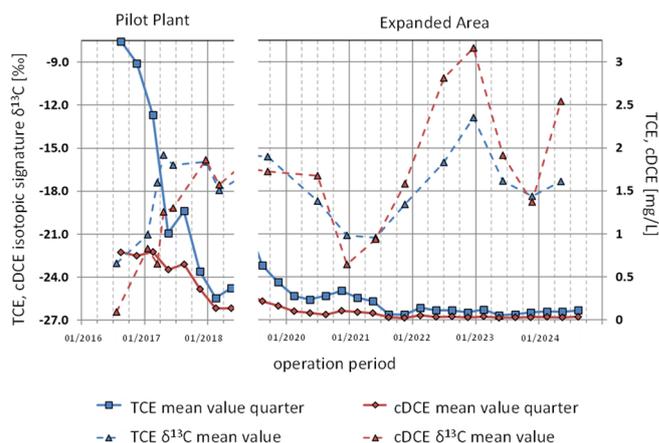


Figure 9. Development of the TCE concentration and the resulting change in the isotopic signature due to the aerobic metabolic biodegradation of the chloroethenes during the pilot plant phase and the expanded field scale treatment. All data available from the monitoring in one quarter were averaged.

During EiCLaR, prior untreated areas were included into the groundwater circulation setup with expansion of the area. The draw-in of groundwater from an untreated area led to an increase of contaminants as well as a change in the physico-chemical (pH, ORP, sulfate) composition of the groundwater across the whole treatment area as well as the isotope profile of the chloroethenes. With ongoing treatment, degradation of the contaminants progressed, and the increased bioactivity allowed for another expansion of the treatment area increasing the oxygenated subsurface volume from $5.7 \times 10^4 \text{ m}^3$ (2018) to $1.5 \times 10^5 \text{ m}^3$ (2024) (Figure 10).

The bioremediation at the SF site is based on the presence of bacteria capable of aerobic TCE degradation and the availability of sufficient oxygen, as demonstrated by the comparison of aerobic and anaerobic areas. Due to changes of the extraction/infiltration configuration, the monitoring well B828 is outside of the groundwater circulation zone since 2022. The TCE-degradation

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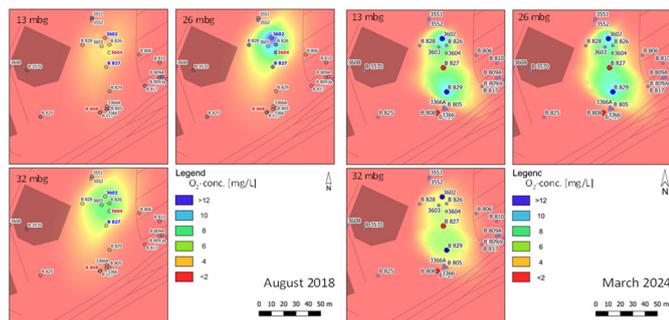


Figure 10. Spread of dissolved oxygen in the subsurface during the pilot test in 2018 and the field scale treatment phase during the EiCLaR project.

stalled as soon as the available oxygen was depleted. As a result, TCE-concentrations increased back to ~0.5 mg/L, most probably due to TCE rebound from matrix diffusion. The stall of biodegradation and lack of oxygen led to a noticeable decrease of the $\delta^{13}\text{C}$ isotopic signature to -20.7‰, and diminishing of the biomarker concentration to $\sim 6.3 \times 10^0$ gene copies/mL.

In comparison, monitoring well B826, which was affected by the groundwater circulation throughout the whole duration, had oxygen available at all times, resulting in TCE-concentrations of $\sim 5 \mu\text{g/L}$ a $\delta^{13}\text{C}$ isotopic signature of -16.9‰ and a biomarker concentration of $\sim 5.0 \times 10^3$ gene copies/mL (Table 2).

The success of the bioaugmentation approach to remediate the site allowed the hydraulic containment to be turned off.

Table 2: Comparison of TCE-concentrations, dissolved oxygen, $\delta^{13}\text{C}$ isotopic signature and biomarker *moC* in the monitoring wells B826 and B828 in January 2024. While B826 was affected by the groundwater circulation at all times, B828 was no longer affected starting in February 2022.

