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MICROBIAL DEGRADATION OF CHLORINATED ETHENES IN HYPORHEIC ZONES: PROCESSES AND DEVELOPMENT OF MITIGATION STRATEGIES

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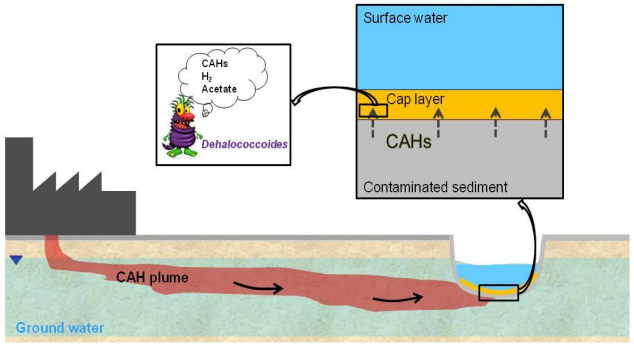

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Bioscience Engineering

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Nothing ever comes to one, that is worth having, except as a result of hard work.

- Booker T. Washington

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Met vriendelijk groeten,

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Summary

Chlorinated aliphatic compounds (CAHs) like tetrachloroethene (PCE), trichloroethene (TCE) and their daughter products *cis*-dichloroethene (cDCE) and vinyl chloride (VC) are major groundwater contaminants threatening water quality and human health. At many industrial sites, CAH contaminated groundwater plumes discharge into surface water bodies like rivers and lakes. Research and technology development aiming at mitigation of contamination of surface water by discharging CAH groundwater plumes is largely directed to source confinement and plume remediation in the aquifer compartment. However, riverbed sediments- known as the hyporheic zone- can support a broad spectrum of natural CAH attenuation processes such as sorption, dilution and biodegradation. Biodegradation is the only mechanism that results into destruction of CAH compounds in the environment and involves bacteria mediated metabolic processes like organohalide respiration (OHR) under anoxic conditions and aerobic degradation under oxic conditions. However, despite the critical role of biodegradation processes in determining contaminant fate and fluxes toward receiving ecosystems, and in estimating the need for additional remedial actions, information on CAH biodegradation processes in hyporheic zones is scarce. Particularly, the potential for a combination of aerobic/anaerobic CAH biodegradation in hyporheic sediments has received limited attention. Therefore, the general objective of this work was to study the underlying processes of as well anaerobic as aerobic microbial degradation of CAHs in hyporheic zones and the development of mitigation strategies to reduce or prevent CAH discharge into receiving surface water bodies. The study focused on the hyporheic zone of a particular industrial site of the Zenne River in Belgium where a VC/cDCE polluted groundwater plume discharges into the river. A previous study showed that the hyporheic zone at that site displays cDCE and VC OHR activity.

Since hyporheic surficial river-bed sediments are often characterized by sharp redox gradients between the oxic benthic sediment and underlying anoxic sediment, it can be hypothesized that it represents an ideal niche for both aerobic and anaerobic VC degraders. To test this hypothesis, in the first part of the study, the fate of VC and the dynamics of bacterial guilds involved in aerobic and anaerobic degradation of VC was studied in microcosms containing surficial sediments of the hyporheic zone of the study location under anoxic and oxygen-exposed static conditions. After degradation of 3 consecutive VC spikes,

quantitative PCR (qPCR) showed that *Dehalococcoides mccartyi* 16S rRNA and VC reductive dehalogenase-encoding genes (*vcrA*, *bvcA*) were enriched more than four orders of magnitude in anoxic microcosms, concomitant with stoichiometric conversion of VC to ethene. In oxygen-exposed microcosms, *etnC* and *etnE* involved in aerobic ethene/VC-oxidation, were enriched more than one order of magnitude with concomitant low or no accumulation of ethene. However, *D. mccartyi* 16S rRNA gene, *vcrA* and *bvcA* copy numbers were also enriched in oxygen-exposed microcosms containing sediment characterized by a high organic carbon content, a small grain size and limited oxygen penetration, whereas they were reduced more than two order of magnitude in oxygen-exposed sediment characterized by low organic carbon content, a larger grain size and extensive oxygen penetration. These results suggest the co-existence and co-activity of anaerobic and aerobic VC degraders in the same small volume of surficial sediment of the Zenne River, and that oxygen distribution, as determined by sediment grain size and organic matter content, affects the local VC degrading bacterial community and VC biodegradation pathway.

In the hyporheic zone, fluctuations in redox conditions are regularly occurring. Penetration of oxygenated surface water into the riverbed sediment in hyporheic zones and resulting redox fluctuations might affect site-specific aerobic and anaerobic microbial guilds and their contribution to degradation of contaminants such as cDCE and VC in discharging groundwater. To test this hypothesis, in the second part of the study, the resistance and resilience of aerobic and anaerobic VC/cDCE degraders to fluctuating redox conditions was studied in microcosms prepared from surficial Zenne sediment. The microcosms were incubated under anoxic static condition and each time when the degradation of 3 consecutive VC/cDCE spikes was completed, the redox and incubation conditions were changed with respect to oxygen exposure and/or shaking. The results show that oxygen exposure under static condition resulted in an 1.5-3.4 folds increased VC degradation rate as compared with anoxic static incubations and concomitant enrichment of the catabolic genes *etnC* and *etnE*. However, under oxygen-exposed shaking condition, real oxidative VC assimilation was only noticed in sediments with low organic carbon content leading to 5 folds higher degradation rate as compared with anoxic incubations. In these sediments, VC/cDCE respiration activity was impaired under oxygen-exposed shaking condition, resulting in a more than two orders of magnitude decay of *D. mccartyi*. The impacted

resilience of organohalide respiring bacteria (OHRB) led to irreversible hindrance of OHR under subsequent anoxic static conditions. Furthermore, oxidative VC assimilators did not show the ability to assimilate cDCE. On the other hand, in the sediments with high organic carbon content where OHRB were protected from oxygen under oxygen-exposed static condition, VC/cDCE removal appeared to be non-assimilative, since in these microcosms ethenotrophic assimilation of the ethene that was produced by VC/cDCE respiration, led to mischaracterized aerobic degradation. These results suggest the role of oxygen penetration and organic carbon distribution in hyporheic zones not only in instant pathway selection but also in long-term adaptation toward assimilative aerobic VC degradation.

In situ bioreactive capping is a promising technology for mitigation of surface water contamination by enhancing biodegradation of contaminants that discharge into the river from either polluted sediments or discharging groundwater. In bioreactive caps, contaminants are transformed into harmless products through microbially mediated reactions. Stimulation of CAHs degradation in bioreactive caps can be achieved through incorporation of solid polymeric organic materials (SPOMs) in the cap in order to provide a sustainable electron source for reductive dechlorination by OHRB. In the third part of the study, the possibility of application of natural SPOMs to stimulate CAH dechlorination in a bioreactive cap approach, was examined. Five different SPOMs, i.e., wood chips, hay, straw, tree bark and shrimp waste, were assessed for their long term applicability as an electron donor for reductive dechlorination of cDCE and VC in sediments of the Zenne River. The partitioning of reducing equivalents between OHR and methanogenesis as well as the dynamics of associated microbial guilds were studied with the aim of finding an electron source that preferentially stimulated OHR over methanogenesis. The initial fast release of fermentation products such as acetate, propionate and butyrate led to 171, 152, and 112 times higher methane production in the microcosms amended with shrimp waste, straw and hay as compared with natural attenuation while no considerable stimulation of VC/cDCE respiration was obtained in any of the SPOM stimulated microcosms. However, in the longer term, accumulation of short chain fatty acids decreased as well as methanogenesis while sustained dechlorination rates of both VC and cDCE were established with concomitant increase in the number of *D. mccartyi* and corresponding catabolic genes *vcrA* and *bvcA* both in the sediment and on the surface of the SPOM materials. The rapid and persistent

colonization of tree bark by *D. mccartyi* combined with 4-12 folds lower stimulation of methanogenesis compared with other stimulated conditions selected tree bark as a SPOM of interest for use in bioreactive caps for long term stimulation of dehalorespiration of CAHs.

In the fourth part of the study, the bacterial community composition was examined in the hyporheic sediment of the Zenne River before and after the installation of a wastewater treatment plant (WWTP) which was expected to decrease the organic load of the river. It was hypothesized that the OHRB that was fueled by the organic-rich untreated wastewater input of the river, might be impacted after the installation of the WWTP due to reduced organic carbon input. Bacterial 16S rRNA gene sequences amplified from DNA extracts of horizontal sediment layers, collected two years before (in 2005) and after WWTP construction (2010 and 2011) were obtained by pyrosequencing. Major differences in bacterial community composition were observed in the sediments of 2010 and 2011 compared with those of 2005 which could be associated with a reduction in organic content. *Proteobacteria* was the most dominant phylum in the sediments of 2005 followed by the *Chloroflexi* phylum that includes OHRB like *D. mccartyi.* The relative abundance of *Chloroflexi* declined dramatically from the average of 14.8% of the qualified bacterial reads in sediments of 2005 to 4.21 and 0.7 % in sediments of 2010 and 2011, respectively. The more oxic and oligotrophic sediments of 2011 were associated with a strong decrease of copiotrophic and anaerobic microbial groups and enrichment of oligotrophic members of *Alpha*- and *Betaproteobacteria.* Moreover, the results indicated lower species richness and diversity in the sediments of 2011. The results of qPCR indicated a more than two orders of magnitude decrease in the number of bacteria and *D. mccartyi* 16S rRNA gene in the sediments with depth and time concomitant with a substantial decrease of anaerobic VC respiration potential in sediments microcosms over time. Although the installation of the WWTP and subsequent reduced organic carbon load led to reduced bacterial biomass and diversity, it also decreasedOHR potential and natural attenuation capacity of the hyporheic sediments in the Zenne River.

Overall, the study shows the role of aerobic assimilative VC degradation in hyporheic sediments and that in addition to OHR, it is important to consider that activity to fully evaluate the importance of biodegradation in mitigation of CAH discharge into surface water. Moreover, it shows the potential of SPOM to be incorporated into *in situ* bioactive

caps as slowly degrading natural solid organic material to stimulate OHR for improved protection of rivers against discharging CAH contaminated groundwater. The results from the last part of the study accentuate the role of organic matter in OHR in hyporheic zones and the impact of river habilitation on that activity. The local implementation of *in situ* bioactive caps or the activity of aerobic CAH degraders can result in river protection from CAH contaminated discharging groundwater.

Samenvatting

Gechloreerde alifatische koolwaterstoffen (CAHs) zoals tetrachloorethyleen (PCE) en trichloorethyleen (TCE), en hun afbraakproducten *cis*-dichloorethyleen (cDCE) en vinyl chloride (VC) behoren tot de belangrijkste grondwatercontaminanten. Op vele industriële terreinen wordt de infiltratie waargenomen van CAH vervuild grondwater in oppervlaktewaters zoals rivieren en meren. Onderzoek en technologie ontwikkeling voor de bescherming van oppervlaktewater waarin CAH gepollueerd grondwater terecht komt, is vnl gericht naar bronremediatie en pluimbehandeling in het grondwatercompartiment. In sedimenten van rivierbeddingen- beter gekend als de hyporheïsche zone- komt echter een breed spectrum van natuurlijke CAH attenuatieprocessen voor zoals sorptie, verdunning en biodegradatie. Biodegradatie van CAHs is het enige mechanisme waarbij CAHs destructief uit het milieu worden verwijderd en kan plaatsgrijpen via bacteriële metabolische processen zoals organohalide respiratie (OHR) onder anoxische omstandigheden en aerobe degradatie onder oxische omstandigheden. Ondanks de kritische rol van biodegradatie in de bepaling van contaminantfluxen naar de ontvangende ecosystemen, en in de inschatting van de nood aan bijkomende remediatie acties, is informatie over de CAH biodegradatie processen in de hyporheïsche zone beperkt. Voornamelijk het potentieel voor de combinatie van aerobe/anaerobe afbraak van CAH in de hyporheïsche zone heeft tot nog toe slechts beperkte aandacht gekregen. De objectieven van dit doctoraat waren daarom om de onderliggende processen betrokken in zowel de anaerobe als aerobe microbiële afbraak van CAHs in de hyporheïsche zone te bestuderen. Bovendien werden remediatiestrategieën ontwikkeld om de influx van CAHs in de ontvangende oppervlaktewaters te verminderen of te voorkomen. De studie concentreerde zich op de hyporheïsche zone van een industriële locatie aan de Zenne rivier in België waar een VC/cDCE verontreinigde grondwaterpluim de rivier infiltreert. Een vorige studie toonde in de hyporheïsche zone van deze locatie de aanwezigheid aan van een cDCE/VC dehalorespirerende biodegradatie activiteit.

Aangezien riviersedimenten in de hyporheïsche zone vaak worden gekenmerkt door het bestaan van scherpe redox gradiënten tussen de oxisch bentische sedimenten en de onderliggende anoxische sedimenten, kan de hypothese gesteld worden dat de hyporheïsche zone een potentiële niche vormt voor zowel aerobe als anaerobe VC afbrekers. Deze hypothese werd getest door in microcosme-experimenten de afbraak van VC en de

dynamiek van anaerobe en aerobe VC afbrekers te onderzoeken in oppervlakteriviersedimenten afkomstig van de hyporheïsche zone van de studielocatie en dit onder anoxische en aan zuurstof blootgestelde statische omstandigheden. Na de afbraak van 3 consecutieve spikes van VC, toonde Quantitatieve PCR (qPCR) aan dat zowel het aantal *D. mccartyi* 16S rRNA genen als de aantallen VC reductieve dehalogenase genen (*vcrA*, *bvcA*) met meer dan vier ordes stegen in anoxische microcosmen en dat dit samenviel met de stoichiometrische omzetting van VC in etheen. In aan zuurstof blootgestelde microcosmen daarentegen werden de genen *etnC* en *etnE*, betrokken in aerobe etheen/VC-oxidatie, aangerijkt met meer dan één orde en met beperkte tot geen accumulatie van etheen. Het aantal *D. mccartyi* 16S rRNA genen en *vcrA* en *bvcA* genen stegen echter ook sterk in aerobe microcosmen van sediment dat gekenmerkt werd door een hoog organisch koolstofgehalte, een kleine korrel grootte en een beperkte infiltratie van zuurstof. Deze genen verminderden echter met meer dan twee ordes in aerobe sedimenten gekenmerkt door een laag organisch koolstofgehalte, een grotere korrel grootte en diepere infiltratie van zuurstof. Deze resultaten suggereren de co-existentie en co-activiteit van anaerobe en aerobe VC afbrekers in hetzelfde kleine volume van het Zenne sediment. Bovendien beïnvloedt de zuurstof distributie, bepaald door de korrel grootte en het organisch koolstofgehalte van de sedimenten, de lokale VC afbrekende bacteriële gemeenschap en VC biodegradatie afbraakweg.

Hyporheïsche zones worden gekenmerkt door reguliere fluctuaties in redoxomstandigheden. Infiltratie van zuustofrijk oppervlakte water in de rivier sedimenten van de hyporheïsche zone en de resulterende redox fluctuaties kunnen eventueel sitespecifieke aerobe en anaerobe microbiële organismen en hun biodegradatie van contaminanten zoals cDCE en VC beïnvloeden. Om deze hypothese te testen, werden de resistentie en heropleving van aerobe en anaerobe VC/cDCE afbrekers onder variërende redox condities bestudeerd in Zenne sedimentmicrocosmen. De microcosms werden geïncubeerd onder anoxische statische condities. Telkens als 3 opeenvolgende VC/cDCE spikes waren afgebroken, werden de redox en incubatie condities veranderd door zuurstof aan de microcosms toe te voegen en/of de microcosms al schuddend te incuberen. De resultaten tonen aan dat in vergelijking met de statische anoxische condities, blootstelling aan zuurstof onder statische condities resulteerde in een verhoging van de VC afbraak snelheid (factor 1,5-3,4) en een aanrijking

van de catabolische genen *etnC* en *etnE.* Alhoewel, bij blootstelling aan zuurstof onder schuddende condities, werd oxidatieve VC assimilatie enkel waargenomen in sedimenten met een laag organisch koolstofgehalte. Bovendien werd onder deze condities een 5 maal hogere afbraak snelheid vastgesteld dan onder anoxische incubaties. In deze sedimenten werd de VC/cDCE respiratie activiteit verlaagd door de blootstelling aan zuurstof onder schuddende condities wat samenging met een afname met meer dan twee ordes van het aantal *D. mccartyi*. Deze impact op de heropleving van de organohalide respirererende bacteriën (OHRB) resulteerde in een irreversibele hindering van OHR onder de daaropvolgende opgelegde anoxische statische condities. Oxidatieve VC assimilerende organismen bleken geen cDCE te degraderen. In sedimenten met een hoog organisch koolstofgehalte daarentegen, waar OHRB beschermd worden tegen zuurstof onder zuurstof rijke statische conditie, bleek de VC/cDCE verwijdering niet-assimilatief vermits in deze microcosms ethenotrofe assimilatie van de etheen die werd geproduceerd door VC/cDCE respiratie resulteerde in een misinterpretatie van aerobe afbraakl. Deze resultaten suggereren dat zuurstof infiltratie en organische koolstof verdeling in hyporheïsche zones niet enkel de selectie van de biodegradatieweg beïnvloedt maar ook een rol speelt in de lange termijn microbiële adaptatie wat betreft assimilatieve aerobe afbraak van VC.

In situ bioreactieve capping is een veelbelovende techniek om de contaminatie van oppervlaktewater door polluenten afkomstig van sedimenten of infiltrerend grondwater te voorkomen. In bioreactieve caps worden de contaminanten door microbiële processen omgezet in onschadelijke producten. Stimulatie van de CAH afbraak in bioreactieve caps gebeurt door de incorporatie van vaste polymerische organische materialen (SPOMs) die optreden als een duurzame electronenbron voor reductieve CAH dehalorespiratie door OHRB. In een derde deel van de studie werd het potentieel onderzocht van natuurlijke SPOMs om CAH dechlorinatie in een bioreactieve cap te stimuleren. Vijf verschillende SPOMs - hout chips, hooi, stro, boomschors en garnaalafval - werden onderzocht wat betreft hun langdurige toepassing als electronendonor voor reductieve dechlorinatie van cDCE en VC in microcosme-experimenten met sedimenten van de Zenne. De verdeling van reducerende equivalenten tussen OHR en methanogenese en de dynamiek van de microbiële groepen gekoppeld aan deze microbiële activiteiten werden bestudeerd. Het doel was om een electronenbron te vinden die eerder OHR stimuleert dan methanogenese. De

initiële snelle vrijstelling van fermentatie producten zoals acetaat, propionaat en butyraat leidde tov de natuurlijke attenuatie conditie tot een 171, 152, en 112 maal hogere methaanproductie en dit voornamelijk in microcosmen waaraan garnaalafval, stro of hooi werd toegevoegd. Bovendien werd er in deze eerste fase van het experiment in geen enkele van de microcosmen VC respiratie gestimuleerd. Op langere termijn verlaagde echter zowel de productie van korte keten vetzuren als de methanogeneseactiviteit terwijl een duurzameactiviteit van VC en cDCE dechlorinatie ontstond. Bovendien namen *D. mccartyi* en zijn catabolische genen *vcrA* en *bvcA* in aantal toe zowel in het sediment als op het oppervlakte van het SPOM materiaal. Gebaseerd op zo wel de snelle en persistente kolonisatie van boomschors door *D. mccartyi* als de 4 tot 12 maal lagere methanogenese activiteit in deze conditie tov de andere SPOMs, lijkt boomschors de te verkiezen SPOM voor lange termijn stimulatie van CAH dehalorespiratie in bioreactieve caps.

In het vierde deel van de studie werd de samenstelling van de bacteriële gemeenschap in de hyporheïsche sedimenten van de Zenne rivier onderzocht voor en na de installatie van een waterzuiveringsstation (WWTP) stroomopwaarts van de studielocatie. Door de installatie van het WWTP wordt verwacht dat de organische belasting van de rivier zou dalen. De hypothese werd bevraagd dat de bacteriële gemeenschap met inbegrip van de OHRB van het sediment dat oorspronkelijk gevoed werd door de organische koolstof rijke belasting van de rivier, beïnvloed zou worden wat betreft samenstelling en structuur na de installatie van de WWTP omwille van de lagere organische belasting. De sequenties van bacteriële 16S rRNA fragmenten, geamplificeerd uit horizontale sediment lagen, werden bekomen door pyrosequencing. Deze sedimentstalen werden genomen voor (in 2005) en na (2010 en 2011) de bouw van de WWTP. Er werden grote verschillen in de bacteriële gemeenschapssamenstelling waargenomen in de sedimenten uit 2010 en 2011 t.o.v. van deze uit 2005. Deze verschillen gingen samen met een daling in het organisch koolstofgehalte. *Proteobacteria* was de meest dominante phylum in de sedimenten van 2005 gevolgd door het *Chloroflexi* phylum waartoe OHRB zoals *D. mccartyi* behoren De relatieve hoeveelheid van *Chloroflexi* daalde zeer sterk van gemiddeld 14,8% van de gekwalificeerde bacteriële reads in de sedimenten van 2005 naar respectievelijk 4,21 en 0,7 % in de sedimenten van 2010 en 2011. De meer oxische en oligotrofe sedimenten van 2011 waren geassocieerd met een sterke daling in copiotrofe en anaerobe microbiële groepen en

een aanrijking van oligotrofe leden van de *Alpha-* en *Betaproteobacteria.* Bovendien toonden de resultaten een verlaagde species rijkdom en diversiteit aan in de sedimenten van 2011. De resultaten bekomen via qPCR toonden een daling met meer dan twee ordes in het aantal bacteria en *D. mccartyi* 16S rRNA gen aan in de sedimenten met de tijd. Dit ging samen met een sterke daling in het anaeroob VC respiratie potentieel van het sediment. Alhoewel de installatie van de WWTP en de daaropvolgende daling in organisch koolstof gehalte resulteerden in een daling in bacteriële biomassa en diversiteit, werd ook een daling in OHR potentieel en natuurlijke attenuatie capacitiet van de hyporheïsche sedimenten van de Zenne waargenomen.

Samenvattend toont deze studie de rol van aerobe assimilatieve VC biodegradatie in hyporheïsche sedimenten en het belang om deze activiteit naast OHR in te schatten om een volledig beeld te krijgen van de bijdrage van biodegradatie in mitigatie van CAH input in het oppervlaktewater. Verder toont de studie het potentieel van de incorporatie van SPOMs als traagafbrekend natuurlijk organisch materiaal in bioreactieve caps om OHR te stimuleren voor een betere bescherming van rivieren tegen infiltrerend CAH verontreinigd grondwater. De resultaten van het laatste deel van de studie tonen nogmaals de rol van organische materie in OHR in hyporheïsche zones aan en toont de invloed van rivierhabilitatie op die activiteit. De lokale applicatie van *in situ* bioactieve caps of de activiteit van aerobe CAH afbrekers kan resulteren in rivierprotectie tegen infiltrerend CAH verontreinigd grondwater.

List of abbreviations

1,1,1-TCA	1,1,1-trichloroethane
1,1-DCA	1,1-Dichloroethane
AkMO	Alkene monooxygenase
CAHs	Chlorinated aliphatic hydrocarbons
cDCE	*cis*-1, 2-dichloroethene
DGGE	Denaturing gradient gel electrophoresis
DOC	Dissolved organic carbon
DNAPL	Dense non-aqueous phase liquid
EaCoMT	Epoxyalkane-coenzyme M transferase
EPA	Environmental Protection Agency
FID	Flame ionization detector
GAC	Granular activated carbon
MMO	Methane monooxygenase
MNA	Monitored natural attenuation
NA	Natural attenuation
OHR	Organohalide respiration
OHRB	Organohalide respiring bacteria
OUT	Operational taxonomic unit
PCBs	Polychlorinated biphenyls
PCDD/Fs	Polychlorinated dibenzo-p-dioxins and dibenzofurans
PCE	Tetrachloroethene
Pd	Palladium
pMMO	Particulate methane monooxygenase
qPCR	Quantitative PCR
RCM	Reactive core mats
RDA	Redundancy analysis
RDase	Reductive dehalogenase
SCFA	Short chain fatty acids
sMMO	Soluble methane monooxygenase
SPOM	Solid polymeric organic material

TCE	Trichloroethene
tDCE	*trans*-1, 2-dichloroethene
TOC	Total organic carbon
T-RFLP	Terminal restriction fragment length polymorphism
VC	Vinyl chloride
VOCs	Volatile organic compounds
WWTP	Wastewater treatment plant
ZVI	Zerovalent iron

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Chapter 1: General introduction and objectives

As urban and industrial development continues to expand around the world's rivers and coastlines, so does the rate of unintentional release of chemical contaminants to subsurface and surface waters. Chlorinated compounds compose a main group of chemicals that have been widely used in various industrial applications resulting in decades long wide-scale contamination of groundwater, surface water and sediment contamination. Chlorinated aliphatic compounds (CAHs) such as tetrachloroethene (PCE) and trichloroethene (TCE) that are used as degreasing and dry-cleaning agents, are the most frequently detected chlorinated organic contaminants in shallow groundwater in industrially developed countries (Wiedemeier *et al*., 1997). Because of the mobility and persistence of CAHs, CAH-contaminated groundwater plumes discharge at many sites into surface water. Exposure to CAHs is of public concern since several of them are classified as potential human carcinogens and some, such as vinyl chloride (VC), are proven human carcinogens (Kielhorn *et al*., 2000).

As such, the development of sound and reliable technologies to protect surface water bodies from discharging groundwater contamination plumes is of interest (Council of the European Community, 2000). Research and technology development aiming at mitigation of contamination of surface water by discharging CAH groundwater plumes is largely directed to source confinement and plume remediation in the aquifer compartment. However, sediments in streams, rivers and lakes represent zones of interaction and transition between groundwater and surface waters, referred to as the hyporheic zone. Hyporheic zones are storage zones for organic carbon and are characterized by physical and chemical gradients enabling a broad spectrum of natural attenuation processes for pollutant removal such as sorption and biodegradation (Biksey & Gross, 2008; Hamonts *et al*., 2009). The use of these processes for protecting surface waters and potentially reduce the need for expensive remedial actions has as such gained increasing interest during the last 10 years. Of all naturally occurring attenuation processes in hyporheic zones, biodegradation is the only that implies destruction and true removal of CAHs from the environment. The identification and quantification of predominant processes of CAH biodegradation in hyporheic zones is therefore critical for determining contaminant fate and fluxes toward the receiving ecosystems, and in estimating the need for additional remedial actions.

This chapter intends to provide background information on intrinsic CAH attenuation processes such as sorption, dilution, volatilization, and biodegradation in hyporheic zones. Particular attention is devoted to the contribution of anaerobic and aerobic CAH degradation in CAH removal. Subsequently, sediment remediation technologies are reviewed with focus on sediment capping. The chapter finally reports on the objectives and outline of the thesis.

1.2 Chlorinated aliphatic hydrocarbons (CAHs)

CAHs are colourless, highly volatile liquids at room temperature, with limited solubility in water, low viscosity and low interfacial tension relative to water. Another unifying feature of CAHs is their high density relative to water, with densities ranging from 1.1 to 1.7 g/ml (Table 1.1) (Löffler *et al*., 2013). These characteristic physical-chemical properties govern chlorinated solvent behaviour after their release into the environment and profoundly impact their environmental distribution and longevity. The limited aqueous solubility of TCE and PCE leads to formation of dense non-aqueous phase liquid (DNAPL) at source zones. DNAPLs compose a long term source of groundwater contamination as slow dissolution of

the source zone can continue for many years under natural conditions resulting in large CAH contaminated groundwater plumes (Johnson & Pankow, 1992). These plumes, especially at industrial sites located near rivers and other freshwater bodies, often discharge in surface water bodies. Of all industrial land-derived contaminants, CAHs have the greatest potential to discharge into surface waters with upwelling groundwater (Ellis & Rivett, 2007).

Table 1.1. Chemical and physical properties of the CAH compounds PCE, TCE, *cis*-dichloroethene (cDCE) and VC.

	PCE	TCE	cDCE	VC
Formula	C_2Cl_4	C_2HCl_3	$C_2H_2Cl_2$	C_2H_3Cl
Water solubility at 25 °C (g/L)[a]	0.15	1.0	3.5	2.7
Boiling point (°C) [a]	121	87	60	-14
Melting point (°C) [a]	-19	-73	-80	-153
Molecular weight (g/mol)	165.8	131.4	96.9	62.5
Density at 20°C (g/ml)	1.62	1.47	1.26	0.91
Henry´s law constant (atm·m^3/mol)[a]	1.3×10^{-2}	1.0×10^{-2}	13.4×10^{-3}	5.6×10^{-2}
Log K_{OC} [a,b]	2.32-2.38	2	1.56-1.69	1.75
Log K_{OW} [a,c]	3.4	2.29	1.86	0.6
Groundwater cleanup standard (µg/L)[d]	40	70	50	5

[a] Data from USEPA
[b] Water-solid partitioning coefficient normalized to organic carbon content
[c] Octanol-water partition coefficient
[d] For Flanders (Vlaamse regering, 2008)

1.3 The importance of hyporheic zone in attenuation of CAHs

The term hyporheic originated from the Greek word "*hypo"* that means"under" or "beneath", and "*rheos"* meaning "a stream" (*rheo* means "to flow") (Smith, 2005). Hyporheic zones are sediments beneath and adjacent to stream and river channels, through which surface water exchanges and mixes with groundwater (Fig. 1.1, Brunke & Gonser, 1997). The exchange between groundwater and surface water basically proceeds in two ways: groundwater wells up into the streambed (discharge) or surface water infiltrates the streambed (downwelling or recharge), depending on the hydraulic head (Vervier *et al*., 1992). Due to differences in the physicochemical properties of groundwater and surface water such as differences in pH, temperature, concentrations of nutrients, mixing of both waters creates redox gradients in the hyporheic zone, supporting diverse biogeochemical processes (Brunke & Gonser, 1997). Specifically, the movement of surface water into the

subsurface provides a vector for dissolved constituents (oxygen, nutrients, and pollutants) to come into direct contact with entrained carbon sources, with microbial communities colonizing the surface of sediment grains, and with a range of biogeochemical conditions (e.g., both oxidative and highly reducing zones) (Hester & Gooseff, 2010). Hence, a variety of nutrients is cycled by different redox-driven biochemical reactions such as aerobic respiration, nitrification, denitrification, metal oxidation, metal reduction, sulphur oxidation, sulphate reduction, methanogenesis, methane oxidation, acetogenesis, and fermentation (Allan, 1995).

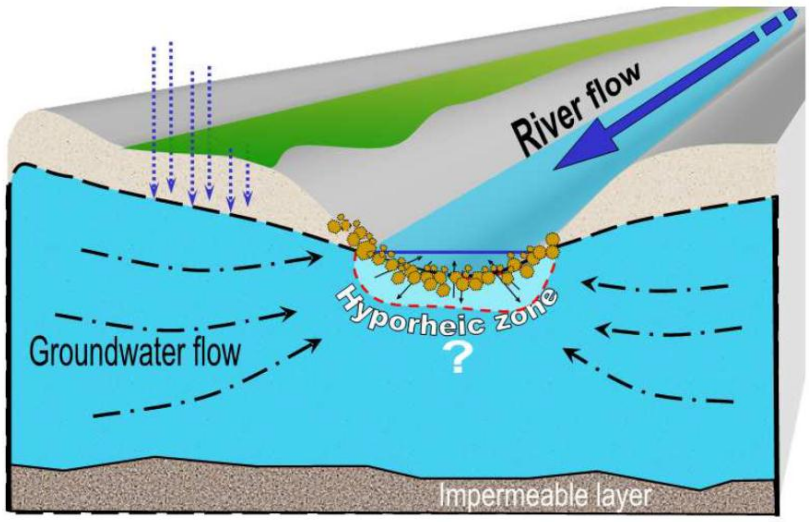

Fig. 1.1. Schematic illustration of an hyporheic zone. The exchange between groundwater and surface water in the hyporheic zone is shown by solid black arrows (from Chou, 2009).

Among the chemical species that are transferred between the water column and the sediment, organic carbon and oxygen are the main mediators of biological and geochemical processes in the sediment zone. Organic carbon is probably the main determinant of trophic complexity in hyporheic foodwebs and ecosystems where primary production by photosynthesis is absent (Gibert & Deharveng, 2002). Moreover, the organic matter in sediments is a principal factor that controls sorption of organic compounds (Cornelissen *et al*., 2005) making especially eutrophic sediments an important sink for hydrophobic organic contaminants such as polychlorinated biphenyl (PCBs) and polychlorinated dibenzo-p-dioxins

and dibenzofurans (PCDD/Fs) entering aquatic ecosystems. The supply of oxygen on the other hand, is essential for aerobic life within the sediment. Among the electron acceptors, oxygen is energetically the most favourable for the oxidation of organic compounds (Ziebis *et al*., 1996). Typically, the concentrations of both oxygen and organic carbon decline with depth in hyporheic zones (Fig. 1.2) and especially in sediments with high organic matter content, a sharp redox boundary exists between the aerobic benthic sediment and underlying anoxic sediment (Brunke & Gonser, 1997). The existence of such oxygen/redox gradients in hyporheic zones is of major interest for biological degradation of contaminants present in discharging groundwater such as CAHs. Indeed, depending on ambient redox conditions, CAHs can be degraded by as well aerobic as anaerobic processes, as outlined in section 1.4.3.

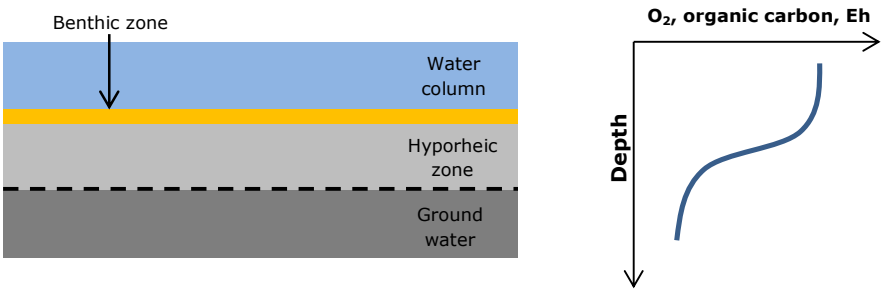

Fig. 1.2. Conceptual cross-section of a river system, made up of water column, benthic zone, and hyporheic zone (left panel). Associated typical gradients of, oxygen, concentration of organic carbon, and redox state variability are presented (right panel) (adapted from Hester & Gooseff, 2010).

1.4 Main attenuation processes

As mentioned above, hyporheic zones are characterized by high organic matter contents, steep chemical/redox gradients and the occurrence of a variety of key stream (bio)geochemical processes by which pollutants can be sorbed, diluted, transformed or destroyed. The main processes contributing to the attenuation of chlorinated solvents present in discharging groundwater in hyporheic zones are sorption, dilution and biodegradation.

1.4.1 Sorption

Once released in water, hydrophobic haloorganic pollutants will partition between the water phase and the solid sediment materials. The sorption of organic pollutants to sediment material is in the first place determined by the K_{oc} of the compound and the organic matter content of the sediment. More highly chlorinated compounds often display a relatively high K_{oc} and will bind more strongly to amorphous organic carbon and black carbon present in the sediment (Bedard, 2003; Armitage, 2008). Accordingly, VC would be much more mobile than PCE and TCE in hyporheic zone. The fate and behavior of chlorinated compounds in hyporheic zones can be highly affected by sorption. Sorption retards contaminant fluxes into the overlaying surface water and maintains a low concentration of readily available toxicants dissolved in the pore water. This creates more favorable conditions for microbial activity in case the chlorinated compounds are toxic for microbiota. On the other hand, sorption limits biodegradation of contaminants by decreasing their aqueous concentration and limiting their accessibility to enzymes and microorganisms (Cornelissen *et al*., 2005). The high organic carbon content of hyporheic sediments was reported to contribute to a higher degree of sorption of discharging CAHs such as TCE compared to the surrounding aquifer (Conant *et al*., 2000; Conant *et al*., 2004; Ellis & Rivett, 2007).

1.4.2 Dilution

The notion that “dilution is the solution to pollution” has led to contamination of many surface waters and rivers due to dumping of waste during the last two centuries. Dilution reduces the actual concentration of the contaminant, but this is at the expense of a larger volume of groundwater/surface water that contains the contaminant. Hence, although there might be decrease of the contaminant concentration in the receiving water bodies due to massive dilution, no substantial change will take place in the overall pollution load in the environment as the contaminant is transferred from one ecosystem to another. Therefore, dilution has been criticized as a means of natural attenuation of contaminants (NRC, 2000; Macdonald, 2000). Dilution of TCE in hyporheic zones has been attributed to surface water-mixing in downwelling zones (Chapman *et al*., 2007; Ellis *et al*., 2007; LaSage *et al*., 2008), or to mixing of the upwelling groundwater with surface water (Chapman *et al*., 2007; LaSage *et al*., 2008). Upon discharge into the surface water, massive dilution and volatilization of the

pollutants occurs, resulting in concentrations below or close to detection limits in most river water samples (Conant, 2004; Ellis & Rivett, 2007; Chapman *et al*., 2007).

1.4.3 Biodegradation

Biodegradation of chlorinated compounds occurs when suitable bacteria, carbon sources, terminal electron acceptors and nutrients are present and factors such as moisture, pH and temperature are conductive (Rivett *et al*., 2011). Depending on the degree of chlorination of the ethene molecule and on the prevailing site redox conditions, and the type of microorganism involved, biodegradation of CAHs can occur by reductive dechlorination or aerobic degradation. The tendency of chloroethenes to undergo reductive dechlorination decreases as the number of chlorine substituents decreases. PCE, with its four chlorine atoms, is a stronger oxidant than the naturally occurring electron accepting species found in groundwater systems, with the exception of oxygen (Vogel *et al*., 1987). The higher halogen substitution leads to electrophilicity of the molecule and its resistance to the electrophilic attack by oxygenases of aerobic bacteria. Conversely, chloroethenes more easily undergo oxidative degradation with decreasing numbers of chlorine substituents (Tiehm & Schmidt, 2011).

1.4.3.1 Reductive dechlorination

Chlorinated ethenes are relatively oxidized compounds due to the presence of electronegative substituents. Hence, steric hindrance and the highly oxidized nature of the PCE carbons (oxidation state of +2) and the TCE carbons (average oxidation state of +1) hamper the attack of oxygenolytic enzyme systems (Löffler *et al*., 2013). In anoxic environments, however, CAHs can undergo reductive dechlorination reactions which can be either metabolically and co-metabolically. In case of metabolic reductive dechlorination, the CAH compound functions as terminal electron acceptors for respiration by specialized reductive dehalogenase (RDase) enzymes (Smidt & de Vos, 2004). This process involves replacement of a chlorine substituent with a hydrogen atom in which 2 electrons are required for each dechlorination step as shown for the 4-step reductive dechlorination of PCE to ethene in Fig. 1.3. As such, dechlorination is accompanied by energy gain and growth (Smidt & de Vos, 2004). Metabolic reductive dechlorination has been named with different terms such as chlororespiration, dechlororespiration, halorespiration, dehalorespiration (Löffler *et al*., 2013). Recent discussions among experts in the field, however, have resulted

in a new term, organohalide respiration (OHR) (Löffler *et al*., 2013) which will also be used throughout this thesis. Co-metabolic reductive dechlorination of CAHs is due to the activity of free and enzyme-bound metal ion containing heat-stable tetrapyrroles and has been reported for iron- and sulphate-reducing bacteria, acetogens and methanogens (Gantzer & Wackett, 1991; El Fantroussi *et al*., 1998). These enzymes are involved in other reactions but fortuitously also transform CAHs, therefore, the process does not yield energy for growth. A special and rare mechanism of metabolic reductive dechlorination is vicinal reduction or dichloroelimination, which can occur when chloroaliphatic compounds have chlorine substituents located on two adjacent saturated carbon atoms (Ritalahti & Löffler, 2004).

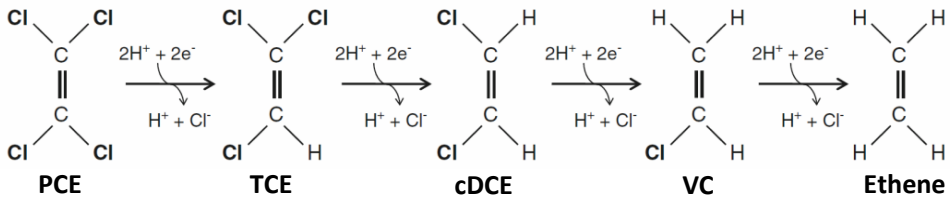

Fig. 1.3. OHR pathway leading to detoxification of PCE and TCE via cDCE and VC to ethene.

