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Cecilie Bang Ottosen
PhD Thesis

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in situ natural and enhanced degradation
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PhD Thesis
December 2020

DTU Environment
Department of Environmental Engineering
Technical University of Denmark

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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk.

Preface

The research presented in this PhD thesis was conducted from August 2017 to October 2020 at the Department of Environmental Engineering, Technical University of Denmark (DTU). Associate Professor Mette M. Broholm (DTU) was the main supervisor and Professor Poul L. Bjerg (DTU) the co-supervisor.

The thesis is organized in two parts: the first part puts the findings of the PhD study into context in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

I **Ottosen**, C.B., Murray, A.M., Broholm, M.M., and Bjerg, P.L., 2019. In situ quantification of degradation is needed for reliable risk assessments and site-specific monitored natural attenuation. Environmental Science and Technology, 53, 1-3.

II Murray, A.M., **Ottosen**, C.B., Maillard, J., Holliger, C., Johansen, A., Brabæk, L., Kristensen, I.L., Zimmermann, J., Hunkeler, D., and Broholm, M.M., 2019. Chlorinated ethene plume evolution after source thermal remediation: Determination of degradation rates and mechanisms. Journal of Contaminant Hydrology, 227, 103551.

III **Ottosen**, C.B., Rønde, V., McKnight, U.S., Annable, M.D., Broholm, M.M., Devlin, J.F., and Bjerg, P.L., 2020. Natural attenuation of a chlorinated ethene plume discharging to a stream: Integrated assessment of hydrogeological, chemical and microbial interactions. Water Research, 186, 116332.

IV **Ottosen**, C.B., Bjerg, P.L., Hunkeler, D., Zimmermann, J., Tuxen, N., Harrekilde, D., Bennedsen, L., Leonard, G., Brabæk, L., Kristensen, I.L., and Broholm, M.M. Degradation of chlorinated ethenes after field scale injection of activated carbon and bioamendments: Application of isotopic and microbial analyses. *Submitted, 2020.*

V **Ottosen**, C.B., Skou, M., Sammali, E., Zimmermann, J., Hunkeler, D., Bjerg, P.L., and Broholm, M.M. Dataset for laboratory treatability experiment with activated carbon and bioamendments to enhance biodegradation of chlorinated ethenes. *Submitted, 2020.*

In this online version of the thesis, paper **I-V** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

In addition, the following publication, not included in this thesis, was also prepared during this PhD study:

Ottosen, C.B., Bjerg, P.L., Broholm, M.M., and Søndergaard, G.L., 2018. Nedbrydningsrater til brug i GrundRisk Risikovurdering – Litteraturstudie [Degradation rates for use in GrundRisk Risk Assessment – Literature study]. Report for the Danish Environmental Protection Agency (EPA), No. 2013.

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I am thankful to Otto Mønsteds Fond for funding my attendance at international conferences, to Torben og Alice Frimodts Fond for funding my external research stay, and to P.A. Fiskers Fond for funding my external research stay and the communication of my research. This has allowed me to partake in the international discussions within the research area. I am also grateful to the Capital Region of Denmark, the Southern Region of Denmark and the Danish Environmental Protection Agency for their funding on collaborative projects. Finally, I am grateful to DTU Environment for funding my PhD studies.

I am thankful for my colleagues at DTU: my wonderful officemates and section members for creating such a great work atmosphere, DTU staff members for their tireless support, and field and laboratory technicians for their experimental assistance.

Lastly, I would like to thank my parents, siblings and partner for continued encouragement and support.

Summary

Chlorinated ethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), are widespread soil and groundwater contaminants. Fortunately degradation, either naturally or enhanced, can provide *in situ* destruction of these contaminants. In order to apply degradation-based remedial strategies to treat chlorinated ethene plumes, documentation and quantification of degradation are required. No method alone can provide the necessary information, and an integrated approach is therefore recommended in order to obtain an overall assessment. An integrated approach combine knowledge obtained by multiple tools, to characterise and quantify degradation, with system understanding (e.g. flow and transport) and conceptualisation. Various tools exist, that can be used in the integrated approach, where molecular diagnostic techniques and compound-specific isotope analysis (CSIA) are the most recent additions to the toolbox. Molecular diagnostic tools, e.g. measurements of the abundance of specific bacteria and functional genes related to degradation of chlorinated ethenes, document the potential for biodegradation. CSIA can document the occurrence of degradation and quantify the extent. These novel techniques show great promise, but have only to a limited extent been applied in an integrated approach for the assessment of *in situ* degradation of chlorinated ethenes. Therefore, the advantages and challenges of the combined approach have yet to be assessed.

This PhD thesis aimed to address this knowledge gap on the applicability of the approach, by advancing and exploring the use of techniques to characterise and quantify *in situ* degradation in diverse settings and scenarios. Particularly focus has been on the application of the advanced isotopic and microbial tools. Three scenarios were investigated: (1) A biostimulated large-scale plume with several degradation pathways, (2) A naturally transient plume at the groundwater-surface water interface, and (3) A plume bioremediated with activated carbon and bioamendments.

In scenario 1, extensive sampling was conducted in a large-scale plume, tracking the lasting impact of a source remediation, which had released dissolved organic carbon that stimulated degradation down-gradient the source zone. Isotopic and microbial data were used to identify degradation pathways (biotic and abiotic), document biodegradation potential and quantify degradation rates throughout the plume. Integrated in a conceptual site model, it was demonstrated that the chlorinated ethenes underwent a destructive process, but documentation of the responsible mechanism(s) was constrained at the plume front.

In scenario 2, a near-stream system, where a groundwater plume consisting of primarily cis-DCE and VC discharged to the stream, was investigated through a multi-scale integrated approach. The study documented a behaviour not commonly observed: a change in dynamics from limited attenuation to significant bioattenuation. The significant bioattenuation was documented by a high abundance of relevant microbial targets, and through significant increases in $\delta^{13}C$ values for cis-DCE and an isotope mass balance. Integrated in a conceptual site model, it could be determined that a short residence time in the near-stream system restricted complete dechlorination.

In scenario 3, a comprehensive investigation was conducted after bioremediation with activated carbon and bioamendments injected across a TCE plume. The injection caused transient dynamics, and only an integrated approach could be used to describe the complex, non-stationary system. Microbial and isotopic data provided understanding on dynamics that could not have been obtained otherwise; Microbial data revealed potential reasons for the degradation stalling at cis-DCE and isotope data enhanced the knowledge on the effect of bioremediation through increased process understanding. The integrated approach effectively documented degradation and described the factors controlling it, yet quantification of degradation was inaccessible with the applied methods, due to the high complexity of the system dynamics.

In conclusion, this PhD study has identified advantages and limitations of applying an integrated approach in order to document and quantify *in situ* degradation in new settings and scenarios. The investigations underlined the added value when including microbial and isotopic techniques, and identified constraints in the use of the tools. Furthermore, the importance of integrating the novel techniques in an overall system understanding and conceptualisation was highlighted. The obtained knowledge can improve future site-specific evaluations of *in situ* natural and enhanced degradation as well as holistic risk assessments.

Dansk sammenfatning

Chlorerede ethener, som for eksempel tetrachlorethen (PCE) og trichlorethen (TCE), er udbredte forureningsstoffer i jord og grundvand. Heldigvis kan disse forureningsstoffer nedbrydes *in situ* enten natuligt eller stimuleret. For at kunne anvende afværgemetoder baseret på nedbrydning, til at behandle faner med chlorerede ethener, er det nødvendigt at kunne dokumentere og kvantificere nedbrydningen. Ingen metode alene kan bidrage med den nødvendige viden, og derfor er en integreret fremgangsmåde anbefalet til at opnå en samlet vurdering. En integreret fremgangsmåde kombinerer viden opnået ved forskellige værktøjer, til at karakterisere og kvantificere nedbrydning, med system forståelse (fx grundvandsstrømning og transport) og konceptualisering. Forskellige værktøjer kan anvendes i den integrerede fremgangsmåde, hvoraf molekylære diagnosticeringsteknikker og stof-specifik isotop analyse (CSIA) er de nyeste tilføjelser til værktøjskassen. Molekylære diagnosticeringsværktøjer, fx måling af niveauet af specifikke bakterier og funktionelle gener relateret til nedbrydning af chlorerede ethener, dokumenterer potentialet for bionedbrydning. CSIA kan dokumentere tilstedeværelsen af nedbrydning og kvantificere omfanget. Disse nye teknikker udviser stort potentiale, men har kun i ringe grad været anvendt i integrerede fremgangsmåder til at evaluere *in situ* nedbrydning af chlorerede ethener. Derfor er der et behov for at vurdere fordele og udfordringer ved den samlede metode.

Denne ph.d. afhandling har haft til formål, at adressere dette videnshul vedrørende anvendelsen af den integrerede fremgangsmåde, ved at videreudvikle og undersøge anvendelsen af teknikker til at karakterisere og kvantificere *in situ* nedbrydning i forskellige miljøer og scenarier. Fokus har især været på anvendeligheden af de avancerede mikrobielle og isotop værktøjer. Tre scenarier blev undersøgt: (1) En biostimuleret stor-skala fane med forskellige nedbrydningsveje, (2) En naturlig afvekslende fane ved grænsefladen mellem grundvand og overfladevand, og (3) En fane bioremedieret med aktiveret kul, bakterier og donor.

I scenarie 1 blev omfattende prøvetagning udført i en stor-skala fane, for at følge den varige påvirkning af en kildeoprensning, hvilket havde frigjort opløst organisk kulstof, som stimulerede nedbrydning nedstrøms kildeområdet. Data fra isotop og mikrobielle analyser kunne anvendes til at identificere nedbrydningsveje (biotisk og abiotisk), dokumentere potentialet for bionedbrydning og kvantificere nedbrydningsrater gennem hele fanen. Ved at integrere data i en konceptuel model for lokaliteten kunne det demonstreres, at de chlorerede

ethener gennemgik en fjernelsesproces, men dokumentation af ansvarlige mekanisme(r) var begrænset ved fanens front.

I scenarie 2 blev et ånært system, hvor en grundvandsfane bestående af primært cis-DCE og VC strømmede ud i åen, undersøgt ved en multi-skala integreret fremgangsmåde. Studiet dokumenterede en ikke ofte set adfærd: en ændring i dynamikken fra begrænset dæmpning af forurening til signifikant bionedbrydning. Den markante nedbrydning blev dokumenteret ved et højt niveau af relevante mikrobielle indikatorer, og ved markante stigninger i δ^{13}C værdier for cis-DCE og en isotop massebalance. Integreret i en konceptuel model for lokaliteten kunne det bestemmes, at en kort opholdstid i det ånære system begrænsede fuldstændig dechlorering.

I scenarie 3 blev en omfattende undersøgelse udført efter bioremediering med aktiveret kul, bakterier og donor injiceret på tværs af en TCE fane. Injiceringen medførte vekslende dynamikker, og kun en integreret fremgangsmåde kunne anvendes til at beskrive det komplekse, ikke-stationære system. Mikrobielle og isotop data bidrog til forståelsen af dynamikken, viden som ikke kunne være opnået på anden måde; Mikrobielle data afslørede potentielle grunde til, at nedbrydningen stoppede ved cis-DCE, og isotop data øgede procesforståelsen til at forklare effekten af bioremediering. Den integrerede fremgangsmåde dokumenterede nedbrydningen, og beskrev faktorerne der kontrollerede den. Derimod var kvantificering af nedbrydningen ikke mulig ved de anvendte metoder, på grund af den høje kompleksitet af processerne i systemet.

Samlet set har dette ph.d. studie identificeret styrker og begrænsninger ved at anvende en integreret fremgangsmåde til at dokumentere og kvantificere *in situ* nedbrydning i forskellige miljøer og scenarier. Undersøgelserne understregede den tilførte værdi ved at inkludere mikrobielle og isotop teknikker, og identificerede begrænsninger ved brugen af værktøjerne. Derudover, blev vigtigheden af at integrere de nye teknikker i en overordnet system forståelse og konceptualisering fremhævet. Den opnåede viden kan forbedre fremtidige lokalitets-specifikke evalueringer af *in situ* naturlig og stimuleret nedbrydning såvel som holistiske risikovurderinger.

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1 Introduction

1.1 Background and motivation

Chlorinated ethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), are widespread contaminants that may pose a risk to valuable groundwater and surface water resources (Moran et al., 2007; Weatherill et al., 2018). PCE and TCE have been used as cleaning and degreasing solvents, and improper handling and disposal have led to point sources that contaminate the underlying soil and groundwater (McCarty, 2010). Over the last decades, remedial techniques to treat chlorinated ethenes source zones and plumes have been developed (Kueper et al., 2014; Stroo, 2010). Monitored natural attenuation and bioremediation, which depend on microorganisms to degrade contaminants, are generally preferable as they are cost-efficient and have a lower impact on the environment than other remediation approaches (Illman and Alvarez, 2009; Lemming et al., 2010). Hence, destruction of chlorinated ethenes can be obtained *in situ* through natural degradation, if the redox conditions are sufficiently reduced to facilitate degradation, or through enhanced bioremediation (Bradley and Chapelle, 2010).

