SCREENING OF THE BACTERIAL REDUCTIVE DECHLORINATION POTENTIAL OF CHLORINATED ETHENES IN CONTAMINATED AQUIFERS

A TECHNICAL ASSISTANCE MANUAL FOR ASSESSMENT OF NATURAL ATTENUATION OF CHLOROETHENES-CONTAMINATED SITES

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DIAGRAMS, TABLES AND FIGURES

LIST OF ACRONYMS

1,1-DCE	1,1-dichloroethene
BCS	Bacterial community structure
CEs	Chlorinated ethenes
c12DCE	cis-1,2-dichloroethene, cis-1,2-DCE
DNRA	dissimilatory nitrate reduction to ammonium
ENA	Enhanced natural attenuation
GWS	Groundwater system
mRNA	Messenger ribonucleic acid
NA	Natural attenuation
NR	Nitrate reduction or dissimilatory nitrate reduction to nitrite
OHRB	Organohalide-respiring bacteria
ORP	Oxidation/reduction potential
OSites	Ordonnance sur l'assainissement des sites pollués
PCE	Per- or tetrachloroethene
PCR	Polymerase chain reaction
RdhA	Reductive dehalogenase catalytic subunit A
rRNA	Ribosomal ribonucleic acid
TEAPs	Terminal electron-accepting processes
TCE	Trichloroethene
t12DCE	trans-1,2-dichloroethene, trans-1,2-DCE
VC	Vinyl chloride
X.PCE, X.TCE, X.c12DCE, X.VC	'X.' means percentage of the following chlorinated ethene compound. *This acronym is used by the statistical analysis software that interprets % as X.*

GLOSSARY

Aerobic: Chemical conditions pertaining to when oxygen is present; also used to refer to bacterial metabolism linked with the presence of oxygen for life.

Aerobic oxidation (direct) or aerobic respiration: Microbial breakdown of a compound in the presence of oxygen during which the compound serves as an electron donor and as a primary growth substrate for the microbe mediating the reaction.

Allochthonous: In ecology this term describes organisms or environmental elements (e.g. rocks) not indigenous of the studied place. They originate from a place other than where they are found.

Anaerobic: Chemical conditions pertaining to when no oxygen is present; also used to refer to bacterial metabolism linked with the absence of oxygen.

Bacterial consortium: A two- (or more) membered bacterial culture or natural assemblage in which all organisms benefit from the activities of each other.

Broken-Stick model: A model that randomly segments a line representing the total variance of a data set and compares them to Eigenvalues associated with principal components in Principal Component Analysis or Multiple Factor Analysis. Broken stick has been recommended as a stopping rule where principal components should be retained as long as the observed Eigenvalues are higher than the corresponding random broken-stick components.

Biogeochemistry: A scientific discipline that involves the study of the chemical, physical, geological, and biological processes and reactions (e.g. identified on terminal electron acceptor compounds) that govern the composition of the natural environment. The biogeochemical cycles are the pathway by which a chemical element changes through the biotic and abiotic parts of an ecosystem.

Catalytic subunit: A protein unit of an enzyme that catalyzes a chemical reaction.

Chlorinated ethenes (CEs): Organohalide compounds in which hydrogens bound to carbon of an ethene molecule are substituted by chlorines. In the present document "Chlorinated ethenes" or "chlorinated ethene compounds" are used as generic names to mention either Perchloroethene (PCE), Trichloroethene (TCE), isomeric forms of Dichloroethene (DCE), or Vinyl Chloride (VC).

Ecosystem: A community of living organisms (plants, animals and microbes) in conjunction with the non-living components of their environment (like air, water and mineral soil), interacting as a system. These biotic and abiotic components are regarded as linked together through nutrient cycles and energy flows (from and referenced in Wikipedia).

Eigenvalues: Eigenvalues associated with Principal Component Analysis indicate how much variation is explained by the data set. They are usually expressed as a percentage of the total variance of the data set.

Electron acceptor: A chemical entity that accepts electrons transferred to it from another compound. It is an oxidizing agent that, by virtue of accepting electrons, is itself reduced in the process.

Guild: Group of organisms that exploit the same class of environmental resources in a similar way without regard for the taxonomic position of each organism. Thus, the guild is the functional unit of the ecosystem, whereas the species is the biological unit of a community. Microbial guilds can objectively be defined as a group of microorganisms using the same energy and carbon sources and the same electron donors and acceptors (from Garcia-Cantizano et al., 2005).

Messenger RNAs (mRNA): Nucleic acid molecules synthetized by the cell machinery from genes present in the chromosomal DNA during gene transcription. mRNAs serve as support, during mRNA translation, for the production of peptides and proteins (e.g. enzymes).

Multivariate statistical analysis: Observation and analysis of more than one statistical outcome variable at a time. In design and analysis, the technique is used to perform trade studies across multiple dimensions while taking into account the effects of all variables on the responses of interest (from Wikipedia).

Natural attenuation (NA): Biological, chemical and physical processes causing, without the intervention of man, a reduction of the mass, load, toxicity, mobility or concentration of a substance in soil and groundwater. These processes involve biodegradation, chemical transformation, sorption, dispersion, diffusion, and volatilization of the substances (EPA, 1999).

Monitored natural attenuation (MNA): Monitoring measures taken for controlling the effectiveness of natural attenuation processes.

Enhanced natural attenuation (ENA): An *in situ* remediation strategy that actively stimulates and supports natural attenuation processes with the input of generally fermentable organic substances using the natural reaction space, hence when man actively intervenes in the NA process.

Phylogenetic analysis: The study of evolutionary relationships among groups of organisms (as species, populations…), which are discovered, for example, through molecular sequencing data.

Polymerase chain reaction (PCR): A molecular biology technique used to amplify a single copy or a few copies of a specific piece of DNA across several orders of magnitude, generating thousands to millions of copies of the original DNA sequence.

R function: A small script with code for a specific function. A master script, generally written by the user according to his needs, will call R functions.

R package: A folder including a set of R functions and datasets requisite for the computing of analysis related to some kind of statistical analysis. For instance, the R package “vegan” (initially developed for VEGetation ANalysis) contains among others all necessary tools for PCA analysis. A master script, generally written by the user according to his needs, will call the specific packages.

Ribosomal RNA (rRNA): In the cell machinery, rRNA constitutes an essential component of the ribosomes. Ribosomes are indispensable constituents of every single cell for protein synthesis. They are formed by two subunits, the large subunit (LSU) and small subunit (SSU). rRNA sequences are currently used to identify relationships and classify organisms.

RV correlation coefficient (RV coeff.): Values that indicate the closeness of two sets of points that may each be represented in a matrix.

Water quality: Describes the condition of the water, including chemical, physical, and biological characteristics, usually with respect to its suitability for a particular purpose such as drinking or swimming.

1. INTRODUCTION

1.1. General context

Water quality in Switzerland is generally good, but various anthropogenic substances, even in low concentrations, are threatening the resources globally. According to the registers compiled by the federal and cantonal authorities, there are around 38,000 polluted sites in Switzerland. Around 60% are industrial sites, and the remaining 40% are landfills and a few accident sites (Federal Council, 2015). Even at low concentrations, allochthonous substances in groundwater can cause serious financial consequences linked to elevated cost of remediation of sites. Since groundwater resources are hard to access, experts may have to face a lack of knowledge about the contaminated sites. All of this contributes, in many cases, to the impossibility of achieving the legally required remediation criteria.

1.2. The chlorinated ethenes

Chlorinated ethenes (CEs) are the organic compounds perchloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE) and vinyl chloride (VC) where hydrogen substitutes of ethene are replaced by chlorines (e.g. PCE has four chlorine substitutes, TCE three, etc.). CEs are used mainly in industry as save-to-use solvents (e.g. degreasing, dry cleaning, extracting) and in plastic manufacturing, especially polyvinyl chloride (Chloronet, 2009). **The mismanagement of the use and elimination of CEs, mainly PCE and TCE, during the 20th century caused their dispersal into the environment.** The behavior of CEs in the environment is governed by different processes related to: i) the distribution of the compound between its various aqueous, gas, or organic phases, ii) the different capacities of transport in the aqueous form, whether it is present in the interstitial air, or as a pure liquid organic phase (DNAPL), and iii) the degradation possibilities of the compound (i.e. biodegradation and/or chemical reactions).

PCE and TCE are the most common CEs, and can be entirely degraded anaerobically (without oxygen). The less chlorinated ethenes (DCE and VC) are degradable under **anaerobic** and aerobic conditions (with oxygen). The complete anaerobic degradation of CEs is a biological reductive dechlorination process during which chlorines are sequentially replaced by hydrogens until the ultimate production of the harmless ethene molecule (Figure 1). During this biodegradation process, CEs assume the role of an **electron acceptor** (like oxygen in classical respiration), and receive electrons taken from an electron donor (e.g. an organic compound). In the field, the organic carbon is fermented, and the product of fermentation is di-hydrogen that will be used as electron donor. This organic matter could either be supplied naturally or derived from other contaminant sources (e.g., petroleum hydrocarbons) that often accompany a CE plume.

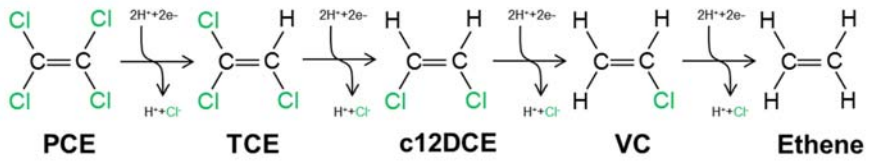


Figure 1 – Sequential reactions of the reductive dechlorination process.
PCE: perchloroethene, TCE: trichloroethene, c12DCE: cis-1,2-dichloroethene, VC: vinyl chloride.

Several bacteria have been isolated that can couple the reductive dechlorination of CEs to energy conservation. These bacteria can differ in their electron donor requirements, kinetics, endpoints of dechlorination, and the maximum tolerated concentrations of chlorinated solvent. In cultivated **consortia**, they allow a complete bacterial degradation of the CEs until ethene. This dechlorination process also happens in the field, but it is slow and often incomplete. Experience has demonstrated that the degree of CE attenuation is influenced by background concentrations of aqueous and solid phase electron acceptors (not only CEs) in different redox conditions. In this sense, and in order to understand the *in situ* occurrence of the dechlorination process, the environmental variables (pH, temperature, redox, etc) as well as concentrations of electron donors and acceptors are traditionally measured during any monitoring of a CE-contaminated site.

1.3. Conceptual framework of this manual and methodological principles

The proposed approach to evaluate the dechlorination processes in the saturated zone of contaminated sites examines aquifers as a coherent and dynamic assembly comprised of a biotic and abiotic part. Indeed, recent advances clearly demonstrate that aquifers are dynamic ecosystems showing complex interactions between physical, chemical and biotic components (Shani, 2012; Goldscheider *et al*., 2006; Humphreys, 2006). These ecosystems are extremely heterogeneous, even at a very small scale, in terms of lithological composition and grain size distribution. The complex geological matrix structurally organizes and modulates the fluxes of carbon and energy through groundwater flow. As a consequence, the heterogeneous nature of the solid habitat, coupled with the fluxes of energy and matter, fixes the boundaries of the development of the microbial communities. The resulting compartmentalization of the habitat allows the occurrence of ecosystem processes in a defined sequence and ensures the development of a high microbial diversity. Microbial communities are able to grow using a complex metabolic network, with a combination of metabolisms using trace organic inputs and the fixation of atmospheric gases (lithotrophy). Then, from an ecological point of view, aquifers can be considered heterogeneous assemblages of discrete macro- and micro-scale habitats that provide a variety of living conditions, which influence the heterogeneous distribution of the microbial community structures and their inherent activities. Thus, the proposed approach basically contains all the elements of a traditional hydrogeological analysis (chemical, physical, etc.), with the addition of complementary data in the form of bacterial analyses. These analyses are conducted in order to understand the relationships and correlations between the living and inert parts of the aquifer, i.e. how the microbes interact with their habitat. The additional data used here come mainly from molecular biology analyses performed by specialized laboratories. They involve research and evaluation of specific actors responsible for the degradation of CEs as well as analysis of bacterial community structures in their entirety by profiling analysis, such as T-RFLP.

The original contribution of this methodology is the use of statistics derived from numerical ecology tools. These tools have been used successfully for many years in the general ecology analyses, and offer unrivaled analytical power for complex data processing. The tools selected in this manual will be mainly used for the analysis of the physicochemistry and bacteriological status of a contaminated aquifer; and the interpretations will be of value to understand existing degradation mechanisms. The conclusions drawn from the analysis will also help to identify the probable reasons for a halt in the reductive dechlorination process and to evaluate potential solutions.

1.4. Keys for using the manual

This document presents, in detail, the proposed approach. The structure of the document is intended for a user, and the document guides the interested party either in "automatic" mode or in “step-by-step” mode for those who want to go deeper. Analyses of the bacterial communities involved in and interacting with the degradation of CEs are presented, and a detailed case study is described at the end of the manual. The appendices include all documents necessary for understanding and implementation of the procedure.

For the numerical analysis, the necessary hardware is a simple desktop computer and a color printer. The software and connected libraries are open access and the detailed procedure script is provided in the appendices.

1.5. Target audience

This document is addressed to anyone involved in finding solutions for the remediation of aquifers contaminated with CEs. Basic knowledge of bacterial respiratory metabolisms and molecular biology will be useful to understand and carry out the proposed techniques. The use of this manual also requires basic computer skills and some familiarity with database management. Knowledge of basic statistics is strongly recommended, and knowledge of multivariate statistics would be beneficial but is not specifically required.

2. OBJECTIVES

The objective of this manual is to offer a practical solution for anyone wishing to examine, in detail, the remediation process (natural or enhanced) in a contaminated site, such as a saturated aquifer polluted by CEs.

First, a step-by-step screening tool for the evaluation of the site status and examination of the presence, or absence, of **natural attenuation** (NA) of the pollutants is presented. Second, tools are presented to understand the potential reasons of a stalling of the CE degradation process. Finally, the proposed tools can contribute to the choice of a remediation technique or strategy and allow its monitoring in time and space.

"How does it work?"

The document presents a procedure based on a multivariate statistical tool dedicated to the analysis of data provided by geological, chemical and biological analyses. In this sense, aquifer ecosystem functioning is examined as a whole. The statistical analysis looks for the best correlation between different data sets and identifies the statistically significant variables. A conceptual model of aquifer functioning regarding CEs degradation is deduced from the analysis. Interpretation of the model can then elucidate the possible stalling of lower CE degradation, and a corrective strategy can be proposed.

The protocol presented in this document can be implemented at different steps of the Swiss procedure for investigation of contaminated sites (Diagram 1). This protocol can be inserted in the "detailed investigation" phase when the "specification of the technical investigation" is established and continued during the elaboration of the "remediation project" phase (FOEN, 2000; FOEN 2001a and b).The choice of the moment to apply the present methodology depends on the site, on the available equipment at the site (e.g. number of sampling wells), and also on technical and financial resources.

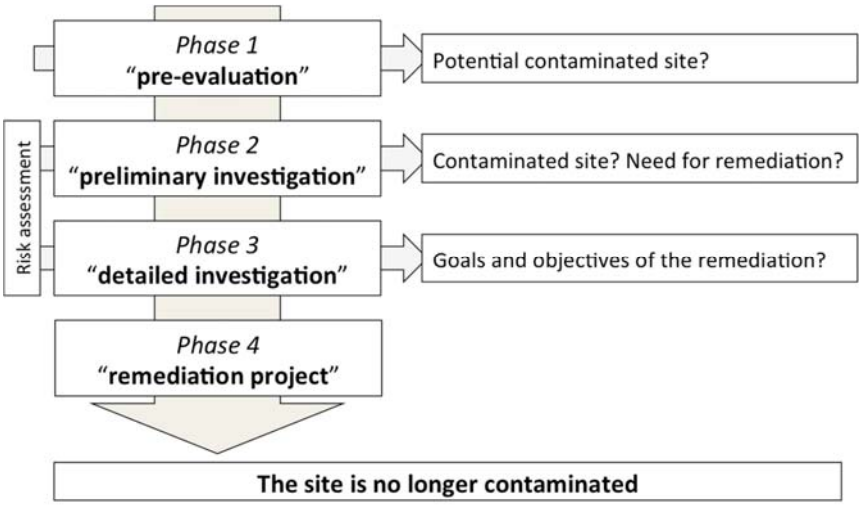


Diagram 1 - Systematic treatment of contaminated site in 4 phases (modified from FOEN, 2001a)

3. STEPS OF THE DIAGNOSTIC TOOL

GWSs are composed of both abiotic and biotic components, which are defined, respectively, as the "habitat" and "biota" i.e. the living organisms (Humphreys, 2006; Goldscheider *et al*., 2006). Evaluation of a site's bioremediation potential is associated with an investigation of habitat and biota components that can include multiple levels of analysis (Figure 2). Bacteria are targeted in biota as they are considered key actors in CE degradation (ChloroNet, 2009) and biogeochemical cycles (Treseder *et al.*, 2012; Rousk and Bengtson, 2014). Integration of data obtained from the exploration of the different levels (Figure 2, e.g. hydrology, hydrochemistry, bacterial community, bacterial genes and enzymes) leads to an overall understanding of the system. The finest appreciation of the biological functioning and CE degradation potential of a GWS will be obtained when the microbiology is characterized at every level and its interaction with abiotic environmental factors is analyzed.

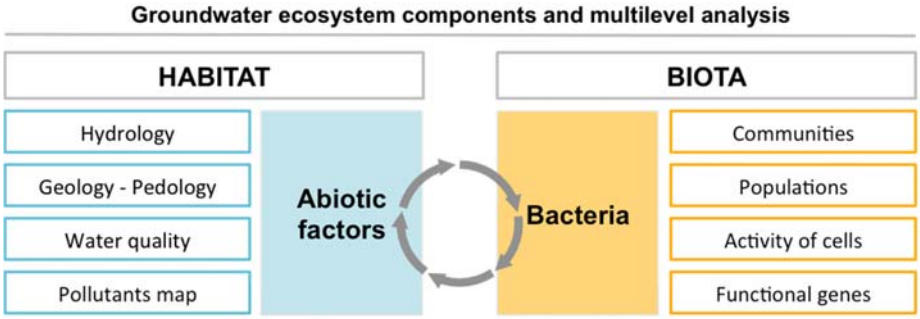


Figure 2 – Groundwater system complexity associated to bioremediation strategy evaluations.
A GWS is composed of habitat and biota components that can be analyzed at different levels, shown in the orange and blue boxes. The central circle of arrows indicates that dynamic interactions occur between elements of the habitat and the biota, which are a driver of GWS functioning.. This diagram should be viewed as a snapshot of a real dynamic situation where all levels and components are interdependent.

On this basis, a step-by-step screening process is presented in Diagram 2. The procedure explores the potential for reductive dechlorination in an aquifer through three gradual steps (boxes in grey on the right of Diagram 2) that answer the following questions:

1) Is *in situ* reductive dechlorination activity present?
2) Is there potential for complete degradation?
3) What are the reasons for possible incomplete reductive dechlorination in the aquifer?

Regarding the technical objectives (color boxes in Diagram 2), the first step is devoted to the acquisition of the key abiotic environmental data of the studied aquifer. The second step describes the characterization of microbiology directly and specifically involved in the reductive dechlorination of CEs (levels of populations, activity of cells and functional genes; Figure 2). The third step completes the analysis of microbiology at a wider scale (communities, Figure 2) that includes not only reductive dechlorination but all bacterial activities of the aquifer. This last step also provides an integrated analysis of the results of microbiology and physico-

chemistry inquiries (biota and habitat, respectively) to assess the global biogeochemical characteristics of the aquifer and their impact upon the biodegradation potential of CEs.

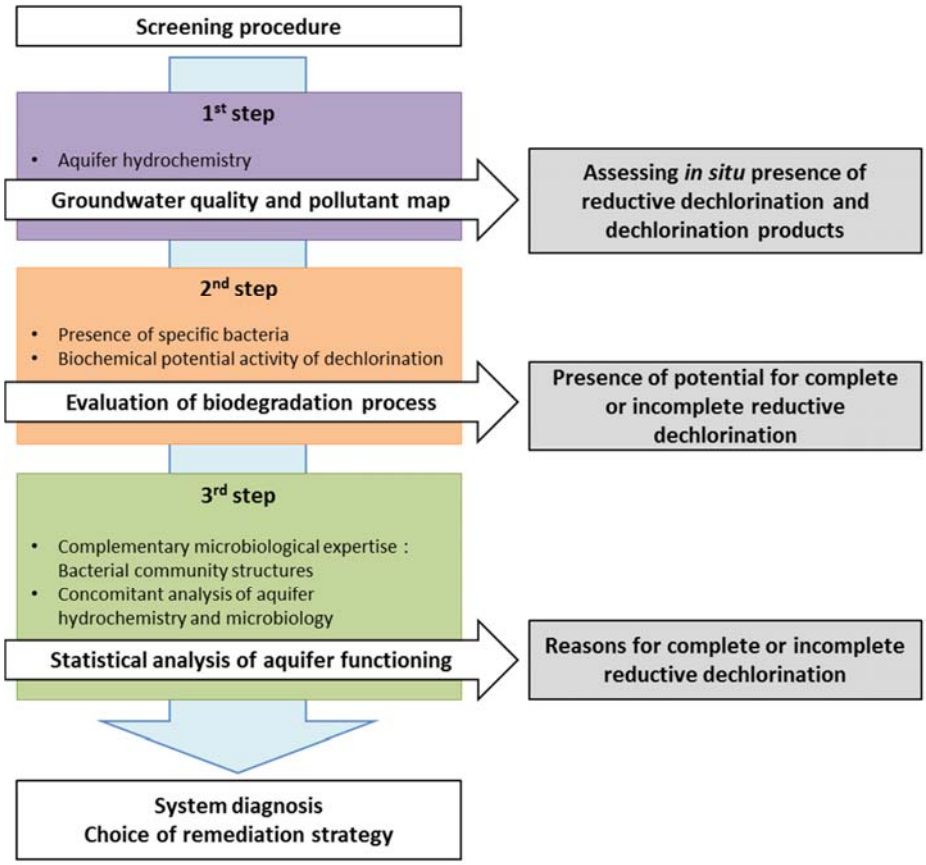


Diagram 2 – Systematic screening process, in three steps, for the evaluation of the potential of reductive dechlorination in an aquifer contaminated with chlorinated ethenes.

4. ASSESSING IN SITU PRESENCE OF REDUCTIVE DECHLORINATION

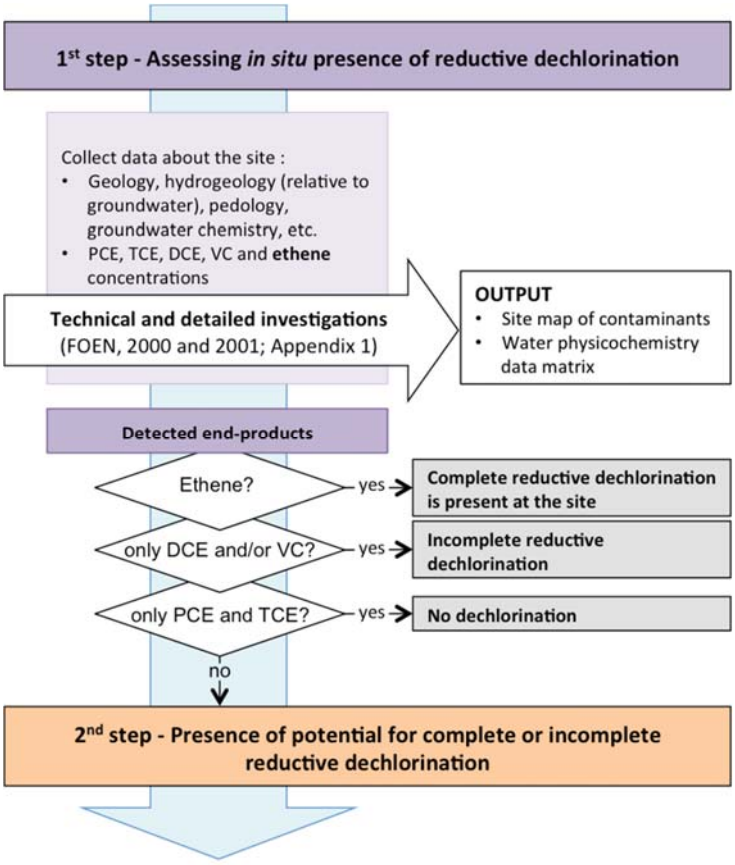


Diagram 3 – The first step of the screening process for evaluation of the potential of biodegradation in aquifer contaminated with chlorinated ethene compounds.

The main objective of the first step of the procedure is to assess the presence or absence of reductive dechlorination inside the GWS. The different tasks to achieve this aim are detailed in Diagram 3. The first aspects to explore are related to the collection of groundwater chemistry data, with a particular interest in concentrations and distributions of PCE, TCE and their daughter degradation products, especially ethene, which is often not analyzed. Collection of supplementary data about the geology, hydrology, and pedology of the site are also helpful (e.g. to give an overview of the water flow dynamic of the aquifer), as are data to evaluate the extent of the polluted area. Consequently, this examination of the site will result in a map of the contamination and the physicochemical data related to the studied aquifer habitat.

4.1. Basis for the screening

The "preliminary investigation" described in phase 2 of the procedure of contaminated site remediation enacted by the FOEN (1994 and 2001) provides preliminary conclusions for

the first step of the current screening process (i.e. is dechlorination observed *in situ* and until which end products?). Whether the site must be remediated based on the findings of the "preliminary investigation" is continued in the FOEN procedure in phase 3 with a "detailed investigation" (Diagram 1). The "detailed investigation" aims to provide accurate information about the type and extent of pollution, as well as damage that is likely to be generated. These data are necessary for the authority to determine the urgency of remediation and its overall goals. It is at this stage that multiple steps from the current screening protocol can take place simultaneously. In some cases, some parts may already have started during the "technical field investigation" of step 3 of the "preliminary investigation" when the latter is needed to rank a site.

4.2. Water sampling strategy or monitoring well network

All sites are different, and there is no one standard way to start an investigation and to successfully achieve the desired objectives. FOEN (2003) has edited a well-detailed assistance manual to delimit polluted sites and define sampling strategies (e.g. regarding the distribution and the number of sampling points). The location and number of monitoring wells should be determined on a site-specific basis. However, because monitoring wells are important parameters to accurately identify contaminant concentrations and determine the overall contribution of biological processes to contaminant decrease, these wells cannot be placed until sufficient knowledge of the aquifer system is obtained. Design of the monitoring network will be determined by the size of the plume, site complexity, source strength, groundwater/ subsurface water interactions, direction of groundwater flow, hydraulic conductivity, etc. In all cases, a higher density monitoring network will yield a greater degree of confidence in the interpretation of the results.

For the current manual, screening of reductive dechlorination can be conducted through a one-time sampling campaign where enough water is collected for completion of the whole procedure (see 5.2.1). Samples shall be taken in the saturated zone from different locations in the site schematically depicted in Figure 3: i) in the area of highest concentration, ii) along a down gradient from the contaminant "source" (if not yet eliminated) or from dense non-aqueous phase liquids (DNAPL), and iii) on a non-impacted area allowing comparison of the geochemistry of uncontaminated groundwater with the contaminated plume.

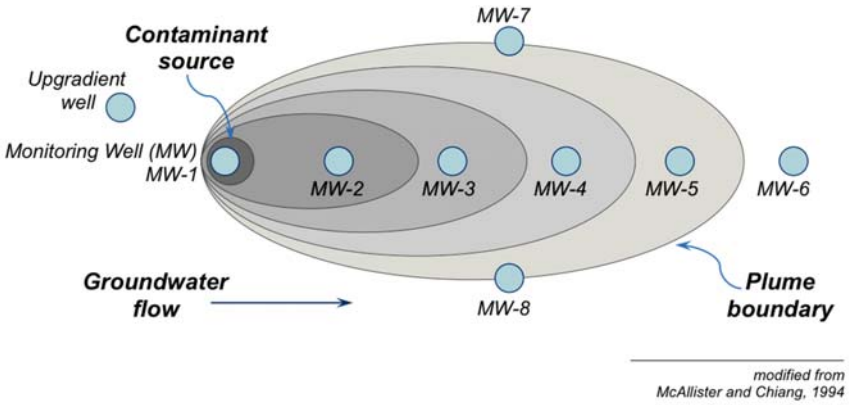


Figure 3 - Groundwater monitoring well network in an ideal plume of pollutant dispersion.

A total lower limit of 10-12 monitoring wells representative of the locations described above is estimated to be enough to allow **multivariate statistical analysis** of the data. If data confidence limits cannot be reached with one-time sampling campaign, for example due to fewer monitoring wells available on the site with no possibility to expand or because of homogeneity of the data, two or more rounds of sampling can be planned with enough time between rounds (several months, see complete example in Shani et al. (2013)) to avoid replicates and allow evolution of the groundwater system parameters.

4.3. Parameters to evaluate for appreciation of *in situ* state of reductive dechlorination

The sequential reductive dechlorination of the initial contaminants PCE and TCE produces well-defined intermediates such as the more toxic c12DCE, the most toxic VC, and the harmless end-product ethene (Figure 1). Formation of the two other dichloroethenes, t12DCE and 1,1-DCE, is also possible but rarely observed. During this process, which supports microbial growth, H_2 is most often used as electron donor and the CEs as electron acceptors. H_2 is typically supplied by the fermentation of organic substrates. PCE or TCE is reduced by the sequential loss of a chlorine atom, and presence of the daughter degradation products in a GWS unambiguously demonstrates that biological degradation occurs through the predominant reductive dechlorination process. This depends on many environmental factors including strongly anaerobic conditions, presence of fermentable substrates for generation of molecular hydrogen, and the appropriate microbial populations to catalyze the reactions.

4.3.1. Contaminant map

For the 1st step, the concentrations related to the pollutant and degradation products that must be measured are marked with * (the others are optional):

- ✓ **PCE* and TCE*** normally the initial contaminants of the site, and highly recalcitrant to oxidation. Their degradation only occurs in strictly anaerobic conditions. TCE can also be degraded by co-metabolism in aerobic conditions (see section 5, Figure 6) but with low efficiency. This is considered a minor degradation pathway.
- ✓ **c12DCE***, t12DCE, 11DCE among the DCE isomers, c12DCE is the main intermediate product of the reductive dechlorination process (more than 80%). Concentrations of t12DCE and 11DCE are generally low.
- ✓ **VC*** under reductive conditions (i.e. allowing sulfate reduction or methanogenesis, see 4.1) DCE is degraded to form VC, a carcinogenic compound, with much higher toxicity than the parent molecules PCE and TCE.
- ✓ **Ethene*** is the harmless end product of reductive dechlorination and the best indicator for the presence of a complete reductive dechlorination process in the saturated zone of a GWS.
- ✓ **Chlorides** (Cl^-) are the end products of both biotic and abiotic degradation of CEs.

The analytic methodology for measurement of CEs in solid and aqueous samples is presented in FOEN (2013), under methods S-8 and E-8, respectively (Appendix 2). Ethene and ethane can be measured with the methodology proposed by Kampbell *et al*., (1989), and chlorides can be measured using ion chromatography.

Results of the measured concentrations are then evaluated with respect to permissible concentration limits listed in the Federal Ordinance on the Remediation of Polluted Sites (Table 1, Appendix 1 of OSites 814.680 available at http://www.admin.ch/opc/fr/classified-

compilation/19983151/index.html). Aquifer zones with concentrations above the limits are mapped, and the PCE/TCE/daughter product distribution along the contaminant plume may be interpreted with the help of hydrogeological data collected from the site.

Table 1 - Overview of contaminant concentration limits (mg/L) presented in the Federal Ordinance on the Remediation of Polluted (OSites 814.680) and other guidance and limit values

Contaminant	Concentration OSites water	Limit value OSEC drinking water	Limit value OMS drinking water	Limit value US-EPA drinking water
Perchloroethene	0.04	0.04	0.04	0.005
Trichloroethene	0.07	0.07	0.02	0.005
Cis-1,2-dichloroethene	0.05*	0.05*	0.05*	0.07
Trans-1,2-dichloroethene	0.05*	0.05*	0.05*	0.1
1,1, dichloroethene	0.03	0.03	-	0.007
Vinyl chloride	0.0001	-	0.0003	0.002

Modified from Chloronet, 2009. OSEC: Ordinance on foreign substances and components, OMS: World health organization, US-EPA: US Environmental Protection Agency. * Limit value referring to the sum of cis and trans-1,2-dichloroethene.

4.3.2. Interpretation of contaminant concentrations and detected end products

The investigation of contaminant concentrations leads to different conclusions based on which CEs, including ethene, are detected. (Diagram 3).

The detection of ethene indicates that the potential for complete reductive dechlorination of PCE/TCE is already present *in situ*. The detection of only DCE and/or VC as degradation end products indicates an incomplete anaerobic degradation process. No detection of ethene, VC or DCE reveals an absence of *in situ* reductive dechlorination activity.