Several groups of organohalide respiring bacteria (OHRB) such as *Desulfomonile*, *Dehalobacter*, *Desulfitobacterium*, *Desulfuromonas* and *Geobacter* are able to degrade PCE and TCE to cDCE by OHR, while bacteria of the *Dehalococcoides mccartyi* group are capable of performing additional steps in the complete reductive dechlorination of PCE to ethene (Smidt & de Vos, 2004; Maphosa *et al*., 2010a). *D. mccartyi* strain 195 is the only reported bacterium that reduces PCE to ethene completely. However, the last dechlorination step from VC to ethene is slow and follows first-order kinetics, suggesting that it is a cometabolic process (Maymó-Gatell *et al*., 1997). *D. mccartyi* strain BAV1 (He *et al*., 2003) and VS (Müller *et al*., 2004) are notable for their ability to reduce VC to ethene for growth. *D. mccartyi* strain FL2 reduces TCE to VC for growth (He *et al*., 2005) and *D. mccartyi* strain GT can reduce TCE to ethene for growth (Sung *et al*., 2006).

OHR of CAHs is mediated by reductive dehalogenase (RDase) enzymes. Reported RDase genes involved in chloroethene reduction are *pceA* encoding PCE reductase in *D. mccartyi*

strain 195 (Magnuson *et al*., 1998) and *D. mccartyi* strain CBDB1 (Fung *et el*., 2007), *tceA* encoding TCE reductase in *D. mccartyi* strain 195 (Magnuson *et al*., 2000) and *D. mccartyi* strain FL2 (He *et al*., 2005), *vcrA* encoding the VC reductase in *D. mccartyi* strain VS (Müller *et al*., 2004) and *bvcA*, encoding the VC reductase in *D. mccartyi* strain BAV1 (Krajmalnik-Brown *et al*., 2004). Incomplete OHR of CAHs occurs when other alternative electron acceptors are present (Wiedemeier *et al*., 1997), suitable electron donor species (such as H_2) are absent (Aulenta *et al*., 2005) or when dechlorinating guilds are absent at contaminated sites (Löffler & Edwards, 2006). Incomplete dechlorination of PCE and TCE leads to the so-called cDCE (Chartrand, *et al*., 2005; Cheng *et al*., 2010) or VC "stall" (Bunge, *et al*., 2007). cDCE is toxic and especially VC is of major concern due to its high carcinogenicity (Bucher *et al*., 2005).

1.4.3.2 Aerobic oxidation

The potential persistence of less-chlorinated daughter products such as cDCE and VC during incomplete dechlorination of PCE/TCE, has long been a concern with the exclusive reliance on OHR. However, compounds like cDCE and VC have been shown to be biodegradable under aerobic conditions. Aerobic oxidation of CAHs can be either cometabolic or growth-coupled (Gossett, 2010). Co-metabolic aerobic degradation is a fortuitous conversion of non-growth substrates due to the action of dioxygenase and monooxygenase enzymes with no clear benefit for the responsible organisms and depends on the presence of substrates that support microbial growth and energy conservation and that induce expression of the appropriate catalytic enzymes (e.g., methane, toluene, ammonia) (Wackett, 1995). Oxidation of CAHs yields unstable chlorinated epoxides that subsequently break down spontaneously. Cometabolic degradation of CAHs has been well-described for methanothrophic bacteria through the action of methane monooxygenase enzymes (Alvarez-Cohen & Speitel, 2001; Forrester *et al*., 2005; Shukla *et al*., 2009) and for a diversity of hydrocarbon oxidising bacteria growing on phenol (Folsom *et al*., 1990), toluene (Chauhan *et al*., 1998), ethene and ethane (Freedman & Herz, 1996; Koziollek *et al*., 1999) using bacterial oxygenase systems. Cometabolism is the only aerobic mode of attack on highly-chlorinated ethenes (PCE, TCE), while the lesser-chlorinated ethenes can also be attacked by metabolic (growth-linked) processes. During metabolic degradation lesser-chlorinated CAHs act as primary substrate and carbon source with oxygen as electron acceptor (Mattes *et al*., 2010). Aerobic oxidation

of VC was first reported in groundwater samples taken from a shallow aquifer (Davis & Carpenter, 1990). Since then, there have been numerous reports on aerobic assimilative oxidation of VC and a wide variety of aerobic VC oxidising bacteria have been isolated belonging to genera such as *Mycobacterium* (Hartmans & de Bont 1992; Coleman *et al*., 2002b), *Pseudomonas* (Verce *at al*., 2000, 2001; Danko *et al*., 2004), *Nocardioides* (Coleman *et al*., 2002b) and *Ochrobactrum* (Danko *et al*., 2004). Aerobic VC-assimilating bacteria are closely related to ethenotrophs in which VC metabolism is associated with ethene metabolism (Mattes *et al*., 2010). The initial reactions in the oxidation of VC and ethene are initiated by an alkene monooxygenase (AkMO) (EtnABCD) encoded by *etnABCD* which yields epoxyethane from ethene and chlorooxirane from VC. These reactive, toxic, and mutagenic epoxides are further metabolized by an epoxyalkane-coenzyme M transferase (EaCoMT) enzyme (EtnE) encoded by *etnE* (Coleman & Spain, 2003; Mattes *et al*., 2005; Mattes *et al*., 2010). The remainder of the VC/ethene pathway is unclear. Fig. 1.4 shows a simplified scheme of the proposed pathways for VC and ethene metabolism. Conversion of VC to chlorooxirane was observed in *Mycobacterium aurum* L1, providing evidence that AkMO was the initial enzyme in the pathway (Hartmans & de Bont, 1992). *Pseudomonas strain* MF1 also converted VC to chlorooxirane and was inhibited by acetylene, which inactivates monooxygenase enzyme (Verce *et al*., 2000). The involvement of AkMO was also reported for oxidation of VC and ethene in *Mycobacterium* strain JS60 and *Nocardioides* sp. strain JS614 (Mattes *et al*., 2005). The EtnE is the second enzyme in aerobic VC and ethene assimilation and requires coenzyme M as a cofactor. Such requirement for coenzyme M has been only described in methanogenic *Archaea* (Jin & Mattes, 2010). EtnE is an important enzyme in the oxidation of C2- and C3-alkenes by alkene degrading bacteria. EaCoMT activity and *etnE* are found in both VC and ethene degrading Mycobacteria (Coleman & Spain, 2003), *Nocardioides* (Mattes *et al*., 2005) and *Pseudomonas* (Danko *et al*., 2006). Interestingly, adaptation of ethane oxidizing bacteria to grow on VC as the sole carbon and energy source in *Mycobacterium* strain JS623, has been associated with two particular missense mutations in the *etnE* gene that lead to higher EaCoMT activity (Jin & Mattes, 2010) suggesting that aerobic VC assimilation evolved from ethane assimilation. Adaptation of ethane assimilating bacteria to grow on VC was shown for various organisms like the Gram-positive

Mycobacterium strains JS622, JS623, JS624, and JS625 (Jin & Mattes, 2008) and the Gram-negative *Pseudomonas* strain DL1 (Vrece *et al*., 2001).

Up to now only one aerobic cDCE-assimilating bacterial isolate, i.e., *Polaromonas* strain JS666 has been reported (Coleman *et el*., 2002a). This isolate also transforms VC, *trans*-1, 2-dichloroethene (tDCE), TCE, and 1,2-DCA, but none of these organochlorines supports growth.

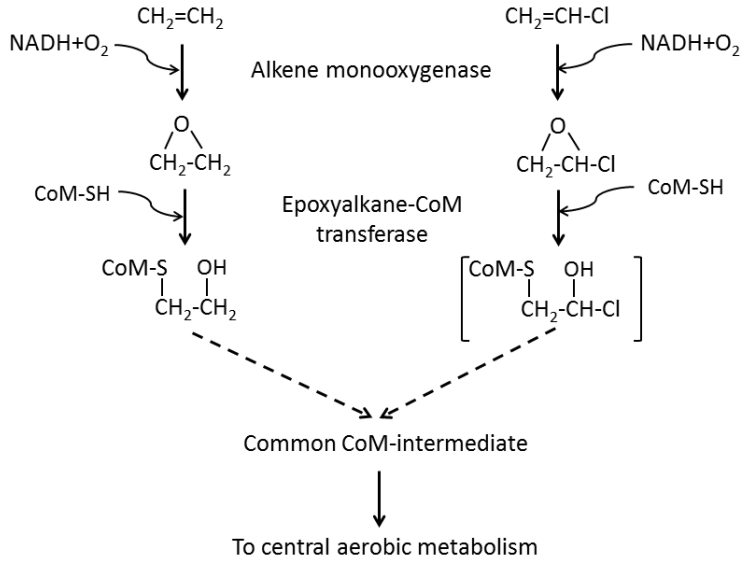

Fig. 1.4. Simplified scheme of proposed pathway for aerobic assimilation of ethene and VC. Solid arrows depict the biochemical reactions that are supported by experimental evidence. Dashed arrows indicate the proposed biochemical reactions, and the proposed metabolic intermediate is enclosed in parentheses (from Mattes *et al*., 2010).

In stream sediments, the existence of aerobic VC and cDCE oxidizers was first shown in microcosm studies by Bradley and Chapelle (1998, 2000). A microcosm study by Abe *et al*. (2009) demonstrated the potential of VC oxidation in streambed sediments consistent with the PCR detection of the *etnE* gene but no aerobic transformation of cDCE was observed over a period of 1.5 years. Coleman *et al*. (2002b) detected aerobic VC biodegradation in samples of groundwater, soil, sediment, and activated sludge showing that aerobic oxidation of VC is a common process at contaminated sites while aerobic cDCE oxidation is rare. The presence of an aerobic CAH degradation potential in oxic surficial sediments of hyporheic

zone is of great interest for the biological degradation of these contaminants when they are present in discharging groundwater. While the deeper anoxic sediments could be conducive to OHR of highly chlorinated parent compounds, the surficial oxic layer could be complementary by aerobic oxidation of the remaining less chlorinated daughter products of anaerobic transformation processes. Such a sequential anaerobic-aerobic degradation process can result in conversion of CAH to harmless products in the upwelling groundwater before it reaches the surface water. An identical interplay between aerobic oxidation and anaerobic reductive processes has been proposed in contaminated aquifers (Lee *et al*., 1998; Tiehm & Schmidt, 2011) but no report exists on the interplay between both processes in degradation of CAHs in the hyporheic zone.

1.5 Quantification of CAH degrading bacteria

During past years, significant efforts have been devoted to study the microbial ecology of CAH biodegradation for instance to analyze and assess the effectiveness of bioremediation efforts for CAH contaminated sites. Recent approaches have been shifting towards the application of culture-independent methodologies (Maphosa *et al*., 2010a). A wide array of methodologies including Quantitative real-time PCR (qPCR), fluorescent *in situ* hybridization, enzyme activity profiles, transcriptomics, proteomics and more recently high-throughput sequencing of (meta)genomic DNA or PCR amplified DNA, has been developed to provide a more holistic insight of microbial processes involved in organohalide degradation (Maphosa *et al*., 2012). qPCR that aims at providing information on cell numbers by estimating the numbers of specific marker genes, is currently the most routinely used molecular tool which continues to provide valuable information for CAH contaminated site management (Maphosa *et al*., 2010a). For instance, qPCR has been routinely used for quantification of dehalogenating bacterial guilds such as *D. mccartyi*, *Dehalobacter*, *Desulfitobacterium*, *Geobacter* and functional catabolic genes involved in reductive dechlorination i.e. *pceA*, *tceA*, *vcrA* and *bvcA* (Amos *et al*., 2008; Maphosa *et al*., 2010b; Hunkeler *et al*., 2011; Wei & Finneran, 2011; Hamonts *et al*., 2012). Moreover, qPCR has provided information on the growth characteristics of *D. mccartyi*, their dehalogenating abilities and determination of which halogenated chemicals can be dechlorinated by specific guilds/biomarkers (Cupples, 2008). Moreover, qPCR has been valuable for site management by demonstrating cause-and-effect relationship between number of CAH degrading bacteria and environmental

variations (Löffler & Edwards, 2006). *D. mccartyi* targeted qPCR has been used to compare bioaugmentation with biostimulation for chlorinated solvent remediation (Lendvay *et al*., 2003). Others have applied qPCR to compare the effectiveness of biostimulation at two different sites (Rahm *et al*., 2006). Lu *et al*. (2006) compared *D. mccartyi* cell densities from field sites with degradation rates to determine if there was a valid association between the two parameters. More recently, qPCR has provided a wealth of information for improving bioremediation efforts of groundwater plumes (Hunkeler *et al*., 2011), clayey till (Scheutz *et al*., 2010), and anoxic bioreactors (Maphosa *et al*., 2010b) by demonstrating a cause-and-effect relationship between dechlorination of chlorinated compounds and detection of associated biomarkers.

In spite of significant numbers of studies applying qPCR for documenting *in situ* groundwater bioremediation, our knowledge about the dynamics of CAH degraders in hyporheic sediments is mostly restricted to bench-scale laboratory studies (Himmelheber *et al*., 2007). Using a PCR method, Abe *et al*. (2009) detected *D. mccartyi* in riverbed sediment at locations with organic carbon content of > 2%. Hamonts *et al*. (2012) noticed dechlorination of cDCE/VC and 1,1-dichloroethane (1,1-DCA) in the riverbed and linked this with the presence of *D. mccartyi* and *Dehalobacter*, respectively. A qPCR assay was recently reported for ethenotrophs which is useful in conducting microbial ecology studies involving ethenotrophs (Jin & Mattes, 2010).

1.6 Contaminated sediment remediation techniques

Compared to the wide variety of techniques used for groundwater aquifer remediation, remedial options for sediment reclamation in surface water bodies are limited and include monitored natural attenuation, dredging, and *in situ* capping.

1.6.1 Monitored natural attenuation

Natural attenuation (NA) processes are in place naturally ongoing physical, chemical, or biological processes that reduce the toxicity, mass and/or mobility of a contaminant without human intervention (Declercq *et al*., 2012). Monitored natural attenuation (MNA) is a remedial technology during which the occurrence of NA is quantified and documented by converging lines of evidence to demonstrate that these processes are sufficiently active to protect human health and the environment (Röling & van Verseveld, 2002). MNA is as such a passive remediation approach which relies on pre-existing conditions. Application of MNA is

of especial interest for low-risk sites with low-level or diffuse contamination, where human health and ecological risks are not immediate or substantial (Magar & Wenning, 2006). Besides low costs and limited human exposure, MNA as a remediation technology has the lowest disruptive effect on the sediments. Next to dilution, MNA relies on physico-chemical processes such as sorption and precipitation which leads to reduced mobility and bioavailability of contaminants. Another important process is biodegradation which is the only NA process that truly removes organic pollutants from the environment. Indigenous microorganisms are the key players of NA and rely on site conditions for their activity. OHR has been reported to be the most important CAH attenuation process in hyporheic sediments (Abe *et al*., 2009; Chapman *et al*., 2007; Hamonts *et al*., 2009) followed by dilution by unpolluted groundwater discharge (Hamonts *et al*., 2009) or surface water mixing (Chapman *et al*., 2007; Hamonts *et al*., 2009). High spatial variability was reported in the degree of CAH biotransformation in hyporheic sediments which was related to organic matter content (Abe *et al*., 2009; Hamonts *et al*., 2012) redox conditions (Abe *et al*., 2009; Hamonts *et al*., 2012) and groundwater discharge rates (Abe *et al*., 2009).

1.6.2 Dredging

Dredging is an anthropogenic excavation activity to remove soft-bottom contaminated sediments from the river bottom and relocate them. This physical sediment mass removal is usually applied to sediments liable to erosion and variations in hydrologic conditions (NRC, 2007). Dredging is a method of choice for chlorinated compounds such as PCBs that bind strongly to organic particles. Heavily PBC-contaminated sediments are frequently dredged and disposed as a preferred remediation method (Gullbring & Hammar, 1993). Dredging was a traditional method of choice for sediment remediation projects of the Environmental Protection Agency (EPA) as it was believed that permanent removal of contaminated sediment is the most effective remediation approach (NRC, 2007; Reible *et al*., 2003). Although dredging can lead to immediate reduction of the contaminants from the target site, disposal of the dredged materials will shift the contamination to another location without reduction in pollution load (Perelo, 2010). Besides the inherent high costs of dredging (Nightingale & Simenstad, 2001), the dredging itself and the transportation and disposal of the dredging material can lead to various adverse impacts on the environment. The excavation process involves intensive disturbance of the local environment (Nightingale

& Simenstad, 2001) which can have important ecological impacts (Bridges *et al*., 2010). Intensive sediment dredging destroys the benthic habitat and impact benthic organisms Another drawback of dredging is the potential release of contaminants from bedded and suspended sediments (Tomaszewski *et al*., 2007). Further, the transport and disposal of contaminated dredged sediment present significant technical and political problems including storage and *ex situ* treatment issues (Nightingale & Simenstad, 2001).

1.6.3 Capping

In situ capping manages contaminated sediment on-site and hence does not need to remove, transport, and dispose contaminated material offering economic, logistical, and ecological advantages compared to dredging (Reible *et el*., 2006). *In situ* capping is a promising technology for reducing exposure of the benthic community to contaminants present in or leaking from the sediments and is achieved through physical isolation of the contaminated sediment from the water column by clean and neutral material media such as sand, gravel and clean sediment (Palermo *et al*., 1998). Passive sand caps provide excellent protection when contaminants are strongly adsorbed to the solid phase and in the absence of rapid contaminant flux processes (Reible *et al*., 2006). Nevertheless, due to the inactive or passive nature of such caps, contaminants are not removed nor destroyed (McDonough *et al*., 2007). Moreover, in case of groundwater seepage, passive sand caps are unable to isolate contaminated sediment from the surrounding environment or decrease contaminant fluxes. To improve contaminant retardation, sorbent materials such as activated carbon, coke by-products, apatite (a principal phosphate mineral in phosphate rocks), and organoclays (consist of bentonite that is modified with quaternary amines) can be added within the cap layer. These types of caps are often termed active caps to differentiate from passive sand layers and aim to reduce contaminant flux to the overlying water by contaminant sequestration offering greater flexibility in contaminated sediment management (Murphy *et al*., 2006; Reible *et al*., 2006; Barth *et al*., 2008; Knox *et al*., 2008; Knox *et al*., 2010). Active caps are generally considered for hot spots containing mobile contaminants in upwelling areas (Barth *et al*., 2008). Organoclays are particularly effective at removing non-polar pollutants such as oil, polychlorinated biphenyls (PCBs), chlorinated solvents, and polycyclic aromatic hydrocarbons (Alther, 2002; Xu *et al*., 1997).

Although effective in sequestration, physico-chemically based active caps are unable of detoxification of organic chlorinated compounds since the compounds are not degraded or transformed. To couple adsorption to degradation, application of granular activated carbon (GAC) impregnated with zerovalent iron/palladium (ZVI/Pd) bimetallic nanoparticles was introduced by Choi *et al*. (2008, 2009a&b). In the hybrid GAC/ZVI/Pd system, the physical adsorption capacity of GAC was combined with the dechlorination destructive reactivity of ZVI/Pd to produce a reactive AC (RAC). The GAC attracts hydrophobic organohalides such as PCBs while ZVI acts as an electron donor to dechlorinate the organohalide through hydrogenation with Pd promoting the reactivity by acting as a noble metal catalyst (Smuleac *et al*., 2010; Choi *et al*., 2009a). Although the use of bimetallic nanoparticles in active caps for remediation of chlorinated compound has received significant attention, it has shortcomings. Those include the high cost of RAC material synthesis and the risk of leaching of Fe and Pd that can cause human health effects and negatively impact the ecosystem (El-Temsah & Joner, 2012; Sanchez *et al*., 2012; Miller *et al*., 2012).

Most recent developments in the field of sediment capping are the *in situ* bioreactive caps in which the capping layer hosts and stimulates microbiota that can degrade pollutants including CAH dehalogenating organisms (Himmelheber *et al*., 2011). For instance, solid polymeric materials can be used in bioreactive caps to provide a long term continuous supply of electron donors for dehalorespiration. Such a layer will form a highly active biotransformation layer in which the electron donor and CAHs are coupled with the dehalogenating microorganisms. As demonstrated in Fig. 1.5, application of a bioreactive cap hosting a layer of solid polymeric organic materials (SPOMs) can satisfy the need for electron donor as documented by Himmelheber *et al.* (2011). These authors applied a bench-scale sand based bioreactive cap seeded with an organohalide respiring consortium for biodegradation of PCE. Accordingly, a *Dehalococcoides*-seeded SPOM-layer is an interesting recipe for bioremediation of the sediments that are not conductive to OHR due the lack of OHRB or electron donors. Such a layer can be incorporated between thin geo-textile membranes (McDonough *et al*., 2007) and installed horizontally in the sediment-surface water interface, covered by a thin sand layer.

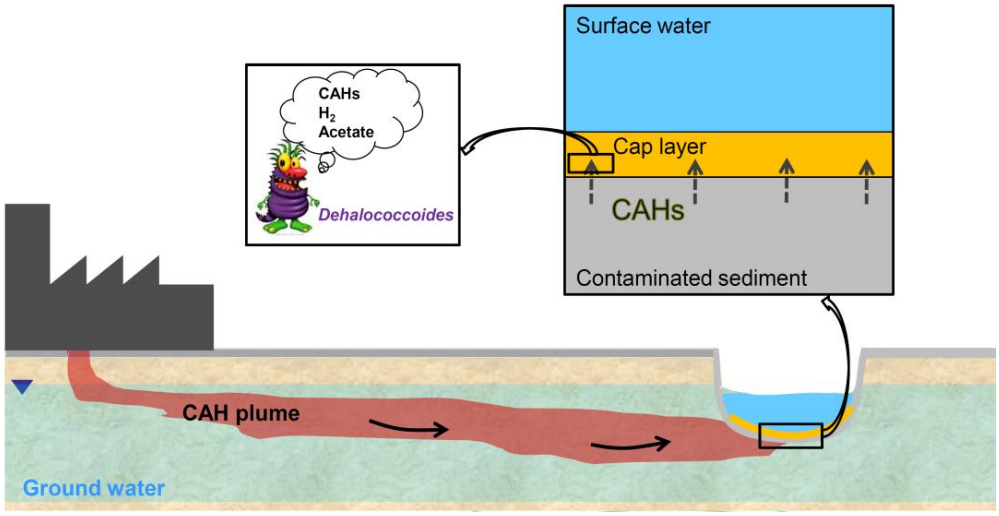

Fig. 1.5. Schematic presentation of the bioreactive cap concept.

1.7 Objectives and outline of the thesis

Past research on contamination with CAHs that discharges into surface water bodies has been mainly directed at source confinement/remediation in groundwater plumes. Hyporheic zones can act as a biological filter reducing or even preventing the transfer of CAHs from groundwater to surface water. However, most of the research performed on microbial biotransformation of CAH in groundwater-surface water interface has exclusively focused on OHR while knowledge on occurrence of aerobic CAH biodegradation in hyporheic zone and its contribution to CAH attenuation is largely unknown. Indeed, in previous studies, highly spatially variable OHR was documented in a short stretch of the Zenne River (Belgium) impacted by discharging CAH-polluted groundwater (Hamonts *et al*., 2009, 2012; Kuhn *et al*., 2009). At some locations in that short stretch of riverbed, it was suggested that CAHs were still leaking into the river since indications for the occurrence of OHR were limited. However, the presence of aerobic degradation potential/capacity in the riverbed sediments of the Zenne River was not studied. Since 2007 a municipal waste water treatment system (WWTP) treats the municipal wastewater that was directly discharging in the Zenne River in the past. This affected the organic load of the river and the sediment that was considered to be of major importance for thriving OHR processes. On the other hand, the decreased organic

carbon load in the surface water led to increased oxygen concentration in the surface water. We hypothesized that infiltration of the oxic surface water into the hyporheic sediments could stimulate aerobic CAH degradation, especially in the top surficial sediment layers at sediment-surface water interface. This hypothesis was further supported by previous $\delta^{18}O$ and $\delta^{2}H$ isotopic measurements conducted between 2004 and 2007 that indicated surface water penetration into the river sediments till 20 cm depth (Hamonts *et al*., 2009). Hence, the aerobic CAH biodegradation at the interface between surface water and sediment might be an important accessory process in removing CAH in discharging groundwater which was otherwise supposed to leak into the Zenne ecosystem. Therefore, the **main objective** of this thesis was to study the underlying processes of as well anaerobic as aerobic microbial degradation of chlorinated ethenes in hyporheic zones and the development of mitigation strategies to reduce or prevent CAH discharge into receiving surface water bodies. The study is based on the results reported by Hamonts *et al*. (2009, 2012) and continues to use sediment materials from the same location in the Zenne River. The **specific objectives** of this work were:

(i) To understand the occurrence, relative contribution, and environmental relevance of OHR versus aerobic degradation of cDCE and VC in hyporheic sediments.

(ii) Assess the possibility of application of SPOMs to stimulate cDCE and VC OHR and assess their potentials to be incorporated in a bioreactive cap system.

(iii) To examine the response of the microbial community dynamics and in particular OHRB to the long-term organic carbon decrease in the hyporheic zone of the Belgian Zenne River due to installation of a WWTP.

The first part of the PhD deals with OHR versus aerobic degradation of cDCE and VC and is handled in Chapters 2 and 3. In Chapter 2, the hypothesis is being examined that both aerobic and anaerobic VC degraders coexist in upper sediment layers of hyporheic zones of Zenne River and that the physicochemical properties of riverbed sediments, in particular those that determine oxygen distribution, represent pivotal factors in determining local VC biodegradation pathways. To this end, we studied the fate of VC in aerobic and anaerobic microcosms containing surficial sediment of the Zenne River in **Chapter 2**. The study showed

that indeed anaerobic VC respiring microbial guilds and aerobic VC degraders co-exist and could be co-active in the surficial riverbed sediment of Zenne hyporheic zone. **Chapter 3** describes the effect of periodic fluctuations in redox conditions as expected to occur in hyporheic zones on biodegradation of cDCE and VC. The results not only improved our knowledge on the effect of changing redox conditions on the resistance and resilience of chloroethene degrading organisms in sediments, but also suggested the possible role of oxygen penetration and organic carbon distribution in hyporheic zones in evolution of ethenotrophs to VC degradation. **In Chapter 4**, the ability of five SPOMs including wood chips, hay, straw, tree bark and shrimp waste to serve as electron donors for OHR of cDCE and VC was evaluated in sediment microcosms and the partitioning of reducing equivalents to OHR versus competing methanogenesis was studied. In **Chapter 5**, we investigated the effect of the long-term decrease of organic carbon inputs in the Zenne River as a consequence of the activity of a recently installed WWTP on the composition of the bacterial community in the hyporheic zone of the river. To this end, we used deep pyrosequencing of 16S rRNA gene fragments to determine the dynamics of the bacterial community in horizontal layers of sediment cores, collected before at after installation of the WWTP and the hypothesis was studied that decreases in organic carbon load results in a decrease in OHRB involved in CAH respiration. **Chapter 6** provides a general discussion on the key findings of Chapters 2 to 5 and proposes research perspectives.

Chapter 2: Small-scale oxygen distribution determines the vinyl chloride biodegradation pathway in surficial sediments of river-bed hyporheic zones

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2.1 Introduction

At many industrial sites exhibiting groundwater contamination with chlorinated aliphatic hydrocarbons (CAHs), polluted groundwater discharges into nearby surface water bodies through hyporheic zone sediments where groundwater and surface water interact. Hyporheic zones are storage zones for organic carbon and are characterized by physical and chemical gradients enabling a broad spectrum of microbial processes (Biksey & Gross, 2008). Oxygen in surface water that infiltrates into the river-beds plays an important role in determining redox zones and microbial processes (Gandy *et al*., 2007). The depth of dissolved oxygen penetration into the sediment is a function of the exchange rates of groundwater and surface water and the oxygen consumption rate by chemolithotrophic and chemoheterotrophic microorganisms, which in turn will depend on organic matter content of the sediment (Triska *et al*., 1993). Often, especially in sediments with high organic matter

content, a sharp redox boundary exists between the oxic benthic sediment and underlying anoxic sediment (Brunke & Gonser, 1997). The existence of such oxygen/redox gradients in hyporheic zones is of major interest for biological degradation of contaminants present in discharging groundwater and in particular for biodegradation of CAHs. Anoxic sediments in deeper layers provide ideal conditions for reductive CAH dechlorination by organohalide respiring bacteria (OHRB) while more oxic sediment layers at the surface water-sediment interface, can be conducive to aerobic degradation of less-chlorinated daughter products (see Fig. 2.1). As such, a scenario of sequential anaerobic-aerobic degradation can be anticipated that results in conversion of CAH to harmless products before the groundwater reaches the surface water.

Of all less-chlorinated CAH daughter products, vinyl chloride (VC) is of major interest due to its high carcinogenicity (Bucher *et al*., 2005). Groundwater VC contamination occurs via anaerobic reductive dechlorination of tetrachloroethene (PCE), trichloroethene (TCE), 1,1,1-trichloroethane and the dehydrohalogenation of 1,2-dichloroethane (Vogel *et al*., 1987). VC is much less oxidized and its reductive dechlorination to the harmless ethene is often inefficient under natural attenuation conditions (Bradley, 2003). In contrast, complete dechlorination of VC via aerobic biodegradation is highly efficient and occurs via either co-metabolic or metabolic processes. Co-metabolic aerobic degradation is a fortuitous conversion with no clear benefit to the responsible organisms and requires a primary substrate to support microbial growth and energy conservation (Alvarez-Cohen & Speitel, 2001). The well-described co-metabolic CAH degradation by methanotrophs (Alvarez-Cohen & Speitel, 2001; Forrester *et al*., 2005; Shukla *et al.,* 2009) is of interest in hyporheic zones since it can be hypothesized that their activity in upper oxic sediment layers is sustained by methane produced by indigenous methanogens in lower anoxic layers (Fig. 2.1). In methanotrophs, methane monooxygenase (MMO), the first enzyme in the pathway of methane oxidation, is responsible for CAH degradation (Shukla *et al.,* 2009). Two different forms of MMO exist i.e., a membrane-bound particulate MMO (pMMO), which is present in almost all methanotrophs isolated so far, and the soluble form (sMMO), which is present in only some methanotrophs (Kolb *et al*., 2003). Both forms are active against CAHs (Lee *et al*., 2006) and both forms need copper for their activity, however, at copper-to biomass ratios

greater than 0.89 µmol copper·(g cell dry weight)$^{-1}$, only pMMO is expressed (Hanson & Hanson, 1996).

Aerobic VC-assimilating bacteria gain energy and carbon from the degradation of VC. Various aerobic VC assimilating bacteria belonging to a wide variety of genera have been isolated. They are ethenotrophs in which VC metabolism is associated with ethene metabolism (Mattes *et al*., 2010). The initial reactions in the oxidation of VC and ethene are initiated by an alkene monooxygenase (EtnABCD) and an epoxyalkane-coenzyme M transferase enzyme (EtnE) (Coleman & Spain, 2003; Mattes *et al*., 2005; Mattes *et al*., 2010).

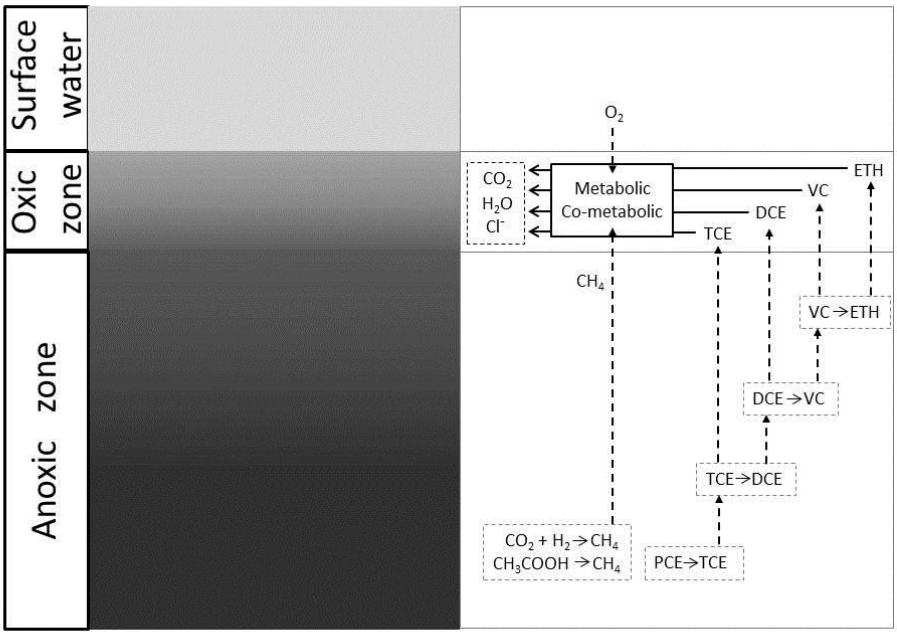


Fig. 2.1. Schematic presentation of hypothetical sequential reductive/oxidative biodegradation in hyporheic zones of riverbed sediment: in deeper anoxic sediment layers, higher chlorinated aliphatic hydrocarbons are reductively degraded to less chlorinated compounds and methane is produced by methanogens. Depending on the degree of dechlorination, different daughter products and methane will reach the oxic surficial sediment layer, where oxygen is present due to penetration from surface water. In the oxic layer, aerobic co-metabolic (mediated by methanotrophs) or metabolic degradation (mediated by aerobic degraders) represent the predominating degradation pathways.

Although the presence of anaerobic VC dechlorinating as well of aerobic metabolic/co-metabolic VC/ethene degrading bacteria has been shown in surficial river sediments, there are, to the best of our knowledge, no studies of the co-existence and activity of both

functional groups of VC degraders in the same sediment and their relative contribution to VC degradation. We hypothesized that both types of degraders co-exist in upper sediment layers of hyporheic zones of rivers that receive VC-contaminated groundwater and that the physico-chemical properties of river-bed sediments, in particular those that determine oxygen distribution, represent pivotal factors in determining local VC biodegradation pathways. To this end, the current study uses a microcosm set-up to examine VC biodegradation in a surficial river sediment that originated from a short stretch of the Zenne River (Belgium) impacted by discharging VC-polluted groundwater. Organohalide respiration (OHR) appeared to be the most important attenuation process of VC between 120 cm and 20 cm depth in those sediments followed by dilution by unpolluted groundwater discharge and surface water mixing (Hamonts *et al*., 2009; Kuhn *et al*., 2009; Hamonts *et al*., 2012). The occurrence of aerobic degradation of residual VC in the upper layers of the sediment was not examined but $\delta^{18}O$ and $\delta^{2}H$ isotopic measurements of the Zenne surface water and discharging polluted ground water suggest that surface water penetrates into the river sediments till 20 cm depth (Hamonts *et al*., 2009). The sediment samples were collected from two different locations containing sediments that differed in physico-chemistry, particularly in organic carbon content and grain size. Sediments were incubated in microcosms in the presence of VC under oxygen-exposed and anoxic non-shaking conditions. Assessment of the biodegradation pathways in the microcosms was performed by monitoring VC removal and accumulation of metabolites as well as determining changes in abundances of biomarkers associated with known microbial guilds and functional catabolic genes involved in OHR and aerobic VC degradation.

2.2 Materials and Methods

2.2.1 Sediment sample collection

Sediment samples were taken from the Zenne River in Machelen-Vilvoorde, Belgium at a previously described site (Hamonts *et al*., 2009). At that site, a VC/*cis*-dichloroethene (cDCE) contaminated groundwater plume of approximately 1.4 km width flows in the northwestern direction into the Zenne River (Fig. 2.2). Sediment samples were collected from the top 20 cm layer of the river-bed sediment at locations post 26 (P26) and post 25 (P25) as indicated in Fig. 2.2, using a 4 cm diameter piston sediment sampler, and transferred immediately into glass jars. Total organic carbon content (TOC) of homogenized sediment samples was

calculated as the fraction of dry matter that was removed at 550°C, after drying the sediment overnight at 105°C. Surface water was collected at P26. At the time of sampling (May 2010) the dissolved oxygen concentration in the surface water was 3.76 mg/L.

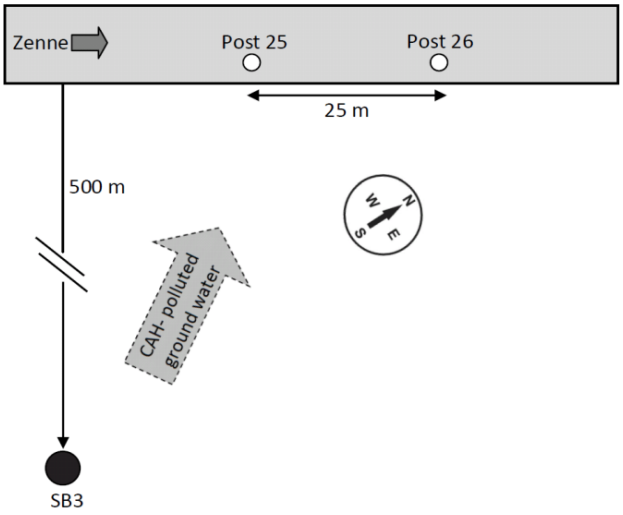


Fig. 2.2. Schematic overview of the test site and locations of post P26, and P25 and the monitoring well SB3.

2.2.2 Sediment microcosm set-up

Sediment microcosms were prepared in 160-mL glass serum bottles containing 37 g (wet) sediment from P26 or P25 and 70 mL of surface water and sealed with Teflon-lined butyl rubber stoppers. All the preparations were performed in an anaerobic glove box (Don Whitley Scientific ltd, West Yorkshire, UK) and under anoxic conditions. Anoxic microcosms (designated as An) were flushed 3 times (each time for 15 min) with oxygen free nitrogen gas to remove most of the residual oxygen. In the oxygen-exposed microcosms (designated as O), sterile oxygen gas (ultra-pure, Air Products, Belgium; filter sterilized over a 0.2 µm filter) was amended via a syringe and a 27-gauge needle at an initial amount of 7% (vol/vol) of the headspace after the withdrawal of an equal volume of headspace. To stimulate the growth of methanotrophs as co-metabolic aerobic VC degraders, in another series of microcosms (designated as OM), 21% (vol/vol) of the headspace was replaced with a methane-oxygen gas mixture at a 2:1 ratio. As a control for abiotic losses, for each treatment, microcosms were included that were inactivated with formaldehyde added at a

final concentration of 1% (v/v). For all treatments, duplicate microcosms were prepared. The bottles were spiked with VC (70 µM in water phase) and incubated at room temperature without shaking. In the course of the experiment, the concentration of oxygen in the liquid phase of O and OM microcosms were retained in the range of 2-3 mg/L by periodic addition of sterile oxygen gas when the concentration of oxygen in the headspace dropped below 6 mg/L. After the first addition of VC was degraded, except for the abiotic control, all microcosms were flushed with nitrogen. In O microcosms, 7% (vol/vol) of the headspace was replaced with oxygen and in OM microcosms, 21% (vol/vol) of the headspace was replaced with a methane-oxygen gas mixture at a 2:1 ratio, followed by addition of VC to all VC-degrading microcosms. The same procedure was followed for the third VC addition.

2.2.3 DNA extraction and Quantitative PCR

After degradation of the third VC spike, the sediment microcosms were thoroughly mixed and ~2 g samples were withdrawn from all duplicate microcosms. DNA was extracted from the samples as described previously (Hendrickx *et al*., 2005). Real-time PCR quantification (qPCR) was performed in an iQ5 iCycler (BioRad, Veenendaal, The Netherlands) using the iQ SYBR Green Supermix kit (BioRad). qPCR measurements were performed in triplicate in 25 µl reactions and no-template controls were included. The complete list of primers used in this study is provided in Table 2.1. Primers were used at a final concentration of 200 nM. Conditions of qPCR for determining *Dehalococcoides mccartyi* 16S rRNA gene copy numbers were 10 min at 95°C, followed by 40 amplification cycles of 15 sec at 95°C, 30 sec at 50°C, and 30 sec at 72°C. Melt curve analysis was performed from 50°C-95°C in steps of 0.5°C and 10 sec at each step. qPCR assays targeting the 16S rRNA gene of bacteria, *Dehalobacter,* and *Polaromonas* were performed using similar protocols as described above except that an annealing temperature of 60°C was applied and that melt curve analysis was done from 60°C-95°C. The qPCR assay targeting reductive dehalogenase genes (*rdh*) was done as follows: 10 min at 95°C, followed by 40 amplification cycles of 15 sec at 95°C, and 1 min at 58°C (for *bvcA* and *vcrA*) or 60°C (for *tceA*). In all cases, T7-SP6 PCR-products amplified from pGEMT-Easy clones carrying the targeted genes, were used as qPCR standards.

The *mcrA* gene was quantified as described by Wilms *et al*. (2007) using genomic DNA of *Methanosarcina mazei* (ATCC BAA-159; GenBank accession number NC_003901), as calibration standard. Methanotrophs were quantified based on the *pmoA* gene which

encodes the hydroxylase component of pMMO, as described by Kolb *et al*. (2003). The *etnC* and *etnE* genes were quantified according to Jin and Mattes (2010) using cloned fragments as calibration standards. Primers for *Mycobacterium* and *Nocardiodes* were designed using Primer 3 software (Rozen & Skaletsky, 2000). Primers have a bias towards cDCE and VC degraders (Jin & Mattes, 2010) but do not completely discriminate against non-degrading strains. Cloned 16S rRNA genes of *Mycobacterium* sp. JS60 and *Nocardiodes* sp. JS614 were used as standards, respectively. Respective gene copy numbers were calculated as copies/g for all analyses. The fraction of *D. mccartyi* to total bacteria was estimated by dividing the number of *D. mccartyi* 16S rRNA gene copies by the copy number of bacterial 16S rRNA genes multiplied by 100.