In anaerobic chlorinated ethene plumes reductive dechlorination, through cis-1,2-dichloroethene (cis-DCE) and vinyl chloride (VC) to the harmless end-product ethene, is the primary biological degradation pathway (Dolinová et al., 2017). Chlorinated ethenes can also undergo abiotic degradation by reactive iron minerals (He et al., 2015). The degradation products of PCE and TCE can be more mobile and toxic, which is the case for VC, and incomplete dechlorination can thus increase the risk (McCarty, 2010). In order to produce reliable risk assessments and evaluations of bioremediation performance, it is therefore essential to document the occurrence, extent and rate of degradation.

Various methods exist that can indicate, document and quantify *in situ* degradation. These include conventional methods such as analysis of redox sensitive parameters as well as chlorinated parent compounds and specific metabolites. Recently, novel microbial and isotopic techniques have been introduced, which can provide advanced process understanding (Illman and Alvarez, 2009). While various tools are available, no stand-alone method exist, that can provide the information required to assess degradation completely (Bombach et al., 2010). Therefore, an integrated approach should be applied to characterise and quantify *in situ* degradation. The term 'integrated approach' has been used in various studies with different perceptions and details. For clarification, in this

thesis the integrated approach is defined as: An overall assessment of *in situ* degradation in a system, through knowledge obtained by various assessment tools integrated with system understanding and conceptualisation (Figure 1).
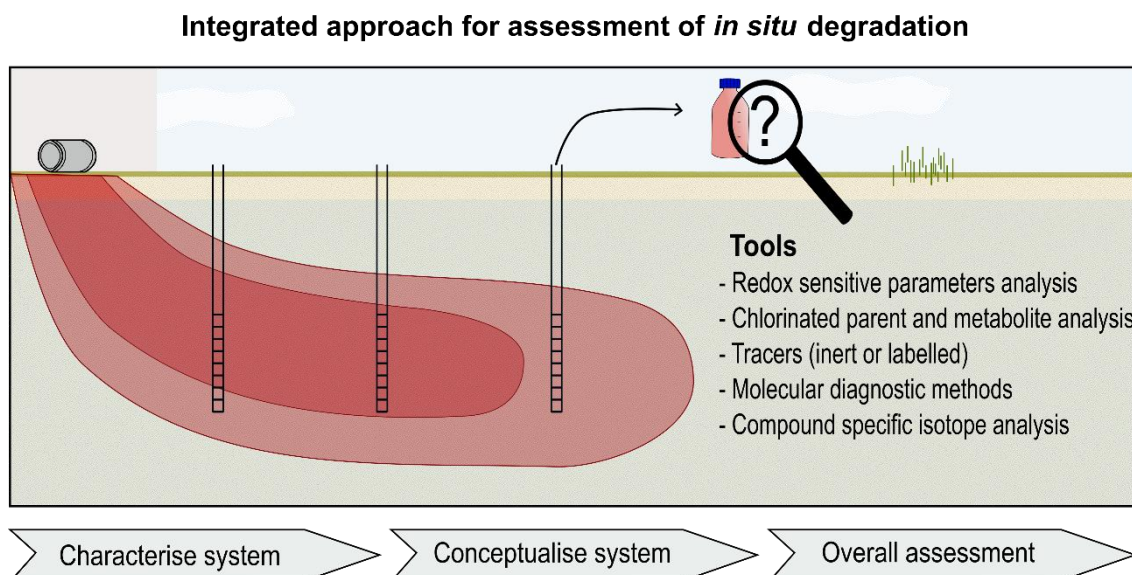
Integrated approach for assessment of *in situ* degradation

Tools
- Redox sensitive parameters analysis
- Chlorinated parent and metabolite analysis
- Tracers (inert or labelled)
- Molecular diagnostic methods
- Compound specific isotope analysis

Characterise system > Conceptualise system > Overall assessment

Figure 1. Principle of the integrated approach for assessment of *in situ* degradation, which integrate chemical and microbial knowledge obtained by various tools with system understanding (i.e. geology, hydrogeology, flow and transport) in a conceptual model, to obtain an overall assessment.

Over the last decade, an integrated approach has been used to: conduct a preliminary screening of degradation potential; monitor natural attenuation (MNA); or evaluate enhanced natural attenuation (ENA) (e.g. Abe et al., 2009; Courbet et al., 2011; Révész et al., 2014). The addition of isotopic and microbial tools has especially provided key information on degradation mechanisms and the degree at which they progress (Hunkeler et al., 2008; Yargicoglu and Reddy, 2015). However, the number of research studies that apply an integrated approach, which include these novel and promising microbial and isotopic tools, are still limited. Hence, to improve future risk assessment and bioremediation performance evaluation, additional research is required to test the integrated approach under diverse conditions; with the aim of assessing the applicability, strengths and limitations, of especially the advanced tools, in documenting and quantifying *in situ* degradation in chlorinated ethene plumes.

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1.2 Research objectives

The aim of this PhD thesis is to advance and explore the use of techniques for *in situ* characterisation and quantification of chlorinated ethenes degradation in contaminant plumes. The specific objectives of the PhD study are:

- Evaluate the existing methods to document and quantify degradation of chlorinated ethenes and describe the state-of-the-art for the scientific field.

  - Identify the need for documentation and quantification of *in situ* degradation (Ottosen et al., 2019-**I**).

- Employ and investigate the integrated approach, with special focus on microbial and isotopic tools, in diverse settings including:

  - A biostimulated large-scale plume with several degradation pathways (Murray et al., 2019-**II**).

  - A naturally transient plume at the groundwater-surface water interface (Ottosen et al., 2020-**III**).

  - A plume bioremediated with activated carbon and bioamendments (Ottosen et al., 2020-**IV** and 2020-**V**).

- Analyse and identify the applicability, benefits and shortcomings of the advanced microbial and isotopic tools in these diverse settings under the framework of the integrated approach.

1.3 Outline of the PhD synopsis

The synopsis is divided into five chapters. Chapter 2, provide a theoretical review that sets the context of the study. Aspects of chlorinated ethenes degradation are described, namely why it is important to understand degradation, and a short overview of degradation pathways is presented. This is followed by an overview of methods to indicate, document and quantify degradation of chlorinated ethenes. Where, novel isotopic and microbial techniques and available quantification methods are elaborated on. Chapter 3, incorporate and discuss the new knowledge acquired in the PhD study. The applicability of the integrated approach is evaluated. That is, the scenarios where it can be used, and a summary of the current research level to assess natural and enhance degradation. Furthermore, the relevance of system conceptualisation and understanding and the integration of the advanced tools are evaluated. Lastly, conclusions are presented in Chapter 4, and perspectives for future research are identified in Chapter 5.

2 Documentation and quantification of chlorinated ethene degradation

2.1 Degradation of chlorinated ethenes

2.1.1 Importance of assessing degradation

Over the last decades, remediation technologies to treat chlorinated solvent plumes have advanced (Stroo, 2010), and remedial strategies based on *in situ* degradation are preferable cost-efficient, low-impact options (Illman and Alvarez, 2009; Lemming et al., 2010). Therefore, bioremediation is increasingly applied for clean-up of chlorinated contaminants (Maphosa et al., 2010). However, the acceptance of biodegradation as a remediation strategy depend on the ability to sufficiently demonstrate that a decrease in contaminant concentrations is caused by destructive biodegradation processes, rather than by non-destructive physical processes such as dispersion and volatilisation (Bombach et al., 2010). Hence, characterisation and quantification of degradation are essential in order to evaluate the success of bioremediation, to obtain a full assessment of contaminant risk, and to perform informed prioritisation of site clean-up (Illman and Alvarez, 2009; Ottosen et al., 2019-**I**).

Although methods are continuously developed and demonstrated for assessment of *in situ* biodegradation processes, and the knowledge and experience on the topic are expanding, degradation rates are rarely quantified at field-scale. First-order degradation rates determined *in situ* for chlorinated ethenes are sparsely available, and the published rates range considerably (Ottosen et al., 2019-**I**). This appears to be a general problem, as the same is the case for emerging organic contaminants in soil and groundwater (Greskowiak et al., 2017). Hence, there is a need for more field-scale investigations applying state-of-the-art techniques to assess and especially quantify *in situ* biodegradation. Furthermore, there is a need to investigate the boundaries for the use of these techniques, and thus the possibility of optimisation, by applying them in various settings and scenarios.

2.1.2 Degradation pathways

Degradation of chlorinated ethenes can occur through biotic and abiotic pathways under anaerobic conditions (Figure 2). Aerobic degradation of cis-DCE and VC is also possible, but chlorinated ethene plumes are often anaerobic when these chlorinated metabolites are present (Mattes et al., 2010). Generally, the primary biological pathway is anaerobic reductive dechlorination, which is

a sequential process, where one chlorine atom is substituted with a hydrogen atom (Dolinová et al., 2017). The extent of degradation through this pathway depends on the redox conditions, the electron donor availability in the aquifer (Bradley and Chapelle, 2010), and the presence of specific bacteria (see Section 2.2.2). Abiotic degradation of chlorinated ethenes may be facilitated by reactive iron minerals, and depend on the presence and/or formation of these minerals and the microorganisms that control the biogeochemical processes (He et al., 2015). Abiotic and biotic processes can occur simultaneously at field sites (e.g. Murray et al., 2019-**II**).
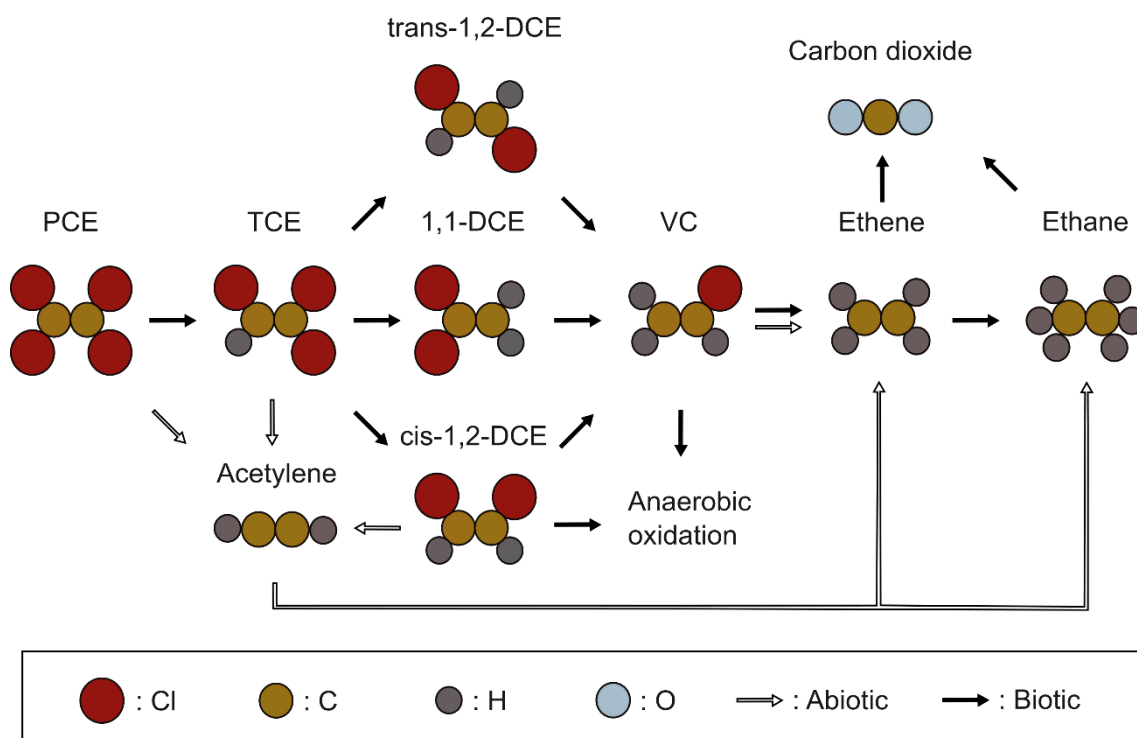

Figure 2. Degradation pathways for chlorinated ethenes under anaerobic conditions. The dominant DCE isomer in anaerobic reductive dechlorination is cis-DCE. Inspired by (Dolinová et al., 2017; He et al., 2015; Mattes et al., 2010).