Well	O ₂ [mg/L]	TCE [mg/L]	Isotopic signature $\delta^{13}\text{C}$ [‰]	Biomarker <i>moC</i> [gene copies/mL]
B826	7.18	0.0005	-16.9	5.0×10^3
B828	1.42	0.4760	-20.7	6.3×10^0

5. APPLICATION OF THE TECHNOLOGY

As a bioremediation approach, the MBR-technology has requirements regarding site properties that need to be met in order to facilitate growth of the bacteria utilized during the contaminant degradation. Aerobic chloroethene degradation is applicable for the chloroethenes TCE, cDCE and VC. Therefore, PCE-contaminated sites need to facilitate the transformation of PCE to the lower chlorinated ethenes via reductive dechlorination in order to be suitable for the MBR-technology in a sequential anaerobic/aerobic approach. Another crucial requirement is the pH-value of the groundwater. The bioaugmentation culture can facilitate TCE-degradation in a range from pH 5 to pH 8 with its optimum at pH 7. Sites with pH-values outside this range are not suitable for the MBR-approach without prior adjustment of the pH-value.

Bioaugmentation approaches to stimulate the reductive dechlorination with *Dehalococcoides* bacteria have already been successfully applied at contaminated sites. Therefore, bioaugmentation approaches at chloroethene contaminated sites are generally accepted by regulatory agencies. With the application of aerobic metabolic TCE degrading enrichment cultures, the formation of toxic intermediates like VC will be avoided and additionally no anaerobic side reactions, such as biological methane or H₂S formation will occur in the aerobic groundwater. By metagenomic analysis of the bioaugmentation culture it could be demonstrated that it does not contain any harmful bacteria or pathogens and only bacteria, specialized on the aerobic TCE degradation will be transferred into the site groundwater.

Once implemented, the technology will result in the mineralization of the contaminant to CO₂ and chloride. Due to the aerobic metabolic nature, the intermediate presence of cDCE and the carcinogenic VC as well as undesirable side reactions, like methanogenesis and sulfate reduction, are avoided. Additionally, waiving the need of an auxiliary substrate, which is the main sink for oxygen in aerobic co-metabolic processes, increases the overall efficiency of the remediation approach.

Application on site can be expanded into prior untreated areas, using the established bioaugmentation zone as inoculum for the expansion. While the process can be scaled even after implementation, limitations are set by the availability of oxygen in the aquifer.

The oxygen demand of the MBR-approach is between 2.7-4.1 mg_{Oxygen}/mg_{TCE}, which can be used to calculate the range of the oxygen requirement to facilitate the degradation of the amount of contaminant. The total amount of required oxygen might exceed the solubility of oxygen (~8 mg/L), which can be circumvented by multiple dosages of oxygen along the contaminant plume or by groundwater circulation wells.

While the costs of remediation are site specific, at the SF-site the MBR-approach allowed the disconnection of the pump and treat setup used as a hydraulic barrier, lowering the contaminant specific removal costs to ~1/3 of the removal costs caused by the pump and treat setup (€/kg_{TCE}).

By omitting *ex situ* treatment, the application footprint is significantly lower compared to established interventions to contain chloroethene concentrations. Additionally, costs, transport and after treatment for process material, as well as miscellaneous costs like sewage charges are avoided, resulting in not only lower costs, but also a lower carbon footprint and therefore lower climate impact of the technology.

With application of the MBR technology, the aquifer once remediated will be in an oxidized state, resulting in a better soil health compared to reductive dechlorination treatment or hydraulic containment of the contamination.

Moreover, research currently focuses on the optimization of oxygen applications. Possible oxygen sources, such as technical oxygen, oxygen pulsed injection, H₂O₂ and Oxygen Releasing Compounds (ORCs) are compared and will be evaluated on the highest efficiency of aerobic TCE degradation.

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6. CONCLUSIONS

The further progression of the bioaugmentation approach to stimulate the aerobic metabolic degradation of chloroethenes was successful. The applicability of the approach has been demonstrated successfully on a variety of different groundwaters using different bioaugmentation approaches.

With the transition from the laboratory scale to technical scale, major milestones for the field implementation have been achieved. The successful field application and scalability of the treated area on site has been demonstrated at the SF-site. With clear advantages over other bioremediation approaches targeting chloroethenes, the aerobic metabolic degradation pathways excel by avoiding unwanted side reactions (e.g. methanogenesis) and potential accumulation of concerning intermediate products like VC, as well as a high oxygen usage efficiency by omitting the need for auxiliary substrates. Due to the *in situ* mineralization of the contaminants, the MBR approach demonstrated a higher sustainability compared to traditional approaches. So far, groundwater circulation with oxygen enrichment as well as the utilization of electrolysis have been applied successfully to stimulate the aerobic metabolic TCE degradation.

With the developed qPCR methods, based on metagenomic analyses, a monitoring method is now available detecting the site potential for aerobic productive TCE degradation, but also reliably tracking MBR approaches.

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