Table 2.1. Overview of DNA oligonucleotide primers used in this study.

Primer	Sequence	Target 16S rRNA / gene	Reference
341F	5′-CCTACGGGAGGCAGCAG-3′	All Bacteria	Muyzer *et al*., 1993
534R	5′-ATTACCGCGGCTGCTGGC-3′		
Dre441F	5′-GTTAGGGAAGAACGGCATCTGT-3′	*Dehalobacter* spp.	Smits *et al*., 2004
Dre645R	5′-CCTCTCCTGTCCTCAAGCCATA-3′		
Dco728F	5′-AAGGCGGTTTTCTAGGTTGTCAC-3′	*D. mccartyi*	Smits *et al*., 2004
Dco944R	5′-CTTCATGCATGTCAAAT-3′		
PO313F	5′-AATGGATGGTACAGAGGGTC-3′	*Polaromonas*	Sun *et al*., 2010b
PO313R	5′-ATTACTAGCGATTCCGACTT-3′		
ME1 fwd	5′-GCMATGCARATHGGWATGTC-3′	*mcrA*	Wilms *et al*., 2007
ME3 rev	5′-TGTGTGAAWCCKACDCCACC-3′		
A189F	5′-GGNGACTGGGACTTCTGG-3′	*pmoA*	Kolb *et al*., 2003
mb661r	5′-CCGGMGCAACGTCYTTACC-3		
TceA1270F	5′-ATCCAGATTATGACCCTGGTGAA-3′	*tceA*	Johnson *et al*., 2005
TceA1336R	5′-GCGGCATATATTAGGGCATCTT-3′		
Bvc925F	5′-AAAAGCACTTGGCTATCAAGGAC-3′	*bvcA*	Ritalahti *et al*., 2006
Bvc1017R	5′-CCAAAAGCACCACCAGGTC-3′		
Vcr1022F	5′-CGGGCGGATGCACTATTTT-3′	*vcrA*	Ritalahti *et al*., 2006
Vcr1093R	5′-GAATAGTCCGTGCCCTTCCTC-3′		
RTC_F	5′-ACCCTGGTCGGTGTKSTYTC-3′	*etnC*	Jin & Mattes, 2010
RTC_R	5′- TCATGTAMGAGCCGACGAAGTC-3′		
RTE_F	5′-CAGAAYGGCTGYGACATYATCCA-3′	*etnE*	Jin & Mattes, 2010
RTE_R	5′-CSGGYGTRCCCGAGTAGTTWCC-3′		
Myco1F	5′-CAGCTCGTGTCGTGAGATGT-3′	*Mycobacterium* spp.	This study
Myco1R	5′-AGACCGGCTTTGAAAGGATT-3′		
Noc1F	5′-CAGAGATGGTGCCCCTTTTA-3′	*Nocardiodes* spp.	This study
Noc1R	5′-AAGGGGCATGATGACTTGAC-3′		

2.2.4 Microsensor analysis

At the end of the third VC spike, O_2 microsensors (tip diameter = 100 µm; response time < 8 s, Unisense, Denmark) were used to determine vertical O_2 partial pressure profiles in sediment microcosms at 0.5 mm intervals. The microelectrode was lowered into the bottles by a motor-driven micromanipulator with computerized depth control capable of maneuvering accurate to 0.1 µm. For calibration, the sensor tip was positioned in water

vigorously bubbled with air (saturated oxygen concentration (8.3 mg/L)) and then transferred into an anoxic solution consisting of 0.1M sodium ascorbate and 0.1M NaOH (0 mg/L oxygen concentration).

2.2.5 Determination of grain size

Sediment grain size distribution was determined using a Beckman-Coulter LS-230 laser diffraction particle size analyzer, designed to measure particles with sizes from 0.04 µm to 2000 µm. The samples were prepared by suspending 4 g of the sediment samples in distilled water by gentle stirring. Before grain-size analysis, all samples were sieved over a 2 mm sieve and the fraction < 2 mm was used for further analysis.

2.2.6 Analysis of volatile components

CAHs concentrations in water samples were analyzed using a Thermo Finnigan Trace GC-MS equipped with a DB5-MS column (J&W Scientific), with an analytical accuracy of 10%. Methane, ethene and ethane concentrations in water samples were determined using a Varian GC-FID (CP- 3800) with a Rt-U plot column (J&W Scientific). Headspace analyses of the microcosms were performed on a Varian GC-FID (CP-3800) equipped with a Rt-U plot column for the detection of methane, ethene and ethane, or a split-splitless injector followed by an Rt-X column (Restek) and a DB-1 column (J&W Scientific) for analysis of CAHs. Standards for chlorinated compounds, ethene, ethane, and methane were prepared by adding a known amount of each compound to a serum bottle with the same headspace to liquid ratio as the microcosm bottles. Concentrations of volatile compounds are expressed as total moles in the bottle divided by the liquid phase (i.e., nominal concentrations) (Aulenta *et al*., 2005). Oxygen was analyzed by injecting 100-μL headspace samples into a Hewlett-Packard (HP) 6890N gas chromatograph equipped with an HP-Plot MoleSieve column (15 m × 0.53 mm; film thickness 25 μm nominal) and a thermal conductivity detector. Helium (6 mL/min) was the carrier gas, and the injector (split at 10:1), oven, and detector were maintained at 90, 40, and 150 °C, respectively. The detection limit for oxygen was ca. 2% (vol/vol).

2.2.7 Statistical analysis

Differences between degradation rates of VC in the anoxic and oxygen-exposed microcosms were compared using a one-way ANOVA analysis and a Tukey-Kramer test at a significance level of 0.05.

2.3 Results

2.3.1 Sediment properties

Sediment of location P26 consisted of black coloured fine grain, with a TOC of 0.73% (w/w). Sediment of P25 was gray in colour with coarse grain and a TOC of 0.31 % (w/w). Both sediments consisted for the majority of medium sand (250-500 µm, Fig. 2.3A). Besides medium sand, the sediment at P26 largely consisted of clay and fine sand (overall 39.11 %), whereas the sediment at P25 contained coarse and very coarse sand as other important fractions (together 42.03 %). In oxygen-exposed microcosms containing P26 sediment, oxygen penetration into the sediment layer as determined at the end of third VC spike by microsensor measurements, was restricted to the upper 2 mm of the sediment (Fig. 2.3B). The smaller grain size and the higher organic carbon load (0.73% (w/w)) of the P26 material apparently resulted in limited oxygen penetration into the sediment, leaving the deeper parts of the sediment anoxic. In contrast, in oxygen-exposed microcosms containing P25 material with a particle size distribution characterized by a higher fraction of coarse sand and lower TOC compared to P26 sediment, oxygen penetrated deeper into the sediment layer (Fig. 2.3B). This could be conducive for aerobic degraders but inhibiting for OHRB.

2.3.2 Occurrence of aerobic and anaerobic VC degradation activity in sediment microcosms

VC was removed rapidly from the microcosms prepared with Zenne river-bed sediments from P25 and P26 under both anoxic and oxygen-exposed conditions (Fig. 2.4A & B). A lag phase of 7 days was observed in all conditions. Since no significant changes in VC concentrations were observed in the abiotic controls, the change in VC concentration observed in experimental microcosms can be attributed to biological activity. Complete dehalogenation of the first VC spike under anoxic conditions in microcosms containing P26 material occurred within 28 days with stoichiometric conversion into ethene and ethane as end products (Fig. 2.4A &C). After the second and third VC spikes, dehalogenation started immediately resulting in complete removal of VC within 14 days. Highly similar VC consumption (Fig. 2.4B) and accumulation of reduced daughter products (Fig. 2.4D) was observed in anoxic microcosms containing P25 material.

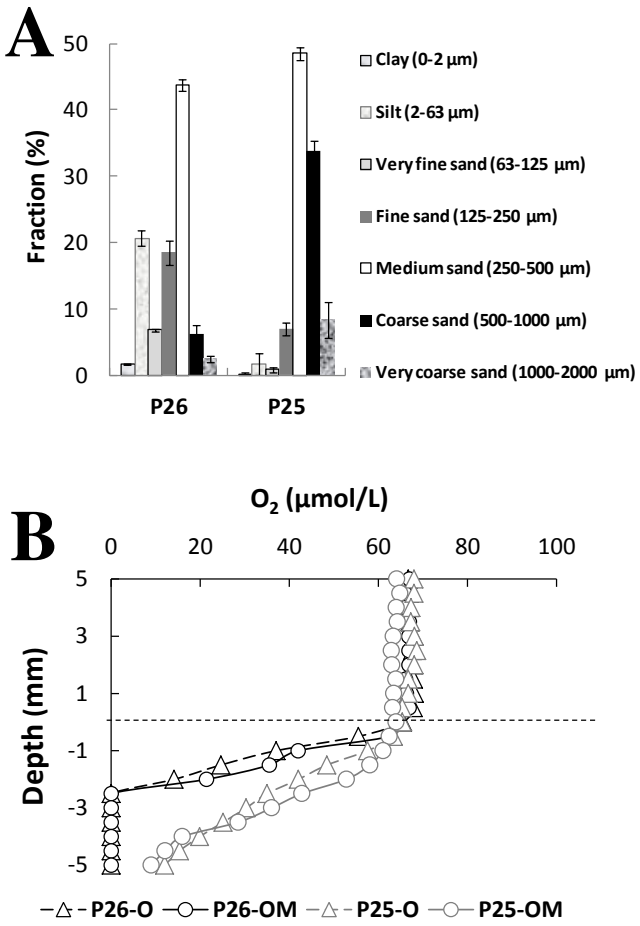


Fig. 2.3. Distribution of grain size in sediments taken from locations P25 and P26 (A) and vertical O_2 profiles in oxygen-exposed microcosms containing sediment from locations P25 and P26 analysed at the end of third VC spike (B). In panel A, presented data are the average of duplicate analysis on two independent samples. The dotted line in panel B indicates sediment-surface water interface at zero-depth. O: oxygen-exposed microcosms; OM: oxygen/methane-exposed microcosm.

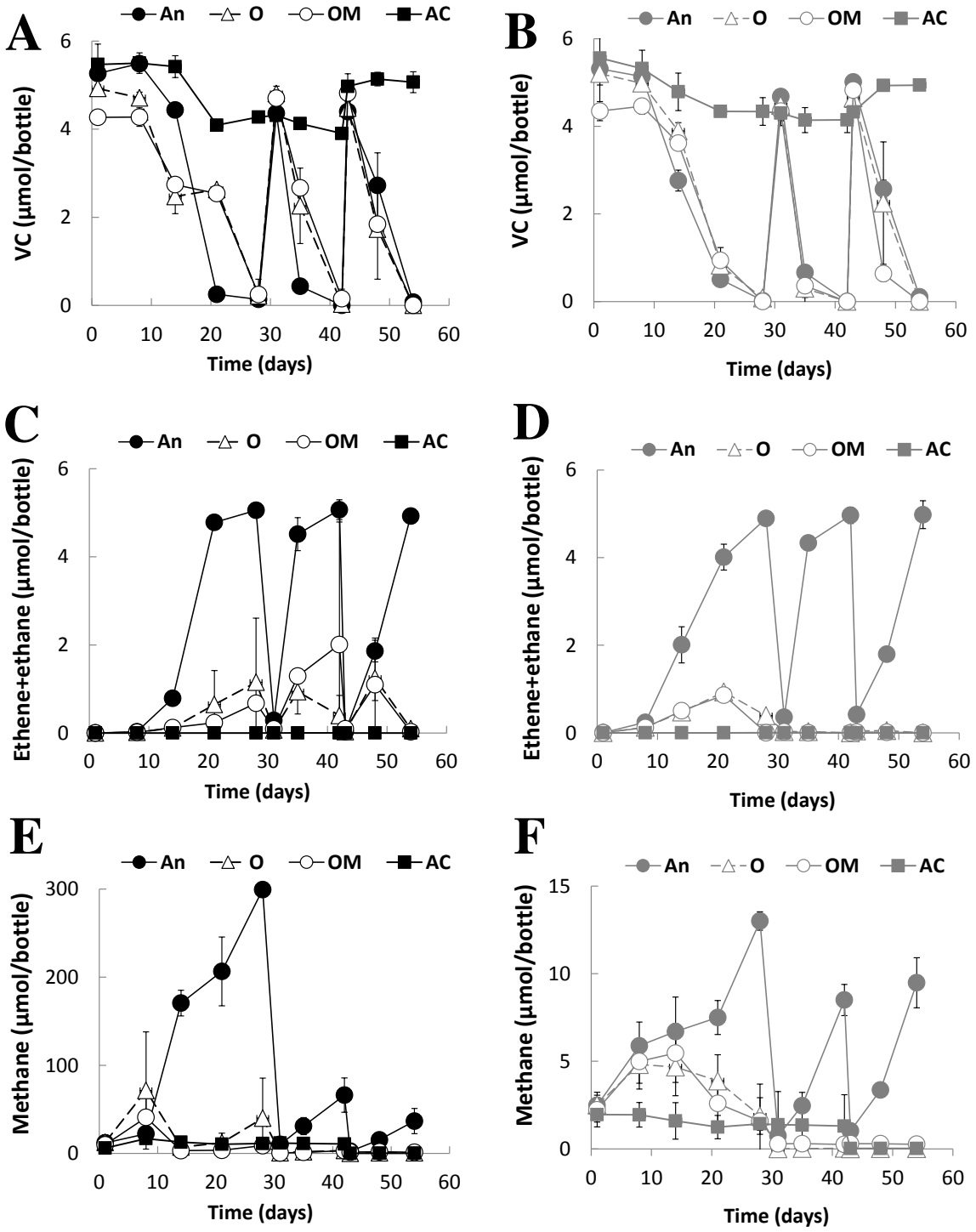


Fig. 2.4. VC degradation (A and B), accumulation of ethene and ethane (C and D) and methane production (E and F) in anoxic and oxygen-exposed microcosms containing sediment from location P26 (panels A, C and E) and P25 (panels B, D and F), respectively. An: anoxic; O: oxygen-exposed microcosms; OM: oxygen/methane-exposed microcosms; AC: abiotic control. Note that for the Y axes of panel E and F different concentration scales are used. All data points represent average values from duplicate microcosms with the error bars showing the standard deviation. If no error bars are shown, the standard deviations were too small to be illustrated.

The average rates of VC degradation after 3 VC spikes in oxygen-exposed microcosms of P26 were comparable to those observed in anoxic microcosms, while in the P25 microcosms, the average degradation rates in oxygen/methane-exposed microcosms were significantly higher than those in anoxic and oxygen-exposed microcosms (Table 2.2). Lower and non-stoichiometric accumulation of reduced daughter products (mainly ethene, Appendix 1) occurred in the oxygen-exposed microcosms containing material from P26 after the first VC spike, and continued after the second and third spike, whether or not methane was added (Fig. 2.4C, Appendix 1A & C). In oxygen-exposed microcosms containing P25 material, ethene production was observed only after the first VC spike (Fig 2.4D, Appendix 1B & D).

Methane production was most pronounced in anoxic microcosms containing P26 material and reached 300 µmol/bottle at the end of first VC spike (Fig. 2.4E) compared with 14 µmol/bottle in anoxic microcosms containing P25 material (Fig. 2.4F). In all oxygen-exposed microcosms, methane production was only noted during degradation of the first VC spike. Addition of methane in OM microcosms did not lead to higher methane level in OM microcosms, possibly due to consumption of methane by methanotrophic populations (Fig. 2.4E & F).

Table 2.2. Average VC degradation rates (k) after 3 VC spikes in microcosms containing P26 and P25 material. Degradation rates were determined by linear regression of concentration/time series. The superscript letters (a-c) indicates significant differences ($P < 0.05$) between conditions based on the Tukey-Kramer test.

Conditions	k [day^{-1}]
P26-anoxic	0.44 ^{a}
P26-oxygen-exposed	0.45 ^{a}
P26-oxygen/methane-exposed	0.38 ^{a}
P25-anoxic	0.38 ^{a}
P25-oxygen-exposed	0.57 ^{ab}
P25-oxygen/methane-exposed	0.63 ^{c}

2.3.3 Dynamics of biomarkers

Abundances of biomarkers of microbial guilds (i.e. *D. mccartyi* and *Dehalobacter*) and functional genes (*tceA*, *vcrA*, *bvcA*) associated with OHR clearly changed after completing degradation of three VC additions at day 54 compared to their abundances at day 0 (Fig. 2.5). The number of *D. mccartyi* 16S rRNA gene copies in anoxic microcosms containing P26

material increased from undetectable levels to $1.53 \times 10^5 \pm 1.86 \times 10^4$ copies/g (Fig. 2.5A) which accounted for approximately 0.2 % of the total bacterial 16S rRNA gene pool. Apparently, the availability of oxygen in oxygen-exposed microcosms containing P26 sediment did not have a profound inhibitory effect on the *D. mccartyi* population size since abundances similar to these in the anoxic microcosms were recorded at day 54. An identical increasing trend was observed in the number of *Dehalobacter* 16S rRNA gene copies (Fig. 2.5A). In accordance with the enrichment of *D. mccartyi,* there was a clear increase in the abundance of *rdh* genes, i.e., *tceA*, *vcrA*, and *bvcA* in microcosms containing P26 material. The most abundant *rdh* gene at day 54 was always *bvcA* with numbers that were generally 2 orders of magnitude higher than those of the *D. mccartyi* 16S rRNA gene. The TCE reductase-encoding *tceA* gene was always the least abundant *rdh* gene (Fig. 2.5A).

The number of *D. mccartyi* 16S rRNA gene copy in anoxic microcosms containing P25 material increased from $8.51 \times 10^2 \pm 5.36 \times 10^1$ copies/g at day 0 to a maximum of $1.59 \times 10^5 \pm 1.1 \times 10^4$ copies/g at day 54 (Fig. 2.5B), which accounted for approximately 0.1 % of the total bacterial 16S rRNA gene pool. In contrast to microcosms containing P26 material, oxygen exposure apparently prevented an increase in abundance of *D. mccartyi* in the oxygen-exposed microcosms containing P25 material as no significant increase in their numbers was observed. A similar trend was noted in the numbers of *rdh* genes, while the number of *Dehalobacter* 16S rRNA gene copies were increased in all microcosms of 25 (Fig. 2.5B).

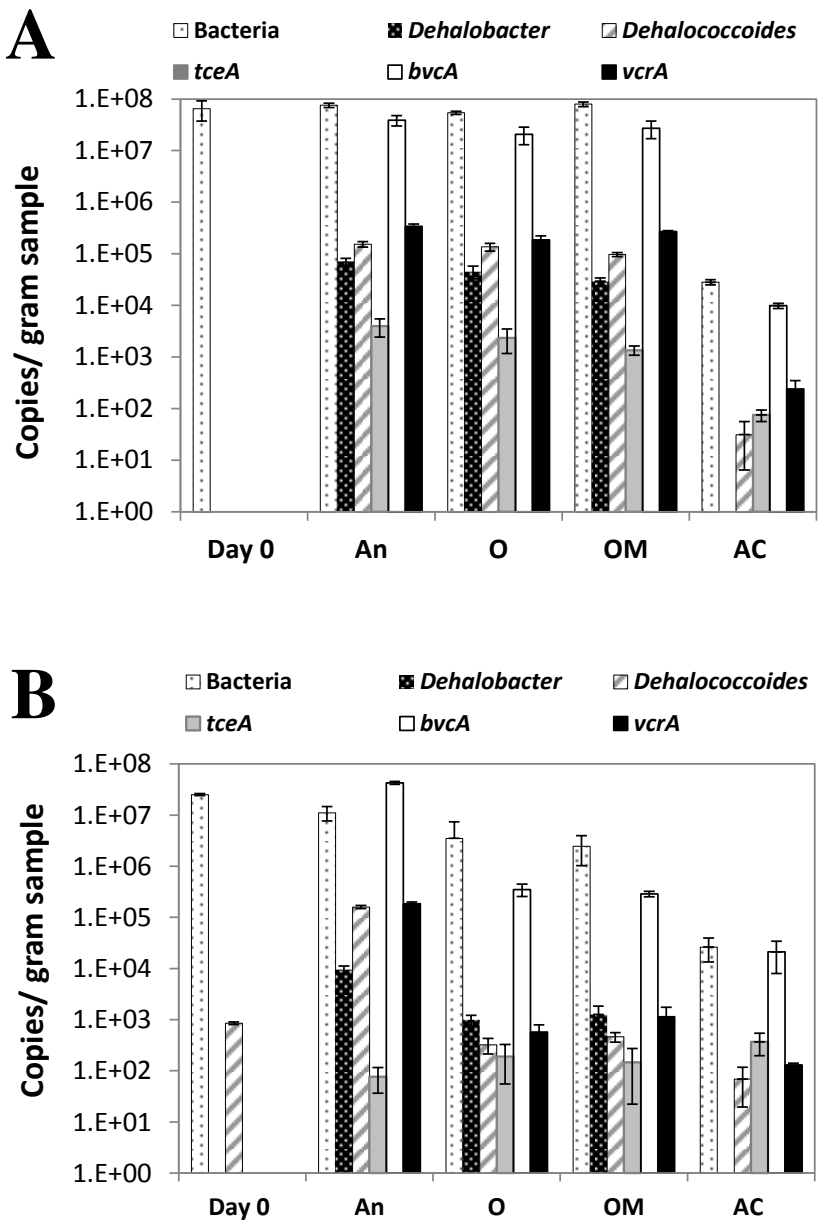


Fig. 2.5. 16S rRNA gene copy numbers of bacteria*, Dehalobacter*, *D. mccartyi* and *rdh* genes on day 0 and day 54 in microcosms containing sediment of locations P26 (A) and P25 (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). An: anoxic; O: oxygen-exposed microcosms; OM: oxygen/methane-exposed microcosms ; AC: abiotic control.

We tested for the presence of members of *Mycobacterium* and *Nocardiodes* by means of 16S rRNA gene targeted qPCR, since VC-assimilating bacterial isolates often belong to these genera (Mattes *et al*., 2010). In addition, the16S rRNA gene of *Polaromonas* was targeted

since members of this genus were shown to aerobically oxidize cDCE as carbon and energy source (Coleman *et al*., 2002a). However, microbial counts associated with metabolic VC degrading organisms indicated no substantial difference between anoxic and oxygen-exposed microcosms (Fig. 2.6).

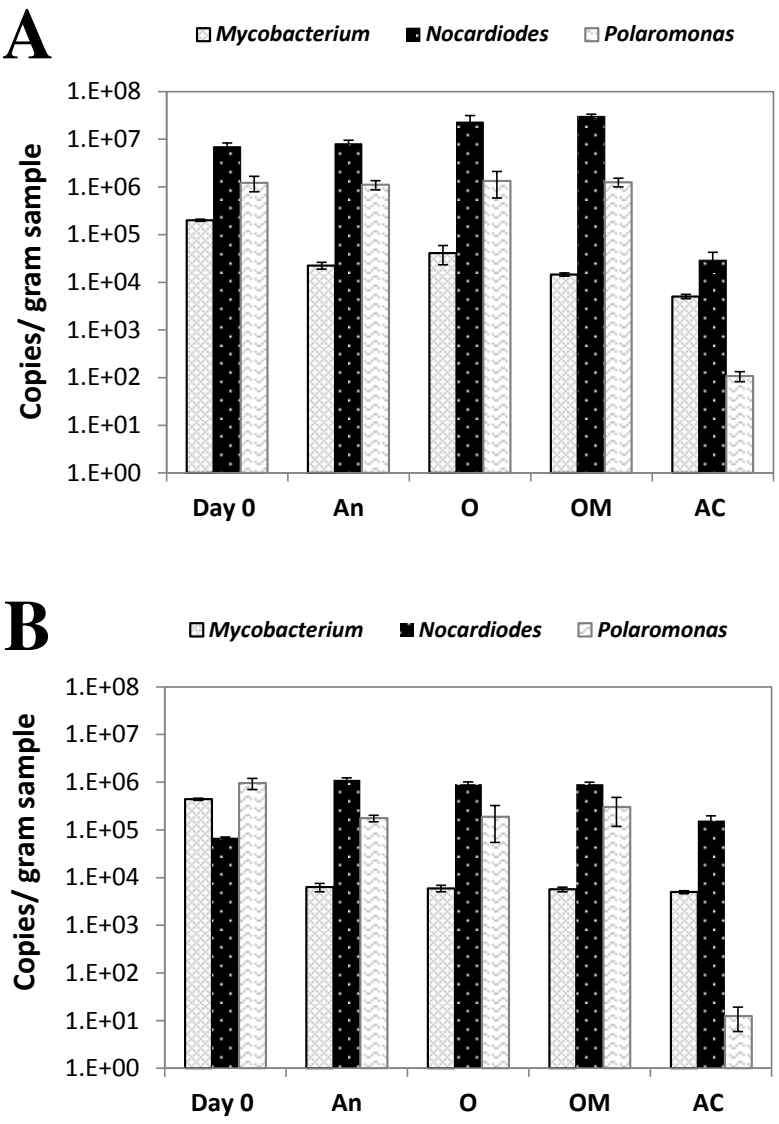


Fig. 2.6. 16S rRNA gene copy numbers of *Mycobacterium*, *Nocardiodes*, and *Polaromonas* on day 0 and day 54 in microcosms containing sediments of locations P26 (A) and P25 (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). An: anoxic; O: oxygen-exposed microcosms; OM: oxygen/methane-exposed microcosms; AC: abiotic control.

Furthermore, we quantified *etnC* and *etnE* as these represent functional genes associated with aerobic VC metabolism. The number of *etnC* did not change (P26) (Fig. 2.7A) or decrease (P25) (Fig. 2.7B) in anoxic microcosms. In contrast, *etnC* was enriched in oxygen-exposed microcosms containing P26 material, i.e., 40-fold in the O microcosms and 120-fold in the OM microcosms (Fig. 2.7A). An increase was also observed in the oxygen-exposed microcosms containing P25 material, i.e. a threefold increase in the O microcosms and a sevenfold increase in the OM microcosms (Fig. 2.7B). An identical trend was observed in *etnE* abundance. While *etnE* numbers decreased slightly in all anoxic microcosms, its numbers increased in oxygen-exposed microcosms containing P26 material, i.e., by a factor of 15 in O microcosms and a factor of 55 in OM microcosms (Fig. 2.7A). In microcosms containing P25 material, *etnE* increased fivefold in O microcosms and 149-fold in OM microcosms (Fig. 2.7B).

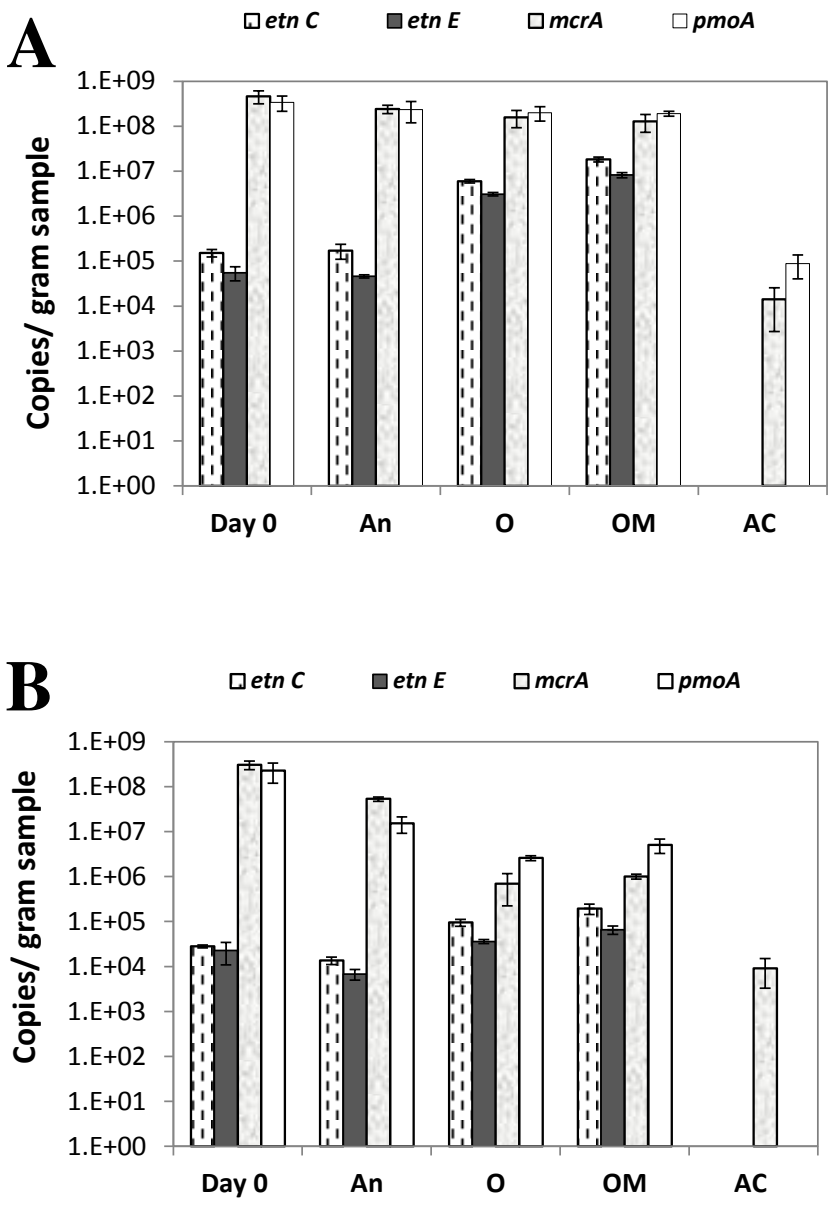


Fig. 2.7. Copy numbers of *etnC*, *etnE*, *mcrA*, and *pmoA* genes on day 0 and day 54 in microcosms containing sediments of locations P26 (A) and P25 (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). An: anoxic; O: oxygen-exposed microcosms; OM: oxygen/methane-exposed microcosms; AC: abiotic control.

In accordance with the trends observed for *D. mccartyi*, methanogen abundance (based on *mcrA* gene copy numbers determined at day 0 and 54) appeared undisturbed in both anoxic and oxygen-exposed microcosms containing P26 material (Fig. 2.7A). On the other hand, the

number of *mcrA* gene copies decreased 6-fold in the anoxic microcosms containing P25 material while its number decreased more than 2 orders of magnitude in oxygen-exposed microcosms containing P25 material (Fig. 2.7B). No significant change in the abundance of methanotrophs (as determined by *pmoA* copy numbers at day 0 and 54) occurred in both anoxic and oxygen-exposed microcosms containing P26 material (Fig. 2.7A) while their numbers declined in microcosms containing P25 material, i.e., 15-fold in anoxic microcosms, 87-fold in O microcosms and 45-fold in OM microcosms (Fig. 2.7B). Apparently, the concomitant production and consumption of CH_4 in oxygen-exposed microcosms of P26 resulted in a stable community of methanotrophs, while suppression of CH_4 production in oxygen-exposed microcosms containing P25 material led to a decline in the number of methane assimilating communities which was less severe in OM microcosms.

2.4 Discussion

This study examined the co-occurrence of anaerobic reductively VC dechlorinating and aerobic VC degrading microbiota in surficial sediment samples taken from the hyporheic zone of a river impacted by VC contaminated groundwater. Moreover, it examined how sediment physico-chemistry impacted the corresponding activities and hence the VC biodegradation pathway. To the best of our knowledge, this is the first report on co-existence and activity of both aerobic and anaerobic VC degraders in the same sediment and their relative contribution to VC degradation as well as the role of sediment characteristics that determine local oxygen distribution and subsequent VC biodegradation pathway.

2.4.1 Co-existence of anaerobic and aerobic VC degrading populations

The results showed the presence of aerobic VC-degraders as well as anaerobic VC dehalogenating populations in the same small volume of sediment. In all microcosms containing P25 or P26 sediment materials, VC was degraded by the indigenous microbial communities under both oxygen-exposed and anoxic conditions with concomitant growth of the corresponding microbial guilds.

The observed accumulation of ethene as a reduced daughter product in oxygen-exposed microcosms containing P26 material after all three VC spikes, showed that in these microcosms anaerobic degradation pathways were never fully inhibited and still contributed to VC degradation (Fig. 2.2C). Accordingly, the molecular data showed that the numbers of

D. mccartyi 16S rRNA and *rdh* genes were highly enriched in oxygen-exposed microcosms of P26 (Fig. 2.3A). Biomarkers related to aerobic VC degradation also increased in oxygen-exposed microcosms containing P26 material (Fig. 2.4A). Based on these observations, it can be suggested that the observed VC disappearance in oxygen-exposed microcosms containing P26 sediment is a result of both anaerobic and aerobic degradation, where at least part of the VC is reductively dehalogenated to ethene. The remainder of the VC and the produced ethene is then degraded by ethenotrophs, which can assimilate these compounds as carbon and energy sources for growth (Mattes *et al*., 2010). In oxygen-exposed microcosms containing P25 material, the accumulation of reduced daughter products associated with anaerobic VC dechlorination ceased after the second and third VC spike (Fig. 2.2D) with only limited additional proliferation of biomarkers associated with OHR (Fig. 2.3B). This is suggestive of a higher contribution of oxidative VC-degraders than anaerobic dechlorinators in oxygen-exposed microcosms containing P25 material compared to the oxygen-exposed microcosms containing P26 material.

The tracked biomarkers provided information on the microbiota involved in VC degradation. VC respiration activity was clearly correlated with the increase in *D. mccartyi* (and hence growth) and both the *bvcA* and *vcrA* gene in both P26 and P25 microcosms showing VC reductive dechlorination activity. Calculated yields of *D. mccartyi* at the expense of VC degradation in anoxic microcosms of P26 and P25 (1.41×10^{10} and 1.37×10^{10} cells/mmole VC, respectively) were generally in the range of values reported previously for cCDE e.g. 0.44×10^{10} cells/mmole (Schaefer *et al*., 2009) and 8.4×10^{10} cells/mmole (He *et al*., 2005) but lower than the values reported for TCE e.g. 1×10^{11} cells/mmole (Duhamel & Edwards, 2007) and 28.6×10^{11} cells/mmole (Yu & Semprini, 2004) which might be due to availability of 3 chlorine molecules in TCE for OHR. The *bvcA* gene has been originally identified in *D. mccartyi* strain BAV1 (Krajmalnik-Brown *et al*., 2004), whereas *vcrA* was identified in strain VS (Müller *et al*., 2004) and strain GT (Sung *et al*., 2006). These microorganisms respire on VC using hydrogen as electron donor. Up to now, strains from the *D. mccartyi* genus are the only organisms known to reductively dehalogenate VC for growth. Therefore, our data suggest that *D. mccartyi* carrying the *bvcA* or *vcrA* gene are responsible for VC reductive dechlorination in the Zenne microcosms. This is supported by earlier data in which sediment free VC dechlorinating cultures enriched from Zenne sediment were shown to be dominated

by *D. mccartyi* (Dijk *et al.,* 2008). On the other hand, *bvcA* numbers were always 2 orders of magnitude higher than *D. mccartyi* numbers. This has been observed before for other sites, albeit with *vcrA* as the dominant gene (Maphosa *et al*., (2010b); van der Zaan *et al*., (2010)). Such observations can be explained by the presence of unknown organohalide respiring microorganisms containing *bvcA* in addition to *D. mccartyi*. For instance, although never associated with VC dechlorination, recently, members of the *Chloroflexi* distantly related to *D. mccartyi* have been identified, which reductively dehalogenate PCE to DCE (Kittelmann & Friedrich, 2008a; Kittelmann & Friedrich, 2008b). Moreover, using an RNA stable isotope probing approach, Kittelmann & Friedrich (2008a) showed that the diversity of organohalide respiring microorganisms in the environment is largely underestimated. Alternatively, *bvcA* might be carried by *Dehalobacter* whose number also increased in the microcosms, although the *Dehalobacter* genus has not yet been associated with harbouring *bvcA* or with VC dechlorination. The distribution of *bvcA* in different dehalogenating bacteria in the same microcosms might not be unusual since the gene was shown to be associated with mobile elements (McMurdie *et al*., 2011).

With regards to the aerobic VC degradation, it appears that in the microcosms of P26, there is assimilative metabolism of VC rather than methane-dependent cometabolic degradation while addition of methane to the oxygen-exposed microcosms of P25 led to significantly higher VC degradation rates. However, methane addition to the microcosms, did not increase the number of *pmoA* in oxygen/methane-exposed microcosms of both locations. In this study, only *pmoA* was monitored as a biomarker of methanotrophs and not *mmoX* (encoding the hydroxylase component of sMMO) since the used VC concentrations were rather high (i.e. 70 µM in water phase) and methanotrophs expressing pMMO have been reported to have a competitive advantage over cells expressing only sMMO at VC concentrations above 10 µM (Lee *et al*., 2006). The failure of an explanation for this might be due to low copper-to-biomass ratios during which methane oxidation is carried out by sMMO rather than pMMO which is active in higher copper-to-biomass ratio (Lontoh & Semrau, 1998). However, neither the copper concentration nor the number of *mmoX* were analyzed in this study. Aerobic VC biodegradation was also not associated with an increase in the number of bacterial genera most commonly associated with aerobic VC metabolism such as *Mycobacterium* and *Nocardiodes*. However, that does not necessarily mean that they are

not involved in VC degradation since the numbers of those genera were relatively high at the start of the experiment and as such growth of VC degrading populations within this genus may have been masked as recently observed during biodegradation of the herbicide linuron in soil by *Variovorax* spp. (Bers *et al*., 2011). Furthermore, aerobic VC metabolic bacteria are taxonomically diverse (Coleman & Spain, 2003). Accordingly, in attempts to isolate VC-assimilating microorganisms, instead of organisms like *Mycobacterium*, Gram-negative aerobic VC degraders belonging to the genera *Pseudomonas* and *Ochrobactrum* were isolated from the sediment sample (S. Atashgahi, unpublished results).

2.4.2 Influence of physico-chemistry on VC degradation pathways

The physico-chemistry of the sediment seems to play an important role in VC biodegradation pathways with oxygen probably as the central mediator. Both sediment samples showed a different penetration depth of oxygen in the oxygen-exposed microcosms. In the oxygen-exposed microcosms containing P26 sediment, the oxygen gradient was much steeper than in the microcosms containing P25 sediment. This can be due to the smaller grain size and higher organic matter content in the P26 sediment compared to the P25 sediment. Smaller grain sizes result in a more compact sediment texture and consequently reduced permeability, which limits vertical mixing and the diffusion of oxygen into the sediment matrix (Maier *et al*., 2000). High organic loads lead to faster consumption of oxygen through respiration by heterotrophic and chemolithotrophic microorganisms, limiting oxygen penetration into the sediment. Therefore, aerobic activity can develop at surficial layer of the sediment, without affecting the activity and survival of strict anaerobes in the deeper parts of the sediment. In the oxygen-exposed microcosms, the differences in oxygen penetration can explain the differences in observed biodegradation pathways between microcosms containing P25 and P26 material. In microcosms containing P25 material, oxygen diffusion into the sediment was higher due to the larger grain size and its consumption was low because of the low organic carbon content. These higher oxygen concentrations probably affected the microbial guilds associated with VC reductive dechlorination and inhibited their activity and growth. *D. mccartyi* as dedicated VC dechlorinators, are strict anaerobes and a brief exposure of *D. mccartyi* cultures to air or oxygen has been shown to completely and irreversibly inhibit CAH dechlorination (Adrian *et al*., 2007; Amos *et al*., 2007). Besides limited oxygen penetration into the sediment layer in

oxygen-exposed microcosms of P26, an alternative reason for the observed survival of *D. mccartyi* could be the presence of anaerobic microniches inside the sediment-associated biofilms. Recent characterization of microscale geochemical conditions inside sediment biofilms showed a significant decrease in redox potential and dissolved oxygen as a function of depth (Nguyen *et al*., 2012). These authors suggested that, even in the presence of air-saturated groundwater, suboxic and perhaps anoxic zones exists in sediment biofilms. The observed sharp oxygen gradient into the sediment biofilm indicated that there was significant oxygen consumption in the biofilm. Such a behavior can be due to the presence of facultative aerobic bacteria colonizing the outer layers of sediment biofilms, which can rapidly consume oxygen, in turn protecting the strict anaerobes in core microniches.

2.5 Conclusion

In conclusion, our results show the co-existence of aerobic VC degraders with anaerobic dehalogenating populations in the same small volume of a river surficial sediment impacted by a VC-contaminated groundwater plume. In addition, it shows that sediment physico-chemistry, by mediating oxygen penetration and possibly local redox conditions, strongly affects the relative contribution of different CAH degradation pathways. As such, aerobic VC biodegradation at the interface between surface water and sediment might be an important accessory process in removing CAH in discharging groundwater in the Zenne ecosystem in addition to the previously shown OHR in deeper sediment layers (Hamonts *et al*., 2009, 2012; Kuhn *et al*., 2009). Further research should emphasize on the spatial distribution of the participating CAH degrading communities including those present *in situ* and on their ecological significance in removing CAH from polluted groundwater before reaching the surface water.

