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Combined use of ISCR and biostimulation techniques in incomplete processes of reductive dehalogenation of chlorinated solvents

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Abstract

Pools of chloroethenes are more recalcitrant in the transition zone between aquifers and basal aquitards than those elsewhere in the aquifer. Although biodegradation of chloroethenes occur in this zone, it is a slow process and a remediation strategy is needed. The aim of this study was to demonstrate that combined strategy of biostimulation and *in situ* chemical reduction (ISCR) is more efficient than the two separated strategies. Four different microcosm experiments with sediment and groundwater of a selected field site where an aged perchloroethene (PCE)-pool exists at the bottom of a transition zone, were designed under i) natural conditions, ii) biostimulation with lactic acid, iii) *in situ* chemical reduction (ISCR) with zero valent iron (ZVI) and under iv) a combined strategy with lactic acid and ZVI. Biotic and abiotic dehalogenation, terminal electron acceptor processes and evolution of microbial communities were investigated for each experiment. The main results where: i) limited reductive dehalogenation of PCE occurs under sulfate-reducing conditions; ii) biostimulation with lactic acid promotes a more pronounced reductive dehalogenation of PCE in comparison under natural conditions, but resulted in an accumulation of *cis*-dichloroethene (cDCE); iii) ISCR with zero-valent iron (ZVI) facilitates a sustained dehalogenation of PCE and its metabolites to non-halogenated products, however, the iv) combined strategy results in the fastest and sustained dehalogenation of PCE to non-halogenated products in comparison of all four set-

ups. These findings suggest that biostimulation and ISCR with ZVI are the most suitable strategy for a complete reductive dehalogenation of PCE-pools in the transition zone.

Key words: anaerobic microcosm experiment; transition zone to the basal aquitard; zero-valent iron (ZVI); compound specific isotopic analysis (CSIA); terminal restriction fragment length polymorphism (T-RFLP).

1. Introduction

Chloroethenes are chlorinated solvents that belong to the group of dense non-aqueous phase liquids (DNAPLs) and have been detected in numerous contamination events (Tiehm and Schmidt, 2011). These compounds have an elevated toxicity (Moran et al., 2006), and in the case of perchloroethene (PCE), trichloroethene (TCE) and vinyl chloride (VC), the risk of cancer increases under exposure (USEPA, 2009).

Parker et al. (2003) described transition zones between granular aquifers and basal aquitards as a reasonable paradigm for the DNAPL source area architecture in granular aquifers. Transition zones are made up of numerous thin silty-clay layers interstratified with coarser-grained layers (i.e., sands and gravels), which decreases the contaminant mobility. Therefore, the higher recalcitrance of DNAPL sources in these zones has far-reaching implications for the environment.

Chloroethenes may be recalcitrant under certain conditions over long periods (several decades or longer). However, they can be degraded under anoxic conditions by biotic reductive dehalogenation (Bradley, 2003; Bradley and Chapelle, 2011; Wiedemeier et al., 1998) carried out by organohalide-respiring bacteria (OHRB, Adrian and Löffler, (2016)). Reductive dehalogenation of chloroethenes occurs sequentially from PCE to TCE to 1,2-cis-dichloroethene (cDCE), which is the most common metabolite in TCE biodegradation (Bouwer, 1994), to VC, and to ethene (Tiehm and Schmidt, 2011; Vogel et al., 1987). Reductive dehalogenation of PCE and TCE to cDCE can be carried out by a wide range of microorganisms such as *Dehalococcoides*, *Geobacter*, *Dehalobacter*, *Desulfitobacterium*, *Sulfurospirillum*, *Anaeromyxobacter*, *Desulfomonile*, *Desulfovibrio*, *Desulfuromonas* and *Dehalogenimonas* spp. (Atashgahi et al., 2016;

Nijenhuis and Kuntze, 2016). However, only *Dehalococcoides* spp. have been described as capable of the complete reductive dehalogenation of PCE/TCE (Maymó-Gatell et al., 1997; Zinder, 2016).

The reductive dehalogenation of PCE and TCE may occur under nitrate- (van der Zaan et al., 2010), Mn- and Fe-reducing conditions as well as under sulfate-reducing and methanogenic conditions (Bouwer, 1994; Bradley, 2003; Bradley and Chapelle, 1996), especially if an excess of electron donors is supplied to achieve substantial dehalogenation (Aulenta et al., 2007). The reductive dehalogenation may be wholly or partially inhibited by competition for electron donors depending on environmental conditions. This competition occurs between OHRB and anaerobic hydrogenotrophic (including reducers of NO_3^-, Mn^{4+}, Fe^{3+} and SO_4^{2-}), autotrophic methanogenic, and homoacetogenic microorganisms (Wei and Finneran, 2011).

High concentrations of chloroethenes in the contaminant source may inhibit microbial activity (National Research Council, 1999; Philips et al., 2013), causing a decrease in the microbial richness of the population due to their toxicity (Haack and Bekins, 2000). This potential inhibition of microbial activity does not affect all chloroethene-biodegrading microorganisms equally, inducing a specialization in the microbial community, which decreases the microbial richness of the population (Sleep et al., 2006). For example, *Dehalobacter restrictus* PER-K23 (Holliger et al., 1998), *Desulfuromonas chloroethenica* TT4B (Krumholz, 1997), *Sulfurospirillum halorespirans* DSM 12446 T (Luijten et al., 2003), and *Dehalococcoides mccartyi* (Maymó-Gatell et al., 2001) are completely inhibited by high concentrations of PCE. By contrast, other species such as *Desulfitobacterium* Y51 (Suyama et al., 2001), *Clostridium bifermentans* DPH-1 (Chang et al., 2000), *Enterobacter agglomerans* MS-1 (Sharma and McCarty, 1996), and *Desulfuromonas michiganensis* BB1 and BRS1 can dehalogenate PCE and/or TCE even at saturation concentrations (Sung et al., 2003). In addition, high concentrations of chloroethenes may inhibit the activity of microorganisms that potentially compete with OHRB, such as, for example, methanogenic populations (Yang and McCarty, 2002).

In situ chemical reduction (ISCR) with zero-valent iron (ZVI) has been proven as an efficient strategy to dehalogenate chloroethenes (Gillham and O'Hannesin, 1994; VanStone et al., 2004). The reaction mechanisms of ZVI to reductively dehalogenate chloroethenes are complex and produce different end-

products depending on the conditions (Elsner et al., 2008; VanStone et al., 2004; Vogel et al., 1987). For example, Orth and Gillham (1996) found that 80% of TCE was mainly dehalogenated to ethene and ethane (in a ratio of 2:1), but with additional products, such as methane, propane, propene, 1-butene and butane. Other researchers detected other products during abiotic reductive dehalogenation of PCE and TCE with ZVI (Campbell et al., 1997), e.g. VC, cDCE, 1,1DCE, methane, chloroacetylene, ethine, ethene, ethane. The great variety of abiotic degradation pathways by ZVI potentially avoids the accumulation of toxic byproducts (such as VC), in contrast with biodegradation, that may build-up in the aquifer (Brown, 2010).

Each remediation strategy presents several limitations. For example, the accumulation of metabolites in the case of biostimulation and the relatively long time of application in the case of monitored natural attenuation (MNA) and ISCR with ZVI. Consequently, different remediation strategies can be applied sequentially (Brown, 2010) or combined (Henry, 2010). It is common to sequentially apply a remediation strategy in the source (such as biostimulation or injection of chemical products) and subsequently apply a MNA in the plume. In other cases, when the biostimulation of chloroethenes with lactic acid lead to an accumulation of cDCE (Lorah et al., 2008), a second strategy is needed to completely dehalogenate this compound, such as bioaugmentation (Ellis et al., 2000), ISCR or oxidative biostimulation. Otherwise, ISCR technologies are usually applied in an organic emulsion, which increases the disponibility of ZVI (Quinn et al., 2005); therefore, it also increases the abiotic dehalogenation of chloroethenes, while OHRBs are stimulated. Recent studies are proving the efficiency of combined injection of ZVI and an organic substrate. For instance, Peng et al. (2017) proved the efficiency of nZVI and biochar injection for p-nitrophenol degradation under anoxic conditions. Also, Kocur et al. (2016) proved that combined injection of nZVI and carboxymethyl cellulose positively impacted remediation of chloroethenes by promoting growth of anaerobes and dechlorinating bacteria.

For a better understanding of the processes affecting the fate of chloroethenes, an integrative set of chemical and biological monitoring tools is needed. For instance, there is a need to monitor the different terminal electron acceptor processes (TEAPs; Puigserver et al. (2016b)). Moreover, compound-specific stable isotope analysis (CSIA) has been applied efficiently as direct proof of the biological degradation of chlorinated solvents and to distinguish the different processes affecting the fate of these pollutants (Elsner, 2010; Hunkeler et al., 2008; Hunkeler and Aravena, 2010; Wiegert et al., 2013). CSIA is based on

109 the evidence that bonds formed by heavy isotopes (^{13}C) are slightly more stable and, consequently,
110 cleaved slower than bonds between lighter isotopes (^{12}C). As a result, the remaining fraction of the
111 substrate becomes isotopically enriched as a reaction proceeds. Different molecular techniques have
112 been used to investigate the complexity of the reductive dehalogenation processes in microbial systems.
113 To characterize the microbial consortia in the presence of chlorinated solvents, terminal restriction
114 fragment length polymorphism (T-RFLP) has been used efficiently in laboratory experiments (Flynn et al.,
115 2000; Mészáros et al., 2013; Révész et al., 2006) and in field studies (Lendvay et al., 2003; Macbeth et al.,
116 2004; Rahm et al., 2006; Richardson et al., 2002).

117 The aim of this article was to define an efficient bioremediation strategy to treat a source of chloroethene
118 in sand layers interbedded with silts (i.e. the transition zone to the basal aquitard). Therefore, a
119 combination of biological and chemical strategies to achieve better efficiency was investigated. Four
120 different microcosm experiments were designed under i) natural conditions, ii) biostimulation with lactic
121 acid, iii) *in situ* chemical reduction (ISCR) with ZVI and under iv) a combined strategy with lactic acid and
122 ZVI. Microcosm experiments have been successfully used to choose the most efficient remediation
123 strategy (ITRC, 2004; Morse et al., 1998; Wiedemeier et al., 1998) and to determine how geochemical
124 conditions would change and microbial communities would adapt (Lu et al., 2009; Puigserver et al., 2016b)

125 The working hypothesis of this investigation was, that the main limiting factors of biological reductive
126 dehalogenation of chloroethenes in the presence of DNAPL are toxicity and electron donor availability.

127

128 **2. Methods**

129 **2.1. Site description and**

130 The area under study is a confined aquifer made up of Pliocene prograding alluvial fan deposits. The site
131 is located in an industrial area in Vilafant (Alt Empordà, NE Spain), approximately 150 km to the north of
132 Barcelona. PCE contamination was detected at the site in 1980 by the Catalan Water Agency (ACA), but it
133 is not known when this originated. The main contaminant is PCE, which was used as a degreaser of vehicle
134 parts at a nearby industrial plant serving the automotive industry. Puigserver et al. (2016a) located the

source of the PCE in a transition zone to a basal aquitard (lower section of the aquifer between the depths of 5.60 and 7.50 m). Although there is evidence that reductive dehalogenation is active, it has been proven that natural attenuation is not a viable strategy in the middle term and that the source should be treated (Puigserver et al., 2016a).

2.2. Design of microcosm experiments

Four remediation strategies were studied: natural attenuation, biostimulation with lactic acid, *in situ* chemical reduction (ISCR) with ZVI and a combined strategy with lactic acid and ZVI. Each experiment consisted of two live (biotic) and two abiotic (autoclaved) controls. An autoclave (Selecta Model Autester 75 E DRY-PV) was used (for four periods of 30 minutes reaching a temperature of 121 °C, a pressure of 1 atm, and saturated vapor conditions) to sterilize the control microcosm bottles containing 1200 mL of groundwater, 250 mL of sediment, and 50 mL of stock solution with 147 mM $HgCl_2$ (Riedel-de Haën, CAS 7487-94-7) as a bactericide, following Trevors (1996). The remaining materials were cleaned and sterilized with methanol (MeOH, Merck, ISO Pro analysis). Experiments were conducted in an anaerobic chamber (Glove-type box, Coy Laboratory Products Inc.).

The sediment used in the experiments was from transition zone to a basal aquitard (borehole B-F2UB, between 6.77 and 7.46 m) made up of sand layers and interbedded silts that are rich in organic matter, Fe and Mn (foc = 0.016%, Mn = 5.7 mmol/g and Fe = 174.1 mmol/g; Puigserver et al., 2016)). Groundwater for the experiments was pumped from conventional well S3 (located 3 m from B-F2UB) and collected in Pyrex bottles (1 L). Sediment and groundwater were stored in a cold room at 4 °C in total darkness until use. Groundwater initially showed oxidizing conditions, with dissolved oxygen, NO_3^- and SO_4^{2-} concentrations of 1.55, 100 and 60 mg/L, respectively, and concentrations of Mn^{2+} and Fe^{2+} below detection (Puigserver et al., 2016a). Dissolved oxygen content was reduced to <0.1 mg/L by purging with N_2 gas (as described by Chen et al. 2008) for 60 min to promote the most favorable conditions for the reductive dehalogenation of chloroethenes.

Each bottle was filled with 850 g of homogenized sediment and 1100 mL of groundwater, which represents 17% for sediment and 55% for groundwater of the total volume of the bottle. No injection of exogenous microorganisms has been made. As the bottles had a capacity of 2000 mL, the remaining 28%

was the anaoxic atmosphere of the chamber (95% N_2 and 5% H_2). In ISCR and the combined strategy microcosm experiments, a total of 5 g of granular ZVI (Panreac Quimica, iron metal fine granulated QP 99% 10-40 mesh) was added. Due to the loss of PCE during the purge of dissolved oxygen, 10 µL of of PCE (Sigma-Aldrich, reagent grade, 99.9%) was added at a final concentration of 130 mM. Bottles were sealed with Mininert® valves (SUPELCO analytical) and insulating tape. Furthermore, in the anaerobic chamber, all bottles were arranged horizontally on shelves and covered by a thick black cloth to be preserved in complete darkness until usage.

Periodically, 2 mL of lactic acid (Sigma-Aldrich, 85%) was injected in the microcosm experiments of biostimulation and combined strategy. Additionally, periodically, 8 mL of stock solution 6% $HgCl_2$ (Riedel – de Haën, 99.5%, 31005) was injected in all control experiments.

Water samples from the microcosm experiments were collected to study the time evolution of 1) concentrations of the main inorganic electron acceptors (SO_4^{2-}, NO_3^- and NO_2^-), acetate, chloroethenes (PCE, TCE, isomers of DCE, and VC), ethine, ethene, ethane, methane, Mn^{2+} and Fe^{2+}; 2) carbon isotope values of chloroethenes; and 3) microbial communities. Sodium azide (N_3Na Fluka, purum pa) was added to the microcosm water samples immediately after being collected to inhibit bacterial activity. Before analysis, vials containing water and gas samples were stored at 4 °C in total darkness. In the case of microbial analysis, a total of 20 mL of aqueous phase was taken with a sterile syringe. Then, water was filtered with a filter system (Swinnex, Millipore) and 0.2 µm filters (IsoporeTM membrane filters, Millipore). Filters were kept in sterile Eppendorf cones and stored at - 20 °C until further extraction and analysis.