2.2 Methods to indicate, document and quantify degradation of chlorinated ethenes

2.2.1 Overview of monitoring approaches

The interest in applying biodegradation in remediation strategies has led to a development of several methods to indicate, document and quantify *in situ* biodegradation (Table 1). These include traditional methods such as analysis of redox sensitive parameters, chlorinated parent compounds and specific metabolites, as well as the novel techniques compound-specific isotope analysis (CSIA) and molecular diagnostic tools (Nijenhuis et al., 2018; Thullner et al., 2012; Yargicoglu and Reddy, 2015).

The redox conditions have major influence on the efficiency of biodegradation, as increasingly reduced conditions are required during the sequential anaerobic reductive dechlorination (Bradley and Chapelle, 2010). Monitoring of the redox conditions in a plume can thus reveal the degradation potential. Monitoring of contaminant patterns can be conducted by several methods: (1) The presence of metabolites provide direct evidence of degradation (Bombach et al., 2010); (2) The contamination pattern for parent compounds and metabolites can indicate the degree of dechlorination (Damgaard et al., 2013a) and (3) Contaminant mass discharge estimations can be used to quantify degradation between transects (Illman and Alvarez, 2009).

Conservative tracers can be used to differentiate degradation processes from other attenuation processes along a flow path. In addition, push-pull test or *in situ* microcosms with isotopically labelled reactive tracers can provide direct evidence and potentially quantification of degradation (Bombach et al., 2010; Hageman et al., 2004). Molecular diagnostic tools based on DNA or RNA extracts can be used to document the potential and occurrence of degradation (Yargicoglu and Reddy, 2015). Finally, CSIA can be used to document and quantify degradation as well as indicate degradation pathways (Hunkeler et al., 2008). These more advanced and promising monitoring methods, focusing on microbial and isotopic techniques, will be elaborated on in the two following sections.

Other methods for assessment of degradation, that are not applied *in situ* but are worth mentioning, are: analytical models describing contaminant fate (see also Section 2.2.4) and laboratory microcosms studies based on field materials to evaluate degradation under controlled conditions (Illman and Alvarez, 2009).

Table 1. Overview of *in situ* methods to assess biodegradation of chlorinated ethenes.

Target	Principle	Advantages	Challenges	Ref.
Redox conditions	Presence of suitable conditions for degradation.	Inexpensive, easy to collect water samples and interpret.	Does not provide direct evidence of degradation.	[1], [2]
Metabolites	Detection of specific degradation products.	Direct evidence of degradation occurrence.	Not always quantitative.	[3], [4]
Contaminant fate	Decreasing concentrations and a development in metabolite contaminant ratio.	Inexpensive, easy to collect water samples and interpret.	Influenced by non-destructive processes. Different phase distribution properties.	[5], [6]
Contaminant mass discharge	Decrease in contaminant mass migrating through cross-sections.	Quantification of degradation.	Require extensive monitoring.	[7], [8]
Normalisation with tracers	Decrease in the reactive contaminant corrected for decrease of the conservative tracer.	Quantification of degradation.	Extensive monitoring required.	[3], [9]
Isotopically labelled tracers	Injection of, or *in situ* microcosm with, isotopically labelled analogues of the contaminants.	Direct evidence and quantification of degradation.	Expensive. Environmental regulations may limit use of labelled tracers.	[3], [10]
Extracted DNA	Presence of bacterial genera and the genetic properties.	Document degradation potential. Spatial and temporal trends can reveal bacterial growth and variations.	Expensive. Interpretation becomes more complex when going from qPCR to sequencing.	[11], [12]
Extracted RNA	Determine the metabolic capacity of the bacteria.	Direct evidence of degradation.	Expensive. Sample collection and analysis procedure more challenging than DNA-based approach.	[11], [13]
Compound-specific stable isotopes	Document enrichment in ^{13}C temporally or spatially.	Direct evidence and potentially quantification of degradation.	Expensive. Uncertainty in enrichment factors.	[14], [15]
Dual isotopes	Determine relationship between C and Cl isotopes.	Indication of the degradation pathway.	Expensive. Limited by availability of literature values.	[14], [13]

[1] (Christensen et al., 2000), [2] (Tarnawski et al., 2016), [3] (Bombach et al., 2010), [4] (Tillotson and Borden, 2017) [5] (Nijenhuis et al., 2018) [6] (Damgaard et al., 2013a), [7] (Illman and Alvarez, 2009), [8] (Courbet et al., 2011), [9] (Rügge et al., 1999), [10] (Hageman et al., 2004), [11] (Yargicoglu and Reddy, 2015), [12] (Scheutz et al., 2008), [13] (Murray et al., 2019-**II**), [14] (Hunkeler et al., 2008), [15] (Morrill et al., 2005).

2.2.2 Molecular diagnostic tools

Microorganisms are the drivers of biogeochemical processes and are essential in remediating contaminated environments (Maphosa et al., 2012; Yargicoglu and Reddy, 2015). Organohalide-respiring bacteria (OHRB), that use for example chlorinated ethenes as electron acceptors to yield energy for growth, provide *in situ* destruction of contaminants in anaerobic environments (Dolinová et al., 2017). Recent advances in molecular diagnostic tools have increased understanding of bioremediation processes, whereof the most favourable technique is quantitative polymerase chain reaction (qPCR) (Jugder et al., 2016). When DNA has been extracted from an environmental sample, a specific, identified gene can be targeted, amplified and quantified using qPCR (Yargicoglu and Reddy, 2015). The target can be bacterial genera (16s rRNA gene) and/or functional genes that catalyse organohalide respiration (reductive dehalogenase genes) (Maphosa et al., 2010), an example is given in Figure 3. Reductive dehalogenase genes are more detailed targets than the bacterial genera, as it clarifies the organohalide respiratory capacity of the bacteria (Hug et al., 2013). Through reverse-transcriptase PCR, the microbial activity can also be determined from RNA extracts, but to obtain this information is more challenging (Yargicoglu and Reddy, 2015).

The bacterial genera that have been identified as significant OHRB are *Dehalococcoides*, *Dehalobacter*, *Desulfitobacterium*, and *Dehalogenimonas* (Nijenhuis and Kuntze, 2016). Several groups of OHRB are known to be able to degrade PCE and TCE (Hug et al., 2013), while *Dehalococcoides mccartyi* strains are the only identified bacteria capable of dechlorinating all chlorinated ethenes to ethene, and for that reason they are the most studied (Molenda et al., 2020). An important finding has been the identification of VC reductase genes, *bvcA* and *vcrA*, from *Dehalococcoides* species facilitating their use as biomarkers in microbial monitoring (Maphosa et al., 2010). Positive correlations between the *in situ* abundance of *Dehalococcoides* biomarker genes and the dechlorination activity has been confirmed (Clark et al., 2018). Until recently, the only identified genus capable of degrading cis-DCE and VC was *Dehalococcoides*, however recently Yang et al., (2017) identified the VC reductase gene, *cerA*, in a *Dehalogenimonas* species. This finding highlights that other unidentified and potential relevant biomarkers likely exist, and it is supported by the fact, that the majority of the known OHRB genomes contain multiple supposed reductive dehalogenase genes, but only a few have been characterised (Molenda et al., 2020).
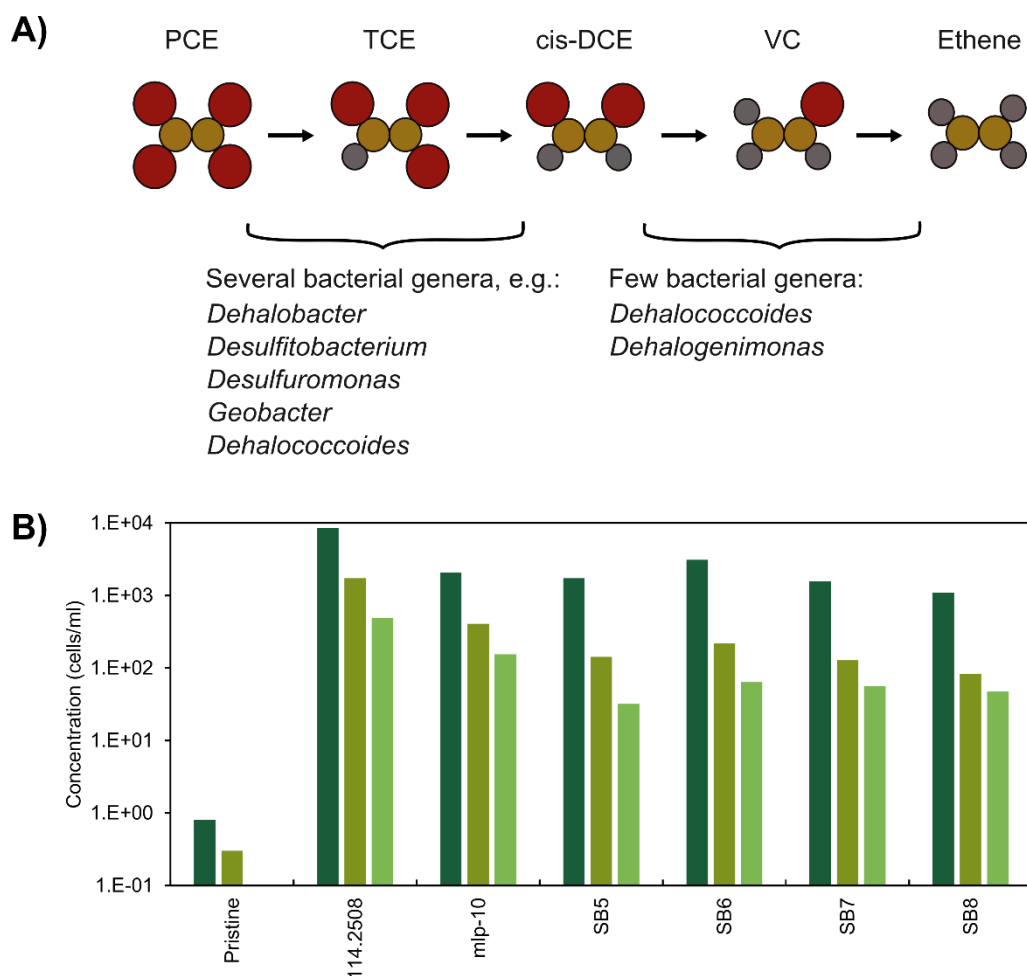
Figure 3. A) Example of bacterial genera that can facilitate anaerobic reductive dechlorination of chlorinated ethenes (Atashgahi et al., 2016; Yang et al., 2017). B) Example of qPCR results for *Dehalococcoides* (■), *bvcA* (■) and *vcrA* (■) from seven groundwater samples from a field site (modified from Ottosen et al., 2020-**III**).

The development of high-throughput methods, i.e. the development of next-generation sequencing and microarrays, has rapidly advanced our understanding of microbial genetics (Yargicoglu and Reddy, 2015). This has led to sequencing of entire OHRB genomes, which allows for classification of similar reductive dehalogenase genes that likely share similar functions (Hug et al., 2013; Molenda et al., 2020). An increasing use of genome sequencing combined with qPCR will lead to exploration of the functional diversity of OHRB, characterisation of new species and reductive dehalogenase genes, and thus improve knowledge on relevant biomarkers in bioremediation monitoring (Hug et al., 2013; Maphosa et al., 2010) – transferring knowledge from science to practice.

Early research focused on characterising the identity and catabolic capacity of the OHRB, recently more emphasis has been given to understanding the structure and function of the microbial community (Maphosa et al., 2012). Either by targeting bacteria through 16S rRNA gene amplicon sequencing (e.g. Murray et al., 2019-**II**) or the entire community genome through metagenomics (e.g. Hug et al., 2012). Gaining an understanding of the community can assist in clarifying occurring processes and microbial interactions, and reveal areas where manipulations can optimise bioremediation (Maphosa et al., 2012). However, it should be recognised that the assessment of the uncharacterised sequenced data is based on predictions that depend on the utilised gene database (Hug et al., 2012).

The knowledge on microbial dehalogenation has expanded significantly in recent years due to technological advances (Nijenhuis and Kuntze, 2016) and new emerging tools, such as proteomics targeting protein biomarkers, are under development (Heavner et al., 2019; Maphosa et al., 2010). While the emerging microbial tools give a holistic picture of reductive dechlorination, generally it is on a scientific level (Jugder et al., 2016), and qPCR is still the most applied microbial diagnostic tool to obtain valuable information for assessment on *in situ* degradation of chlorinated ethenes (e.g. Niño de Guzmán et al., 2018; Ottosen et al., 2020-**III**; Scheutz et al., 2008).