Chapter 3: Resistance and resilience of aerobic/anaerobic chloroethene degrading microbiota to redox fluctuations in riverbed hyporheic sediments affected by a chloroethene contaminated groundwater plume

3.1 Introduction

Chlorinated aliphatic hydrocarbons (CAHs) are among the most frequently detected groundwater contaminants. At many industrial sites, CAH-contaminated groundwater discharges into surface water through the groundwater-surface water interface within the riverbed referred to as the "hyporheic zone". Hyporheic zones are storage zones for organic carbon and are characterized by physical and chemical gradients governing a broad spectrum of microbial processes (Biksey & Gross, 2008). In recent years, hyporheic zones have been recognized as a critical transition zone that can act as a physico-chemical and biological filter reducing or even preventing the transfer of CAHs from groundwater to surface water (Conant *et al.,* 2004; Ellis *et al*., 2007; Hamonts *et al.,* 2009; Hamonts *et al.,* 2012). The high organic matter content and anoxic conditions in deeper sediment layers can be conducive to partial or complete biological reductive dechlorination of CAHs for instance

by sustaining the activity of organohalide respiring bacteria (OHRB) like *Dehalococcoides mccartyi* (Smidt & de Vos, 2004). On the other hand, more oxic layers at the surface water-sediment interface provide conditions for aerobic degradation of less-chlorinated CAHs (see Chapter 2). The combination of anoxic deeper sediment layers that govern reductive dechlorination and an overlaying oxic surface layer that sustains complementary aerobic degradation forms a dual-layer biobarrier for natural attenuation of a CAH contaminated discharging groundwater plume. Such a system, however, is highly dynamic as the delineation of oxic/anoxic zones varies in space and time depending on the environmental conditions that determine the surface water level. For instance, during high precipitation events, surface water levels will increase and downwelling migration of oxygenated surface water will change the redox conditions in the hyporheic zone from anoxic to oxic over short distances. After the precipitation event, generally, the anoxic groundwater recovers its normal upwelling flow. Due to this flow reversal and concomitant local redox fluctuation, portions of the hyporheic zone are repeatedly exposed to oscillating redox conditions (Brunke & Gonser, 1997).

Chapter 2 introduced a new concept regarding the CAH-biodegradation capability of hyporheic zones and indicated that anaerobic VC respiring microbial guilds and aerobic VC degraders can co-exist and be co-active in the hyporheic sediments. This was shown for surficial river bed sediment of the eutrophic Zenne River in Belgium affected by a chloroethene contaminated groundwater plume. Sediment samples taken from this river showed activities of VC reductive dechlorination as well as aerobic VC biodegradation. The observed activity and biodegradation pathway depended on whether oxic or anoxic conditions were installed but also on the sediment characteristics that control oxygen penetration in the sediment such as grain size and in particular organic carbon content. For instance, in sediments with high organic carbon content, reductive dechlorination activity prevailed even under an oxic atmosphere due to rapid oxygen depletion in the sediment governed by organic carbon utilization. Since microbial mechanisms for degradation of chloroethenes are fundamentally redox processes, periodic fluctuations in redox conditions and oxygen exposure (that are expected to occur in hyporheic zones) might stall reductive dechlorination by precluding the activity of obligatory OHRB (Amos *et al*., 2008) and/or stimulating the aerobic biodegradation of lower chlorinated ethenes by aerobic degraders

when oxic conditions prevail and vice versa when anoxic conditions prevail. Moreover, adverse conditions might lead to decay of the affected organisms which might affect subsequent activity when conditions are again optimal. However, knowledge on the effect of changing redox conditions on the resistance and resilience of chloroethene degrading organisms in hyporheic sediment zones is scarce although this knowledge is of paramount importance to evaluate the role of hyporheic sediments in mitigation of discharge of CAHs from groundwater into surface water.

In the current study, we investigated the response of hyporheic sediment-associated microbial communities involved in biodegradation of VC/cDCE to periodic redox fluctuations. To this end, microcosms were set up that contain surficial river sediments collected from the two locations of the Zenne river-bed (Belgium), i.e., Post 26 (P26) and Post 25 (P25), that were investigated in Chapter 2. VC/cDCE biodegradation was followed under subsequently changing redox conditions and DNA-based biomarkers of known microbial guilds and functional catabolic genes involved in anaerobic reductive and aerobic VC/cDCE degradation were used as indicators of the observed biodegradation profiles.

3.2 Materials and Methods

3.2.1 Site material

Sediment samples used in this study were taken from the Zenne River in Machelen-Vilvoorde, Belgium. A groundwater plume mainly contaminated with cDCE and VC is flowing from the nearby aquifer into the Zenne River. Details of the test site can be found elsewhere (Hamonts *et al*., 2009). All samples were collected in May 2010. River-bed sediment samples up to a depth of 20 cm were collected from two locations designated as P26 with a high total organic carbon content (TOC) and P25 with low TOC. Sediment samples were transferred into air-tight glass jars and transported to the laboratory in a cooler. Surface water samples were collected as grab samples in glass bottles, without leaving a headspace. The characteristics of sediment and surface water used in this study were reported in Chapter 2.

3.2.2 Microcosm set-up and reversibility test

Sediment microcosms were prepared in duplicate in an anaerobic glove box (Don Whitley Scientific ltd, West Yorkshire, UK) in 160-mL glass serum bottles and contained 37 g (wet) sediment and 70 mL of surface water and sealed with Teflon-lined butyl rubber stoppers.

The microcosms contained either sediment from P26 or P25 and were used to assess either VC or cDCE biodegradation. Microcosms containing sediment from P26 used to assess VC/cDCE degradation and from P25 used to assess degradation of VC underwent the following subsequent treatments (as outlined in Fig. 3.1A): First, either VC or cDCE was added to the corresponding microcosms at final concentrations of 5 mg/L in liquid phase and their degradation was followed under static condition (treatment designated as anoxic static-1). When VC/cDCE was fully dechlorinated, the head space of the microcosms was flushed 3 times for 15 min with oxygen free nitrogen and spiked again with VC/cDCE (5 mg/L). When three consecutive spikes of VC/cDCE were fully dechlorinated, the material in the microcosms was exposed to oxygen by injection of sterile oxygen gas (ultra-pure, Air Products, Belgium; filter sterilized over a 0.2-µm filter) at an initial concentration of 7% (vol/vol) of the headspace after the withdrawal of an equal volume of headspace (treatment designated as oxygen-exposed static, see Fig. 3.1A). VC/cDCE degradation was followed for 3 consecutive VC/cDCE spikes (5 mg/L). In a third treatment, the microcosms were converted back to anoxic conditions (treatment designated as anoxic static-2) and degradation of 3 spikes of VC/cDCE (5 mg/L) were again followed to study resilience of the OHRB. In a subsequent treatment, culture conditions were still kept anoxic but 3 consecutive spikes of VC/cDCE (5 mg/L) degradation was followed while the vials were incubated shaking horizontally on a rotary shaker (120 rpm) (treatment designated as anoxic shaking). Then, the microcosms were spiked again with VC/cDCE (5 mg/L) and their degradation monitored for 3 consecutive spikes under anoxic static conditions (treatment designated as anoxic static-3). In the next treatment step, the biodegradation of 3 spikes of VC/cDCE (5 mg/L) was followed under oxic shaking conditions by providing oxygen at 7% (vol/vol) of the headspace and horizontal shaking (treatment designated as oxygen-exposed shaking). In the last treatment step, degradation of 3 spikes of VC/cDCE (5 mg/L) was followed under anoxic static conditions (treatment designated as anoxic static-4). Microcosms containing sediment of P25 used to assess cDCE biodegradation underwent a similar scheme but only till the “anoxic static-2” step (Fig. 3.1B). During all treatment, the microcosm were incubated at room temperature. At the end of third VC/cDCE spike under anoxic static-1, oxygen-exposed static, anoxic shaking, oxygen-exposed shaking, and anoxic static-4, liquid samples were

withdrawn and filtered over a 0.45-µm filter for DOC measurements, unless otherwise stated.

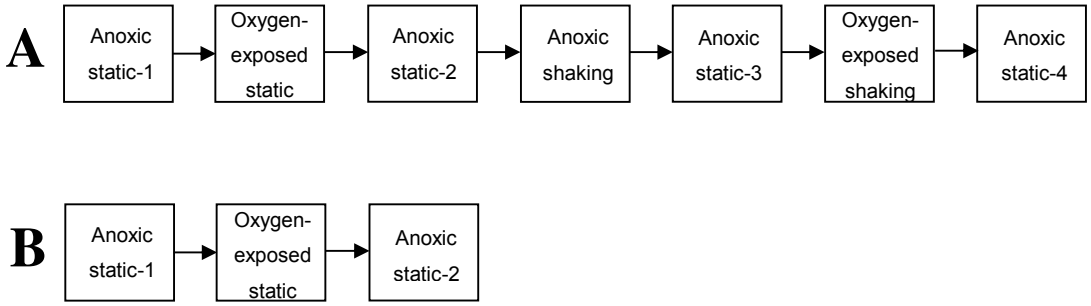


Fig. 3.1. Schematic presentation of experimental strategy used in the reversibility experiments with VC/cDCE spiked microcosms containing P26 sediment material and VC spiked microcosms containing P25 material (A) and cDCE spiked microcosms containing P25 material (B).

3.2.3 DNA extraction and quantitative PCR

Samples for DNA extraction were withdrawn as approximately 2 gram slurries from all duplicate microcosms after thorough mixing. Samples were taken always at the end of the third VC/cDCE spike, unless otherwise stated. DNA was extracted as described previously (Hendrickx *et al*., 2005). Real-time PCR quantification (qPCR) amplifications targeting *D. mccartyi* 16S rRNA gene and catabolic genes *bvcA*, *vcrA*, *etnC* and *etnE* were performed as described in Chapter 2.

3.2.4 Analysis of volatile components

Concentrations of cDCE, VC, ethene and oxygen were determined via head-space analysis as described in Chapter 2. Dissolved organic carbon (DOC) was determined from samples as the difference between total dissolved carbon and dissolved inorganic carbon, measured with a Shimadzu TOC-5000 analyzer equipped with an ASI-5000 auto-sampler.

3.3 Results

3.3.1 Dynamics of VC degradation and associated biomarkers

High VC biodegradation rates were observed under initial anoxic conditions in microcosms prepared from both locations (Fig. 3.2 A&C panel a; Table 3.1). At the end of each of the three VC spikes, all VC was converted stoichiometrically to ethene suggesting that VC was degraded by organohalide respiration (OHR). Concomitantly, the number of *D. mccartyi* 16S

rRNA gene copies and VC reductive dehalogenase (RDase)-encoding genes *vcrA* and *bvcA* increased from undetectable levels at day 0 (Fig. 3.2 B&D panel t0) to 10^4-10^5 copies/gram (Fig. 3.2 B&D, panel a).

When three spikes of VC were degraded, the headspace of the microcosms was converted to oxygen-exposed conditions (oxygen-exposed static). In both P25 and P26 microcosms, VC was degraded but production of ethene was only noticed during the degradation of the first VC spike. As observed in Chapter 2, *etnC* and *etnE* numbers increased. The disappearance of VC with only limited production of ethane and the concomitant enrichment in the numbers of *etnC* and *etnE* under oxygen-exposed conditions suggested that aerobic mineralization of VC commenced instantly once the microcosms were amended with oxygen (Fig. 3.2 A,B,C & D panel b). Oxygen exposure under static conditions did not affect DOC concentrations in the microcosms prepared from both locations as compared with those recorded at the end of the former anoxic incubation (Table 3.2).

After three spikes of VC were degraded under oxygen-exposed static conditions, the microcosms were converted back to anoxic static conditions (anoxic static-2). Formation of ethene was resumed during degradation of the first VC spike in both P25 and P26 microcosms (Fig. 3.2 A&C panel c). Since oxygen penetration into the sediment layer under static conditions was limited (see Chapter 2), we intended to subsequently examine the effect of oxygen exposure while shaking. To exclude that the observed effects were rather due to the shaking than to improved penetration of oxygen, the effect of shaking was first tested under anoxic conditions. Shaking under anaerobic conditions resulted into slightly decreased degradation rates (Fig. 3.2 A&C panel d; Table 3.1) and a slight decrease in the community size of *D. mccartyi* (Fig. 3.2 B&D, panel d), especially in microcosms containing sediment of P25. The subsequent return to static incubation (anoxic static-3), however, resulted into rapid re-establishment of degradation rates (Fig. 3.2A&C panel e; Table 3.1) and the *D. mccartyi* population size (Fig. 3.1 B&D panel e). When the microcosms were then incubated under oxygen-exposed shaking conditions, high VC degradation rates were noticed in microcosms containing P25 sediment (Fig. 3.2C panel f, Table 3.1) with a concomitant increase in *etnC* and *etnE* numbers (Fig. 3.2D, panel f). In contrast, in the P26 sediment microcosms, a significant decrease in VC degradation rate was observed, with no significant change in the number of *etnC* while *etnE* numbers increased (Fig. 3.2 A&B panel

f). Shaking in the presence of oxygen, however, resulted in DOC concentration decrease in both the P25 and P26 microcosms (Table 3.2). Moreover, in as well the P25 microcosms as the P26 microcosms, shaking under oxygen-exposed conditions resulted into a large reduction in the number of biomarkers associated with VC reductive dehalogenation, i.e., *D. mccartyi*, *bvcA* and *vcrA*. This was translated into the VC degradation profiles that were recorded after the microcosms were again converted to the anoxic static conditions (anoxic static-4). Removal of VC and accumulation of ethene in P26 microcosms showed a long lag phase and only started after 5 weeks of incubation (Fig. 3.2A panel g). However, after the second and third VC spike, near-complete revival of reductive dechlorinators was observed as can be deduced from high VC degradation rates, stoichiometric accumulation of ethene (Fig. 3.2A panel g) and enrichment of *D. mccartyi* (Fig. 3.2B panel g). In contrast, in P25 microcosms, neither VC degradation (Fig. 3.2C panel g) nor revival of *D. mccartyi* (Fig. 3.2D panel g) was noticed.

Table 3.1. First order VC/cDCE degradation rates observed in the VC/cDCE spiked microcosms containing P25 and P26 sediment. The rates were determined by linear regression of concentration/time series during degradation of the third spike of VC/cDCE unless otherwise indicated. Presented data are the average of duplicate analyses.

Culture conditions	k [day^{-1}]			
	P26 VC	P25 VC	P26 cDCE	P25 cDCE
Anoxic non-shaking-1	0.81	0.37	0.70	0.27
Oxygen-exposed non-shaking	1.26	1.26	0.15	0.01
Anoxic non-shaking-2	0.36	0.13	0.49	0 [a]
Anoxic shaking	0.19	0.03	0.16	-
Anoxic non-shaking-3	0.45	0.30	0.28	-
Oxygen-exposed shaking	0.01[a]	1.86	0.01[a]	-
Anoxic non-shaking-4	0.22	0	0.31	-

[a] Determined at the end of first spike

Table 3.2. DOC measurements in the VC/cDCE spiked microcosms containing P25 and P26 sediment after degradation of the third spike of VC/cDCE unless otherwise indicated. Presented data are the average of duplicate analyses.

Culture conditions	DOC (mg/L)			
	P26 VC	P25 VC	P26 cDCE	P25 cDCE
Anoxic non-shaking-1	17.94	18.72	19.02	18.72
Oxygen-exposed non-shaking	16.33	17.43	18.97	13.43
Anoxic non-shaking-2	ND [a]	ND	ND	12.62 [b]
Anoxic shaking	17.32	19.78	19.18	-
Anoxic non-shaking-3	ND	ND	ND	-
Oxygen-exposed shaking	11.16	10.81	10.5	-
Anoxic non-shaking-4	11.31	11.51	11.9	-

[a] Not done

[b] Determined at the end of first spike

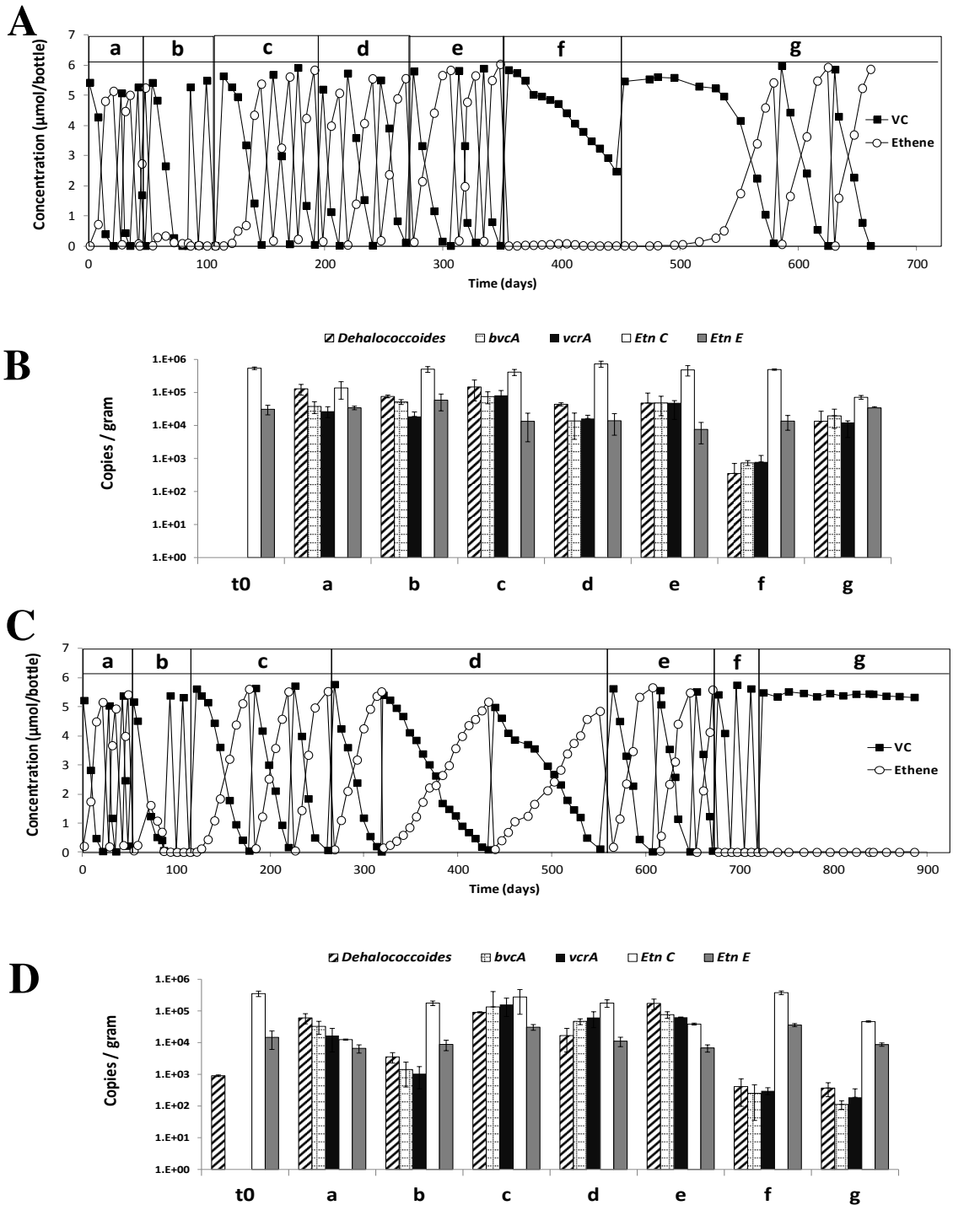


Fig. 3.2. VC (■) degradation and accumulation of ethene (○) (A, C) and concomitant changes in numbers of the *D. mccartyi* 16S rRNA gene, *bvcA*, *vcrA*, *etnC* and *etnE* genes (B, D) in VC spiked microcosms prepared from sediment materials of P26 (A, B) and P25 (C, D). Presented data in graph A and C are the average of duplicate analysis on two independent samples. For all conditions, the numbers of the gene markers were determined in samples taken just before the treatment conditions were changed. Each bar represents the average of triplicate qPCRs performed on one sample of each duplicate microcosm (n = 6). Panels: t0: time zero; a: anoxic static-1; b: oxygen-exposed static; c: anoxic static-2; d: anoxic shaking; e: anoxic static-3; f: oxygen-exposed shaking; g: anoxic static-4.

3.3.2 Dynamics of cDCE degradation and associated biomarkers

In cDCE spiked microcosms containing as well P26 as P25 sediments, cDCE degradation profiles were identical to those observed for VC degradation during initial anoxic static incubation, with stoichiometric conversion of cDCE to ethene (Fig. 3.3A&C panel a). Under subsequent oxygen-exposed static conditions, in P26 microcosms, high cDCE degradation rates maintained (Table 3.1), but with higher accumulation of ethene compared with the VC spiked microcosms of P26 (Fig. 3.3A panel b) and even with higher numbers of *D. mccartyi* (Fig. 3.3B panel b) as compared to those recorded after anoxic incubation (Fig. 3.3B panel a). In contrast, in cDCE spiked P25 microcosms, the observed cDCE degradation profile under oxygen-exposed static conditions was completely different. After the first cDCE spike, high cDCE degradation was coupled to pronounced accumulation of ethene showing activity of anaerobic OHRB (Fig. 3.3C panel b). After the second cDCE spike, however, cDCE degradation rates dramatically decreased and no cDCE removal was noted after the third spike (Fig. 3.3C panel b). Concomitant with this stall of cDCE removal, there was a more than two orders of magnitude decrease of *D. mccartyi*, *vcrA* and *bvcA* numbers (Fig. 3.3C panel b). Moreover, long-term oxygen exposure resulted in reduction of DOC only in microcosms of P25 as compared with the DOC measured under anoxic static condition (Table 3.2). To investigate the revival of reductive dechlorinators, the headspace of the microcosms was flushed with nitrogen (leading to the sudden drop of cDCE in P25 microcosms at the end of the treatment under oxygen-exposed static conditions, Fig. 3.3C panel b) to establish anoxic condition. After cDCE addition under subsequent anoxic static condition (anoxic static-2), cDCE degradation only resumed in P26 microcosms with stoichiometric conversion of cDCE to ethene during degradation of the second and third spikes (Fig. 3.3A panel c) and a concomitant increase in *D. mccartyi*/*vcrA*/*bvcA* numbers (Fig. 3.3B panel c). No degradation of cDCE (Fig. 3.3C panel c) or revival of *D. mccartyi* (Fig. 3.3D panel c) was noted in P25 microcosms even after a prolonged incubation of 280 days. Hence, the experiments with P25 microcosms were stopped and no further manipulation was performed. In microcosms containing P26 material, the cDCE dechlorination trends observed under subsequent anoxic shaking and anoxic static-3 conditions were identical to those recorded for VC biodegradation in the VC spiked P26 microcosms, i.e., shaking under anoxic conditions slightly decreased the degradation rate (Fig. 3.3A panel d) and the number of biomarkers (in

particular *vcrA* and *etnE*) (Fig. 3.3B panel d), while subsequent incubation under anoxic static conditions alleviated the effects of shaking (Fig. 3A&B panel e). No substantial cDCE removal was noted under oxygen-exposed shaking conditions (Fig. 3.3A panel f) although the number of *etnE* increased (Fig. 3.3B panel f). As observed for VC spiked microcosms, oxygen exposure under shaking conditions the reduced DOC concentrations in these microcosms (Table 3.2). During the subsequent incubation under anoxic static conditions (anoxic static 4), cDCE dechlorination resumed after 6 weeks with stoichiometric ethene production after the second and third cDCE spike (Fig. 3.3A panel g) and concomitant increase in *D. mccartyi*, *bvcA* and *vcrA* numbers (Fig. 3.3B panel g).

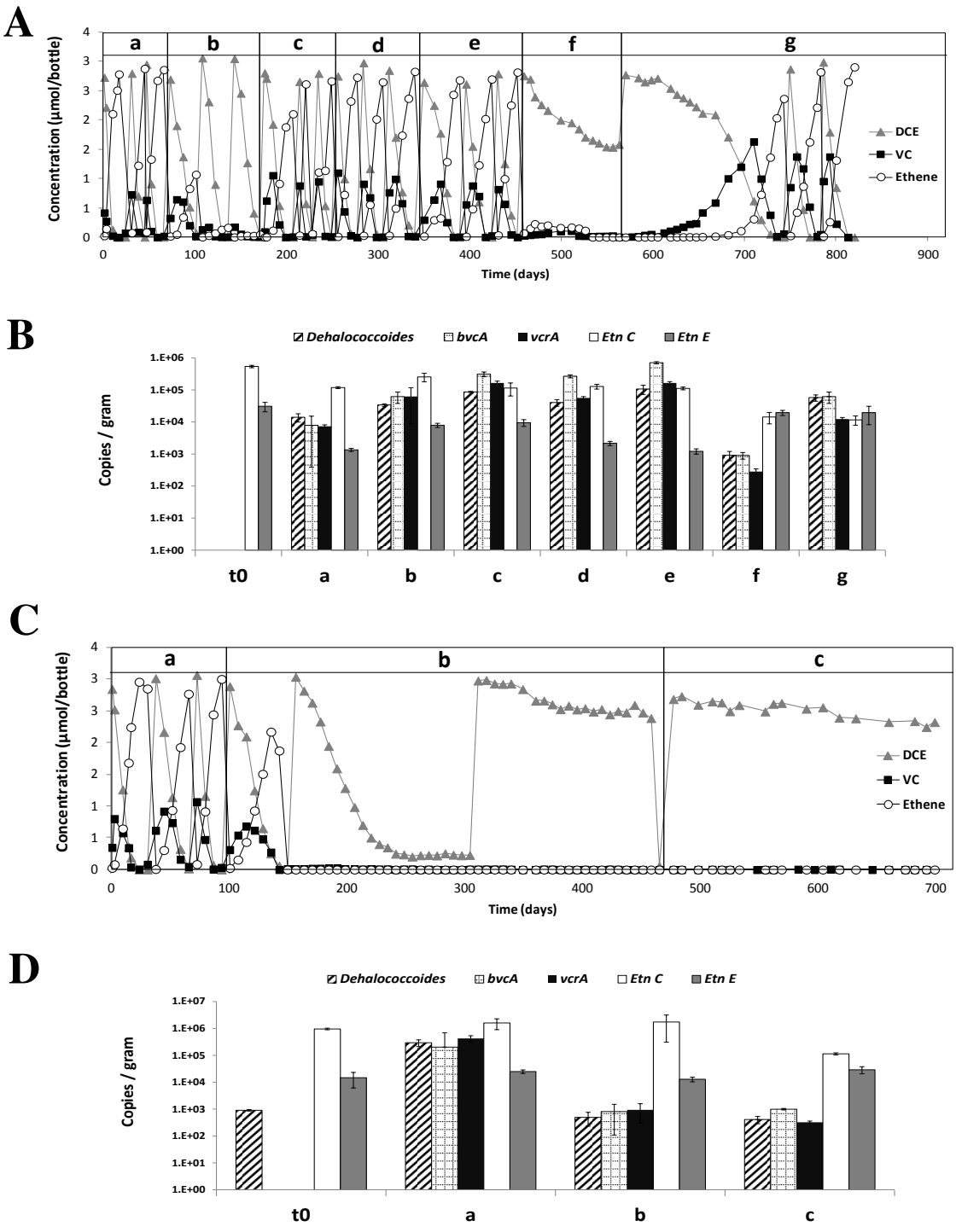


Fig. 3.3. cDCE (Δ) degradation and accumulation of VC (■) and ethene (○) (A, C) and concomitant changes in numbers of the *D. mccartyi* 16S rRNA gene, *bvcA*, *vcrA*, *etnC* and *etnE* genes (B, D) in cDCE spiked microcosms prepared from sediment materials of P26 (A, B) and P25 (C, D). Presented data in graph A and C are the average of duplicate analysis on two independent samples. For all conditions, the numbers of the gene markers were determined in samples taken just before treatment conditions were changed. Each bar represents the average of triplicate qPCRs performed on one sample of each duplicate microcosm (n = 6). Panels: t0: time zero; a: anoxic static-1; b: oxygen-exposed static; c: anoxic static-2; d: anoxic shaking; e: anoxic static-3; f: oxygen-exposed shaking; g: anoxic static-4.

3.4 Discussion

Our previous study showed that surficial stream sediment may benefit from both anaerobic and aerobic pathways for degradation of VC (see Chapter 2). Depending on ambient redox conditions, chlorinated ethenes can serve either as electron donors or terminal electron acceptors for microbial energy metabolism. Hence, we hypothesized that due to flow reversal in the streambed and subsequent local redox fluctuation, microbial mechanisms for degradation of chloroethenes, which are fundamentally redox processes, can be strongly affected.

Rapid mineralization of VC and *c*-DCE was observed under initial anoxic conditions confirming the high organohalide respiration capacity in microbial communities indigenous to both locations P26 and P25. During subsequent oxygen-exposed static incubation, the removal of VC in microcosms of both locations, and of cDCE in P26 microcosms, showed high resilience of aerobic degraders against long term incubation under strict anoxic conditions as reported earlier by Schmidt and Tiehm (2008) using ground water microcosms and Gossett using mixed enrichment cultures (2010). However, besides enrichment of *etnC* and *etnE* in VC/cDCE spiked microcosms of P26, the accumulation of reduced daughter products in these microcosms especially when amended with cDCE concomitant with a stable (VC spiked microcosms) or even enriched (in cDCE spiked microcosms) number of *D. mccartyi* and *vcrA* and *bvcA* indicated mixed aerobic/anaerobic degradation in P26 microcosms under oxygen-exposed conditions. In contrast, in VC-spiked microcosms containing P25 material under oxygen-exposed static conditions, the number of *D. mccartyi*, *vcrA* and *bvcA* dropped more than one order of magnitude with no accumulation of reduced daughter products while pronounced VC removal was coupled to enrichment of *etnE/etnC* numbers indicating that VC biodegradation in the sediments of P25 appears to be strictly associated with aerobic VC assimilators. In contrast to VC, cDCE degradation under static oxygen-exposed conditions was not observed in P25 microcosms, suggesting the lack of an aerobic cDCE degradation capacity in sediments of this location. Unlike aerobic VC assimilating bacteria, assimilative aerobic cDCE degradation has been rarely observed (Mattes *et al*., 2010).

When the oxygen-exposed microcosms were again converted to anoxic conditions (anoxic static-2), stoichiometric conversion of VC (in microcosms containing as well P25 as P26 material) and of cDCE (only in microcosms containing P26 material) to ethene was observed

after the second and third spike with concomitant enrichment of *D. mccartyi* indicating the recovery of OHR as the main degradation pathway in those microcosms and an oxygen resistant fraction of *D. mccartyi*. This is in accordance with the results obtained in Chapter 2 where it was suggested that the low organic carbon and larger grain size in sediment microcosms of P25 led to extensive oxygen penetration and consequently affected *D. mccartyi* numbers negatively while this was not the case for P26 material that contains high TOC and fine grain size. This suggests that under oxygen-exposed static conditions, the main VC/cDCE degraders in P26 microcosms were reductive dechlorinators that were protected from oxygen inhibition in deeper sediment layers or sediment aggregates/biofilms. However, in contrast to VC biodegradation, no resilience of cDCE dechlorinators was noted in microcosms containing P25 material as cDCE biodegradation in these microcosms did not reinstall under anaerobic conditions. This could be due to the prolonged oxygen exposure (370 days) during the oxygen-exposed static step in cDCE amended microcosms compared to VC amended microcosms or alternatively, due to the presence of different VC and cDCE OHRB with different sensitivity toward oxygen in P25 sediments. An alternative explanation is the lower DOC concentrations and hence potential lower availability of potential sources of natural electron donors required for OHR. Indeed, the DOC concentrations were highly reduced after long-term oxygen exposure in these microcosms (Table 3.2). Under the anoxic shaking conditions, degradation rates were negatively affected especially during degradation of the third cDCE spike (Table 3.1). Due to vigorous shaking and the resulting mechanical disturbance, the integrity of sediment particle-associated biofilms can be disrupted. Such a disturbance can affect cellular respiration, viability and overall bacterial community structure by rendering them susceptible to substances such as chlorinated ethenes (Chu & Alvarez-Cohen, 1999).

Interestingly, while under oxygen-exposed shaking conditions, substantial and rapid VC removal was observed in VC spiked P25 microcosms which was is in line with the oxidative assimilative VC degradation capacity in these microcosms observed under oxygen-exposed static conditions, only slow and limited removal of VC/cDCE was observed in P26 microcosms suggesting rather co-metabolic aerobic degradation than metabolic aerobic degradation. This suggests presence of different aerobic VC degrader populations at locations P25 and P26, i.e., VC assimilating ethenotrophs at P25 and VC cometabolic

ethenotrophs at P26. Interestingly, it has been suggested that VC assimilating bacteria are ethene-assimilating bacteria that adapted to growth on VC as a sole carbon source (Verce *et al*., 2001; Jin & Mattes, 2008). The switch from ethene to VC as substrate is due to point mutations in the alkene/epoxide catabolic gene i.e. *etnE* (Jin *et al*., 2010). This might point toward the role of oxygen penetration and organic carbon distribution in the evolution of aerobic VC assimilating populations in hyporheic zones. At P25, the site properties such as low organic carbon load, coarse grain size and extensive oxygen penetration into the sediment will result into a limited anaerobic degradation potential. This is in accordance with the relatively high VC concentrations (up to 2600 µg/L VC) found at location P25 at 20 cm depth (Hamonts *et al*., 2009). This implies exposure of ethenotrophs to relatively high VC concentrations in the oxic zones at the P25 location and hence the occurrence of selective conditions for VC assimilators. In contrast, at P26, due to the high anaerobic dechlorination activity in deeper sediment layers, negligible VC concentration reached the top sub-oxic and oxic sediment layers (from non-detectable up to 81 µg/L, Hamonts *et al*., 2009) decreasing the selective conditions for adaptation of ethenotrophs to VC assimilation. Instead, non-VC degrading ethenotrophs are selected due to the production of ethane from VC dechlorination. Moreover, the limited oxygen penetration into the sediments of P26 due to the compact structure and high organic carbon content (which leads to rapid oxygen consumption) (Chapter 2), would negatively impact growth of aerobic VC assimilators. These findings emphasize the need for the development of new quantitative molecular methods to distinguish between real VC-assimilating bacteria and ethenotrophs that degrade VC only co-metabolically. This can also be of critical importance for field applications and CAH-contaminated site management where disappearance of VC is interpreted as aerobic VC degradation. As shown here, the unexplained disappearance of VC at some sites, could be either due to its actual assimilation or a combination of reductive dechlorination of VC to ethene and its further oxidation to CO_2 by ethenotrophs, leading to mischaracterized “aerobic degradation” of VC. Therefore, caution should be taken in assessment of effectiveness of aerobic VC degradation at a specific site. Until development of such molecular tools capable of distinguishing genotypic features between aerobic VC degraders and ethenotrophs, application of alternative/complimentary approaches such as the recently developed compound-specific isotope analysis of ethene is highly advised to reduce

the uncertainty by distinguishing between actual VC assimilation and apparent VC disappearance, and lack of mass balance that is related to biotransformation of ethene (Mundle *et al*., 2012).

The revival of reductive dechlorinators was noted only in VC/cDCE spiked microcosms containing P26 material when microcosms were converted from oxygen-exposed shaking to anoxic static-4 but not in P25 microcosms. However, an identical trend in the reduction of DOC was noticed in all microcosms at the end of the oxygen-exposed shaking and anoxic non-shaking-4 incubations. Therefore, the suppression of OHR in the P25 microcosms is likely not due to the exhaustion of primary electron donors in these microcosms. Apparently the *D. mccartyi* population was protected to some degree in sediments of P26 where we previously noted high organic carbon load and low and compact grain size (Chapter 2) indicating that the observed resilience of *D. mccartyi* was due to the site properties. Interestingly, in cDCE-spiked P26 microcosms, accumulation of VC started 6 weeks after oxygen removal while ethene accumulation took place only after 15 weeks of incubation (Fig. 3A, panel g). This is in line with the results of Amos *et al*. (2008) who showed strain specific response to oxygen i.e. *D. mccartyi* strains such as isolates GT and BAV1 that metabolically dechlorinate VC to ethene were more susceptible to oxygen inhibition than strain FL2 capable of cDCE-to-VC reduction. Accordingly, we also observed a slightly better recovery of *bvcA* gene numbers at the end of anoxic static-4 VC dechlorination step.

3.5 Conclusion

In contrast to the resistance and resilience of aerobic degraders toward strict anoxic conditions, a high sensitivity of *D. mccartyi* was observed to oxygen exposure which is consistent with previous reports using pure and enrichment sediment free cultures (Adrian *et al*., 2007; Amos *et al*., 2008). However, as shown with the sediments of P26, the site physico-chemical properties might shield *D. mccartyi* against local redox fluctuations. Therefore, at locations with high organic carbon load, infiltration of oxygenated surface water into hyporheic sediments is probably not detrimental to *D. mccartyi*. However, these properties might have affected the possible adaptation of ethenotrophs to aerobic VC assimilation. Therefore, we noticed a highly possible role of appropriate VC concentrations and sufficient exposure time governed by site physico-chemical properties as the selective pressure in the phenomenon of spontaneous evolution of ethenotrophs to VC degradation.

Chapter 4: Evaluation of solid polymeric organic materials for use in bioreactive sediment capping to stimulate the degradation of chlorinated aliphatic hydrocarbons

4.1 Introduction

Contamination with chlorinated aliphatic hydrocarbons (CAHs) poses serious threats to groundwater quality in industrialized countries (Abelson, 1990). In case of limited attenuation in the aquifer and riverbed sediment, discharges of CAH contaminated groundwater into surface water systems present an additional environmental concern (Hamonts *et al*., 2009). *In situ* capping is a promising technology for reducing the exposure of biota present in the surface water column to contaminants present in or leaking from the sediments. Passive *in situ* capping is achieved by isolating the contaminated sediment through coverage with clean material media such as sand gravel and clean sediment (Go *et al*., 2009). Such barriers are based on physical separation and are especially useful for containment of sediments contaminated with strongly sorbed hydrophobic pollutants. They are, however, not effective for mitigating contamination of the surface water that is affected

by discharging groundwater plumes containing mobile persistent contaminants (Himmelheber *et al*., 2007). An innovative alternative *in situ* capping technique that addresses particularly sites affected by mobile contaminants is active capping, a technology that aims at sequestering or transforming contaminants that are easily released from the sediment (Reible *et al*., 2006; Knox *et al*., 2008; Himmelheber *et al*., 2011). The focus in the development of reactive caps has been on the application of physicochemical processes for contaminant removal primarily by retarding the contaminants through sorption. The drawback of this approach is that the risk to human and environmental health returns once the capping material is saturated (Sun *et al*., 2010a). Therefore, recent developments in active sediment capping include the *in situ* bioreactive capping approach in which the capping layer hosts and stimulates microorganisms that can degrade the pollutants (Himmelheber *et al*., 2011).

A major biological process that contributes to biodegradation of CAHs is organohalide respiration (OHR). In this process, specific bacterial groups such as *Dehalococcoides mccartyi*, use suitable electron donors, which is often hydrogen, for respiration on CAH which results in their dechlorination (Smidt & de VOS, 2004). Well known examples are the stepwise dechlorination of the groundwater pollutants tetrachloroethene (PCE) and trichloroethene (TCE) to ethene through *cis*-dichloroethene (cDCE) and vinyl chloride (VC). OHR is stimulated by the addition of a substrate that is converted by fermentation into the electron donor needed for OHR. Stimulation of OHR of CAHs in bioreactive caps can as such be achieved by incorporation of suitable solid polymeric organic materials (SPOM), which allows a long term production of the OHR supporting electron donors, including H_2. However, other H_2 consuming anaerobic organisms like sulfate, and ferric iron-reducing bacteria, homoacetogens and methanogens will compete with organohalide respiring bacteria (OHRB) for the produced H_2. Especially methanogens are considered as unwanted and main competitors of OHRB such as *D. mccartyi* due to their exclusive dependence on reducing equivalents such as H_2 and acetate (Smatlak *et al*., 1996; Wei & Finneran, 2012).