Characterization of microbial communities was based on only one of the duplicates of the active experiments. To assess the reproducibility of the experiments, duplicates of natural attenuation and ISCR experiments were performed. The bacterial community of time 0 (8 days from the beginning of the experiments) of the natural attenuation experiment was taken as the initial bacterial community. Subsequently, four bacterial communities were sequenced by clone library to characterize the dehalogenating bacterial community.

2.3. Chemical analysis

All chemical analyses were conducted in the laboratories of Scientific-Technical Services at the University of Barcelona. Gas chromatography-mass spectrometry (GC-MS) was used to determine chloroethenes in water samples by head-space analysis. The limits of quantification of PCE, TCE, cDCE, tDCE, 1,1-DCE and VC were 2.16, 1.92, 1.68, 1.68, 1.62 and 1.31 µg/L, respectively (i.e., 0.0130, 0.0146, 0.0173, 0.0173, 0.0167 and 0.0210 µmol/L). Carbon isotope analyses on chloroethenes were performed using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS, Delta Plus XP model, Thermo Fisher Scientific) to determine $\delta^{13}C$ values in the chloroethenes of the water samples. These analyses were performed in duplicate and followed a protocol that involved removal of VOCs by direct adsorption from the aqueous phase (Palau et al., 2007). Extraction of each sample was performed by inserting an adsorbent fiber (Supelco; SPME Fiber Assembly 75 µm Carboxen PDMS) into 20 mL of water stored in a glass vial (SUPELCO analytical) closed with a septum of silicone and with the water sample in continuous agitation for 30 min to adsorb the VOCs on the fiber. The standards used were PCE, TCE, and cDCE (Sigma-Aldrich) determined using Elemental Analyzer Flash EA 1112 coupled to an IRMS delta C Thermo Fischer Scientific. The carbon isotope composition is reported in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (Coplen et al., 2006). The isotope fractionation was calculated by application of the Rayleigh-eq (eq 1) where R represents the isotope ratio ($R = {}^{13}C/{}^{12}C$), C expresses the chloroethene concentration, subscripts 0 and t refer to the beginning and a later time point t of the degradation process, and ε is the enrichment factor, correlating changes in concentration to changes in isotope composition. The weighted average of the chloroethenes (CEs) isotope signature or isotope mass balance, $\delta^{13}C_{\Sigma(CEs)}$ (eq 2), is used to assess if sequential reductive dehalogenation ends in chlorinated or non-chlorinated products (Aeppli et al., 2010), where χ represents the mole fraction. As a consequence of the incomplete sequential reductive dehalogenation, $\delta^{13}C_{\Sigma(CEs)}$ remains constant. On the other hand, $\delta^{13}C_{\Sigma(CEs)}$ increases beyond the source $\delta^{13}C$-value of PCE in the case of dehalogenation to nonchlorinated products.

$$\ln(R_t/R_0) = \varepsilon \times \ln(C_t/C_0) \quad (1)$$

$$\delta^{13}C_{\Sigma(CEs)} = \delta^{13}C_{PCE}\chi_{PCE} + \delta^{13}C_{TCE}\chi_{TCE} + \delta^{13}C_{cDCE}\chi_{cDE} + \delta^{13}C_{tDCE}\chi_{tDCE} + \delta^{13}C_{1,1DCE}\chi_{1,1DCE} + \delta^{13}C_{VC}\chi_{VC} \quad (2)$$

215 NO_3^-, NO_2^- and SO_4^{2-} were analyzed using ion chromatography (IC) following EPA protocol 9056, with a
216 limit of quantification of 0.1 mg/L. Fe^{2+} and Mn^{2+} were analyzed using absorbance spectrophotometry
217 (Reactive tests 14761 and 14770 for Fe^{2+} and Mn^{2+}, respectively, using Spectroquant NOVA60, Merck),
218 with a limit of quantification of 0.005 mg/L. Acetate was analyzed using HPLC (Agilent 1100) following the
219 protocol for organic acids, with a limit of quantification of 1 mg/L. CO_2 was removed by a $CaCO_3$ trap to
220 determine the concentrations of methane, ethane, ethene and ethine. Semiquantitative concentration of
221 gases was determined using gas chromatography (GC).

222

223 **2.4. Molecular analysis**

224 Molecular analyses were conducted to verify the presence of bacterial communities in water samples and
225 to analyze their role in the biotransformation of chloroethenes. The analyses were performed at the
226 laboratories of Helmholtz Centre for Environmental Research–UFZ (Leipzig-Germany). Genomic DNA was
227 extracted from filters using Kit Ultra Clean Soil DNA (MoBio) following the manufacturer's protocol to
228 perform terminal-restriction fragment length polymorphism (T-RFLP) and clone library analysis.

229 PCR was used to amplify part of the 16S rRNA genes from Eubacteria. The PCR mix per reaction contained
230 10 µL de GoTaq® Green Master Mix (Promega), 0.5 µL (each) forward and reverse primers (10 µM,
231 Promega), 1.5 µL from the template and 7.5 µL molecular-grade water (Promega, Madison, WI, USA).
232 Eubacterial primers 27f (Lane, 1991) and 1492r (Lane, 1991) were used to amplify nearly the complete
233 16S rRNA gene using the following scheme: 95 °C (15 min); followed by 25 cycles of 95 °C (45 s), 52 °C (45
234 s) and 72 °C (120 s); and completed with an additional 15 min at 72 °C. If there was a positive signal, the
235 same conditions of PCR were repeated with fluorescent primer 27FAM in order to perform T-RFLP
236 analysis. If there was a negative sign, a second round of PCR for T-RFLP analysis employing universal primer
237 1378r (Heuer et al., 1997) and fluorescent primer 27FAM was completed. The same master mix was used
238 with the addition of 1 µL from the PCR product. The PCR scheme was 95 °C (15 min); followed by 30 cycles
239 of 95 °C (45 s), 52 °C (45 s) and 72 °C (120 s); and completed with an additional 15 min at 72 °C. The PCR
240 product was purified using purification Kit Wizard® for Genomic DNA (Promega). A total of 50 ng of
241 purified DNA was restricted twice for each sample with three different restriction enzymes (HaeIII, HhaI

242 and MspI, Thermo Scientific) and their respective buffers. Dry DNA was dissolved with Hi-DiTM Formamid
243 (Applied Biosystems) using the standard GeneScan™ 500 ROX™ and was analyzed using an ABI 3100
244 Genetic Analyzer (Applied Biosystems) and the Genemapper 3.7 Software (Applied Biosystems).

245 Clone libraries of four water samples were established to analyze the bacterial community. Clones of four
246 water samples were sequenced to characterize the microbial communities responsible of reductive
247 dehalogenation. These samples correspond to a 1) bacterial community of a natural attenuation
248 experiment of T5 after 267 days at which time point reductive dehalogenation of PCE and TCE was
249 detected; 2) a bacterial community of ISCR with a ZVI experiment of T5 at which time point reductive
250 dehalogenation of PCE and TCE was detected (267 days); 3) a bacterial community of the combined
251 strategy experiment of T1 at which time point reductive dehalogenation of PCE and TCE was detected (22
252 days); and 4) a bacterial community of a combined strategy experiment of T5 at which time point
253 reductive dehalogenation of cDCE and VC was detected (267 days). These four samples were chosen to
254 distinguish the bacterial community responsible for the reductive dehalogenation of chloroethenes and
255 to identify restriction fragments (RFs) from T-RFLP. The PCR products obtained with primers 27f and 1492r
256 and extracted genomic DNA as previously described were ligated into the pGEM-TEasy™ vector (Promega,
257 Madison, WI, USA) and were transformed into competent *E. coli* JM109 cells. Procedures of plasmid
258 extraction, amplification, grouping into OTUs, purification, and sequencing were performed following the
259 protocol from Imfeld et al. (2010).

260

261 **2.5. Molecular data treatment**

262 T-RFLP results were used to determine the microbial diversity (microbial richness). Microbial diversity was
263 assessed with the number of RF greater than 50 bp and greater than 1% of the total area. From the three
264 different results obtained (one for each restriction enzyme), the larger was taken as valid. The actual
265 microbial diversity is 3 or 4 times higher than the number of RFs, according to Liu et al. (1997) and Marsh
266 et al. (2000).

267 The density of the microbial community (degree of development) was estimated qualitatively by checking
268 the presence or absence of a signal in the first round of PCR with primers 27f-1492r. Therefore, bacterial

communities were characterized by a high degree of development if there was a sign in the first round and with a low degree of development if there was only a sign in the second round of PCR.

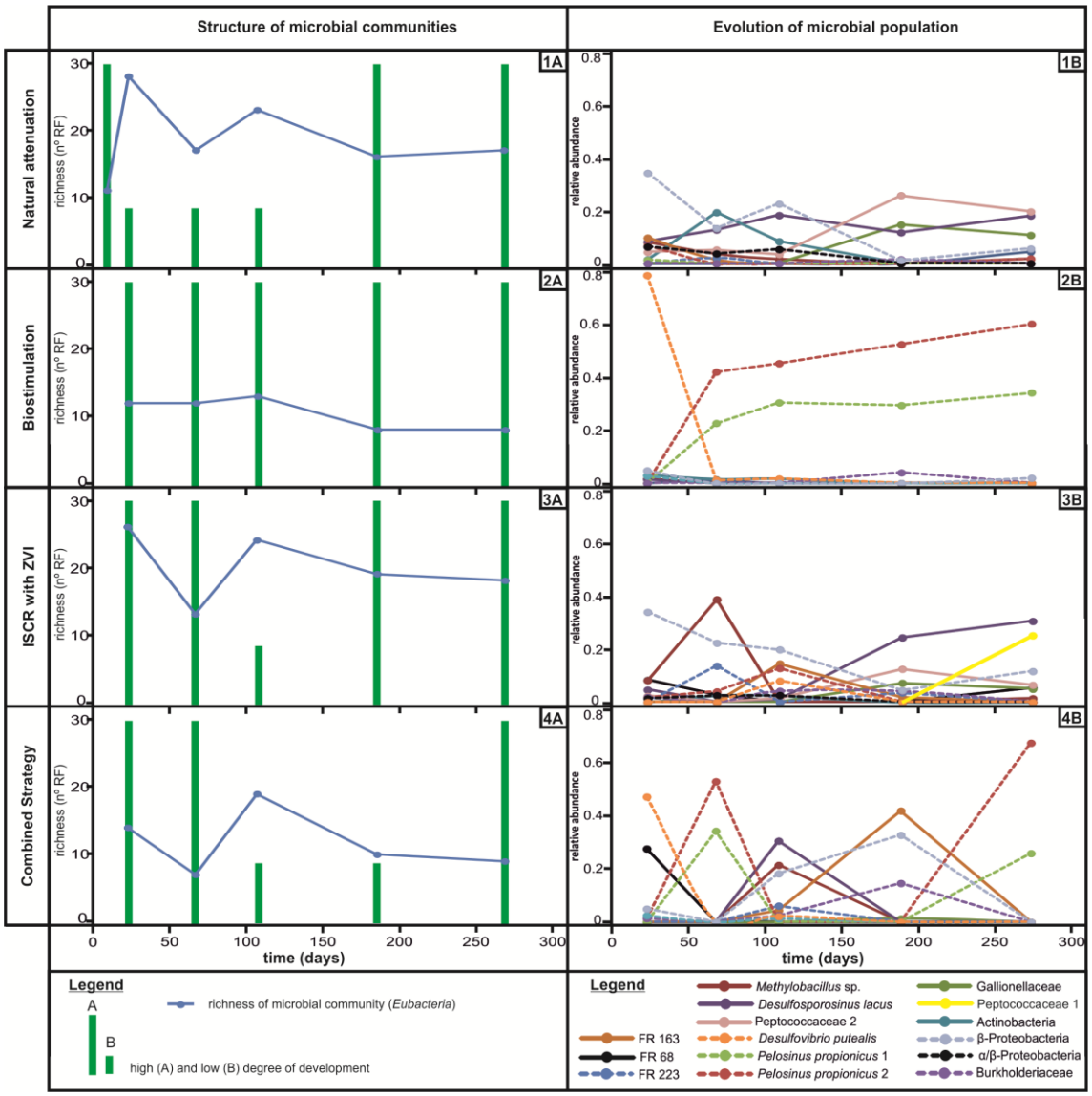
Sequences from clone library analysis were compared to sequences from databases using the BLASTN search tool (http://www.ncbi.nlm.nih.gov/blast/) and mapped onto the NCBI taxonomic hierarchy using the metagenome analyzer (MEGAN) to define the most likely ancestor for each query sequence (version 5.2.3; Huson et al., 2011). Sequences were virtually digested with restriction enzymes HaeIII, HhaI and MspI. When there was a 100% positive match between T-RFLP results and virtual digestion, RFs were positively identified. If there was no positive match, RFs were identified through the T-RFLP database or remained as unknown RF.