The finding of incomplete *in situ* reductive dechlorination has already been observed at several PCE/TCE contaminated sites, and has been attributed to different factors, including: i) insufficient anaerobic conditions to support reductive dechlorination activity, ii) a lack/absence of bacterial populations capable of reductive dechlorination of c12DCE to VC and ethene (Lorah and Voytek, 2004; Dowideit *et al*., 2010), and iii) a lower reaction rate of the last steps of the sequential degradation process that create an apparent accumulation of DCE and/or VC (Middeldorp *al.*, 1999; Figure 1). The reasons why PCE/TCE dechlorination is not detected or is incompletely realized *in situ*, with apparent or actual accumulation of DCE and/or VC, will be evaluated in the next steps of this screening procedure.

Further from the contaminant source, VC and c12DCE can migrate into **aerobic** areas (Figure 5) where direct oxidation of this compound can occur and produce CO_2 (Figure 6). This indicates that remediation can be achieved by different, successive processes depending on the conditions. To avoid misinterpretation of a non-detection of ethene, stability of the site hydrochemistry should be monitored (FOEN, 2004) and degradation rates should be evaluated. The case where ethene production is measured *in situ*, determines whether a **monitored natural attenuation** (MNA) strategy for remediation of the site is possible. MNA as remediation alternative can be proposed upon the confirmation that the transformation processes are taking place at a rate protective of human health and the environment. The evaluation should include a reasonable expectation that these processes will continue at an acceptable rate for an acceptable period of time (EPA, 1999; EPA, 1998). This last evaluation and determination of success are not further dealt with in this protocol; however literature has been published on this subject (EPA, 1998; Chapelle et al., 2003; ADEME, 2007; Grandel et Dahmke, 2008, Umweltbundesamt, 2011).

4.4. Parameters for evaluation of GWS physicochemistry and redox conditions

4.4.1. Predominant terminal electron-accepting processes

A reliable approach for screening bacterial degradation potential of CEs relies on the identification of the predominant Terminal Electron-Accepting Processes (TEAPs) occurring in a GWS (Chapelle *et al.*, 1995). TEAPs are related to the bacterial oxidative/reductive respiration processes. In any environment in which microbial activity occurs, there is a progression from oxic to anoxic conditions, and different bacteria along this progression use a specific sequence of compounds as terminal electron acceptors for respiration. Under aerobic conditions, oxygen is the final electron acceptor. In the absence of oxygen, bacteria are able to transfer electrons to other oxidized inorganic compounds (e.g. NO_3^-, SO_4^{2-}) and use them as terminal electron acceptors. To illustrate this, Figure 4 presents a schematic view of the sequential TEAPs defining aquifer oxidation/reduction conditions: reactions 1 to 6 correspond, respectively, to aerobic respiration, denitrification including nitrate reduction, manganese reduction, iron reduction, sulfate reduction and methanogenesis. Each reaction happens successively in the previously stated order, and once an electron acceptor is depleted, a new redox reaction using another acceptor occurs. The electron acceptor that leads to the next largest generation of energy during the reaction will dominate (EPA, 2000; Figure 4, right). Consequently, this succession of respiring oxidative/reductive processes (TEAPs) affects the chemistry and redox conditions of groundwater in all aquifer systems.

Each respiring process occurs in a specific redox potential (Eh) range, which decreases from aerobic respiration until methanogenisis (Figure 4, left). The processes also require, as electron donors, different ranges of dihydrogen concentrations (or an equivalent reducer) (Figure 4, right).

In this context, the occurrence of CE reductive dechlorination depends on redox conditions of the aquifer established by the inhabiting bacterial populations implicated in TEAPs and the availability of inorganic electron acceptor compounds along the contamination plume (Figure 5). Reductive dechlorination occurs from a specific redox potential slightly less than that of denitrification (Figure 4, in green) and within the required specific ranges of dihydrogen availability.

When the redox potential, Eh, is approximately 500 mV, PCE will be degraded to VC with a low concentration of reducing equivalents (<1 nM H_2). The required reducing equivalent concentration is higher to reduce VC to ethene (>1-10 nM H_2). Other TEAPs with lower redox potentials (e.g. manganese reduction) take advantage of this high requirement and can outcompete the VC reduction process because they require lower concentrations of reducer equivalent (<1 nM H_2). Conversely, the dechlorination process competes for electron donors with bacterial TEAPs that require the same concentration range of dihydrogen, such as c12DCE reduction and manganese reduction. Competition also occurs when low reducing conditions are achieved in the aquifer, as in the case of VC dechlorination competition with sulfate reduction and methanogenesis for reduction equivalents.

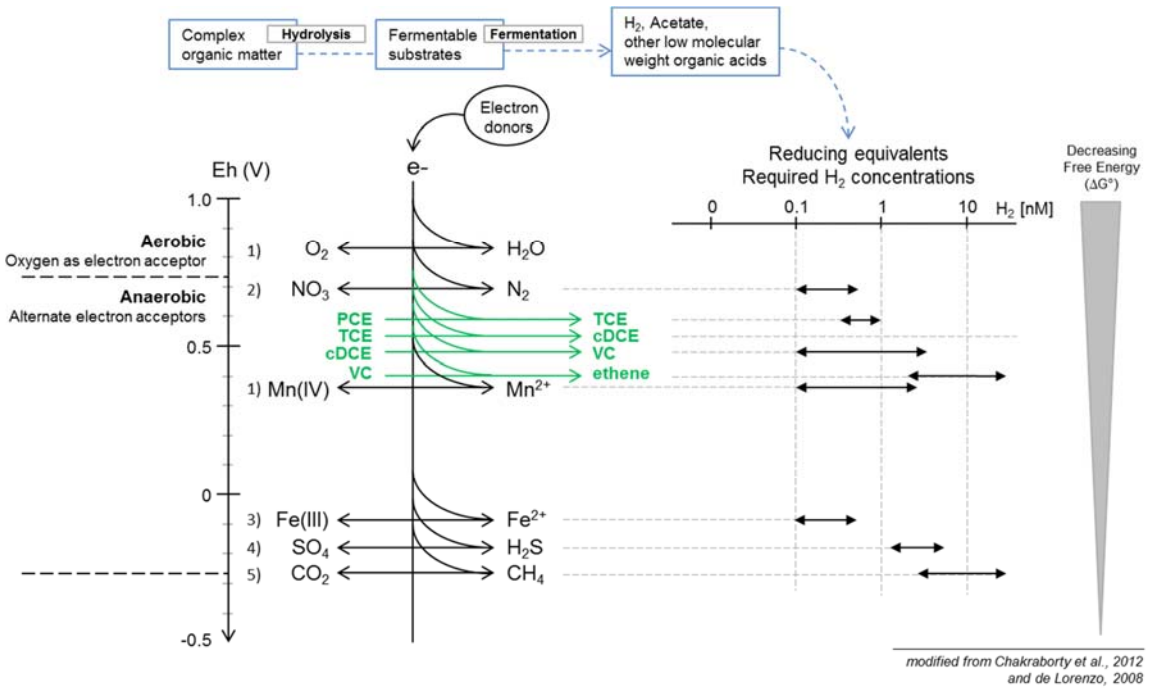


Figure 4 – Ecological succession of terminal electron acceptor processes (TEAPs) linked to bacterial respiring processes.
1) Aerobic respiration, 2) Denitrification, 3) Manganese reduction, 4) Iron reduction, 5) Sulfate reduction 6) Methanogenesis. Oxidation-reduction potentials of the reactions are shown on the left (Eh in Volts). Ranges of dihydrogen concentrations required for each TEAP are shown on the right. Reductive sequential CE dechlorination processes are depicted in green. Organic matter degradation pathways providing equivalent reducing potential in the system are schematized in the blue flow scheme at the top of the figure.

The reductive dechlorination process is included in the ecological succession of TEAPs. Consequently, determining TEAPs that occur in an aquifer, documenting their spatial distribution, and understanding how they affect concentrations of contaminants are central to assess and predict the possibilities of CE dechlorination.

The distribution of TEAPs in a GWS can be assessed by measuring the availability of electron acceptors (e.g. NO_3^-, Mn(IV), Fe(II), SO_4^{2-}) and/or by showing the distribution of their reduced forms (e.g. NO_2^-, Mn(II), Fe(II), S^{2-}/H_2S). For this purpose, some important parameters need to be measured to complement the CE concentration measurements.

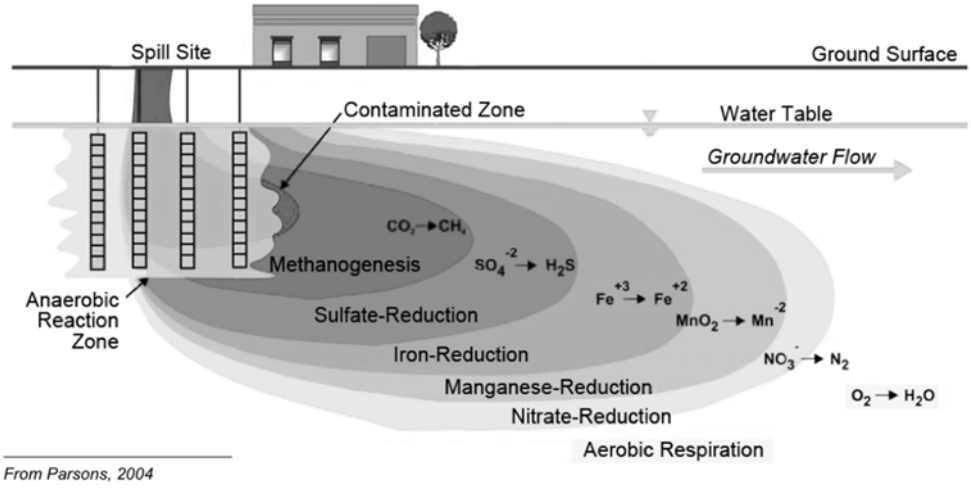
4.4.2. Parameters to evaluate the redox conditions of the GWS

In the current procedure, details regarding the necessary parameters are given to clarify the existing data acquisition methodology. The relative procedures and documentation for data acquisition are available in the following documents:

1) Sampling of groundwater in relation to contaminated sites (FOEN, 2003)
2) Analytical methods in the field of waste and polluted sites (FOEN, 2013)
3) Contaminated sites, evaluation of risk. Specifications for the technical investigation of contaminated sites (FOEN, 2000)
4) Practical guidelines for the protection of groundwater (FOEN, 2004)

For the water sampling procedure, see Section 5.2.1. Again, it is important to note that all water samples needed throughout the whole screening procedure must be collected at the same time to obtain results that can be analyzed in a comprehensive and integrative way.

Figure 5 - Redox zones of a typical contaminant plume.



A plume moving with groundwater flow will typically develop distinct redox zones. In a conceptual contaminant plume, a "redox gradient" is established due to dilution from the source, movement of CEs with groundwater flow, and microbial activities (i.e. TEAPs).

4.4.2.1. General parameters and descriptions

Essential parameters, which must be measured, are marked below with *. Others are complementary and useful, but not essential:

- ✓ **Oxidation/reduction potential * (ORP):** "Redox" potential is directly measured by a field electrode device. **Eh** is the ORP value corrected relative to the standard hydrogen electrode.
- ✓ **pH ***: pH has a great impact on dechlorinating microorganisms. For example, KB-1™ a natural dechlorinating microbial consortium that contains **phylogenetic** relatives of *Dehalococcoides ethenogenes* (Major *et al*., 2002), did not show any dechlorination activity below pH 5 or above pH 10, optimum pH for dechlorination being between 6.0 and 8.0.
- ✓ **Temperature *:** Lower temperatures limit dechlorination activity
- ✓ **Electrical conductivity *** (**eC**): eC indicates global ion concentration in solution.
- ✓ **Dissolved oxygen * (dO_2):** Dissolved oxygen is the electron acceptor used by aerobic microorganisms to degrade organic matter. Anaerobic bacteria are inhibited by an O_2 concentration higher than 0.5 mg/L, and reductive dechlorination cannot occur in this condition. When dO_2 decreases due to organic matter respiration, anaerobic bacteria successively use other electron acceptors for respiration as indicated in Figure 4. Each terminal electron acceptor, when used, modifies the groundwater's ORP status, and environmental redox conditions become more and more reduced.

ORP/Eh, pH, temperature, conductivity and dO_2 measurements are obtained in the field using portable electric power units. These parameters are standard for groundwater quality analysis (Appendix 1) and are not specific to contaminated site investigations.

4.3.2.2. Chemical parameters

- ✓ **nitrate NO_3^- *** , **nitrite NO_2^-***, **ammonium NH_4^+** : Nitrogen is found in groundwater as dissolved organic nitrogen, NO_3^-, NO_2^- or NH_4^+. Low concentrations of NO_3^- in saturated zones indicate possible anaerobic bacterial respiration like dissimilatory nitrate reduction to nitrite (NR) and denitrification ($NO_3^- > NO_2^- >>> N_2$). Denitrification takes place at oxic/anoxic

interfaces, as part of the NO_3^- along a soil profile comes from leaching and microbial oxidation of NH_4^+, called nitrification ($NH_4^+ > NO_2^- > NO_3^-$), which occurs in the upper oxic environment. NO_3^- are considered as weakly available at concentrations below 1 mg/L. Under NO_3^- depleted conditions, reductive dechlorination of PCE to TCE can occur. NO_2^- is an intermediate of both denitrification and nitrification, which are respectively anaerobic and aerobic processes. Thus, the presence of NO_2^- at oxic/anoxic interfaces (i.e. slightly reducing conditions) can indicate either incomplete denitrification (stopped after NR) and/or incomplete nitrification (stopped after oxidation of ammonium). This situation must be interpreted with caution and in relationship to other measured parameters. When anaerobic redox conditions (i.e. strongly reducing) are combined with high amounts of carbon, bacteria may reduce NO_3^- to NH_4^+ by dissimilatory nitrate reduction to ammonium (DNRA). Nevertheless, NH_4^+ is found naturally in GWSs as a result of anaerobic decomposition of organic material (Böhlke et al., 2006), and high NH_4^+ concentrations are a common sign that surface water influenced by anthropogenic activities is infiltrating to groundwater (Lindenbaum, 2012). Consequently, interpretation of NH_4^+ concentrations in a GWS must also be done with caution.

- ✓ **reduced manganese Mn(II) *:** An Mn(II) measurement is indicative of manganese(IV) reduction. Manganese(IV) reduction is restricted to conditions where SO_4^{2-} concentrations are low or absent (EPA, 1999). Redox conditions of Manganese(IV) reduction are favorable for and in competition with DCE to VC degradation since the same H_2 concentration range is required for both reactions (MacMahon *et al.*, 2008; Figure 4).
- ✓ **reduced iron Fe(II) *:** When manganese oxides become limiting, iron(III) reduction to iron(II) is the predominant TEAP. It seems that iron reduction doesn't occur until all Mn(IV) oxides are depleted. When groundwater is under iron reducing conditions, reductive dechlorination of PCE and TCE to c12DCE and VC is possible (Figure 4, same range of required H_2 concentrations).
- ✓ **sulfate SO_4^{2-} *:** When SO_4^{2-} become the main electron acceptor, redox conditions are reduced and complete reductive dechlorination (PCE until ethene) is possible. The EPA technical protocol for evaluating NA (EPA, 1998) indicates that SO_4^{2-}concentrations must be lower than 20 mg/L to avoid competition between sulfate reduction and reductive dechlorination.
- ✓ **sulfide H_2S *(S^{2-}):** S^{2-} is a dissolved gas and a product of sulfate reduction. Sulfides easily react and precipitate with metal ions such as ferrous iron and can therefore be difficult to measure in some GWSs.
- ✓ **methane CH_4 *:** Stable molecule and easily to measured methane indicates highly reduced conditions characteristic of methanogenesis and favorable for reductive dechlorination. Methanogenesis can be observed even if SO_4^{2-} is not depleted. The EPA protocol asserts that a methane concentration higher than 1 mg/L indicates competition between methanogenesis and reductive dechlorination and leads to VC accumulation on site.
- ✓ **carbon dioxide CO_2 *:** CO_2 is an oxidized electron acceptor of methanogenesis and also an end product of CE oxidation (see section 5, Figure 6).
- ✓ **total organic carbon (TOC) * and chemical oxygen demand** (**COD):** TOC and COD are evaluations of the global carbon content in groundwater. The non-purgeable organic carbon (NPOC) measurement procedure, which includes few or no volatile organic compounds, must be used to prevent organochlorine compounds from contributing to the measured value. Measurement of organic carbon content is linked to the evaluation of available electron donors (reducer equivalents) in a GWS for the TEAPs (Figure 4, blue

boxes) that lead aquifers to optimal, anaerobic conditions for biodegradation of CEs. A low TOC concentration will contribute to a lower efficiency of CE biodegradation.

5. POTENTIAL FOR REDUCTIVE DECHLORINATION OF CEs

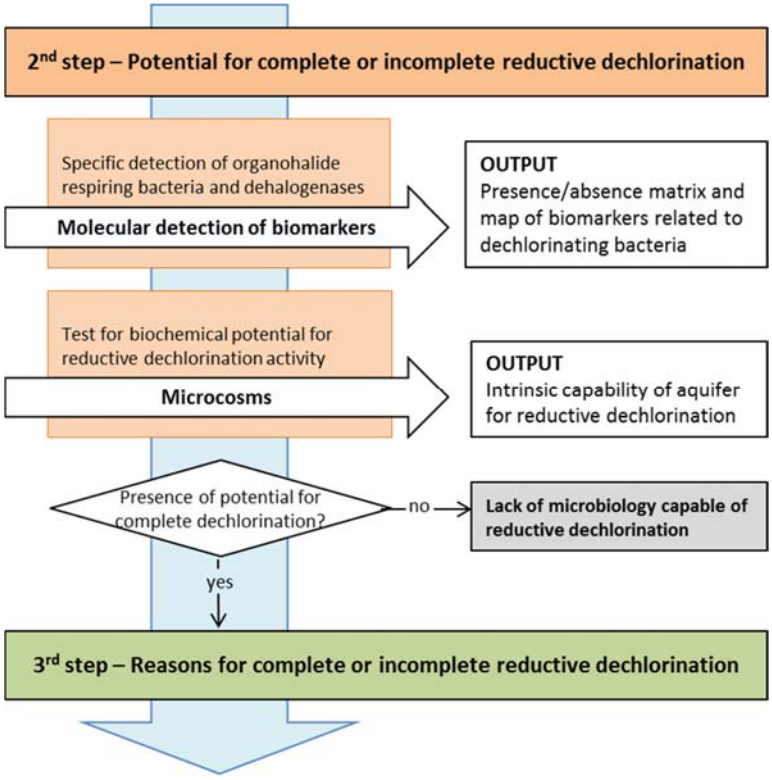


Diagram 4 - Second step of the screening process for the evaluation of the reductive dechlorination potential in an aquifer contaminated with chlorinated ethenes.

In this step, the bacteria implicated in the reductive dechlorination processes, the organohalide-respiring bacteria (OHRB), are studied directly from water or aquifer sediment samples. The general objective is to evaluate the inherent potential for biological reductive dechlorination of CEs in a GWS (Diagram 4).

The proposed analysis determines whether OHRB are present, and if so then assesses whether they express the ability to completely dechlorinate CEs. This is achieved by completing the following technical objectives:

- Detect, with molecular tools, the presence of bacteria and enzymes known to be involved in the process of reductive dechlorination
- Assess the intrinsic capacity of aquifer samples to express the biochemical activities of reductive dechlorination through microcosm tests

The results obtained from completion of these two objectives can clarify observations made during step 1. For example, they may indicate that incomplete degradation is due to the absence of some OHRB and/or the lack of the enzymatic machinery required for complete dechlorination (Diagram 4, grey boxes).

5.1. Looking for biomarkers in aquifer samples: Organohalide-respiring bacteria and reductive dehalogenase genes detection

5.1.1. Biodegradation processes of chlorinated-ethenes (Bradley, 2003)

Different bacterial processes are implicated in CE degradation (Figure 6):

1) **Metabolic processes** are those in which CEs are used as electron acceptors in **reductive dechlorination** in the saturated zone (see also, Figure 1) or as electron donors in **oxidation reactions** (oxidative pathway) used to obtain energy and produce biomass. **Aerobic oxidation** of VC occurs at a higher rate than anaerobic reductive dechlorination. VC oxidation may be important in downstream aerobic areas, where a GWS's redox conditions return to its natural state (Figure 5).

2) **Co-metabolic processes** are defined as reactions in which a non-specific enzyme produced for microbial metabolism fortuitously reduces a chlorinated molecule. No benefit returns to the implicated microorganisms.

3) **Anaerobic oxidation processes** are those in which VC and DCE can be directly oxidized under iron- and manganese-reducing conditions, respectively. Alternatively, VC can be degraded into acetate via oxidative acetogenesis. The produced acetate can be mineralized to CO_2 and CH_4 through acetoclastic methanogenesis and to CO_2 via microbial humic acids reduction. It can also be used as an electron donor and/or carbon source by other anaerobic bacterial processes, i.e. TEAPs including reductive dechlorination.

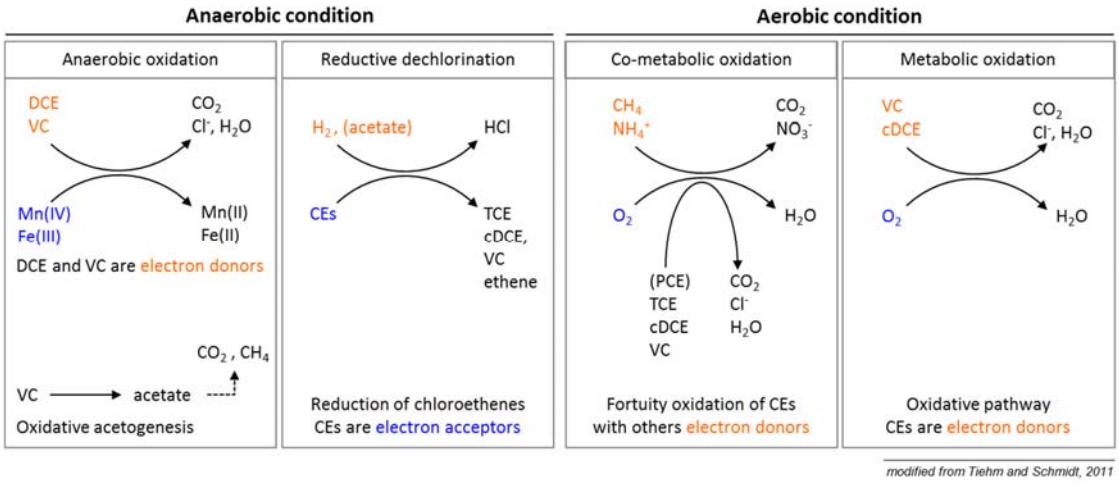


Figure 6 – Bacterial reductive and oxidative processes for degradation of chloroethenes (CEs). Electrons donors are shown in orange, and electrons acceptors in blue. PCE: perchloroethene, TCE: trichloroethene, cDCE: cis-1,2-dichloroethene, VC: vinyl chloride, Cl^-: chloride, NO_3^-: nitrate, NH_4^+: ammonium, CH_4: methane.

The abiotic pathway is a 4th type of process where CEs are reduced by chemical reaction with active compounds. Abiotic agents that may enhance the dechlorination of CEs are zero-valent metals, sulfide minerals or green rusts (Tobiszewski and Namiesnik, 2012). Natural abiotic dechlorination occurs rarely and is slower than bacterial reductive dechlorination.

It is not possible to distinguish the four reactions in the field. Nevertheless, reductive dechlorination of CEs by organohalide respiration (OHR) (Figure 7-B) does occur in the anoxic, saturated zones of an aquifer. This pathway was demonstrated and is recognized as the most

efficient CE degradation process that takes place in contaminated aquifers (Chloronet, 2009; EPA, 2000).

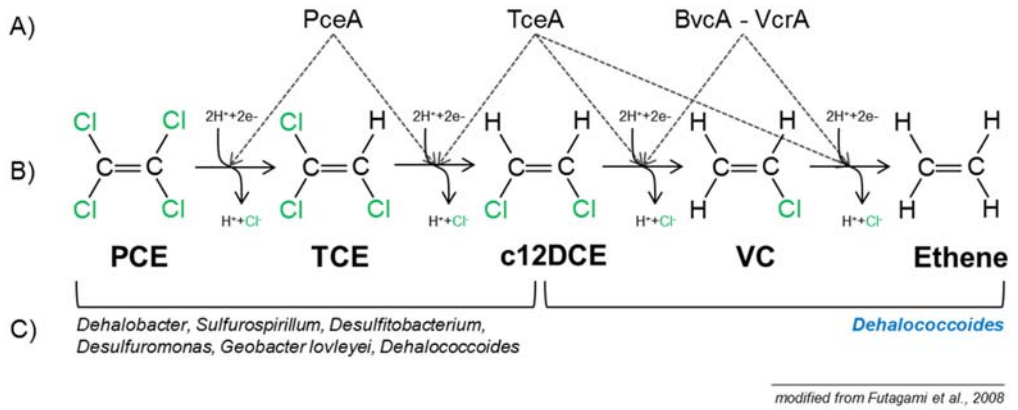


Figure 7 – Schematic overview of the anaerobic reductive dechlorination of chloroethenes.
A) the catalytic reductive dehalogenase enzymes (RdhA) implicated in each reaction, **B**) the sequential steps of the degradation process, **C**) the two different groups of bacterial genera involved in the sequential reaction. The reductive dechlorination efficiency decreases as the chlorination degree of the ethene module decreases. Conversely, the fewer chlorine atoms present, the more readily the molecules can be oxidized, though this is mainly in the unsaturated aerobic zone or near the unsaturated/saturated zone interface.

5.1.2. Biomarkers of the reductive dechlorination process

Microorganisms capable of reductive dechlorination of CEs are called organohalide-respiring bacteria (OHRB). Their ability for dechlorination is highly strain-dependent (Futagami *et al*., 2008). Current knowledge of OHRB diversity and physiology were increased by molecular microbial ecology, genomics, and cellular metabolism studies (Hiraishi *et al*., 2008; Maphosa *et al*., 2010). The results of these efforts are represented in Figure 8. OHRB that can grow with chloroethenes as terminal electron acceptors include *Dehalobacter*, *Dehalococcoides*, *Desulfitobacterium*, *Desulfuromonas*, *Geobacter*, and *Sulfurospirillum* strains (Figure 7-C and Figure 8). While most of these strains can use PCE or TCE as electron acceptors, only some *Dehalococcoides* strains are known to perform reductive dechlorination of c12DCE and VC to ethene (Figure 7-C). These isolates are metabolically specialized to use only H_2 as an electron donor and CEs as an electron acceptor to support their growth., Few bacteria are able to realize the last steps of the reductive dechlorination process, and consequently, even if all actors in the sequential degradation process are present in the saturated zone, PCE to DCE reductive dechlorination kinetics are faster than DCE to ethene dechlorination kinetics. This can result in natural, transient DCE and VC accumulation in an aquifer.

It is currently possible to detect and monitor OHRB in the environment through the use of biology tools such as **Polymerase Chain Reaction** (PCR). OHRB can be detected by specific amplification of genes that code for the 16S **ribosomal RNA** subunit (16S rRNA gene) of bacteria directly implicated in CE dechlorination. Amplification of target genes that code for the key enzymes of organohalide respiration, the reductive dehalogenases, also allow monitoring of the CE dechlorination pathway. Reductive dehalogenase enzymes consist of a **catalytic subunit** called RdhA and a small putative membrane anchor subunit called RdhB. Studies have shown that there are various clusters of RdhAs with similar functions (Figure 7-A). The following RdhAs are associated with the CE sequential degradation pathway: PceA for the

tetrachloroethene reductase, TceA for the trichloroethene reductases and BvcA and VcrA both for DCE and vinyl chloride reductases (Futagami *et al*., 2014; Maphosa *et al*., 2010).

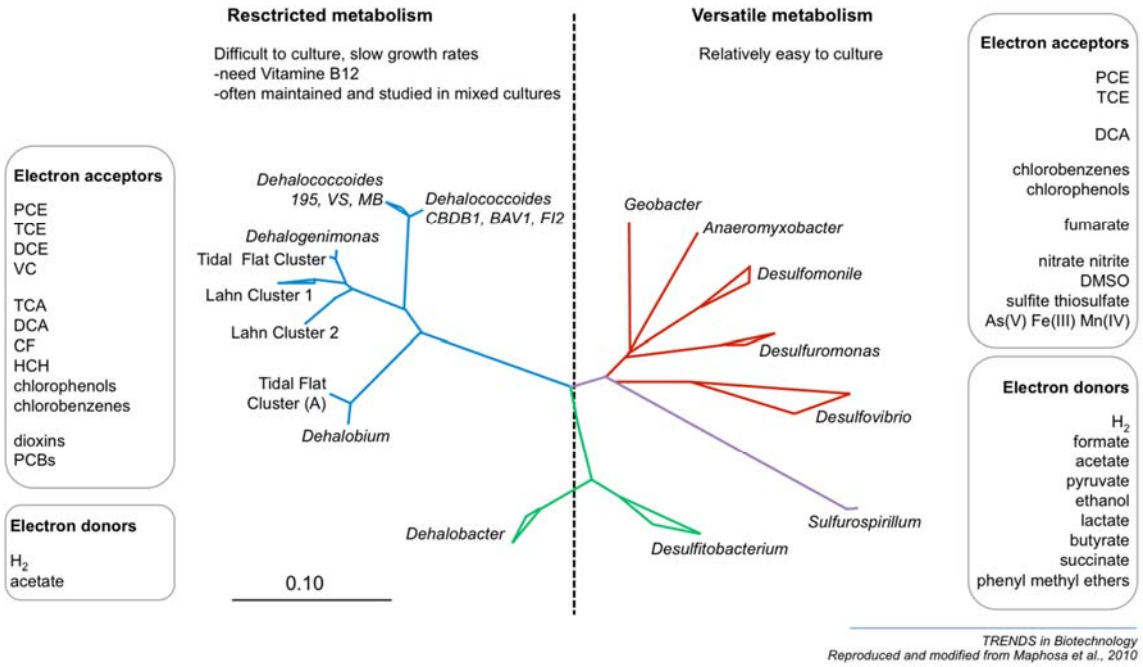


Figure 8 – Phylogenetic tree of organohalide respiring bacteria based on bacterial 16S rRNA gene sequences.
The reference bar at the bottom center indicates the branch length that represents 10% sequence divergence. Electron donors and acceptors are listed in the text boxes and are grouped according to their chemical nature and complexity. Color key: Chloroflexi (red), Deltaproteobacteria (blue), Epsilonproteobacteria (purple), Firmicutes (green). Abbreviations: CF, chloroform; DCA, dichloroethane; DCE, dichloroethene; HCH, hexachlorocyclohexane; PCB, polychlorinated biphenyls; PCE, tetrachloroethene; TCE, trichloroethane; TCA, trichloroethene; VC, vinyl chloride.

5.2. Evaluation for potential of reductive dechlorination by specific detection of the presence of known OHRB and reductive dehalogenase genes

At this stage, the proposed analyses are carried out on DNA extracts from water samples. Results of this first molecular analysis will indicate the presence of bacterial guilds implicated in reductive dechlorination. These data can be obtained relatively rapidly, however the presence of bacteria does not mean that they are or could be active. Regarding the molecular investigation of bacterial activity, one path is to analyze gene expressions by targeting **messenger RNA** (mRNA) that code for proteins and the enzymatic machinery of the cell. This investigation yields data closer to the real activity than DNA analysis, but is more expensive in terms of laboratory materials and more complicated in terms of technical expertise in molecular biology. The choice to work on DNA is made to facilitate the knowledge transfer. The data obtained with this investigation are powerful for evaluation of a GWS's dechlorination potential, and extensive experiments can be realized at any time.

5.2.1. Water sampling: collection, transportation and storage.

The general procedure for sampling contaminated groundwater is currently well described (FOEN, 2013; EPA, 2008). Biology-specific analyses are detailed in Shani (2012). Water samples for microbial analysis must be collected at the same time as the water samples for chemical analysis in the 1st step of the screening procedure. Groundwater samples are collected using a peristaltic pump (e.g. Type P2.52, Eijkelkamp, Giesbeek, The Netherlands) and PTFE tubes (inner diameter 4 mm, Semadeni SA, Switzerland). Each well is purged at a flow rate of 100 mL/min, and physicochemical parameters (temperature, pH, electrical conductivity, ORP) are continuously measured through a flow cell device. Purging lasts for at least one well volume and continues until the physicochemical parameters stabilize. Groundwater samples are then collected from each well directly at the pump exit at the low flow rate of 100 mL/min. Water is sampled for chemical analysis (FOEN, 2013), and 1 L is sampled for molecular biology analyses by completely filling a 1 L polypropylene Nalgene® bottle (Thermo Fisher Scientific, USA) without headspace. For microcosm experiments, two sterile 40 mL serum glass vials (type 27051 SUPELCO, Sigma-Aldrich, Buchs SG, Switzerland) are filled with water and hermetically sealed to avoid sample contact with air. After collection, aqueous samples should be protected from light and refrigerated for transport from the field. In the laboratory, all groundwater samples are stored in the dark and at 4°C until analysis, which should be done immediately or within a maximum of 48 hours after sampling.