Although the application of SPOMs as a slow release electron source for biostimulation of reductive dechlorination has been reported (Wu *et al*., 1998; Vera *et al*., 2001; Kassenga *et al*., 2003; Brennan *et al*., 2006), this has not been related with the microbial ecology of such systems. For instance, it is not known how the presence of SPOMs affects the microbial

community and especially OHRB, such as *D. mccartyi*, as the main mediators of CAH degradation, and methanogens as the main competitors of OHRB for reducing equivalents. Moreover, information on whether these bacteria are colonizing the capping material and in particular the SPOMs, is lacking. In this study, the long term potential of five different solid organic materials as electron donors to stimulate reductive dechlorination of cDCE and VC was evaluated. The SPOM materials were chosen from waste products with the dual aim of stimulation of reductive dechlorination and waste recycling. We hypothesized that different SPOMs will result in different degradation patterns and stimulation effects of *D. mccartyi* and methanogens. SPOMs were introduced into sediment microcosms prepared from the Zenne river-bed sediment impacted by discharging VC/cDCE polluted groundwater. The sediments have previously been shown to contain VC/cDCE OHRB including *D. mccartyi* (Hamonts *et al*., 2012; Atashgahi *et al*., 2013). The intermediate volatile short chain fatty acids (SCFAs) formed during the hydrolysis/fermentation of SPOMs were identified and their impact on the abundance of *D. mccartyi* and the genes involved in CAH respiration, archaea and methanogens in the sediment and on the SPOMs, were quantified by quantitative PCR (qPCR) assay. Finally, the efficiency of the different SPOM in stimulating VC dechlorination when installed at the Zenne site as 40 cm capping layer, was evaluated *in silico* using a 1D numerical model.

4.2 Materials and Methods

4.2.1 Materials

River-bed sediment samples were taken from the Zenne River in Machelen-Vilvoorde, Belgium in May 2010 at a site that has been previously described (Hamonts *et al*., 2009). At that site, a VC/*c*-DCE contaminated groundwater plume of approximately 1.4 km width flows in the northwestern direction into the Zenne River (Fig. 4.1). The sediment samples were obtained from the top 80 cm river-bed sediment at the post 25 location. Groundwater was obtained from the SB2 groundwater extraction well near the right river bank as outlined by Hamonts *et al*. (2012). The SPOMs used were wood chips, hay, straw, tree bark, and shrimp waste containing 48, 42, 46, 40, and 38% carbon, respectively, and 0.07, 0.44, 0.09, 0.28 and 8.94% nitrogen, respectively.

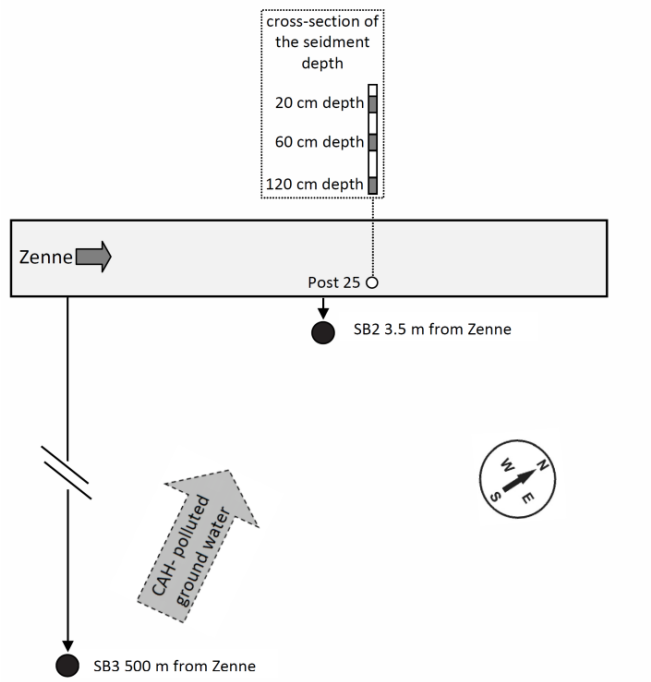


Fig. 4.1. Schematic representation of the locations of interstitial water samplers (ISW) (grey cylinder at small open dot), at post 25 and monitoring wells SB2 (large black dots) monitored in this study. SB3 shows the area close to plume source. Flow directions of the CAH plume and Zenne River are indicated by arrows.

4.2.2 Sediment microcosm set-up

Microcosms were prepared in 160-mL glass serum bottles containing 20 g wet and well-mixed sediment obtained from the top 80 cm depth of Zenne river-bed, 70 mL of ground water and a particular SPOM. The amounts of SPOM added to the microcosms were different for each SPOM, but were chosen to obtain an equal amount of initially released dissolved organic carbon (DOC) in all setups. To determine this, in a preliminary experiment, 1 g of each SPOM, cut into pieces of 4-5 mm, was incubated at room temperature on a shaker (50 rpm) in 100 ml of groundwater in 500 mL glass vials. After 1 and 8 hours of incubation, samples were taken and analyzed for the DOC concentration. Afterwards, based on the DOC release from wood chips that was used as the reference, the amount of each SPOM was determined. The SPOMs, cut into pieces of 4-5 mm, were placed into non-sorptive and permeable membrane pockets made from polyamide membrane 49 PA 6/5 (Hendrickx *et al.*, 2005) and introduced into the corresponding sediment microcosms in duplicate. All bottles were sealed with Teflon-lined butyl rubber stoppers followed by

addition of VC at final concentrations of 70 µM in liquid phase and incubated at room temperature in an anaerobic glove box (Don Whitley Scientific ltd, West Yorkshire, UK) under N_2 atmosphere. Time course liquid samples were withdrawn and filtered over a 0.45-µm filter for DOC/SCFAs measurements. After degradation of the first VC spike, microcosms were flushed with N_2 followed by a second VC spike at identical initial concentration. After three consecutive additions of VC, cDCE was added to microcosms and its degradation was followed for another three consecutive cycles, unless otherwise stated. To study longevity of SPOM effects, the polyamide bags were replaced with new bags containing fresh SPOMs and the microcosms were used for long-term cDCE degradation. Each time when cDCE was degraded, the microcosms were flushed with N_2 and cDCE was replenished.

4.2.3 Sampling for molecular analysis and DNA extraction

Before the initiation of the experiment, approximately 0.5 g of each SPOM was used for DNA extraction in duplicate. After dechlorination of the third VC spike, the sediment microcosms were decapped in the anaerobic glove box. The polyamide bags containing the SPOMs were opened and few pieces of SPOM were withdrawn from both replicates. The SPOMs were washed with sterile water to remove sediment particles and used for DNA extraction. Afterwards, the bottles were capped again, mixed thoroughly, opened and approximately 2 gram slurry samples were withdrawn from all duplicate microcosms. The same procedure was followed at the end of third cDCE spike and at the end of the long-term cDCE dechlorination cycles. DNA was extracted from the samples as described previously (Hendrickx *et al*., 2005).

4.2.4 Quantitative PCR

Real-time quantitative PCR (qPCR) targeting Bacteria, *D. mccartyi*, and reductive dehalogenase genes *vcrA* and *bvcA* was performed as described in Chapter 2. qPCR targeting archaea and the methanogenic families *Methanosarcinaceae* and *Methanosaetaceae* was performed using the primer sets described by Yu *et al*. (2005) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The primer sets used in qPCR for targeting archaea, *Methanosarcinaceae* and *Methanosaetaceae* numbers are reported in Table 4.1. The reaction mixture of 20 µL consisted of 10 µL of GoTaq® qPCR Master Mix (Promega, Madison, Wis), 3.5 µL of nuclease-free water and 0.75 µL of each primer (final concentration of 375 nM) and 5 µL of template DNA. The qPCR program consisted of a predenaturation

step of 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and simultaneous annealing and extension for 1 min at 60 °C. All DNA-samples were analyzed in triplicate. The fraction of *D. mccartyi* as compared to total bacteria was estimated by dividing the number of *D. mccartyi* 16S rRNA gene copies by the copy number of bacterial 16S rRNA genes multiplied by 100. The ratios of *D. mccartyi* to archaea, archaea to bacteria, *vcrA* to *D. mccartyi*, and *bvcA* to *D. mccartyi* were determined in the same way.

Table 4.1. Overview of DNA oligonucleotide primers used in this study.

Primer	Sequence	Target 16S rRNA gene	Reference
ARC787F	5´-ATTAG ATACC CSBGT AGTCC-3´	Archaea	Yu *et al*., 2005
ARC1059R	5´-GCCAT GCACC WCCTC T-3´		
Msc380F	5´-GAAAC CGYGA TAAGG GGA-3´	*Methanosarcinaceae*	Yu *et al*., 2005
Msc828R	5´-TAGCG ARCAT CGTTT ACG-3´		
Mst702F	5´-TAATC CTYGA RGGAC CACCA-3´	*Methanosaetaceae*	Yu *et al*., 2005
Mst862R	5´-CCTAC GGCAC CRACM AC-3´		

4.2.5 Model Development

The efficiency of the different capping materials when installed at the Zenne site, was evaluated *in silico* by means of a 1D numerical model using PHREEQC (Parkhurst & Appelo, 1999). The model consists of 75 cells with a cell size of 2 cm to describe 10 cm of aquifer, 1 m of sediment and a capping layer of 40 cm. The site has been monitored intensively regarding CAH concentrations and temperature values in the sediments and those results were used in the model (Hamonts *et al*., 2012; Ebrahim *et al*., 2012). The domain is characterized by a Darcy velocity of 2 cm/day and a small dispersivity of 0.1 m. An average measured groundwater temperature of 12 °C was implemented as temperature. The influent cDCE and VC concentrations were set at 22 µg/L and 1222 µg/L, respectively, which corresponds with the largest concentration measured at the bottom of the sediment during the entire monitoring period. First order degradation rates for cDCE and VC in the aquifer were obtained by calibration to observed concentrations. The degradation rates in sediment and in the capping materials were obtained by linear regression of concentration time series in batch experiments at 12, respectively 20 °C. The observed degradation rates were recalculated to 12 °C using the Arrhenius equation assuming that the rate is halved with a temperature decrease of 10 °C (Appendix 2).

4.2.6 Analytical methods

Total mass of CAHs, methane, ethene and ethane were quantified by means of gas chromatography (GC) in a GC apparatus equipped with a flame ionization detector as described in Chapter 2. DOC was determined from samples as the difference between total dissolved carbon and dissolved inorganic carbon, measured with a Shimadzu TOC-5000 analyzer with ASI-5000 auto-sampler. Total Nitrogen was detected after all particulate carbon was removed by filtration (pore size 0.45 µm). Carbon and nitrogen content of the SPOMs were determined using the method reported in Nieuwenhuize *et al*. (1994). SCFAs (C2-C5) were analyzed in ether extracts from aqueous solutions as previously described (Calli *et al*., 2008).

4.3 Results

A minimum DOC value of 60 mg/L was previously reported to be required to support dechlorination of 23 µM PCE beyond cDCE in microcosm studies (Lee *et al*., 1997). Therefore, the amount of DOC released from different SPOMs as a source of carbon and electrons after 1 h of incubation in ground water was assessed in this study (Table 4.2). Wood chips material released 50.4 mg/l of DOC, and was used as a reference, and 1, 0.173, 0.725, 3.33, and 1.37 g of wood chips, hay, straw, tree bark, and shrimp waste, respectively, were added to microcosms to reach equal initial DOC values.

Table 4.2. Natural DOC release from SPOMs after 1 hour shaking in groundwater.

SPOMs	DOC (mg/L)
Wood chips	50.4
Hay	289.52
Straw	68.88
Tree bark	15.16
Shrimp waste	36.7

After the first VC spike, microcosms amended with tree bark and shrimp waste showed the shortest lag phase (around 7 days) and high initial degradation rates (Fig. 4.2). Microcosms amended with straw and wood chips showed the longest lag phase of 28 days after which high VC dechlorination rates were established in both microcosms (Fig. 4.2A). Although dechlorination was also observed in microcosms that were not amended with exogenous electron donor, addition of SPOMs in general led to 3-4 times higher degradation rates

compared to incubations without SPOM (Table 4.3). After the second VC spike, dechlorination started immediately with pronounced rates of VC, with highest rates observed in the microcosms amended with tree bark and shrimp waste (Table 4.3). Accordingly, complete VC removal was obtained within 14 days. The produced ethene was further reduced to ethane in microcosms containing hay, straw and tree bark (Fig. 4.2C). An identical trend was observed after the third VC spike with the exception of microcosms amended with wood chips as those apparently lost their stimulatory effect (Fig. 4.2A).

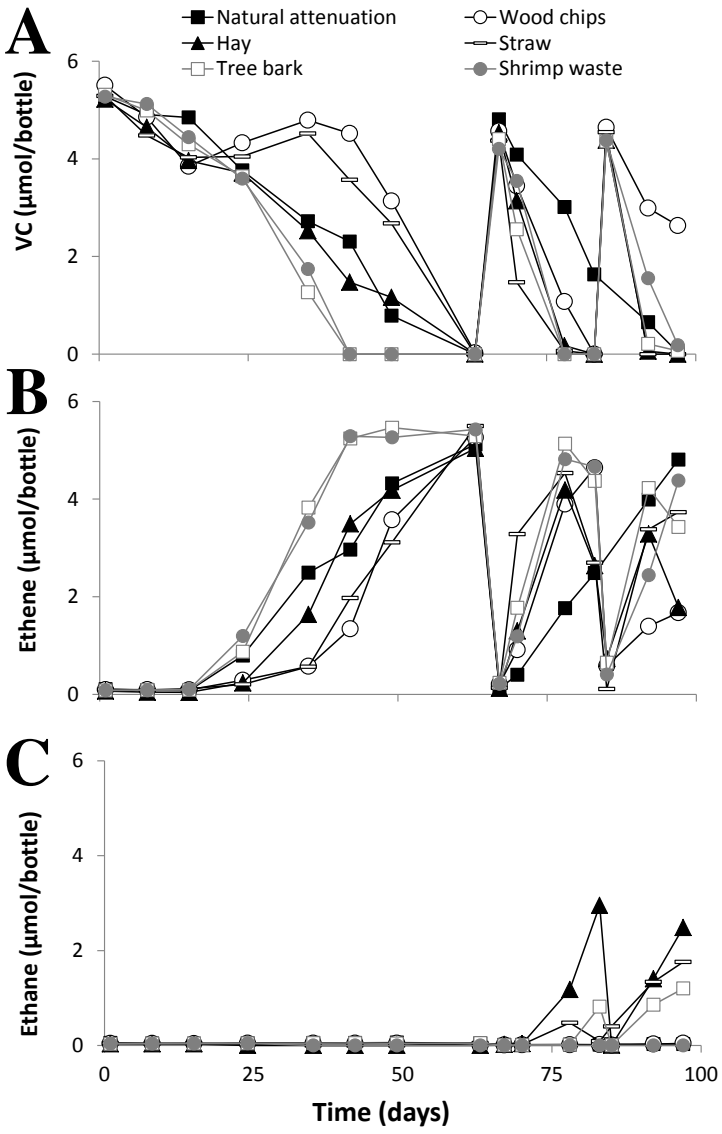


Fig. 4.2. Anaerobic dechlorination of VC (A) and accumulation of ethene (B) and ethane (C) in sediment microcosms containing different solid polymeric organic materials (SPOMs) as a source of carbon and electrons. Data shown are average values obtained from duplicate microcosms. Error bars are not shown for clarity.

Table 4.3. First order VC/cDCE degradation rates obtained by linear regression of concentration time series in batch experiments during first addition of SPOMs to microcosms. Presented data are the average of duplicate analysis.

Conditions	k [day^{-1}]					
	Natural attenuation	Wood chips	Hay	Straw	Tree bark	Shrimp waste
1st VC spike	0.05	0.19	0.17	0.22	0.20	0.20
2^{ed} VC spike	0.21	0.35	0.38	0.38	0.58	0.59
3th VC spike	-	0.05	0.61	0.87	0.43	0.26
1st cDCE spike	0.07	0.03	0.54	0.60	0.50	0.18
2^{ed} cDCE spike	-	-	0.74	0.80	0.53	0.16
3th cDCE spike	-	-	0.36	0.57	0.45	0.24

The stimulation of CAH degradation by SPOMs remained evident after VC was replaced by cDCE as the target CAH (Fig. 4.3A). The microcosms amended with hay, straw, and tree bark sustained high rates of cDCE dechlorination for three consecutive spikes (Table 4.3), with no accumulation of VC (Fig. 4.3B) and dechlorination proceeded to ethene (Fig. 4.3C) and eventually to ethane as compared with unamended microcosms (Fig. 4.3D). In contrast, the microcosms amended with shrimp waste showed intermediate degradation rates with all cDCE spikes whereas, wood chips amended microcosms did not show stimulated cDCE degradation as compared with the unamended microcosms.

During hydrolysis and fermentation of organic matter released from the different SPOMs, different profiles and amounts of SCFAs were produced (Fig. 4.4). Microcosms amended with shrimp waste showed the highest accumulation of SCFAs followed by straw and hay. Acetate showed the highest concentration with a peak at day 14, followed by propionate and butyrate. Propionate production was exceptionally high with an increasing trend till day 60 in the microcosms amended with straw (Fig. 4.4B). Production of other SCFAs such as isobutyrate, valerate, isovalerate, caproate and isocaproate was negligible (data not shown). Overall, the SPOM amended microcosms showed maximum accumulation of SCFAs (and DOC, Fig. 4.5) during the first 60 days of experiment.

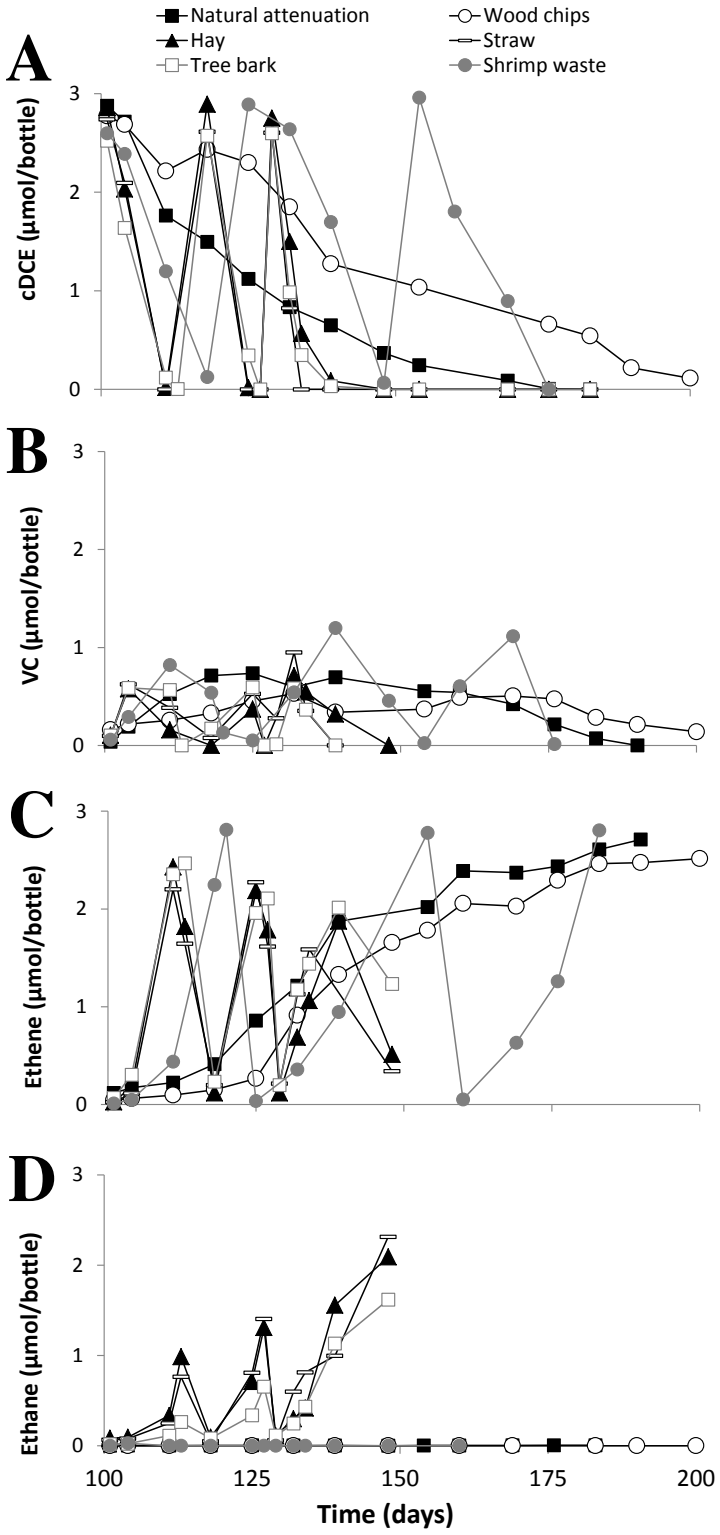


Fig. 4.3. Anaerobic dechlorination of cDCE (A) to VC (B), ethene (C) and ethane (D) in sediment microcosms containing different SPOMs as a source of carbon and electrons. cDCE was added at day 100 when degradation of VC was completed in microcosms. Data shown are average values obtained from duplicate microcosms. Error bars are not shown for clarity.

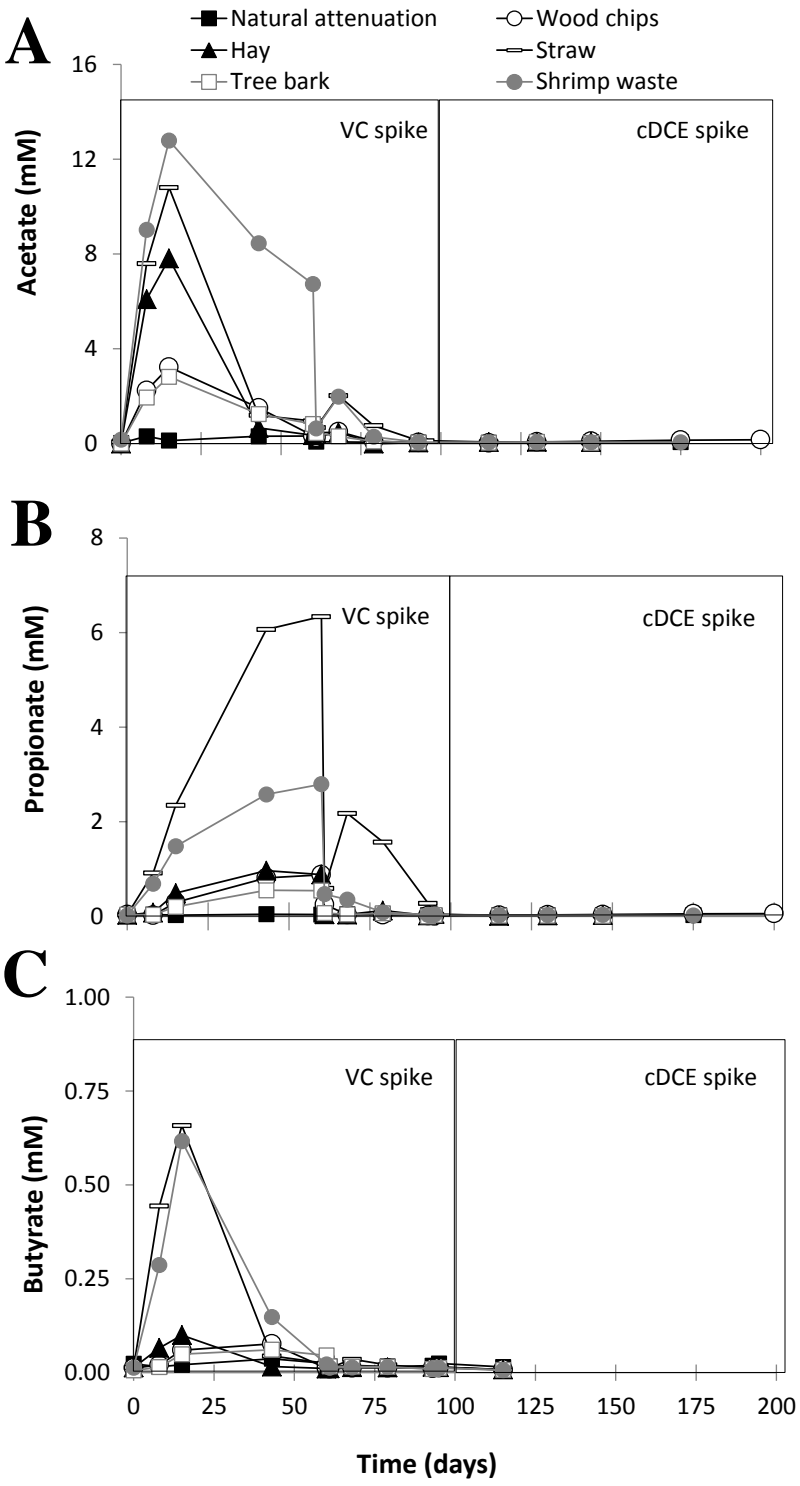


Fig. 4.4. Production of acetate (A), propionate (B) and butyrate (C) in sediment microcosms containing different SPOMs during VC and cDCE dechlorination cycles. Data shown are average values obtained from duplicate microcosms. Note that different concentration scales are used for the Y axes of panels A, B and C. Error bars are not shown for clarity.

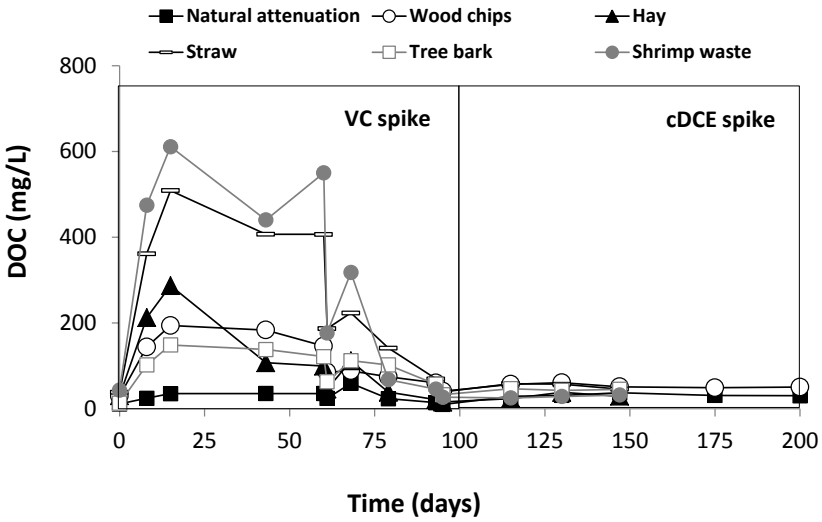


Fig. 4.5. Production of DOC in sediment microcosms containing different SPOMs during VC and dechlorination cycles. Data shown are average values obtained from duplicate microcosms. Error bars are not shown for clarity.

No substantial methane production was noticed in the first 14 days of the experiment. However, extensive methanogenesis between day 14 and 60 resulted into 171, 152, and 112 times higher methane production in microcosms amended with shrimp waste, straw and hay, respectively, as compared with natural attenuation, while this ratio was only 28 in microcosms amended with tree bark (Fig. 4.6).

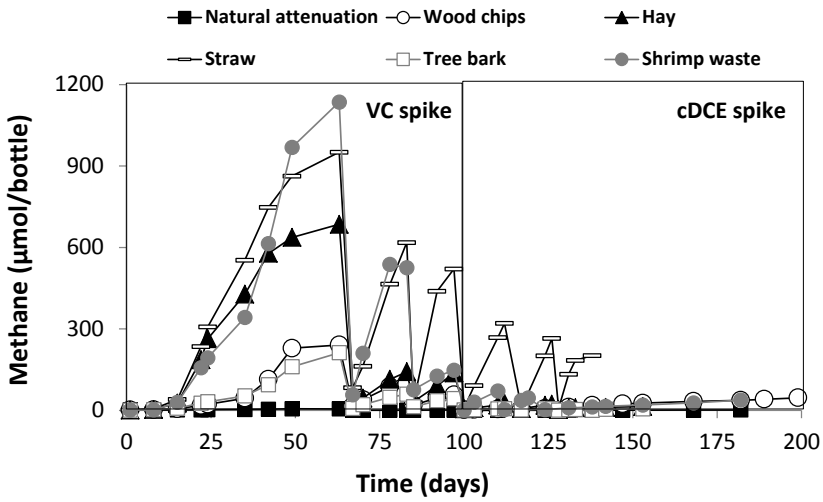


Fig. 4.6. Methane in microcosms containing different SPOMs during VC and cDCE dechlorination cycles. Data shown are average values obtained from duplicate microcosms. Error bars are not shown for clarity.

After the end of the first VC spike at day 60, regardless of the SPOM, the concentration of SCFAs (Fig. 4.4) and DOC (Fig. 4.5) decreased steadily till day 100 (end of third VC spike), concomitant with a sharp decrease in methanogenesis (Fig. 4.6). Accordingly, after day 100, when VC was replaced with cDCE as target CAH, a dramatic decrease in methanogenesis was noticed in all microcosms (Fig. 4.6) while still high cDCE dechlorination rates were sustained in microcosms amended with straw, hay and tree bark (Table 4.3). It is likely that the initial fast release of easily degradable materials led to extensive methanogenesis, while long-term incubation appears to confine methane production without affecting dechlorination rate. This hypothesis was further tested by a second round of SPOM addition into the microcosms and by following cDCE dechlorination cycles for another period of 1 year. During that period, 23-72 % of the total methane produced in all of the SPOM amended microcosms was formed during the first cDCE spike (Table 4.4) and was concomitant with a high SCFAs/DOC production (Appendix 3). While methane production decreased over time, cDCE degradation rates were sustained in microcosms amended with the lignocellulosic materials hay, straw, and tree bark indicating that these SPOMs do not become more recalcitrant over time and that their slow degradation maintains sufficiently reducing and nutrient-rich conditions to promote reductive dechlorination (Table 4.4). In contrast, extensive degradation of shrimp waste appeared to cause depletion of fermentation intermediates in a shorter time period leading to diminished cDCE degradation compared with the lignocellulosic materials. Straw amended microcosms degraded the highest mass of cDCE with the highest degradation rates. However, the ratio between the amount of methane and ethene+ethane produced was 101.7 µmol/µmol in these microcosms while this ratio was only 8.01 in microcosms amended with tree bark (Appendix 4).

Table 4.4. First order cDCE degradation rates and proportion of methane production in microcosms during long term cDCE degradation for 1 year.

Spike number	Natural attenuation		Wood chips		Hay		Straw		Tree bark		Shrimp waste	
	k[a] [day^{-1}]	CH_4[b] (%)	k [day^{-1}]	CH_4 (%)	k [day^{-1}]	CH_4 (%)	k [day^{-1}]	CH_4 (%)	k [day^{-1}]	CH_4 (%)	k [day^{-1}]	CH_4 (%)
1	0.08	33.8	0.20	63.4	0.24	68.1	0.41	26.3	0.31	23.32	0.24	72.9
2	0.09	27.3	0.15	19.1	0.76	8.94	0.75	8.01	0.76	5.82	0.74	13.2
3	0.08	22.3	0.04	10.1	1.62	5.34	1.63	7.44	1.16	6.27	0.36	5.65
4	0.08	16.5	0.02	7.31	1.13	5.31	2.03	5.06	1.14	10.9	0.34	3.51
5	-	-	-	-	0.58	3.04	1.64	7.90	0.59	5.28	0.58	1.34
6	-	-	-	-	0.59	3.06	1.17	5.05	0.58	5.73	0.58	1.35
7	-	-	-	-	0.58	1.37	1.16	3.98	0.58	3.42	0.15	0.87
8	-	-	-	-	0.59	1.70	1.16	2.84	0.59	6.22	0.08	0.78
9	-	-	-	-	0.58	1.26	1.17	2.83	0.57	5.46	0.11	0.04
10	-	-	-	-	0.33	0.30	1.17	2.84	0.42	1.31	0.06	0.18
11	-	-	-	-	0.34	0.46	1.16	2.93	0.58	0.91	0.03	0.06
12	-	-	-	-	0.49	0.64	1.59	3.04	0.49	9.99	-	-
13	-	-	-	-	0.58	0.42	1.16	2.17	0.58	3.23	-	-
14	-	-	-	-	0.58	0.05	1.16	2.16	0.59	1.14	-	-
15	-	-	-	-	0.59	0.03	1.15	2.21	0.58	1.81	-	-
16	-	-	-	-	0.58	0.03	1.16	1.13	0.58	1.49	-	-
17	-	-	-	-	0.37	0.02	0.82	2.37	0.59	4.32	-	-
18	-	-	-	-	0.33	0.01	1.18	1.9	0.57	3.35	-	-
19	-	-	-	-	-	-	1.16	2.24	-	-	-	-
20	-	-	-	-	-	-	1.16	2.07	-	-	-	-
21	-	-	-	-	-	-	0.58	2.01	-	-	-	-
22	-	-	-	-	-	-	0.57	1.94	-	-	-	-
23	-	-	-	-	-	-	0.57	1.64	-	-	-	-

[a] Degradation rates obtained by linear regression of concentration time series in batch experiments

[b] Methane proportion for each condition obtained by dividing the amount of methane produced at the end of each spike to total amount of methane produced during long term repeated batches

4.3.1 Dynamics of biomarkers

qPCRs was performed to quantify the number of 16S rRNA gene copies of total bacteria, archaea, *Methanosarcinaceae*, *Methanosaetaceae* and *D. mccartyi*, and functional genes associated with reductive dechlorination of VC (*vcrA* and *bvcA*), to assess the dynamics in the population sizes of microbial guilds involved in OHR and methanogenesis in the sediment and associated with the SPOM materials. At the end of the VC dechlorination cycles, the 16S rRNA gene copy numbers of bacteria (Fig. 4.7) and archaea (Fig. 4.8) increased in all microcosms, and in particular on SPOM martials where their abundance increased generally more than two orders of magnitude.

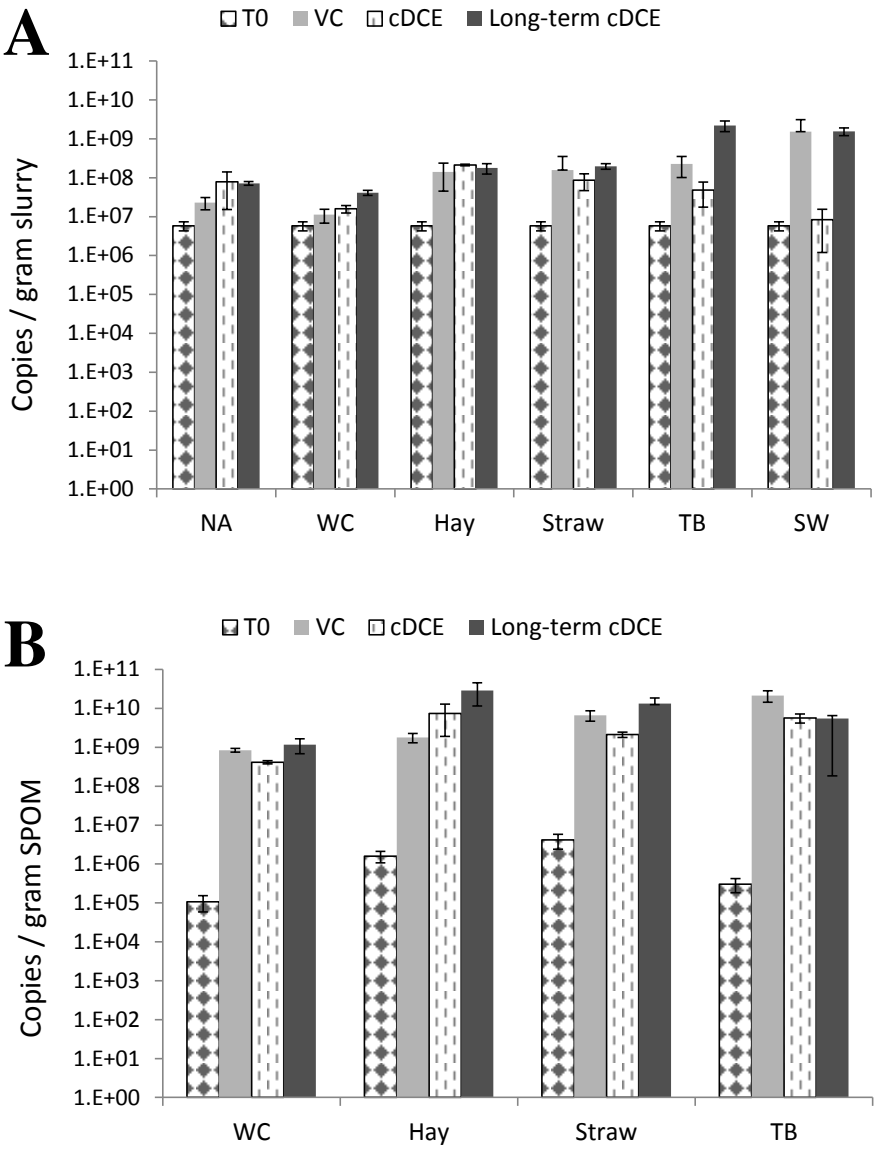


Fig. 4.7. 16S rRNA gene copy numbers of total bacteria on day 0 and at the end of biodegradation cycles of VC, cDCE, and long-term cDCE dechlorination in sediment slurry (A) and SPOMs (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). NA: natural attenuation; WC: wood chips; TB: tree bark and SW: shrimp waste.

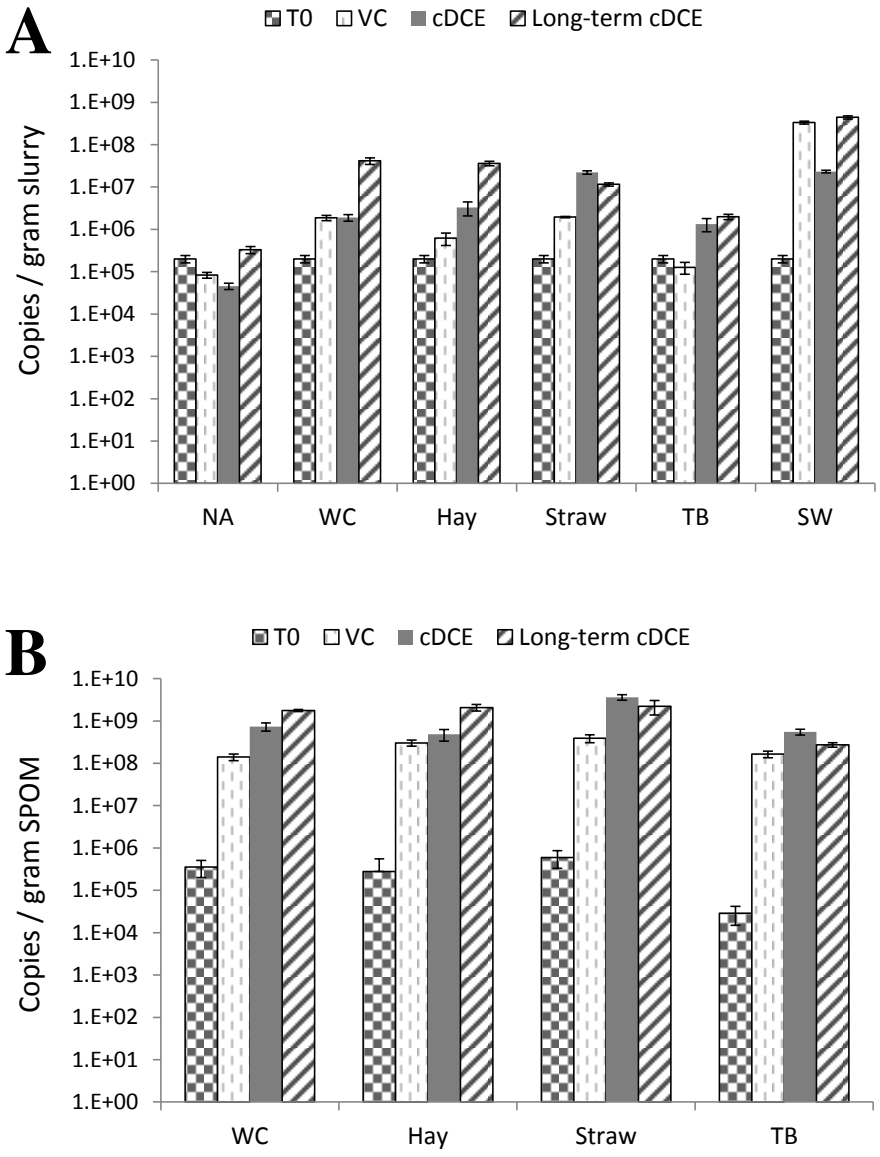


Fig. 4.8. 16S rRNA gene copy numbers of total archaea on day 0 and at the end of biodegradation cycles of VC, cDCE, and long-term cDCE dechlorination (A) and SPOMs (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). NA: natural attenuation; WC: wood chips; TB: tree bark and SW: shrimp waste.

Although growth of *D. mccartyi* was also noticed in the non-amended microcosms, addition of lignocellulosic material led to a higher relative abundance of *D. mccartyi* 16S rRNA gene copies (*D. mccartyi*/bacteria), especially in the microcosms amended with tree bark where this ratio was 21-fold higher compared with non-amended microcosms (Fig. 4.9A, Table 4.5).

An identical trend was noted for the relative abundances in the VC reductive dehalogenase-encoding genes *vcrA* and *bvcA* (Appendix 5 & 6) and their ratio over *D. mccartyi* (Table 4.5), which was the highest in both the sediment slurry and on SPOM samples withdrawn from the microcosms amended with tree bark.