3. Results and discussion

3.1. Natural attenuation

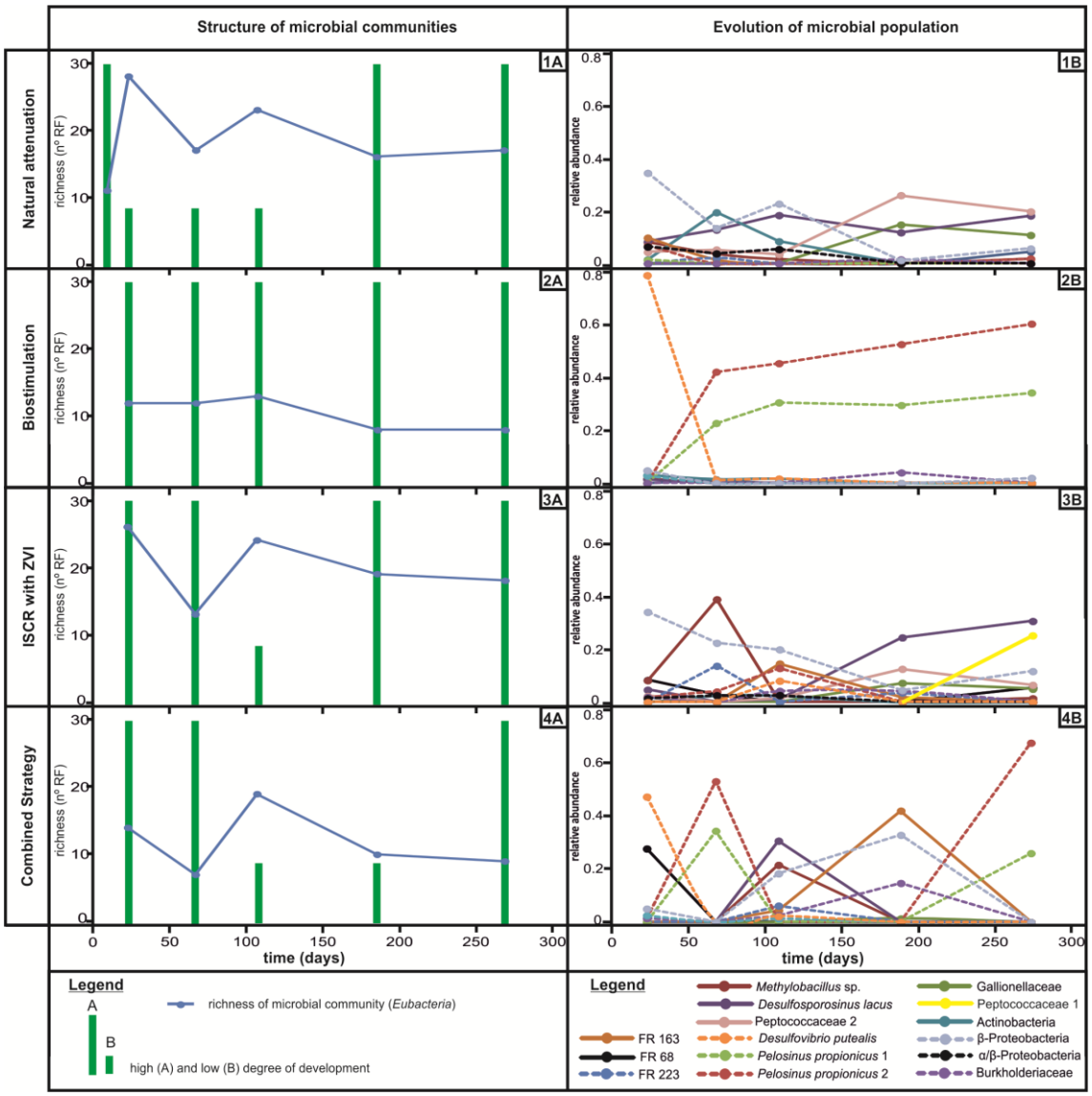
Biotic reductive dehalogenation processes were active from day 106, with a decrease in PCE concentration (from 130 to 66 µM) and a respective increase in TCE, cDCE and 1,1DCE concentration (Figure 1.2A) and a slight shift to more positive values of $\delta^{13}C_{PCE}$ (from -26.5‰ to -22.7‰) and $\delta^{13}C_{TCE}$ (from -31.2 ‰ to -26.5 ‰) (Figure 1.3A). These reductive dehalogenation processes seem to be most efficient under sulfate-reducing conditions (Figure 1.1A, from day 185). Biotic reductive dehalogenation led to a small isotopic fractionation of PCE ($\varepsilon < -1‰$, Table 1), similar to carbon isotope fractionation measured at the studied site (Herrero et al., n.d.) as well as to the literature (Hunkeler and Morasch, 2010). Further products of biotic reductive dehalogenation of cDCE (e.g. VC and ethene) were not detected (Figure 1.2A and Table 1) and chloroethenes remained balanced (Table 1). Abiotic controls show no variation in PCE, nitrate and sulfate concentrations, absence of TCE, cDCE and other metabolites of PCE and an increase of Mn^{2+}.

Bacterial communities associated with the sulfate reduction and reductive dehalogenation of PCE and TCE are characterized by a well-developed bacterial community (



292

293 Figure 2.1A), a mid-to-high richness (



294

295 Figure 2.1A) and the predominance of the Firmicutes phylum (

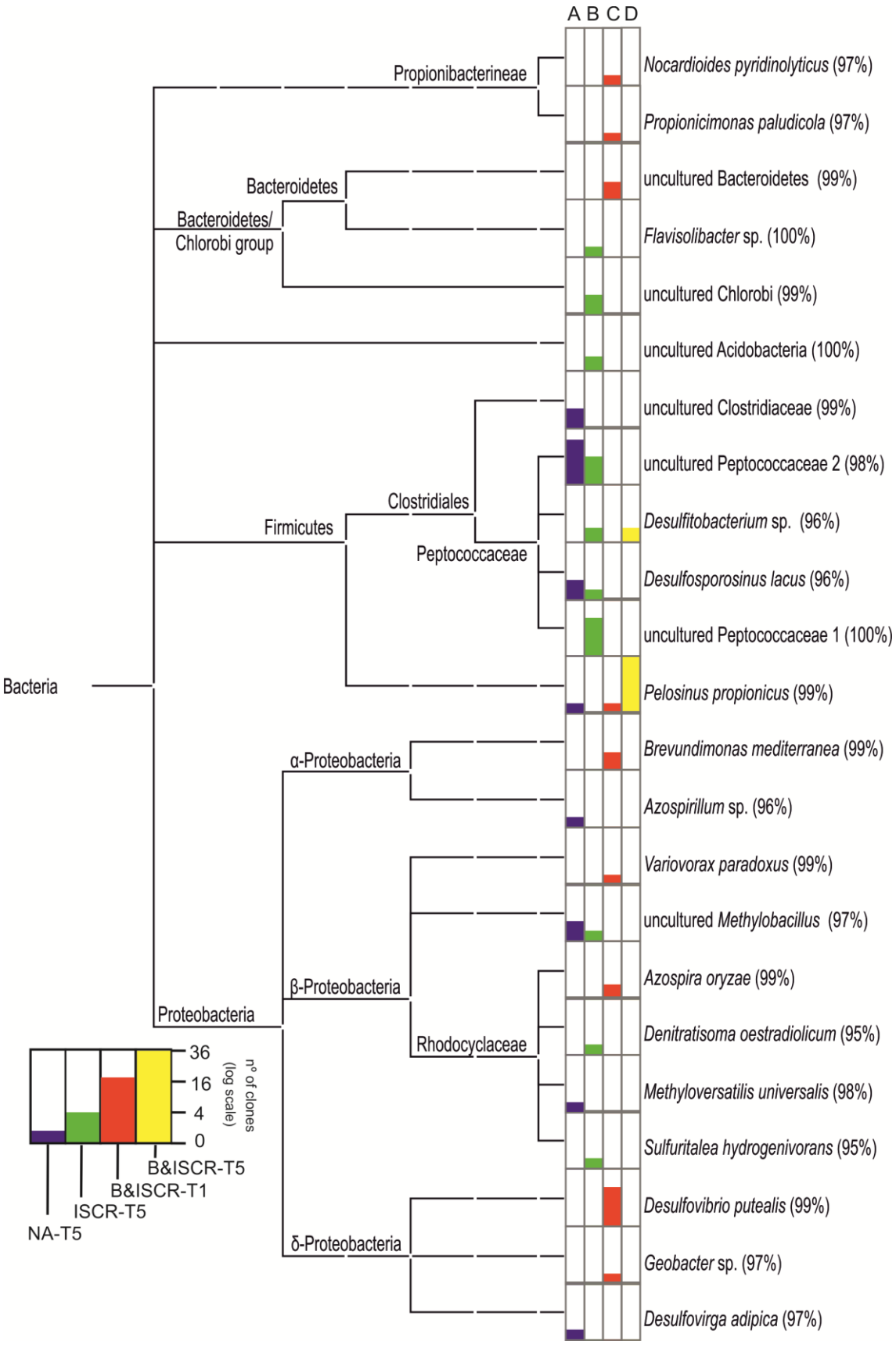


Figure 3.A). Specifically, these bacterial communities were characterized by the presence of fermenting microorganisms of the Peptococcaceae family (

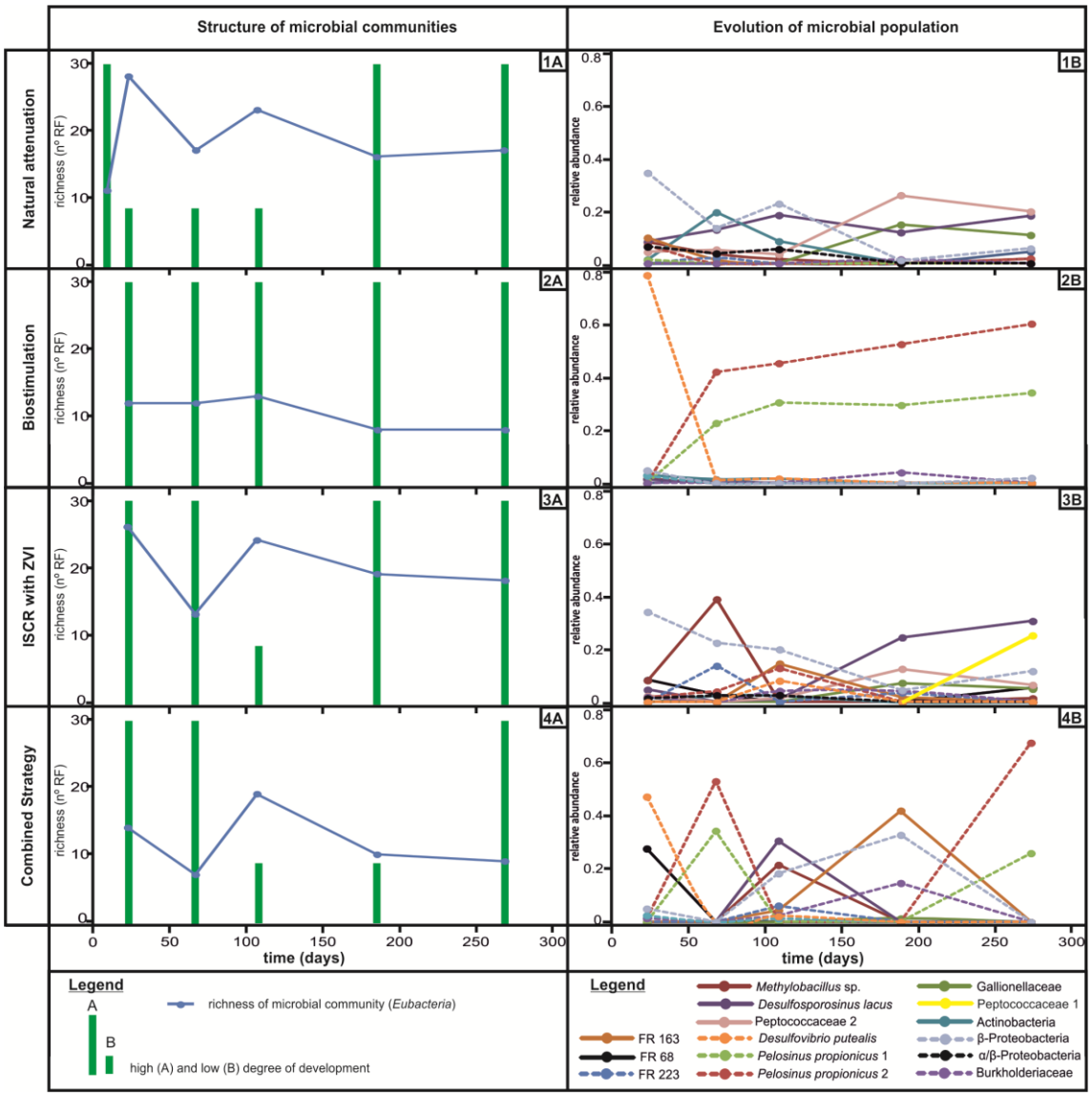


Figure 2.1B) (Patil et al., 2014) and bacteria related to *P. propionicus* (Boga et al. 2007 and Shelobolina et al. 2007), sulfate reducers *D. lacus* and *D. adipica* (Robertson et al., 2001) and Fe reducers in the Gallionellaceae family (Hallbeck and Pedersen, 1991). No OHRBs were detected; thus, it can be assumed that these microorganisms were a proportionally smaller part of the bacterial community (as described by Sercu et al., 2013), although some of the sulfate-reducing bacteria may degrade TCE and PCE, as described by Bagley and Gossett (1990), Löffler et al. (2003) and Mohn and Kennedy (1992).

The biotic reductive dehalogenation process does not occur or is not significant prior to sulfate reduction because NO_3^-, Mn^{4+} and Fe^{3+} compete with PCE and TCE as electron acceptors. PCE concentration decreases and TCE and cDCE concentration increases (Figure 1.2A) only when sulfate reduction is detected from day 185 (Figure 1.1A). Therefore, denitrification and Fe and Mn reduction processes are

thermodynamically more favored than the reductive dehalogenation process. During denitrification and the reduction of Fe and Mn, bacterial communities are well developed (high degree of development from day 185,