2.2.3 Compound-specific isotope analysis (CSIA)

Over the last two decades, analyses of stable isotopes have been applied to evaluate natural and enhanced degradation of chlorinated ethenes at field scale (e.g. Elsner et al., 2010; Hunkeler et al., 2005; Sherwood Lollar et al., 2001; Ottosen et al., 2020-**IV**). Carbon is the most frequently analysed isotope in organic contaminants and it consists of two stable isotopes ^{12}C and ^{13}C (Hunkeler et al., 2008). The relative abundance between the heavy and light isotope is commonly expressed in δ-notation in per mil (‰) relative to an international standard, to ensure inter-laboratory comparability (Schmidt et al., 2004).

During degradation of chlorinated ethenes the $\delta^{13}C$ value will become more positive, as the process generates an enrichment in ^{13}C, because chemical bonds to the light isotope are more easily broken than bonds to the heavy isotope (Braeckevelt et al., 2012). Hence, a qualitative assessment of degradation can be obtained by an increase in the $\delta^{13}C$ value downgradient a source zone, as phase transfer and transport processes generally are associated with minor isotope fractionation (e.g. Hunkeler et al., 2011b, 2004; Slater et al., 2000).

Nevertheless, there are some scenarios where caution should be taken, i.e. when DNAPL is present or in non-stationary systems (Chartrand et al., 2005; Ottosen et al., 2020-**IV**).

For sequential degradation an increase in the $\delta^{13}C$ value for the metabolite can be related to production as well as degradation, but if the value is compared to the original signature of the parent compound it can be qualitatively evaluated which process is the dominant (Figure 4) (Hunkeler et al., 1999). Commonly it is not feasible to obtain the original signature of the source, and it is approximated by: using literature reported values for pure products; the most negative values measured at the site; or an isotope mass balance (Hunkeler et al., 2011a, 2008).
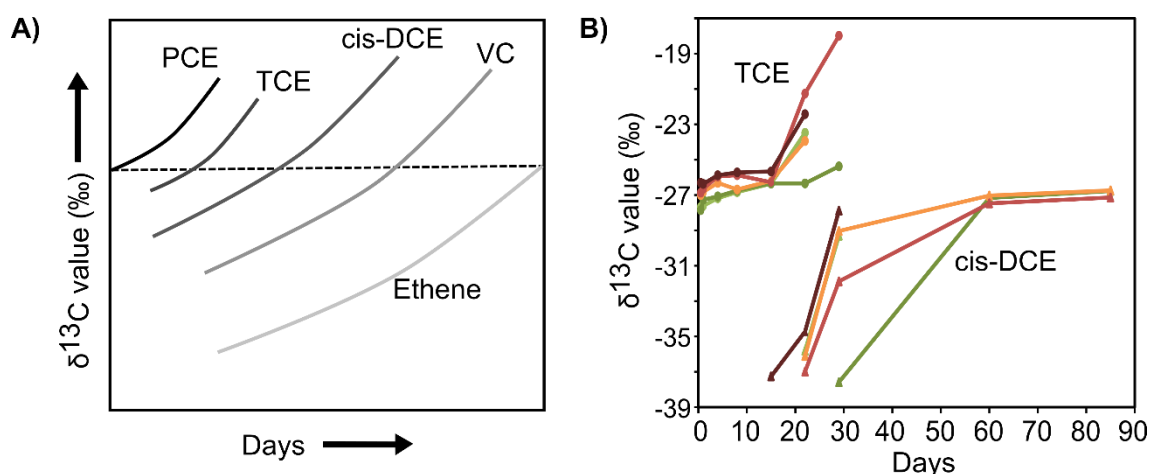

Figure 4. A) Schematic illustration on carbon isotope development during sequential degradation of chlorinated ethenes (inspired by Hunkeler et al. (1999)). Initially the metabolite is depleted in ^{13}C compared to their precursor, and eventually the metabolite become enriched in ^{13}C from the precursor and through further degradation. B) Example from laboratory treatability experiment with enhanced degradation of TCE to cis-DCE, where each color represent a distinct microcosm (modified from Ottosen et al., 2020-**V**).

The degradation-induced change in the isotope ratio, called stable isotope fractionation, can be expressed by the enrichment factor, ε (Meckenstock et al., 2004). The enrichment factor is a key parameter as it enables quantification of degradation for a specific compound and pathway (see section 2.2.4). Enrichment factors are typically determined in controlled laboratory experiments, and a range of values are reported in the literature (Hunkeler et al., 2008). The variation is important to consider, as the uncertainty of the enrichment factor reflects the uncertainty of the quantification estimates, and the largest enrichment factor should be used for a conservative estimate (Thullner et al., 2012).

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Another valuable feature of stable isotope analyses is that it provide information on transformation mechanisms, e.g. aerobic or anaerobic and abiotic or biotic, providing an essential link between laboratory and field studies (Elsner, 2010). The kinetic isotope effects are position-specific, and can therefore be used to identify degradation pathways (Elsner et al., 2005). Distinction between pathways is crucial information to constrain ε values for quantification purposes. Isotope fractionation may be masked by non-fractionating steps, such as mass transfer into cell membranes. Isotope fractionation for different elements are often masked to the same extent. Hence, dual isotope slopes can be used to designate the transformation mechanism (Elsner, 2010; Ojeda et al., 2020). In the last decade, multi-element analysis of C and Cl has emerged as an approach to distinguish transformation pathways for chlorinated ethenes (Zimmermann et al., 2020). Another advantage with C-Cl dual isotope plots is that it can provide site specific enrichment factors (Figure 5) (Murray et al., 2019-**II**).
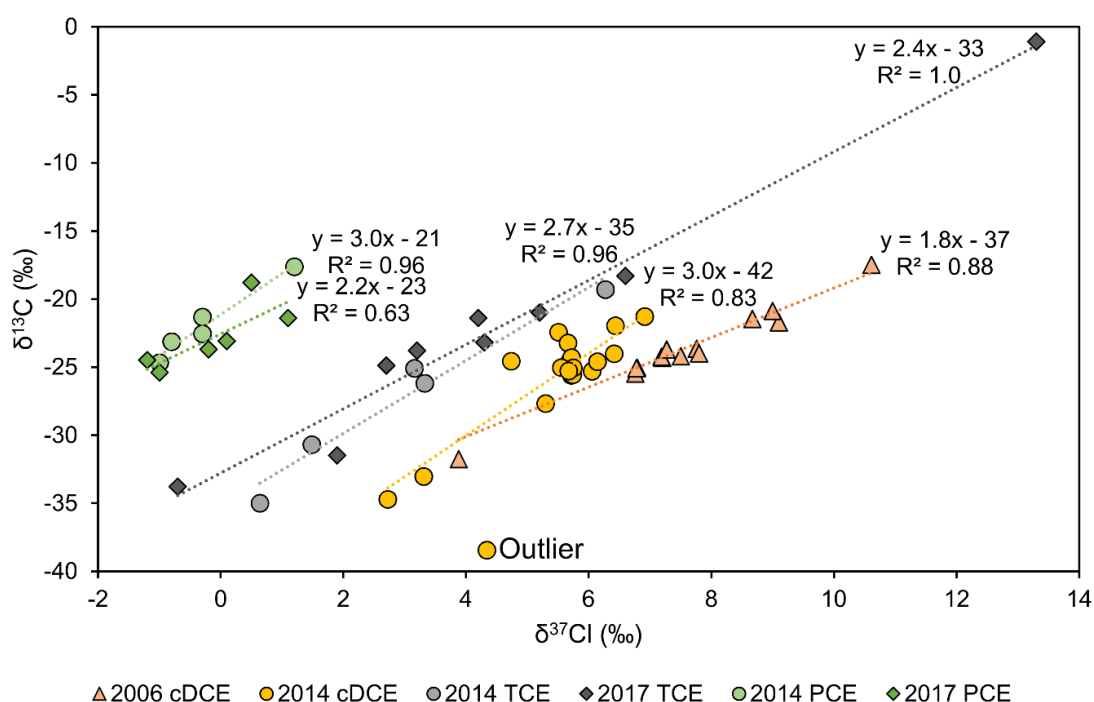
Figure 5. *In situ* C-Cl dual isotope plots for PCE, TCE and cis-DCE at different years (Murray et al., 2019-**II** with permission). The dual plot for PCE and TCE, strongly indicate that PCE was transformed by anaerobic reductive dechlorination, and the offset of the intercepts on the δ^{13}C axis provide carbon enrichment factors for this transformation step. The slopes for TCE and cis-DCE could not be directly related to specific transformation pathways when compared with literature reported slopes.

In summary, CSIA has become a well-established tool to document, characterise and quantify *in situ* degradation of chlorinated ethenes (Thullner et al., 2012), and the method is now emerging to other contaminant groups (Elsner and Imfeld, 2016). CSIA to assess *in situ* degradation is widely applied in research and has also advanced to practical use at contaminant site management (Nijenhuis et al., 2016) though there is still need for further integration into field management practices (Ojeda et al., 2020). To strengthen the tool, additional insights are required on the influences from non-fractionating steps (Elsner, 2010). Furthermore, there is a requirement for expanded availability of dual CSIA and data, identification of methods to produce compound-specific reference standards, and standardisation of the approach to obtain the dual-slopes (Ojeda et al., 2020; Zimmermann et al., 2020).

2.2.4 Quantification of degradation extent and rates

Exploration of methods to quantify degradation of chlorinated solvents dates back several decades. Initial methods to estimate first order degradation rates based on field data were (Wiedemeier et al., 1999):

- Conservative tracers

- Methods that assume steady-state equilibrium of the plume

- Mass balance methods

A conservative tracer, which is not degraded in the field site environment, can be used to correct mass loss though physical processes (i.e. dispersion, dilution, volatilisation and sorption) for the compound of interest. The degradation rate for the contaminant can then be determined by the first order decay equation (if the data fit the model) when the concentration at the up-gradient point (C_0) and the solute travel time between the two points are determined (Hageman et al., 2004; Rügge et al., 1999). A simplified version, where a graphical method that plot the natural log concentration against time or distance from the source along a flow line, has also been used. This has led to some ambiguity in the early determined rates as some describe attenuation and others biodegradation only (Newell et al., 2002). The uncertainty is problematic because these early determined rates constitute a large portion of the published rates (Ottosen et al., 2019-**I**).

Assuming that a contaminant plume is in steady-state, a combination of analytical solutions for contaminant transport with *in situ* concentration trends, can be used to estimate first order degradation rates (Wiedemeier et al., 1999).

This method has developed to incorporate compound-specific stable carbon isotope data (Höhener et al., 2015).

A mass balance approach can be used if an extensive monitoring well network is available. The total mass of an individual contaminant is monitored over time though interpolation and integration, and is then used to determine the degradation rate (Courbet et al., 2011; McAllister and Chiang, 1994).

The advance of CSIA offers a valuable alternative to quantifying degradation of chlorinated ethenes. A good agreement has been found between biodegradation rates determined by conventional and isotopic methods, and typically the latter provide a more conservative and possible more accurate estimate (Abe and Hunkeler, 2006; Hunkeler et al., 2008; Morrill et al., 2005). The difference between the initial and final $\delta^{13}C$ values for the contaminant of interest facilitate determination of the fraction of biodegradation along a flow line:

$$D = 1 - f = 1 - \exp\left(\frac{\Delta\delta^{13}C}{\varepsilon}\right) \tag{1}$$

Furthermore, if the pore water velocity is known along the flow line, then the degradation rate, k, can be estimated:

$$k^{12}_{compound} = \frac{-\left(\frac{1000}{\varepsilon}\right) \cdot \ln(\frac{\delta^{13}C/\,1000 + 1}{\delta^{13}C/\,1000 + 1})}{R_{compound} \cdot L/v_{p,compound}} \tag{2}$$

Where R is the retardation coefficient for the compound, L the distance between the two points, and v_p the pore water velocity. Quantification of degradation through CSIA can only be conducted for compounds, where no precursors are present. This is to assure, that the increase in $\delta^{13}C$ values are due to degradation and not production, as both production and degradation of an intermediate leads to an increase in the $\delta^{13}C$ value (Figure 4) (Badin et al., 2016). Optimal results are obtained with site-specific enrichment factors, as the enrichment factors reported in literature can vary significantly (Murray et al., 2019-**II**). Incorporation of ^{13}C-labels into compounds of interest, can be used to quantify degradation *ex situ* or *in situ* by monitoring the labels in the metabolites over time in microcosms or as tracer tests. However, this approach is work and cost intensive and is rarely used to evaluate biodegradation at field-scale (Fischer et al., 2016).