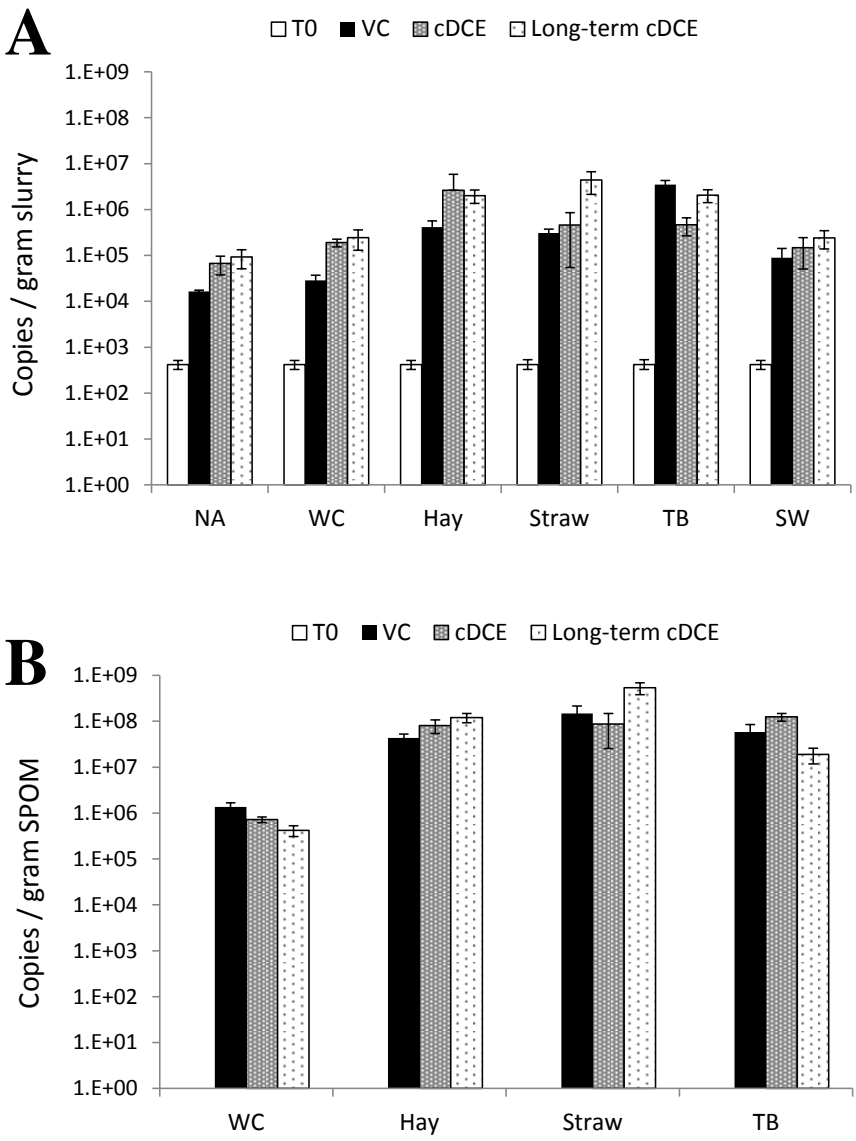


Fig. 4.9. 16S rRNA gene copy numbers of *D. mccartyi* on day 0 and at the end of biodegradation cycles of VC, cDCE, and long-term cDCE dechlorination (A) and SPOMs (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). NA: natural attenuation; WC: wood chips; TB: tree bark and SW: shrimp waste.

Table 4.5. Heat map of the ratio of biomarkers in the sediment slurry and SPOM samples. The minimum values are shown in red, midpoints values in yellow, and the maximum values in green.

Sample type	Target biomarkers	Conditions	Ratio (%)[a]			
			T0[b]	VC[c]	cDCE[d]	LT-cDCE[e]
Slurry	*Dehalococcoides* /bacteria	Natural attenuation	0.01	0.07	0.09	0.13
		Wood chips	0.01	0.26	1.20	0.59
		Hay	0.01	0.29	1.23	1.13
		Straw	0.01	0.20	0.53	2.24
		Tree bark	0.01	1.53	0.97	0.09
		Shrimp waste	0.01	0.01	1.77	0.02
	Dehalococcoides /archaea	Natural attenuation	0.21	19.86	147.20	28.08
		Wood chips	0.21	1.53	10.06	0.59
		Hay	0.21	67.39	80.10	5.55
		Straw	0.21	15.71	2.07	38.32
		Tree bark	0.21	2748.92	34.79	103.99
		Shrimp waste	0.21	0.03	0.64	0.05
	aechaea/bacteria	Natural attenuation	3.45	0.36	0.06	0.46
		Wood chips	3.45	16.71	11.97	100.60
		Hay	3.45	0.44	1.54	20.41
		Straw	3.45	1.25	25.72	5.85
		Tree bark	3.45	0.06	2.79	0.09
		Shrimp waste	3.45	21.64	276.33	28.25
	vcrA/Dehalococcoides	Natural attenuation	0.00	81.97	41.92	176.02
		Wood chips	0.00	19.54	7.77	6.63
		Hay	0.00	2.85	3.98	24.70
		Straw	0.00	1.47	164.62	3.07
		Tree bark	0.00	11.21	47.86	4.61
		Shrimp waste	0.00	19.79	41.65	47.93
	bvcA/Dehalococcoides	Natural attenuation	0.00	213.66	190.18	190.74
		Wood chips	0.00	66.61	62.17	9.12
		Hay	0.00	15.18	19.19	28.59
		Straw	0.00	3.54	5447.27	7.75
		Tree bark	0.00	204.47	198.83	15.06
		Shrimp waste	0.00	53.02	51.56	69.78
SPOMs	*Dehalococcoides* /bacteria	Wood chips	0.00	0.16	0.17	0.04
		Hay	0.00	2.45	1.10	0.42
		Straw	0.00	2.22	4.07	4.01
		Tree bark	0.00	0.27	2.19	1.78
	Dehalococcoides /archaea	Wood chips	0.00	0.01	0.00	0.00
		Hay	0.00	0.14	0.17	0.06
		Straw	0.00	0.38	0.02	0.24
		Tree bark	0.00	0.35	0.23	0.35
	aechaea/bacteria	Wood chips	335.64	16.70	178.43	150.52
		Hay	17.45	17.04	6.55	7.24
		Straw	14.49	5.85	170.98	16.60
		Tree bark	9.44	0.77	9.66	5.00
	vcrA/Dehalococcoides	Wood chips	0.00	34.56	23.42	77.43
		Hay	0.00	7.99	8.70	166.03
		Straw	0.00	2.43	14.93	60.38
		Tree bark	0.00	41.72	101.33	1389.73
	bvcA/Dehalococcoides	Wood chips	0.00	19.25	47.25	37.11
		Hay	0.00	33.89	27.13	204.90
		Straw	0.00	6.20	118.33	29.93
		Tree bark	0.00	141.22	228.73	1081.19

[a] The ratio was estimated by dividing the number of Dehalococcoides 16S rRNA gene copies by the copy number of bacterial and archaeal 16S rRNA genes multiplied by 100

[b] Time zero

[c] End of third VC spike

[d] End of third cDCE spike

[e] End of long term cDCE spikes

After degradation of the third VC spike, the ratio of archaea/bacteria was lowest in both sediment slurry and on SPOM samples in microcosms amended with tree bark (Table 4.5), which was in line with the lowest methane production in these microcosms (Fig. 4.6). In contrast, the ratio of archaea/bacteria was the highest in the sediment slurry samples from microcosms amended with shrimp waste which is in agreement with the highest methane production in these microcosms (Fig. 4.6). The apparent extensive growth of archaea in the microcosms amended with shrimp waste (Fig. 4.8A) resulted also into the lowest ratio of *D. mccartyi*/archaea (Table 4.5). However, due to the degradation of shrimp waste, no SPOM could be sampled to study the localization of the target biomarkers on shrimp waste. Among the lignocellulosic materials, microcosms amended with wood chips had the lowest ratio of *D. mccartyi*/archaea, which is in line with the lowest degradation rates observed in these microcosms.

At the end of the cDCE degradation cycles at day 200, among the amended microcosms, the *D. mccartyi*/archaea ratio was the highest in sediment slurry and on SPOM samples in the microcosms amended with hay and tree bark, although it should be noted that this ratio was lower than in the sediment slurries of unamended microcosms (Table 4.5). In accordance with the dramatic decrease in SCFAs concentrations (Fig. 4.4) and methane production (Fig. 4.6), the abundance of archaea (Fig. 4.8A), *Methanosarcinaceae* (Appendix 7A) and *Methanosaetaceae* 16S rRNA gene copies (Appendix 8A) decreased more than one order of magnitude only in the sediment slurry of the microcosms amended with shrimp waste. Nevertheless, due to the concomitant decrease of bacterial 16S rRNA gene copies (more than two orders of magnitude) the ratio of archaea/bacteria was still the highest in the sediment slurry of the microcosms amended with shrimp waste (Table 4.5). The ratio of archaea/bacteria increased more than 20-fold in both sediment slurry and on SPOM samples withdrawn from microcosms amended with straw, which was due to further proliferation of archaea in these microcosms (Fig. 4.8) which relates to the continuous production of methane by the end of the third cDCE spike (Fig. 4.6).

At the end of the long-term cDCE degradation experimental period, the microcosms amended with tree bark had the highest ratio of *D. mccartyi*/archaea and the lowest ratio of archaea/bacteria in both sediment slurry and on SPOM samples (Table 4.5). On the contrary,

microcosms amended with wood chips showed the lowest ratio of *D. mccartyi*/archaea and the highest ratio of archaea/bacteria among the lignocellulosic materials.

4.3.2 Prediction of reactive transport of CAH in *in situ* caps

The modelling results show that VC concentrations above the threshold of 5 µg/L would discharge in the surface water if no additional measures are being taken (Fig. 4.10). In case of a pore water velocity of 5.4 cm/day, a capping layer of 40 cm using hay, straw, tree bark or shrimp waste as SPOMs would suffice to bring the VC concentration below the threshold level.

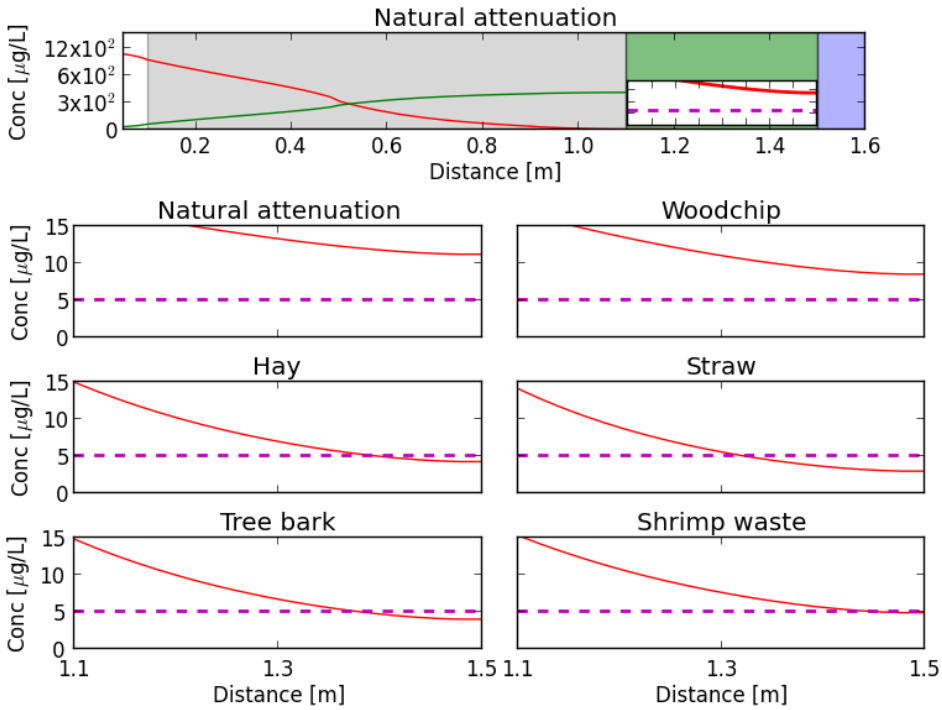


Fig. 4.10. An analysis of the different capping materials applied at the Zenne site, Belgium. The graphs show the concentrations of VC (red) and ethene (green). The concentration of cDCE was much lower than VC and is not included. The top graph shows the modelled domain with the different compartments indicated by the background colours with the aquifer in white, the sediment in gray, the capping material in green and the river in blue. Under natural attenuation, application of a sand cap was considered. The insert shows the concentration in the capping material at a more detailed scale, with the legal threshold for VC in groundwater indicated by the dashed line. The bottom graphs show the concentration profiles in the respective capping materials. A distance of 0 m (x-axis) was defined as a depth of 1.1 m from the sediment-surface water interface without cap.

4.4 Discussion

We examined whether SPOMs can be incorporated into bioreactive sediment caps to stimulate bioremediation of CAH-contaminated sediments by means of OHR, and whether the channeling of reducing equivalents to methanogenesis can present technical and environmental concern. This was achieved by studying the effect of the presence of SPOMs on reductive dechlorination of CAHs and the dynamics of population of OHRB and methanogenic archaea in sediments that have been previously exposed to CAH contamination.

During the first two weeks, extensive accumulation of SCFAs (Fig. 4.4) and DOC (Fig. 4.5) was noticed in SPOM containing microcosms, which could be due to direct release and fermentation of sugars, amino acids, and proteins (Robinson-Lora & Brennan, 2009). Accumulation of SCFAs was likely noticed due to the initial low numbers of SCFAs consuming microorganisms such as *Methanosarcina* (Appendix 7A) as the main acetoclastic methanogens (De Vrieze *et al*., 2012). This is in line with the fact that no accumulation of methane as the product of methanogenesis was noticed in this phase. Another reason for such a delay in onset of methanogenesis might be the dominance of iron- and sulfate-reduction which typically take place during the early stages of anoxic incubations and has been reported in anoxic paddy soil amended with straw rice (Glissmann & Conrad, 2000). Although no accumulation of ethene as the product of reductive dechlorination was noticed during the first two weeks (Fig. 4.2B), a slight decrease in VC concentration was evident in SPOM amended microcosms which might be due to anaerobic VC oxidation coupled to iron-reduction (Bradley & Chapelle, 1996). The onset of extensive methane production at day 14 in microcosms amended with shrimp waste, straw and hay (Fig. 4.6) was concomitant with a decrease in acetate and butyrate concentrations (Fig. 4.4). In contrast, no considerable stimulation of VC dechlorination was obtained in SPOM amended microcosms during this period of high methane production suggesting dominance of methanogenesis in the early stage of the experiment. The clear stimulation of dechlorination was obtained in SPOM amended microcosms after the second and third VC spikes was coupled to a substantial enrichment of *D. mccartyi* (Fig. 4.9) and VC catabolic genes (Appendix 5 & 6) at day 100. Although the ratio of *D. mccartyi*/archaea in the slurry samples of the unamended

microcosms was higher than microcosms amended with shrimp waste, wood chips and even straw (Table 4.5), the obtained degradation rates in the unamended microcosms were not sufficient to reduce the VC concentration below the threshold level (Fig. 4.10). The fact that *D. mccartyi* was not detected on the SPOM materials before incubation and the extensive colonization of SPOM samples by *D. mccartyi* in the course of the experiment suggest that the SPOMs can host sediment born *D. mccartyi* populations capable of CAH biotransformation.

The sustained high degradation rates during subsequent cDCE addition (Fig. 4.3) with dramatic decrease of methanogenesis (Fig. 4.6) after day 100 showed that long-term incubation might lead to the dominance of OHRB instead of methanogens. Accordingly, during the long-term cDCE degradation experimental period with new addition of SPOMs, confined methanogenesis and sustained dechlorination rates over time especially in tree bark amended microcosms were recorded (Table 4.4), suggesting that long-term incubation favors dechlorinating communities over methanogens. It can be speculated that after depletion of immediately available easily degradable molecules (organic acids, alcohols, …) with concomitant predominance of methanogens as evident by the fact that most methane is produced in the initial phase after SPOM addition (Table 4.4), anaerobic degradation of more complex materials such as cellulose by cellulolytic and fermentative microbes provide a long-term slow release source of acetate and H_2, which is sufficient to sustain reductive dechlorination. The success of reductive dechlorination in this phase could be due to the competitive advantage of *D. mccartyi* over methanogens in conditions of low H_2 fluxes, as based on H_2 half-saturation rate constants since *D. mccartyi* strains are excellent scavengers of H_2 under H_2 limiting conditions (Fennell & Gossett, 1998). Hydrogen concentrations were not measured in our experiments but Brennan (2003) showed that the majority of the total electron donating capacity released during chitin fermentation was acetate while hydrogen represented less than 0.01%. Accordingly, other reports showed that the major fraction of electron equivalents during fermentation is routed to organic products and not to H_2 (Aulenta *et al*., 2007; Fennell *et al*., 1997), even though the measured concentrations of hydrogen do not necessarily reflect hydrogen consumption rates (Löffler *et al*., 1999).

Although straw amended microcosms degraded the highest mass of cDCE with the highest degradation rates (Table 4.4), the ratio between the amount of methane and ethene+ethane

produced was 12.7-fold higher in these microcosms than in microcosms amended with tree bark (Appendix 4) while the *D. mccartyi* population size (Fig. 4.9) and reductive dehalogenation gene numbers (Appendix 5 & 6) were identical in both microcosms and the ratio of *D. mccartyi*/archaea was higher in microcosms amended with tree bark both in the sediment slurry and on the SPOM (Table 4.5). This shows that the major fraction of reducing equivalents from the straw was routed to production of organic intermediates exceeding the requirement for reductive dechlorination, which can lead to stimulation of methanogenesis and increase of biochemical oxygen demand in the water. Recently, Wei and Finneran (2012) reported that adding 10-times more acetate did not increase the rate or extent of TCE reduction, but only increased methane production. Taking into account that other main SCFAs such as propionate and butyrate are expected to be converted to acetate, CO_2, formate and hydrogen by syntrophic microbial communities (Stams & Plugge, 2009), the excess of electron donors will be consumed for methane production which is unwanted for several reasons. The impact of methanogenesis is not merely restricted to the consumption of reducing equivalents and production of the greenhouse gas methane. Extensive methanogenesis *in situ* can also strip volatile CAHs by the formed methane bubbles (Vroblesky *et al*., 1989, Viana *et al*., 2007), compromise the physical stability of the bioreactive cap and as such provide additional pathways for contaminant release (Reible *et al*., 2006). Therefore, control of methanogenesis is important to guarantee effectiveness of a SPOM-based bioreactive caps.

4.5 Conclusion

Taking into account that lignocellulosic materials are agricultural by-products/wastes with little or no economic value, their use into bioreactive caps is of interest with the dual aim of pollutant degradation and waste load reduction. However, it should be noted that due to differences between SPOMs, site characteristics, and contaminants of concern, the selection of an appropriate SPOM can have critical impact on the success of bioreactive sediment capping technology. As shown here, addition of wood chips did not translate to a better dechlorination, and most of the reducing equivalents produced from addition of straw and hay were channeled to methanogenesis. Our results showed that tree bark is the best SPOM to stimulate CAH degradation in the sediments of the Zenne site. Rapid and persistent colonization of the tree bark by a *D. mccartyi* community shows that a SPOM-based layer

can potentially form a sustainable biotransformation layer in a bioreactive cap in which the release of electron donor and presence of CAHs is coupled with the dehalogenating microorganisms in the same place. We hypothesize that *D. mccartyi* native to the sediment colonized the SPOM which suggests that *D. mccartyi* eluted from deeper sediment by the upwelling groundwater can colonize the bioreactive layer in an *in situ* application. Previously, localization of microorganisms indigenous to aquatic sediments on the overlaying sand cap material was shown in up-flow column experiments (Himmelheber *et al*., 2009). In conclusion, the results of this study showed that although lignocellulosic materials are promising candidates to be incorporated into bioreactive caps, before embarking into full-scale field bioreactive capping efforts, bench-scale screening of SPOMs is necessary to stimulate the target microbial groups without generating new environmental problems.

Chapter 5: Shifts in bacterial community composition and function in the hyporheic zone of an eutrophic river after installation of an upstream wastewater treatment plant

5.1 Introduction

Rivers in urban areas are often heavily impacted by untreated wastewater discharge containing excessive organic carbon loads (Carey & Migliaccio, 2009). Wastewater treatment plants (WWTPs) are one of the most common systems of modern environmental biotechnological measures to treat municipal wastewater before it discharges into receiving water bodies by removing a large fraction of nutrients, especially the organic load (Gücker *et al*., 2006). Allochthonous organic carbon has long been known to fuel river biogeochemical activities (Tank *et al*., 2010), which mainly take place in the riverbed sediment, either at or just below the surface, a zone known as the hyporheic zone (Boulton *et al*., 1998). The high organic matter content in hyporheic zones results into steep chemical/redox gradients going from oxic to anoxic that allows for multiple critical reactions in biogeochemical cycling of primary elements including the transformation of complex organic matter, processing of

heavy metals, and pollutant attenuation (Biksey & Gross, 2008). Because of the absence of light, food webs in the hyporheic zone are based primarily on the entrainment of organic carbon deposited from the surface water (Baker *et al*., 1999). Hence, long-term decrease of organic carbon loads in the receiving surface water due to wastewater treatment can potentially impact microbial activities that are dependent on organic carbon in the hyporheic zone.

One of the critical reactions mediated by high organic carbon content in hyporheic zones is natural attenuation of contaminants. For instance, in recent years, the hyporheic zone has received a lot of attention because of its capacity to retard or degrade contaminants like chlorinated aliphatic hydrocarbons (CAHs) present in upwelling contaminated groundwater plumes (Ellis & Rivett, 2007; Abe *et al*., 2009; Hamonts *et al*., 2009, 2012). The organic carbon in the hyporheic zone is crucial for this activity since it provides a natural supply of electron donors and anoxic conditions for reductive dechlorination of CAHs by organohalide respiring bacteria (OHRB) (Smidt & de Vos, 2004; Hamonts *et al*., 2012). Of all bacterial groups mediating CAH organohalide respiration (OHR), *Dehalococcoides mccartyi* species belonging to the *Chloroflexi* phylum, are the most important as they are the only OHRB that can completely dechlorinate CAHs to ethene (Smidt & de Vos, 2004).

In the present study, we investigated the bacterial community composition across sediment depth in the hyporheic zone of a stretch of the Belgian Zenne River that is impacted by a discharging groundwater plume containing vinyl chloride (VC), before and after the upstream installation of a WWTP. In previous studies, we have shown that in that river stretch, the highly reducing and organic rich sediments were conducive to natural attenuation of CAHs present in the discharging groundwater mainly by OHR (Hamonts *et al*., 2009, 2012; Kuhn *et al*., 2009). The WWTP was installed approximately one km upstream of the test site treating the sewage that was previously discharged directly into the Zenne River. The implementation of the WWTP resulted in important physico-chemical changes in surface water composition such as a decrease in organic carbon content and an increase in dissolved oxygen (Carpentier *et al*., 2013). We hypothesized that the decrease in organic carbon content in the surface water could result in decrease of organic carbon content in the hyporheic zone, subsequently leading to shifts in bacterial community composition and in particular in the OHRB that are dependent on organic carbon-derived electron donors and

reducing conditions. To address this hypothesis, sediment cores were collected in 2005 (2 years before construction of the WWTP in 2007), 2010 and 2011, and the bacterial community composition in vertical sediment profiles was analyzed by pyrosequencing of PCR amplified partial 16S rRNA genes and by targeted quantitative PCR (qPCR). The relationship between surface water characteristics, sediment characteristics like organic carbon load, the bacterial community dynamics and VC respiration potential was examined.

5.2 Materials and methods

5.2.1 Site description

The Zenne River is a small river with a total length of 103 km. It was notorious for being one of Belgium's most polluted rivers, since all effluents from the Brussels Capital Region used to be discharged into it without treatment (Carpentier *et al*., 2013). In March 2007, the completion of a new WWTP alleviated this problem. The study site is a short transect of the Zenne River located in an industrial area near Brussels, Belgium (Fig. 4.1, Chapter 3) (Hamonts *et al*., 2009). At the study site, the Zenne River is relatively straight, 12-15 m wide, 0.5-2 m deep and has a stream flow of 5-10 m^3/s in dry weather conditions (Hamonts *et al*., 2012). The aquifer was contaminated with tetrachloroethene (PCE), trichloroethene (TCE) and 1,1,1-trichloroethane (1,1,1-TCA), originating from several sources. Based on monitoring from October 2004 till January 2007, it was noted that a groundwater plume mainly contaminated with cDCE and VC is flowing from the nearby aquifer into the Zenne River (Hamonts *et al*., 2009). Since 2009 the concentration of CAHs that reach the river has appeared to decrease while the concentration of contaminants has been rather stable in the source area, which might be due to plume diversion. It is unclear whether this is due to increased OHR activity during plume flow from source towards the river, or rather due to plume diversion.

5.2.2 Sample collection

Sediment core samples were collected in December 2005, May 2010 and September 2011 from the top 80-cm layer of the riverbed sediment at post 25 (P25) using a 4-cm-diameter piston sediment sampler. At all time-points, duplicate sediment cores were collected at a distance of approximately 50 cm from each other. One of the sediment cores was preserved immediately on dry ice to conserve the spatial structure of the sediment layers. These cores

were kept at -20 °C until DNA extraction in November 2011 as described below. The other core was directly transferred to the lab in a cooler and used to set up microcosms to study the OHR potential. Groundwater, surface water and sediment pore water sampling and preservation was performed as reported (Hamonts *et al*., 2009, 2012). Briefly, groundwater samples were collected from monitoring wells SB-1 and surface water samples were collected from the river as grab samples in glass bottles, without leaving a headspace. Electrical conductivity, dissolved oxygen, pH and redox potential were measured regularly (Table 5.2) in the surface water using an electrometric multimeter (MultiLine F/SET3, WTW, Weilheim, Germany) and appropriate electrodes (TetraCon 325, CellOx 325 and Sen Tix 41 from WTW, Weilheim, Germany and Oxitrode Platinum from Hamilton, Bonaduz, Switzerland, respectively). Sediment interstitial water (ISW) was collected from Teflon pore water samplers that were permanently installed in the test area near the right riverbank at two different depths in the river sediment (20 and 60 cm) as reported (Hamonts *et al*., 2012).

5.2.3 Microcosm set up

Riverbed sediment microcosms were prepared with samples taken from the top (0-20 cm depth) sediment layer. The microcosms were prepared in an anaerobic glove box (Don Whitley Scientific ltd, West Yorkshire, UK) under anoxic conditions. Approximately 37 g (wet) sediment was transferred to 160-ml glass serum bottles containing 70 ml groundwater from monitoring well SB-1. All microcosms were prepared in duplicate. Two additional microcosms prepared from the sediments of 2011 were supplemented with 1 g tree bark as a solid organic carbon and electron donor source as reported in Chapter 4. All microcosms were spiked with VC at a final concentration of 2 mg/L aqueous concentration. The microcosms containing sediment of 2005 were first allowed to degrade residual VC that was present in the groundwater, before VC was added.

5.2.4 DNA extraction, PCR-DGGE and qPCR

The sediment cores were divided into 1-cm slices till a depth of 60-70 cm, and at 5 cm intervals, sediment samples of approximately 2 g were used for DNA extraction as described by Hendrickx *et al*. (2005). DGGE fingerprinting of bacterial 16S rRNA gene was performed on all 40 samples as described by Uyttebroek *et al*. (2006). qPCR targeting Bacteria and *D. mccartyi* was performed on 20 selected samples as described in Chapter 2.

5.2.5 Bacterial 16S rRNA gene amplicon pyrosequencing

Bacterial 16S rRNA genes were first amplified from the DNA extracts using the 27F/1492R primer set (Lane, 1991) followed by a second PCR using barcoded primers (Table 5.1) in a GS0001 thermocycler (Gene Technologies, Braintree, United Kingdom). The barcoded amplicons covered the V1-V2 region of the bacterial 16S rRNA gene and were generated using the 27F-DegS primer (van den Bogert *et al*., 2011) appended with the titanium sequencing adaptor A and an 8 nucleotide sample specific barcode (Hamady *et al*., 2009) at the 5' end as the forward primer, and an equimolar mix of the two reverse primers, 338R I and II (Daims *et al*., 1999; Table 5.1), that carried the titanium adaptor B at the 5' end. Sequences of both titanium adaptors were kindly provided by GATC Biotech (Konstanz, Germany). The PCR mix (100 µl final volume) contained 20 µl of 5× HF buffer (Finnzymes, Vantaa, Finland), 2µl PCR Grade Nucleotide Mix (Roche Diagnostic GmbH, Mannheim, Germany), 1µl of Phusion hot start II High-Fidelity DNA polymerase (2U/µl) (Finnzymes), 500 nM of the reverse primer mix and the forward primer (Biolegio BV, Nijmegen, The Netherlands), 2 µl (i.e. 40 ng) template, and 65 µl nuclease free water. PCR was performed using the following conditions: 98°C for 30 s to activate the polymerase, followed by 30 cycles consisting of denaturation at 98 °C for 10 s, annealing at 56 °C for 20 s, elongation at 72 °C for 20 s, and a final extension at 72 °C for 10 min. Five µl of the PCR products, approximately 450 bp in length, were analyzed by 1% (w/v) agarose gel electrophoresis, containing 1× SYBR® Safe (Invitrogen, Carlsbad, CA, USA) and purified from gel using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics) according to the manufacturer's instructions. The DNA concentration of gel-purified amplicons was measured by Qubit 2.0 Fluorometer (Life Technologies, Darmstadt, Germany), and purified PCR products were mixed in equimolar amounts and run again on an agarose gel prior to excision and purification by using a DNA gel extraction kit (Millipore, Billerica, MA, USA). Amplicons obtained from the 20 samples were analysed simultaneously by pyrosequencing on half a plate using an FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany).

Table 5.1. Oligonucleotide primers and sequence adapters used for this study.

Primer [a]	Oligonucleotide sequence [b]	Reference
Adaptor A	5´- CCATCTCATCCCTGCGTGTCTCCGACTCAG -3´	Provided by GATC-Biotech
Adaptor B	5´- CCTATCCCCTGTGTGCCTTGGCAGTCTCAG -3´	Provided by GATC-Biotech
27F-DegS	5´- GTTYGATYMTGGCTCAG -3´	Van den Bogert *et al*., 2011
338R-I	5´- GCWGCCTCCCGTAGGAGT -3´	Daims *et al*., 1999
338R-II	5´- GCWGCCACCCGTAGGTGT -3´	Daims *et al*., 1999
27F	5´- AGA GTT TGA TCC TGG CTC AG -3´	Lane, 1991
1492R	5´- GGT TAC CTT GTT ACG ACT T -3´	Lane, 1991

[a] Primer names may not correspond to original publication
[b] M = A or C; R = A or G; W = A or T; Y = C or T

5.2.6 Analysis of the pyrosequencing data

Pyrosequencing data were analysed using the QIIME 1.5.0 pipeline (Caporaso *et al*., 2010). Low quality sequences were removed using default parameters, and operational taxonomic units (OTUs) were defined at a 97% identity level. A representative sequence from each OTU was aligned using PyNAST (DeSantis *et al*., 2006). The taxonomic affiliation of each OTU was determined at a confidence threshold of 80% using the BLAST algorithm (Altschul *et al*., 1990). Possible chimeric OTUs were identified using QIIME's Chimera Slayer (Haas *et al*., 2011) and subtracted from the previously generated OTU list, producing a non-chimeric OTU list. The relative abundance of each taxon was estimated by dividing the number of the reads of each taxon by the total number reads of all taxons multiplied by 100.

5.2.7 Analytical methods

Concentrations of CAHs, ethene, and ethane were determined via head-space analysis on Varian GC-FID (CP-3800) as described in Chapter 2. Total organic carbon (TOC) and total nitrogen (TN) were measured using the oxidative digestion method (C/N analyzer EA1110). Dissolved organic carbon (DOC) was determined as described in chapter 3.

5.2.8 Data analyses

In order to relate the changes in microbial communities at order level to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 5 software package (Biometris, Wageningen, The Netherlands). The environmental variables tested were DOC and oxygen in surface water, TOC and TN in the sediment cores, DOC, and sulphate in IWS, and time and depth. Nitrite and nitrate in ISW was not considered as their

levels were always below detection limit. A Monte Carlo permutation test based on 499 random permutations was used to determine which of the experimental variables significantly contributed to explaining the observed variance in composition of microbial communities. The community structure was visualized via ordination triplots with scaling focused on intersample differences. For all statistical analyses, correlations were considered significant at a *P* value of < 0.05.

The similarity among bacterial communities at the order level between different years and depths was calculated by Bray-Curtis analysis using complete linkage with PRIMER6 software (Clarke & Warwick, 2001).

5.3 Results

5.3.1 Physico-chemical characterization of the study site

Obvious trends in concentrations of cations and anions, pH, or conductivity in the surface water were not discernible during the experimental period (Table 5.2). Surface water temperature was in the range of 7.4-18.2 °C. Overall, the concentrations of nitrite and nitrate increased in the surface water over time. High DOC values and strongly reducing conditions prevailed in the Zenne surface water before the construction of the WWTP in March 2007 (Table 5.2). After the establishment of the WWTP, dissolved oxygen increased to above 3 mg/L in 2007 and on average above 4 mg/L after 2008. DOC of the surface water decreased to values below 20 mg/L in 2010 and below 10 mg/L in 2011. However, DOC values in ISW collected by means of the permanent pore water samplers did not show a dramatic change over time and depth (Table 5.3). The concentrations of sulphate in ISW decreased over time while the concentration of nitrite and nitrate were always below detection limit (Table 5.3).

Table 5.2. Physico-chemical parameters of surface water. The dates when sediment cores were taken for microbial analysis are shown in bold.

Date	Concentration (mg/L)									DOC	O_2	Temp	pH	Cond [c]
	PO_4	SO_4	NO_2	NO_3	Cl	Fe	Na	Mg	K	(mg/L)	(mg/L)	(°C)		(µS/cm)
Dec 2005	**ND**[a]	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**32**	**1.2**	**ND**	**ND**	**ND**
May 2006	2.32	214	BD[b]	0.68	258	0.5	90	15	18	70	**ND**	17.8	7.88	1126
Dec 2006	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.9	14.5	7.62	1576
Mar 2007	0.67	103	BD	7.5	95	ND	ND	ND	ND	ND	3.6	12.9	7.62	1542
May 2007	1.97	58	BD	BD	148	0.58	80.3	14.9	16.2	ND	3.7	13.2	7.58	1368
Nov 2007	0.82	101	BD	6.7	79	0.09	44.7	13.7	9.6	ND	ND	ND	ND	ND
Oct 2008	ND	73	BD	BD	61	0.1	ND	ND	ND	ND	5.9	15.7	8.46	952
Feb 2010	ND	65	ND	13.9	92	0.08	63	11	10	15.9	5.5	11.5	7.32	911
May 2010	**ND**	**111**	**0.56**	**10.4**	**104**	**0.02**	**81**	**17**	**13**	**19.7**	**4.2**	**13.9**	**7.47**	**1122**
Sep 2010	1.8	102	BD	6.14	53	0.01	71	14	15	13.1	4.4	18.2	7.42	962
Jan 2011	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	6.5	7.4	7.42	1070
May 2011	ND	100	ND	ND	140	0.17	ND	ND	ND	6.55	4.7	18	7.38	1211
Sep 2011	**1.1**	**78**	**0.15**	**2.4**	**79**	**0.01**	**75**	**12**	**9.7**	**7.9**	**5.3**	**17.1**	**7.5**	**777**

[a] ND: Not determined

[b] BD: Below detection limit

[c] Cond: Conductivity

TOC decreased dramatically over time and also with sediment depth. For the samples taken in 2005, TOC values decreased from above 1 % in the surficial sediment samples to below 0.2 % in the core samples from 55 cm to 70 cm (Fig. 5.1). Especially the TOC of the core sample from 65 cm was exceptionally high reaching to 1.98 %. The TOC values of the samples of 2010 ranged between 0.4 % to 0.1 %, whereas the TOC content of the samples of 2011 did not surpass 0.16 %. An identical trend was observed for TN content in the samples (Fig. 5.1).

Table 5.3. Physico-chemical parameters of interstitial water sampled with the permanent pore water samplers in the river bed.

Date	Sampling depth (cm)	Concentration (mg/L)				
		DOC	SO_4	NO_2	NO_3	VC
Dec 2005	20	5	170	BD [a]	BD	0.02
	60	4	198	BD	BD	1.2
May 2010	20	3.7	145	BD	BD	0
	60	3.5	144	BD	BD	0
Sep 2011	20	6.3	140	BD	BD	0
	60	2.6	140	BD	BD	0

a BD: Below the detection limit

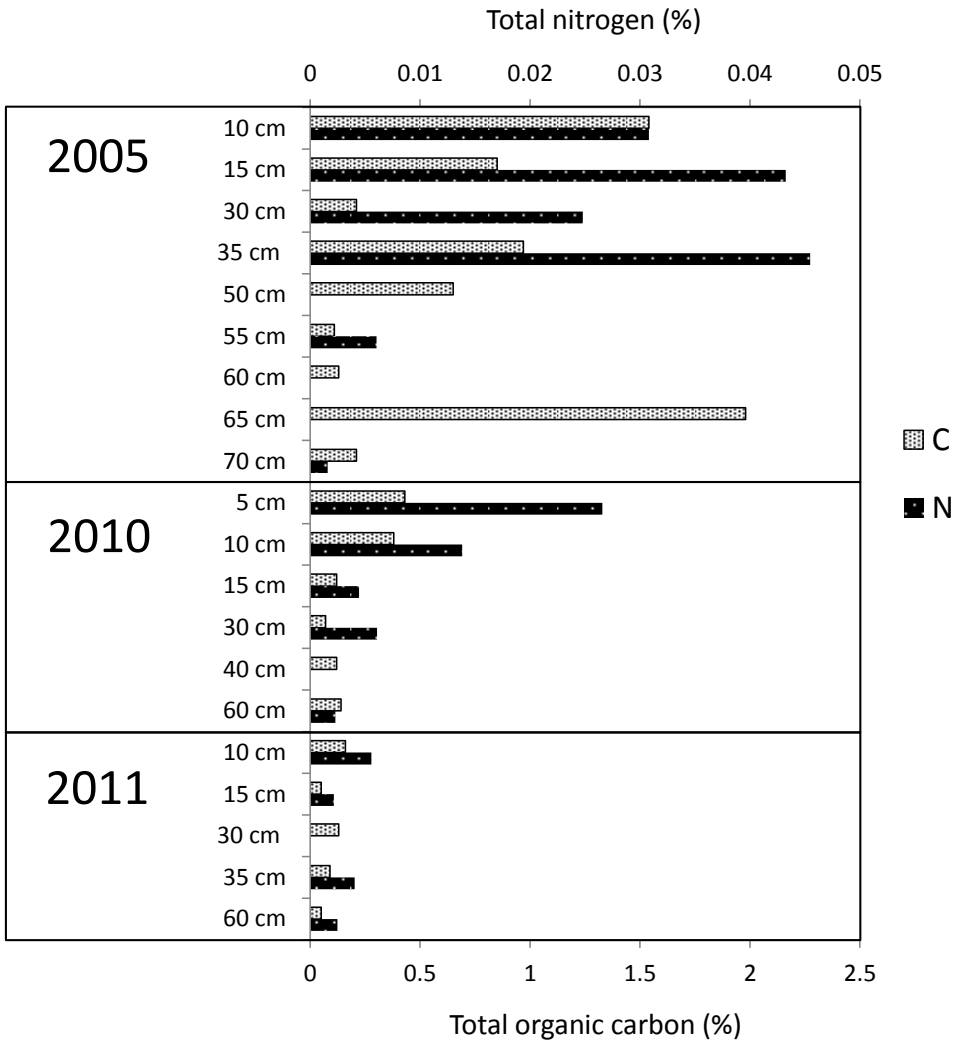


Fig. 5.1. Total organic carbon and total nitrogen content at different depths in the sediment core samples taken in 2005, 2010 and 2011.

5.3.2 Occurrence of VC respiration activity in sediment microcosms

The results of VC dechlorination in microcosms prepared from top 20 cm sediments and SB1 groundwater are shown in Fig. 5.2. Rapid dechlorination of VC was noticed in microcosms containing sediment material sampled in 2005 and in 2010 (Fig. 5.2A) with stoichiometric ethene production within 28 days (Fig. 5.2B). VC dechlorination was much slower in microcosms prepared with sediment materials sampled in 2011 and 70 days were needed for complete VC dechlorination to ethene. Addition of tree bark to sediments of 2011 reduced the lag phase to 14 days and all VC was dechlorinated stoichiometrically to ethene

within 35 days suggesting that organic carbon as a source of electron donors was limiting for dechlorination in samples of 2011 compared to those of 2005 and 2010.