5.2.2. Sample filtration and DNA extraction

Groundwater samples are filtered under a laminar flow hood with a filtration system (Filter Funnel Manifolds, Pall Corporation, USA) and a 0.2 µm pore size, sterile polycarbonate membrane (IsoporeTM Membrane Filters, Millipore, USA) or with Nalgene® bottle-top sterile filter units (Sigma-Aldrich, Buchs SG, Switzerland). The complete water sample volume of 1 L should be filtered. Several membranes can be used if necessary, then stored in a sterile container. Following filtration, membranes are submitted to DNA extraction using the protocol detailed in Appendix 3 or kept at -20°C for long term storage.

5.2.3. PCR specific detections of OHRB and RdhA genes

As mentioned previously, the specific PCR detection of OHRB 16S rRNA genes and genes associated with the catalytic subunit of reductive dehalogenase (RdhA) are indicative of the presence of bacterial guilds involved in CE reductive dechlorination. Presence of OHRB and related genes is assessed by PCR amplifications with the primers listed in Table 2 by following the protocol in Appendix 4.

5.3. Evaluation of the intrinsic reductive dechlorination potential of a GWS using microcosm experiments

In this 2nd phase of the 2nd step of the procedure (Diagram 4), groundwater samples are placed in optimal conditions for the biochemical expression of reductive dechlorination. All or some intermediate compounds of degradation (TCE, DCE, VC and ethene) will be produced according to the OHRB guilds presents in the water sample. The complete procedure for preparation of microcosms is detailed in Appendix 5. Briefly, the anaerobic medium is prepared as detailed in Holliger *et al*., (1993) except that fermented yeast extract solution is replaced by 0.1 g/L of peptone. For the preparation of one microcosm, 5 mL of PCE dissolved in hexadecane (100mM) are added to 50 mL of sterilized anaerobic medium. Through a 0.2 µm filter, 1 mL of electron donor mixture (equal parts ethanol, butyrate, and propionate,

each at 100mM) is also added. The gas phase in the microcosm is aseptically changed with N_2/CO_2 (4:1, vol/vol) before the final step of inoculation with 5 mL of groundwater sample. The microcosms are incubated in the dark at 30°C for at least 3 months. The gas phase in the microcosm is sampled regularly and analyzed with GC for the presence of dechlorination products TCE, DCE, VC, and ethene.

Table 2 - Organisms and genes specifically targeted to evaluate reductive dechlorination potential.

Target organism/gene	Primer	Sequence (5'-3')	References
Dehalococcoides sp. *	DHC587f	GGACTAGAGTACAGCAGGAGAAAAC	Hendrickson *et al*., 2002
	DHC1212r	GGATTAGCTCCAGTTCACACTG	''
Dehalobacter sp.*	Dre441f	GTTAGGGAAGAACGGCATCTGT	Smits *et al*., 2004
	Dre645r	CCTCTCCTGTCCTCAAGCCATA	''
Sulfurospirillum sp. *	DHSPM576f	GCTCTCGAAACTGGTTACCTA	Ebersole *et al*., 2003
	DHSPM1210r	GTATCGCGTCTCTTTGTCCTA	''
Desulfuromonas sp. *	Desulfo494f	AGGAAGCACCGGCTAACTCC	Bond *et al*., 2002
	Desulfo1050r	CGATCCAGCCGAACTGACC	''
Desulfitobacterium sp.*	Dsb406f	GTACGACGAAGGCCTTCGGGT	Smits *et al*., 2004
	De2r	CCTAGGTTTTCACACCAGACTT	Lanthier *et al*., 2001
Geobacter lovley	Geo73f	CTTGCTCTTTCATTTAGTGG	Duhamel and
	Geo485r	AAGAAAACCGGGTATTAACC	Edwards, 2006
*vcr*A **	vcrA880f	CCCTCCAGATGCTCCCTTTA	Behrens *et al*., 2008
	vcrA1018r	ATCCCCTCTCCCGTGTAACC	''
*bvc*A**	bvcA227f	TGGGGACCTGTACCTGAAAA	''
	bvcA523r	CAAGACGCATTGTGGACATC	''
Eubacteria	27f	AGAGTTTGATCMTGGCTCAG	Lane 1991
	519r	GWATTACCGCGGCKGCTG	Turner *et al*., 1999

* gene coding for the small 16S ribosomal RNA subunit (16S rRNA gene) is targeted to specifically detect the mentioned bacterial genus. ** genes coding in *Dehalococcoides* sp. for the reductive dehalogenase enzymes implicated in the dechlorination of c12DCE into VC then ethene.

5.4. Basis for interpretation of the microbial analyses

Whether there is biochemical potential for complete dechlorination can be elucidated from the presence or absence of OHRB and the RdhA genes. This is particularly important if, in step 1 of the procedure, the *in situ* contaminant concentrations show that PCE and/or TCE are not degraded or that the degradation is incomplete with observed accumulation of c12DCE and/or VC. Several scenarios may occur:

a) If the tested biomarkers are not detected, in particular the genus *Dehalococcoides* and the RdhA genes, the biochemical potential required for complete dechlorination is missing (Diagram 4, grey box). If microcosm experiments also show an inability to induce complete dechlorination of PCE, it can be concluded that an accumulation of c12DCE and/or VC will occur.

b) Conversely, the whole set of biomarkers may be not detected in PCR while a biochemical potential for complete degradation is observed in microcosms. Like any other measurement technique, PCR has a detection threshold. A negative PCR result does not prove the absence of the target; it can indicate a quantity that is too low to be detected. This undetected potential by molecular tools would be revealed through the microcosm experiments.

A positive result from microcosm experiments could also suggest that the potential for dechlorination exists at the tested site, but that it is carried out by yet unknown bacteria and genes not targeted by the PCR analysis. Currently only some *Dehalococcoides* strains are

known to completely dechlorinate PCE to ethene (Figure 7-C), and the reductive dechlorination process appears to be accomplished by **bacterial consortia** where each population performs one or several of the sequential dechlorination steps (e.g. Holliger and Schumacher, 1999; Gu *et al*., 2004 Yang *et al*., 2005). Complete dechlorination of CEs has been observed without evidence of usually "wanted" biomarkers, especially *Dehalococcoides* strains (Flynn *et al*., 2000). This suggests that consortia of non-*Dehalococcoides* dechlorinating strains can achieve complete dechlorination, and moderation is advised regarding the assertion that *Dehalococcoides* strains are necessary for complete dechlorination.

c) Another possible case is detection of all or part of the biomarkers using PCR with no activity exhibited in microcosm experiments. This result suggests that microorganisms associated with the targeted biomarkers do not have functional enzyme systems or that items are missing for sequential dechlorination steps (Diagram 4, grey box).

d) In the last case, all tested biomarkers may be detected, including OHRB and RdhA genes (Figure 7-A), and complete reductive dechlorination may have been induced in microcosm experiments. This is a reliable indication that the necessary biochemistry is present in the tested GWS to realize complete dechlorination. If the whole process was not observed *in situ* during analysis in step 1, it could be because the redox conditions of the tested aquifer are not yet favorable for reductive dechlorination or an inherent problem linked to the structure of the aquifer exists. These latter aspects will be specifically investigates in step 3 of the screening procedure.

6. AQUIFER BIOGEOCHEMISTRY

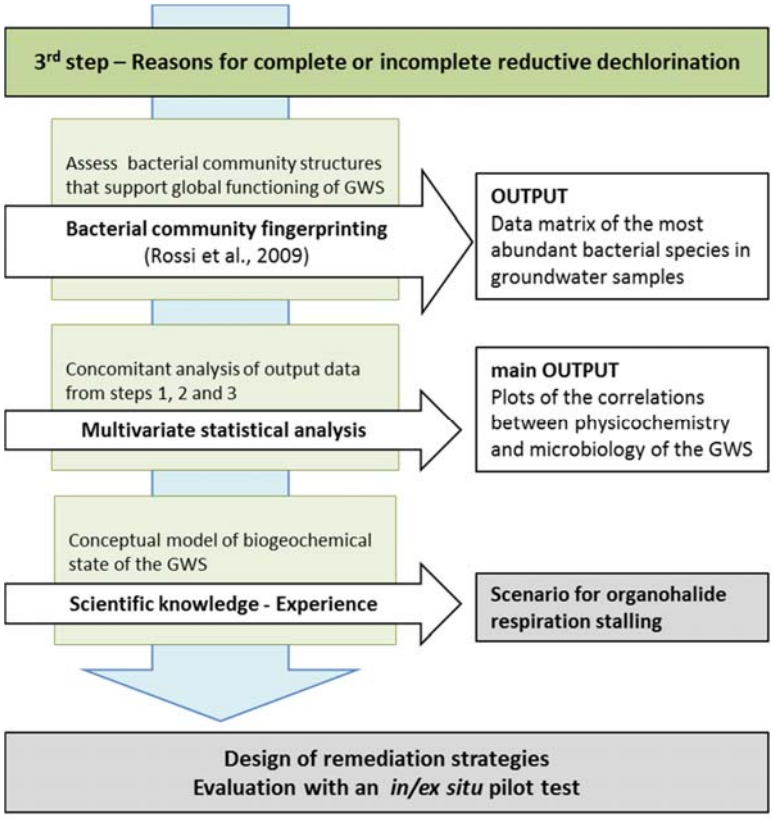


Diagram 5 - Third step of the screening process for the evaluation of the dechlorination potential in aquifers contaminated with chlorinated ethenes.

In this step, reductive dechlorination processes are placed in the framework of interactions with other natural bacterial processes (TEAPs) occurring in a GWS. The general objective is to evaluate the reasons why the reductive dechlorination of CEs is perturbed, usually observed *in situ* by the absence of ethene production and stalling of the degradation process at c12DCE and/or VC (Diagram 5).

The technical objective of this 3rd step is the analysis of the global bacterial community that lives in and interacts with the aquifer habitat (Figure 2) and also supports OHRB guild activities. For this community fingerprinting, a molecular method is used and provides multiple useful "barcode" characteristics of the bacterial community structure (BCS) at the different sampling locations. Raw data from the descriptions of the habitat (output of step 1) and the biota (outputs of step 2 and 3) are analyzed in an integrative manner. The interrelationships between bacterial communities and environmental variables are evaluated using unambiguous multivariate statistical techniques. Through interpretation of the integrative data analysis results, a dynamic conceptual model of an aquifer's biogeochemisty can be built. Such a model is also based on up-to-date scientific knowledge of energetic mechanisms, such as the ecological succession of TEAPs; thermodynamic and kinetic aspects; and minimum hydrogen threshold requirements. Finally, hypotheses and scenarios to explain

lower CE accumulation are formulated. The investigator's experience and intuition are helpful at this stage.

6.1. Assessment of the bacterial community structures of the groundwater system

Knowledge of microbial communities and factors that influence microbial community composition is fundamental for biotechnological applications such as groundwater remediation. It is important to understand the critical role played by ecological interactions among OHRB and other members of the microbial community in anaerobic dehalogenation in nature. Indeed, the reactions involved in reductive dechlorination of chlorinated solvents require the input of electrons from external donors. Most isolated bacterial populations that metabolically use CEs as terminal electron acceptors require hydrogen as the terminal electron donor (see Figure 8). Thus it is necessary, at the microbial community level, that fermentative bacteria are present. These bacteria degrade complex carbon sources and produce, in return, smaller molecules and hydrogen to support growth and establishment of OHRB and other bacterial TEAPs (Figure 4). It is the interaction of all these bacterial populations that form the bacterial community and develop an aquifer's dechlorination potential.

In this procedure, BCS is assessed using terminal restriction fragment length polymorphism analysis (T-RFLP). T-RFLP is an interesting and robust tool for BCS studies that allows quick analysis of high sample throughput at low cost. In a T-RFLP analysis, the 16S rRNA gene is usually amplified by PCR, wherein one or both primers are labeled with a fluorescent marker. The PCR product is composed of a mixture of 16S rRNA gene copies, labeled at one or both ends with the fluorescent markers, from the different bacteria present in the water sample. The PCR product is then digested with a restriction enzyme, and the restriction fragments are separated by electrophoresis on polyacrylamide or capillary gel. The marked terminal restriction fragments (T-RFs) are detected by an automated DNA sequencer, and the lengths of the T-RFs are estimated. The resulting T-RFLP bacterial community fingerprint is a characteristic set of T-RFs. The length of a T-RF depends on the position in the gene of the first site recognized by the restriction enzyme. Therefore, differences in the 1st restriction site position will generate different T-RFs lengths and indicate different bacterial populations of the community (Figure 9). The differences between samples of populations that compose the microbial community are evaluated by comparing the presence and relative abundance of T-RFs in the T-RFLP profiles.

T-RFLP analysis must be carried out on the same DNA extracts obtained during the 2nd step of the screening procedure to enable integrative analysis of the data. Each DNA extract may be processed via T-RFLP in triplicate for a more robust analysis. A complete T-RFLP procedure protocol is available in Rossi *et al*., (2009) (Appendix 6).

6.2. Preparation of data for statistical analysis

Data from the previous analyses should be handled and prepared for saving in *.csv file format. A list of acronyms that must to be used to name the variables in the *.csv files are given in Table 3. For a user that does not have any knowledge of the R language, which will be used in the data analysis software, this precaution is necessary to allow the statistic script (Appendix 9) to run completely and without error; for other users, it will be possible to adapt the script as needed.

Other parameters that are not listed in Table 3 can be added to the *.csv file without any problems, as long as the data header name does not conflict with the acronyms of the listed variables.

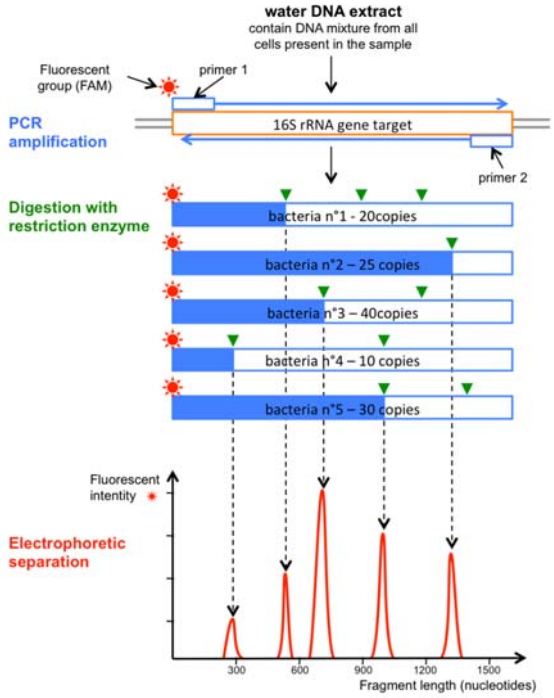


Figure 9 – Schematic procedure of terminal restriction fragment length polymorphism analysis. It consists of 4 steps: i) PCR amplification of 16S rRNA genes from a complex sample, ii) digestion of the PCR products with a restriction enzyme, iii) electrophoretic separation of the enzymatic products of digestion and iv) recovery of size and abundance data for each separated marked terminal fragment

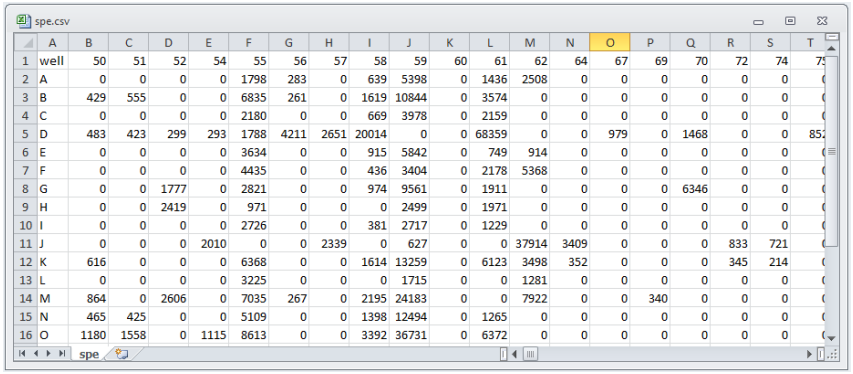
Table 3 – List of the variable names and acronyms to be used in the *.csv files.

Acronym	Teaps variables	Acronym	CE accumulation	Acronym	Others variables
PCE	perchloroethene	X.PCE	%PCE	Eh	redox potential
TCE	trichloroethene	X.TCE	%TCE	pH	acidity
c12DCE	cis-1,2-DCE	X.c12DCE	%c12DCE	tC	temperature (t°C)
VC	vinyl chloride	X.VC	%VC	eC	electric conductivity
ethene	ethene			TOC	total organic carbon
NO3	nitrate (NO_3^-)			Cl	chloride (Cl^-)
NO2	nitrite (NO_2^-)				
NH4	ammonium ($NH4^+$)				
MnII	manganese(II) (Mn(II))				
FeII	iron(II) (Fe(II))				
SO4	sulfate (SO_4^{2-})				
H2S	hydrogen sulfide (H_2S)				
CH4	methane (CH_4)				
CO2	carbon dioxide (CO_2)				
dO2	dissolve oxygen (dO_2)				

6.2.1. T-RFLP profiles

A T-RFLP profile from a water sample contains two types of information: i) the T-RFs positions in base pairs and ii) the amount of detected fluorescence for each T-RF (T-RF peak

area and height). These data are extracted from the electropherogram using the GeneMapper v4.0 software (ABI). The *.txt output files from the GeneMapper software that contain T-RFLP data are opened in Excel software. Data profiles are aligned semi-manually using Treeflap crosstab macro1 (Rees et al., 2004) by crosstabbing the T-RFs' peak area and size (rounded to the nearest 1 in setup options). This means that T-RFs in a profile that differ by less than 0.5 bp are considered identical and assigned to the average size. The resulting crosstab must be copied and pasted without link in a new Excel sheet to be modified. Peaks with an area smaller than 50 relative fluorescence units (RFU) are discarded. T-RFLP profiles that show less than 100,000 RFU in the total of all peak areas are discarded. T-RFs shorter than 50 bp and larger than 500 bp are discarded due to inconsistent results and lack of precision in their sizing respectively (max. size of the PCR product is 510 pb). To reduce potential biases induced by the inconsistent reproducibility of T-RFs that show very small amounts of fluorescence, T-RFs less than 300 RFU are suppressed when they are not present in all replicate profiles of a sample. When all data profiles are treated, the final excel sheet is saved under comma separated value (.csv) format with the name spe.csv (Figure 10). It is important to always save T-RFLP data prepared for statistical analysis under this name, as the file will be recalled from the statistical analysis script using this name.


spe.csv

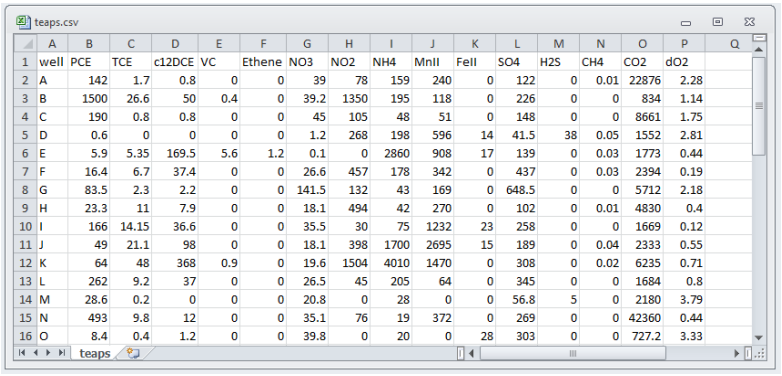
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
1	well	50	51	52	54	55	56	57	58	59	60	61	62	64	67	69	70	72	74	75
2	A	0	0	0	0	1798	283	0	639	5398	0	1436	2508	0	0	0	0	0	0	0
3	B	429	555	0	0	6835	261	0	1619	10844	0	3574	0	0	0	0	0	0	0	0
4	C	0	0	0	0	2180	0	0	669	3978	0	2159	0	0	0	0	0	0	0	0
5	D	483	423	299	293	1788	4211	2651	20014	0	0	68359	0	0	979	0	1468	0	0	852
6	E	0	0	0	0	3634	0	0	915	5842	0	749	914	0	0	0	0	0	0	0
7	F	0	0	0	0	4435	0	0	436	3404	0	2178	5368	0	0	0	0	0	0	0
8	G	0	0	1777	0	2821	0	0	974	9561	0	1911	0	0	0	0	6346	0	0	0
9	H	0	0	2419	0	971	0	0	0	2499	0	1971	0	0	0	0	0	0	0	0
10	I	0	0	0	0	2726	0	0	381	2717	0	1229	0	0	0	0	0	0	0	0
11	J	0	0	0	2010	0	0	2339	0	627	0	0	37914	3409	0	0	0	833	721	0
12	K	616	0	0	0	6368	0	0	1614	13259	0	6123	3498	352	0	0	0	345	214	0
13	L	0	0	0	0	3225	0	0	0	1715	0	0	1281	0	0	0	0	0	0	0
14	M	864	0	2606	0	7035	267	0	2195	24183	0	0	7922	0	0	340	0	0	0	0
15	N	465	425	0	0	5109	0	0	1398	12494	0	1265	0	0	0	0	0	0	0	0
16	O	1180	1558	0	1115	8613	0	0	3392	36731	0	6372	0	0	0	0	0	0	0	0

spe

Figure 10 – spe.csv final data matrix in Excel.
First column contains name of the samples, first line refers the T-RFs size in base pair (bp). Data correspond to the value in RFU of each peak area.

6.2.2. Physicochemical data

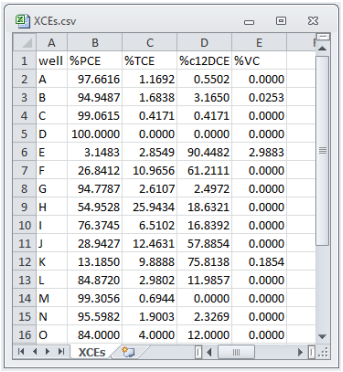
The measured chemical and physicochemical parameters have to be prepared in three different *.csv files: teaps.csv, XCEs.csv and others.csv. The teaps.csv file contains CE (including ethene) concentration data and chemical parameters related to TEAPs, i.e. optimally PCE, TCE, c12DCE, t12DCE, VC, ethene, NO_3^-, NO_2^-, NH_4^+, Mn(II), Fe(II), SO_4^{2-}, H_2S/S^{2-}, CH_4, CO_2, dO_2 (Figure 11). In addition, for the file teaps.csv, all contaminant concentrations must be expressed in the same unit (µg/L). Percentage of PCE, TCE, DCE and VC can be calculated from the sum of contaminant concentrations. These calculated parameters are conserved in a new table, XCEs.csv, and will be indicative of accumulation of the different CEs. The others.csv file (Figure 13) contains all other measured environmental parameters (i.e. TOC, ORP/Eh, pH, temperature, conductivity, sampling depth, etc.).



teaps.csv

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	well	PCE	TCE	c12DCE	VC	Ethene	NO3	NO2	NH4	MnII	FeII	SO4	H2S	CH4	CO2	dO2	
2	A	142	1.7	0.8	0	0	39	78	159	240	0	122	0	0.01	22876	2.28	
3	B	1500	26.6	50	0.4	0	39.2	1350	195	118	0	226	0	0	834	1.14	
4	C	190	0.8	0.8	0	0	45	105	48	51	0	148	0	0	8661	1.75	
5	D	0.6	0	0	0	0	1.2	268	198	596	14	41.5	38	0.05	1552	2.81	
6	E	5.9	5.35	169.5	5.6	1.2	0.1	0	2860	908	17	139	0	0.03	1773	0.44	
7	F	16.4	6.7	37.4	0	0	26.6	457	178	342	0	437	0	0.03	2394	0.19	
8	G	83.5	2.3	2.2	0	0	141.5	132	43	169	0	648.5	0	0	5712	2.18	
9	H	23.3	11	7.9	0	0	18.1	494	42	270	0	102	0	0.01	4830	0.4	
10	I	166	14.15	36.6	0	0	35.5	30	75	1232	23	258	0	0	1669	0.12	
11	J	49	21.1	98	0	0	18.1	398	1700	2695	15	189	0	0.04	2333	0.55	
12	K	64	48	368	0.9	0	19.6	1504	4010	1470	0	308	0	0.02	6235	0.71	
13	L	262	9.2	37	0	0	26.5	45	205	64	0	345	0	0	1684	0.8	
14	M	28.6	0.2	0	0	0	20.8	0	28	0	0	56.8	5	0	2180	3.79	
15	N	493	9.8	12	0	0	35.1	76	19	372	0	269	0	0	42360	0.44	
16	O	8.4	0.4	1.2	0	0	39.8	0	20	0	28	303	0	0	727.2	3.33	

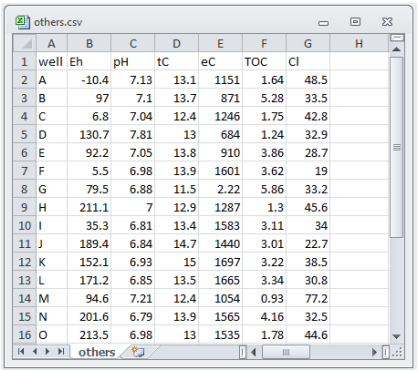
Figure 11 – teaps.csv final concentration matrix in Excel.
First column contains name of the samples, first line contains names of CEs and inorganic compounds related to TEAPs. The acronyms used are PCE: perchloroethene, TCE: trichloroethene, c12DCE: cis-1,2-dichloroethene, VC: vinyl chloride, NO3: nitrate, NO2: nitrite, NH4: ammonium, MnII: manganese(II), FeII: iron(II), SO4: sulfate, CH4: methane.



XCEs.csv

	A	B	C	D	E
1	well	%PCE	%TCE	%c12DCE	%VC
2	A	97.6616	1.1692	0.5502	0.0000
3	B	94.9487	1.6838	3.1650	0.0253
4	C	99.0615	0.4171	0.4171	0.0000
5	D	100.0000	0.0000	0.0000	0.0000
6	E	3.1483	2.8549	90.4482	2.9883
7	F	26.8412	10.9656	61.2111	0.0000
8	G	94.7787	2.6107	2.4972	0.0000
9	H	54.9528	25.9434	18.6321	0.0000
10	I	76.3745	6.5102	16.8392	0.0000
11	J	28.9427	12.4631	57.8854	0.0000
12	K	13.1850	9.8888	75.8138	0.1854
13	L	84.8720	2.9802	11.9857	0.0000
14	M	99.3056	0.6944	0.0000	0.0000
15	N	95.5982	1.9003	2.3269	0.0000
16	O	84.0000	4.0000	12.0000	0.0000

Figure 12 – XCEs.csv final data matrix in Excel.
First column contains name of the samples, first line contains the CE percentages labels.



others.csv

	A	B	C	D	E	F	G	H
1	well	Eh	pH	tC	eC	TOC	Cl	
2	A	-10.4	7.13	13.1	1151	1.64	48.5	
3	B	97	7.1	13.7	871	5.28	33.5	
4	C	6.8	7.04	12.4	1246	1.75	42.8	
5	D	130.7	7.81	13	684	1.24	32.9	
6	E	92.2	7.05	13.8	910	3.86	28.7	
7	F	5.5	6.98	13.9	1601	3.62	19	
8	G	79.5	6.88	11.5	2.22	5.86	33.2	
9	H	211.1	7	12.9	1287	1.3	45.6	
10	I	35.3	6.81	13.4	1583	3.11	34	
11	J	189.4	6.84	14.7	1440	3.01	22.7	
12	K	152.1	6.93	15	1697	3.22	38.5	
13	L	171.2	6.85	13.5	1665	3.34	30.8	
14	M	94.6	7.21	12.4	1054	0.93	77.2	
15	N	201.6	6.79	13.9	1565	4.16	32.5	
16	O	213.5	6.98	13	1535	1.78	44.6	

Figure 13 – others.csv final data matrix in Excel.
First column contains name of the samples, first line contains names of environmental parameters. The acronyms used are Cl: chlorine, F: fluorine, PO4: phosphate, K: potassium, Corg: total organic carbon (TOC), pH: acidity, tC: temperature, turb: turbidity, Eh: oxido/reduction potential, depth: sampling depth.

The first line of the tables presents names of the measured parameters; avoid concentration units, dots, and subscript or superscript characters in names (see acronyms listed in Table 3). The first column contains the sample names that must be in the same order as in the spe.csv file. These names need to be unique, even if the samples come from the same sampling well. Then the table is filled with the measured values, where dots, not commas, are used as the decimal separator. Optimally the table should not present missing values. It

is important that all parameters are documented for each monitoring well; otherwise it will be necessary to suppress a whole line or column for the analysis. Measured concentrations below the detection limit should be considered as null concentrations and noted as 0 in the table. Parameter columns with null values for all samples should be deleted. This information should be kept elsewhere as a reminder that the parameter was measured but not detected, as may be the case for the ethene concentration parameter. Finally, each excel sheet is saved in .csv format under the precise name needed to call it from the statistic script.

6.3. Multivariate statistical analysis for integrative analysis of bacterial and environmental data

The objective for collection of the above bacterial and physicochemical data is to examine aquifer ecosystem functioning as a whole regarding CE dechlorination (section 3). The Multiple Factor Analysis (MFA[1]) was chosen to explore the data and achieve this comprehensive analysis (Borcard *et al*., 2011). MFA is a general type of Principal Component Analysis (PCA[2]). Its goals are to analyze several data sets of variables collected on the same set of observations and to evaluate the relationships between variables or groups of variables (Abdi et al., 2013). In our investigation of aquifers, MFA explores data tables in which water samples are described by several inter-correlated quantitative, dependent variables. MFA allows the simultaneous exploration of the four data sets of variables: i) the BCS dataset (spe.csv), ii) the dataset that indicates the reduction states of the contaminant as well as environmental variables related to TEAPs (teaps.csv), iii) the CE accumulation dataset (XCEs.csv), and iv) the remaining environmental data (others.csv). Data from biomarker detection should not be included in MFA since these PCR results, in contrast to the other matrices that present quantitative data, are qualitative presence/absence data. Interpretation of the resulting MFA could be delicate and introduce confusion, and therefore these data should not be used for MFA.

Each water sample is characterized by its own values of measured parameters. Graphically, these measurements place the water samples in a multidimensional space defined by a number of axes related to the number of measured variables (Figure 14a). The goal of MFA is to extract the important information from the whole dataset and represent it in a set of new orthogonal axes called MFA dimensions or components (Figure 14b). These new axes point to the directions of maximal variance or dispersion of the whole dataset. In MFA, just like in standard PCA, the importance of a dimension is reflected by its **Eigenvalue** percentage, which indicates how much of the total variance of the data is explained by this dimension. MFA will define as many axes as are needed to describe the total dispersion of samples. Commonly, the first two dimensions are those that describe most of the variance in the data and are used for coherent graphical representation in 2D of the MFA results (Figure 14c). MFA also defines the contribution of the former variables in this new MFA space, i.e. the contribution of the former variables to explain the variance of the data.

[1] Multiple Factor Analysis in R software: http://factominer.free.fr/advanced-methods/multiple-factor-analysis.html

[2] Principal component analysis explained visually :http://setosa.io/ev/principal-component-analysis/

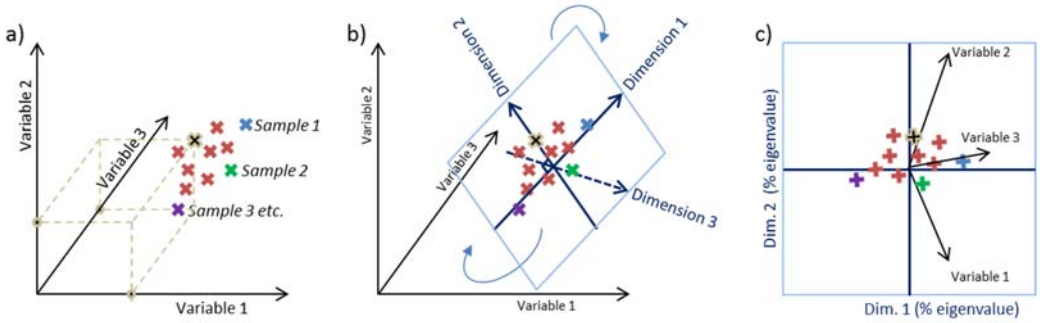


Figure 14 – Schematic construction of PCA graphical representations, and by analogy of MFA.
a) original data in 3D, b) delineation of new axes by MFA and c) 2D graphical output of the statistical analysis

MFA visually represents i) the samples' similarity based on their combined bacterial and physicochemical characteristics, ii) the distribution of predominant redox conditions in a GWS, iii) the relationships between the predominant redox conditions and the distribution/degradation state of CEs, and iv) the relationships between environmental parameters and bacterial communities, including bacteria involved in reductive dechlorination.

As the statistical analysis is a limiting aspect of the procedure, and its interpretation is the corner stone of the screening process, the MFA medthod is described with particular attention.