3 Integrated approach for assessment of *in situ* degradation

Aquifer systems are heterogeneous and dynamic, and complex interactions occur between biogeochemistry and contaminant fate (Meckenstock et al., 2015). Therefore, it is recommended to integrate chemical and microbial knowledge obtained by various tools with system understanding to evaluate degradation processes, and thus achieve a more reliable and robust assessment. Integrative degradation studies can be designed in multiple ways, and the appropriate combination of tools depend on the aim and compounds of interest (Bombach et al., 2010). The extent of investigations highly depend on the potential risk the site pose, i.e. the level of contamination and whether it is near areas of special interest (Illman and Alvarez, 2009). Three lines of evidence have been recommended by U.S. EPA to assess natural attenuation: (1) Decreasing concentration and/or contaminant mass over time at appropriate sampling points; (2) Hydrogeological and geochemical characterisation to demonstrate degradation potential and rate of occurring processes; (3) Field or microcosm studies to document the occurrence of degradation at the site (Wiedemeier et al., 1998).

3.1 Application of an integrated approach

The integrated approach can be applied in different scenarios to obtain knowledge on degradation processes and the factors that control them (Figure 6). The integrated approach can be used to: conduct a preliminary screening of degradation potential; to obtain the required evidence in monitored natural attenuation; and to assess the performance of a bioremediation strategy.

When screening for the degradation potential (Figure 6A), the degree of necessary details increase with the complexity of the system, and advanced tools can with advantage be included in assessments of complex systems (e.g. Ottosen et al., 2020-**III**). The evaluation is strengthened by the use of advanced tools, and though they are more expensive, they also provide additional valuable information that could lower the total cost of site clean-up by producing a more robust conceptual model and guiding appropriate remedial actions (Hunkeler et al., 2008). If the conceptual model point towards limited degradation (e.g. chlorinated parent compound concentrations are stable and no metabolites are detected), extended monitoring should not be conducted and monitored natural attenuation (MNA) is not a remedial option.
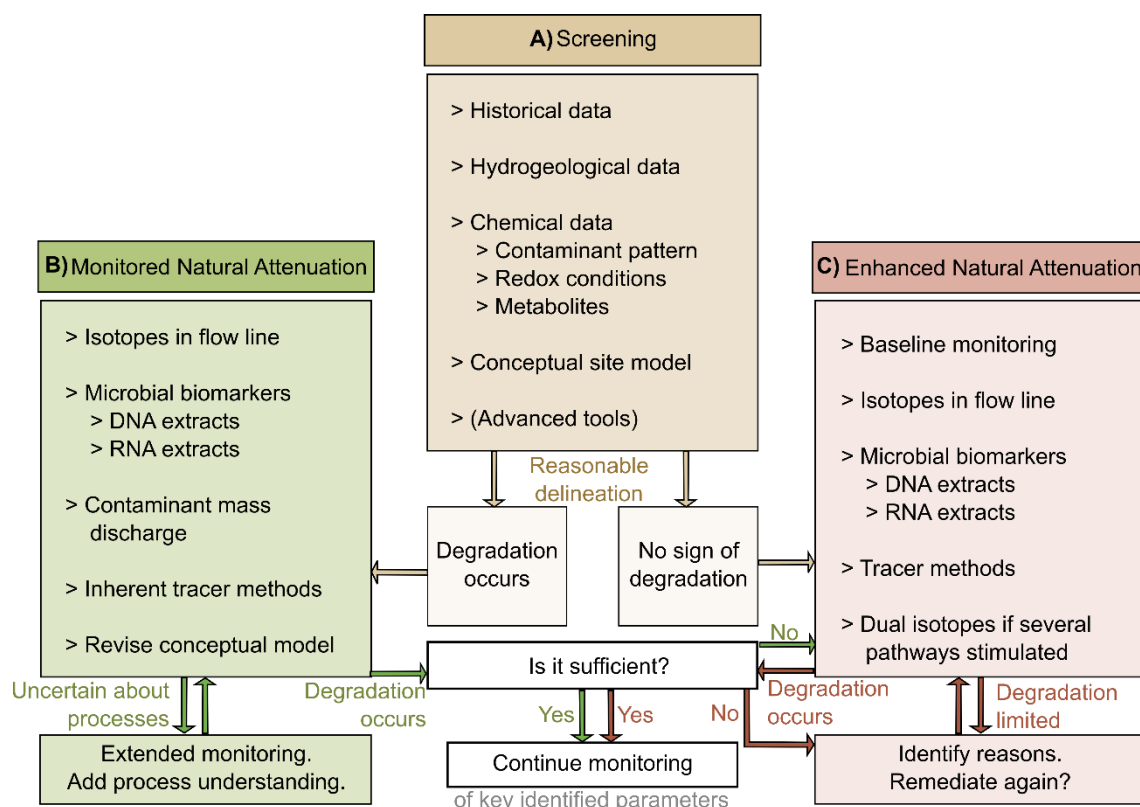
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Figure 6. Different applications stages (A-C) of an integrated approach to assess *in situ* degradation of chlorinated ethenes. Relevant tools for each application stage are provided, but the investigations are not limited to those, likewise all tools are not a requirement.

In contrast, if the screening reveal that degradation occurs then MNA can be considered and assessed by an integrated approach (Figure 6B). The monitoring approach for MNA has moved from a period of qualitative evaluations of *in situ* processes to a period with quantitative assessments of the processes at field-scale (Wilson, 2010). The advanced tools can provide knowledge on important processes and mechanism(s), which can be used to assess natural attenuation and the rate at which it progresses (e.g. Abe et al., 2009). Furthermore, the integrated approach can identify possible reasons for incomplete dechlorination and support decision of additional remedial actions.

If the natural degradation is insufficient to clean-up the contamination, bioremediation should be considered. The integrated approach to assess enhanced bioremediation likewise benefit from including additional advanced techniques (Figure 6C). Because bioremediation interfere with the processes in the plume, characterisation and conceptualisation of the changes in the system are essential (Ottosen et al., 2020-**IV**), and laboratory treatability experiments under controlled conditions can enhance process understanding (Ottosen et al., 2020-

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V). The integrated approach can provide the required information to evaluate the performance of the remedial strategy (Révész et al., 2014).

3.2 Research applications of an integrated approach at field-scale

The practical aspect of applying an integrated approach is highly linked to the research aspect. The practical use of the approach facilitate identification of unknown areas that require further research, while the research provide knowledge on the applicability of the tools in different scenarios and settings.

As indicated above, advanced tools can provide essential information of *in situ* degradation in various scenarios. While numerous field studies are published, that apply the novel isotopic or microbial techniques to assess *in situ* degradation, field studies that combine them in an integrated approach are still less common (e.g. Blázquez-Pallí et al., 2019a; Nijenhuis et al., 2007; Ottosen et al., 2020-**III**, 2020-**IV**). Recent research reveals that the two parameters are linked, i.e. the enrichment factor vary considerably depending on bacterial groups (Cichocka et al., 2008), and it is therefore important to understand the interplay of these promising techniques at field scale. Therefore, this chapter focus on research studies that apply an integrated approach which include both of these novel techniques.

3.2.1 Natural attenuation

The first applications of an integrated approach, including isotopic and microbial techniques, to assess *in situ* biodegradation were applied to examine natural attenuation, and over the years several field studies have been published (Table 2). A spatial understanding of the natural degradation is obtained at a snapshot in time, and some studies moreover include a temporal assessment (Carreón-Diazconti et al., 2009; Courbet et al., 2011; Imfeld et al., 2008; Ottosen et al., 2020-**III**). The focus has primarily been to characterise and document the occurrence of natural degradation and fewer studies have also quantified the degradation (Abe et al., 2009; Carreón-Diazconti et al., 2009; Courbet et al., 2011; Ottosen et al., 2020-**III**). The research illustrates that valuable information can be obtained by the advanced tools (Table 2). The abundance of specific bacteria and functional genes reveal degradation potential for several pathways and illustrate spatial variation. The compound-specific δ^{13}C values facilitate characterisation, documentation and quantification of degradation as well as indication of multiple sources. Hence, the integrated approach can be used to document and quantify *in situ* natural degradation.

An example of a study, where an integrated approach was applied to assess the temporal developments and to quantify bioattenuation, is the near-stream investigation by Ottosen et al. (2020-**III**). A contaminant groundwater plume primarily consisting of cis-DCE and VC, which discharge to the stream (Grindsted stream), was examined. Hydrogeological, chemical and microbial data obtained by state-of-the-art techniques were combined to achieve a conceptual understanding of the system over a 7 year monitoring period (Figure 7). The investigations provided unusual information, as it exhibited a specific attenuation behavior not commonly observed; the system changed from limited attenuation occurring for several years to a situation where significant bioattenuation occurred.

For the first period, the limited attenuation through the system was documented by a reach scale mass balance, where compound molar ratios were nearly constant. Quantification of the in-stream contaminant mass discharge confirmed the two different stages for the system. Furthermore, the advanced techniques provided strong evidence for biodegradation in the near-stream system in the second period. *Dehalococcoides* with *vcrA* and *bvcA* were present in high abundances in the near-stream plume core (Figure 3B), compared to literature reported levels for effective dechlorination. Together with sufficiently reduced redox conditions and donor availability the microbial data indicated strong potential for anaerobic reductive dechlorination.

Furthermore, CSIA for cis-DCE, VC and the non-chlorinated end-product ethene could be used to document degradation. A significant enrichment in ^{13}C isotopes for especially cis-DCE provided strong evidence for degradation of this compound. In addition, an increase in the carbon isotope mass balance values illustrated complete dechlorination for a portion of the chlorinated ethenes. Hence, the isotopic and microbial data provided the documentation for the increased degradation and the zone at which it primarily occurred. The study, furthermore illustrated the importance of applying an integrated approach, as the conceptualization of the site revealed that short a residence time restricted the extent of dechlorination. Thus, the evaluation of the biodegradation potential illustrates a capacity for biological remediation technologies if the limitation of the residence time is overcome. For that, continued monitoring is required to support the latest records of increased bioattenuation.

Table 2. Overview of field studies that use an integrated approach, including isotopic and microbial techniques, to monitor *natural attenuation* in contaminant plumes* and sources**.

Study	Details
(Nijenhuis et al., 2007) (Imfeld et al., 2008) †	[*,**] The δ^{13}C values described the dechlorination process. *Dehalococcoides, Desulfuromonas, Desulfitobacterium* and *Dehalobacter* were present. Chlorinated ethene concentrations influenced the bacterial community structure. Laboratory microcosms confirmed biodegradation potential for complete dechlorination to ethene, but restricted by donor or nutrient limitations.
(Abe et al., 2009)	[*] Investigations at the GW-SW interface. The δ^{13}C values facilitated depth-specific assessment of the degree of degradation in different redox zones, and quantification of a degradation rate. *Dehalococcoides* presence limited, gene related to aerobic VC degradation more pronounced. Aerobic microcosm laboratory studies demonstrated the potential of VC oxidation.
(Carreón-Diazconti et al., 2009)	[*] The δ^{13}C values reflected incomplete dechlorination, and indicated extent of biodegradation. *Dehalococcoides* with *vcrA* was present. Bacteria capable of degrading PCE and TCE were also present. Microcosm confirmed degradation stalling at DCE.
(Courbet et al., 2011)	[*] The δ^{13}C values documented degradation of DCE and VC, and spatially limited degradation of TCE. *Dehalococcoides* with *vcrA* and *bvcA* were present and active, and less so *tceA*. Mass balance supported existence of natural attenuation, and combined with CSIA revealed that other attenuation process than degradation occurred.
(Hunkeler et al., 2011a)	[*,**] The δ^{13}C and δ^{37}Cl values distinguished different zones of degradation for the individual compounds. *Dehalococcoides* signal detected in the front of the plume.
(Damgaard et al., 2013b)	[**] The δ^{13}C values documented spatial distribution of chlorinated ethene degradation, and suggested both biotic and abiotic degradation. *Dehalobacter* and *Dehalococcoides* with *vcrA* and *bvcA* were present in biozones. Sampling of intact cores enabled high resolution investigation.
(Blázquez-Pallí et al., 2019b)	[*] The δ^{13}C values documented incomplete dechlorination and suggested two distinct sources. *Dehalococcoides* with *vcrA* was present. Microcosm studies showed potential for bioenhancement.
(Ottosen et al., 2020-**III**)	[*] Investigations at the GW-SW interface. The δ^{13}C values documented temporal dechlorination of cis-DCE and VC. *Dehalococcoides* with *bvcA* and *vcrA* were present. Mass balance quantified natural attenuation processes.