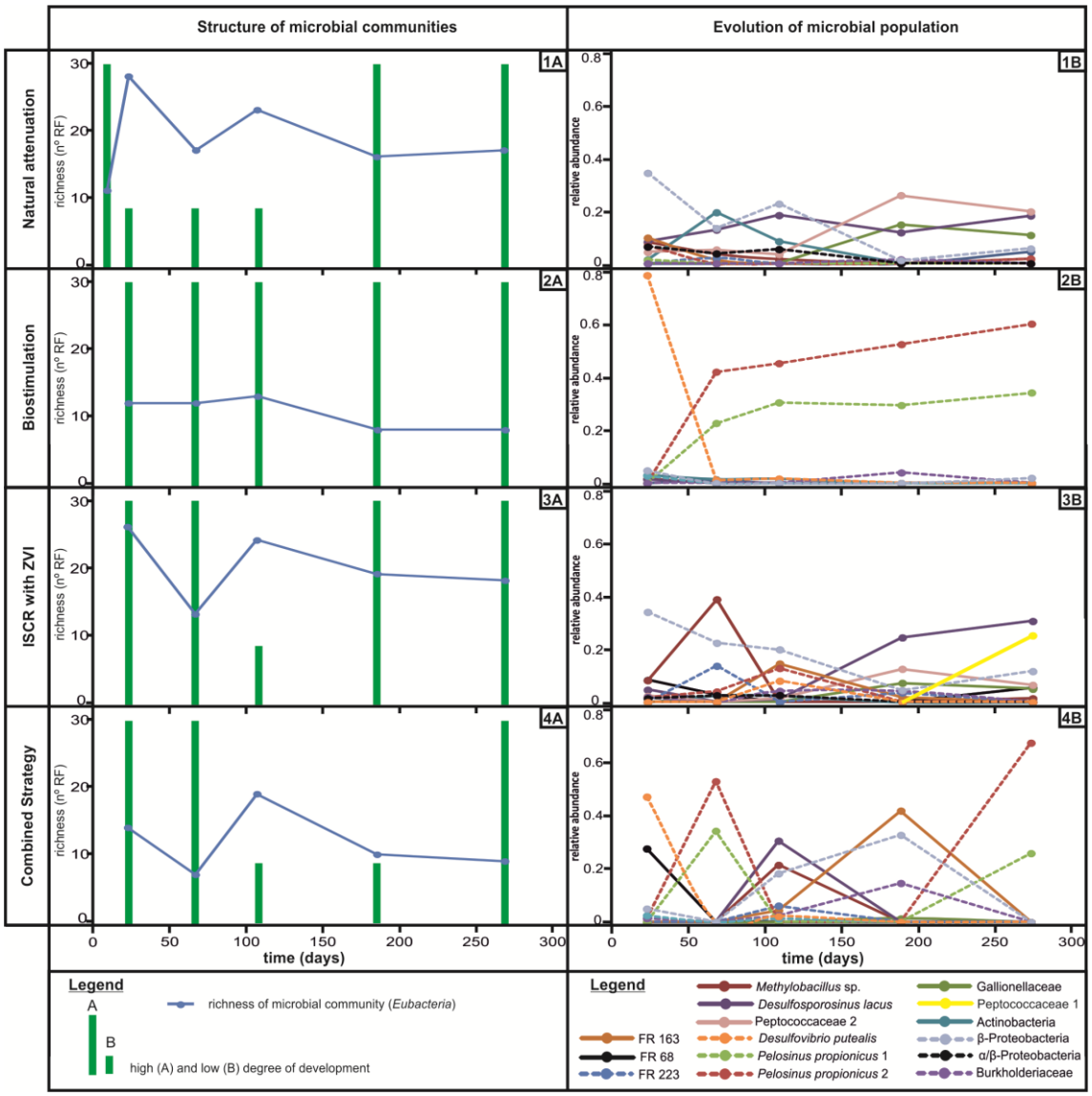
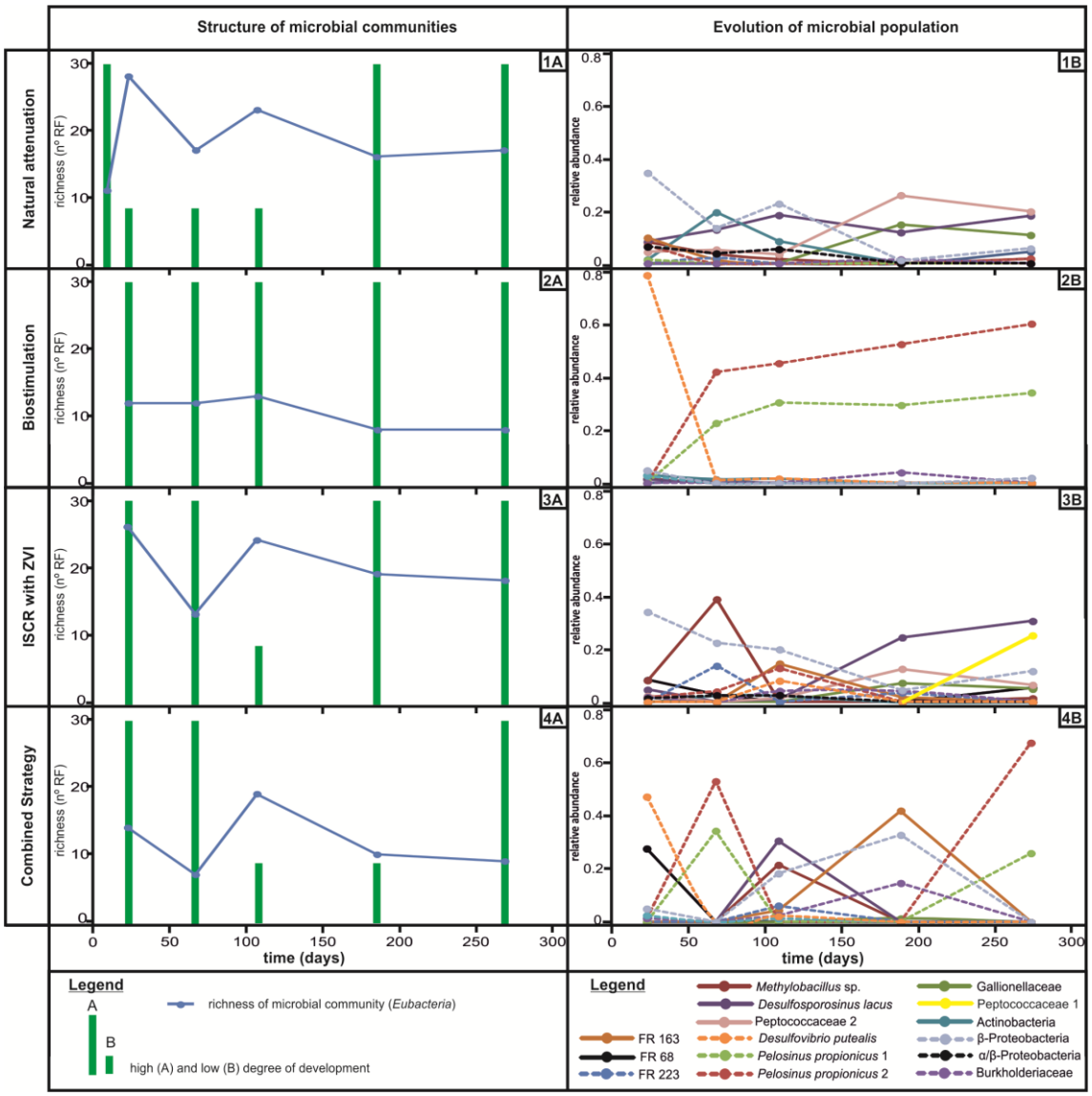


Figure 2.1A), and several populations have been identified whose functions are unknown, with the exception of the fermenting bacteria of the Peptococcaceae family (



316

317 Figure 2.1B). Subsequently, a bacterial community undergoes a lag phase (a less-developed bacterial

318 community,

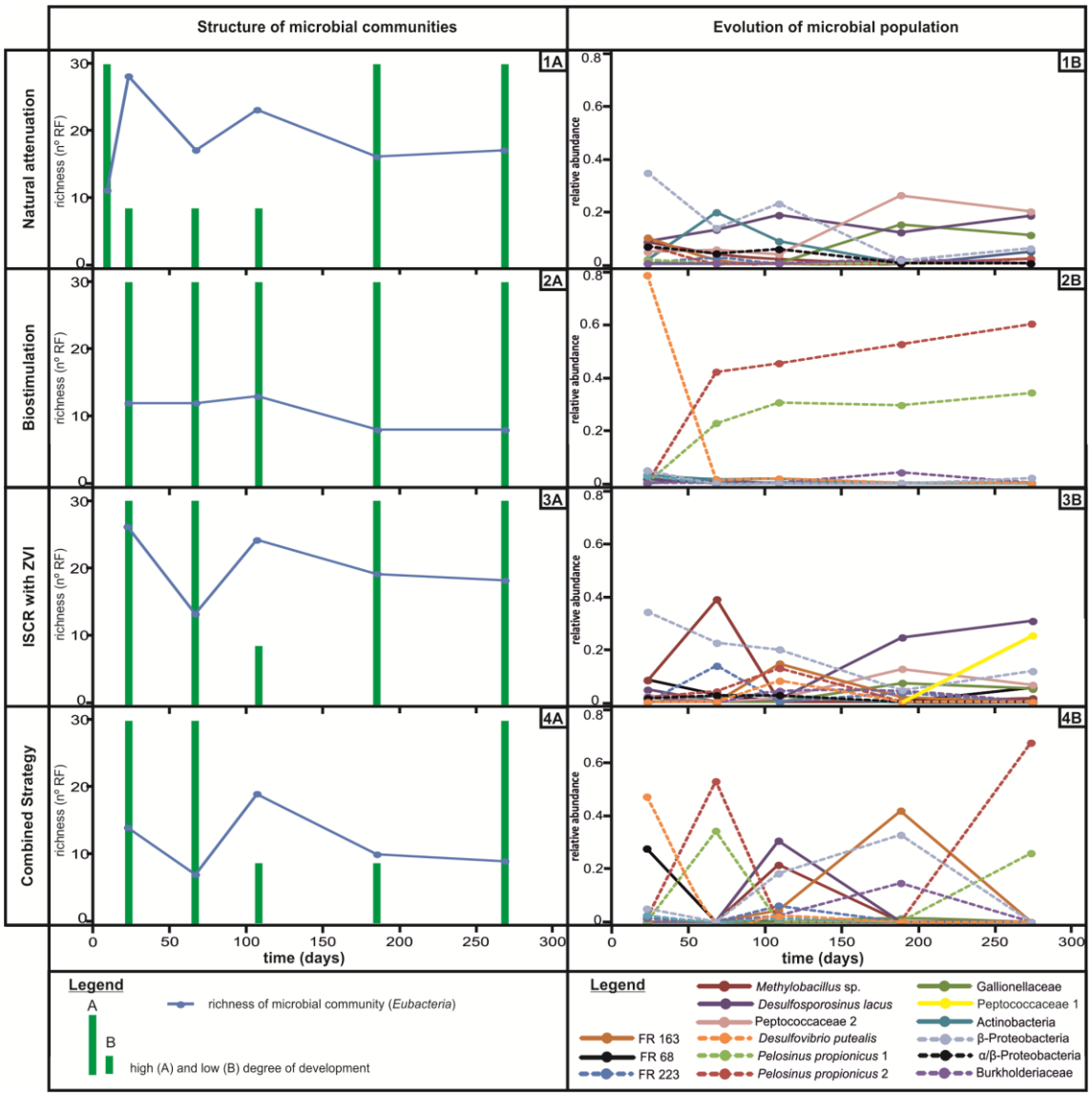


Figure 2.1A), with a variation in its structure and a predominance of metal-reducing Gallionellaceae and sulfate-reducing *D. lacus* (

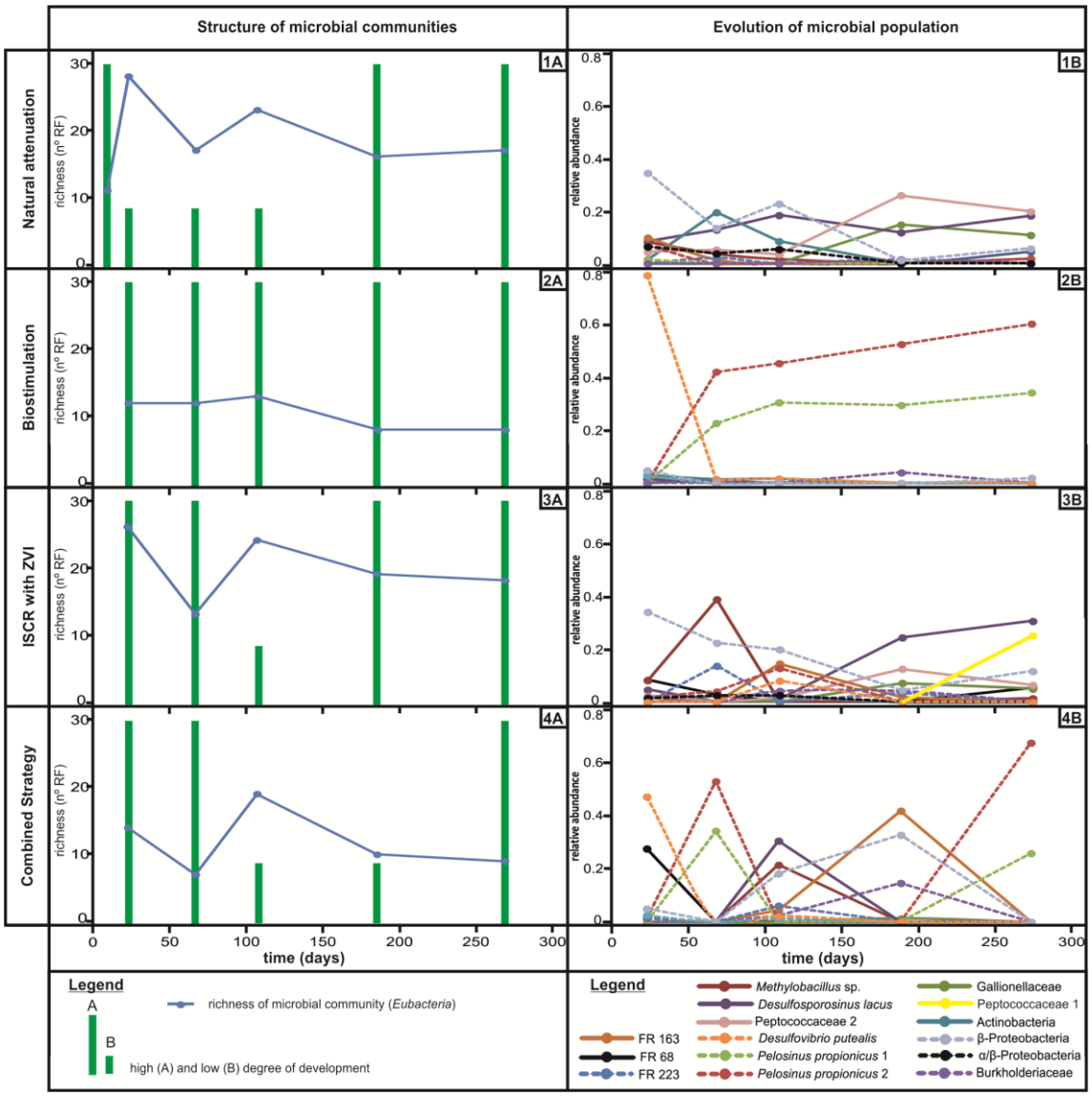


Figure 2.1B).

3.2. Biostimulation

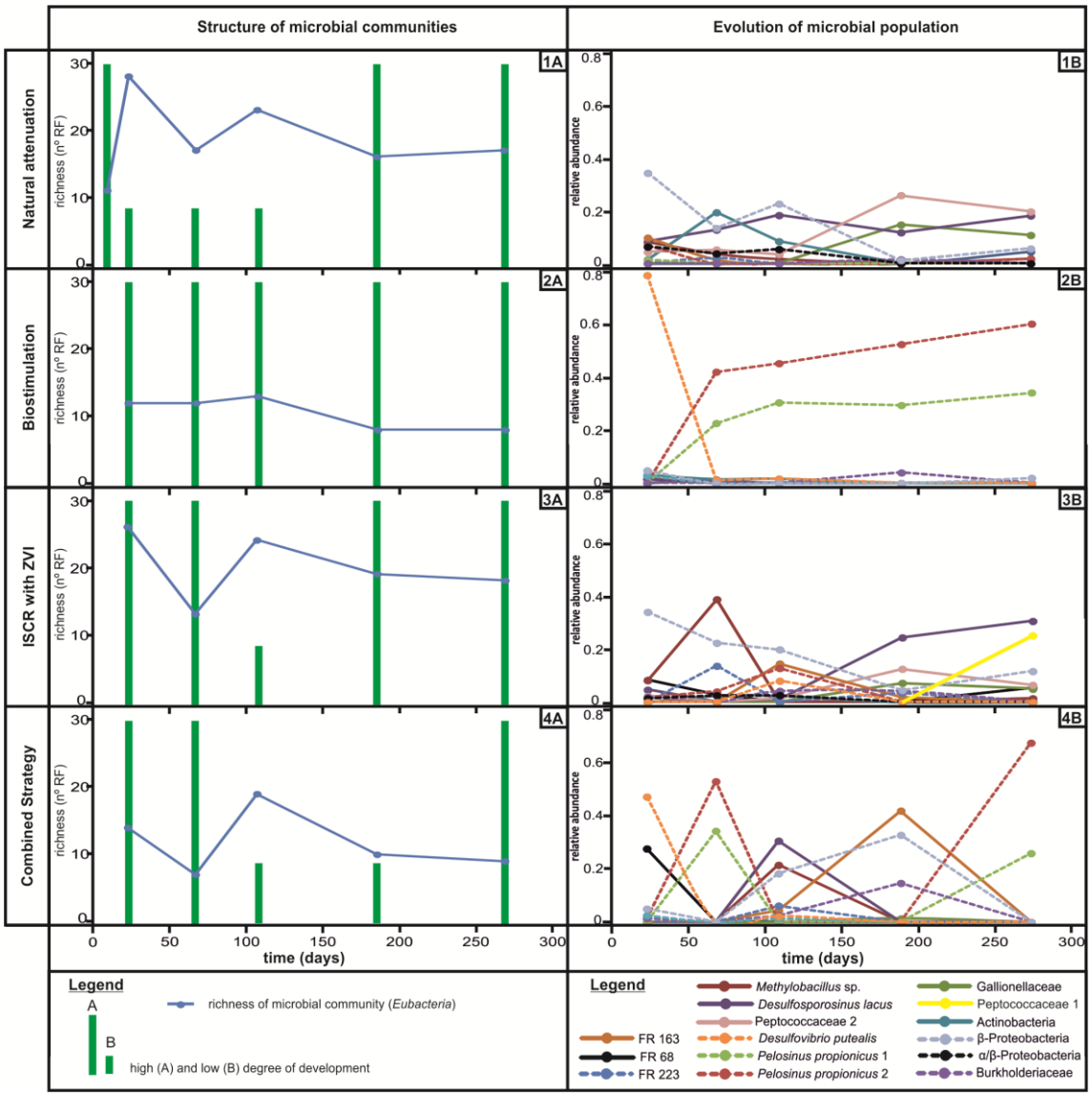
In the microcosm experiments where lactic acid was added, reduction processes such as denitrification, iron, manganese and especially sulfate reduction occurred very quickly (Figure 1.1B). The evolution of methane (Table 1) as well as acetate confirmed methanogenic and acetogenic conditions, respectively.