6.3.1. Procedure for the statistical analysis

MFA is realized using R software. This software must be installed on the users' computer. It is freeware that can be downloaded from the CRAN website. The installation procedure and instructions for the creation of an 'R working folder' are given in Appendix 7. When the software installation is done, the **R packages** listed in Table 4 should be installed. These packages only need to be installed once; thereafter the user will be able to load them from the MFA script. They can be easily installed by running the following code line in the R console:

```
> install.packages (c('ade4', 'vegan', 'FactoMineR'))
```

Then the **R function** 'evplot.R' should be saved in the user 'R working folder', as described in Appendix 8. This evplot.R function will be used to create plots of eigenvalues and percentage of variation as well as to test MFA axes validity with the broken-stick model (see section 7.4.2)

Table 4 – List of the R packages to install

Name of the R package	Meaning of the R package
ade4 (CRAN link)	# Multivariate data analysis and graphical display
vegan (CRAN link)	# Ordination methods, diversity analysis and other functions for community and vegetation ecologists.
FactoMineR (CRAN link)	# Multivariate exploratory data analysis and data mining # Include function for the MFA analysis

Finally, the R code script for MFA is presented in Appendix 9. The latter should be saved in the user 'R working folder' as described in the appendix.

At this stage, the user should check that the following six files are present in the 'R working folder': i) 0_MFA_LBE_ofev.R, ii) evplot.R, iii) spe.csv, iv) teaps.csv, v) XCEs.csv and vi) others.csv. MFA can then be run. Two ways are proposed to compute the analysis, a "step-

by-step way" and an "easy way". First, the user must verify that the R working directory corresponds to the "R working folder" (Appendix 7), then choose one of the approaches described below.

Step by step way: The user opens the MFA script from the R software and executes line-by-line. The user can read the comments (after each # symbol) placed throughout the script that describe the lines of code and guide the user through the script.

Easy way: If the user is not interested with the details of the analysis, the following line of code can be written in the R console to let the script execute alone:

```
> source("0_MFA_LBE_ofev.R ")
```

The MFA output files generated by the script are saved in the 'R working folder' under the names listed in Table 5.

Table 5 – List and brief descriptions of the MFA R script output files

Output file name	Brief description
1) 1_Test_Axes.png	Show graphically axes validity of the MFA dimensions
2) 2_Plot_CorrelationCircle.png	Show correlation between variables and their contribution in the MFA dimensions 1 and 2
3) 3_Plot_CorrelationCircle_0.05.png	MFA Correlation circle showing only significant variables (with a p-value < 0.05) that explain the MFA model
4) 4_Plot_IndFactMap.png	Plot of the water samples in the MFA dimensions 1 and 2
5) 5_Table_Correlations_BCS_Env.csv	Table of the correlations and associated p-value between the BCS and groups of environmental variables, and between BCS and variables take separately.

6.4. Keys for reading MFA plots

The file '1_Test_Axes.png', which is produced during the MFA, provides a way to evaluate the statistical validity of MFA axes that describe GWS data. If MFA axes are not validated, interpretation of the results will be wrong. If validated, the axes are robust and the MFA results are reliable. An example will be detailed in Section 7.4.2. Then the two main, useful output plots of the MFA are the **'Correlation circle'** and the **'Individual factor map'** (Table 5, output files 2, 3 and 4 respectively). A case study of these output plots is presented in Figure 15 to facilitate comprehension of the reading keys listed below. The case study data imitate a contaminated site where no ethene production is detected.

Both the '**Correlation circle**' and '**Individual factor map**' graphs are composed of a two-dimensional plane defined by the two principal dimensions, MFA1 and MFA2. These axes are associated with an **Eigenvalue** percentage that corresponds to the variance among samples explained by each axes. For example, in Figure 15, the MFA axes together explain 39.13% of the variance between samples, 22.07% along the MFA1 axis and 17.06% along the MFA2 axis.

The '**Correlation circle**' graph (Figure 15) depicts the projection of the **original measured variables** as **grey, green, red** and **blue vectors** that correspond respectively to the analyzed groups of variables **BCS**, **TEAPs**, **%CEs** and **Others** (section 6.2). Each parameter is projected in the defined MFA two-dimensional plane according to their respective importance to explain the variability of the data. With this graphical output of MFA, all parameters can be observed and compared in a unique referential, the 'Correlation circle' showing the **intra- and inter-relationships of the physicochemical and the microbiological parameters**.

The keys for reading the 'Correlation circle' are:

- **The longer a vector is,** the more the associated **variable presents dissimilar values and explains the variance among samples**. This does not necessarily imply that a long-length vector corresponds to a parameter with high values, but rather to a variable whose values are heterogeneous and different from one sample to another. To appreciate the magnitude of the data it is essential to return to raw data.
- Collinear vectors pointing in the **same direction** indicate parameters that are **positively correlated** in a GWS (i.e. when a parameter is present at high concentration, the other is also at high concentration). For example, this is the case for the NH_4^+ and TOC vectors or the Fe(II) and VC vectors in Figure 15.
- Vectors pointing in **opposite directions** from the origin of the graph indicate parameters that are **negatively correlated** in a GWS (i.e. when a parameter is present at elevated concentration the other is at low concentration). This is the case for the NO_3^- and NO_2^- vectors or the %PCE and %VC vectors in Figure 15.
- **Perpendicular vectors** indicate parameters that are **independent** one from another. This means that no trend links the values of one parameter to the value of the other. For example, in Figure 15, the perpendicular position of the tC and Eh vectors indicates that temperature is not linked to oxidation-reduction potential.

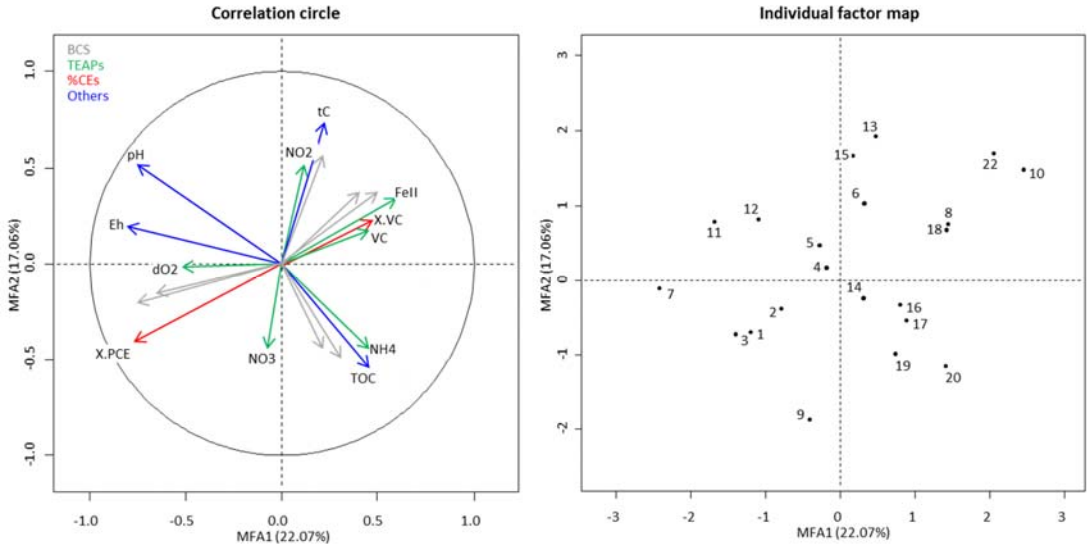


Figure 15 – Multiple factor analysis (MFA) plots presented as a case study to aid understanding of the reading keys and interpretation of the MFA multivariate statistical analysis.
In the 'Correlation circle', grey vectors correspond to the projection of the T-RF positions of the T-RFLP profiles (section 6.1) (i.e. interpretable as presence of a bacterial population). Other abbreviations: Eh, oxidation-reduction potential; dO2, dissolved oxygen; X.PCE, percentage of perchloroethene among total CEs; NO3, nitrate; TOC, total organic carbon; NH4, ammonium; VC, vinyl chloride; X.VC, percentage of vinyl chloride among total CEs; FeII, iron(II); tC, temperature, NO2, nitrite. In the 'Individual factor map', points n°1 to 20 correspond to the sample projections, which summarize the results obtained from the four variable groups, (BCS, TEAPs, %CEs and Others) for each sample.

The 'Individual factor map' (Figure 15) represents the projection of each sample in the MFA dimensions, depicted by gathering the results obtained from the four variable groups, BCS, TEAPs, %CEs and Others. This means that the position of a sample on the graph is the barycenter of the data of all bacteriological and physicochemical parameters measured for this sample. The 'Individual factor map' shows the similarity or dissimilarity between water

samples according to their relative positions. The keys for reading the 'Individual factor map' are:

- The closer two samples are, the more similar they are in their physicochemistry and microbiology and vice versa. For example, in Figure 15 the close samples 8 and 18 are similar, and the distant samples 7 and 10 are dissimilar.
- Samples close to the center of the MFA graph are few or are not explained by the MFA axes. This is the case for samples 4 and 14 in Figure 15. This means that the measured parameters (taken as a whole) are not discriminant factors and do not elucidate the biogeochemistry of the sample.

The 'Correlation circle' and the 'Individual factor map' are different output representations of the same MFA and can be read together. This means that:

- The direction indicated by a vector in the 'Correlation circle' (Figure 15, left) is associated to samples in the same graph area on the 'Individual factor map' (Figure 15, right). The variable associated with the vector indicates the main discriminant parameter for samples in this area of the graph (Figure 15, right). For example, samples 18, 8, 22 and 10 occupy the area pointed to by the Fe(II), %VC, and VC vectors. This means that water samples 18, 8, 22 and 10 are different from the others by the simultaneous presence of Fe(II) and vinyl chloride, as well as vinyl chloride accumulation.
- A vector that is collinear to one of the MFA axes indicates that this parameter substantially impacts the distribution of the samples along the MFA dimension. For example, the collinearity of the dO_2 vector with the MFA1 axis indicates that the dissolved O_2 concentration is the main factor that explains sample dispersion along the MFA1 axis (i.e. samples located in the left part of the graph present higher dissolved O_2 concentrations than samples in the right part). Similarly, the distribution of the samples along the MFA2 axis is explained in particular by the vertically positioned NO_2^- and NO_3^- vectors. The sample dispersion along the MFA2 axis is due to differences in NO_2^- and NO_3^- concentrations.

6.5. Guidelines for MFA interpretation and setting up the GWS biogeochemical model

The MFA output graph interpretation is the cornerstone for identifying both competition between bacterial metabolisms and reductive dechlorination and the reasons for complete or incomplete dechlorination in a GWS. In this sense, the reading keys presented above are the foundation for identifying the important factors that affect aquifer biogeochemical functioning, such as:

- The predominant TEAPs present in a GWS
- Competition for electron donors that occurs between TEAPs and CE biodegradation (linked to 4.4.1 section and Figure 4)
- The overall connections between measured environmental and microbiological parameters

For example, interpretation of the 'Correlation circle' in Figure 15 indicates that 5 predominant TEAPs occur in the GWS (for theory help, see Section 4.3.2.2):

(1) The Eh and dO_2 vectors on the left part of the 'Correlation circle' indicate that the presence of oxygen is concomitant with a high oxidation-reduction potential. These parameters are indicative of the **aerobic respiration process**. This also means that the right part of the graph is probably related to anaerobic bacterial processes.

(2) The presence of the NO_2^- vector in the right upper quarter of the 'Correlation circle' may indicate that the **dissimilatory nitrate reduction process** (NR) takes place in part of the aquifer. In this case, NO_3^- is reduced at least into NO_2^-. The position of the NO_2^- vector in the right part of the 'Correlation circle' that is related to anaerobic bacterial processes (see point 1) corroborates this interpretation. However, as mentioned in Section 4.3.2.2., an explanation for the presence of NO_2^- must be interpreted with caution and formed in conjunction with other forms of nitrogen. The opposite area of the graph, indicated by the NO_3^- vector, signifies that another part of the GWS is characterized by NO_3^- available for denitrification, but probably in low concentrations (should be confirmed with raw data), and oxic conditions (observed in point (1)) may limit the process.

(3) The NH_4^+ vector in the right lower part of the graph, collinear to the TOC vector, indicates that the presence of organic carbon is correlated to NH_4^+. The bacterial process that produces NH_4^+ under anaerobic conditions (observed in point (1)) and requires large amounts of carbon as an energy source is the **dissimilatory nitrate reduction to ammonium process** (DNRA). The origin of NH_4^+ in the aquifer should nevertheless be taken into consideration to avoid misinterpretation. For example, NH_4^+ could originate from anaerobic degradation (ammonification) of organic material or from anthropogenic activities.

(4, 5) The VC and %VC vectors are in collinear alignment with the Fe(II) vector. This indicates that the microbial processes of **VC reductive dechlorination** and **iron(III) reduction** are in competition with each other and that due to VC accumulation iron(III) reduction inhibits VC reductive dechlorination. The iron(III) respiration processes requires similar ranges of reducing equivalents as DCE reductive dechlorination (electron donor H_2, Figure 4). This would explain a slowdown of DCE degradation. Second, iron(III) reduction activities require lower H_2 concentrations than VC reductive dechlorination (Figure 4). This means that iron(III) reduction wins the competition under low redox conditions, and VC accumulates.

Consequently, the reason that lower CE dechlorination stalls in this example is that dechlorination of VC to ethene is limited or blocked in the system by iron oxide respiration.

Lastly, **RV correlation coefficients**, which are correlations between the environmental and bacterial community datasets, and the associated p-values, are calculated in MFA; the output is located in '5_Table_Correlations_BCS_Env.csv'. The RV coefficients can reveal significant correlations between bacterial communities/populations with ongoing biogeochemical processes or identify a microbiological lack. An example of these results will be detailed in the next section of this document, which describes a concrete application of the screening procedure through a complete case study.

7. CASE STUDIES

APPLICATION OF THE SCREENING PROCEDURE FOR THE EVALUATION OF THE NATURAL ATTENUATION POTENTIAL IN CE-CONTAMINATED AQUIFERS

7.1. Case study 1 - Former dry-cleaning facility, Canton of Solothurn, Switzerland

This first case study was detailed in Shani *et al.*, (2013). The complete document can be downloaded from the website of the journal Environmental Science and Technology (doi: 10.1021/es304017s). A short summary of this site investigation is provided here. The site (Figure 16) was a former dry-cleaning facility operated from the mid-1940s until 1987. A major subsurface PCE contamination event occurred in 1969.

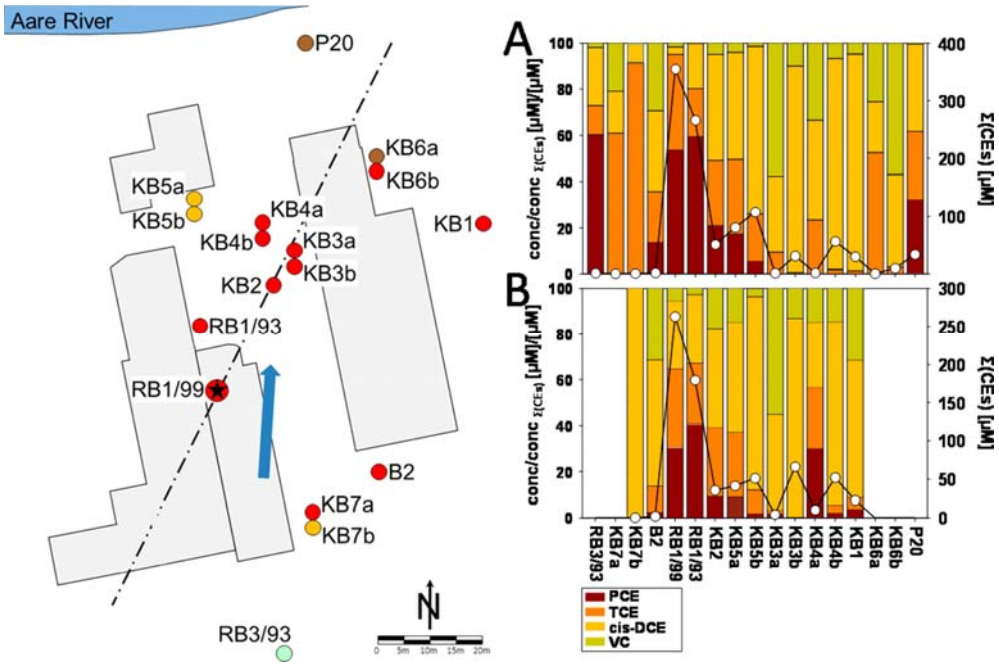


Figure 16 - Map of the Solothurn test-site.
Monitoring wells are shown as colored circles (left). The source of the PCE contamination is shown as a star. The general groundwater flow direction is indicated with a blue arrow. The circle colors indicate the local oxidation–reduction status at the monitoring location. Green: oxic/nitrate-reducing, orange: nitrate to iron-reducing, red: iron-reducing and brown: methanogenic. Concentrations of CEs measured from two sampling campaigns (A: April and B: October 2008 respectively) are shown on the left. The figure is extracted from the Shani *et al.*, 2013 publication.

VC accumulated *in situ* overall, as shown previously with stable isotope techniques (Aeppli et al., 2010), while complete reductive dechlorination and the corresponding ethene production occurred locally. Shani et al. (2013) followed a procedure similar to the present protocol to investigate the reasons for this.

The procedure involved the 3 steps: i) collection of the aquifer's biogeochemical data, ii) confirmation of biochemical potential through the CE reductive dechlorination process via PCR detection of both RdhA and OHRB 16S rRNA genes and via microcosm studies, iii) elucidation of the mechanisms responsible for incomplete *in situ* dechlorination of CEs via the correlations between BCS and the environmental variables (TEAPs and Others) determined by MFA. In order to validate the mechanisms proposed in the third and last step of the protocol, this study additionally presents a deeper bacterial analysis, which identifies the bacterial populations associated with the terminal restriction fragments (T-RFs) that significantly correlated with TEAP variables.

The MFA showed that TEAPs shaped the structure of the bacterial community (BCS) in general. VC and iron(III) reduction were both keys and antagonistic microbiological processes in this aquifer. An interpretation of this result could be that the iron-reducing bacteria's higher affinity for hydrogen, as compared to VC-reducing bacteria (Figure 4), would explain the inefficient complete dechlorination and the local VC accumulation. The detailed molecular analyses of BCS showed that the bacterial populations that correlated significantly with VC reduction were those affiliated to the genus *Dehalococcoides* and to the "Lahn Cluster" (included in the class Dehalococcoidetes). Conversely, T-RFs affiliated with the genus *Rhodoferax*, known to harbor iron-reducing bacterial species, were anti-correlated to "Lahn Cluster" T-RFs. These results supported the hypothesis that competition between reductive dechlorination and iron-reduction was the bacterial-dependent mechanism that limited the natural attenuation of VC in the aquifer.

In conclusion, this first integrative environmental and microbial data analysis is a helpful example that identifies the reasons for accumulation of toxic dechlorination intermediates.

7.2. Case study 2 - Former municipal discharge site, Canton of Geneva, Switzerland

7.2.1. Site information

This case study was carried out on a PCE-contaminated site near Geneva, Switzerland, and the monitoring and study was entrusted to BG Consulting Engineers (BG). The site is currently occupied by family gardens and was originally a municipal landfill (probably a place of wild repository). The site map is presented in Figure 16, where the main investigation area is surrounded with green lines (around 13000 m^2). At the time of the investigation, the site was equipped with 24 monitoring wells, two of which could be sampled at two different depths (MIP05 and MIP06).

The soil profile analysis of a NNW-SSE transect (Figure 17) indicated that the groundwater system is composed of upper and lower aquifers with different groundwater flow directions (Figure 18a and b). They are separated by a horizon of compacted sandy loamy gravel with low permeability, but fractures in some parts of the horizon allow water to flow from the upper to the lower aquifer.

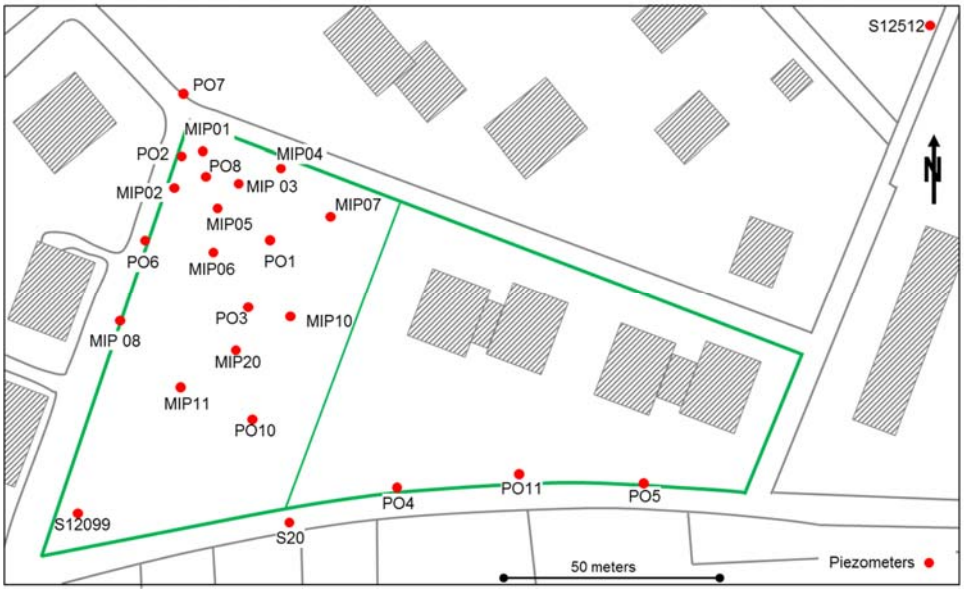


Figure 17 – Map of the studied site contaminated with chlorinated ethenes and distribution of the monitoring wells.

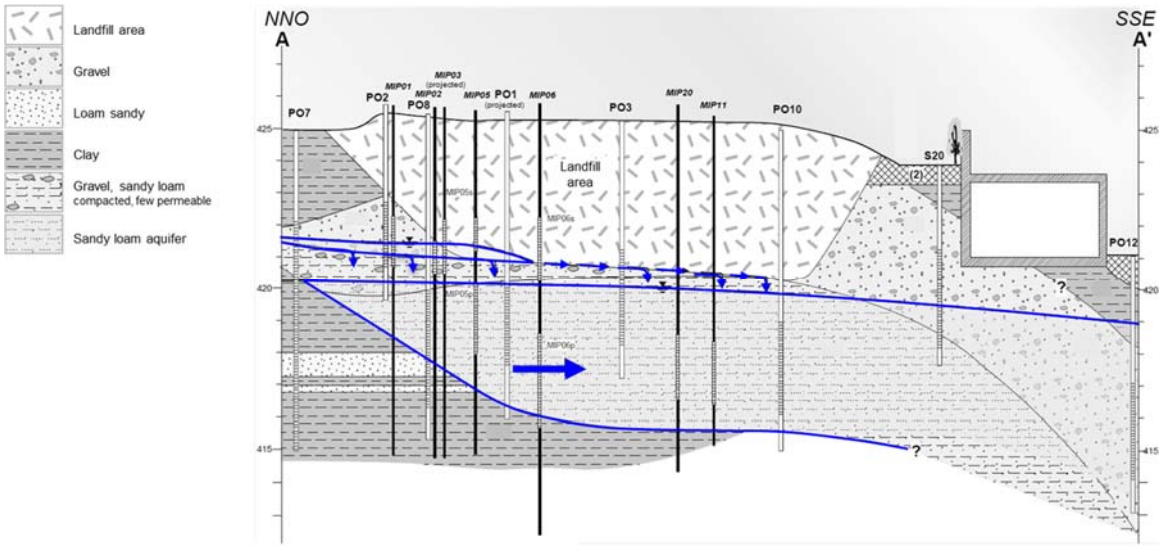


Figure 18 – Soil profile of a NNW-SSE transect of the studied site contaminated with chlorinated ethenes, and projection of the piezometer positions on the transect plane.
Blue lines indicate the limits of the upper and lower aquifers.

7.2.2. Assessing in situ presence of reductive dechlorination – Step 1

Twenty-five water samples were collected from 24 piezometers (MIP01, MIP02, MIP03, MIP04, MIP05-upper, MIP05-lower, MIP06-lower, MIP07, MIP08, MIP10, MIP11, MIP20, PO1, PO2, PO3, PO4, PO5, PO6, PO7, PO8, PO10, PO11, S12099, S12512, S20), and the physicochemical parameters listed in section 3 of the procedure were analyzed. The **physicochemical data** is given in Appendix 10A. The **contaminant maps** presented in Figures 19 and 20 were produced based on the results of CE concentration measurements.

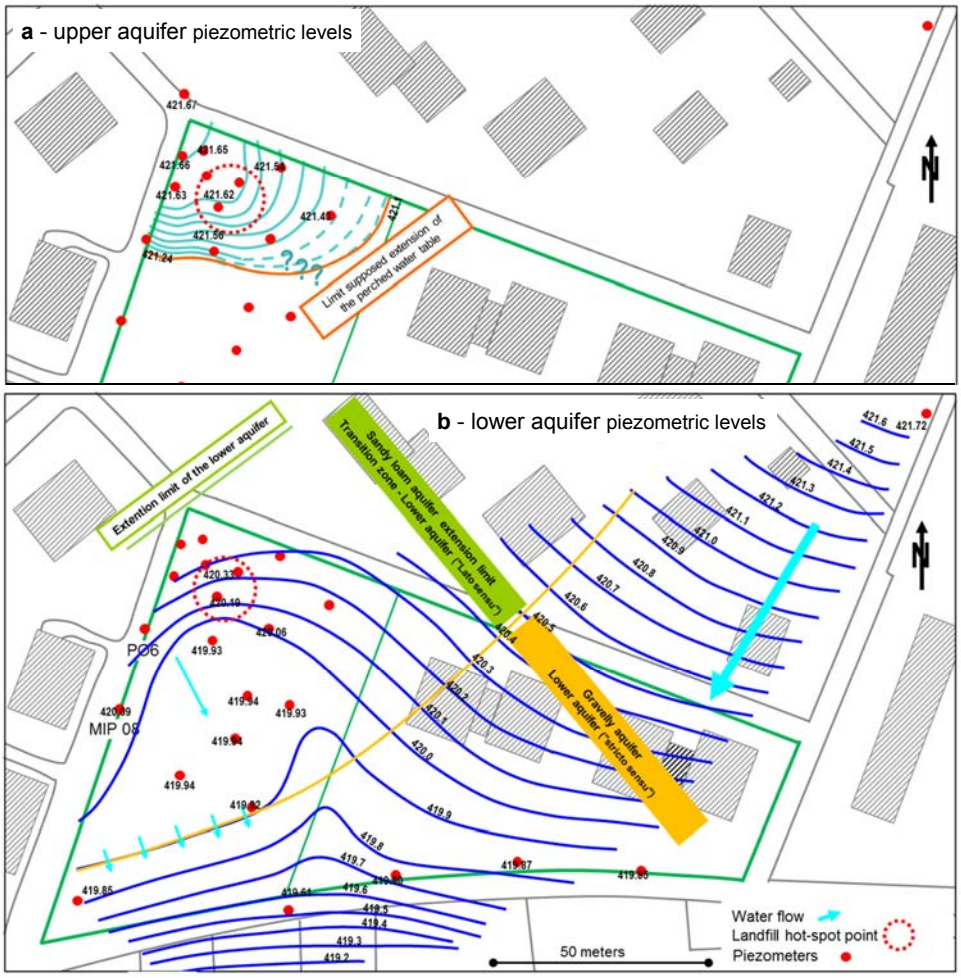


Figure 19a and 19b – Piezometry and groundwater flow direction in the upper (a) and lower (b) aquifers of the contaminated site

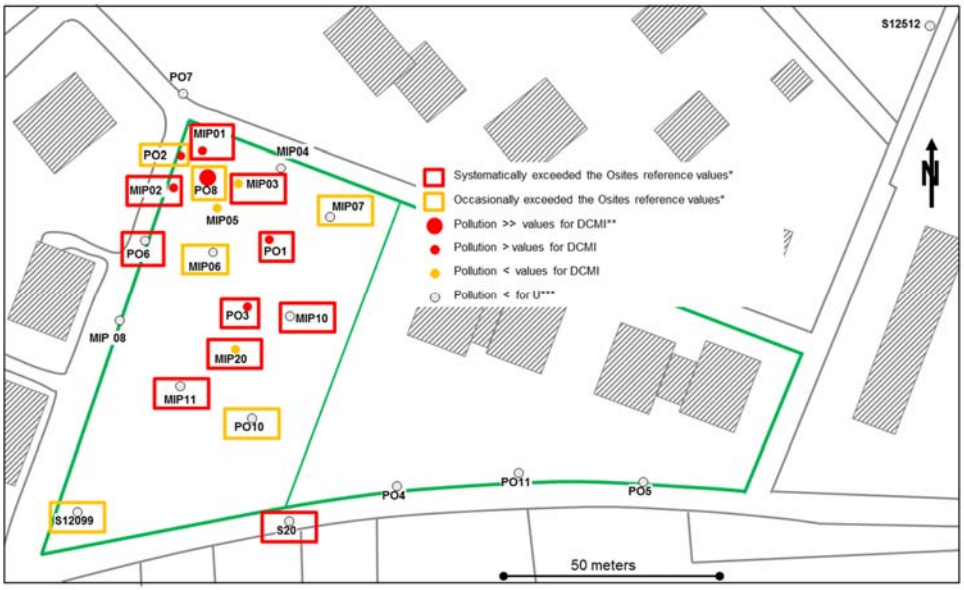


Figure 20 – Global distribution of the contamination, and evaluation of the pollution level in regard to groundwater system protection and norm in deposit of uncontaminated excavated materials in soil.
***** Ordinance on the remediation of contaminated sites (OSites 814.680), values for groundwater. ****** Landfills for inert materials, ******* Deposit for uncontaminated excavated materials: indicative values for grounds in Ordinance on the treatment of wastes (OTD 814.600) and in FOEN (1999).

On this site, PCE, c12DCE and VC concentrations were above legal limits. PCE was dechlorinated until c12DCE and VC along the groundwater flow direction. This created a contaminant plume, from the NNW to SSE axis, where c12DCE and VC concentrations were 10- and 50-fold higher than legislation limits, respectively (Figure 20). No ethene production was detected at the site. **Hence, the reductive dechlorination process occurred at the site, but is incomplete.**

7.2.3. Evaluation of the biochemical potential for reductive dechlorination - Step 2

7.2.3.1. Detection of biomarkers

A total of 25 water samples were analyzed for the biomarkers for OHRB and RdhA genes listed in 4.2.3. The results are summarized in Figure 21 and detailed in Table 6. All biomarkers were detected at the site (Table 6), and at least one of the biomarkers was detected per well. The two most commonly detected genera of OHRB were *Desulfitobacterium* and *Desulfuromonas*, from which strains are known to dechlorinate PCE to c12DCE (Figure 7-C and Figure 8). One or both of these genera were found alone, without detection of other OHRB or RdhA genes, in 52% of the samples. This was the case for the wells PO10 and MIP20; where the highest c12DCE concentration was reported and only one OHRB was detected (*Desulfitobacterium* or *Desulfuromonas*, Table 6). These results indicate that part of the site lacked the bacterial guild required for complete dechlorination.

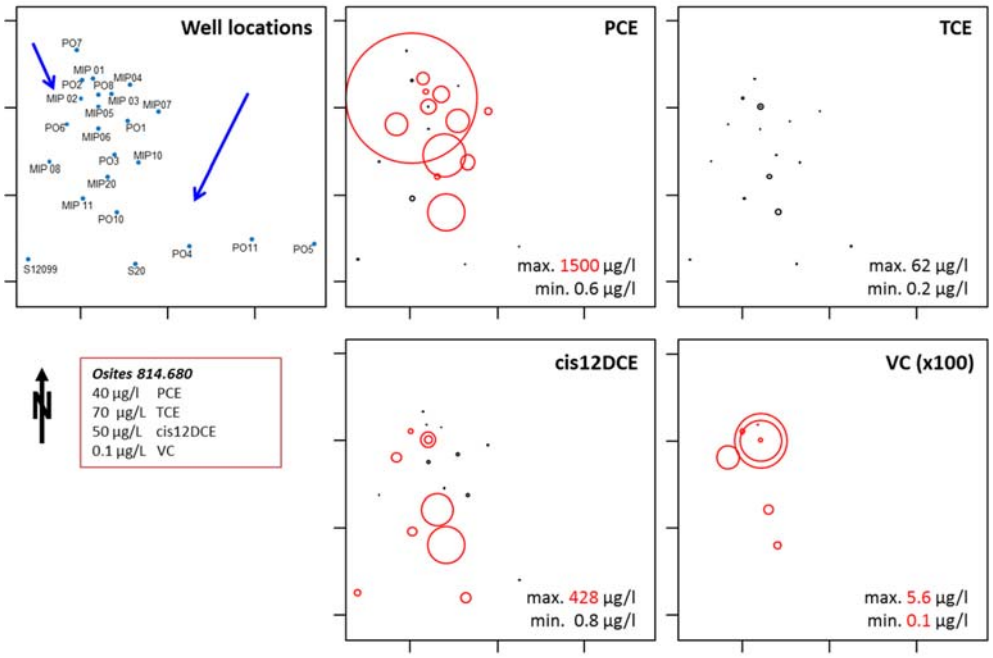


Figure 21 – Map of the contaminants PCE, TCE, c12DCE and VC produced at the site.
CE concentrations are plotted as circles with a size proportional to their concentration values. Red circles correspond to concentrations that exceed the OSites limits (shown in the red box). Black circles correspond to non-zero concentrations below OSites limits. The scale used to plot the VC concentration is 100 fold higher than the one used for the other CEs.