† These two references are put together as they were conducted at the same site, and the latter used the dataset from the first in their assessment. Furthermore, the conclusions are coherent between the two studies.
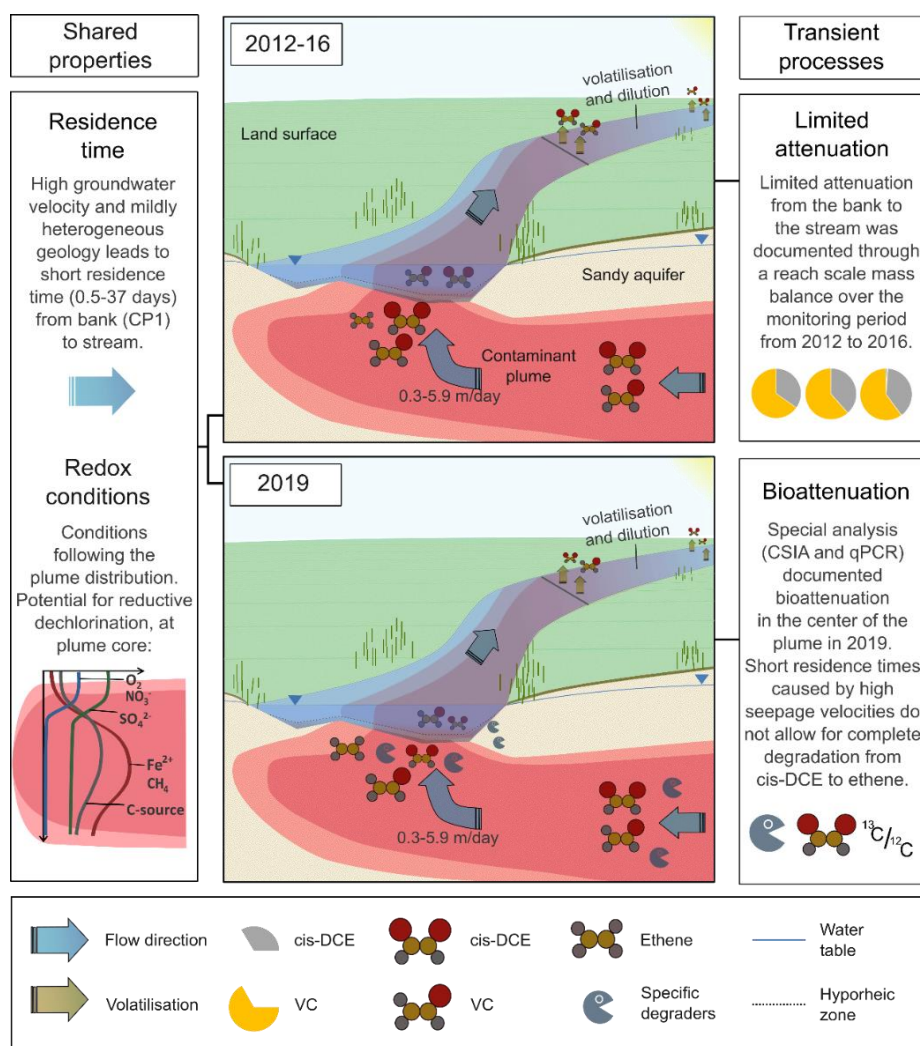
Figure 7. Conceptual model of the chlorinated ethene attenuation at the near-stream system in Grindsted over a monitoring period of 7 years (Ottosen et al., 2020-**III** with permission).

3.2.2 Enhanced natural attenuation

Within the last decade the integrated approach, including isotopic and micro-bial techniques, has also been applied to assess *in situ* enhanced biodegradation (Table 3). During enhanced natural attenuation the processes in the plume are altered, which make the interpretation more complex, and thus enhance the requirement of an integrated approach to describe the occurring processes. Most commonly the integrated approach has been used to assess enhanced bi-odegradation in plumes with biostimulation and/or bioaugmentation (e.g. Blázquez-Pallí et al., 2019a; Murray et al., 2019-**II**; Révész et al., 2014). The integrated approach has also been used in sequential studies, where an initial screening of degradation potential was followed by a full scale enhanced bio-remediation (Blázquez-Pallí et al., 2019b, 2019a). Furthermore, the integrated

approach has also been used to examine the effect of a source zone treatment that mobilized dissolved organic carbon which caused spatial and temporal developments in chlorinated ethenes degradation in the downgradient plume (Badin et al., 2016; Murray et al., 2019-**II**). Generally, the combined data provide knowledge of the remediation efficiency by determining the spatial development in degradation and indicate potential reasons for critical areas where degradation stall. Microbial and isotopic data can support assessments of system responses to and success of bioremediation applications.

Recently an integrated approach, that include the advanced isotopic and microbial techniques, has also for the first time been applied to assess degradation at a field site where bioamendments were injected together with liquid activated carbon (Ottosen et al., 2020-**IV**). The scientific basic for the remedial strategy is recognised, i.e. the activated carbon should retain plume spreading though sorption and increase contact time between bacteria and contaminants, but methods to actually document degradation are required (Fan et al., 2017). At the field study, the amendments were injected to establish a treatment zone intercepting a TCE plume, and long-term monitoring was conducted with high sampling frequency (Ottosen et al., 2020-**IV**).

The injection induced transient, complex processes in the downgradient plume, which could only be understood by the use of an integrated approach. The injection of activated carbon and bioamendments enhanced degradation from TCE to primarily cis-DCE. Developments in OHRB abundances, combined with system characterisation, enabled an assessment of possible reasons for the observed stall of degradation at cis-DCE. The abundance of *Dehalococcoides*, which was the central bacteria in the culture, only increased slowly in the injection zone, to the same levels as downgradient indigenous *Dehalococcoides*. This indicate unideal conditions for the injected bacteria, which could be related to the low pH of the added donor. Furthermore, the data indicated that further dechlorination could be limited by zones where redox conditions were not reduced enough and by nutrient deficiency. Hence, the microbial data combined with system characterisation delivered essential knowledge on the injection effect and indicated critical zones and reasons for the incomplete dechlorination (Ottosen et al., 2020-**IV**).

Table 3. Overview of field studies that use an integrated approach, including isotopic and microbial techniques, to monitor *enhanced natural attenuation* in contaminant plumes* and sources**.

Study	Explanation
(Damgaard et al., 2013a)	[*,**] Biostimulation and bioaugmentation. Enhanced degradation of TCE with spatial variation. A significant increase in abundance of active *Dehalococcoides* with *vcrA* and *bvcA* observed. The $\delta^{13}C$ values of chlorinated ethenes confirmed degradation. Core samples revealed bioactive zones. Estimate of average mass reduction was 24% after 4 years.
(Révész et al., 2014)	[*] Biostimulation and bioaugmentation. Enhanced degradation of TCE, with decreasing efficiency over distance from the injection well. An increase in the abundance of *dehalococcoides* with *vcrA* and *Geobacter* followed the same trend. The $\delta^{13}C$ values corresponded well with concentration developments, and the isotope mass balance revealed dechlorination beyond VC for some time. The monitoring revealed a slowdown in treatment efficiency after a year.
(Badin et al., 2016)	[*] Stimulated by mobilisation of DOC after thermal remediation of source. Enhanced degradation of PCE to predominantly cis-DCE. Abiotic and biotic degradation of cis-DCE. The $\delta^{13}C$ values of chlorinated ethenes identified the occurrence and extent of degradation, and dual C-Cl isotope slopes indicated degradation pathways. *Dehalococcoides* was present and active in first half of the plume. 16S rRNA sequencing enabled identification of relative abundance of different bacteria.
(Atashgahi et al., 2017)	[*] Biostimulation. Enhanced degradation of cis-DCE but not complete. Abundance of *Dehalococcoides* with *vcrA* and *bvcA* increased. The $\delta^{13}C$ values of cis-DCE and VC documented a variation in degradation. 16S rRNA sequencing revealed that the biostimulation significantly affected bacterial community.
(Blázquez-Pallí et al., 2019a)	[*] Biostimulation. Laboratory microcosm confirmed biostimulation potential. Enhanced dechlorination of PCE and TCE to degradation products at field scale. The $\delta^{13}C$ values of chlorinated ethenes confirmed degradation and isotope mass balance documented dechlorination beyond VC in most wells. Metagenomic data revealed shift in microbial composition towards dominance of fermentative bacteria.
(Murray et al., 2019-**II**)	[*] Same site as (Badin et al., 2016) monitored 3 years later. Main differences/additional information: *Dehalococcoides* with *vcrA* and *bvcA* and *Dehalogenimonas* with *cerA* were present. The $\delta^{13}C$ values of PCE and cis-DCE facilitated estimation of degradation rates over distance and time.
(Ottosen et al., 2020-**IV)**	[*] Biostimulation, bioaugmentation and addition of liquid activated carbon. Insufficient sorption to retain plume spreading. Enhanced degradation of TCE to primarily cis-DCE. Abundance of *Dehalococcoides* with *vcrA* and *bvcA* and of TCE-degraders increased. The $\delta^{13}C$ values of TCE masked by several effects, the $\delta^{13}C$ values for cis-DCE confirmed stalling degradation.

Valuable information was also obtained through compound-specific stable isotope data. The δ^{13}C values for TCE and cis-DCE behaved unexpectedly, while δ^{13}C values for TCE indicated limited degradation, δ^{13}C values for cis-DCE indicated almost complete degradation from TCE to cis-DCE, as the values for both compounds were close to the initial signature. Hence, the δ^{13}C values did not correspond with the TCE degradation documented by metabolite production and accumulation. In addition, the δ^{13}C values for TCE behaved differently in the down-gradient part of the plume, as a small increase in δ^{13}C values was observed over time. Therefore, a conceptual model that combined system characterisation and isotope trends was formed (Figure 8)(Ottosen et al., 2020-**IV**).

The conceptual model of the isotope trends illustrated that δ^{13}C values for TCE were masked by TCE with the original signature. TCE with the original signature was added by inflow of the up-gradient plume into the injection zone, and by desorption from the sediment, as enhanced degradation caused gradient changes in the treatment zone. This addition of TCE disguised the isotope fractionation caused by degradation. The relatively long screens used to monitor the aqueous phase, mixed water from zones with limited degradation and significant degradation, thereby δ^{13}C values for the two contaminants became the same. In addition, this provide another masking effect on isotope fractionation caused by degradation. Combined with system characterisation of flow and transport, the difference between the injection zone and the down-gradient plume can also be explained. The masking effect caused by desorption and screen mixing were similar in the two zones, whereas the masking effect of the inflowing TCE was observed in the injection zone only, for the time of monitoring. Hence, the masked δ^{13}C values for compounds present in the plume prior to remediation (here TCE) revealed zones with insufficient treatment, whereas it complicated quantification of degradation. In contrast, for compounds not present in the plume prior to remediation (here cis-DCE and VC) CSIA could be applied as done by common practice. Thus, the conceptualisation provided essential process understanding, and revealed benefits and limitations for CSIA in a non-stationary system (Ottosen et al., 2020-**IV**).
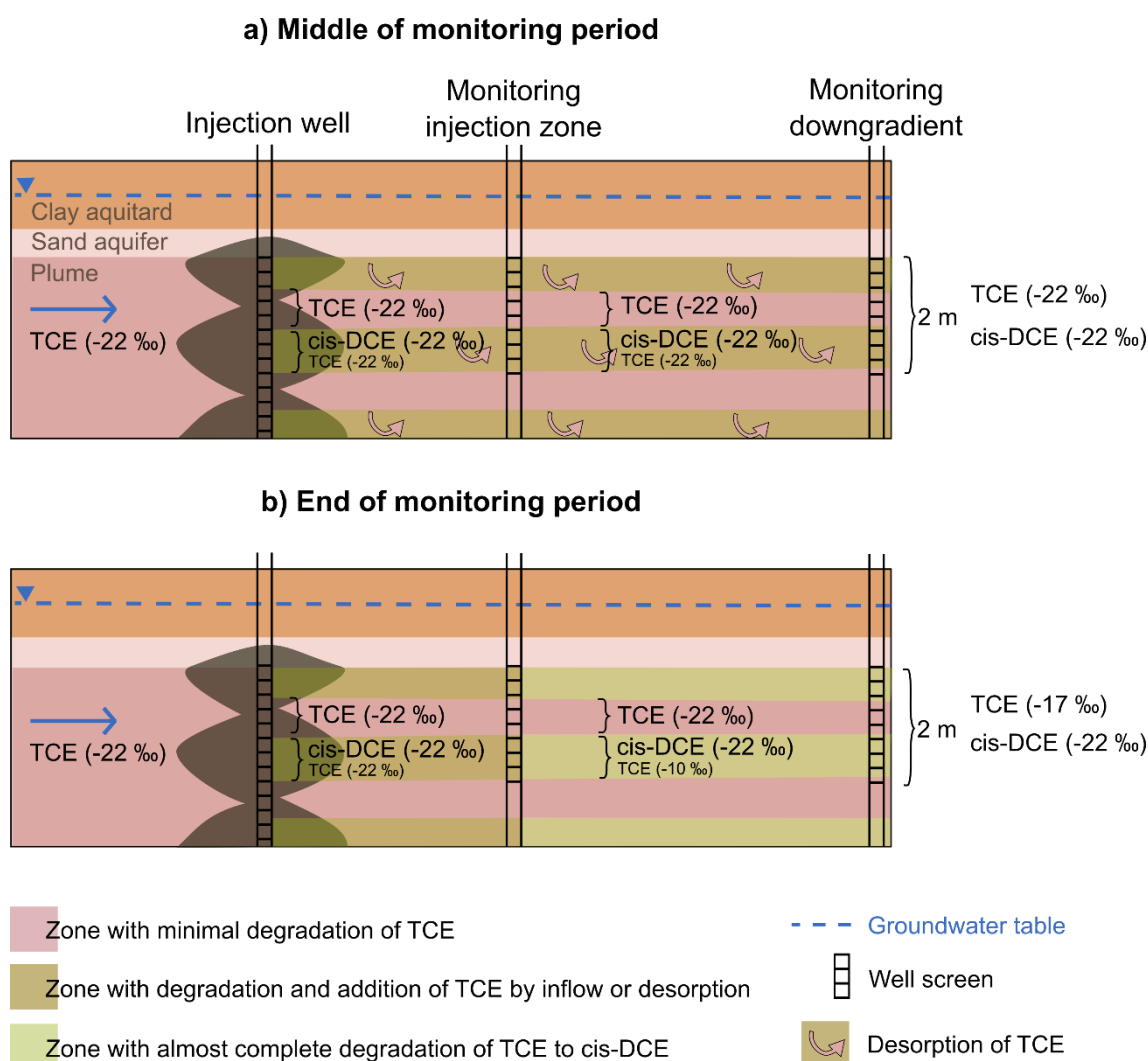
Figure 8. Conceptual model presenting a schematised description of the isotope trends at a field site after injection of liquid activated carbon and bioamendments (Ottosen et al., 2020-**IV**). The δ^{13}C values for TCE are masked by (1) TCE with the original signature flowing into the treatment area, (2) TCE with the original signature desorbing from the sediment due to degradation-enhanced gradient changes, and (3) mixing of zones with different signatures in long monitoring screens. The identified advantages and challenges for CSIA are also relevant for plumes with reactive barriers without the use of activated carbon.