In comparison to the natural attenuation set-up the reductive dehalogenation of PCE and TCE started already at day 22 with a rapid reduction in the concentration of dissolved PCE (from 130 µmol/L to values of 1 µmol/L), transitory formation of TCE that reaches values of 4 µmol/L and a final formation of cDCE of 130 µmol/L, which was not further degraded. Isotopic fractionation were observed for PCE, TCE and cDCE

(Figure 1.3B), with an enrichment factor of PCE of -2.0‰ ± 0.3 (Table 1),, which is in the range of carbon isotope enrichment factors previously published (Hunkeler and Morasch, 2010). Both TCE and cDCE started with a lighter isotopic composition than the initial value of $\delta^{13}C_{PCE}$ followed by a shift towards more positive values in which the $\delta^{13}C_{cDCE}$-value reached the initial value of $\delta^{13}C_{PCE}$ confirming the inhibition in cDCE-degradation (Figure 1.3B and chloroethenes isotopically balanced, Table 1). Due to the absence of reductive dehalogenation of cDCE, VC, ethane, ethene and ethine were absent (Figure 1.2A and Table 1).

Abiotic controls of biostimulation experiments, show the same results as natural attenuation experiments, with no variation in PCE, nitrate and sulfate concentrations, absence of metabolites of PCE and an increase of Mn^{2+}.

342 A dehalogenating bacterial community was characterized by a high degree of development (



343

344 Figure 2.2A), low microbiological richness (

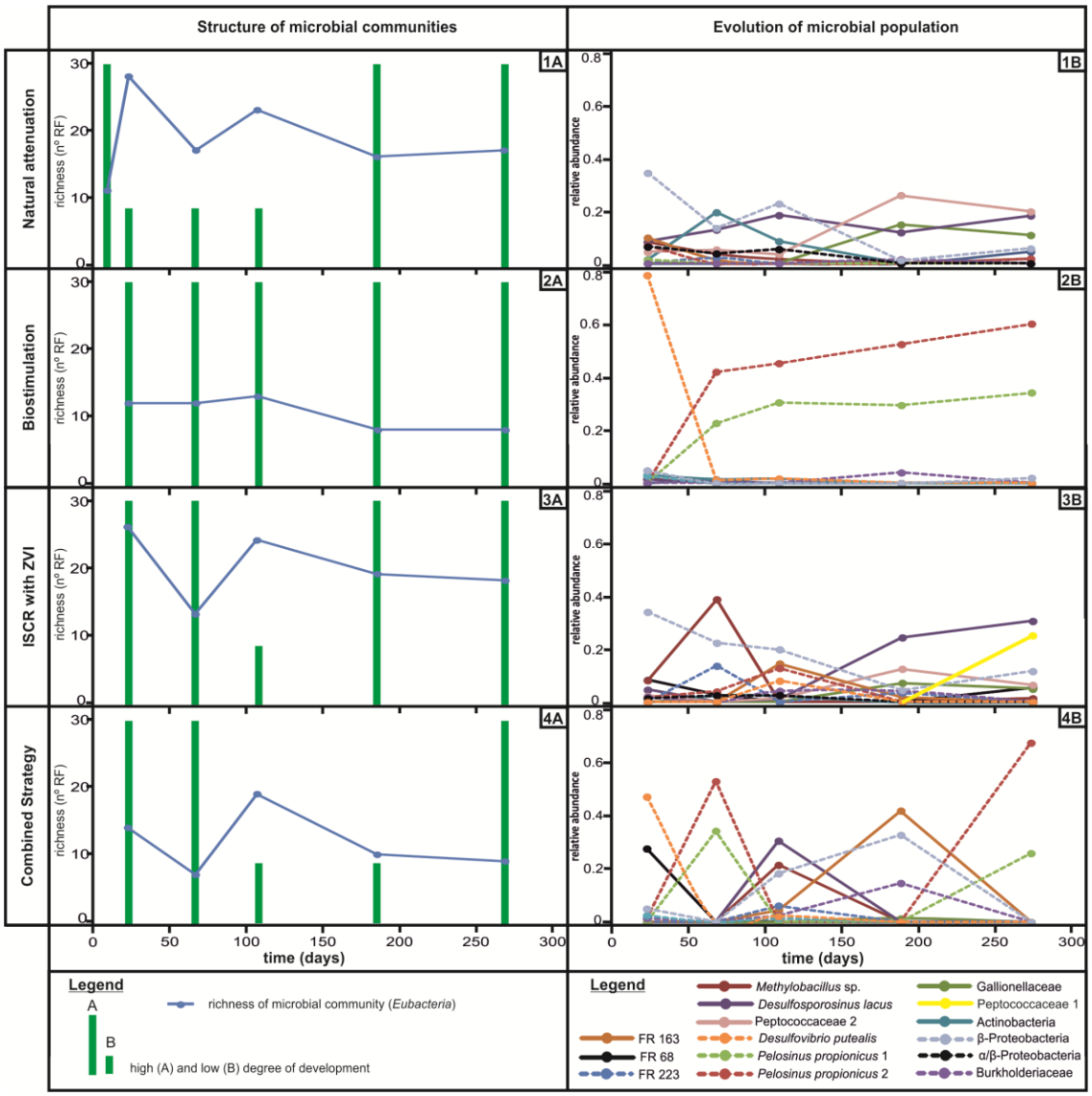


Figure 2.2A) and the dominance of bacteria related to *Desulfovibrio putealis* (

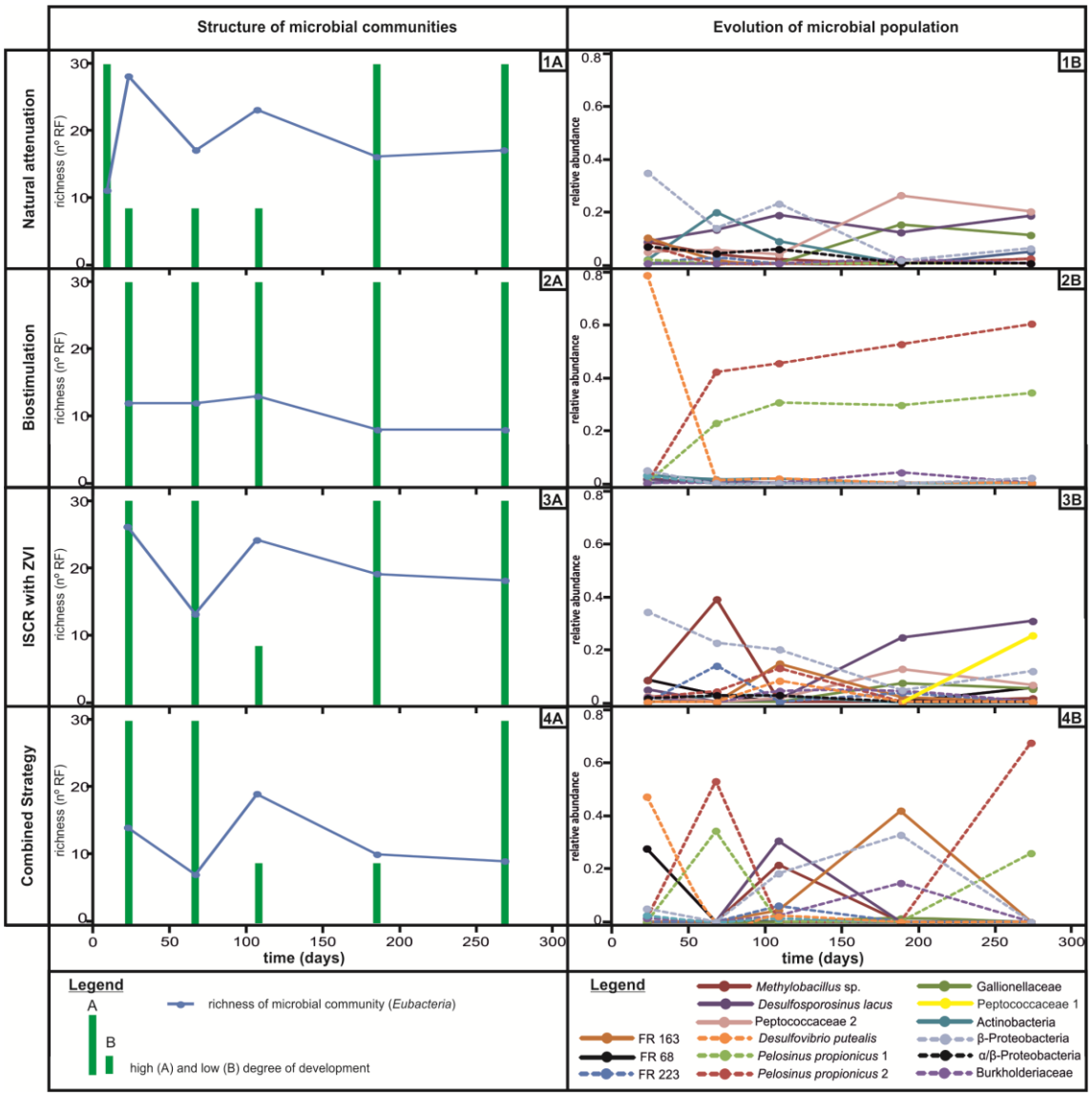


Figure 2.2B). *D. putealis* is a sulfate reducer tolerant of high concentrations of PCE and a producer of H_2 from the oxidation of lactic acid (preferred substrate for these microorganisms, Heidelberg et al. (2004)). This microorganism acts as facultative OHRB (Löffler et al., 2003). This finding is consistent with 1) a low proportion of fermenting microorganisms (*Bacteroidetes* and *P. propionicus*, Figure 4) as *D. putealis* is able to use lactic acid as a carbon source and 2) a low presence of OHRB because at the same time, these bacteria could use PCE and TCE as electron acceptors.

The final concentration of cDCE was equivalent to the initial PCE concentration, and therefore there is no evidence for degradation beyond cDCE. During this period, a bacterial community can have a high degree of development and low richness (

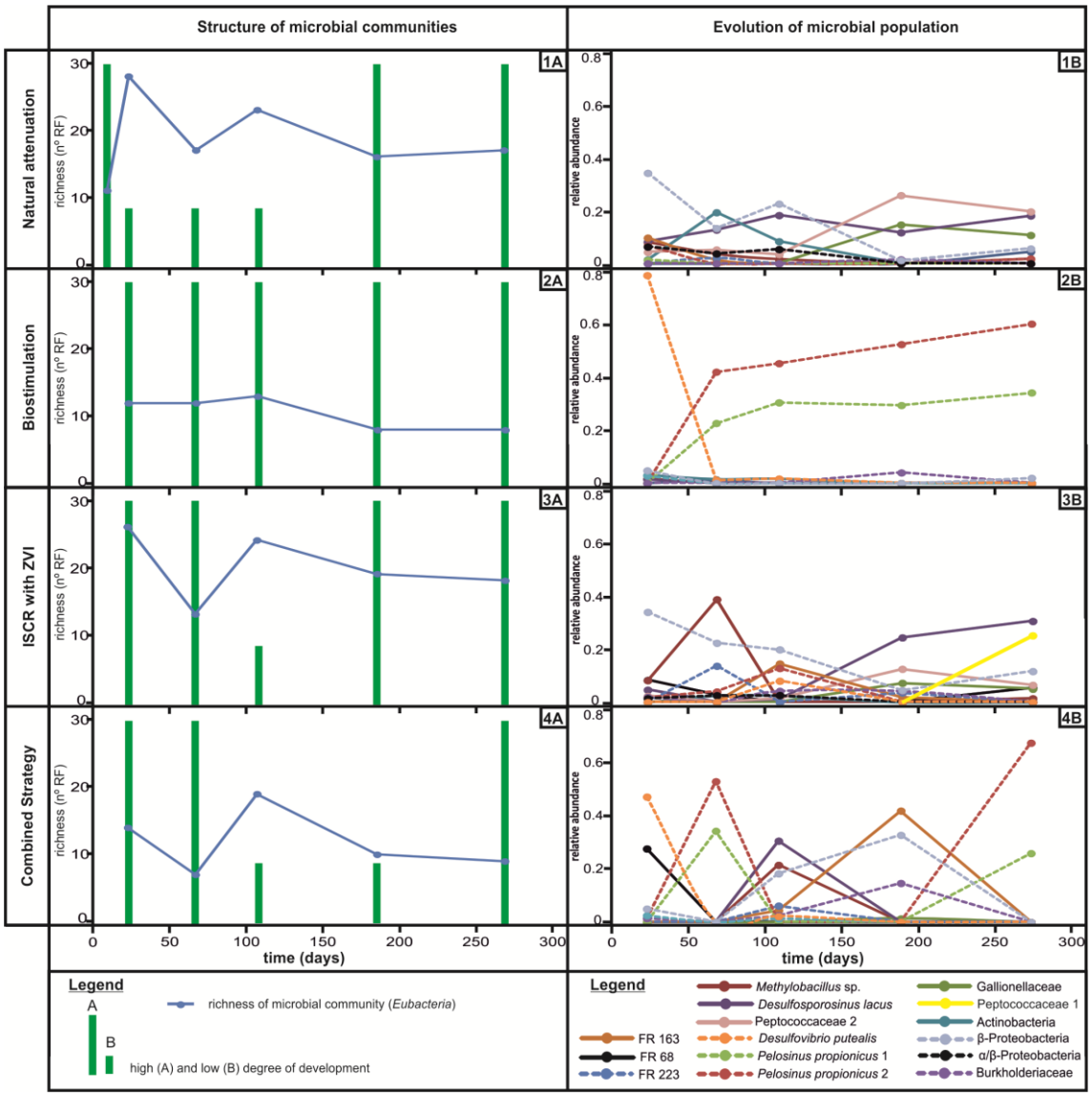


Figure 2.2A) due to the prevalence of fermenting bacteria related to P. propionicus (