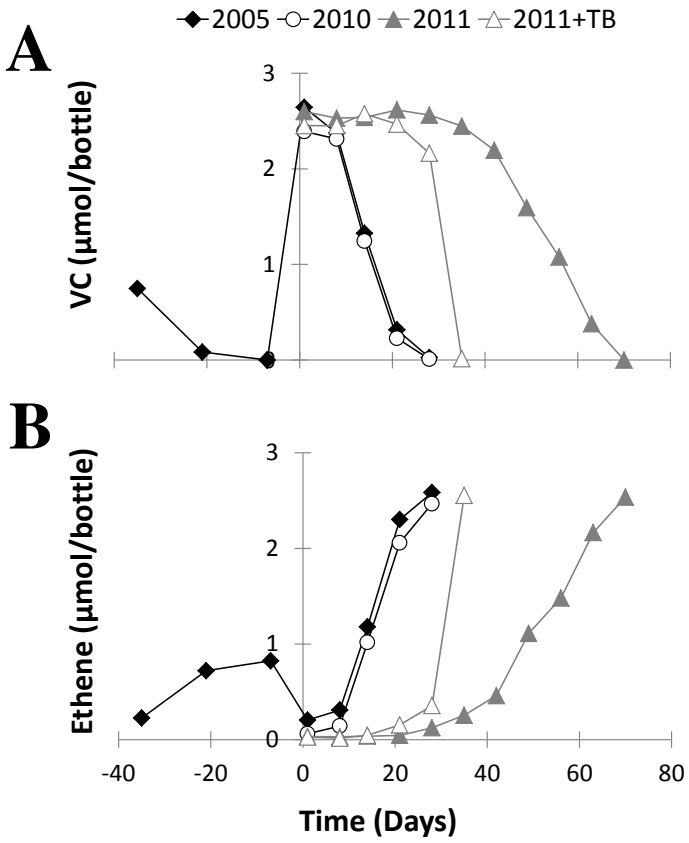


Fig. 5.2. VC dechlorination (A) and accumulation of ethene (B) in microcosms prepared from top 20 cm river-bed sediment from 2005, 2010, 2011 and 2011 stimulated with tree bark (TB). Sediment bottles of 2005 were re-spiked with VC on day 28. The day of VC spike into the microcosms was considered as the day zero on the x-axis.

5.3.3 Sequencing and quality control

Prior to pyrosequencing, a total of 40 DNA extracts obtained from the sediment core slices at 5 cm intervals, was analyzed by bacterial 16S rRNA gene based PCR-DGGE. Based on the obtained patterns (Appendix 9), a total of 20 slice samples representing samples with clear differences in DGGE profiles was selected for community 16S rRNA gene pyrosequencing employing the V1-V2 region of the 16S rRNA gene. A total of 181,647 reads was obtained for all 20 samples. After quality filtering and chimera removal, a total of 162,939 reads was

recovered ranging from 3506 to 20694 reads per sample (Table 5.4), with an average of 8,147 reads per sample and an average read length of 317 bp. The relative abundance of each taxon was estimated by dividing the number of the reads of each taxon by the total number reads of all taxa, multiplied by 100.

Table 5.4. Number of reads in each sample, species diversity, richness estimates and diversity coverage obtained at genetic distances of 3% by normalizing the number of sequences of each sample to the fewest reads among all samples (3,506 reads).

Sample name	Number of reads per sample	Shannon index (H´)	Chao1	Diversity coverage (%)[a]
2005-10cm	3969	5.34	920.9	40.65
2005-15cm	4321	5.26	993.9	40.22
2005-30cm	3506	5.67	1578.2	37.79
2005-35cm	11301	5.67	1170.9	34.94
2005-50cm	5697	6.47	1034.1	44.25
2005-55cm	3820	7.29	2022.8	38.86
2005 60cm	4746	6.29	992.3	42.83
2005-65cm	12141	6.06	1743.6	31.49
2005-70cm	4681	7.14	1758.7	45.09
2010-05cm	6949	9.22	6991.7	23.07
2010-10cm	6805	9.44	7990.3	21.28
2010-15cm	7979	9.22	6690.1	24.62
2010-30cm	8693	9.36	6251.8	26.60
2010-40cm	8372	9.69	7697.5	23.53
2010-60cm	7933	9.79	9308.1	20.46
2011-10cm	20694	4.39	425.9	46.37
2011-15cm	15492	5.04	567.9	48.69
2011-30cm	8460	3.73	185.3	50.42
2011-35cm	9114	3.82	187.9	49.41
2011-60cm	8266	3.94	238.2	53.36

[a] The diversity coverage was estimated by dividing the average observed species by average expected species multiplied by 100

5.3.4 Bacterial richness and diversity indices

In order to estimate taxon richness and evenness among the samples, the number of sequences of each sample was normalized to the lowest number of the reads among all samples (3,506 reads) using QIIME 1.5.0 pipeline that performs normalization based on random removal of reads from each sample until 3,506 reads remained. In the sediment core sampled in 2005, the bacterial diversity (Shannon indices) and richness (Chao1) generally increased with depth (Table 5.4). The diversity coverage in the samples of 2005 ranged between 31.5 to 45.1% at a genetic distance of 3%. The bacterial communities in the

samples of 2010 were highly diverse with a Shannon index of diversity ranging from 9.22 to 9.79 with no depth related trend. The high richness in these samples (data not shown) led to a lower species coverage of 20.46-24.62% (at 3% genetic distance). The lowest diversity and richness was noticed in samples of 2011 which decreased with depth (Table 5.4).

5.3.5 Phylum-level taxonomic distribution

Overall, thirty different phyla were identified from the sequences using the QIIME analysis pipeline. *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Nitrospirae* were the six most abundant phyla in all samples (Fig. 5.3). *Proteobacteria* was the dominant phylum in samples of 2005 with an average abundance of 51.6% of the qualified bacterial reads followed by the *Chloroflexi* (on average 14.8% of the qualified bacterial reads). The relative abundance of *Proteobacteria* was variable along the vertical sediment profiles of the core taken in 2005 but in general its abundance increased with increasing depth (Fig. 5.3). In comparison, however, the *Chloroflexi* phylum showed a stronger depth related stratification. Members of the *Chloroflexi* were the most abundant at the surface and declined steadily with depth. *Firmicutes* and *Nitrospira*e phyla were predominantly present in the deeper horizons of the sediment cores of 2005, i.e., between 50 cm to 60 cm, and at 70 cm. The proportion of the *Proteobacteria* phylum strongly increased in the sediment cores of 2010 reaching an average abundance of 75.77% of the qualified reads. Generally, all six main phyla in the samples of 2010 showed identical and rather stable depth related stratification. *Bacteroidetes* was the second most abundant phylum reaching to an average abundance 10.45% of the reads. In contrast, compared with the samples of 2005, the relative abundance of *Chloroflexi*, *Firmicutes*, and *Nitrospirae* decreased sharply reaching to an average of 4.21, 2.92, and 0.18% of the reads in the samples of 2010, respectively. In accordance with the trend observed in the samples of 2010, the proportion of *Proteobacteria* further increased in the samples of 2011 reaching on average 80% of the qualified reads over the different layers. Except for the deepest sediment layer (60 cm), *Chloroflexi* dropped to 0.7 % in the samples of 2011 (Fig. 5.3).

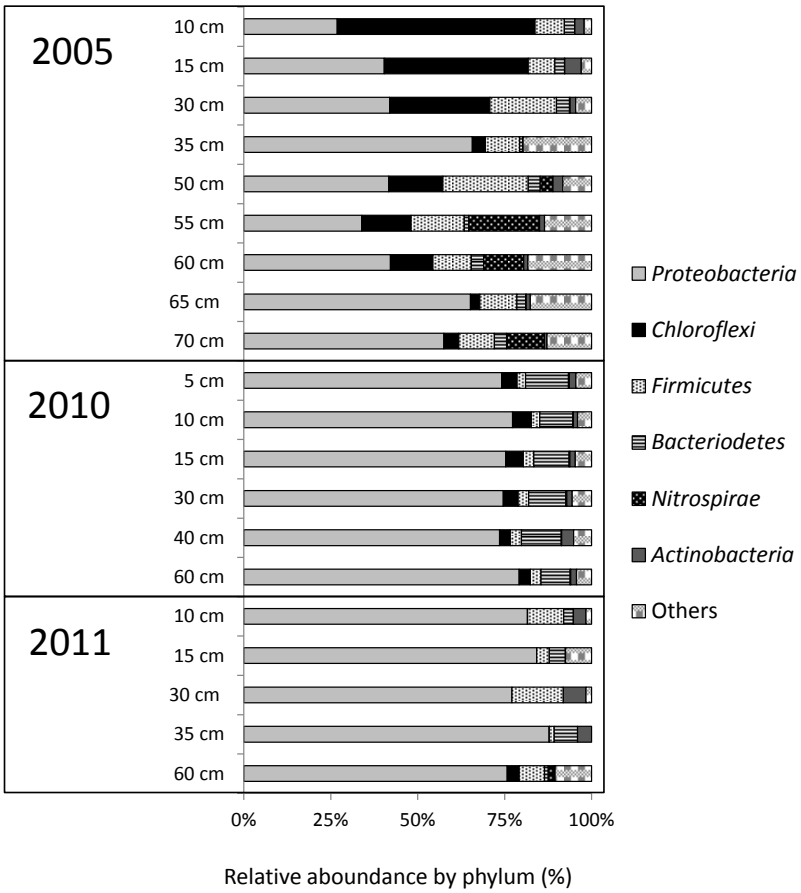


Fig. 5.3. Relative abundance of the dominant bacterial phyla at different depths in the sediment core samples taken in 2005, 2010 and 2011.

5.3.6 Class-level taxonomic distribution

The 162,939 classifiable reads were affiliated with 92 classes of which the top 11 most abundant classes (representing on average 88.8 % of all reads) (Fig. 5.4). Compared with the samples of 2005, where all 5 *Proteobacteria* classes were distributed between samples of different depth, *Alphaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria* became the most dominant classes in sediments of 2010 and 2011. Differences in the proteobacterial community composition were, however, most evident for the *Epsilonproteobacteria*. This taxon was found especially in the deeper horizons of the sediments of 2005 comprising on average 7.1% of all the reads and declined steadily to 0.03% and 0.001% of the community in the samples of 2010 and 2011, respectively (Fig. 5.4). The *Anaerolineae* class comprised the majority of the reads of the *Chloroflexi* and showed a

depth-related decrease in abundance in the samples of 2005 (Fig. 5.4), which was in line with the profiles observed for the *Chloroflexi* at the phylum level (Fig. 5.3). *Dehalococcoidetes* to which *D. mccartyi* belongs formed a minor fraction within the *Chloroflexi* sequences (Fig. 5.5). Its relative abundance within the *Chloroflexi* phylum decreased from 2.3 % in 2005 to 0.4 % in 2010 and 0.001 % in 2011, in agreement with the strongly decreasing 16S rRNA gene counts as determined by qPCR (Fig. 5.7). Members of the *Clostridia* as the main class of the *Firmicutes* increased in relative abundance with depth in samples of 2005 with a peak at 50 cm depth where they comprised up to 21.6 % of the reads. An identical trend in samples of 2005 was also evident for the members of *Nitrospira* as the major class of *Nitrospirae* phylum with a relative abundance peak of 20.03 % at 50 cm depth.

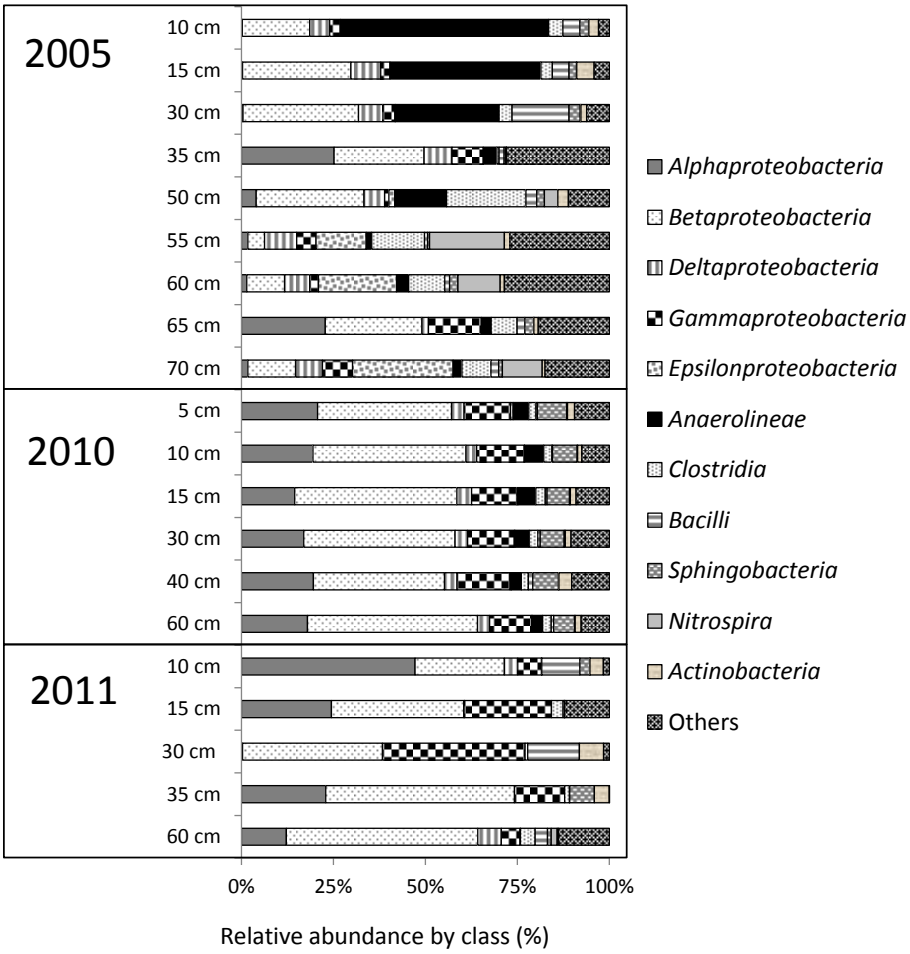


Fig. 5.4 Relative abundance of the dominant bacterial classes at different depths in the sediment core samples taken in 2005, 2010 and 2011.

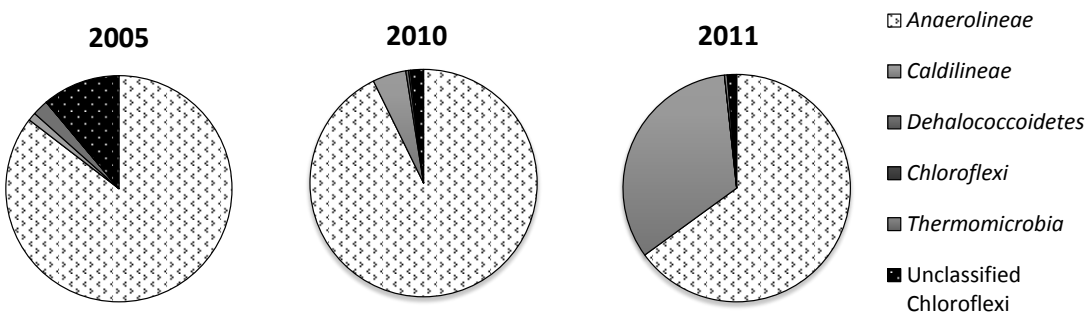


Fig. 5.5. Comparison of relative abundances of *Chloroflexi* classes in the sediment core samples taken in 2005, 2010, and 2011.

5.3.7 Order-level taxonomic distribution

At the order level, the 22 most abundant orders accounted for 67-99% of the classified sequences in different samples (Fig. 5.6). The two orders of *Anaerolineales* (from *Anaerolineae* class) and *Rhodocyclales* (*Betaproteobacteria*) were the most abundant in the top sediment layers of 2005 and their abundance decreased with increasing depth and with time. Members of *Clostridiales* (*Clostridia*) and *Lactobacillales* (*Bacilli*), *Campylobacterales* (*Betaproteobacteria*), *Desulfobacterales* (*Deltaproteobacteria*) and *Nitrospirales* (*Nitrospira*), which were abundant in the samples of 2005, decreased in relative abundance over time. In contrast, the *Burkholderiales* (*Betaproteobacteria*) and *Xanthomonadales* (*Gammaproteobacteria*) were enriched in the samples of 2010 and 2011. In samples of 2011, the relative abundance of *Burkholderiales* increased with depth reaching more than 50% of the classified sequences in the bottom layers (Fig. 5.6). The orders of *Rhodobacterales* (*Alphaproteobacteria*), *Nitrosomonadales* (*Betaproteobacteria*) and *Sphingobacteriales* (*Sphingobacteria*) were specifically enriched in the samples of 2010 while alphaproteobacterial orders of *Sphingomonadales* and *Rickettsiales* were enriched in samples of 2011 (Fig. 5.6).

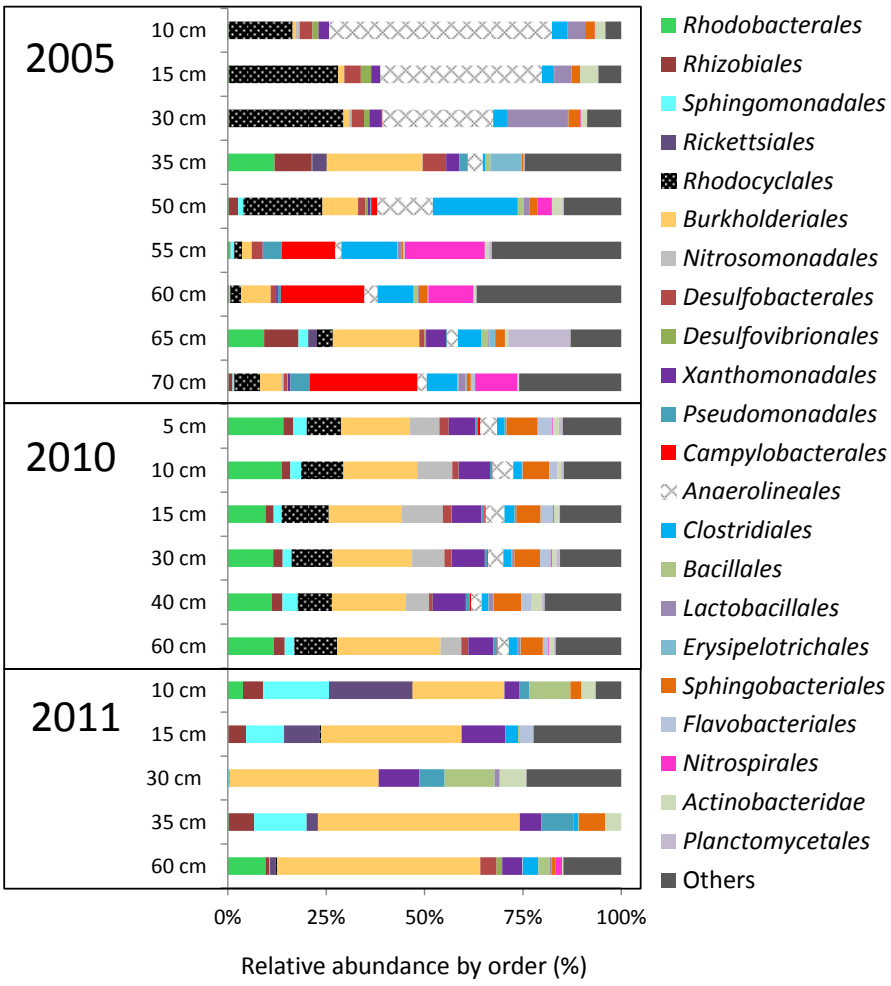


Fig. 5.6. Relative abundance of the dominant bacterial order at different depths in the sediment core samples taken in 2005, 2010 and 2011.

5.3.8 Quantification of bacteria and *D. mccartyi* in sediment cores

The highest bacterial 16S rRNA gene numbers were detected in surficial layers of the sediment core taken in 2005 with numbers above 10^8 copies per gram of sediment (Fig. 5.7). Bacterial 16S rRNA gene numbers declined steadily with depth till 10^5 per gram of sediment in deeper layers. In contrast, the core sample from 2010 did not show a clear depth related trend and the bacterial 16S rRNA gene abundance varied between 10^5 to 10^7 copies per gram of sediment. Bacterial 16S rRNA gene numbers in the core taken in 2011, however, were the lowest with abundances from 10^3 to 10^5 copies per gram sediment. *D. mccartyi* 16 rRNA gene was detected in most of the layers of the core taken in 2005 as well in the one taken in 2010 (Fig. 5.7). In both cores, *D. mccartyi* 16S rRNA gene abundance was the highest

in surficial layers where they reached 10^3 to 10^4 16 rRNA gene per gram of sediment (Fig. 5.7). In contrast, *D. mccartyi* 16S rRNA gene was detected in only one of the layers of the core sampled in 2011.

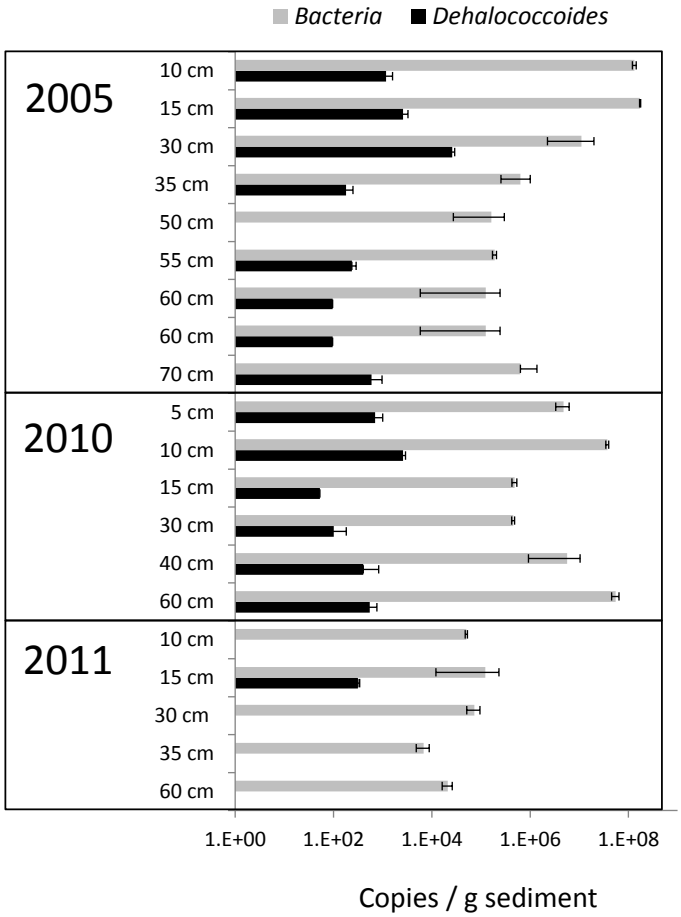


Fig. 5.7. 16S rRNA gene copy numbers of bacteria and *D. mccartyi* at different depth in the sediment core samples taken in 2005, 2010 and 2011 as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample.

5.3.9 Correlation between environmental parameters and microbial communities

We performed RDA to study the relationship of environmental parameters and bacterial community composition at order level. DOC and oxygen in surface water and time were found to contribute significantly to explaining the observed variation in community composition (P = 0.004, 0.01, and 0.01 respectively) (Fig. 5.8). *Burkholderiales* was strongly correlated with oxygen and time in surface water while it was negatively correlated with

DOC in surface water. An identical trend was evident for *Rickettsiales* and *Sphingomonadales* which were also negatively correlated with TOC and TN in the sediment. In contrast, *Lactobacillales* and *Anaerolineales* were strongly correlated with the DOC level in surface water and sulphate in ISW while being negatively correlated with oxygen in surface water (Fig. 5.8).

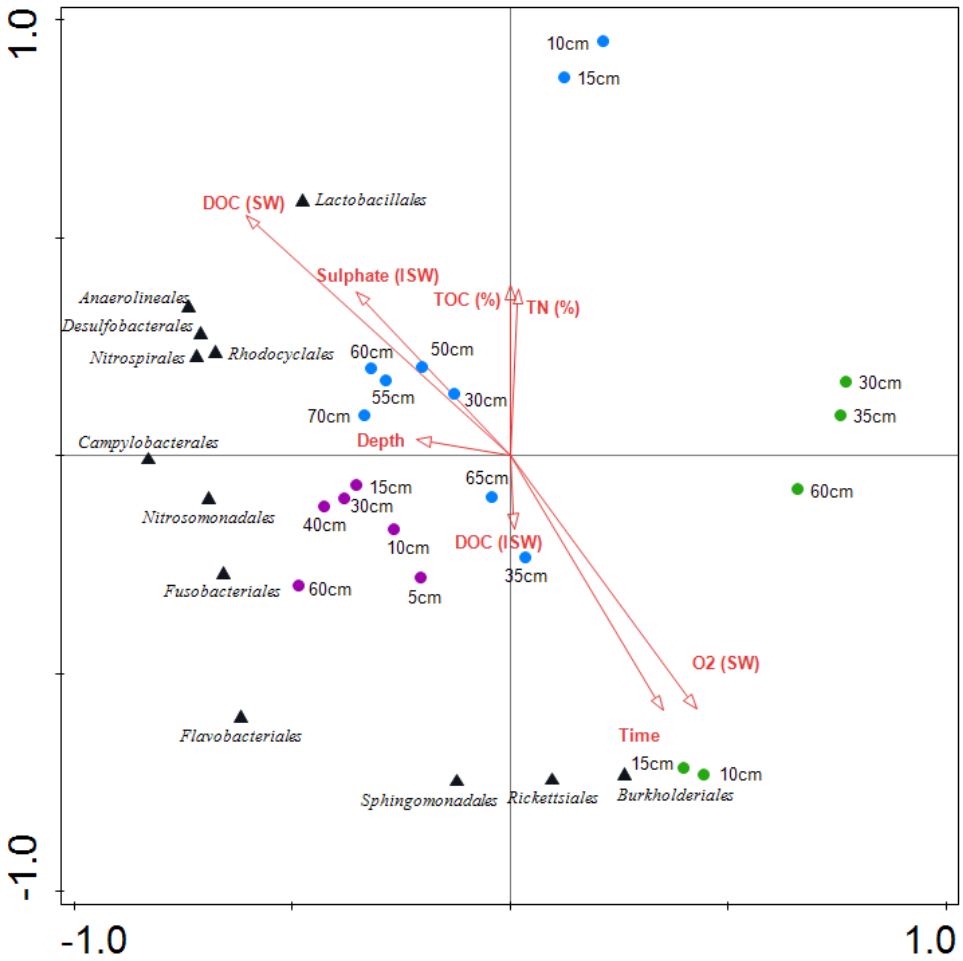


Fig. 5.8. Redundancy analysis triplot showing the relationship between microbial composition at order level and environmental parameters. Orders are indicated with black triangles, environmental parameters with red arrows and the samples of 2005, 2010, and 2011 are shown in blue, purple and green circles, respectively. The eigenvalues of the first (*x*) and second (*y*) canonical axes are 0.343 and 0.163, respectively. The arrow length corresponds to the variance that can be explained by the environmental variable. The direction of an arrow indicates an increasing magnitude of the environmental variable. The perpendicular distance between orders and environmental variable axes in the plot reflects their correlations. The smaller the distance, the stronger the correlation. SW: surface water; ISW: interstitial water.

A Bray Curtis similarity cluster was computed to compare the similarities of the microbial communities at the order level among different years and depths (Fig. 5.9). In general, bacterial communities were clustered rather by year rather than by depth. Only for the 2005 samples 2 groups could be distinguished according to depth, i.e., the more shallow samples and the deeper samples. The bacterial communities in the samples of 2010 clustered tightly together, indicating a high degree of similarity among them. Moreover, the 2010 cluster also harboured two samples of 2005, i.e., 2005.35 cm and 2005.65 cm suggesting a substantial dissimilarity of the bacterial communities in these two samples compared with the rest of the samples of 2005 (Fig. 5.9). The samples of 2011 clustered together and were more related to those of 2010 than to those of 2005.

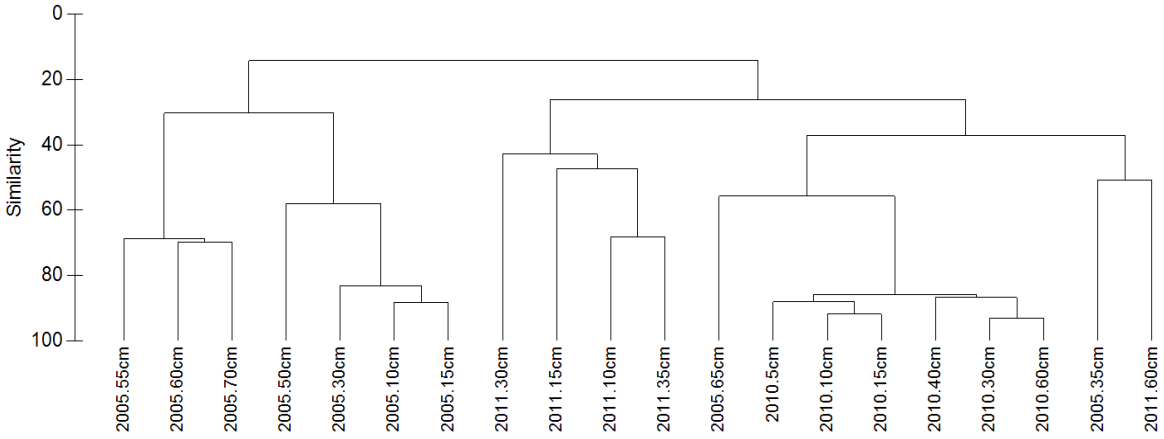


Fig 5.9. Clustering of samples. Bray-Curis similarity index was calculated using the relative abundance of bacterial communities at order level, and hierarchical clustering was calculated using PRIMER 6.

5.4 Discussion

The objective of this study was to assess the impact of the implementation of WWTP on the microbial community structure and VC respiration potential in the hyporheic zone of the Zenne River impacted with VC-contaminated groundwater plume. We hypothesized that the reduction in organic load in the river as a consequence of the water treatment would have important effects on the sediment community and its functionality especially OHRB.

The high organic carbon content in the surface water in 2005 appear to cause severe depletion of dissolved oxygen and high enrichment of organic carbon in the shallow sediment. Hence, it was not surprising to see the highest bacterial counts in the surficial sediments of 2005, which showed nearly exponential decrease in microbial biomass with sediment depth (Fig. 5.7). An identical decrease in microbial biomass parallel to a decrease in carbon content was reported in soil (Eilers *et al*., 2012).

The analysis of bacterial communities showed substantial differences in community structure, evident even at the phylum level. *Proteobacteria* was the most abundant phylum throughout time, and its relative abundance increased after installation of the WWTP. Drury *et al.* (2013) reported a reduction of the relative abundance of proteobacterial sequences within bacterial communities in the sediment downstream of a WWTP as compared to upstream sediment communities. Within the *Proteobacteria*, especially the relative abundance of the *Betaproteobacteria* became enriched in proportion in the sediments over time. Interestingly, its sub-class taxa showed diverging dynamics in our study. For instance, the proportion of *Burkholderiales* sequences showed an increase in time especially in the deeper sediment horizons, whereas the relative abundance of *Rhodocyclales* decreased. This pattern might be explained by the concept of copiotrophic (fast-growing) versus oligotrophic (slow-growing) bacteria (Fierer *et al*., 2007; Li *et al*., 2012). It can be hypothesized that the substantial decrease of organic carbon and in particular the easily accessible ones in the surface water and sediments of Zenne River favored oligotrophic members of the community, which are more competitive under conditions of low nutrient availability. For example, *Burkholderia*, a genus within the *Burkholderiales* that is highly enriched in proportion in all samples of 2011, has versatile catabolic traits that enable them to degrade recalcitrant compounds and survive in environments with limited nutrient availability (Suárez-Moreno *et al*., 2011). A low availability of organic nutrients has also been shown to favor certain members of the *Alphaproteobacteria* (Eiler *et al*., 2003; Pinhassi & Berman, 2003). In line with these reports, we noted a marked increase in the relative abundance of *Sphingomonadaceae* whose members are considered to be adapted to conditions of low availability of metabolic substrates (Pang & Liu, 2006). In contrast to *Alpha*- and *Betaproteobacteria*, *Epsilonproteobacteria* were almost non-detectable in the sediment samples of 2011. *Campylobacterales* as the main order in the class of *Epsilonproteobacteria*

were exclusively noticed in the deeper horizons of the sediment samples from 2005. Campylobacters have been isolated from a variety of aquatic systems (Sails *et al*., 2002) and are commonly reported as causes of gastroenteritis (Skirrow, 1994). Their decrease in the samples of 2010 and 2011 might be due to effectiveness of WWTP in removing pathogens (Drury *et al*., 2013). In accordance with enrichment of oligotrophic microbial communities, *Actinobacteria* increased in abundance over time. Members of this phylum play a major role in organic matter turnover and carbon cycling by decomposing recalcitrant carbon sources including cellulose and chitin (Acostamartinez *et al*., 2008). In general, it can be concluded that the high loads of organic carbon coupled to intense microbial activity have led to depletion of oxygen in the hyporheic sediments of 2005, which stimulated bacterial communities that are active under anoxic and sub-oxic condition. Hence, bacterial fermenters, such as *Lactobacillales* and *Clostridiales*, sulphate-reducing *Deltaproteobacteria*, and denitrifying members of *Rhodocyclales* and *Nitrospirales* had the highest abundance in samples of 2005. Accordingly, a strong correlation was observed between bacterial communities of these orders and organic carbon load and the surface water and sediment (Fig. 5.8). In contrast, members of *Sphingomonadales*, *Xanthomonadales*, *Pseudomonadales*, and *Rickettsiales* that are known to be active under oxic conditions, were more prevalent in the more oxic sediments of 2011 with positive correlation to oxygen in surface water and time, and negative correlation to organic carbon load in surface water and sediment (Fig. 5.8).

When we compared the microbial community diversity in the sediments over different years, we noticed remarkable differences both with sediment depth and over time. Based on the Shannon diversity indices (ranged between 5.34 and 7.29), the samples of 2005 fall within the range of values reported previously using a high throughput pyrosequencing for reservoir sediments (Röskea *et al*., 2012), mangrove sediment (dos Santos *et al*., 2011) and even soil (Will *et al*., 2010). However, the diversity increased with sediment depth which is in contrast with other studies on soil microbial communities (Will *et al*., 2010; Eilers *et al*., 2012), while it is expected to decrease with depth. Tokeshi (1999) suggested that the decrease of organic carbon load will increase interspecific competition for the available and more recalcitrant resources, which can in turn lead to biodiversity reduction. The samples of 2010 showed an exceptionally high diversity and richness but no depth related stratification

leading to high similarity observed between these samples (Fig. 5.9). Previous studies on the bacterial communities of hyporheic sediments revealed season-depended community dynamics using 16S rRNA gene based finger printing techniques with limited resolution such as DGGE and terminal restriction fragment length polymorphism (T-RFLP) (Feris *et al*., 2004; Sliva & Williams, 2005; Febria *et al*., 2012), but did not report the uniform evenness at different sediment depths found here for the 2010 sediment cores. In contrast to the sediment of 2010, lower species richness and diversity was recorded in the sediment core of 2011.

Our pyrosequencing data showed a clear decrease in the relative abundance of *Chloroflexi* with increasing sediment depth in sediments of 2005. This is in contrast to previous reports for sediments of man-made lakes (reservoirs) (Röskea *et al.,* 2012) where *Chloroflexi* relative abundance increased with increasing depth. Moreover, the relative abundance of *Chloroflexi* decreased over time and hence with decreasing organic carbon and increasing oxygen levels in the surface water. Similarly, Drury *et al*. (2013) reported significant decreases in the relative abundance of *Chloroflexi* in the riverbed sediment downstream of a WWTP. In our study, in line with the substantial decrease of *Dehalococcoidetes*, numbers of *D. mccartyi* 16S rRNA gene were the highest in the sediments of 2005, while except for one sample, *D. mccartyi* 16S rRNA gene was not detected in the 2011 samples (Fig. 5.7). Moreover, we noticed a reduced relative abundance of fermenting bacterial groups that are the drivers of OHR by producing hydrogen electron donors for OHRB (Maphosa *et al*., 2012). The decreased content of organic carbon and reduced relative abundance of *Chloroflexi, Dehalococcoidetes* and fermenters noted in this study could explain the reduced VC respiration capacity in the microcosms prepared with sediments of 2011. A substantial increase of VC OHR potential in the microcosms of 2011 after addition of tree bark as an electron source indicates that the lack of organic carbon was the main factor limiting VC OHR in the sediments of 2011 rather than long-term VC absence in the study area (Table 5.3). This is further supported by recent microcosm experiments with the Zenne aquifer materials where addition of a liquid organic carbon source increased the number of *D. mccartyi* 16S rRNA copies from a non-detectable level to 10^5-10^6 copies/gram (Atashgahi *et al*., submitted for publication).

Although the reduction in organic carbon load in the surface water and in the sediment is a plausible explanation for the observed differences in community structure before and after WTTP installation, it should be noted that the obtained results do not neglect possible contributions of other factors. Sediment microbial communities have been shown to be spatially diverse and correlated with seasonal physicochemical parameters such as temperature (Sliva & Williams, 2005; Hullar *et al*., 2006; Febria *et al*., 2010, 2012), DOC (Sliva & Williams, 2005; Febria *et al*., 2010, 2012), nitrate (Lowell *et al*., 2009; Febria *et al*., 2012), and phosphate (Febria *et al*., 2012). For instance, the presence of *Burkholderiaceae* in the hyporheic epilithic biofilms in fall has been related to leaf litter degradation (Huller *et al*., 2006) based on their capability on leaf litter decomposition (Gulis & Suberkropp, 2003). Accordingly, the relative abundance of *Burkholderiaceae* increased in the sample taken in Sep 2011. However, in our study, the relative abundance of *Burkholderiaceae* is increasing with depth while the impact of leaf litter on microbial communities is expected to take place in surficial sediments. Another impacting factor could be the exact location of the sediment sampling. Although, during the course of sampling the attempt was to get sediment cores from approximately the same location by sampling within a 0.5 m range, we cannot exclude differences between core characteristics and community structure even within this sampling zone.

5.5 Conclusion

Application of 16S rRNA gene based next-generation sequencing provided detailed insight on the dynamics of the bacterial community of hyporheic zones of a eutrophic river before and after upstream installation of a WWTP. Copiotrophs adapted for rapid metabolism were favoured in the surficial sediments of 2005, which was rich in organic carbon. In contrast, we observed enrichment of 16S rRNA gene sequences similar to those of slow-growing oligotrophs that appear to be favoured specially in the lower sediment horizons of 2011 due to their capabilities to survive in resource poor locations. As shown here, operation of a WWTP can have an important impact on downstream hyporheic bacterial community structure by reducing organic carbon load, bacterial biomass and diversity which might lead to fundamental altering of bacterial composition and functionality in hyporheic zones. Accordingly, we noticed reduced VC respiration potential in the sediments after installation of WWTP which can lead to reduced natural attenuation capacity in the hyporheic zone

against CAH contamination in discharging groundwater. On the other hand, we cannot exclude that other factors than the reduction of organic carbon load in the river and in its sediment over time contributed to the observed community differences.

Chapter 6: Conclusions and future perspectives

The main goal of this PhD was to improve our understanding of the underlying processes of biotic CAH removal in the hyporheic zone of river sediments at locations where CAH contaminated groundwater discharges into the river and development of mitigation strategies to reduce or prevent CAH discharge. To this end, this research consisted of three parts:

In the first part, the aim was to understand and quantify the occurrence, relative contribution, and environmental relevance of reductive dechlorination versus aerobic degradation of cDCE and VC in hyporheic sediments impacted by CAH contaminated groundwater. Therefore, we studied a new concept of co-existence and co-activity of aerobic and anaerobic CAH degraders and their resistance and resilience to varying redox conditions that take place in the surficial riverbed sediments of the Zenne River in Belgium at a site

where CAH contaminated groundwater discharges into the river. The study shed more light on the evolution of aerobic VC-assimilators from ethenotrophs in natural ecosystems.

The second part aimed at the development of mitigation strategies to reduce or prevent CAH discharge into receiving surface water bodies. We tested the potential of five different SPOMs to function as a long term source of electrons for OHRB in bioreactive caps. Through detailed studies of CAH dechlorination and dynamics of microbial guilds and associated biomarkers, we found significant differences between the SPOMs regarding the stimulation of OHRB and methanogens as the main competitors of OHRB for reducing equivalents in the Zenne riverbed sediment.

The third research goal was to evaluate the impact of a WWTP operation that was recently installed upstream of the studied Zenne location on the bacterial community composition and in particular on the OHRB. The performed research determined a striking change on the bacterial community over long time and decreased bacterial diversity and richness. In line with the decreasing organic load, the VC respiration potential decreased in the hyporheic sediments.