A laboratory treatability experiment was conducted under controlled conditions and supported system understanding at field-scale (Ottosen et al., 2020-**V**, 2020-**IV**), exemplifying that laboratory studies can be a valuable tool for process understanding in the integrated approach, especially for new remedial technologies like this. To summarise, an integrated approach can be used to characterise and document *in situ* degradation enhanced by activated carbon and bioamendments, however quantification of degradation is complicated for certain compounds.

3.3 System understanding and conceptualisation

The full value of the methods to characterize and quantify degradation is only achieved, provided that the conditions, the results were obtained under, are understood. Hence, system characterisation and conceptualisation are essential aspects of the integrated approach. It should be ensured that the sampling design reflects the processes sufficiently in time and space. Furthermore, conceptual models for different scenarios can support risk assessment by extrapolating the learnings to a larger framework.

3.3.1 Characterisation of flow and transport

Understanding the processes that control the contaminant transport in groundwater is essential, in order to conceptualise biodegradation processes and the rate at which they progress.

Firstly, characterisation of flow and transport provide essential knowledge on residence times and thus the time at which degradation can take place. The residence time in a system is greatly influenced by the hydrogeological setting and require site-specific measurements. Different tools exist to describe the hydrogeology in aquifers and at the groundwater-surface water interface (Annable et al., 2005; Cremeans et al., 2020; Layton et al., 2017). Residence times can be the main factor controlling contaminant attenuation, and should therefore be carefully evaluated together with biodegradation processes (Figure 7) (Ottosen et al., 2020-**III**). Thus, even when microbial and isotopic data provide strong evidence for degradation, characterization of system flow and conditions are necessary to determine factors that control degradation, and allows for identification of possible ways to optimise the process.

Secondly, characterisation of flow and transport allows for estimation of degradation rates, when combined with knowledge on contaminant fate (Abe et al., 2009; Aeppli et al., 2010; Morrill et al., 2005). An example is the ~2 km long chlorinated ethenes plume described by Murray et al. (2019-**II**). Hydrogeological characterisation revealed a decreasing pore water velocity along the central flow line (Figure 9). Coupled with $\delta^{13}C$ values, and retardation of the individual compounds, degradation rates could be estimated for PCE (0.08 to 0.35 yr^{-1}) and cis-DCE (0.01-0.07 yr^{-1}) in different parts of the plume over time and distance. Determination of degradation rates for cis-DCE revealed insufficient progress to completely reduce cis-DCE in the part of the plume with most optimal redox conditions. Thus, isotope data can, when combined with system characterisation, provide the third line of evidence required for the assessment of attenuation – documentation and quantification of degradation. This study,

thus adds to the small but growing library of *in situ* degradation rates available in the literature, which can support decision of ranges used in risk assessments.
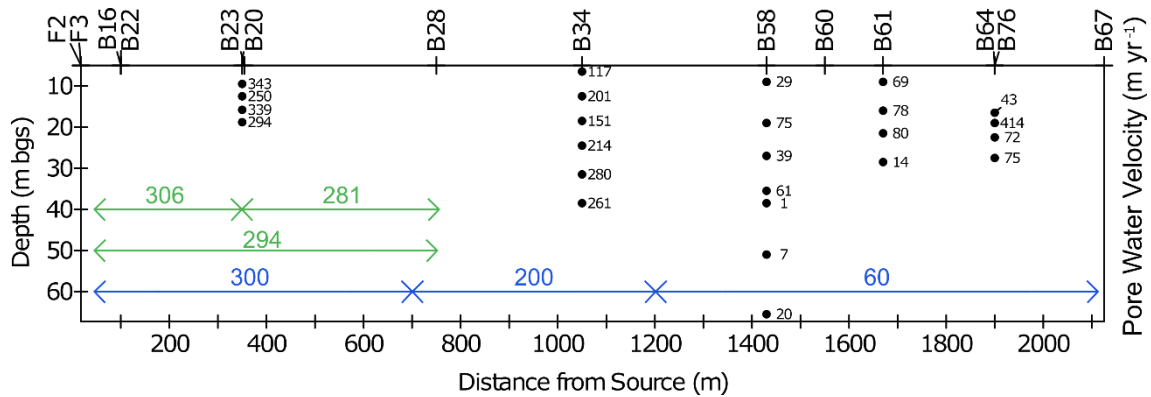

Figure 9. Pore water velocities along the central flow line in the chlorinated ethene plume at the Rødekro site (Murray et al., 2019-**II** with permission). The arrows indicate discrete flow zones used for assessment of overall transport (blue) and to estimate time-based degradation rates (green).

Lastly, characterisation of flow and transport support determination of amendment distribution after bioremediation efforts. Thereby, it can be assessed when and where enhancement effects are expected and to what extent (Révész et al., 2014). Comprehensive knowledge on compound and amendment distribution is essential in order to achieve useful conceptual understanding of the effect and success of bioremediation (Ottosen et al., 2020-**IV**).

In order to obtain the full value of flow and transport characterisation, the variance in geological properties (e.g. hydraulic conductivity) of the contaminated aquifer should be depicted in the overall assessment. Therefore, adequate sampling resolution is required for these parameters as well as others. Furthermore, transient processes that induce possible changes in flow, caused by hydraulic gradients, should be considered, i.e. water abstraction or surface water level.

3.3.2 Spatial and temporal resolution

Aquifers are dynamic and heterogeneous systems, hence to obtain adequate process understanding, samples should be collected at sufficient intervals and resolutions (Meckenstock et al., 2015). The appropriate spatial resolution of sampling points depends on several factors, including the scale of the plume, the heterogeneity of the geological settings, whether degradation is natural or enhanced, and the aim of the investigations.

In large scale plumes, diverse biogeochemical conditions, that influence the occurrence of *in situ* biodegradation, can occur along a vertical gradient

(Imfeld et al., 2011) or along a horizontal flow line of the plume (Murray et al., 2019-**II**). However, high-resolution discrete sampling is not feasible throughout plumes that extents several kilometres. For that reason, prioritised sampling can be conducted at several depths and distances, to depict compartments and enclosed process throughout the plume. In contrast, in small-scale plumes, high-resolution sampling is more achievable. This is relevant, where steep gradients are observed at the interface of different geological layers (Damgaard et al., 2013b; Wanner et al., 2018) and where different water compartments are mixed (Ottosen et al., 2020-**III**; Weatherill et al., 2018) in order to obtain adequate information of dominant processes. Isotopic and microbial techniques can be used to obtain process understanding and characterize mechanism(s) at both scales, where the detail of the sampling design reflect the detail of the assessment.

In systems altered by a bioremediation intervention, large spatial variations may be observed even in mildly heterogeneous systems (Figure 10). Thus, to obtain an accurate assessment of a bioremediation performance the sampling resolution should sufficiently cover the dynamics of the system and high-resolution sampling in prioritised locations may highly increase process understanding. This was the case, at the site with injection of activated carbon and bioamendments, where depth discrete sampling revealed more discretised isotopic trends and supported the conceptual model of isotope trends at the site (Figure 8) (Ottosen et al., 2020-**IV**). Generally, the appropriate spatial resolution depend on the stationarity of the plume processes and building a conceptual model can help deduce if a sufficient understanding has been obtained.

The intervals at which samples are collected are also central, and likewise depend on the stationarity of the plume processes. Degradation can be relatively consistent over numerous years, i.e. a plume that remain more or less steady state (Badin et al., 2016; Murray et al., 2019-**II**), or significant long-term changes may be observed (Ottosen et al., 2020-**III**). From the perspective of risk assessments both scenarios are important to be able to document and quantify. Furthermore, continued sampling provide additional valuable information, e.g. time series data on microbial targets abundances allows for documentation of bacterial growth (Scheutz et al., 2008). As with the spatial design, high-interval sampling could also be relevant. It is important to consider whether short-term variations can influence the long-term assessments. In addition, in transient systems short-term monitoring over long periods may well be necessary to follow the development of system dynamics and thus the potential for biodegradation (Figure 10) (Ottosen et al., 2020-**IV**).
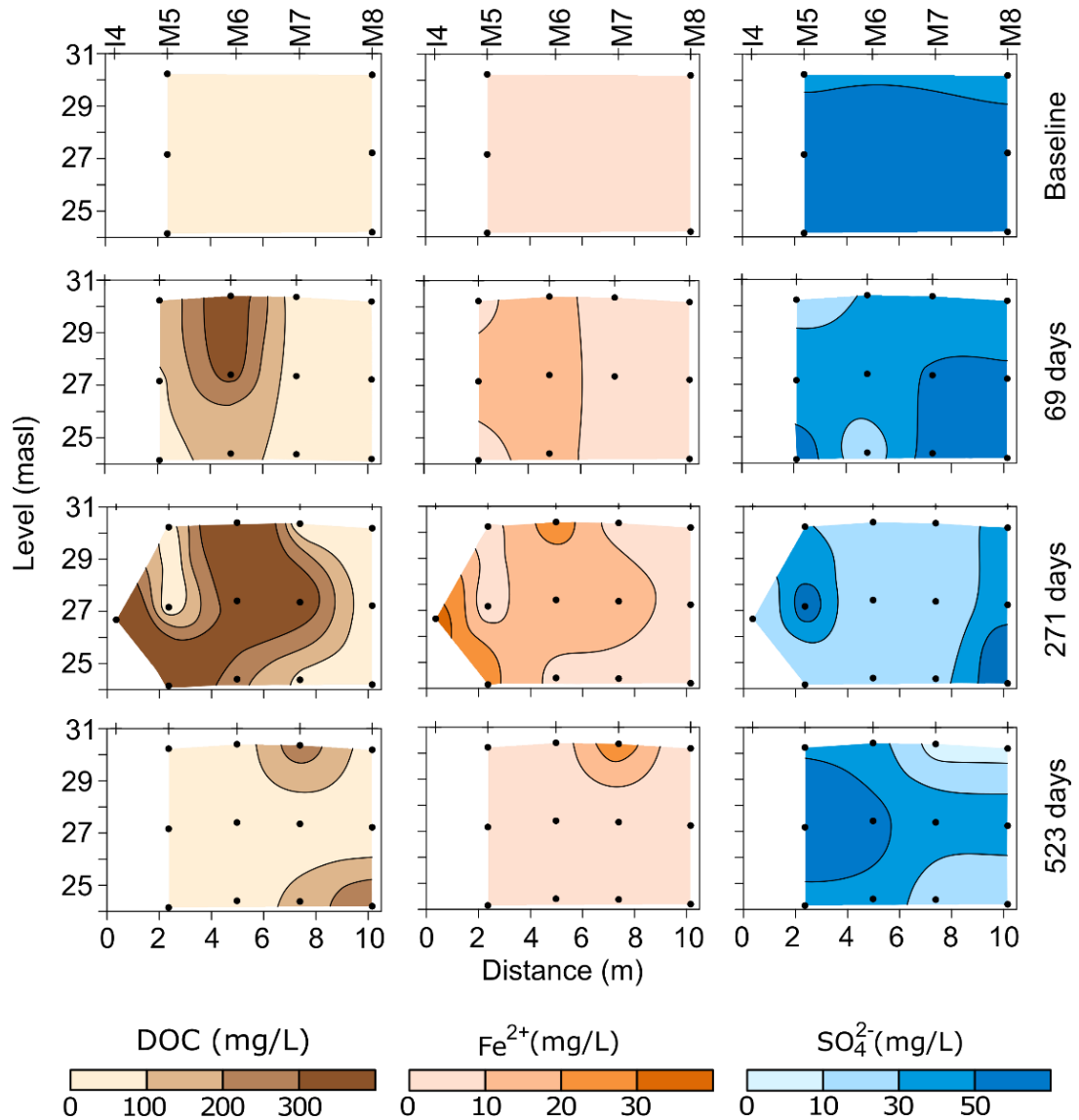
Figure 10. Distribution of dissolved organic carbon and selected redox parameters along a down-gradient flow line after injection of activated carbon and bioamendments (modified from Ottosen et al., 2020-**IV**). The dots indicated the middle of a 2 m screen, I = injection well and M = monitoring well. The data show great temporal and spatial variation after the injection.