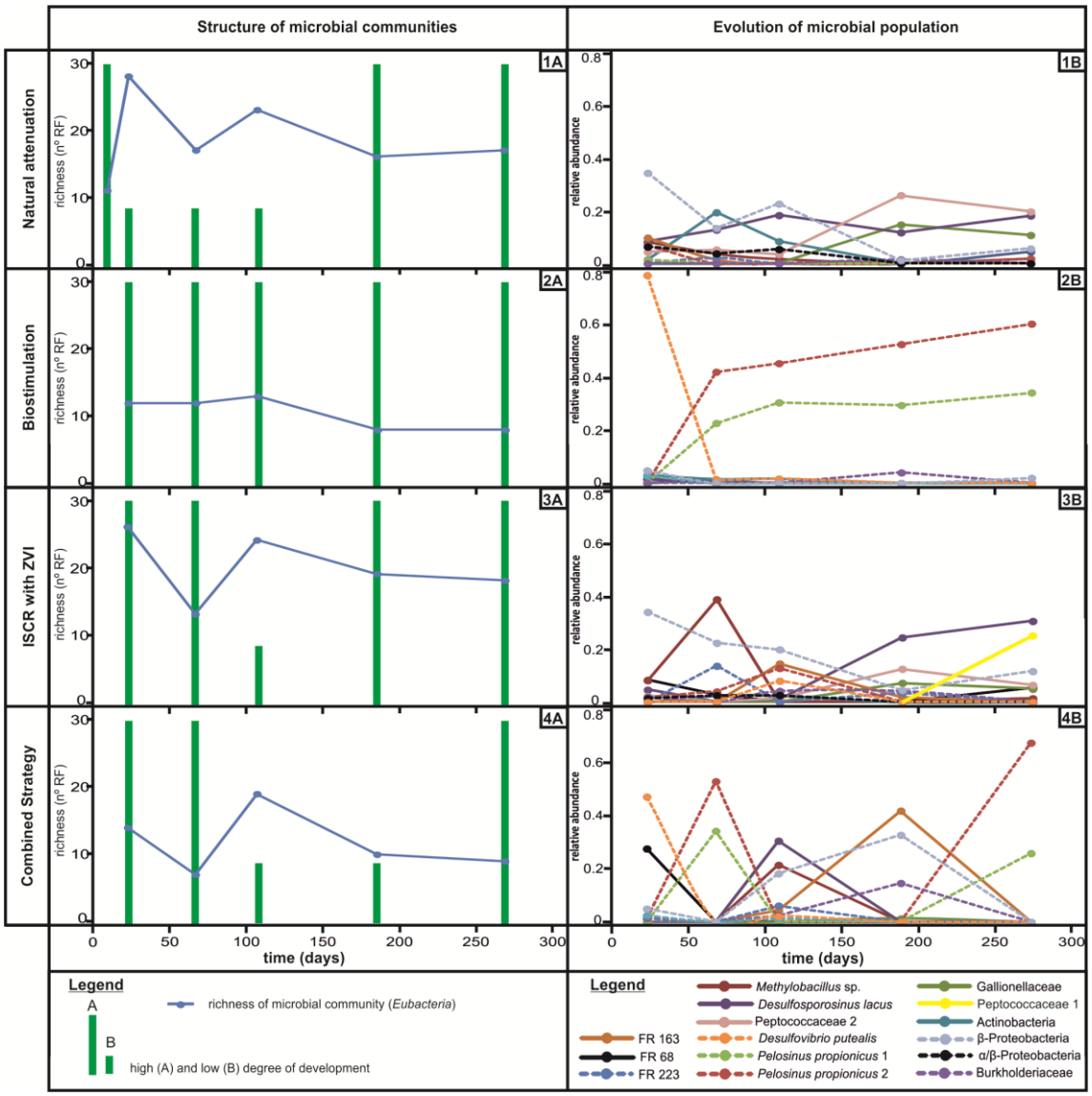


Figure 2.2B) (Boga et al., 2007; Shelobolina et al., 2007). This phenomenon can be explained either by a potential inhibition of Dehalococcoides spp. due to the toxicity of high concentrations of cDCE or due to the absence of Dehalococcoides spp. capable of dehalogenating cDCE and VC ISCR under natural attenuation conditions

3.3. ISCR under natural attenuation conditions

There were two different dehalogenation processes occurring in this set-up: chemical reduction of PCE conducted by ZVI and biotic reductive dehalogenation of PCE and TCE. The reductive dehalogenation of PCE in this set-up (Fig. 1.2C), was more pronounced than natural attenuation set-up (Fig. 1.2A) and less pronounced than biostimulation set-up (Fig. 1.2B). This was confirmed by the comparison of biotic and abiotic controls, in which the biotic controls reveal a higher removal of PCE (83%), than the control

experiments (48%), in which only the abiotic reduction of PCE was present. In addition, the higher percentage of degradation in the active experiments is not only because of biotic reductive dehalogenation but also because of the presence of TEAP (e.g., denitrification and Mn, Fe and sulfate reduction, Figure 1.1C) that allow ZVI to react preferably with chloroethene.

Biotic reductive dehalogenation of PCE is continuous during the experiment (Figure 1.2C) with a significant increase in TCE and cDCE (Figure 1.2C). In addition, in control experiments, there is a progressive decline in PCE, with an increase in TCE concentrations up to 10 µmol/L and the presence of tDCE (data not shown). In the active experiments, the production of methane, ethane and ethene occurred (Table 1). Otherwise, ethene and methane were not detected in control experiments, instead, there was production of ethine (Table 1). Active experiments showed a similar enrichment factor (ε value of -3.6‰ ± 0.7) to control experiments (ε value of -3.2‰ ± 0.5, Table 1). Moreover, $\delta^{13}C_{\sum(CEs)}$ (Table 1) supported that the production of non-chlorinated products was higher in active experiments than in control experiments.

The evolution of bacterial communities showed, in a similar way to the natural attenuation experiments (s. 3.1), two periods of high bacterial activity, separated by a lag phase (

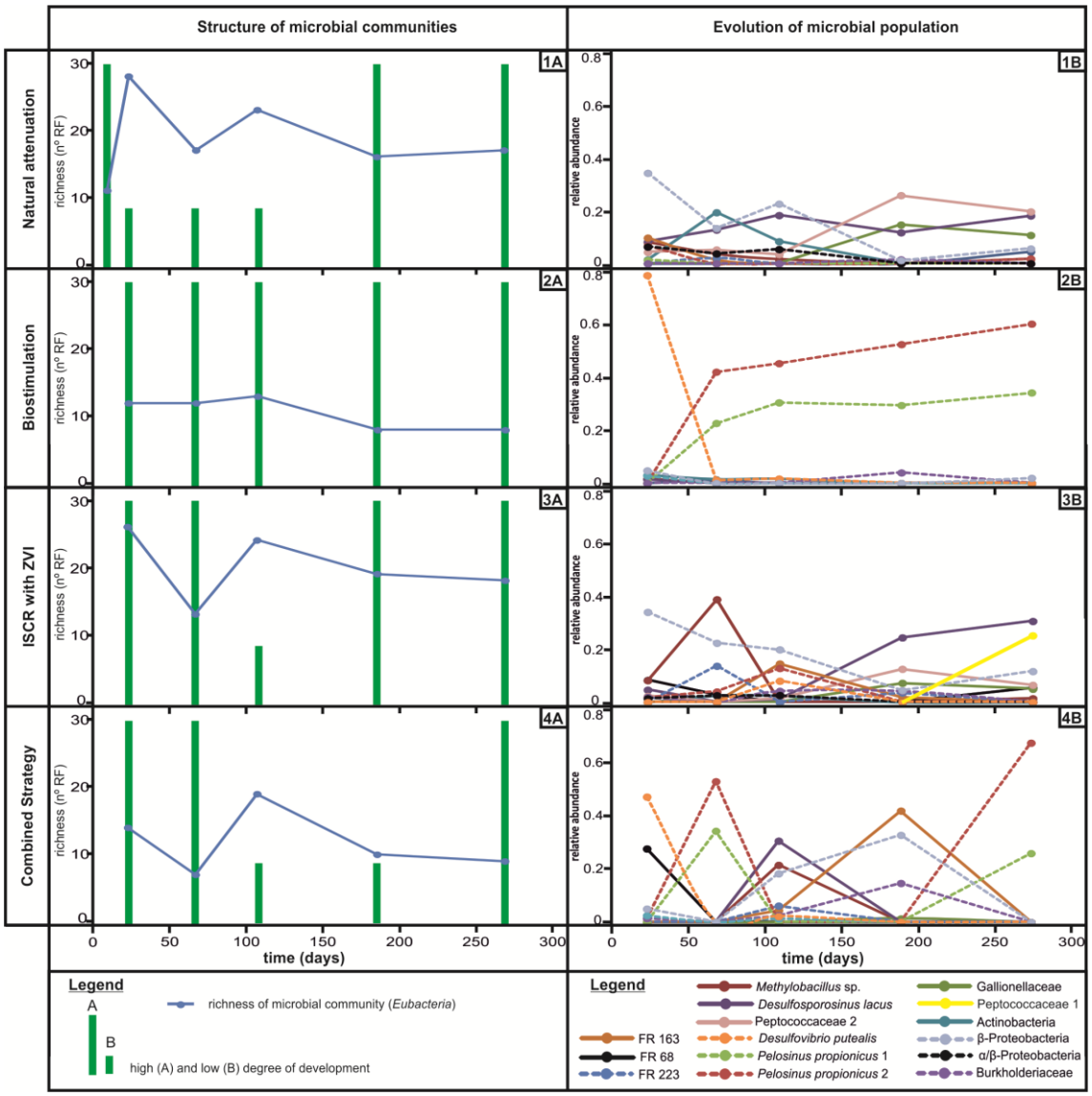


Figure 2.3A). The first period is characterized by high microbial activity, denitrification and Fe and Mn reduction (Figure 1.1C) and a co-dominance and/or alternate dominance of *Methylobacillus* sp., *β*-Proteobacteria (

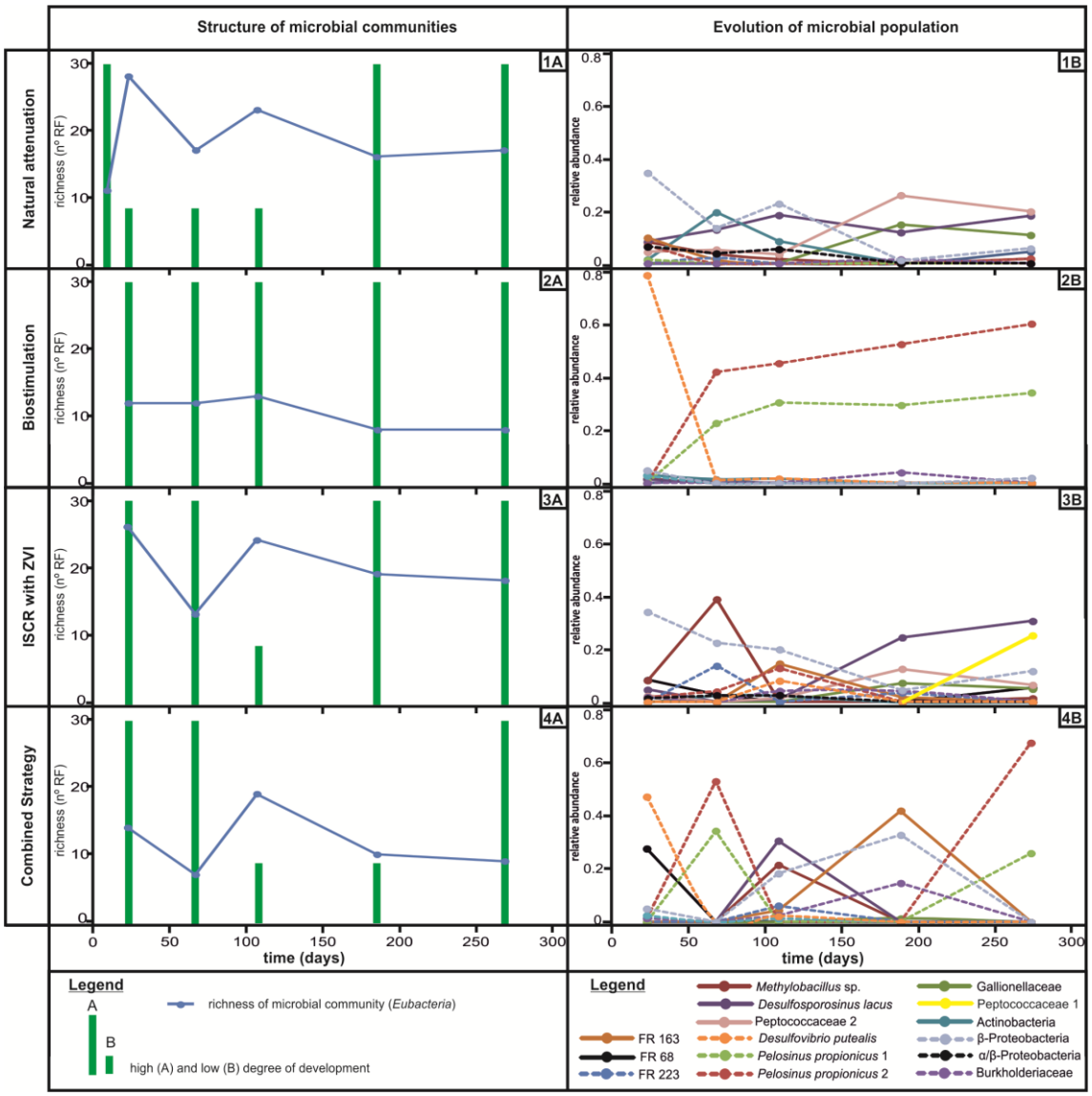


Figure 2.3B). The second period with high bacterial activity is characterized by biotic reductive dehalogenation of PCE and TCE (Figure 1.2C), potentially acetogenic and methanogenic metabolism (Figure 1.1C and Table 1), as well as the dominance of *D. lacus*.