Conversely, the genus *Dehalococcoides*, from which there are strains known to dechlorinate CEs to ethene, was detected in 4 samples, and the *bvcA* and *vcrA* genes, which code for VC dehalogenase catalytic subunits, were detected in 6 samples. Detections occurred close to the contamination source and equally in the upper and lower aquifers. However,

only dechlorination to VC was observed in these wells, and both VC and c12DCE concentrations were high. Hence, the detection of *Dehalococcoides* sp. and RdhA genes without concomitant ethene production suggests that environmental conditions were inadequate for reductive dechlorination of c12DCE and VC or that the species detected did not possess the complete dechlorination functionality.

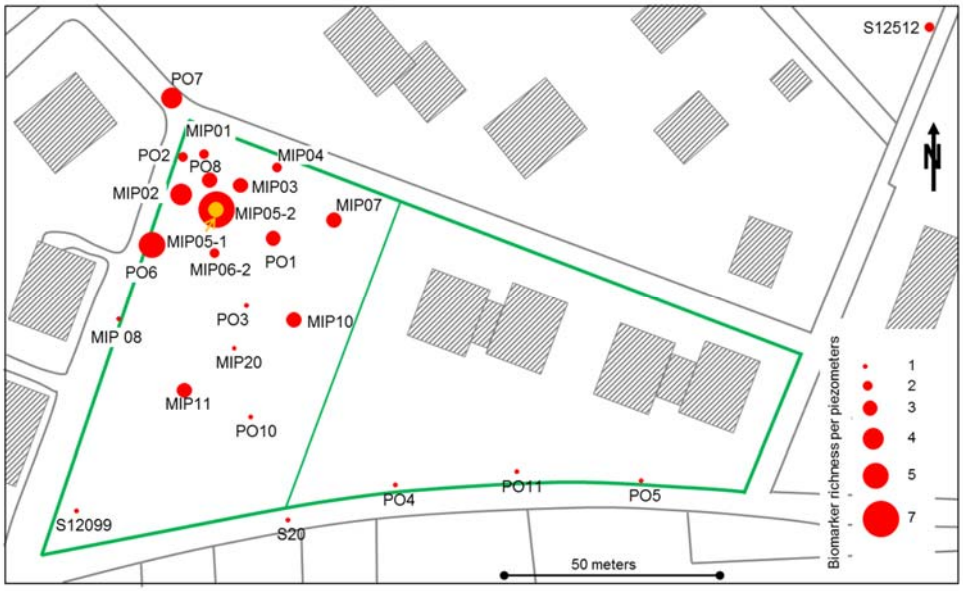


Figure 22– Map of biomarkers detected at the site.
The size of the circle is proportional to the number of markers identified at each well. The yellow circle distinguishes the two sampling depths (1 upper, 2 lower) of well MIP05. No water sample could be retrieved from MIP06-1.

In conclusion, the reductive dechlorination biomarker assessment showed that OHRB and RdhA genes were present in some wells and demonstrated that the actors and biochemical potential for complete dechlorination of PCE to ethene was present in this aquifer. It was difficult to know if the observed incomplete dechlorination was due to adverse environmental conditions or an incorrect assessment of the biological function. Microcosm experiments can clarify which cause limited PCE degradation.

7.2.3.2. Microcosm experiments

Sediments were sampled from soil cores from newly drilled wells: PO7, PO8, PO10, and PO11. Samples were taken from the saturated zones of the cores. Microcosms experiments were prepared as described in Appendix 5, except that instead of water, 15-20 mL of sediment were taken as inoculum. Microcosms were cultivated for 4 months at 37°C. 1 mL of electron donor solution (MixII, Appendix 5) was added in each microcosm every three weeks after GC analysis of the microcosm gas composition. After 73 days of cultivation, all microcosms reduced PCE to c12DCE. Only sample PO7-(8.10) was able to reduce PCE to VC. Microcosms were followed for 130 days, and the last measurements are presented in Table 7. After 122 days, 4 of 7 microcosms developed the ability to reduce PCE to VC, and after 130 days, two microcosms, PO8 and PO10, started to produce ethene. Figure 23 shows the microcosms at the end of the experiment.

Table 6 – Presence (+) / absence (-) of biomarkers assessed by specific PCR on DNA extracted from aquifer water samples.

Well name	Dehalococcoides sp.	Dehalobacter sp.	Sulfurospirillium sp.	Desulfuromonas sp.	Desulfitobacterium sp.	Geobacter sp.	bvcA	vcrA
MIP01	-	-	-	+	+	-	-	-
MIP02	+	-	+	+	+	-	-	-
MIP03	-	-	-	+	+	+	-	-
MIP04	-	-	-	+	-	-	+	-
MIP05-1	+	+	-	-	+	-	-	-
MIP05-2	+	+	-	+	+	+	+	+
MIP06-2	-	-	-	+	+	-	-	-
MIP07	-	-	-	+	+	+	-	-
MIP08	-	-	-	+	-	-	-	-
MIP10	-	-	-	+	+	+	-	-
MIP11	-	-	-	+	+	-	-	+
MIP20	-	-	-	-	+	-	-	-
PO1	-	-	-	+	+	-	-	+
PO2	-	-	-	+	+	-	-	-
PO3	-	-	-	-	+	-	-	-
PO4	-	-	-	+	-	-	-	-
PO5	-	-	-	+	-	-	-	-
PO6	+	-	+	+	+	-	-	+
PO7	-	-	-	+	+	-	+	+
PO8	-	+	-	+	+	-	-	-
PO10	-	-	-	+	-	-	-	-
PO11	-	-	-	+	-	-	-	-
S12099	-	-	-	+	-	-	-	-
S12512	-	-	-	+	+	-	-	-
S20	-	-	-	+	-	-	-	-

-1 and -2 refer to upper and lower aquifer samples, respectively, of wells MIP05 and MIP06.

Table 7 – Dechlorination products detected in the gas phase of microcosms after 122 and 130 days of incubation.
Sample names are followed by the sampling depth in meters in parentheses.

Time	Sample	PCE	TCE	DCE	CV	ethene
122 days	PO7-(7.20)	X	X	X		
	PO7-(8.10)	X	X	X	X	
	PO8-(6.20)	X	X	X	X	
	PO8-(7.10)	X	X	X	X	
	PO10-(7.50)	X	X	X		
	PO10-(9.30)	X	X	X	X	
	PO11-(8.70)	X	X	X		
130 days	PO7-(7.20)	X	X	X		
	PO7-(8.10)	X	X	X	X	
	PO8-(6.20)	X	X	X	X	X
	PO8-(7.10)	X	X	X	X	
	PO10-(7.50)	X	X	X		
	PO10-(9.30)	X		X	X	X
	PO11-(8.70)	X	X	X		

Figure 23 – The organohalide-respiring bacteria (OHRB) enrichment culture microcosms from aquifer sediments after 130 days.

Because PCR detection was conducted with water samples and the microcosm experiments were conducted with sediments, the results of these two methods cannot be directly compared. Nevertheless, the main conclusion was that in optimal conditions, the studied GWS expressed the biochemical potential for complete dechlorination of PCE to ethene.

7.2.4. Biogeochemistry of the aquifer: elucidate the reason for the observed incomplete reductive dechlorination of PCE – Step 3

7.2.4.1. Bacterial community structure investigation

T-RFLP analysis was realized on 23 DNA extracts from the groundwater (the same DNA as the biomarker detection). Data were handled as described in the procedure, and raw T-RF data, including number, position and detected intensity per T-RFLP profile, are presented in Appendix 10B. Samples MIP05-1 and PO11 were excluded as they gave T-RFLP profiles whose total intensities were less than 100,000 RFU. PO7 was also excluded as this sample came from a well identified after the sampling campaign as outside of the main aquifer (Figure 17).

7.2.4.2. Statistical analysis of the entire data set

The data were submitted to statistical analysis as presented in the procedure. The meaning of MFA is to compare samples defined by different groups of variables. Recall that the data sets are structured in different groups: i) **"BCS"**, which corresponds to the T-RFs of the BCS described in each sample by T-RFLP analysis (spe.csv, Appendix 10B); ii) **"TEAPs"**, which corresponds to water chemistry analysis results related to TEAPs (teaps.csv, Appendix 10A); iii) **"%CEs"**, which corresponds to CE accumulation (XCEs.csv, Appendix 10A); and iv) **"Others"**, which corresponds to environmental parameters not related to TEAPs, such as pH, Eh, etc. (others.csv, Appendix 10A).

The first figure obtained relates to the MFA axes validity analysis (Figure 24). MFA axes were sorted in descending order by their Eigenvalues, which represent the portion of the total variance explained by the sample variance. According to Borcard *et al.* (2011), there are two ways to evaluate which axes are important and representative for visualization of data: i) the *Kaiser-Guttman criterion* (KGC), which calculates the mean of all eigenvalues and indicates interpretable axes that present eigenvalues larger than this mean (Figure 24, left) or ii) the *Broken stick model* (BSM), which presents random eigenvalues (Figure 24, right, red), and compares them to eigenvalues extracted from the MFA model. MFA axes with eigenvalues larger than those generated by BSM can be interpreted. BSM is more restrictive than KGC, so it is more robust and is recommended for this analysis. If only one axis is validated, then data interpretation will only be correct along this axis. The two first axes are always those with the highest Eigenvalues, i.e. those that will best represent the variance between samples. Calculation of eigenvalue percentages quantifies the proportion of the variance explained by each MFA axis (axis percentages), and the percentage sum for the two first axes gives the proportion of variance shown by the graphical representation of the MFA model (Figures 24 and 10).

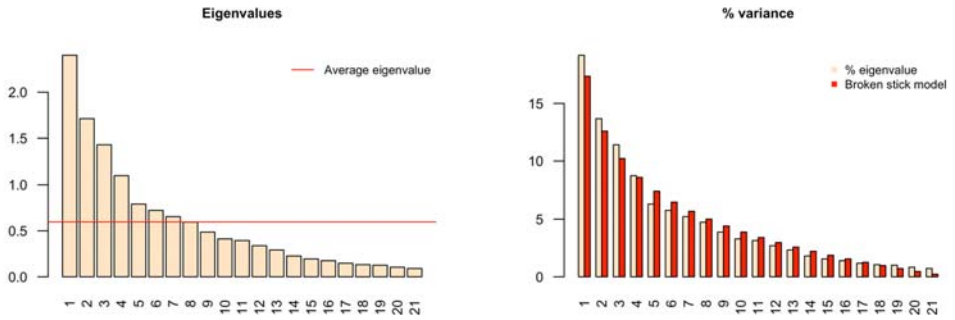


Figure 24 – Representation of Kaiser – Guttman criterion (left) and Broken-stick model (right) tests of MFA axes. Output file: 1_MFA_AxesValidity.png

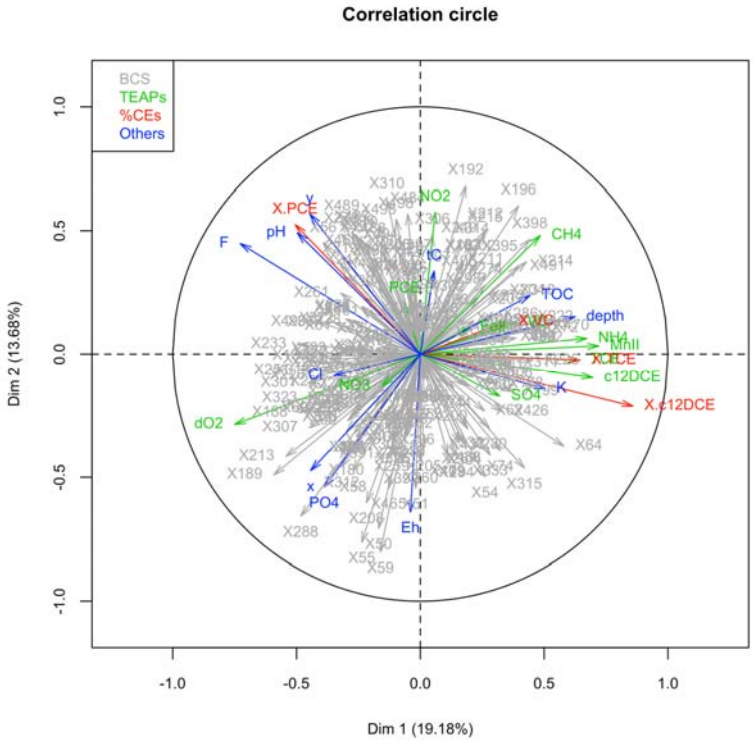


Figure 25 – Correlation circle, graphical output file: 2_MFA_CorrelationCircle.png

Figure 25 is the second graph that was generated by the MFA analysis and corresponds to file "2_MFA_CorrelationCircle.png". This MFA representation is difficult to read since the numerous grey vectors related to the T-RFs of the BCS group partially hide the other variables. This MFA graph shows that the bacterial population vectors (X50 to X500) point in all directions of the correlation circle, and some are close to some environmental vectors. The links between the BCS and environmental parameters will be discussed in 7.2.4.3.

Figure 26 is the 3rd graph that was generated by the MFA analysis and corresponds to the file "3_MFA_CorrelationCircle_0.05.png". It provides a suitable and legible representation of the results. This correlation circle shows only the variables that significantly ($p < 0.05$) contribute to one or both MFA axes. These variables are most likely to explain the variance between samples in the first two dimensions of the MFA, i.e. are the most important physicochemical and bacterial parameters that drove the aquifer redox conditions at the sampling time. This MFA representation focuses on the predominant variable links that are essential

to explore in order to evaluate the potential for natural CE dechlorination in a contaminated aquifer.

The correlation circle and the individual factor map (Figure 27), where samples are plotted instead of the variable vectors, can be read at the same time. Each sample has a point of coordinates in each group of variables, and the individual factor map plots the barycenter of a sample (Figure 27, black dots) based on its coordinates in each group of variables.

7.2.4.3. Interpretation of the statistical analysis output plots

The objective during MFA interpretation is to establish a model of the predominant redox conditions in the aquifer in relation to the CE degradation state and the support provided by the bacterial community structures. Interpretation is based on the bacterial and physicochemical characterizations, and during interpretation, the studied GWS is considered as a whole (Figure 2).

MFA interpretations were done using the guidelines provided in the procedure (section 5.4.). Altogether, the MFA dimensions explained 32.8% of the variance. The sample distribution along axis 1 of the MFA was mainly characterized by the variables: TCE and c12DCE concentrations; TCE and c12DCE percentages; and Mn(II), NH_4^+ and TOC concentrations. The sample distribution along axis 2 was mainly characterized by NO_2^- and Eh, and the BCS variables are mostly correlated with axis 2.

The MFA model indicates that there were different local geochemical conditions in the studied GWS. The Eh and dO_2 vectors (Figure 26) indicate that samples in the lower left quarter of the MFA (Figure 26) were characterized by higher redox potential and dissolved O_2 values than the other samples. The redox potential values of the aquifer ranged between -107.6 mV to 286.5 mV (Appendix 10A), where 9 out of 10 samples registered above 0 mV. 70% of the samples had a dissolved O_2 concentration higher than 0.5 mg/L. This means that, globally, the aquifer was under slightly reducing conditions, though locally, aerobic/aero-tolerant or anaerobic bacterial processes were possible.

The NO_2^- vector, along axis 2 (Figure 26) and in opposite part of the Eh vector, indicates that samples in the upper part of the MFA (Figure 27) were locally characterized by NO_3^- reducing conditions (NO_3^--> NO_2^-). NO_2^-concentrations reached as high as 3940 µg/L (Appendix 10).

The NH_4^+ vector, which belongs to axis 1 on the right (Figure 26), suggests that in other areas of the aquifer, where NH_4^+ concentrations were as high as 4000 µg/L (Appendix 10), dissimilatory nitrate reduction to ammonium (DNRA) could have taken place. DNRA was documented in anoxic marine sediments (Tiedje, 1998) and is possible in GWSs (Smith at al., 1991), but normally plays a minor role since NH_4^+ is stable only in reduced conditions. In the specific context of the studied site, NH_4^+ could also have come from organic mineralization in the upper part of the soil in the landfill area, as is indicated by the concomitant presence of the TOC vector. The NO_3^-concentration was high in many samples (> 5mg/L, Appendix 10), and only when this concentration was lower were the NH_4^+ or NO_2^- concentrations often high. This corroborates the interpretation that nitrate-reducing conditions were present.

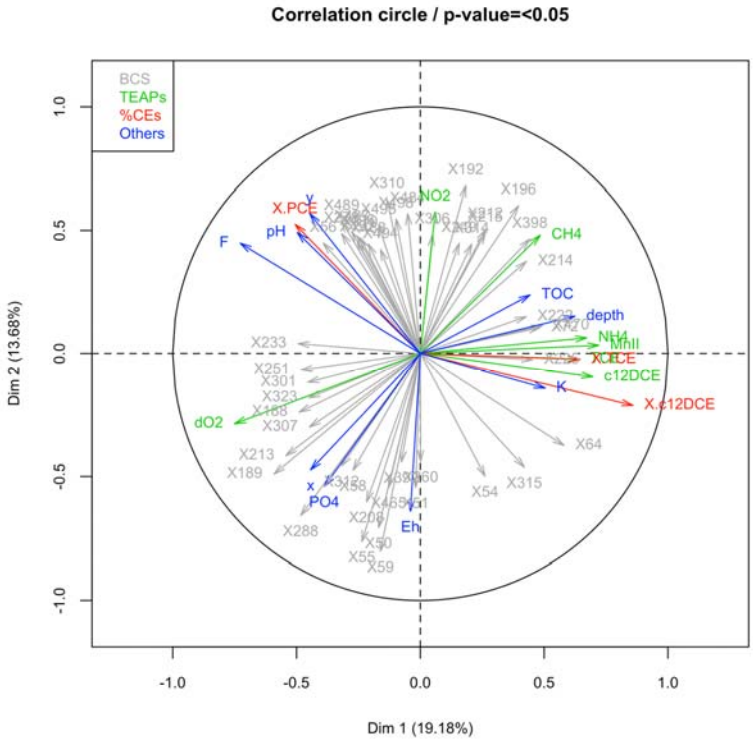


Figure 26 – Correlation circle, graphical output file: 3_MFA_CorrelationCircle_0.05.png.
Grey vectors correspond to the projection of the T-RF positions of the T-RFLP profiles. Eh, oxidation-reduction potential; dO2, dissolved oxygen; X.PCE / X.TCE / X.cDCE, percentage of perchloroethene / trichloroethene / cis-1,2-dichloroethene respectively among total CEs; NO2, nitrite; TOC, total organic carbon; NH4, ammonium; PO4, phosphate; x, position of well along north-south axis; F, Fluoride; pH, ; CH4, methane; MnII, manganese(II); depth, depth of the water sampling; c12DCE, cis-1,2-dichloroethene ; K, potassium ion.

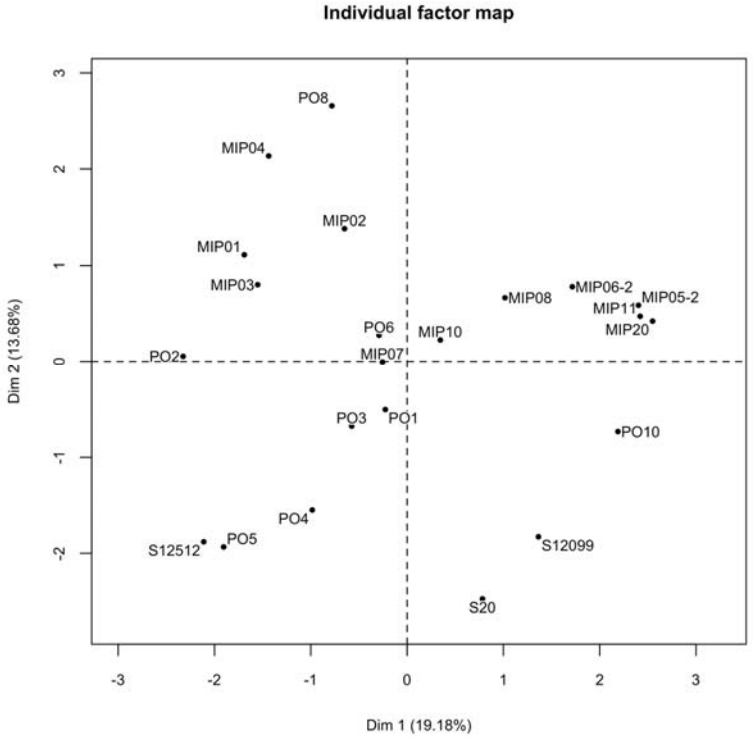


Figure 27 – Individual map factor, graphical output file: 4_MFA_IndFactMap.png.

The CH_4 vector (Figure 25) indicates that in some samples its presence is discriminant, however it is important to note that the CH_4 concentration was low at the site (< 50 µg/L, Appendix 10). Its origin could be microbial or the landfill itself. If microbial, CH_4 would have originated from methanogenic archaea, but only in localized microsites as the general redox potential (Eh min. -10 mV) was not reduced enough for methanogenesis.

Concomitant presence of the Mn(II), TCE, %TCE, c12DCE and %c12DCE vectors gives the most important information regarding the dechlorination activity in the GWS. The spatial distribution of these vectors provides the key for the interpretation of the main mechanisms that occurred in the aquifer. The simultaneous occurrence of the Mn(II), TCE and c12DCE vectors indicates that samples plotted in the right part of the MFA (Figure 26) were under manganese-reducing and reductive dehalogenation conditions. Figure 4 (section 3.4.1) shows that these two processes require the same amount of reducing equivalents to occur. At this site, manganese reduction was in competition with TCE and c12DCE degradation. Consequently, the kinetics of the reductive dechlorination process was locally disturbed by the reduction of manganese oxides, and the weak VC and undetected ethene concentrations (Appendix 10) at the site were a consequence of this reductive dechlorination inhibition. TCE accumulated locally, however the TCE concentration never exceeded the limit set by OSites. This indicates a slight negative difference between the supply and the degradation rates of the TCE pool. However, this is much more marked for the c12DCE pool, which was highly accumulated at the site (Figure 20).

7.2.4.4. Analysis of relationships between environmental variables and BCS

The relationships between the environmental variables and the BCSs were explored in this case study in order to highlight the processes predominantly sustained by the microbiology. For this, RV correlation coefficients (RV coeff.) were calculated from the MFA model. The RV correlation coefficient indicates the closeness of the two sets of values, and the associated p-value indicates the robustness of the correlation. Table 8 presents the RV correlation coefficient calculated between the BCS and the other groups of variables (TEAPs, %CEs and Others), and between the BCS and each environmental parameter individually. To understand the meaning of a correlation between the BCS and a parameter, it is necessary to keep in mind that the number of different types of bacteria and their relative abundance in a sample defines the structure of a bacterial community. This measure of the relationship between the BCS matrix and one environmental parameter is used to show schematically that when a chemical concentration changes from one sample to another, the global structure of the bacterial community also changes, and vice versa.

Table 8 shows that only the group of variables “Others” was significantly correlated with the BCS matrix. Among the variables of this group, the parameter Eh was the only one significantly linked to the BCS matrix. This result shows, as expected, that microbial communities clearly affected the global redox potential of the GWS and vice versa.

The groups TEAPs and %CEs were not globally correlated with BCS. In these groups, only the variables %TCE, NO_2^-, and CH_4 presented significant correlations with BCS. The robust RV correlation coefficient confirms that CH_4 concentrations, although low, were linked to biological activity. As the CH_4 concentration is low and the redox potential is high at the site, CH_4 should be consumed by methane-utilizing bacteria (methanotrophs, Hanson and Hanson, 1997) even though it is produced locally by methanogens in saturated microsites in the soil. The significant connection between the BCS and NO_2^- concentration indicates that the bacterial communities supported nitrate reduction in the GWS area of concern. Nitrate reduction engages specialized microorganisms as obligate respiratory bacteria that belong to

genera such as *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Paracoccus denitrificans*, *Pseudomonas* and *Thiobacillus*.

Table 8 – RV correlation coefficients and the associated p-values calculated between the bacterial community dataset and i) each defined group of environmental variables (TEAPs, %CEs, Others) and ii) each environmental variable separately.

Variables	RV coeff.	p-value	Significance
TEAPs	0.3756	0.2087	ns
%CEs	0.3190	0.0601	ns
Others	0.4830	0.0056	**
PCE	0.1245	0.8006	ns
TCE	0.1042	0.9141	ns
c12DCE	0.0970	0.9519	ns
VC	0.2021	0.0954	ns
NH_4^+	0.1271	0.7045	ns
NO_3^-	0.1435	0.5977	ns
NO_2^-	0.2587	0.0074	**
SO_4^{2-}	0.1306	0.6713	ns
Fe(II)	0.1447	0.5134	ns
Mn(II)	0.1449	0.5227	ns
CH_4	0.3046	0.0085	**
dO_2	0.2331	0.0598	ns
%PCE	0.1666	0.3198	ns
%TCE	0.2454	0.0325	*
%c12DCE	0.1784	0.2483	ns
%VC	0.2054	0.0506	ns
Eh	0.3051	0.0099	**
pH	0.2207	0.0713	ns
t°C	0.1802	0.2439	ns
TOC	0.1382	0.5748	ns
Chloride	0.1694	0.3200	ns

Degree of significance based on the calculated p-value: **, significant p-value < 0.01; *, moderately significant p-value < 0.05; ns, non-significant. RV coeff. : RV correlation coefficient. This table is generated from the output files: 5_MFA_Correlations_BCS _gp-var.csv.

Mn(II), TCE and c12DCE, which highlighted within the interpretation of the 'Correlation circle', were not significantly correlated with the BCS matrix. Different microorganisms can reduce Mn(IV) and Fe(III); for example, the genera *Bacillus*, *Geobacter*, and *Pseudomonas* harbor manganese-reducing bacteria (Lovley, 2006). However, these bacteria have a versatile metabolism and are not dedicated to the manganese reduction process. In fluctuating conditions, these bacteria are able to adapt their respiratory metabolism and use alternative terminal electron acceptors. These bacterial populations are non-discriminants for manganese reduction which translates into unspecific correlations with the manganese reduction process. The same observations can be made about the bacteria implicated in the first steps of the reductive dechlorination process (e.g. *Sulfurospirillum*, *Desulfitobacterium*, *Desulfuromonas*, Figure 8, versatile metabolisms). These statements should elucidate why the Mn(II) and CE vectors were not yet directly associated to a characteristic BCS, even if the oxidation-reduction potential was within the range where manganese(IV) reduction predominates (around 200 mV). Then, when the aquifer reaches a stable, specific redox condition over a long period of time, specialized and obligate e.g. manganese-reducing bacteria (Myers and Nealson, 1988) could emerge in the BCS.

7.2.5. Conclusions - Scenarios for incomplete *in situ* reductive dechlorination

The first step of the procedure, the analysis of the contaminant distribution and their degradation state, has shown that reductive dechlorination was present at the site, but that the process was incomplete. The second step, screening for the presence of OHRB biomarkers and their biochemical potential, has indicated that the GWS contains the genetic markers as well as the functional capacity to perform complete dechlorination. The third step, the analysis of the GWS biogeochemical state, has deepened the understanding of the aquifer functioning at the time of the inventory. The main identifiable explanation for incomplete dechlorination is that the local redox conditions at the site were not sufficiently reducing. The presence of non-negligible concentrations of electron acceptors other than CEs mobilized the reducing power (H_2) to the detriment of reductive dechlorination. Manganese reduction interfered locally by slowing the reductive dechlorination of TCE and c12DCE. Therefore, until manganese oxides are completely reduced, dechlorination of c12DCE will be inhibited or at least occur at a low rate.

Based on the results of the procedure, it is most improbable that complete natural attenuation of PCE will occur in a reasonable timeframe. It is probable that the system will naturally tend towards global accumulation of c12DCE and local accumulation of VC. The presence of specific biomarkers linked to the last stage of PCE dechlorination (*vcrA* and *bvc*A), and the evidence of ethene production obtained in microcosms, indicates that under favorable conditions complete removal could be achieved globally. CEs may be completely eliminated if the redox conditions are modified in the aquifer by adding a suitable substrate (electron donor). Hence, **enhanced natural attenuation** (ENA) is a conceivable remediation strategy.

REFERENCES

Abdi, H., and Williams, L.J. (2010) Principal component analysis, Wiley Interdisciplinary Reviews: Computational Statistics, Vol. 2(4): 433–459 - doi: 10.1002/wics.101

Abdi, H., Williams, L., and Valentin, D. (2013). Multiple factor analysis: principal component analysis for multitable and multiblock data sets.WIREs Comp. Stat. 5, 149–179.

ADEME (2007) Natural attenuation of chlorinated aliphatic compounds in aquifers - Methodological Guide - ADEME Collection "Know to Act" - R&D program MACAOH (Modeling, Attenuation, Characterization in Aquifers of OrganoHalogens), Côme J.M., Ropars M., Kaskassian, S., Quintard M., Vogel T., Razakarisoa O., Nex F., Schäfer G., Haeseler F., 230 pp. (*available in French*) http://www.ademe.fr

Aeppli, C., Hofstetter, T. B., Amaral, H. I. F., Kipfer, R., Schwarzenbach, R. P., Berg, M.(2010) Quantifying in situ transformation rates of chlorinated ethenes by combining compound-specific stable isotope analysis, groundwater dating, and carbon isotope mass balances. Environmental Science and Technology. 44, 3705– 3711

Behrens, S., Azizian, M.F., McMurdie, P.J., Sabalowsky, A., Dolan, M.E., Semprini, L., and Spormann, A.M. (2008) Monitoring abundance and expression of "Dehalococcoides" species chloroethene-reductive dehalogenases in a tetrachloroethene-dechlorinating flow column. Applied and Environmental Microbiology. 74 (18), 5695–5703.

Böhlke, J.K., Smith, R.L., Miller, D.N. (2006) Ammonium transport and reaction in contaminated groundwater: Application of isotope tracers and isotope fractionation studies. Water Resources Research. 42. http://dx.doi.org/10.1029/2005WR004349

Bond, D.R., Holmes, D.E., Tender, L.M., and Lovley, D.R. (2002) Electrode-reducing microorganisms that harvest energy from marine sediments. Science. 295 (5554), 483–485.

Borcard, D., Gillet, F., and Legendre, P. (2011) Numerical Ecology with R, Springer. ISBN 978-1-4419-7975-9 (Print) 978-1-4419-7976-6 (Online).

Bradley, P.M. (2003) History and Ecology of Chloroethene Biodegradation: A Review. Bioremediation Journal. 7(2): 81-109, doi:10.1080/ 713607980

Chapelle, F.H., McMahon, P.B., Dubrovsky, N.M., Fujii, R.F., Oaksford, E.T., and Vroblesky, D.A. (1995) Deducing the distribution of terminal electron-accepting processes in hydrologically diverse groundwater systems. Water Resources Research. 31: 359-371.

Chapelle, F., Widdowson, M.A., Brauner, J.S., and Mendez, E. (2003). Methodology for estimating times of remediation associated with monitored natural attenuation. U.S. Geological Survey Water-Resources Investigation Report 03-4057. 51 pages. http://pubs.usgs.gov/wri/wri034057/.

Chloronet (2009) Guide of Chlorinated hydrocarbons (HCC): Properties and behavior in the environment. FOEN. 50p. (*available in French and German*) http://www.bafu.admin.ch/chloronet/06273/index.html?lang=fr

Dowideit, K., Scholz-Muramatsu, H., Miethling-Graff, R., Vigelahn, L., Freygang, M., Dohrmann, A.B. and Tebbe, C.C. (2010) Spatial heterogeneity of dechlorinating bacteria and limiting factors for *in situ* trichloroethene dechlorination revealed by analyses of sediment cores from a polluted field site. FEMS microbiology ecology. 71, 444-59.

Duhamel M. and Edwards E.A. (2006) Microbial composition of chlorinated ethene-degrading cultures dominated by Dehalococcoides. FEMS Microbiology Ecology. 58(3): 538-549.

Ebersole R. and Hendrickson E. (2003) Nucleic acid fragments for the identification of dechloprinating bacteria. Patent WO/2003/064, 695.