In the end, the characteristics of a plume system can never be fully concealed, but the accuracy and usefulness of the system understanding are highly dependent on the sampling design. In areas where no sampling points are located, assumptions needs to be made, and therefore it is vital to obtain a conceptual understanding that combine all acquired information.

3.3.3 Conceptualisation

Conceptualisation of the processes and mechanisms in a chlorinated ethene plume is a prerequisite for site-specific documentation and quantification of *in situ* degradation, as well as in order to obtain a more holistic description of experiences in various systems.

Conceptual site models are beneficial at near-stream systems where numerous processes may occur simultaneously (Freitas et al., 2015) and to distinguish the dominant attenuation processes over time and space (Figure 7) (Ottosen et al., 2020-**III**). Conceptual models are advantageous at sites with enhanced degradation, for detailed process understanding in these complex systems (Badin et al., 2016), and to determine the dynamics that influence the progressions in the measured parameters (Figure 8) (Ottosen et al., 2020-**IV**). Conceptual site models are furthermore valuable at sites where multiple degradation pathways occur simultaneously (Elsner et al., 2010), and for definite identification that destruction of the chlorinated ethenes occur (Murray et al., 2019-**II**).

An example, where a conceptual site model was essential in order to document and quantify *in situ* degradation is the investigations by Murray et al. (2019-**II**) at Rødekro. The investigations indicated occurrence of both biotic and abiotic mechanisms, but the responsible pathway could not be distinguished for cis-DCE. However, the conceptual model obtained by the integrated approach (Figure 11) provided the required knowledge on processes and mechanism(s) to document that a destructive process occurred.

The Rødekro site has been monitored over a period of 11 years (Badin et al., 2016; Hunkeler et al., 2011a; Murray et al., 2019-**II**) and is a good depiction of the continued development of the advanced microbial and isotopic techniques. Over the duration of the site investigations the new the VC reductase gene, *cerA*, has been discovered in *Dehalogenimonas* (Yang et al., 2017). Therefore, these microbial targets were included in the microbial analysis at latest monitoring campaign, to obtain a more comprehensive understanding on the biodegradation potential. Furthermore, the advancement of sequencing techniques allowed for a more complete understanding on selected relevant guilds in the microbial community by 16Sr RNA gene amplicon sequencing. Organohalide-respiring bacteria were relatively abundant throughout the plume, but the capability for complete dechlorination decreased with distance from the source Murray et al. (2019-**II**).
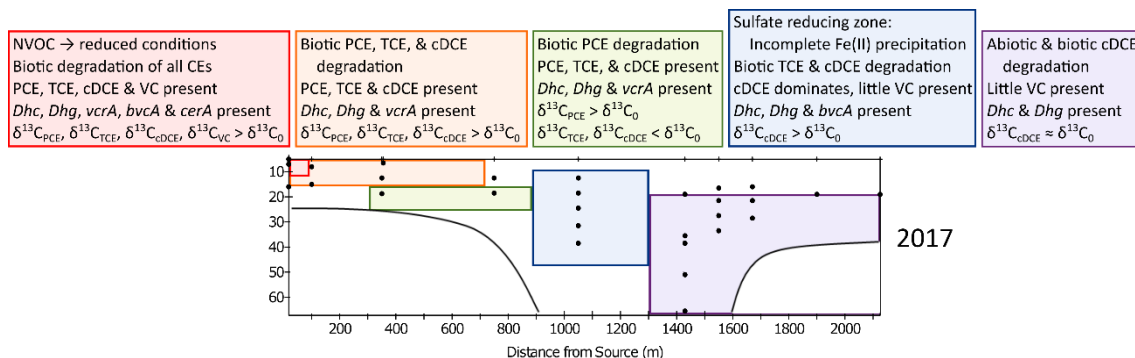
Figure 11. Conceptual model of conditions and degradation mechanisms in different compartments along the central flow line at the Rødekro site (CE =chlorinated ethene, Dhc=Dehalococcoides, Dhg=Dehalogenimonas) (modified from Murray et al., 2019-**II**).

Likewise, over the duration of the site investigations, studies that examine C-Cl dual isotope slopes for different pathways and conditions have increased. This allowed for a more detailed discussion of potential degradation pathways at latest monitoring campaign. Furthermore, it underlined that it is still a field under development, as the pathway could not be clearly distinguished with the obtained slopes for TCE and cis-DCE. In contrast, the integration of isotopic and microbial data in the conceptual site model provided the required information in order to document and quantify degradation Murray et al. (2019-**II**).

Besides providing the required evidence for evaluation of *in situ* degradation, the knowledge obtained from the integrated approach is also relevant on a broader perspective. A holistic framework depicting relevant processes and mechanisms in contaminated groundwater for different scenarios, e.g. landfill leachate plumes (Bjerg et al., 2011) and at groundwater-surface water interactions (Conant et al., 2019), enhance risk assessment and help decision making. This way, comprehensive research can enhance process understanding and the applicability of the assessment tools, which is relevant for more practical purposes, where the degree of detail is lower. In the last two decades, the development of the approach has made it an established methodology – especially with the progression in isotopic and microbial techniques – and the unanswered questions are continuously examined and retorted.

4 Conclusions

The aim of this PhD thesis was to advance and explore the use of techniques applied for *in situ* characterization and quantification of chlorinated ethenes degradation in contaminant plumes. The integrated approach, including microbial and isotopic techniques, provided valuable knowledge and learnings with regards to its applicability, advantages and constraints in diverse settings (A, B, C below).

A) A biostimulated large-scale plume with several degradation pathways:

Extensive sampling was used to track the lasting impact of a source remediation in the down-gradient large-scale plume. Microbial analysis provided knowledge on the potential for reductive dechlorination, and identified relative abundances of the entire microbial community, in different compartments of the plume. CSIA, coupled with flow and transport properties, facilitated quantification of degradation rates. C-Cl dual isotope data could to some extent aid identification of degradation mechanisms, but the deduction was limited by the availability of slopes in literature. The integrated approach identified several degradation pathways (biotic and abiotic) and demonstrated that the chlorinated ethenes underwent a destructive process, but was constrained in documenting the responsible mechanism(s).

B) A naturally transient plume at the groundwater-surface water interface:

Documentation and quantification of degradation in the near-stream system was achieved using an interdisciplinary, integrated approach. Microbial analysis indicated a great potential for anaerobic reductive dechlorination, CSIA documented the occurrence of degradation, and both techniques revealed spatial deviations. Knowledge on the residence time, integrated in a conceptual site model, allowed for assessment of whether the progress of degradation was sufficient to eliminate the risk. This study added valuable knowledge on chlorinated ethene fate at the groundwater-surface water interface, as it documented a behaviour not commonly observed, namely the evolution of limited to significant degradation - valuable in the holistic interpretation of near-stream degradation.

C) A plume bioremediated with activated carbon and bioamendments:

An integrated approach was necessary in order to assess biodegradation in the complex, non-stationary system. The development in microbial targets abundance characterised possible reasons for the stalled dechlorination, and

indicated critical zones. CSIA revealed zones with insufficient treatment of the contamination, i.e. where physical processes had a long term influence on the isotope trends. Isotope data, combined with flow and transport data, improved process understanding and the effect of the bioremediation. The integrated approach successfully documented the degradation and the controlling factors, however it was constrained in quantifying degradation for certain compounds.

To summarise, in all investigated scenarios the integrated approach provided the information needed to assess *in situ* degradation. However, some constraints in the applicability of the approach were also identified, either due to system complexity or unidentified features of the techniques. Implications for future research are therefore provided in the next section. This PhD study highlighted the value of applying isotopic and microbial techniques to characterise, document and quantify degradation at field scale, and underlined the importance of integrating these advanced tools in an overall conceptualisation of mechanism(s) and processes.

5 Research perspectives

Although the scientific research on methods to characterise and quantify *in situ* degradation are progressively developed and improved, there are still some challenges and ambiguities. The following five research perspectives have been identified:

1. Expanded application of the integrated approach in various environments and plume complexities.

The literature study on published degradation rates revealed that first-order degradation rates for chlorinated ethenes, as well as for relevant groundwater contaminants in general, are sparsely available and range substantially. There is a need for an expanded use of the integrated approach. This will improve knowledge on ways to characterise and quantify degradation at various field settings in site-specific evaluations. Furthermore, an increased number of field-based degradation rates will facilitate determination of realistic ranges to obtain accurate risk assessments. Scientific applications should focus on determining the advantages and limitations of the tools in even more settings and scenarios, and identify ways to overcome potential challenges.

2. Investigate the process interplay at sites with multiple and simultaneous degradation pathways.

At sites where several degradation mechanisms occur, the integrated approach can be used to document that the chlorinated ethenes undergo destruction, but the responsible mechanism(s) can be challenging to distinguish. Future research should focus on complex iron geochemistry systems, to enhance the understanding of abiotic and biotic interactions. Furthermore, research should expand the dual C-Cl isotope slope library, and investigate how different conditions/processes may influence the approach, e.g. simultaneously occurring degradation pathways.

3. Examine link between short-term and long-term developments in transient systems (e.g. near-stream system).

In systems with natural variations in processes, such as where groundwater and surface water interchange, the sampling interval is of high importance. Future research should examine the effect of seasonal changes and river stage on long-term developments in attenuation for sites that exhibit different behaviours. Furthermore, there is a need to increase the number of studies on chlorinated

ethenes fate at the groundwater-surface water interface, in order to test the applicability of the tools to characterise and quantify degradation in diverse settings and attenuation behaviours, to improve the holistic representation and identify knowledge gaps.

4. Improve tools to characterise and quantify biodegradation in non-stationary plumes after implementation of bioremediation barrier.

The alteration of a natural system, through injection of amendments across a plume, can result in highly transient processes and conditions, and are for that reason complicated to evaluate. Currently even state-of-the-art techniques can not quantify biodegradation in the aqueous phase for compounds present prior to the remedial intervention. Future research should focus on developing and testing supplementary tools towards this end. Microbial and isotopic analysis for the sorbed phase show great potential for further investigations to advance process understanding in these altered systems. Finally, techniques needs to be developed and tested to assess distribution of amendments to achieve a complete system conceptualisation.

5. Extrapolate knowledge acquired on techniques to characterise and quantify degradation of chlorinated ethenes to other compounds groups.

Documentation and quantification of *in situ* degradation of chlorinated ethenes is a well-developed scientific field. Studies in chlorinated ethenes degradation have discovered how advanced tools can be used to evaluate mechanism(s) and processes. Therefore, research on emerging groundwater contaminants could benefit from extending the obtained knowledge on assessment tools from chlorinated ethenes studies to investigations of these other compounds. Application of advanced tools to characterise and quantify degradation of emerging groundwater contaminants will require some developments. Isotope analysis have to be established for these compounds and possibly also analysis of other elements enclosed in them. Furthermore, development of accurate isotopic reference data for other elements may be needed. Lastly, it may require research in possible degradation pathways and the bacterial genera that execute them.

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