The bacterial community present during sulfate reduction and reductive dehalogenation of PCE and TCE has, similar to that of the natural attenuation experiment (s. 3.1), 1) a high degree of development; 2) an increase in richness; 3) a dominance of Firmicutes phylum (

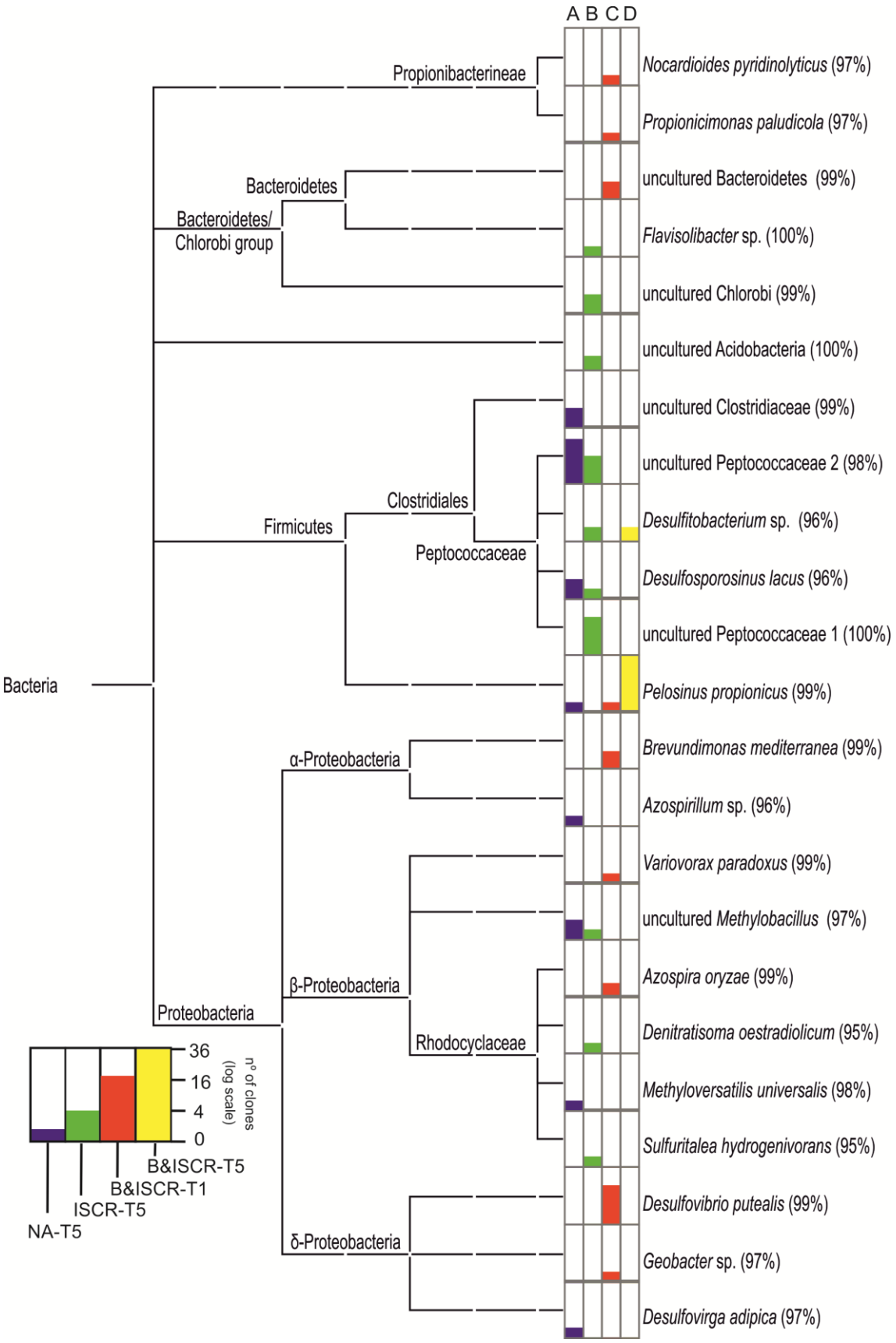
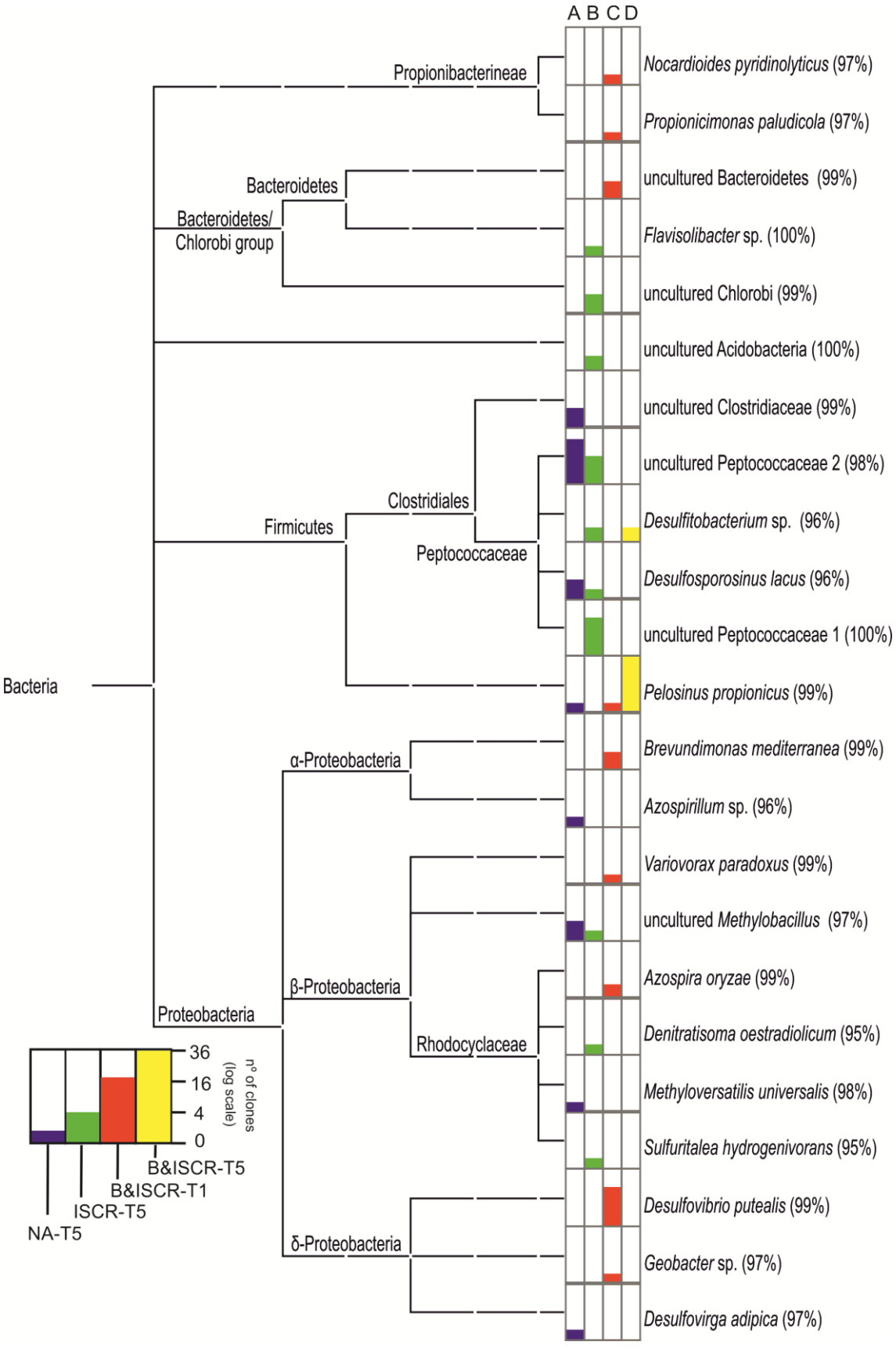


Figure 3.A); and 4) the presence of *D. lacus*, Peptococcaceae and Gallionellaceae (Figure 4), which have a fermenting, sulfate-reducing and metal-reducing metabolism, respectively (Hallbeck and Pedersen, 1991;

398 Patil et al., 2014; Tischer et al., 2013). In addition, both set-ups have an unidentified strain related to
399 *Methylobacillus* sp., which, despite being described as a strict aerobic species (Yordy and Weaver, 1977),
400 must have at least facultative metabolism and an important role as it remained until the end of both
401 experiments.

402 There is a greater diversity of phyla in ISCR experiments (



403

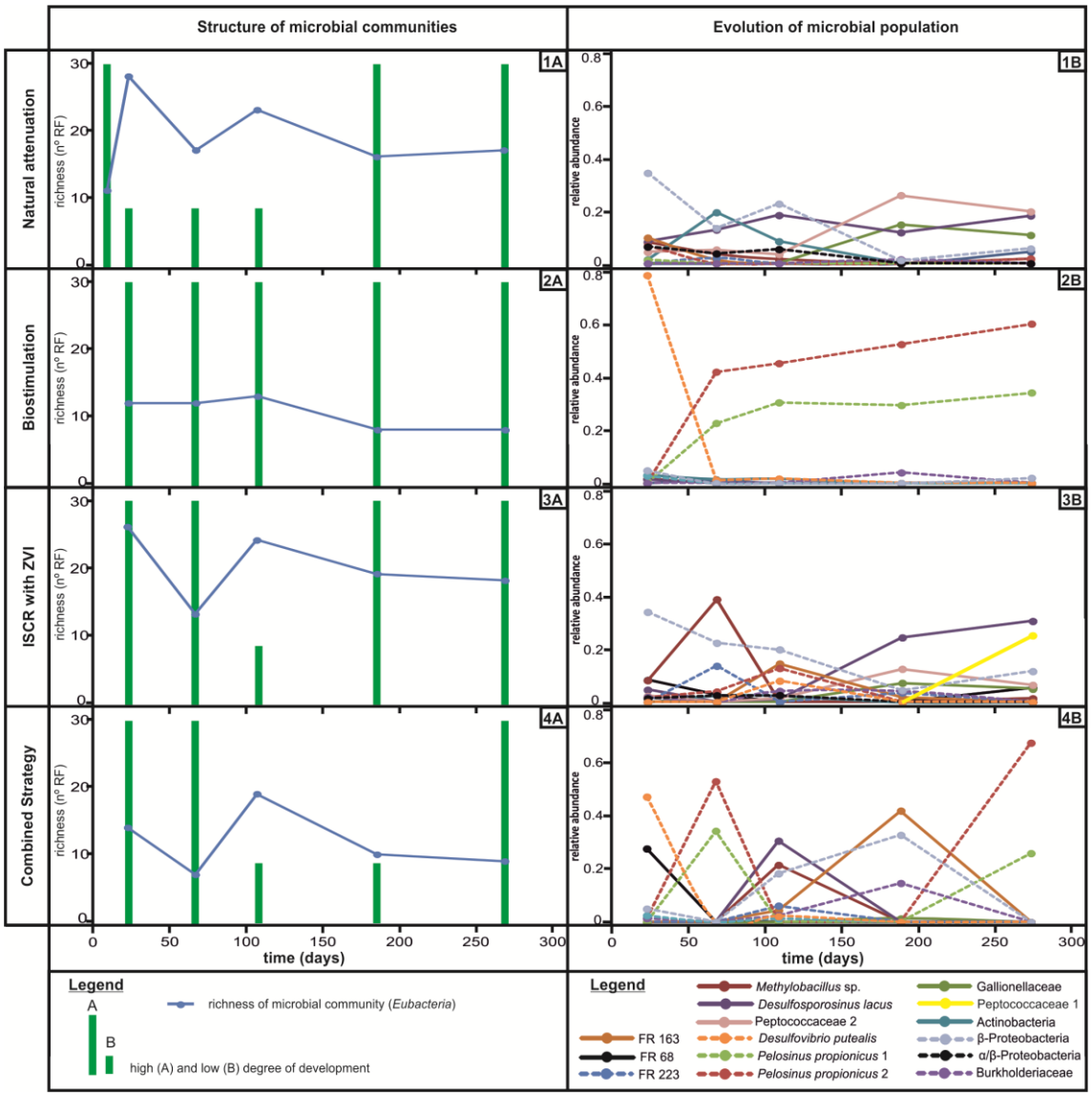
404 Figure 3.A) than natural attenuation experiments, caused by the presence of ZVI and higher degradation

405 of chloroethenes. This suggests that Acidobacteria and Chlorobi (Figure 4) are favored by the oxidation of
406 ZVI. In addition, there is the presence of *Desulfitobacterium* (Figure 4) as a potential reductive
407 dehalogenator of PCE and TCE (Rouzeau-Szynalski et al., 2011).

408 **3.4. ISCR with biostimulation conditions**

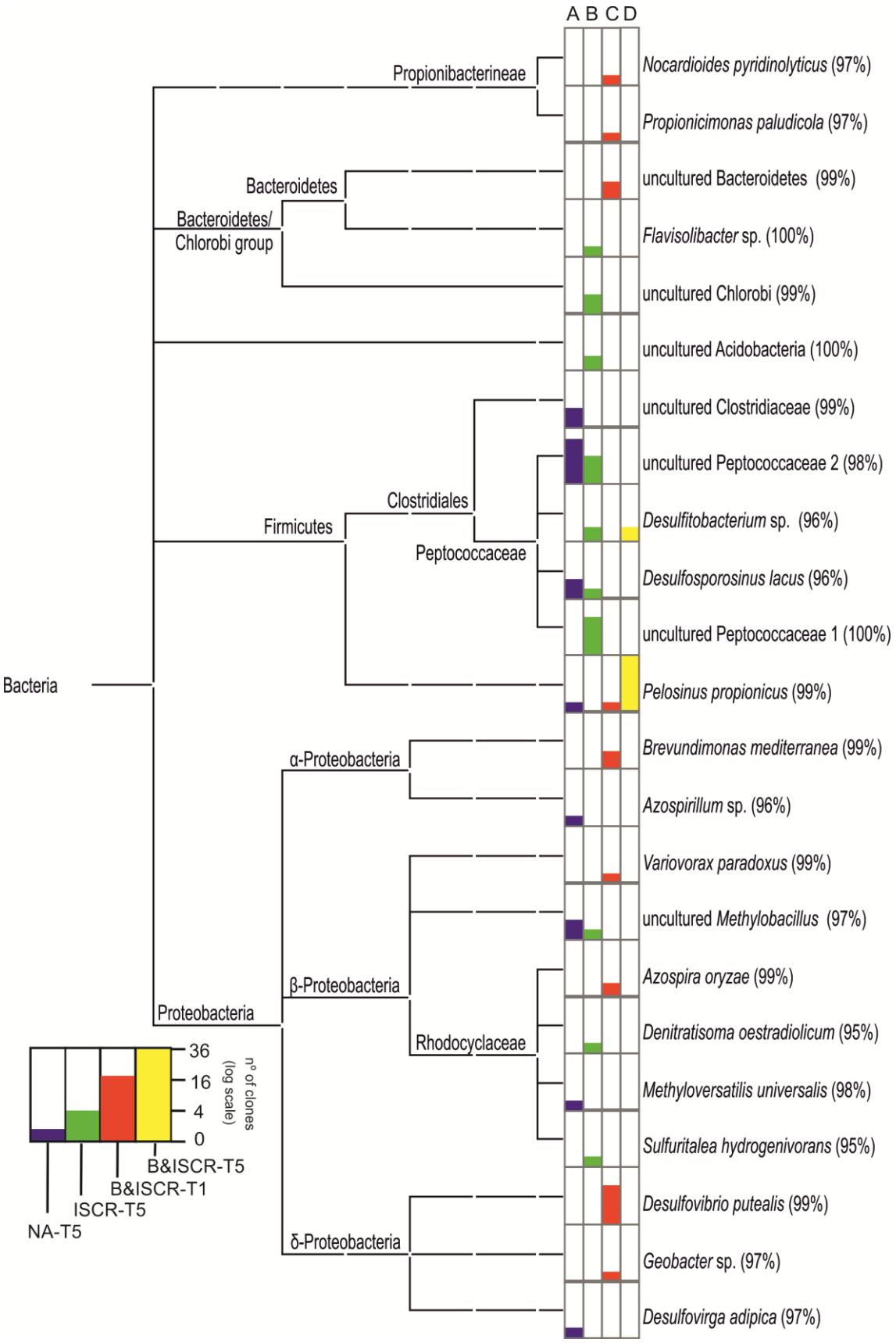
409 Similar to the biostimulation experiments (s. 3.2), redox conditions pass quickly to acetogenic and
410 methanogenic (at day 22, concentration of NO_3^- and SO_4^{2-} was practically zero, the concentration of Mn^{2+}
411 and Fe^{2+} was already high and the concentration of acetate was near 1200 mg/L, Figure 1.1D) in the
412 ISCR/biostimulation set-up. Therefore, these conditions promote the reductive dehalogenation of PCE to
413 TCE, and later to cDCE and 1,1DCE, to be fast in comparison to the other set-ups (Figure 1.2D). However,
414 unlike the biostimulation experiments (s.3.2), there is further transformation to non-chlorinated
415 products, higher in biotic conditions ($\delta^{13}C_{\sum(CEs)}$ = - 21.3 ‰ ± 0.6) than in abiotic conditions ($\delta^{13}C_{\sum(CEs)}$ = -
416 24.4 ‰ ± 0.7). The shift in the isotopic composition of PCE (ε_{PCE} value of -2.5‰ ± 0.5, Table 1) is in between
417 the ε_{PCE} of biostimulation and ISCR experiments. Therefore, dehalogenation processes affecting PCE are
418 potentially a mixture of the processes occurring in Biostimulation and ISCR set-ups.