EPA (2008) Sampling guidance for unknown contaminants in drinking water. EPA-817-R-08-003, 71pp., http://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=P1002CNX.txt

EPA (2000) Engineered Approaches to *In situ* Bioremediation of Chlorinated Solvents: Fundamentals and Field Applications. EPA 542-R-00-008, 144pp. http://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=10002UIJ.txt

EPA (1999) Microbial Processes Affecting Monitored Natural Attenuation of Contaminants in the Subsurface. EPA/540/S-99/001, 18pp. http://nepis.epa.gov/Exe/ZyPDF.cgi/10002E30.PDF?Dockey=10002E30.PDF

EPA (1998) Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water. EPA/600/R-98/128, 248 pp. http://nepis.epa.gov/Exe/ZyPDF.cgi/30003ONO.PDF?Dockey=30003ONO.PDF

Federal Council (2015) Environment Switzerland 2015, Report of the Federal Council, Bern, series: State of the environment. n° er2015, 144pp. . (*available in English, French, German and Italian*) http://www.bafu.admin.ch/publikationen/publikation/01794/index.html?lang=en

Federal Environment Agency (Umweltbundesamt) (2011) Consideration of natural attenuation in remediating contaminated sites, 42pp. http://www.umweltbundesamt.de/en/publikationen/consideration-of-natural-attenuation-in-remediating

FOEN (2013) Analytical methods in the field of waste and polluted sites. Series: Environment in practice. n° UV-1334-F, 80 pp. (*available in French and German*) http://www.bafu.admin.ch/publikationen/publikation/01752/index.html?lang=fr

FOEN (2000) Contaminated sites, endangering evaluation - Specifications for the technical investigation of contaminated sites. Series: Environment in practice. n° VU-3406-F, 25pp. (*available in French and German*) http://www.bafu.admin.ch/publikationen/publikation/00499/index.html?lang=fr

FOEN (2003) Sampling of groundwater in relation to contaminated sites. Series: Environment in practice. n° VU-3413-F, 28pp. (*available in French and German*) http://www.bafu.admin.ch/publikationen/publikation/00505/index.html?lang=fr

FOEN (2001a) Contaminated sites: register, evaluate, remediate. Series: Non-specialist publications. n° DIV-3400-E: 49pp. (*available in French, German*, *Italian and English)* http://www.bafu.admin.ch/publikationen/publikation/00495/index.html?lang=en

FOEN (2001b) Development of remediation projects of contaminated sites. Series: Environment in practice. n° VU-3410-F: 36pp. (*available in French, and German)* http://www.bafu.admin.ch/publikationen/publikation/00502/index.html?lang=fr

FOEN (2004) Practical guidelines for the protection of groundwater. Series: Environment in practice. n° VU-2508-F, 133p. (*available French, German and Italian*) http://www.bafu.admin.ch/publikationen/publikation/00378/index.html?lang=fr

FOEN (1994) Management concept of contaminated sites for Switzerland. Objectives and measures Series: Environmental notebook. *n°220, SRU-220-F* , 34pp. (*available in French)*

FOEN (1999) Directive for valorization, treatment and storage of excavation materials and spoil (Directive on excavation materials). VU-3003-F, 20 pp.

Futagami, T., Goto, M., and Furukawa, K. (2008) Biochemical and genetic bases of dehalorespiration. Chemical Record. 8, 1-12.

Futagami, T., Goto, M., and Furukawa, K. (2014) Chapter 4, Genetic System of Organohalide-Respiring Bacteria. In H. Nojiri *et al*. (eds.), Biodegradative Bacteria: How Bacteria Degrade, Survive, Adapt, and Evolve. doi:10.1007/978-4-431-54520-0_4

Garcia-Cantizano, J., Casamayor, E.O., Gasol, J.M., Guerrero, R., Pedros-Alio, C. (2005) Partitioning of CO_2 incorporation among planktonic microbial guilds and estimation of in situ specific growth rates. Microbial Ecology. 50: 230–241. doi: 10.1007/s00248-004-0144-9

Grandel, S., and Dahmke, A. (2008) Leitfaden – Natürliche Schadstoffminderung bei LCKW-kontaminier- ten Standorten. KORA Themenverbund 3: Chemische Industrie, Metallverarbeitung. Institut für Geo- wissenschaften, CAU Kiel, ISBN 978-3-00-026094-0.

Goldscheider, N., Hunkeler, D., Rossi, P. (2006) Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods. Hydrogeology Journal. 14(6), 926-941.

Hiraishi, A. (2008) Biodiversity of dehalorespiring bacteria with special emphasis on polychlorinated biphenyl/dioxin dechlorinators. Microbes and Environments. 23: 1-12.

Hendrickson, E.R., Payne, J.A., Young, R.M., Starr, M.G., Perry, M.P., Fahnestock, S., Ellis, D.E., and Ebersole, R.C. (2002) Molecular analysis of Dehalococcoides 16S ribosomal DNA from chloroethene-contaminated sites throughout north America and Europe. Applied and Environmental Microbiology. 68(2), 485-495.

Holliger, C., Schraa, G., Stams, A.J.M., and Zehnder, A.J.B. (1992) Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Applied and Environmental Microbiology. 58, 1636-1644.

Holliger, C., Schraa, G., Stams, A.J.M., and Zehnder, A.J.B. (1993) A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Applied and Environmental Microbiology. 59, 2991-2997.

Humphreys, W.F. (2006). Aquifers: the ultimate groundwater-dependent ecosystems. Australian Journal of Botany. 54, 115-132.

Kampbell, D.H., Wilson, J.T., Vandegrift, S.A. (1989) Dissolved oxygen and methane in water by a GC heaspace equilibrium technique. International Journal of Environmental Analytical Chemistry. 36, 249-257.

Lane D.J. (1991) 16S/23S rRNA sequencing In: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt, E. and Goodfellow, M., Eds.), pp. 115–175. Wiley, Chichester, UK.

Lanthier, M., Villemur, R., Lepine, F., Bisaillon, J.G. and Beaudet, R. (2001) Geographic distribution of *Desulfitobacterium frappieri* PCP-1 and *Desulfitobacterium* spp. in soils from the province of Quebec, Canada. FEMS Microbiology Ecology. 36 (2-3), 185–191.

Lindenbaum J. (2012) Identification of sources of ammonium in groundwater using stable nitrogen and boron isotopes in Nam Du, Hanoi. Dissertations in Geology at Lund University, No. 300, 38 pp.

Lorah, M., and Voytek, M. (2004) Degradation of 1,1,2,2-tetrachloroethane and accumulation of vinyl chloride in wetland sediment microcosms and *in situ* porewater: biogeochemical controls and associations with microbial communities. Journal of Contaminant Hydrology. 70, 117-145.

Lovley, D. (2006) Dissimilatory Fe(III)- and Mn(IV)-Reducing Prokaryotes. The Prokaryotes, CHAPTER 1.21, 2:635–658, doi:10.1007/0-387-30742-7_21

Major, D., McMaster, M., Cox, E., Edwards, E., Dworatzek, S., Hendrickson, E., Starr, M., Payne, J. and Buonamici, L. (2002) Field Demonstration of Successful Bioaugmentation to Achieve Dechlorination of Tetrachloroethene to Ethene. Environmental Science Technology. 36, 5106-5116.

Maphosa, F., de Vos, W., and Smidt, H. (2010) Exploiting the ecogenomics toolbox for environmental diagnostics of organohalide-respiring bacteria. Trends in biotechnology. 28, 308–16.

McMahon, P., and Chapelle, F. (2008) Redox processes and water quality of selected principal aquifer systems. Ground water. 46, 259–71.

Middeldorp, P., Luijten, M., Pas, B., Eekert, M., Kengen, S., Schraa, G. and Stams, A. (1999) Anaerobic Microbial Reductive Dehalogenation of Chlorinated Ethenes. Bioremediation Journal. 3, 151-169.

Myers, CR., and Nealson, KH. (1988) Bacterial Manganese Reduction and Growth with Manganese Oxide as the Sole Electron Acceptor. Science, Vol. 240: 1319-1321. doi:10.1126/science.240.4857.1319

Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L. and Grabowski, G. (2002). The Simple Fool's Guide to PCR, Version 2.0. Privately published, Univ. Hawaii. http://palumbi.stanford.edu/SimpleFoolsMaster.pdf

Parsons Corporation. (2004) Principles and Practices of Enhanced Anaerobic Bioremediation of Chlorinated Solvents. For AFCEE, NFESC, ESTCP, USACE. 022/738863/28.doc. http://costperformance.org/remediation/pdf/principles_and_practices_bioremediation.pdf

Rees, G., Baldwi,n D., Watson, G., Perryman, S. and Nielsen, D. (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. Antonie Van Leeuwenhoek, 86(4): 339-347. doi: 10.1007/s10482-004-0498-x.

Rossi, P., Gillet, F., Rohrbach, E., Diaby, N., Holliger, C. (2009) Statistical assessment of the variability of the T-RFLP analysis applied to complex microbial communities. Applied and Environmental Microbiology. 75, 7268–7270. doi:10.1128/AEM.00135-09 http://aem.asm.org/content/early/2009/09/11/AEM.00135-09.full.pdf+html

Rousk, J., and Bengtson, P. (2014) Microbial regulation of global biogeochemical cycles. Frontiers in Microbiology, 5. http://dx.doi.org/10.3389/fmicb.2014.00103

Shani, N. (2012) Assessing the Bacterial Ecology of Organohalide Respiration for the Design of Bioremediation Strategies, EPFL thesis n° 5379, doi:10.5075/epfl-thesis-5379

Shani, N., Rossi P. & Holliger C. (2013) Correlations between environmental variables and bacterial community structures suggest Fe (III) and vinyl chloride reduction as antagonistic terminal electron-accepting processes. Environmental science & technology 47, 6836–6845. doi:10.1021/es304017s

Smits, T.H., Devenoges, C., Szynalski, K., Maillard, J. and Holliger, C. (2004) Development of a real-time PCR method for quantification of the three genera Dehalobacter, Dehalococcoides, and Desulfitobacterium in microbial communities. Journal of Microbiological Methods. 57(3), 369–378.

Smith, R.L., Howes, B.L., Duff, J.H. (1991) Denitrification in nitrate-contaminated groundwater: occurrence in steep vertical geochemical gradients. Geochimica et Cosmochimica Acta. 55, 1815–1825.

Tiedje, J.M. (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. p. 179-244. In A.J.B. Zehnder (ed), Environmental Microbiology of Anaerobes. John Wiley and Sons, N.Y.

Tiehm, A., and Schmidt, K.R. (2011). Sequential anaerobic/aerobic biodegradation of chloroethenes-aspects of field application. Current Opinion in Biotechnology. 22, 415–421. doi:10.1016/j.copbio.2011.02.003

Tobiszewski, M., and Namieśnik, J. (2012) Abiotic degradation of chlorinated ethanes and ethenes in water. Environmental Science and Pollution Research. 19, 1994–2006.

Treseder, K., Balser, T., Bradford, M., Brodie, E., Dubinsky, E., Eviner, V., Hofmockel, K., Lennon, J., Levine, U., MacGrego,r B., Pett-Ridge, J., Waldrop, M. (2011) Integrating microbial ecology into ecosystem models: challenges and priorities. Biogeochemistry. 109, 7–18.

Turner, S., Pryer, K.M., Miao, V.P.W., and Palmer, J.D. (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. Journal of Eukaryotic Microbiology. 46, 327–338.

Weissbrodt, D.G., Shani, N., Sinclair, L., Lefebvre, G., Rossi, P., Maillard, J., Rougemont, J., Holliger, C. (2012) PyroTRF-ID: a novel bioinformatics methodology for the affiliation of terminal-restriction fragments using 16S rRNA gene pyrosequencing data. BMC Microbiology. 12, 306. doi:10.1186/1471-2180-12-306

APPENDIX 1

GROUNDWATER QUALITY

Indicative values for groundwater quality presented in appendix A1 of "Practical guidelines for the protection of groundwater " (FOEN, 2004) and modified from appendix 2 of the "Federal Ordinance on the Remediation of Polluted Sites, OSites 814.201".

Paramètres physiques

Température	écart d'au moins 3 °C par rapport à l'état naturel
Turbidité	<1 UT/F
pH	écart d'au moins 0,5 par rapport à l'état naturel

Constituants principaux et secondaires

Calcium (Ca^{2+})	40 mg/l de plus qu'à l'état naturel
Magnésium (Mg^{2+})	10 mg/l de plus qu'à l'état naturel
Sulfates (SO_4^{2+})	<40 mg/l (1)
Silice dissoute (H_4SiO_4)	10 mg/l de plus qu'à l'état naturel
Baryum (Ba^{2+})	0,5 mg/l de plus qu'à l'état naturel
Sodium (Na^+)	25 mg/l de plus qu'à l'état naturel
Potassium (K^+)	5 mg/l de plus qu'à l'état naturel
Chlorures (Cl^-)	<40 mg/l (1)
Fluorures (F^-)	0,5 mg/l de plus qu'à l'état naturel
Bromures (Br^-)	0,05 mg/l de plus qu'à l'état naturel
Nitrates (NO_3^-)	<25 mg/l (1)
Ammonium (NH_4^+)	<0,1 mg/l (1) dans des conditions oxydantes <0,5 mg/l (1) dans des conditions anoxiques
Nitrites (NO_2^-)	0,05 mg/l de plus qu'à l'état naturel
Phosphates (o-PO_4^{3-})	0,05 mg/l de P de plus qu'à l'état naturel
Cyanures (CN^-)	<0,025 mg/l
Saturation en oxygène (O_2)	>20%
Fer (Fe) dissous	0,3 mg/l de plus qu'à l'état naturel
Manganèse (Mn) dissous	0,05 mg/l de plus qu'à l'état naturel
Carbone organique dissous (DOC)	<2 mg/l (1)

[1] Valeurs figurant en annexe 2, ch. 22, Œaux.

Eléments en traces

Bore (B)	<0,05 mg/l de plus qu'à l'état naturel
Zinc (Zn) dissous	<5 µg/l
Cuivre (Cu) dissous	<2 µg/l
Nickel (Ni) dissous	<5 µg/l
Plomb (Pb) dissous	<1 µg/l
Cadmium (Cd) dissous	<0,05 µg/l
Mercure (Hg) dissous	<0,01 µg/l
Arsenic (As)	<5 µg/l
Sélénium (Se)	<5 µg/l
Chrome (Cr)	<2 µg/l

Substances organiques naturelles et de synthèse

Composés organiques halogénés adsorbables (AOX)	<10 µg/l (1)
AOX, y compris HHV	<10 µg/l
Phénols volatils (entraînables à la vapeur)	<5 µg/l
Hydrocarbures aromatiques monocycliques (BTEX)	<1µg/l pour chaque substance (1)
Hydrocarbures aliphatiques	<1 µg/l pour chaque substance (1)
Hydrocarbures halogénés volatils (HHV)	<1 µg/l pour chaque substance (1)
Chlorure de vinyle	<0,1 µg/l
Pesticides	<0,1 µg/l pour chaque substance (1), somme <0,5 µg/l
Composés nitro-aromatiques totaux	<0,5 µg/l
EDTA et complexants de structure analogue	<5 µg/l pour chaque substance
NTA	<3 µg/l
Hydrocarbures aromatiques polycycliques (HAP)	<0,1 µg/l pour chaque substance (1)
Benzo(a)pyrène	<0,01 µg/l
Méthyl-tert-butyléther (MTBE)	<2 µg/l
Chlorophénols	absence d'odeur
Pentachlorophénol	<0,1 µg/l
Chlorobenzène	<1 µg/l pour chaque substance
Di(2-éthylhexyl)-phtalate (DEHP)	<1 µg/l
Amines aromatiques	<0,1 µg/l pour chaque substance, somme <0,5 µg/l
Triazoles benzéniques	<1 µg/l pour chaque substance
Acides halogénés	<0,5 µg/l pour chaque substance

[1] Valeurs figurant en annexe 2, ch. 22, Œaux.

APPENDIX 2

CHLORINATED ETHENES ANALYSES

Extract from FOEN (2013) Analytical methods in the field of waste and polluted sites. Series: Environment in practice. n° UV-1334-F, 80 pp

S-8 Hydrocarbures halogénés des échantillons solides

Paramètres	1,2-dibrométhane, 1,1-dichloréthane, 1,2-dichloréthane, 1,1-dichloréthène, *cis-1,2-dichloréthène*, trans-1,2-dichloréthène, *dichlorométhane* (*chlorure de méthylène*), 1,2-dichloropropane, 1,1,2,2-tétrachloréthane, *tétrachloréthène* (*PER*), *tétrachlorométhane* (*tétrachlorure de carbone*), *1,1,1-trichloréthane*, *trichloréthène* (*TRI*), *trichlorométhane* (*chloroforme*), chlorure de vinyle, chlorobenzène, 1,2-dichlorobenzène, 1,3-dichlorobenzène, 1,4-dichlorobenzène, 1,2,4-trichlorobenzène
Conservation et entreposage	Prélever les échantillons autant que possible sans échauffement et les transporter au laboratoire dans des bouteilles étanches refroidies (récipients en verre à couvercle étanche). L'échantillon peut aussi être recouvert sur site d'une couche de méthanol. Vérifier la qualité du méthanol utilisé en mesurant sa teneur en HCC. Il est nécessaire de consigner le poids exact de l'échantillon et la quantité de solvant utilisée. Un échantillon distinct sera prélevé pour déterminer la matière sèche.
Préparation des échantillons	Ne pas présécher les échantillons. Si nécessaire, prébroyer les échantillons humides et refroidis et les peser directement dans les bouteilles d'extraction.
Traitement des échantillons	Peser au moins 20 g d'une prise aliquote dans des bouteilles d'extraction à verrouillage étanche. Ajouter du méthanol (20–50 ml) et agiter intensivement pendant au moins 1 h ou extraire par ultrasons. Ensuite laisser décanter pendant la nuit et agiter encore une fois brièvement avant de prélever la prise de méthanol.
Méthode de mesure	Diluer une prise aliquote de l'extrait de méthanol avec de l'eau. Détermination avec chromatographie gazeuse avec Headspace statique ou dynamique, ou microextraction sur phase solide (SPME), avec détection ECD/FID ou MS.
Expression du résultat	en µg/kg d'échantillon sec (105° C), pour chaque substance (Ms déterminée sur un échantillon distinct), ou somme des 7 substances selon la directive sur les matériaux d'excavation.
Seuil de quantification	0,01 mg/kg Ms, pour chaque substance
Bibliographie	[5]: EPA 5021, 8260
Autres méthodes	SPME Headspace pour autant que l'on soit assuré que toutes les substances ci-dessus soient détectées avec une sensibilité suffisante. Purge and Trap pour une meilleure sensibilité et une palette plus large de substances.

Italiques: hydrocarbures chlorés volatils selon la directive sur les matériaux d'excavation[2]

E-8 Hydrocarbures halogénés des échantillons aqueux

Paramètres	1,2-dibrométhane, 1,1-dichloréthane, 1,2-dichloréthane, 1,1-dichloréthène, cis-1,2-dichloréthène, trans-1,2-dichloréthène, dichlorométhane (chlorure de méthylène), 1,2-dichloropropane, 1,1,2,2-tétrachloréthane, tétrachloréthène (PER), tétrachlorométhane (tétrachlorure de carbone), 1,1,1-trichloréthane, trichloréthène (TRI), trichlorométhane (chloroforme), chlorure de vinyle, chlorobenzène, 1,2-dichlorobenzène, 1,3-dichlorobenzène, 1,4-dichlorobenzène, 1,2,4-trichlorobenzène
Conservation et entreposage	Prélever les échantillons directement dans les flacons des passeurs d'échantillons – pas d'espace gazeux – de l'instrument de mesure correspondant. Acidifier avec HCl ou $NaHSO_4$. Transport et entreposage des échantillons au froid.
Préparation des échantillons	Pas de préparation spéciale. En règle générale, détermination directement à partir du flacon contenant l'échantillon.
Traitement des échantillons	Enrichissement dans le Headspace ou via Purge and Trap. Fait normalement partie de la méthode de mesure.
Méthode de mesure	Headspace statique ou Purge and Trap, respectivement microextraction sur phase solide (SPME), avec détection FID-ECD ou MS.
Expression du résultat	en µg/l pour chaque substance
Seuil de quantification	0,0001 mg/l (0,1 µg/l) (Purge and Trap), 0,001 mg/l (1 µg/l) (Headspace)
Bibliographie	[4]: EPA 524.4
Autres méthodes	SPME Headspace GC-MS pour autant que l'on soit assuré que toute la palette de substances ci-dessus puisse être détectée avec une sensibilité suffisante.

APPENDIX 3

DNA EXTRACTION PROTOCOL

DNA isolation from filters using Maxwell 16 Instrument (Promega)

Materials: Maxwell 16 Tissue DNA purification Kit (Promega, Cat.#. AS1030)
Maxwell 16 Instrument (Promega, Cat.# AS2000) configured with the Maxwell 16 SEV Hardware Kit (Promega, Cat.# AS1200)

Materials and additional solutions to be supplied by the user:

- **n-butanol p.a**. CAS n° CAS n° 71-36-3
- **SDS 10%** (w/v) Dodecyl Sodium Sulfate (SDS) - CAS n°151-21-3
  Prepared solutions using ultrapure water
  (e.g. from Direct-Q® 8 Ultrapure Water System, Merck Millipore)
  Do not sterilized
  Stock at room temperature to avoid crystallization
- **Phosphate Buffer 0.12M, pH8** Mix 94.7% of Na_2HPO_4 0.12M - CAS n° 7558-79-4
  with 5.3% NaH_2PO_4 0.12M - CAS n° 7558-80-7
  Prepared solutions using ultrapure water
  Sterilized the solutions by autoclaving 15' at 120°C
  Stock at 4°C (max. 6 months)
- **Proteinase K solution** Specific activity 2.5U/mg (Macherey-Nagel, ref. 740506)
  Adjust concentration regarding specific activity if using another provider
- **1.5mL sterilized storage microtubes** for final purified DNA samples
  (e.g. Eppendorf Safe-Lock Tubes™, ref. 0030 123.328)
- **50mL "Falcon" tubes** ensured for n-butanol and centrifugation resistance, this last is also link with the centrifuge model (e.g. Starstedt tubes, ref. 62.547.254)
- **Automated pipettes** (e.g. Socorex, Acura® manual 825 adjustable volume micropipettes,)
- **Sterilized cottoned tips** (e.g. Promega, Promega Barrier Tips)
- **Sterilized tweezers**

Protocol:

1. Place 2 filters from a same sample in a 50 mL "Falcon" tube *(under a laminar flow hood)*
2. Add 10mL of Phosphate Buffer and 1mL of SDS 10% *(under a laminar flow hood)*
3. Mix and incubate at 70°C for 30min.
4. Add 50µl of proteinase K solution *(under a laminar flow hood)*
5. Mix and incubate at 65°C for 30min.
6. Remove the filter from the falcon tube *(under a laminar flow hood)*
7. Mix the remain 11mL of sample solution with 40mL of n-butanol and vortex *(under a fume hood)*
8. Centrifuge at 5000rpm, 5min. at RT
9. Remove upper n-butanol phase, there still remain n-butanol *(under a fume hood)*

The next steps take place with the Maxwell 16 Instrument by using the Maxwell 16 Tissue DNA purification Kit (refer for details to the Promega protocol for users)

10. Place Cartridges into the cartridge preparation rack, one for each sample and max. 16 per purification run.
11. Transfer 400µl of the remaining aqueous phase obtained in step 9 into the 1st well of the cartridge
12. Add 250 µL of elution buffer (provide in the Promega kit) into the blue Elution Tube (provide in the kit), one for each prepared Cartridges.
13. Unsure that the Maxwell16 robot is in SEV RSRC mode, and program the Maxwell 16 instrument by selecting RUN, DNA, Tissue (see also point 7.B of Promega protocol for usage details)
14. Transfer Cartridges containing samples and plungers from the preparation rack onto the Maxwell 16 platform
15. Place a blue Elution Tube for each cartridge into the Elution Tube Slots at the front of the platform
16. Press RUN/STOP, the platform will retract and the instrument will begin the purification run (running time ~45 min)

APPENDIX 4

PCR PROTOCOL

- All PCR reagents are provided by PeqLab Biotechnologie GmbH (Germany) provide all PCR reagents for this protocol, except if mentioned differently in the text.
- Amplification reactions are carried out in a Thermal Cycler T3 Biometra (Biolabo Scientific Instruments, Switzerland) or peqSTAR 96 Universal gradient (Peqlab Biotechnologie GmbH, Germany) .

The 16S rRNA genes of OHRB are amplified in a nested PCR procedure. In the first step, 16S rRNA gene pools are amplified in 30 µl reaction mixtures (Table A) using Eub28f (5'-GAGTTTGATCNTGGCTCAG-3') / Univ1492r (5'-GGYTACCTTGTTACGACTT-3') primers. PCR products of the first PCR are diluted prior to be used for the second amplification step. In the second amplification step, taxon-specific primers (Table 2, main document) were used to amplify the 16S rRNA gene of known OHRB in 10 µl reaction mixtures (Table B).

Table A - 1st PCR reaction

PCR components	Initial concentration	Final concentration	for 30 µl reaction final volume
Ultrapure type1 sterilized water	-	qsp. final reaction volume	14.10 µl
Buffer S with $MgCl_2$ include	10X 15mM	1X 1.5mM	3.00 µl
dNTPs	10mM each	800µM	0.60 µl
Enhancer P	5X	1X	6.00 µl
Eub28f	10µM	0.5µM	1.50 µl
Univ1492r	10µM	0.5µM	1.50 µl
Taq polymerase peqGOLD	5U/µL	add 1/100 of the final reaction volume	0.30 µl
DNA extract	2ng/µL	add 1/10 of the final reaction volume	3.00 µl

dNTPs : is a generic term referring to the four deoxyribonucleotide triphosphates: dATP, dCTP, dGTP and dTTP. These are the building blocks of DNA.

PCR cycle program for the first PCR reaction

1. 94°C for 5' — initial denaturation
2. 30 cycles of
   - 94°C for 30'' — *denaturation*
   - 50°C for 45'' — *annealing*
   - 72°C for 1' — *elongation*
3. 72°C for 10' — final elongation
4. 4°C forever — *storage*

Table B - 2nd PCR reaction

PCR components	Initial concentration	Final concentration	for 10 µl reaction final volume
Ultrapure type1 sterilized water	-	qsp. final reaction volume	4.10 µl
Buffer Y with $MgCl_2$ include	10X 20mM	1X 2.0mM	1.00 µl
dNTPs	10mM each	3.2mM	0.80 µl
Enhancer P	5X	1X	2.00 µl
Primer 1	10µM	0.5µM	0.50 µl
Primer 2	10µM	0.5µM	0.50 µl
Taq polymerase peqGOLD	5U/µL	add 1/100 of the final reaction volume	0.10 µl
DNA extract	2ng/µL	add 1/10 of the final reaction volume	1.00 µl

PCR cycle program for the second PCR reaction

1. 94°C for 5' initial denaturation
2. 30 cycles of 94°C for 30'' *denaturation*
   55°C for 45'' *annealing*
   72°C for 45'' *elongation*
3. 72°C for 10' final elongation
4. 4°C forever *storage*

The VC reductase genes vcrA and bvcA (GenBank YP003330719 and AY563562) are amplified in 10 µl reaction mixtures (Table C).

Table C – Reaction mixture for PCR amplification of vcrA and bvcA genes

PCR components	Initial concentration	Final concentration	for 10 µl reaction final volume
Ultrapure type1 sterilized water	-	qsp. final reaction volume	3.60 µl
Buffer S with $MgCl_2$ include	10X 20mM	1X 1.5mM	1.00 µl
dNTPs	10mM each	1.2mM	0.30 µl
Enhancer P	5X	1X	2.00 µl
Primer 1	10µM	1µM	1.00 µl
Primer 2	10µM	1µM	1.00 µl
Taq polymerase peqGOLD	5U/µL	add 1/100 of the final reaction volume	0.10 µl
DNA extract	2ng/µL	add 1/10 of the final reaction volume	1.00 µl

PCR cycle program for vcrA and bvA direct amplification

1. 94°C for 4'30'' initial denaturation
2. 40 cycles of 94°C for 30'' *denaturation*
   55°C for 45'' *annealing*
   72°C for 30'' *elongation*
3. 72°C for 10' final elongation
4. 4°C forever *storage*

After amplification reaction, the PCR products were checked for size in comparison to 100bp ladder (Promega) on 1.5% agarose gels stained with GelRed (Stratec Molecular, Germany) after electrophoresis (5Volt/cm during 60') in TEA 1X and U.V. photographed using a transil-luminator device. The expected sizes for the different resulting PCR products are listed in **Table D**.

PCR product for	Expected size
Bacteria	1480 pb
Dehalococcoides sp.	627 pb
Dehalobacter sp.	227 pb
Sulfurospirillum sp.	634 pb
Desulfuromonas sp.	556 pb
Desulfitobacterium sp.	213 pb
Geobacter lovleyi	412 pb
vcrA	139 pb
bvcA	247 pb

Presence of a PCR product at the right size on the gel indicates presence of the corresponding organism in the sample, and can be record as a positive result. Inversely, absence of a PCR signal indicates that the targeted organism is absence/lower than the detection limit of the PCR methodology, and can be record as a negative result. All presence/absence PCR results are finally grouped in a table for further analysis.

APPENDIX 5

ENRICHMENT CULTURE

Medium for anaerobic enrichment cultures of aquifer water samples

Enrichment of reductive dechlorinating bacteria/OHRB as other anaerobic microorganisms, need oxygen-free conditions during cultivation. The main things that you have to keep in mind are:

- Make all the solutions in anaerobic water
- Work in a "sterile" space e.g. around a Bunsen burner
- Put overpressure in the head space of your cultures, flushing with N_2 and/or CO_2

Materials

- N_2, CO_2, H_2 gas station
- Hand crimper (Sigma-Aldrich, product # 22316-U)
- 100mL Serum Bottles (Sigma-Aldrich, product #33110-U)
- 20mm Aluminum crimp seals (Sigma-Aldrich, product #27230-U)
- Septum stopper in material compatible with chlorinated solvents (e.g. in Butyl, Bellco, #2048-11800, in Viton)
- Syringes (Terumo, 2-part syringes 20 mL #SS+T20ES1, 10 mL #SS+T10ES1, 5mL # SS+T05ES1, 2 mL #SS+T02S1)
- Needles (Terumo, brown #NN-2623R, blue #NN-2325R, Orange #NN-2516R)
- Sterile 0.22 µm pore size filters preconditioned in filtration system for syringe (Sarstedt, Filtropur S 0.22 µm, #83.1826.001)

Anaerobic medium preparation

In a 100 mL serum bottles pressurize closed with N_2 gas to 0.5 bar overpressure (15 cycles of gas exchanges i.e. vacuum/gas injection) and autoclaved (20' at 121°C), sterilely add:

45 mL	**Solution A** (inorganic phosphates + redox indicator + organic nitrogen and carbon)
1.25 mL	**Solution B** (vitamins + trace elements)
2.50 mL	**Solution C** (carbonates + reducing agent solution)
1.25 mL	**Solution D** (Ca and Mg)

Exchange gas of the bottle using around 15 cycles of vacuum / 80%N_2:20%CO_2 gas injection

Add 5 mL	**PCE** (100 mM) prepared in Hexadecane (electron acceptor)
Add 1 mL	**MixII** (substrate, electron donors)
Add 5 mL	aquifer water as **inoculum**

- Inoculum must be inject in culture medium without contact with PCE in Hexadecane
- 1 mL of MixII should be add every week or two weeks

Solution A

Solution A contains in final concentration:		**Example for 1 liter of Solution A:**	
4.2 mM	$K_2HPO_4 * 3H_2O$	0.958g	$K_2HPO_4*3H_2O$
1.4 mM	$NaH_2PO_4 * 2H_2O$	0.218g	$NaH_2PO_4*2H_2O$
0.5 $mg.L^{-1}$	Resazurin (from a Resazurin at 0.5 $g.L^{-1}$)	0.1g	Peptone

0.1 $g.L^{-1}$	Peptone	1 mL	Resazurin solution (0.5 $g.L^{-1}$)
			Adjust volume to 1 liter with Mili-Q water

- Prepare the Solution A in a 2L Herlen-Meyer
- Boil during 20'-30' the solution to degas, then cool to room temperature with bubbling of 80%N_2:20%CO_2 gas in the solution
- Distribute 45mL of **Solution A** in the 100 mL serum bottles
- Seal the bottles with stoppers (Butyl) and crimp seals
- Change atmosphere in the bottle with 15 cycles of gas exchange with 80%N_2:20%CO_2 and finish with 0.5 bar overpressure in the bottles.
- Autoclave 15 min at 121°C
- Conservation 9 ±1 months at room temperature, in the dark. If the initial blue-purple color turns to pink the solution is passed and should be discarded
- The 0.5 g.L-1 Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye solution (oxygen indicator), should be stock at 4°C, exp. 2 years ±2 months

Solution B

In a serum bottle containing 20mL **anaerobe Milli-Q water**, add through a 0.2µm sterile filter:
1 ml of Trace element solution - **Solution IV**
1 ml of Vitamin solution – **Solution V**
1 ml of Vitamin B1 solution – **Solution VI**
1 ml of Vitamin B2 solution – **Solution VII**
1 ml of Vitamin B12 solution – **Solution VIII**

Anaerobe Milli-Q water:

- Boil during 20'-30' Milli-Q water and then cool to room temperature with bubbling of N_2
- Dispense 20 mL of the water in 100mL serum bottles
- Seal the bottles with the stoppers (Butyl or Viton) and crimp seals
- Change atmosphere in the bottle with 15 cycles of gas exchange with N_2 and finish with 0.5 bar overpressure in the bottles
- Autoclave 20' at 121°C
- Keep anaerobe Milli-Q water at RT (exp. 1 year ± 1month)

Trace element solution - **Solution IV**

Solution IV contains in final concentration:		**Example for 1 liter of Solution IV:**	
1.711 mM	EDTA	500 mg/l	EDTA
10.06 mM	$FeCl_2*4H_2O$	2000 mg/l	$FeCl_2*4H_2O$
505.3 µM	$MnCl_2*4H_2O$	100 mg/l	$MnCl_2*4H_2O$
798.5 µM	$CoCl_2*6H_2O$	190 mg/l	$CoCl_2*6H_2O$
513.5 µM	$ZnCl_2$	70 mg/l	$ZnCl_2$
15 µM	$CuCl_2*2H_2O$	2.6 mg/l	$CuCl_2*2H_2O$
41 µM	$AlCl_3$	5.5 mg/l	$AlCl_3$
97 µM	H_3BO_3	6 mg/l	H_3BO_3
171 µM	$Na_2MoO_4*2H_2O$	41.4 mg/l	$Na_2MoO_4*2H_2O$
101 µM	$NiCl_2*6H_2O$	24 mg/l	$NiCl_2*6H_2O$
		Add 1 liter	Mili-Q water

- In a 1L Becher dissolve EDTA in a volume of Milli-Q water equivalent to 3/4 of the final volume.
- Dissolve Iron(II) Chloride HCl, then add to the EDTA solution.
- Calibrate a pH meter between 4 and 7, and adjust EDTA solution to pH 7 with concentrated HCl.
- Add trace elements, by dissolving them one by one
- Make up to the desire final volume with Milli-Q water

Vitamin solution – **Solution V**

Solution V contains in final concentration:		**Example for 1 liter of Solution V:**
0.20 µM	Biotin (vitamin H)(p-aminobenzoic)	50 mg
1.57 µM	P-aminobenzoate (Na-salt)	250 mg
0.21 µM	Pantothenate (Na-salt)	50 mg
0.04 µM	Folic acid (dihydrate)	20 mg
0.24 µM	Lipoic acid (thioctic acid)	50 mg
0.59 µM	Pyridoxine (B6 vitamin)	100 mg
4.50 µM	Nicotinamide	550 mg
		Dissolve in 1 L Milli-Q water

Vitamin B1 solution – **Solution VI**

100mg/l	Thiamine HCl (Hydrochloride)
	Dissolve in Milli-Q water

Vitamin B2 solution - **Solution VII**

50mg/l	Riboflavine (B2 vitamine)
	Dissolve in Milli-Q water

Vitamin B12 solution - **Solution VIII**

50mg/l	Cyanocobalamin (vitamin B12)
	Dissolve in Milli-Q water

For Solution V, VI, VII and VIII:

- Prepared 4X100 mL serum bottles pressurize closed with N_2 gas to 0.5 bar overpressure (15 cycles of gas exchanges to remove normal atmosphere) and autoclaved (15' at 121°C).
- Filtrate 50 mL of solution through a 0.22 µm filter in serum bottle (one bottle for each solution).
- Cover each bottle with an aluminum sheet to avoid photolysis
- Stock at 4°C (exp. 2 years ± 1 month)

Solution C

- To 49 mL of solution IX, add 1 mL of solution X
- Conservation: strictly 10 days at 4°C
- Solution C is pink at the beginning then turn transparent as redox decrease with Na_2S

Solution IX :

Solution IX contains in final concentration:		**Example for 1 liter of Solution IX:**
110mM	NH_4HCO_3	9.01 g
0.9 M	$NaHCO_3$	76.11 g
		Dissolve in 1 L Milli-Q water

- Boil the solution to dissolve the powder and then cool to room temperature with bubbling of N_2
- Dispense 49 mL in 100mL serum bottles
- Seal the bottles with stoppers (in Butyl or Viton) and crimp seals
- Change atmosphere in the bottle with 15 cycles of gas exchange with 80%N_2:20%CO_2 and finish with 0.5 bar overpressure in the bottles.
- Autoclave 15' at 121°C
- Conservation 12 ±1 months at room temperature, in the dark

Solution X :

- Dissolve 24.02 g of washed Na_2S*9H_2O in 100 mL of Milli-Q water, and flush with N_2
- Prepared empty 100 mL serum bottles pressurize closed with N_2 gas to 0.5 bar overpressure and autoclaved.