6.1 Reductive dechlorination versus aerobic CAH-degradation

Previous studies on the riverbed sediments of the Zenne River revealed the occurrence of VC reductive dechlorination by OHRB between 120 cm and 20 cm depth in those sediments (Hamonts *et al*., 2009, Kuhn *et al*., 2009, Hamonts *et al*., 2012). $\delta^{18}O$ and $\delta^{2}H$ isotopic measurements of the Zenne surface water and discharging polluted groundwater suggested that surface water penetrates into the river sediments till 20 cm depth (Hamonts *et al*., 2009) which might stimulate the aerobic degradation of VC. Hence, we hypothesized that besides anaerobic OHRB, aerobic bacterial guilds could also perform biodegradation of VC in the discharging groundwater and hence contribute to natural attenuation of the VC pollution. The research introduced a new concept regarding the CAH-biodegradation capacity in the hyporheic zones and indicated that OHRB and aerobic VC degraders can co-exist and be co-active in the hyporheic sediments (Chapter 2). We also noticed that sediment physico-chemistry, by mediating oxygen penetration into the sediment, strongly affected the relative contribution of different CAH degradation pathways. The rapid VC removal and strong reduction of biomarkers associated with OHR in oxygen-exposed microcosms prepared from the sediments of location P25 showed a high contribution of

aerobic VC-degraders to VC removal in P25 microcosms. The course sediment structure, low organic matter and extensive oxygen penetration into the P25 sediments might be the reason for this. This indicates that at the interface between surface water and sediment, aerobic VC biodegradation might be an important accessory process in VC removal from discharging groundwater in the Zenne ecosystem in addition to the previously shown OHR in deeper sediment layers (Hamonts *et al*., 2009, Kuhn *et al*., 2009, Hamonts *et al*., 2012). However, based on the results, we could not determine the relative contribution of aerobic ethene-assimilators versus VC-assimilators to disappearance of VC and/or ethene especially in the sediments of P26 where oxygen exposure did not affect proliferation of OHRB while the number of *etnE* and *etnC* increased simultaneously. This knowledge gap has significant implications for contaminated site management using MNA or bioremediation. Indeed, at some sites, the disappearance of VC might be misinterpreted as aerobic VC degradation, while as suspected in the sediments of P26, disappearance of VC might be due to initial reductive dechlorination of VC to innocuous ethene, and subsequent aerobic assimilation of ethene by ethenotrophs which have high affinity toward ethene (Mattes *et al*., 2010). Until development of molecular methods to distinguish between real VC-assimilating bacteria and ethenotrophs, application of the recently developed compound-specific isotope analysis of ethene (Mundle *et al*., 2012) could assist in determining whether VC is directly oxidised to CO_2 (VC assimilation) or ethene biotransformation happened as an intermediate between VC and CO_2 (assimilation of reductively produced ethene).

The study described in Chapter 3 stemmed out of the findings of Chapter 2. While in Chapter 3 we studied the resistance and resilience of anaerobic reductive dechlorinators and aerobic bacterial guilds towards redox fluctuations, we could also shed light on the underlying mechanisms of CAH disappearance under oxic conditions. After long-term incubations under anoxic conditions, oxygen exposure resulted in VC/cDCE removal with concomitant enrichment of the catabolic genes *etnC* and *etnE*, showing the high resistance and resilience of aerobic degraders to long-term strict anoxic conditions. In contrast, we noticed high sensitivity of *Dehalococcoides mccartyi* to oxygen exposure which is consistent with previous reports using pure and sediment free enrichment cultures of *D. mccartyi* (Adrian *et al*., 2007; Amos *et al*., 2008). However, as suggested with the sediments of P26, the site physico-chemical properties might shield *D. mccartyi* against local redox fluctuations. We also found

that the OHRB responsible for the VC-to-ethene dechlorination step are more susceptible to oxygen inhibition than those that dechlorinate cDCE-to-VC. Hence, long-term oxidative stress might lead to selection of bacteria similar to the cDCE respiring *D. mccartyi* strain FL2 that has been reported to be resistant against oxidative stress (Amos *et al*., 2008) and high temperature (Fletcher *et al*., 2007). However, this strain is unable of metabolic VC dechlorination which might lead to undesirable accumulation of VC during natural attenuation/bioremediation of CAHs.

However, as we noticed in the sediments of P25, the impaired OHR might also lead to long-term exposure of ethenotrophs to elevated VC concentrations in the surficial oxic sediment layers leading to adaptation of ethenotrophs to VC assimilation which is reported to be due to point mutations in the alkene/epoxide catabolic gene *etnE* (Jin *et al*., 2010). Although we could not find any aerobic VC assimilation potential in the sediments of P26, several VC-assimilators were isolated from the microcosms prepared from the sediments of P26. The isolates were Gram-negative aerobic VC degraders belonging to the genera *Pseudomonas* and *Ochrobactrum* (S. Atashgahi, unpublished results). This suggests that in spite of the lack of aerobic VC degradation capacity in the sediments of P26, long term VC exposure under selective enrichment conditions can lead to development of aerobic VC assimilators from ethenotrophs. This observation support the hypothesis that evolution of VC assimilators from ethenotrophs is a likely process in the sediments of Zenne River especially in the sediments of P25 where conditions seems to be conducive for such an evolution i.e. long-term exposure to VC from anoxic deeper sediments and to oxygen from oxic surface water. To study this hypothesis, further molecular-ecological research should examine the frequency of occurrence of such point mutations in the *etnE* gene (Jin *et al*., 2010) in the sediments of P25 and P26 both in the microcosms and *in situ*. Direct sequencing of *etnE* PCR amplicons derived from these environments by means of next generation sequencing can be used for this purpose. A higher frequency of point mutations in *etnE* in the sediments of P25 microcosms as compared with the sediments of P26 microcosms is expected.

6.2 Development of mitigation strategies to reduce or prevent CAH discharge into receiving surface water bodies

Previous studies on the riverbed sediments of Zenne showed that river sediments cannot be assumed as a highly efficient zone of natural attenuation, as at some locations CAHs were

still leaking into the river (Hamonts *et al*., 2009, Kuhn *et al*., 2009, Hamonts *et al*., 2012). Hence, *in situ* capping of the sediment, i.e. the placement of a cap layer on top of the polluted sediment, was proposed to increase the biobarrier efficiency of the riverbed sediment. *In situ* bioreactive capping approach is a new development in the field of sediment capping in which the capping layer hosts and stimulates microbiota that can degrade the pollutants present in discharging groundwater (Himmelheber *et al*., 2011). Our hypothesis was that stimulation of CAHs OHR in bioreactive caps can be achieved by incorporation of suitable SPOM which allow a long term slow release/supply of electron donors for OHRB. Moreover, it increases the contact time between OHRB and the pollutant. The results of this study showed that lignocellulosic materials that are mainly agricultural by-products/wastes, have high potential to be incorporated in bioreactive caps. The obtained degradation rates using hay, tree bark and straw in the sediment microcosms showed that at the pore water velocity observed in the Zenne riverbed sediment (5.4 cm/day), a cap layer of 40 cm length would be sufficient to lower the VC concentration below the threshold concentration of 5 µg/L (Chapter 4). Moreover, rapid and persistent colonization of the SPOMs by a *D. mccartyi* community in this study showed that the SPOM layer can potentially form a biotransformation layer in a bioreactive cap in which the electron donor and CAHs is coupled with the dehalogenating microorganisms in the same place. This further suggests that *D. mccartyi* eluted from deeper sediment by the upwelling groundwater can colonize the bioreactive layer in an *in situ* application. It should be noted that due to decrease of CAH pollutant concentrations in discharging groundwater at the studied Zenne site, application of a bioreactive cap is not needed anymore at that location. However, the obtained knowledge can be used in other rivers impacted by plumes contaminated with CAHs or sediments contaminated with other relevant compounds known to be dechlorinated by OHR e.g. polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs).

The obtained results showed that the selection of an appropriate SPOM can have critical impact on the success of bioreactive sediment capping technology. Accordingly, addition of wood chips did led to a higher OHR capacity while most of the reducing equivalents produced from addition of straw and hay ended up in extensive methanogenesis, surpassing the need for dechlorination. Our results showed that tree bark is the best SPOM to stimulate

CAH degradation in the sediments of the Zenne site. Therefore, bench-scale screening of SPOMs is necessary to find a candid material in terms of stimulation of the target microbial groups without generating new environmental problems. However, besides the type of SPOM, other factors such as site characteristics and type of target contaminants might play an important role in the successful application of bioreactive caps. Therefore, although the bench-scale tests are of interest for selecting the most promising material, ultimately, there is a need to conduct long term, monitored field tests to assess the performance of a SPOM-based bioreactive cap. When applied *in situ*, a bioreactive cap hosting a layer of lignocellulosic such as tree bark can be incorporated between thin geo-textile membranes (McDonough *et al*., 2007) and installed horizontally in the sediment-surface water interface, covered by a thin sand layer. The sand layer would be helpful in deployment of low-density SPOMs, protection of membranes and providing an extra layer for microbial colonization. A similar concept has been introduced as the reactive core mats (RCM) intended for retardation of contaminant breakthrough to the overlying biologically active zone and overlying water column (Meric D, 2010) (Fig. 6.1). Alternatively, sand can be mixed with SPOMs to reach the proper density and placed in between geo-textile membranes as a single layer.

Fig. 6.1. Schematic overview of reactive core mat application in the field. The RCM is applied by rolling it onto the sediment surface from rolls that typically contain 4.5 m wide by 30 m long sheets. Ends of each section are anchored at the shoreline to provide higher stability and tensile strength. After application, the RCM is typically covered by at least 15 cm of sand to ensure its stability and to provide a new habitat for benthic organisms (from Meric D, 2010).

Application of a SPOM-based bioreactive cap will satisfy the delivery of the necessary electrons as documented by Himmelheber *et al*. (2011) who applied a bench-scale sand-based bioreactive cap seeded with a consortium of OHRB for biodegradation of PCE. Accordingly, a *Dehalococcoides*-seeded SPOM layer could be an approach for bioremediation of sediments that do not show a CAH dechlorinating activity due to the lack of OHRB. The key for success of capping technology in the case of a bioaugmented SPOM layer will be the sustained presence/activity of the seeded bacterial community. An alternative could be activation of microbial communities indigenous to the sediment which have the metabolic capability to perform biodegradation. This can be achieved by *ex situ* amendment of some

sediment from target site with appropriate electron donors and an alternate halogenated electron acceptors/co-substrates (haloprimers) to the sediment under treatment. These structurally related compounds exhibit a "priming" effect towards the target compounds. The application of alternate halogenated compounds as priming agents to stimulate the activity of natural sediment-born microbial communities has been shown for PCBs (Van Dort *et al*., 1997; Krumins *et al*., 2009; Park *et al*., 2011), and PCDD/Fs (Albrecht *et al*., 1999; Ahn *et al*., 2005; Ahn *et al*., 2007). The activated sediment can be reamended to the original contaminated location as a layer under a SPOM-based bioreactive layer cap or even in direct combination with a the SPOM layer as the degrading inoculum.

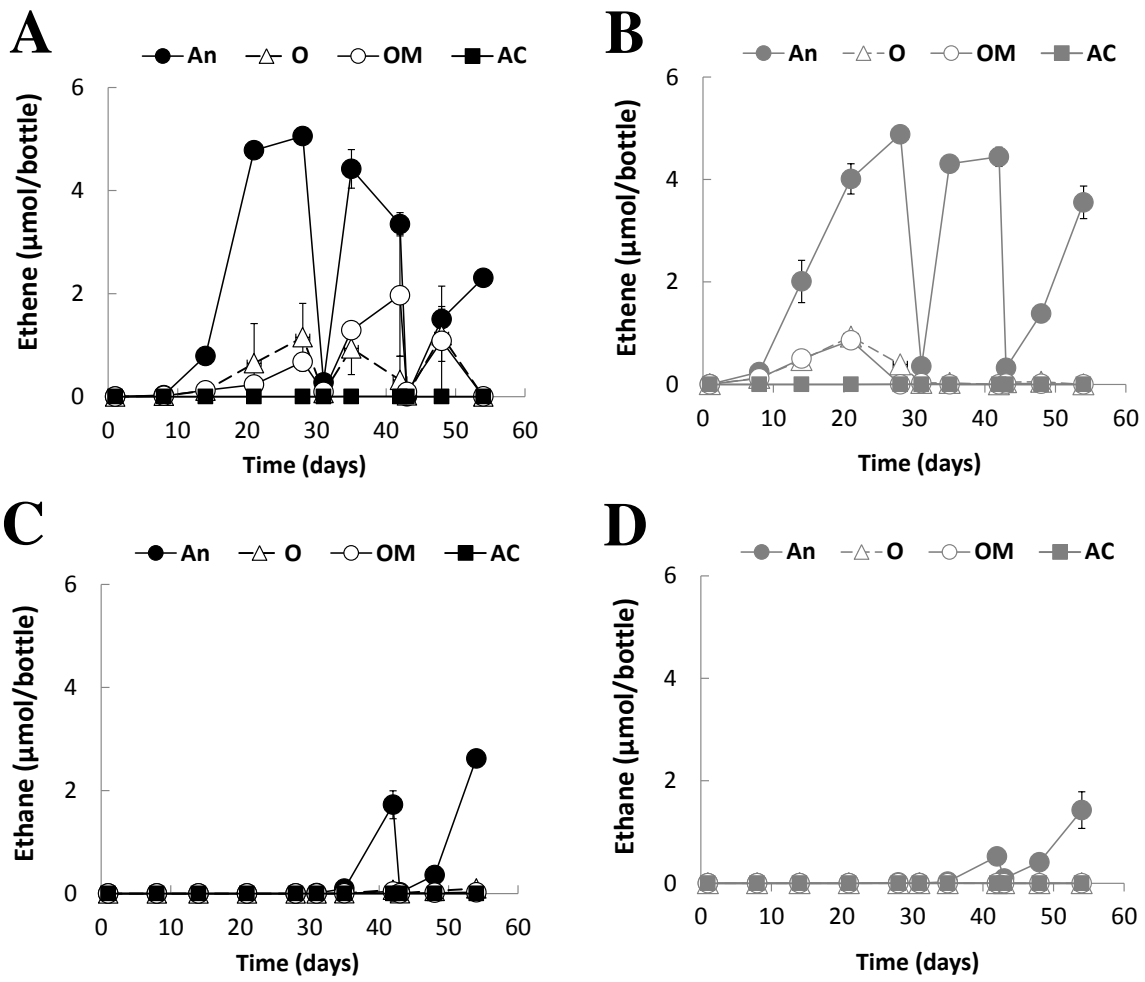
In situ capping whether passive or active cap design does offer some inherent advantages over dredging. First, the cost to cap is typically only 30 percent of the cost to dredge and dispose (Evison *et al*., 2004). An important criteria for the applicability of an *in situ* cap is material cost. As the agricultural by-products/wastes, the lignocellulosic feedstock can be used as an abundant low-cost source for biotechnological applications such as bioreactive sediment capping. For example, lignocellulosic feedstock such as straw, wood residues, energy crops, ... cost $50-60/ton which is much cheaper than routinely used materials in active capping such as apatite ($135/ton), coke ($145/ton), and AquaBlok ($170/ton) (Reible, 2004). However, besides material costs, other aspects should also be taken into account e.g. site characterization costs, design, construction and installation costs, monitoring and labor cost.

6.3 The impact of a WWTP operation on bacterial communities in hyporheic sediment

Modern WWTPs efficiently remove organic carbon from wastewater leading a significantly lower input of organic material in the river water and sediment (Gücker *et al*., 2006). Accordingly, after installation of the WWTP in upstream of the study location in 2007, there was a steady decline of organic carbon load both in the surface water and the sediments of the Zenne River. Considering the fact that food webs in the hyporheic zone are based primarily on the entrainment of organic carbon deposited from the surface water (Baker *et al*., 1999), such a dramatic decline of organic load might justify the shifts noted in the bacterial community composition of the Zenne hyporheic sediments. The decrease of organic carbon load as the main resource in hyporheic sediments will increase interspecific

competition over the residual but more recalcitrant resources (Tokeshi, 1999), favoring oligotrophic members of the community, which are more competitive under conditions of low nutrient availability. However, the obtained results do not neglect possible contribution of other factors to the observed bacterial diversity profiles such as seasonal physicochemical parameters (Sliva & Williams, 2005; Febria *et al*., 2010) and the necessity for additional sediment sampling. Hence, it would have been also of major interest to have analysed more samples before the installation of WWTP to see if a similar relation scan be found between TOC content and community structure as found in the sample of 2005 explored in this study. Indeed, previous studies on the sediment cores from April 2005 and May 2006 showed that organic carbon load (as determined by loss of ignition) was 10 times higher in the top 20 cm sediment layer compared with the deeper layer from 20-60 cm (Hamonts *at al*., 2012). Accordingly the number of *D. mccartyi* 16S rRNA gene decreased from 7.16×10^3 copies/g sediment in 10-20 cm depth to non-detectable level in 50-60 cm depth (Hamonts *at al*., 2012). However, these sediment cores were not analysed for bacterial community dynamics using tag pyrosequencing. Additional future sampling is needed over a wider area to acquire comprehensive information about the organic matter content and the microbial community dynamics in the Zenne River sediments.

Appendix 1



Chapter 2. Accumulation of ethene (A and B) and ethane (C and D) in anoxic and oxygen-exposed microcosms containing sediment from location P26 (panels A and C) and P25 (panels B and D), respectively. An: anoxic, O: oxygen-exposed microcosms amended with oxygen, OM: oxygen/methane-exposed microcosms, AC: abiotic control. All data points represent average values from duplicate microcosms, and standard deviations are included. If no error bars are shown, the standard deviations were too small to be illustrated.

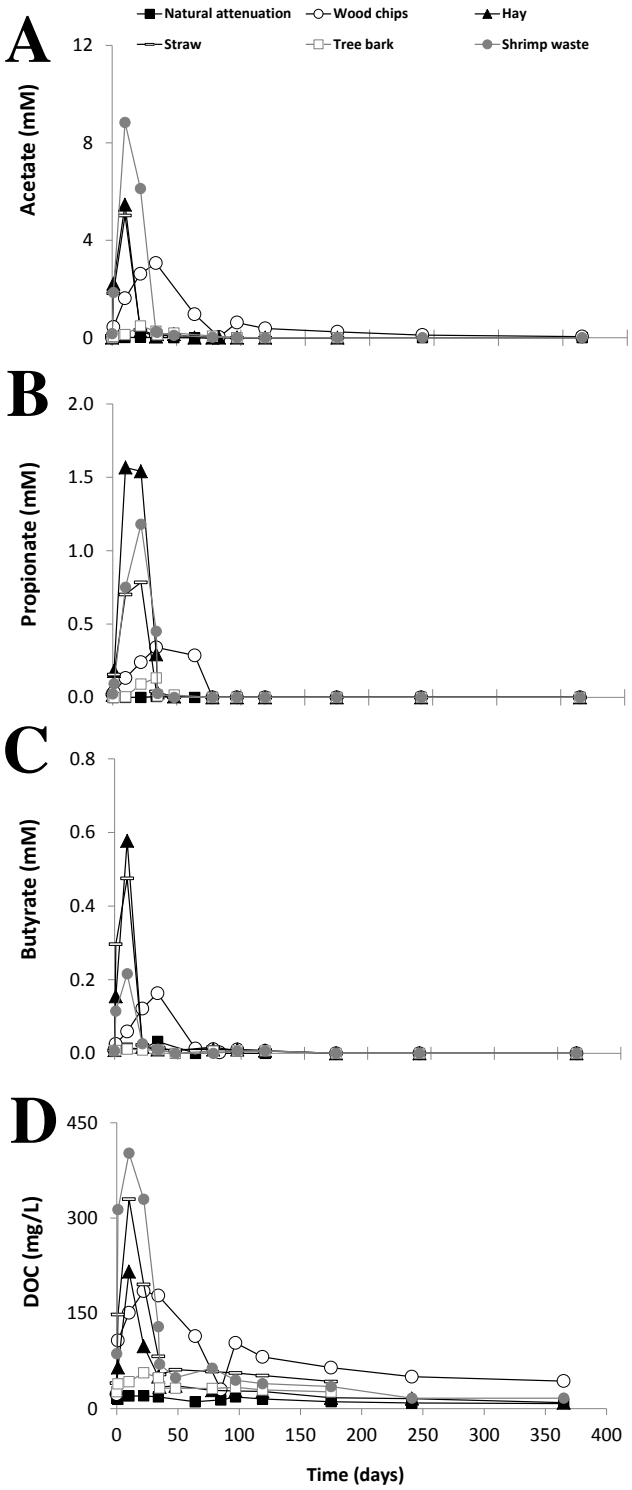
Appendix 2

Chapter 4. First order degradation rates of cDCE and VC obtained by linear regression of log-transformed concentration data. Degradation rates of aquifer, bottom- and top-sediment was obtained from experiments performed by Hamonts *et al*. (2012) and the rest of the rates were obtained from batch experiments in this study during first addition of capping materials (VC, obtained from Table 4.3) and second addition of capping materials (cDCE, obtained from Table 4.4). The degradation constants for the capping materials were set to the average degradation rate observed during the different spikes of the batch experiment.

Compartment	cDCE [day^{-1}]	VC [day^{-1}]
Aquifer [a]	0.004	0.004
Bottom sediment	0.105 ± 0.042	0.088 ± 0.069
Middle sediment	0.319 ± 0.273	0.359 ± 0.361
Top sediment	0.656 ± 0.087	0.593 ± 0.343
Capping (Sand)	0.048 ± 0.004	0.075 ± 0.065
Capping (Wood chip)	0.024 ± 0.009	0.113 ± 0.087
Capping (Hay)	0.347 ± 0.186	0.224 ± 0.128
Capping (Straw)	0.644 ± 0.220	0.284 ± 0.196
Capping (Tree bark)	0.361 ± 0.122	0.234 ± 0.111
Capping (Shrimp waste)	0.172 ± 0.141	0.201 ± 0.120

[a] The degradation rate in the aquifer was arbitrarily set to 0.001 day^{-1} since no degradation was observed in batch microcosms with aquifer material when no additions were made

Appendix 3



Chapter 4. Production of acetate (A), propionate (B), butyrate (C) and DOC (D) in sediment microcosms containing different solid polymeric organic materials (SPOMs) during second long-term cDCE dechlorination cycles. Data shown are average values obtained from duplicate microcosms. Note that different concentration scales are used for the Y axes of panels A, B and C.

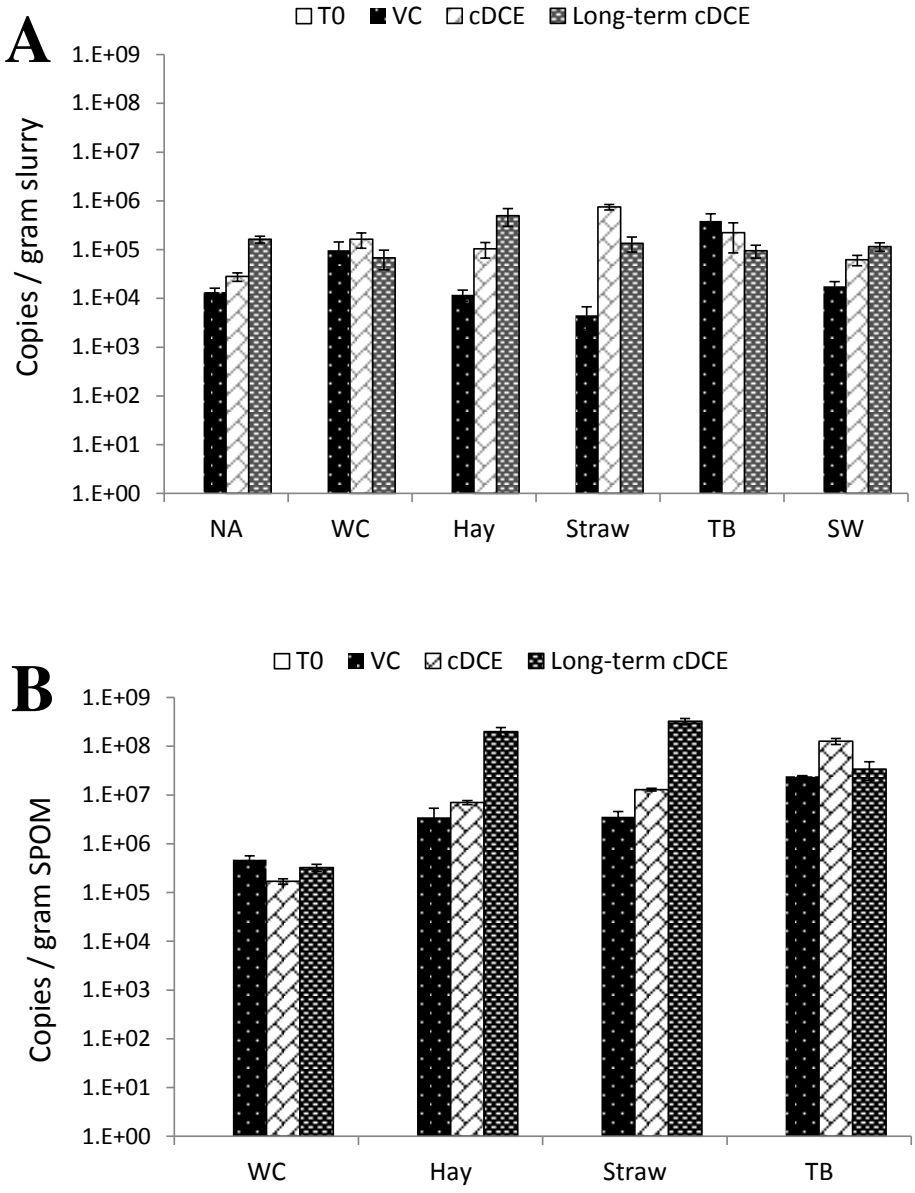
Appendix 4

Chapter 4. cDCE dechlorination results with different SPOMs after 1 year of incubation.

Conditions	spike number	Total cDCE added (μmol)	Total ETH[a]+ETA[b] produced (μmol)	Total MET[c] produced (μmol)	ETH+ETA/cDCE ratio (mol/mol)	MET/ETH+ETA ratio (mol/mol)
NA[d]	4	10.81	10.04	1.33	0.93	0.13
Wood chips	4	10.91	10.03	933	0.92	93.1
Hay	18	49.99	48.52	1663	0.97	34.3
Straw	23	62.69	61.68	6274	0.98	101.7
Tree bark	18	50.37	49.19	393.8	0.98	8.01
Shrimp waste	11	30.35	27.71	2338	0.91	84.4

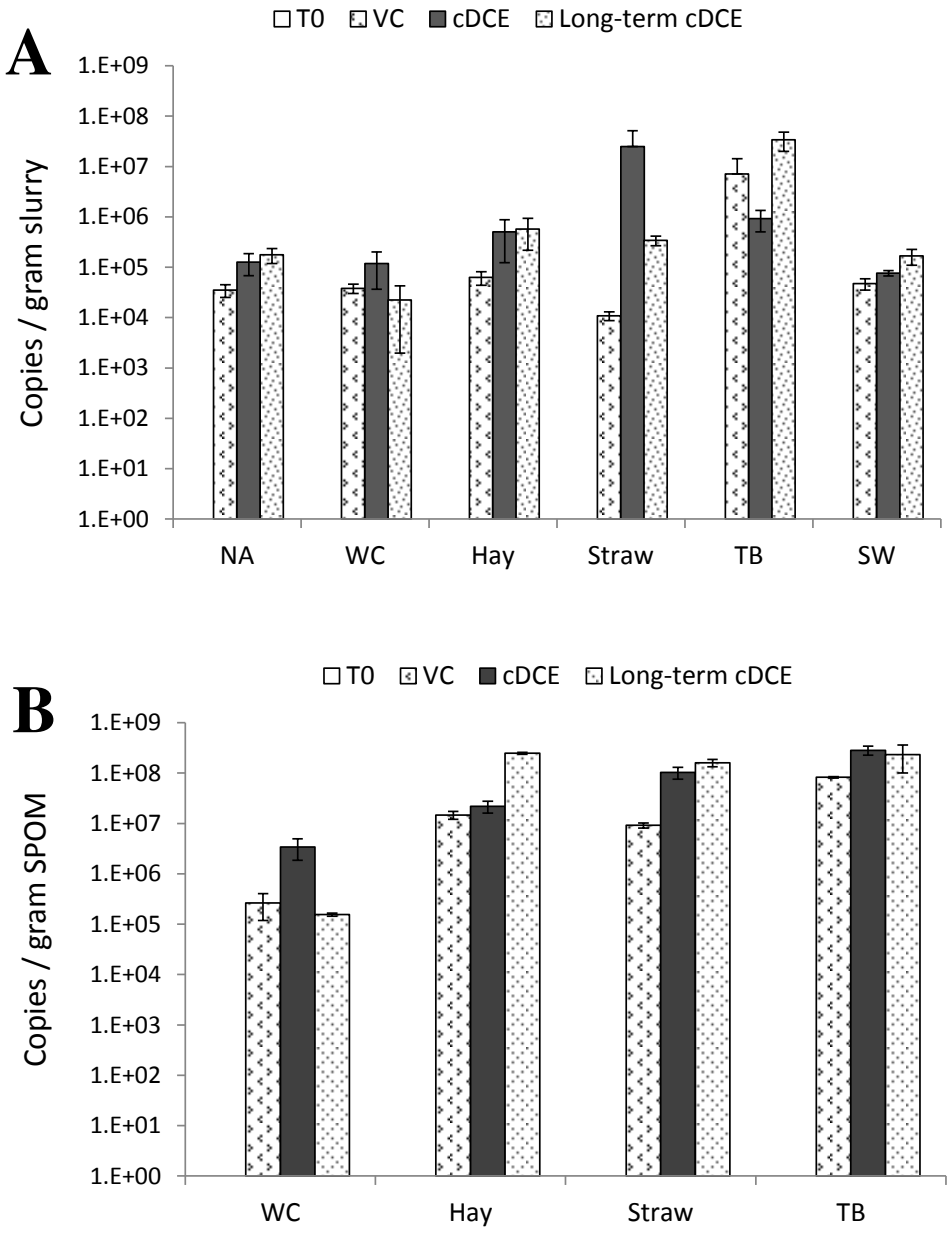
[a] Ethene
[b] Ethane
[c] Methane
[d] Natural attenuation

Appendix 5



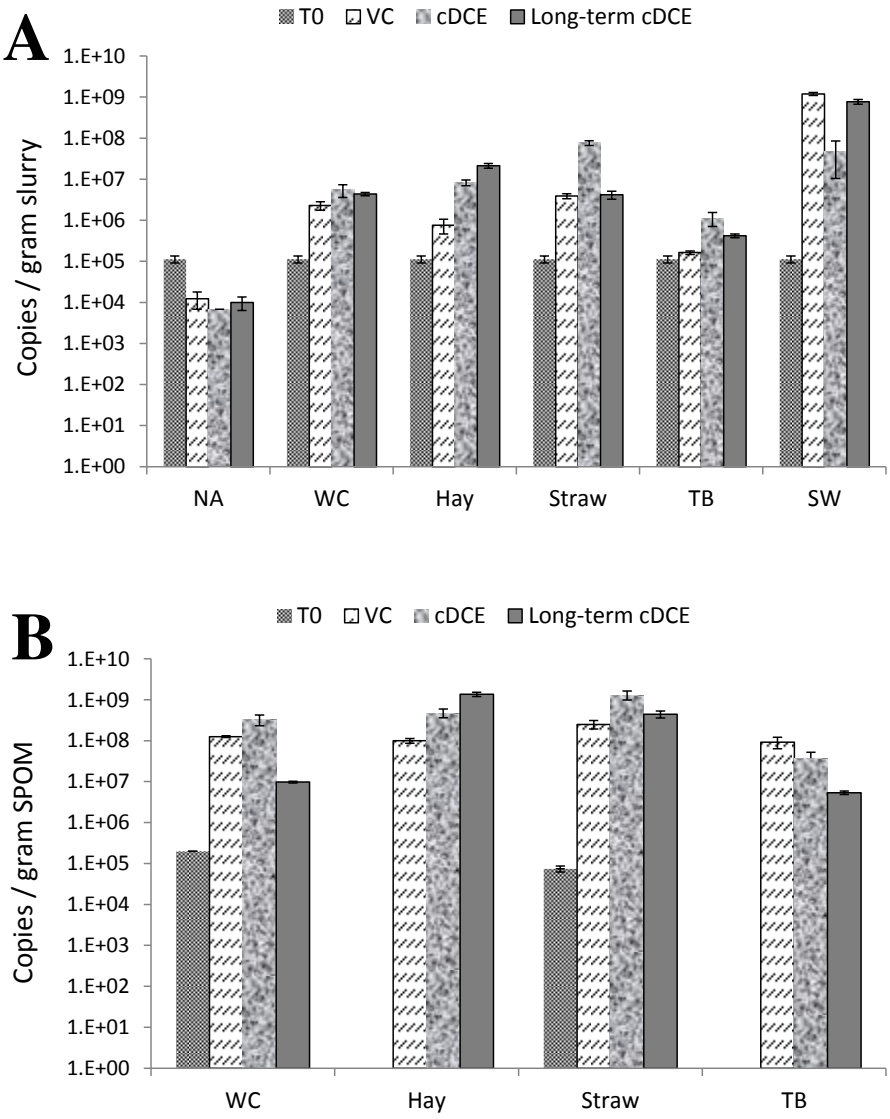
Chapter 4. Copy numbers of *vcrA* gene on day 0 and at the end of biodegradation cycles of VC, cDCE, and long-term cDCE dechlorination in sediment slurry (A) and solid polymeric organic materials (SPOMs) (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). NA: natural attenuation; WC: wood chips; TB: tree bark and SW: shrimp waste.

Appendix 6



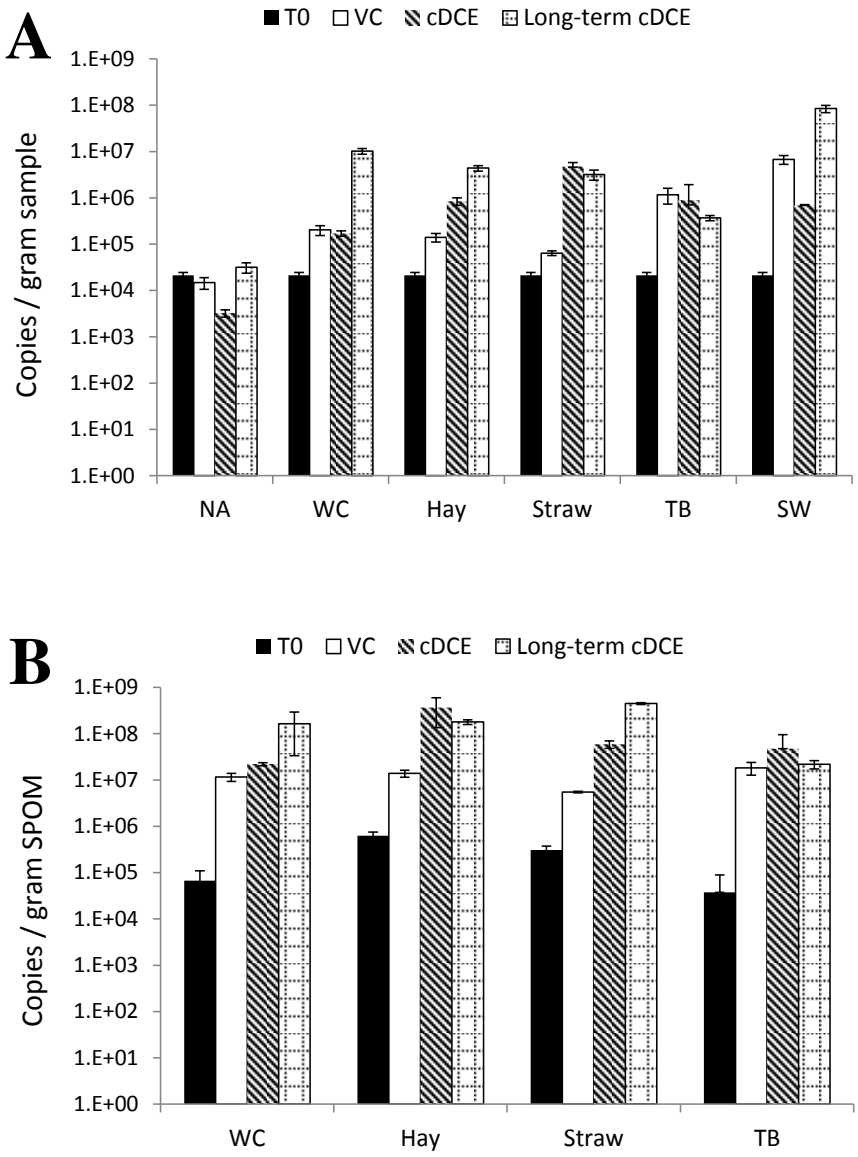
Chapter 4. Copy numbers of *bvcA* gene on day 0 and at the end of biodegradation cycles of VC, cDCE, and long-term cDCE dechlorination in sediment slurry (A) and solid polymeric organic materials (SPOMs) (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). NA: natural attenuation; WC: wood chips; TB: tree bark and SW: shrimp waste.

Appendix 7



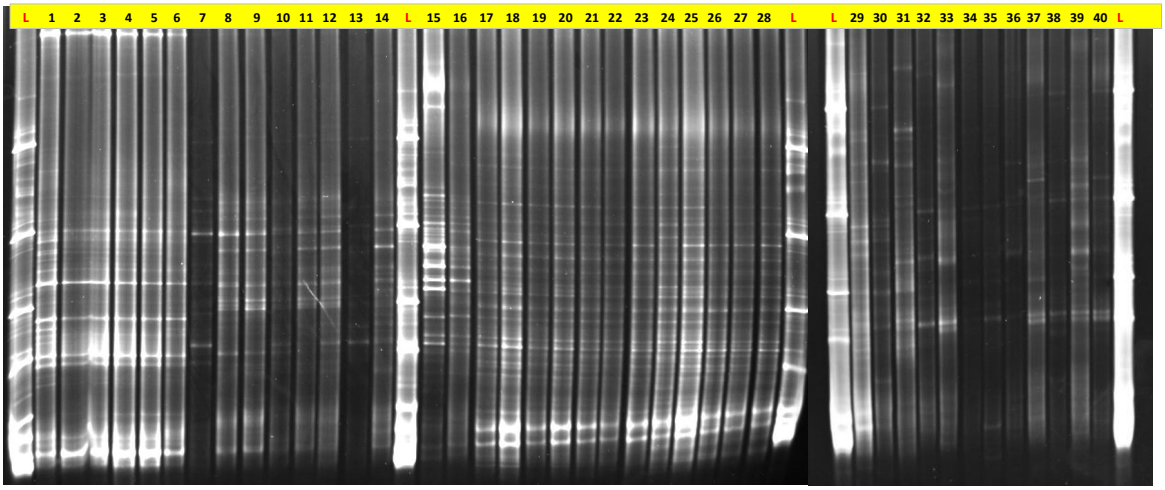
Chapter 4. 16S rRNA gene copy numbers of *Methanosarcina* on day 0 and at the end of biodegradation cycles of VC, cDCE, and long-term cDCE dechlorination in sediment slurry (A) and solid polymeric organic materials (SPOMs) (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). NA: natural attenuation; WC: wood chips; TB: tree bark and SW: shrimp waste.

Appendix 8



Chapter 4. 16S rRNA gene copy numbers of *Methanosaeta* on day 0 and at the end of biodegradation cycles of VC, cDCE, and long-term cDCE dechlorination in sediment slurry (A) and solid polymeric organic materials (SPOMs) (B) as determined by qPCR. Each value represents the average value obtained from triplicate qPCRs performed on one sample of each of the duplicate microcosms (n = 6). NA: natural attenuation; WC: wood chips; TB: tree bark and SW: shrimp waste.

Appendix 9



Chapter 5. DGGE profiles obtained from each 5 cm interval of the sediment cores from 2005, 2010 and 2011. The samples in each line are: L: ladder, 1: 2005-5 cm, 2: 2005-10 cm, 3: 2005-15 cm, 4: 2005-20 cm, 5: 2005-25 cm, 6: 2005-30 cm, 7: 2005-35 cm, 8: 2005-40 cm, 9: 2005-45 cm, 10: 2005-50 cm, 11: 2005-55 cm, 12: 2005-60 cm; 13: 2005-65 cm, 14: 2005-70 cm, L: ladder, 15: 2007-top, 16: 2007-buttom, 17: 2010-5 cm, 18: 2010-10 cm, 19: 2010-15 cm, 20: 2010-20 cm, 21: 2010-25 cm, 22: 2010-30 cm, 23: 2010-35 cm, 24: 2010-40 cm, 25: 2010-45 cm, 26: 2010-50 cm, 27: 2010-55 cm, 28: 2010-60 cm, L: Ladder 29: 2011-5 cm, 30: 2011-10 cm, 31: 2011-15 cm, 32: 2011-20 cm, 33: 2011-25 cm, 34: 2011-30 cm, 35: 2011-35 cm, 36: 2011-40 cm, 37: 2011-45 cm, 38: 2011-50 cm, 39: 2011-55 cm, 40: 2011-60 cm, L: ladder.

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