- Assemble a syringe, a 0.22 µm syringe filter and a needle
- Filtrate sterilely the solution with in the serum bottle
- Conservation: 6 ±1 months at 4°C

Solution D

Solution C contains in final concentration:

30mM $CaCl_2*2H_2O$
20mM $MgCl_2*6H_2O$

Example for 1 liter of Solution C:

4.4g de de $CaCl_2*2H_2O$
4.06g de $MgCl_2*6H_2O$
Add 1000 mL Milli-Q water

- Dispense 200 mL in 500 mL serum bottles
- Seal the bottles with the stoppers (Butyl or Viton) and crimp seals
- Change atmosphere in the bottle with 15 cycles of gas exchange with N_2 and finish with 0.5 bar overpressure in the bottles
- Autoclave 20' at 121°C
- Keep at room temperature (exp. 2 years ± 1month)

Substrate preparation – electron donors Mix II

- Prepared empty 100 mL serum bottles pressurize closed with N_2 gas to 0.5 bar overpressure and autoclaved (15' at 121°C)
- Mix equal volume (1:1:1) of: Ethanol 100mM
  Propionate 100mM
  Butyrate 100mM
- Filtrate sterilely the mix with an assembled syringe/0.22 µm filter/needle in the serum bottle
- Keep at 4°C (exp. 1 years ± 1month)

Ethanol 100mM:
606.20µl absolute ethanol
Add 100 mL Milli-Q water

Propionate 100mM:
917.85µl n-butyric acid
Add 100ml Milli-Q water

Butyrate 100mM:
746.82µl Propionic acid
Add 100 mL Milli-Q water

For each solution:

- Dispense in 100 mL serum bottle
- Seal the bottles with the stoppers (Butyl or Vitton) and crimp seals
- Change atmosphere in the bottle with 15 cycles of gas exchange with N_2 and finish with 0.5 bar overpressure in the bottles
- Autoclave 121°C, 20min
- Keep at 4°C, exp. 1 year ± 1 month

PCE solution – electron acceptors

Tetrachloroethene/Perchloroethene (PCE)
Molecular weight PCE 165.834 g/mol - Density PCE 1.623 g/ml - 1L pure PCE is 9.787 mol/L

- Add 98.98 mL Hexadecane to a 200 mL serum bottle
- Close with a Viton stopper and flush with N_2
- Autoclave
- Add 1.02 mL pure PCE with a syringe
- Final concentration of the stock solution 100 mM
- Conservation 2 years ±1 month)
- Add 10% (v/v) in the culture medium for a final concentration at 40µM

APPENDIX 6

T-RFLP PROCEDURE

Partial reproduction of the supplementary material (Document_1) from:
Rossi P, Gillet F, Rohrbach E, Diaby N, Holliger C (2009) Statistical assessment of the variability of the T-RFLP analysis applied to complex microbial communities. Appl Environ Microbiol 75:7268–7270. Available at http://aem.asm.org/content/75/22/7268.full .

Standardized T-RFLP analysis

PCR products were generated with a combination of FAM-labelled Eub8f and Univ518r[1] primers according to the following conditions: 50 µl PCR reactions were composed of 5 µl 10x PCR buffer (Promega), 1.2 µl of both primer at 10 µM primer, 4 µl of 10mM dNTPs, 2.5 U of GoTaq DNA polymerase (Promega) and 0.1 ng/µl template DNA (final concentration), completed with sterilized and UV-treated MilliQ water (Millipore). PCR amplifications were conducted in a PTC200 Peltier Thermal Cycler (MJ Research) as follows: initial denaturing step at 94°C (4.5 min), followed by 25 cycles of 0.5 min denaturation at 94°C, 1 min annealing at 56°C, 1 min elongation at 72°C and a final elongation step of 10 min at 72°C. PCR products were examined in a 1.5% agarose gel to confirm the specificity of the amplification reaction. PCR products were purified with the EZNA Cycle-pure purification kit (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. An aliquot of 200 ng of purified PCR products was digested with 1 unit of *HaeIII* (Promega) at 37°C for 4 h in a 10 µl reaction volume. 1 µl of digested sample was mixed with 8.5 µl of HiDi formamid (ABI) and 0.5 µl of GS600-LIZ standard (ABI). Samples were denatured by heating to 95°C for 2 min by cooling on ice for 5 min.

The denatured samples were loaded onto an ABI 3130xl DNA capillary sequencer equipped with 50 cm long capillaries (80 µm inner diameter) and POP 7 electrophoresis matrix (dye set G5). The electrophoresis conditions were optimized according to existing built-in data and to information provided by the manufacturer (ABI, personal communication). The oven temperature was set to 60°C. A pre-run (15 kVolts, 180 sec) was followed by the loading of the samples (1.6 kVolts, 30 sec) and the fragment analysis (15 kVolts, 2500 sec). Negative extraction and amplification control samples were composed of sterilized and UV treated water. The resulting electrophoregrams were analyzed with GeneMapper v4.0 (ABI). Peak recognition was carried out automatically using a built-in ALFP peak recognition algorithm.

[1] Primer sequences are given in Table 2 section 4 of the screening procedure

APPENDIX 7

INSTALLATION OF R SOFTWARE

1) Go on CRAN website: http://cran.r-project.org/
2) In the "Download and Install R" first box of the web page, chose your operating system (OS) – Linux, Mac OS, or Windows
3) Than select the file link corresponding to the current version of your OS
4) The installation file downloads on your computer
5) Double click on the file to start the installation and follow the installer instructions, chose standard installation.
6) When installation is finished, open R software and change the working directory:

First, in Explorer (Windows) or Finder (Mac) create in your documents folder a new folder name "R_working_folder". This folder will contain all material linked to R.
Then from the R software follow one of the option below:
Option 1: Through the menu go to 'File' (Windows) or 'Misc.' (Mac) and select 'Change Working Directory', and find the appropriate folder "R_working_folder"
Option 2: Write directly in the R console the code:

```
> setwd("...")  #in which, the "..." is the pathway to reach your R folder
```

e.g., under Windows:

```
> setwd("C:/Users/User Name/Documents/R_working_folder")
```

or under Macs:

```
> setwd("/Users/User Name/Documents/ R_working_folder ")
```

You can check that working directory has been correctly set by writing the function:

```
> getwd()
```

The **working directory needs to be defined at the beginning of each new R session** (R software doesn't remember it) to ensure R find the R function files that you need for the analyses (see Appendix 8) as well as your data *.csv files. It will be also in this folder that you will find the output files from R analyses under the file names mentioned in the current manual.

7) Follow the next steps of the R analysis procedure in the main document in § 6.3 'download the needed R packages')

APPENDIX 8

DETAIL OF 'EVPLOT.R' FUNCTION

In R software click on **Menu/File/New Document** to open a blank editor window
Copy-Past in the window the evplot script below
Then click on **Menu/File/Save As...** to save the document as '**evplot.R' in your R working folder.** This R functions evplot.R can also be downloaded here[1].

evplot script:

```
# Plot eigenvalues and % of variation of an ordination object
# Kaiser rule and broken stick model
# Usage:
# evplot(ev)
# where ev is a vector of eigenvalues

# License: GPL-2
# Author: Francois Gillet, 25 August 2012

evplot <- function(ev)
{
	# Broken stick model (MacArthur 1957)
	n <- length(ev)
	bsm <- data.frame(j=seq(1:n), p=0)
	bsm$p[1] <- 1/n
	for (i in 2:n) bsm$p[i] <- bsm$p[i-1] + (1/(n + 1 - i))
	bsm$p <- 100*bsm$p/n
	# Plot eigenvalues and % of variation for each axis
	op <- par(mfrow=c(2,1))
	barplot(ev, main="Eigenvalues", col="bisque", las=2)
	abline(h=mean(ev), col="red")
	legend("topright", "Average eigenvalue", lwd=1, col=2, bty="n")
	barplot(t(cbind(100*ev/sum(ev), bsm$p[n:1])), beside=TRUE,
		main="% variation", col=c("bisque",2), las=2)
	legend("topright", c("% eigenvalue", "Broken stick model"),
		pch=15, col=c("bisque",2), bty="n")
	par(op)
}
```

[1] https://www.dropbox.com/sh/bse2oo0dbag332g/AABwxSFLpmB5n7ik0p7QiJMwa?dl=0

APPENDIX 9

DETAIL OF THE MFA SCRIPT

In R software click on **Menu/File/New Document** to open a blank editor window
Copy-Past in the window the script below
Then click on **Menu/File/Save As...** to save the document as '0_MFA_LBE_ofev.R **' in your R working folder** . This script can also be downloaded at here[1].

```
#*************************************************************************
## Script to compute MFA analysis associated with the screening procedure
# for evaluation of reductive dechlorination potential and reasons for
# lower CEs stalling
#
## content
# 0 prepare R environment
# 1 Import data
# 2 Transformations of the data and standardization
# 3 Multiple factor analysis (MFA) and multiple plots
# 4 Test of the MFA components/axes validity
# 5 Produce output files of MFA mutiple plots
# 6 Test and select variables that significantly explain the mfa model
# 7 Produce a clean correlation circle MFA plot with only the significant
#   variables
# 8 RV correlations and associated p-values between the BCS group and
#   individual variables of TEAPs, %CES, and Others groups
## Author: Sonia-Estelle Tarnawski
#*************************************************************************

# 0 Prepare R working environment
#*******************************

# if not already done, install the R packages, if yes skip this 1st step
# install.packages (c('ade4', 'vegan', 'FactoMineR'))

# Verified the current pathway of the working directory
getwd()

# Load the required packages (after installation)
# vegan must be loaded after ade4 to avoid some conflicts
library(ade4)
library(vegan)
library(FactoMineR)

# Change working directory for your "R_working_folder" if not already done
#(as indicated in Appendix 7)
# Verify that evplot.R and your data files: spe.csv, teaps.csv, XCEs.csv,
# others.csv are in your "R_working_folder"

# Load additional functions
source("evplot.R")
```

[1] https://www.dropbox.com/sh/bse2oo0dbag332g/AABwxSFLpmB5n7ik0p7QiJMwa?dl=0

```
# 1 Import data
#**************

## Bacterial community structures dataset (T-RFLP)
spe <- read.csv2("spe.csv", dec=".", row.names=1)

## Environmental data splits in the 3 explicative data sets:
## teaps, %CEs and others
teaps <- read.csv2("teaps.csv", dec=".", row.names=1)
CEs <- read.csv2("XCEs.csv", dec=".", row.names=1)
others <- read.csv2("others.csv", dec=".", row.names=1)


# 2 Transformations of spe data (decostand function of the vegan package)
#************************************************************************

## Transform raw t-RFs peak area data(population abundance) in relative
## abundance per sample
spe <- decostand(spe, "total", MARGIN=1)
## Verification of data transformation, sum of the line must be equal to 1
apply(spe,1,sum)
## Create a new excel file with the transformed data
write.table(spe, file = "spe-relative.csv", sep = ";", col.names = NA)

## Hellinger standardization of the relative abundance species data.
## Hellinger transformation minimize consideration of double zero as
## resemblance between samples
spe.hel <- decostand(spe, "hellinger")


# 3 Multiple factor analysis (MFA) - video explaining MFA in french
# https://www.youtube.com/watch?v=ny4IxoLgdeY
# ************************************************************************

## MFA on 4 groups of variables:
## Concatenate data of the 4 groups of variables (spe.hel, teaps, CEs and
## others)
tab4 <- data.frame(spe.hel, teaps, CEs, others)

## tab4 exploration:
# dimension of the data table tab4 (number of lines X number of columns)
dim(tab4)
# number of variables per group, respectively spe,teaps, %CES and others
(grn <- c(ncol(spe.hel), ncol(teaps), ncol(CEs), ncol(others)))
# view of the first lines of tab4
head(tab4)

## Close the any previous graphic windows
graphics.off()

## Compute the MFA and generate multiple plots
t4.mfa <- MFA(tab4, group=grn, type=c("c","s","s","s"), ncp=3,
name.group=c("BCS","TEAPs","%CEs","Others"))

## take time to have a look on these first plots generated by the MFA
## size of graphs can be changed by expanding the window using the mouse
```

```
# 4 Test of the MFA axes validity
# ******************************

## Eigenvalue results of the MFA and percentage of variance explained
# show how to call different results of the mfa
t4.mfa
# call eignevalues
t4.mfa$eig

## Test of axis validity
# Select only first column of the eigenvalue results
ev <- t4.mfa$eig[,1]
names(ev) <- 1:nrow(t4.mfa$eig)

## Close the previous graphic windows
graphics.off()

## Graph of Eigenvalue per dimension of the MFA
## Average eigenvalue (Kaiser Guttman criterion)/Broken Stick Model results
evplot(ev)
# are the 1st et 2nd dim. significant axes? red histograms must be smaller
# than the light pink one on the bottom graph

## if the two first axes are validated, continue the analysis
## if not, stop the analysis, rethink about the data and the possibility
## to complement them with another round of sampling


### To save a '1_Test_Axes.png' file of the graph of 'Eigenvalue per
### dimension of the MFA, average eigenvalue and broken stick model
### results', execute the following code lines:
{
# Apply Kaiser-Guttman criterion to select axes
ev[ev > mean(ev)]
# Broken stick model (MacArthur 1957)
n <- length(ev)
bsm <- data.frame(j=seq(1:n), p=0)
bsm$p[1] <- 1/n
for (i in 2:n)
bsm$p[i] = bsm$p[i-1] + (1/(n + 1 - i))
bsm$p <- 100*bsm$p/n
# Plot eigenvalues and % of variation for each axis, generate *.png file
png(filename="1_Test_Axes.png", width=10, height=4, units="in", res=500)
par(mfrow=c(1,2))
# Eigenvalues/Mean Eigenvalues
barplot(ev, main="Eigenvalues", col="bisque", las=2, cex.axis=0.8,
cex.name=0.8, cex.main=0.8)
abline(h=mean(ev), col="red")    # average eigenvalue
legend("topright", "Average eigenvalue", lwd=1, col=2, bty="n", cex=0.7)
# % Eigenvalue/BSM
barplot(t(cbind(100*ev/sum(ev),bsm$p[n:1])), beside=T, main="% variance",
col=c("bisque",2), las=2, cex.axis=0.8, cex.name=0.8, cex.main=0.8)
legend("topright", c("% eigenvalue", "Broken stick model"), pch=15,
col=c("bisque",2), bty="n", cex=0.7)
dev.off()
}
```

```
# 5 Produce output files of MFA multiple plots
# ****************************************

### General comments about the different ways to save graphic
### representations: Code lines to save graphics in high quality *.png
### files, useful for writing reports, are given in the current scripts.
### You can also save any graph, by clicking on with the right mousse
### button, copy/paste it on e.g. Microsoft PowerPoint or Word; or save it
### in low quality pdf format, by clicking on the graph window, then go in
### the R menu 'File' and select 'Save as'


## To draw different graphs of the MFA and keep some clearest in *.png
## format execute the following code lines :

##### Correlation circle #####
#** Correlation circle (different colors than the default ones)
plot(t4.mfa, choix="var", habillage="group", palette=pal-
ette(c("black","darkgrey","green3","red","blue")), cex=.8, cex.main=1,
cex.lab=.8, cex.axis=.8, shadowtext=TRUE)
# The open graph window shows how it looks like. Save the graph in a
# '2_Plot_CorrelationCircle.png' file in your working folder by executing
# the following codes:
{
png(filename="2_Plot_CorrelationCircle.png",width=7, height=7, units="in",
res=500)
plot(t4.mfa, choix="var", habillage="group", palette=pal-
ette(c("black","darkgrey","green3","red","blue")), cex=.8, cex.main=1,
cex.lab=.8, cex.axis=.8, shadowtext=TRUE)
dev.off()
}
##### Individual factor map #####
# Display 'Individual factor map' plotting samples barycenter in black
plot(t4.mfa, choix="ind", habillage="none", cex=.8, cex.main=1, cex.lab=.8,
cex.axis=.8, shadowtext = TRUE, invisible="quali")
# Save this graph in a .png format in your working folder:
{
png(filename="4_Plot_IndFactMap.png",width=7, height=7, units="in", res=500)
plot(t4.mfa, choix="ind", habillage="none", cex=.8, cex.main=1, cex.lab=.8,
cex.axis=.8, shadowtext = TRUE)
dev.off()
}


# 6 Test and select variables that significantly explain the MFA model
# ************************************************************************

## Select variables presenting correlations with one or both axes of the
## MFA with p-value < 0.05
aa <- dimdesc(t4.mfa, axes=1:2, proba=0.05)
aa # show the results


# 7 Produce a clean 'Correlation circle' MFA plot with only the significant
# variables at p-value < 0.05
# ************************************************************************

## Close the previous graphic windows
graphics.off()
```

```
## Display the graph
plot(t4.mfa, choix="var", habillage="group", title= "Correlation circle / p-
value =<0.05", cex=.8, select= c(rownames(aa$Dim.1$quanti),
rownames(aa$Dim.2$quanti)), unselect="transparent", cex.main=1, cex.lab=.8,
cex.axis=.8, palette=palette(c("black","darkgrey","green3","red","blue")))
## Save the graph under the name '3_Plot_CorrelationCircle_0.05.png' in your
## working folder:
{
png(filename="3_Plot_CorrelationCircle_0.05.png", width=7, height=7,
units="in", res=500)
plot(t4.mfa, choix="var", habillage="group", title= "Correlation circle / p-
value=<0.05", cex=.8, select= c(rownames(aa$Dim.1$quanti),
rownames(aa$Dim.2$quanti)), unselect="transparent", cex.main=1, cex.lab=.8,
cex.axis=.8, palette=palette(c("black","darkgrey","green3","red","blue")))
dev.off()
}

# 8 RV correlations and associated p-values between the BCS group and
# individual variables of the TEAPs, %CEs and Others groups
#*************************************************************************
##### for this step, It is important that the acronym names that you used
##### for the variables being the same that the ones proposed in the
##### screening procedure (Table 3) to be able to use the follow code lines
##### without generated error.

## Use the 'tab4' define at the beginning of the script as data references
## and call the names of the column
## of the different groups as remind if you need to change some names in
## the script
colnames(teaps)
colnames(CEs)
colnames(others)

#********** RV coeff.
## RV global correlations between BCS and other groups
TEAPs <- coeffRV(spe.hel, scale(teaps))$rv
XCEs <- coeffRV(spe.hel, scale(CEs))$rv
OTHERS <- coeffRV(spe.hel, scale(others))$rv

# RV coefficient between BCS and individual variables (X) is given by
# the code line general formula: coeffRV(spe.hel, scale(X))$rv

PCE <- coeffRV(spe.hel, scale(tab4$PCE))$rv
TCE <- coeffRV(spe.hel, scale(tab4$TCE))$rv
cDCE <- coeffRV(spe.hel, scale(tab4$c12DCE))$rv
VC <- coeffRV(spe.hel, scale(tab4$VC))$rv
XPCE <- coeffRV(spe.hel, scale(tab4$X.PCE))$rv
XTCE <- coeffRV(spe.hel, scale(tab4$X.TCE))$rv
XcDCE <- coeffRV(spe.hel, scale(tab4$X.c12DCE))$rv
XVC <- coeffRV(spe.hel, scale(tab4$X.VC))$rv
NH4 <- coeffRV(spe.hel, scale(tab4$NH4))$rv
NO3 <- coeffRV(spe.hel, scale(tab4$NO3))$rv
NO2 <- coeffRV(spe.hel, scale(tab4$NO2))$rv
SO4 <- coeffRV(spe.hel, scale(tab4$SO4))$rv
FeII <- coeffRV(spe.hel, scale(tab4$FeII))$rv
MnII <- coeffRV(spe.hel, scale(tab4$MnII))$rv
CH4 <- coeffRV(spe.hel, scale(tab4$CH4))$rv
dO2 <- coeffRV(spe.hel, scale(tab4$dO2))$rv
Eh <- coeffRV(spe.hel, scale(tab4$Eh))$rv
pH <- coeffRV(spe.hel, scale(tab4$pH))$rv
tC <- coeffRV(spe.hel, scale(tab4$tC))$rv
TOC <- coeffRV(spe.hel, scale(tab4$TOC))$rv
```

```
Chloride <- coeffRV(spe.hel, scale(tab4$Cl))$rv

## Group RV results in a single table
tabRV <- data.frame(TEAPs, XCEs, OTHERS, PCE, TCE, cDCE, VC, XPCE, XTCE,
XcDCE, XVC, NH4, NO3, NO2, SO4, FeII, MnII, CH4, dO2, Eh, pH, tC, TOC,
Chloride)
tabRV # display the table

#********** p-values (PV)
## PV correlations between BCS and other groups
TEAPs <- coeffRV(spe.hel, scale(teaps))$p.value
XCEs <- coeffRV(spe.hel, scale(CEs))$p.value
OTHERS <- coeffRV(spe.hel, scale(others))$p.value

# p-value associated to RV coeff between BCS and individual variables (X)
# is given by the code line general formula: coeffRV(spe.hel, scale(X))$p-
# value. Important: keep the order of the variables same that for RV
# coeff. calculation

PCE <- coeffRV(spe.hel, scale(tab4$PCE))$p.value
TCE <- coeffRV(spe.hel, scale(tab4$TCE))$p.value
cDCE <- coeffRV(spe.hel, scale(tab4$c12DCE))$p.value
VC <- coeffRV(spe.hel, scale(tab4$VC))$p.value
XPCE <- coeffRV(spe.hel, scale(tab4$X.PCE))$p.value
XTCE <- coeffRV(spe.hel, scale(tab4$X.TCE))$p.value
XcDCE <- coeffRV(spe.hel, scale(tab4$X.c12DCE))$p.value
XVC <- coeffRV(spe.hel, scale(tab4$X.VC))$p.value
NH4 <- coeffRV(spe.hel, scale(tab4$NH4))$p.value
NO3 <- coeffRV(spe.hel, scale(tab4$NO3))$p.value
NO2 <- coeffRV(spe.hel, scale(tab4$NO2))$p.value
SO4 <- coeffRV(spe.hel, scale(tab4$SO4))$p.value
FeII <- coeffRV(spe.hel, scale(tab4$FeII))$p.value
MnII <- coeffRV(spe.hel, scale(tab4$MnII))$p.value
CH4 <- coeffRV(spe.hel, scale(tab4$CH4))$p.value
dO2 <- coeffRV(spe.hel, scale(tab4$dO2))$p.value
Eh <- coeffRV(spe.hel, scale(tab4$Eh))$p.value
pH <- coeffRV(spe.hel, scale(tab4$pH))$p.value
tC <- coeffRV(spe.hel, scale(tab4$tC))$p.value
TOC <- coeffRV(spe.hel, scale(tab4$TOC))$p.value
Chloride <- coeffRV(spe.hel, scale(tab4$Cl))$p.value

## Group p-value results in a single table
tabPV <- data.frame(TEAPs, XCEs, OTHERS, PCE, TCE, cDCE, VC, XPCE, XTCE,
XcDCE, XVC, NH4, NO3, NO2, SO4, FeII, MnII, CH4, dO2, Eh, pH, tC, TOC,
Chloride)
tabPV # display the table

## Table with RV coeff results in column 1 and p-value in column 2
tabRV.PV <- rbind(tabRV, tabPV, deparse.level = 2)
tabRV.PV <- t(tabRV.PV)
tabRV.PV

## Keep the above table as output file ‘5_Table_Correlations_BCS_Env.csv in
## your working folder:
write.table(tabRV.PV, file = "5_Table_Correlations_BCS_Env.csv ", sep = ";",
col.names = NA)


##### END #####
```

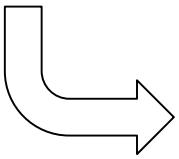
APPENDIX 10A

CASE STUDY – PHYSICOCHEMICAL DATA

Concentrations of the contaminants, chemical compounds related to TEAPs and some of other parameters have been measured in the water samples. Measurement unit is given in the first line of the table. The x, y expressed in meters gives the position of the piezometers in the field from an origin arbitrary chosen

Raw data:

	µg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	µg/L	mg/L	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L	µg/L	mg/L	mg/L	-	°C	mg/L	mV	m	m	m	mS/cm
	PCE	TCE	c12DCE	t12DCE	VC	ethene	ethane	NH_4^+	NO_3^-	NO_2^-	SO_4^{2-}	SO_3^{2-}	H_2S, S^{2-}	FeII	MnII	CH_4	Cl^-	F^-	PO_4^{3-}	K^+	TOC	pH	tC	dO_2	Eh	depth	x	y	eC
MIP01	142.0	1.7	0.8	0.9	0	0	0	159.0	39.0	78.0	122.0	0	0	0	240.0	0.01	48.5	0.2	39.5	10.40	1.64	7.13	13.1	2.28	-10.4	4.3	44.08	89.92	1151
MIP02	1500.0	26.6	50.0	2.8	0.4	0	0	195.0	39.2	1349.5	226.0	0	0	0	118.0	0	33.5	0.2	0	13.90	5.28	7.10	13.7	1.14	97.0	6.8	38.62	81.00	-
MIP03	190.0	0.8	0.8	0.2	0	0	0	48.0	45.0	105.0	148.0	0	0	0	51.0	0	42.8	0.2	46.0	10.20	1.75	7.04	12.4	1.75	6.8	4.3	52.08	82.92	1246
MIP04	0.6	0	0	0	0	0	0	198.0	1.2	268.0	41.5	0	0	14.0	596.0	0.05	32.9	0.3	0	3.43	1.24	7.81	13.0	2.81	130.7	6.8	60.23	86.92	684
MIP05-2	5.9	5.35	169.5	1.05	5.6	0	0	2860.0	0.1	0.0	139.0	0	0	17.0	908.0	0.03	28.7	0	0	9.29	3.86	7.05	13.8	0.44	92.2	6.8	46.46	77.38	-
MIP06-2	16.4	6.7	37.4	0.6	0	0	0	178.0	26.6	457.0	437.0	0	0	0	342.0	0.03	19.0	0	51.5	20.50	3.62	6.98	13.9	0.19	5.5	9.5	46.38	67.54	1601
MIP07	83.5	2.3	2.2	0.1	0	0	0	43.0	141.5	132.0	648.5	0	0	0	169.0	0	33.2	0	56.0	30.50	5.86	6.88	11.5	2.18	79.5	5.8	72.77	75.31	2
MIP08	23.3	11	7.9	0.2	0	0	0	42.0	18.1	494.0	102.0	0	0	0	270.0	0.01	45.6	0	0	3.07	1.30	7.00	12.9	0.40	211.1	8.0	24.69	53.08	1287
MIP10	166.0	14.15	36.6	0.6	0	0	0	75.0	35.5	30.0	258.0	0	0	23.0	1232.0	0	34.0	0	70	21.70	3.11	6.81	13.4	0.12	35.3	6.8	63.85	52.69	1583
MIP11	49.0	21.1	98.0	1.2	0	0	0	1700.0	18.1	398.0	189.0	0	0	15.0	2695.0	0.04	22.7	0	47.5	13.80	3.01	6.84	14.7	0.55	189.4	8.5	39.31	36.92	1440
MIP20	64.0	48	368.0	4.5	0.9	0	0	4010.0	19.6	1504.0	308.0	0	0	0	1470.0	0.02	38.5	0	49.5	20.00	3.22	6.93	15.0	0.71	152.1	8.5	50.38	46.62	1697
PO1	262.0	9.2	37.0	0.5	0	0	0	205.0	26.5	45.0	345.0	0	0	0	64.0	0	30.8	0.1	15.0	21.50	3.34	6.85	13.5	0.80	171.2	7.5	59.38	71.00	1665
PO2	28.6	0.2	0	0	0	0	0	28.0	20.8	0	56.8	0	0	0	0	0	77.2	0.2	79.5	2.25	0.93	7.21	12.4	3.79	94.6	4.5	39.23	89.08	1054
PO3	493.0	9.8	12.0	0.9	0	0	0	19.0	35.1	76	269.0	0	0	0	372.0	0	32.5	0.2	82.5	22.00	4.16	6.79	13.9	0.44	201.6	6.5	53.46	55.92	1565
PO4	8.4	0.4	1.2	0	0	0	0	20.0	39.8	0	303.0	0	0	28.0	0	0	44.6	0	109.0	5.31	1.78	6.98	13.0	3.33	213.5	5.5	86.62	15.77	1535
PO5	0	0	0	0	0	0	0	30.0	20.0	0	133.0	0	0	0	0	0	43.5	0.2	76.0	2.26	0.68	7.16	15.5	6.27	244.6	6.0	141.69	16.77	994
PO6	254.0	6.1	108.0	0.6	2.3	0	0	1960.0	1.8	14.0	15.0	0	0	0	49.0	0.01	6.2	0.2	31.5	3.62	2.88	7.16	13.0	2.43	242.5	4.7	32.31	69.54	667
PO7	0.6	0	0	0	0	0	0	146.0	0.1	1119.5	181.0	0	0	0	153.0	0.13	30.3	0.3	0	5.78	1.76	7.83	15.7	1.54	-107.6	9.5	36.62	102.54	871
PO8	61.4	1.1	3.2	0.4	0.1	0	0	239.0	5.4	3940.0	91.2	0	0	10.5	159.0	0.02	17.7	0.3	39.5	7.77	3.37	7.31	16.9	2.59	61.6	8.5	45.15	84.08	980
PO10	428.0	62	428.0	4.9	0.7	0	0	4000.0	7.2	189.0	261.5	0	0	13.0	2562.0	0.04	38.0	0	32.0	20.55	3.52	6.91	10.5	0.31	24.5	7.0	54.38	30.92	1736
S12099	23.0	12.4	69.2	0.8	0	0	0	104.0	29.8	0	165.5	0	0	0	1265.0	0	28.9	0	0	22.10	2.20	6.81	12.9	1.19	249.3	8.5	15.23	10.00	1411
S12512	0	0	0	0	0	0	0	28.0	21.0	0	75.0	0	0	0	0	0	22.8	0.1	225.0	1.03	0.62	7.13	12.0	5.43	286.5	7.0	206.23	118.38	990
S20	8.5	9.5	120.0	2.3	0	0	0	302.0	31.5	0	267.0	0	0	0	40.0	0	30.2	0	98.5	20.40	2.68	6.91	12.6	1.43	274.4	5.5	62.46	8.00	1576



teaps.csv:

well	PCE	TCE	c12DCE	VC	NH4	NO3	NO2	SO4	FeII	MnII	CH4	dO2
MIP01	142	1.7	0.8	0	159	39	78	122	0	240	0.01	2.28
MIP02	1500	26.6	50	0.4	195	39.2	1349.5	226	0	118	0	1.14
MIP03	190	0.8	0.8	0	48	45	105	148	0	51	0	1.75
MIP04	0.6	0	0	0	198	1.2	268	41.5	14	596	0.05	2.81
MIP05-2	5.9	5.35	169.5	5.6	2860	0.1	0	139	17	908	0.03	0.44
MIP06-2	16.4	6.7	37.4	0	178	26.6	457	437	0	342	0.03	0.19
MIP07	83.5	2.3	2.2	0	43	141.5	132	648.5	0	169	0	2.18
MIP08	23.3	11	7.9	0	42	18.1	494	102	0	270	0.01	0.4
MIP10	166	14.15	36.6	0	75	35.5	30	258	23	1232	0	0.12
MIP11	49	21.1	98	0	1700	18.1	398	189	15	2695	0.04	0.55
MIP20	64	48	368	0.9	4010	19.6	1504	308	0	1470	0.02	0.71
PO1	262	9.2	37	0	205	26.5	45	345	0	64	0	0.8
PO2	28.6	0.2	0	0	28	20.8	0	56.8	0	0	0	3.79
PO3	493	9.8	12	0	19	35.1	76	269	0	372	0	0.44
PO4	8.4	0.4	1.2	0	20	39.8	0	303	28	0	0	3.33
PO5	0	0	0	0	30	20	0	133	0	0	0	6.27
PO6	254	6.1	108	2.3	1960	1.8	14	15	0	49	0.01	2.43
PO8	61.4	1.1	3.2	0.1	239	5.4	3940	91.2	10.5	159	0.02	2.59
PO10	428	62	428	0.7	4000	7.2	189	261.5	13	2562	0.04	0.31
S12099	23	12.4	69.2	0	104	29.8	0	165.5	0	1265	0	1.19
S12512	0	0	0	0	28	21	0	75	0	0	0	5.43
S20	8.5	9.5	120	0	302	31.5	0	267	0	40	0	1.43

XCEs.csv:

well	%PCE	%TCE	%c12DCE	%VC
MIP01	97.66	1.17	0.55	0
MIP02	94.95	1.68	3.16	0.03
MIP03	99.06	0.42	0.42	0
MIP04	100	0	0	0
MIP05-2	3.15	2.85	90.45	2.99
MIP06-2	26.84	10.97	61.21	0
MIP07	94.78	2.61	2.50	0
MIP08	54.95	25.94	18.63	0
MIP10	76.37	6.51	16.84	0
MIP11	28.94	12.46	57.89	0
MIP20	13.19	9.89	75.81	0.19
PO1	84.87	2.98	11.99	0
PO2	99.31	0.69	0	0
PO3	95.60	1.90	2.33	0
PO4	84	4	12	0
PO5	0	0	0	0
PO6	68.46	1.64	29.11	0.62
PO8	92.75	1.66	4.83	0.15
PO10	46.34	6.71	46.34	0.08
S12099	21.82	11.76	65.65	0
S12512	0	0	0	0
S20	6.06	6.77	85.53	0

others.csv:

well	Eh	pH	tC	TOC	Cl	F	PO4	K	depth	x	y
MIP01	-10.4	7.13	13.1	1.64	48.5	0.2	39.5	10.4	4.3	44.08	89.92
MIP02	97	7.1	13.7	5.28	33.5	0.2	0	13.9	6.8	38.62	81
MIP03	6.8	7.04	12.4	1.75	42.8	0.2	46	10.2	4.3	52.08	82.92
MIP04	130.7	7.81	13	1.24	32.9	0.3	0	3.43	6.8	60.23	86.92
MIP05-2	92.2	7.05	13.8	3.86	28.7	0	0	9.29	6.8	46.46	77.38
MIP06-2	5.5	6.98	13.9	3.62	19	0	51.5	20.5	9.5	46.38	67.54
MIP07	79.5	6.88	11.5	5.86	33.2	0	56	30.5	5.8	72.77	75.31
MIP08	211.1	7	12.9	1.3	45.6	0	0	3.07	8	24.69	53.08
MIP10	35.3	6.81	13.4	3.11	34	0	70	21.7	6.8	63.85	52.69
MIP11	189.4	6.84	14.7	3.01	22.7	0	47.5	13.8	8.5	39.31	36.92
MIP20	152.1	6.93	15	3.22	38.5	0	49.5	20	8.5	50.38	46.62
PO1	171.2	6.85	13.5	3.34	30.8	0.1	15	21.5	7.5	59.38	71
PO2	94.6	7.21	12.4	0.93	77.2	0.2	79.5	2.25	4.5	39.23	89.08
PO3	201.6	6.79	13.9	4.16	32.5	0.2	82.5	22	6.5	53.46	55.92
PO4	213.5	6.98	13	1.78	44.6	0	109	5.31	5.5	86.62	15.77
PO5	244.6	7.16	15.5	0.68	43.5	0.2	76	2.26	6	141.69	16.77
PO6	242.5	7.16	13	2.88	6.2	0.2	31.5	3.62	4.7	32.31	69.54
PO8	61.6	7.31	16.9	3.37	17.7	0.3	39.5	7.77	8.5	45.15	84.08
PO10	24.5	6.91	10.5	3.52	38	0	32	20.55	7	54.38	30.92
S12099	249.3	6.81	12.9	2.2	28.9	0	0	22.1	8.5	15.23	10
S12512	286.5	7.13	12	0.62	22.8	0.1	225	1.03	7	206.23	118.38
S20	274.4	6.91	12.6	2.68	30.2	0	98.5	20.4	5.5	62.46	8

APPENDIX 10B

CASE STUDY – BACTERIAL COMMUNITY STRUCTURE DATA

Data of the T-RFs position in base pair is given in the first column of the table, and then comes the sample names. The table is filled with the value of the peak area of each t-RF in fluorescent intensity unit. For the statistical analysis this table must be transposed to create the spe.csv file.

t-RFs (bp)	MIP01	MIP02	MIP03	MIP04	MIP05-2	MIP06-2	MIP07	MIP08	MIP10	MIP11	MIP20	PO1	PO2	PO3	PO4	PO5	PO6	PO8	PO10	S12099	S12512	S20
50	0	429	0	483	0	0	0	0	0	0	616	0	864	465	1180	597	0	0	2393	568	531	1420
51	0	555	0	423	0	0	0	0	0	0	0	0	0	425	1558	0	0	0	2465	626	664	693
52	0	0	0	299	0	0	1777	2419	0	0	0	0	2606	0	0	0	0	0	1351	335	305	763
53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4320	0	0	0	0	0	0
54	0	0	0	293	0	0	0	0	0	2010	0	0	0	0	1115	0	0	0	796	469	0	1943
55	1798	6835	2180	1788	3634	4435	2821	971	2726	0	6368	3225	7035	5109	8613	5105	1821	2713	21281	5357	8248	11851
56	283	261	0	4211	0	0	0	0	0	0	0	0	267	0	0	0	0	0	0	0	0	0
57	0	0	0	2651	0	0	0	0	0	2339	0	0	0	0	0	0	8649	0	0	0	0	0
58	639	1619	669	20014	915	436	974	0	381	0	1614	0	2195	1398	3392	1931	0	0	8338	2005	1534	3494
59	5398	10844	3978	0	5842	3404	9561	2499	2717	627	13259	1715	24183	12494	36731	21325	3248	1456	80096	19632	13916	36519
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	193	0	0	0	528	0
61	1436	3574	2159	68359	749	2178	1911	1971	1229	0	6123	0	0	1265	6372	6227	0	4736	0	2345	0	0
62	2508	0	0	0	914	5368	0	0	0	37914	3498	1281	7922	0	0	0	15473	0	8027	0	3916	14124
63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	731	0	0	0	0	0
64	0	0	0	0	0	0	0	0	0	3409	352	0	0	0	0	0	0	0	1722	496	0	1219
67	0	0	0	979	0	0	0	0	0	0	0	0	0	0	0	0	717	0	0	0	0	0
69	0	0	0	0	0	0	0	0	0	0	0	0	340	0	0	0	589	0	0	0	830	0
70	0	0	0	1468	0	0	6346	0	0	0	0	0	0	0	0	0	400	457	2314	0	488	0
71	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72	0	0	0	0	0	0	0	0	0	833	345	0	0	0	0	0	0	0	0	0	0	0
74	0	0	0	0	0	0	0	0	0	721	214	0	0	0	0	0	0	0	890	440	578	0
75	0	0	0	852	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	767
78	0	0	0	0	0	0	0	0	0	0	499	0	0	0	0	0	0	0	0	0	0	0
89	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2004	0
90	3193	0	0	0	0	0	0	0	2309	0	0	0	0	0	0	0	0	0	549	0	0	0
106	0	0	0	0	0	0	0	0	0	0	0	0	0	352	0	0	0	0	0	0	0	0
107	0	0	0	0	0	0	0	0	0	0	298	0	0	0	0	0	0	0	0	0	692	0
111	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	346	0	0	0	0	0
127	314	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
128	487	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	308	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
130	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	472	0
132	463	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
134	0	0	0	641	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
135	978	0	0	0	0	0	0	0	793	0	0	0	0	0	0	0	0	0	422	0	1398	0
137	337	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
139	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	309	0	0	0	0	0
143	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
145	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	950	0
149	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	357	0	0	0	0	0
154	0	0	0	0	0	0	423	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
157	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3648	0	0	0	0	0	0	0
158	0	0	0	0	22268	0	0	0	0	0	329	0	296	0	0	0	0	0	0	0	0	0
163	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	401	0	0	0
170	675	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	687	0	0	0	0
171	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	403	0	0	0	0	0
172	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	604	0
173	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	615
178	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	513	0	0	0
179	0	0	0	0	0	0	0	0	0	0	0	0	0	1575	0	0	213	0	0	0	0	0
180	334	0	0	0	0	0	0	0	0	0	0	0	0	0	0	478	0	0	559	0	349	0
181	0	0	0	0	0	0	0	0	0	466	0	0	483	0	0	0	331	0	0	0	0	0
182	0	0	0	0	0	0	0	0	0	0	594	0	0	0	0	0	0	405	0	0	0	0
183	1306	711	0	0	0	0	0	0	0	0	0	0	641	0	0	0	10329	0	0	0	1373	0
184	0	540	0	314	0	0	0	0	0	0	0	0	0	0	0	0	11900	0	0	0	0	377
185	0	0	0	0	0	0	0	0	0	512	492	0	0	1031	0	0	0	0	351	0	0	0
186	0	0	0	0	0	0	0	0	0	0	0	0	0	1420	0	0	4785	0	1238	0	0	0
187	0	0	0	0	0	0	0	0	0	349	0	0	0	0	0	0	0	258	0	0	0	0
188	575	0	0	464	0	0	0	0	0	0	0	0	817	0	502	411	224	1849	855	0	374	515
189	2020	1130	541	9100	517	310	1279	1565	1153	564	1790	644	2813	1589	1170	1083	5436	875	1421	888	2702	3206
190	0	0	0	0	0	0	0	0	0	0	0	0	0	1459	0	0	6297	0	0	0	349	0
191	0	0	0	0	277	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	455	0
192	13348	6160	1756	12429	496	7421	4636	25442	5996	39300	34407	0	784	858	0	0	1029	48612	612	0	1615	0
193	0	0	0	0	364	0	0	0	1142	0	0	0	0	658	0	0	2786	0	385	0	387	0
194	355	1747	0	0	0	0	351	0	798	1410	0	0	0	0	0	0	1517	0	0	0	618	0
195	12257	5594	2350	7808	19081	0	601	0	1024	0	817	0	355	0	445	0	472	0	11353	0	1031	0
196	4470	7893	1858	39286	1009	19100	2999	34617	4307	49997	43869	0	0	0	2006	0	1346	21178	11095	0	0	0
197	467	0	0	0	7578	0	0	0	0	0	0	0	0	0	0	0	1032	10625	902	0	773	0
198	0	594	0	0	0	0	935	0	0	0	0	0	1849	0	0	0	1352	0	0	0	530	0
199	944	465	0	0	7290	608	0	0	556	0	0	0	0	0	0	0	740	0	741	371	421	917
200	0	0	0	0	740	637	0	0	0	5369	12854	9227	877	9369	0	0	140	0	845	0	1290	625
201	907	0	0	0	1357	0	571	0	0	4399	9298	0	0	7197	0	0	10465	0	605	0	0	458
202	871	605	0	0	4699	694	972	0	0	0	474	0	0	0	0	0	1869	296	0	0	0	0
203	975	0	0	0	240	0	0	0	0	0	0	0	0	0	0	0	0	0	995	0	297	0
204	0	0	0	750	2724	0	0	0	0	772	0	0	0	0	0	0	819	0	0	0	0	0
205	0	0	0	0	0	0	0	0	0	0	450	0	658	0	0	0	0	0	968	0	305	454

t-RFs (bp)	MIP01	MIP02	MIP03	MIP04	MIP05-2	MIP06-2	MIP07	MIP08	MIP10	MIP11	MIP20	PO1	PO2	PO3	PO4	PO5	PO6	PO8	PO10	S12099	S12512	S20
206	587	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	670	942	232	0	0	0
207	333	291	0	0	0	0	0	0	0	609	0	0	0	0	0	0	1230	0	0	0	493	0
208	590	371	451	7971	5794	958	1155	675	0	713	1121	428	2676	349	944	1843	1159	0	1206	1224	2131	3498
209	0	0	0	3831	22759	6145	0	0	0	0	0	0	348	0	453	0	7654	1060	0	0	0	439
210	0	189	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9541	0	0	356	0
211	0	0	0	2436	750	1781	0	9277	882	14863	9803	0	1050	0	707	0	32918	9425	0	0	0	607
212	5812	19649	6963	24396	610	29736	16262	13593	0	35674	85669	0	0	0	0	0	0	1587	0	0	930	0
213	53114	12994	6339	0	1639	0	10257	0	3387	0	0	2025	6477	1880	3813	7758	2879	1134	6823	2748	3454	15317
214	0	0	2207	22182	8771	36980	12062	2537	3524	56360	9753	0	1691	0	1621	0	8618	6155	16741	0	1133	0
215	8562	11019	3736	8601	1886	5363	1746	11233	3492	21007	15423	0	2370	0	1586	0	2985	1002	7930	543	256	0
216	0	386	0	0	1164	969	0	0	0	0	0	2668	0	262	0	0	0	0	0	0	0	0
217	36711	0	3025	0	0	8683	693	0	490	1546	0	0	0	0	0	0	0	0	0	0	0	0
218	569	0	1768	0	0	197	0	0	839	0	624	0	0	0	0	0	0	0	628	0	1467	0
219	420	1569	0	911	0	580	0	0	0	0	408	0	835	0	0	0	2900	512	0	0	552	0
220	3333	1015	0	3241	0	0	0	0	379	0	0	1025	304	0	497	339	794	0	10420	0	529	289
221	2428	928	0	1183	749	520	929	0	701	0	1069	0	0	0	350	0	0	0	0	316	0	1205
222	0	0	0	1045	1035	0	0	0	0	0	447	0	0	0	0	0	0	0	1687	0	0	0
223	751	0	0	0	0	1660	0	0	0	0	0	0	0	530	0	0	1533	425	1598	0	264	0
224	369	0	0	561	0	543	0	0	1310	0	0	0	519	0	0	0	0	0	0	0	3096	657
225	2403	0	0	0	4890	335	0	0	0	403	0	0	0	0	0	0	840	0	11459	0	0	347
226	873	306	550	0	0	0	526	0	0	0	0	0	1355	854	0	0	2691	0	2024	0	1606	0
227	3511	1137	0	888	391	0	4296	0	472	0	0	0	1000	0	0	0	0	489	2879	0	793	0
228	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1304	0	0	0	0	0
229	389	0	0	0	0	0	0	0	0	0	1010	0	321	0	0	0	839	0	0	0	4114	0
230	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	357	0	1193	0	0	337
231	503	375	0	0	1317	972	1360	0	0	0	0	0	870	0	0	0	354	0	0	0	3069	0
232	428	0	0	0	0	784	0	0	0	0	0	0	289	0	0	0	0	0	15115	0	465	0
233	1095	245	0	0	0	0	0	0	0	0	0	0	616	0	0	0	1078	0	0	0	465	0
234	0	0	0	0	1143	0	0	0	517	0	0	0	442	0	0	0	0	0	249	0	0	0
235	818	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1290	0	0	0	885	0
236	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	859	0	0	0	740	0
237	1578	362	0	0	0	0	0	0	0	0	0	0	0	0	0	0	898	0	0	0	0	480
238	2674	1240	491	0	382	0	712	0	1430	498	0	0	722	0	0	0	0	0	701	0	1071	0
239	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1198	0	1099	0	0	0
240	0	642	0	0	0	0	0	0	0	744	0	0	0	0	0	0	448	0	0	0	0	467
241	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6540	0	0	0	0	0
242	0	360	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	241	0	190	0
243	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2361	0	2205	0
244	366	0	0	0	694	520	0	0	509	0	0	0	0	0	0	0	1844	0	4156	0	1662	0
245	543	842	0	452	0	0	0	0	0	0	0	0	0	0	0	0	231	0	1080	0	0	0
246	0	0	0	0	1735	0	3648	0	0	0	0	0	0	0	0	0	4407	1124	0	0	988	0
247	3326	2264	747	0	0	0	0	0	0	0	0	0	0	0	0	0	2968	0	0	0	0	0
248	0	0	0	0	830	1009	0	487	0	0	403	0	0	388	0	0	0	0	0	0	0	0
249	0	774	360	7843	5987	6150	1776	2112	0	1349	434	0	975	0	0	0	2472	0	0	0	359	0
250	4522	0	0	539	0	0	0	0	648	0	0	0	0	0	0	0	0	387	6464	391	0	1364
251	544	8011	1493	0	399	0	0	0	0	0	0	0	1065	0	0	349	0	0	0	0	5279	304
252	0	0	0	0	0	3416	0	0	0	0	287	0	0	0	0	0	729	0	0	0	891	0
253	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	449	0	1283	0	0	0
254	2541	1234	0	0	0	0	524	0	1550	0	0	0	0	0	0	0	509	0	0	0	3569	0
255	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2842	0	0	0	0	0
256	0	0	0	0	456	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	364	0
257	0	0	0	0	0	0	0	0	0	0	0	0	980	0	0	0	1532	0	482	0	0	655
258	0	0	0	386	0	0	0	0	0	0	0	0	0	0	0	0	0	0	495	0	581	0
259	855	0	0	0	0	0	0	0	0	0	0	0	0	0	730	0	0	0	0	0	0	1206
260	0	905	0	0	1118	342	0	0	0	0	0	4398	755	689	0	0	1450	0	1586	399	2409	5741
261	1263	565	0	578	0	0	1886	0	750	0	0	0	0	0	0	0	953	353	0	0	551	0
262	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	364	0	595	0
263	0	294	0	0	0	0	0	0	0	0	0	0	0	0	0	0	467	0	0	0	0	666
264	0	0	0	0	5531	0	0	0	0	0	0	0	481	0	0	0	0	0	0	0	0	0
265	585	503	0	0	3470	0	0	0	0	0	0	0	604	0	0	0	430	0	689	334	1980	0
266	614	337	0	708	254	0	0	0	2303	0	4543	376	415	905	0	0	0	0	4812	0	2170	0
267	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	790	0	258	0	0	1319
268	0	0	0	0	1076	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
269	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1046	0	0	0	0	0
270	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	800	0
271	0	0	0	0	0	0	1219	0	0	0	0	0	0	0	0	0	0	0	591	0	472	0
272	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	516	0	0	0	0	0
273	0	0	0	334	1365	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
274	1205	0	0	2992	9229	3028	0	0	0	636	0	0	0	0	0	0	0	0	0	0	323	0
277	0	0	0	0	0	0	0	0	1107	0	0	0	0	0	0	0	0	0	692	0	0	0
278	4375	1647	0	773	0	0	0	0	0	0	0	0	0	0	0	0	0	267	0	0	0	0
279	338	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	482	0	0	0	1263	0
280	429	0	0	0	0	0	0	0	0	0	0	1620	0	0	0	0	621	0	0	0	364	0
281	301	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
283	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	569	0	0	0	0	0
284	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1046	0
285	0	0	0	0	0	381	0	0	0	0	0	0	0	0	0	0	1624	0	0	0	0	0
286	0	0	0	401	2750	0	0	0	0	0	0	0	0	0	0	0	1053	0	347	0	0	0
287	0	0	0	332	0	0	611	0	0	0	0	0	0	0	0	0	0	0	2018	0	0	0
288	3219	1392	1323	2600	1290	1780	1901	992	0	469	1067	817	7327	908	1647	3666	6176	1261	4488	1663	4440	7330
290	0	495	0	0	0	0	0	0	0	0	0	0	1080	0	0	0	3714	0	354	0	448	0
291	1124	0	0	0	0	0	0	0	0	0	0	636	0	0	0	0	0	0	0	0	0	0
292	0	0	0	0	78924	9216	0	0	1375	0	0	0	0	0	0	0	0	0	0	0	900	371
293	415	636	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	730	0
294	0	0	0	0	1399	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	348	2067
295	1970	0	0	523	24263	592	16277	0	877	0	0	0	0	0	0	0	0	0	795	0	0	0
296	0	0	0	0	0	0	0	0	0	0	1653	0	0	0	0	0	0	0	515	0	2249	0
297	0	678	0	914	0	0	0	0	0	0	0	985	0	0	0	0	420	0	0	0	1145	0
298	1706	0	0	0	2228	696	0	0	0	0	0	0	0	0	0	0	1255	0	0	0	0	0
299	0	0	0	0	779	0	0	0	0	0	0	0	902	0	0	0	0	0	0	0	706	0
300	0	816	0	420	1846	0	0	0	0	0	0	0	0	0	0	0	0	0	422	0	371	0
301	1998	0	0	395	0	0	0	0	0	0	0	0	8270	0	0	0	3745	0	1138	0	5213	0
302	0	0	0	1078	972	0	0	0	1288	0	0	0	0	0	0	0	0	0	0	0	0	0
303	1870	1105	357	0	3233	382	0	0	0	1574	0	0	351	0	0	0	3233	0	0	0	824	436
304	0	0	0	0	0	0	0	0	0	0	336	0	0	0	0	0	300	0	0	0	0	2154
306	0	2181	401	0	1130	397	0	0	0	0	403	0	0	0	0	0	0	670	0	0	0	0
307	0	0	0	3621	0	0	0	0	0	0	0	0	1495	0	0	642	991	0	0	714	1315	0
308	595	0	0	442	6214	510	1298	0	0	750	0	0	0	0	0	0	0	0	0	0	456	1391
309	0	0	0	0	0	0	0	0	581	0	0	0	0	464	0	0	276	0	837	0	1904	0
310	0	1190	0	22174	0	651	1637	2097	0	0	0	0	0	0	0	0	0	5575	0	0	0	0
311	750	0	0	0	514	1202	0	0	0	2508	1970	0	0	0	0	0	2182	0	0	0	548	565
312	1925	959	592	797	4255	674	280	0	0	0	0	449	1707	493	0	1152	640	0	834	648	1368	2176
313	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	482	0
314	1203	1271	782	8069	1574	3130	3262	641	0	5059	1286	486	787	0	0	0	0	5074	3012	1826	386	0

t-RFs (bp)	MIP01	MIP02	MIP03	MIP04	MIP05-2	MIP06-2	MIP07	MIP08	MIP10	MIP11	MIP20	PO1	PO2	PO3	PO4	PO5	PO6	PO8	PO10	S12099	S12512	S20
315	422	0	0	0	0	1893	701	774	983	2888	1114	0	0	414	0	0	271	0	4678	20089	0	15803
316	1638	0	394	0	0	0	551	0	0	0	0	596	0	0	0	0	297	0	0	0	1056	0
317	0	0	0	0	1954	0	0	0	0	0	0	0	479	0	0	0	0	0	0	0	0	0
318	0	0	0	906	6727	2297	0	0	0	0	0	0	0	0	0	0	353	0	0	0	0	0
320	924	0	0	0	0	0	0	0	0	479	0	0	0	0	0	0	0	0	0	0	435	0
321	0	574	0	0	0	0	0	0	591	0	0	0	0	0	0	0	1093	0	0	0	2229	0
322	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	983	0
323	0	0	0	0	0	0	0	0	0	0	0	0	565	0	0	0	0	0	0	0	248	0
324	0	473	0	0	0	0	0	0	0	0	0	844	0	0	0	0	1354	0	0	0	814	240
325	1073	0	0	0	614	0	0	0	0	0	0	607	0	0	0	0	1287	0	0	0	1245	1882
326	0	0	0	0	0	0	0	0	0	0	0	228	0	0	0	0	0	0	0	0	578	0
328	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	356	0	0	0	691	0
329	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	764	0
330	0	0	0	0	750	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
332	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	386	0	0	4352	0	0
333	0	0	0	0	0	0	0	0	0	418	0	0	0	0	0	0	0	0	630	0	0	3175
334	0	0	0	0	554	0	0	0	0	0	0	0	0	0	0	0	481	0	0	0	0	0
336	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1193	0	1015	0
337	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	431	0	0	0	0	0
338	0	0	0	0	0	0	22198	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
340	0	0	0	0	1256	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
367	0	0	0	591	0	0	0	0	0	0	0	0	2147	0	0	0	1450	0	0	0	0	0
372	0	423	544	575	754	0	1004	0	0	0	851	504	1489	0	0	0	1285	429	801	0	1031	0
373	1037	0	0	0	0	947	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1003
374	0	777	0	0	0	0	0	0	0	0	0	0	0	0	0	0	489	316	0	0	0	0
375	0	0	0	0	0	0	0	0	0	0	0	0	635	0	0	0	0	0	0	0	0	0
376	0	0	0	0	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
380	0	0	0	402	0	0	0	0	0	0	0	0	0	590	0	0	0	0	0	0	0	0
385	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1482	0	0	0	0	0
387	0	0	0	906	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
388	395	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
389	0	0	0	0	0	0	0	0	0	0	476	0	0	0	0	0	0	0	0	0	0	0
390	0	0	0	362	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
391	0	0	0	324	0	0	0	0	0	0	0	0	678	0	0	469	0	0	0	508	0	0
392	0	0	0	0	371	0	0	0	0	0	0	0	0	562	0	0	482	0	0	0	757	996
393	0	0	0	0	0	0	0	0	0	0	992	0	0	0	0	0	0	0	0	0	0	0
394	0	0	0	7604	0	0	0	0	0	0	0	0	0	695	0	0	398	0	0	0	0	0
395	0	0	0	0	499	884	1488	1350	0	2594	1552	0	0	0	0	0	729	3472	0	0	385	0
396	0	0	0	0	0	0	4262	422	0	0	769	877	1125	0	0	530	1555	0	0	0	356	0
397	4089	1469	1223	13673	0	0	0	3110	0	0	18177	0	502	566	0	0	587	565	681	0	0	1765
398	1199	3825	937	0	8864	4889	2849	3913	0	22873	7349	385	0	0	0	0	317	2992	0	0	456	0
399	820	2340	530	519	0	1531	629	0	0	0	0	0	0	536	0	0	4822	0	5494	0	0	0
400	0	0	0	2227	0	667	0	2351	0	0	1474	0	0	0	0	0	0	0	428	0	726	0
401	4355	2151	457	0	0	0	1017	0	0	495	0	1971	0	0	0	0	0	0	0	0	0	0
402	0	0	0	0	0	3355	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
403	3169	834	556	0	0	0	0	874	0	13970	27355	13618	647	17609	0	0	0	0	0	0	0	1502
404	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1680	0	0	0	0	0
406	3032	0	0	0	6142	628	1240	0	0	0	0	0	0	0	0	0	407	419	0	0	0	0
407	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	589	0	0	0	846	0
408	0	0	0	0	0	0	0	0	0	0	0	970	693	0	0	0	0	0	0	0	457	0
409	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	917
410	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
413	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	412
414	0	0	0	0	1722	349	0	0	0	0	0	0	0	0	0	0	0	0	0	0	436	0
415	0	0	0	0	0	452	0	0	0	0	0	0	1054	0	0	0	0	0	1507	0	652	0
422	0	0	0	0	0	0	0	410	0	0	999	0	0	0	0	0	0	0	0	0	0	0
425	517	0	0	481	0	0	0	0	0	0	480	0	0	0	0	0	0	0	0	0	0	0
426	0	0	0	0	0	0	0	0	0	1037	0	0	0	0	0	0	0	0	0	0	0	468
427	576	0	0	398	0	0	0	0	0	0	562	0	0	0	0	0	0	0	0	0	0	0
428	610	496	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
437	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1603	0	0
438	0	0	0	0	0	0	936	0	0	0	0	0	0	0	0	0	0	0	598	0	0	1365
442	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
444	0	0	0	0	0	0	0	0	0	0	559	0	0	0	0	0	0	0	0	0	0	0
448	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	434	0	0	0	0	0
449	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1344	0	0	0	0	0
452	0	0	0	0	0	0	0	0	0	0	0	0	1007	0	0	0	0	0	0	0	0	0
459	930	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
461	0	0	0	0	0	0	0	0	0	0	0	0	0	1074	0	0	0	0	0	0	0	0
462	0	0	0	0	0	0	0	0	0	0	584	0	0	0	0	0	0	0	0	0	0	0
463	461	526	0	0	0	510	0	0	0	0	2074	0	0	640	0	0	0	0	0	0	0	0
465	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1133	0	0	0	2095	949	0
466	1197	1061	1230	0	1140	948	1291	0	0	0	809	0	2260	865	0	0	3794	1030	2037	0	0	2737
468	0	0	0	2592	0	0	0	0	23781	0	0	0	0	457	0	0	0	0	0	0	0	0
469	2964	476	0	632	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
470	0	0	0	0	536	0	0	0	0	0	1816	0	0	0	0	0	0	0	0	0	0	0
475	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	697	0
476	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2004	0
478	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	479	0	0	0	0	0
482	508	0	0	0	0	0	728	0	0	0	0	0	0	0	0	0	829	0	0	0	0	0
483	1304	1036	0	0	0	0	0	0	0	413	0	0	0	0	0	0	0	0	0	0	0	0
484	0	0	0	886	0	0	0	0	0	0	358	0	0	0	0	0	0	508	0	0	0	0
485	0	0	0	315	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
486	1907	0	0	800	0	0	2039	0	0	0	0	0	0	0	0	0	0	0	0	931	0	0
487	369	0	0	917	0	0	0	0	0	0	702	0	483	0	0	0	0	393	0	0	0	0
488	0	0	0	1492	0	0	0	0	0	0	983	0	778	0	0	0	0	645	0	0	0	0
489	1233	0	0	274	0	0	0	0	0	0	0	0	0	0	0	0	0	586	0	0	0	0
490	494	0	0	0	0	0	0	0	0	0	0	0	316	0	0	0	0	0	0	0	0	0
491	0	0	0	516	6731	1275	0	0	0	0	295	0	0	0	0	0	0	531	0	0	0	0
492	481	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	308	0	0	0	0
494	0	3877	853	17365	0	3708	721	0	0	0	15617	0	9038	0	0	0	2114	0	0	0	0	0
495	22915	0	5138	115084	10400	17851	2996	0	0	0	82251	0	51611	0	0	0	0	11563	0	0	0	0
496	106904	23872	0	0	0	0	0	0	17005	0	0	0	0	0	0	0	0	62910	0	0	456	0
497	0	4374	2683	0	1264	0	0	568	16025	0	0	0	0	0	0	0	0	5451	0	0	0	1268
498	0	363	350	851	3328	0	0	0	0	0	0	0	0	0	0	0	0	40896	0	0	0	0
499	0	0	0	0	0	0	0	0	589	0	0	0	0	0	0	0	605	0	0	0	0	0