419 The bacterial community responsible for the reductive dehalogenation of PCE and TCE is characterized by
420 low richness and a high degree of development (



421

422 Figure 2.4A), and Proteobacteria was a predominant phylum (



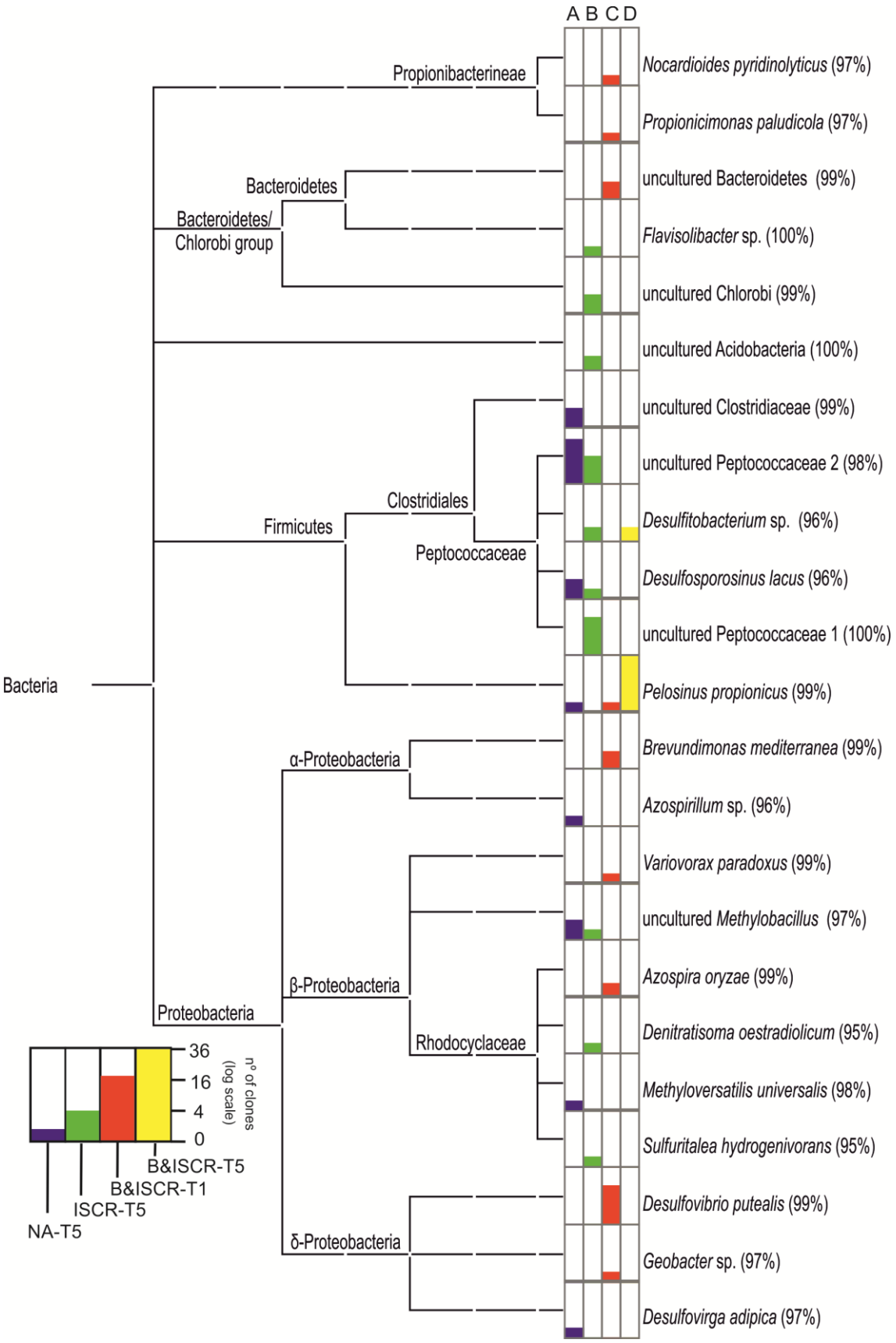
423

424 Figure 3.C). This bacterial community has a predominance of sulfate-reducing bacteria related to

425 *Desulfovibrio putealis* (Figure 4) and the presence of several microorganisms, among them, metal and

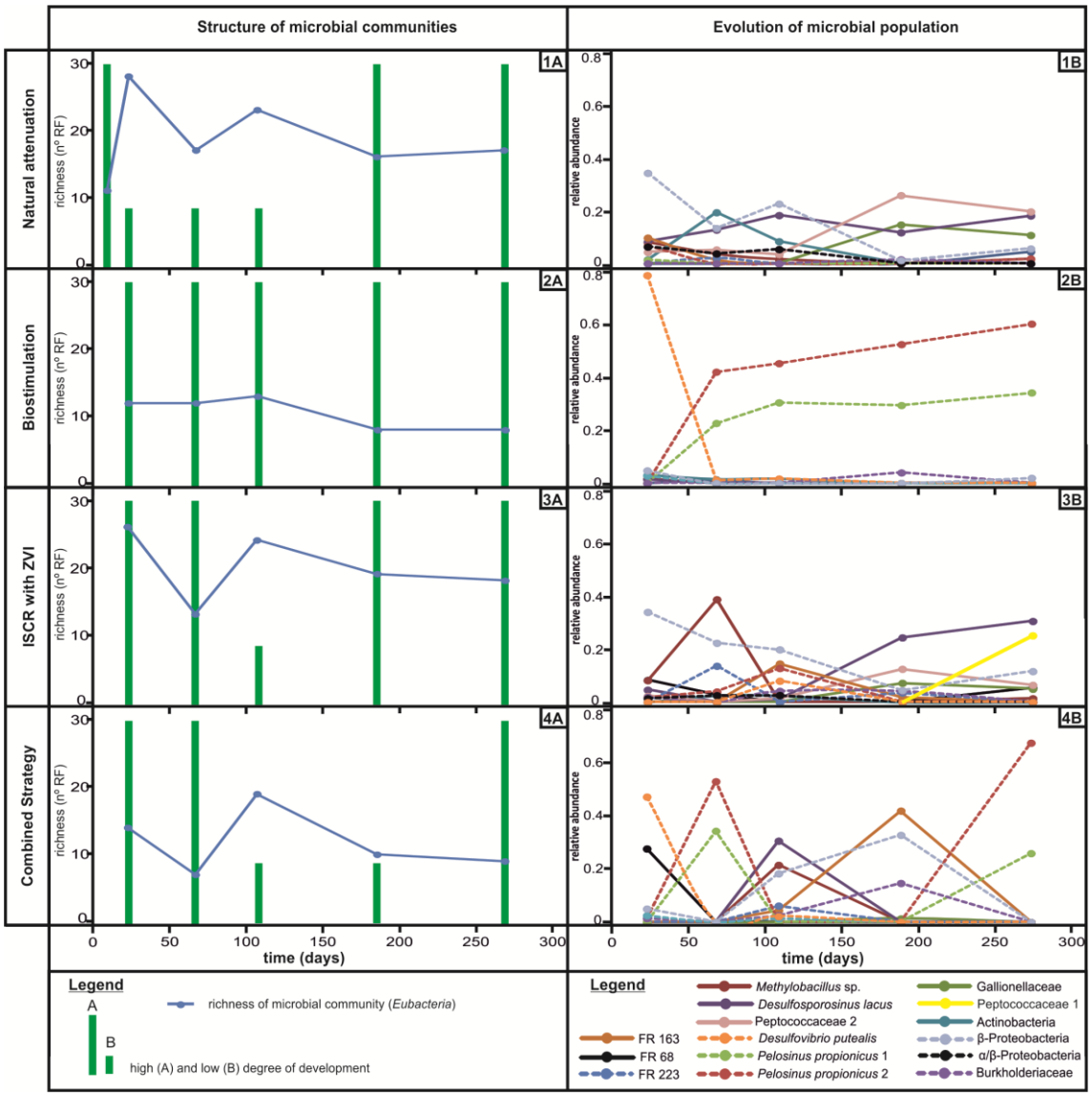
426 sulfate reducer (and potential OHRB) *Geobacter* spp., fermenting bacteria *Propionicimonas paludicola*,
427 *Pelosinus propionicus* and uncultured *Bacteroidetes* (Figure 4). This microbial community shares the same
428 characteristics as the dehalogenating bacterial community of PCE and TCE described in section 3.2.

429 Once the major fraction of chloroethenes is cDCE (Figure 1.2D), abiotic dehalogenation and biotic
430 reductive dehalogenation of cDCE and VC simultaneously occur and are not differentiable. Nevertheless,
431 and similar to the ISCR experiments with ZVI (s.3.3), there is a presence of ethene, ethane and methane
432 in the active experiments (Table 1), while in the control experiments, ethine and methane are present
433 (Table 1). During dehalogenation of cDCE, the bacterial community evolves, similar to the biostimulation
434 experiments (s. 3.2), to a fermenting bacterial community formed exclusively by the Firmicutes phylum (



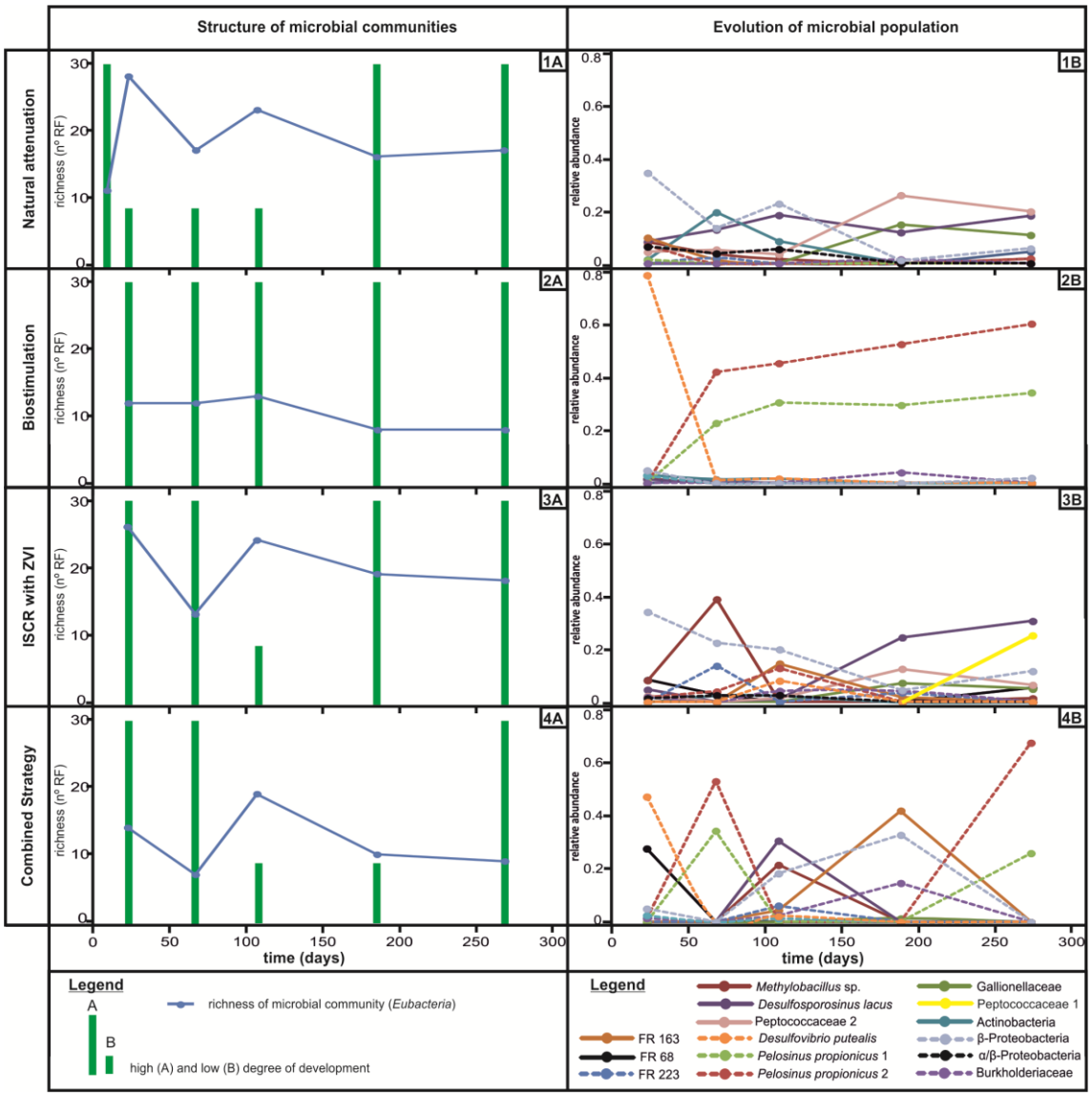
435

436 Figure 3.D) and dominated by bacteria related to *P. propionicus* (



437

438 Figure 2.4B and Figure 4), but it has a lower degree of development (



439

440 Figure 2.2A and 4A) and a lower prevalence of this microorganism (

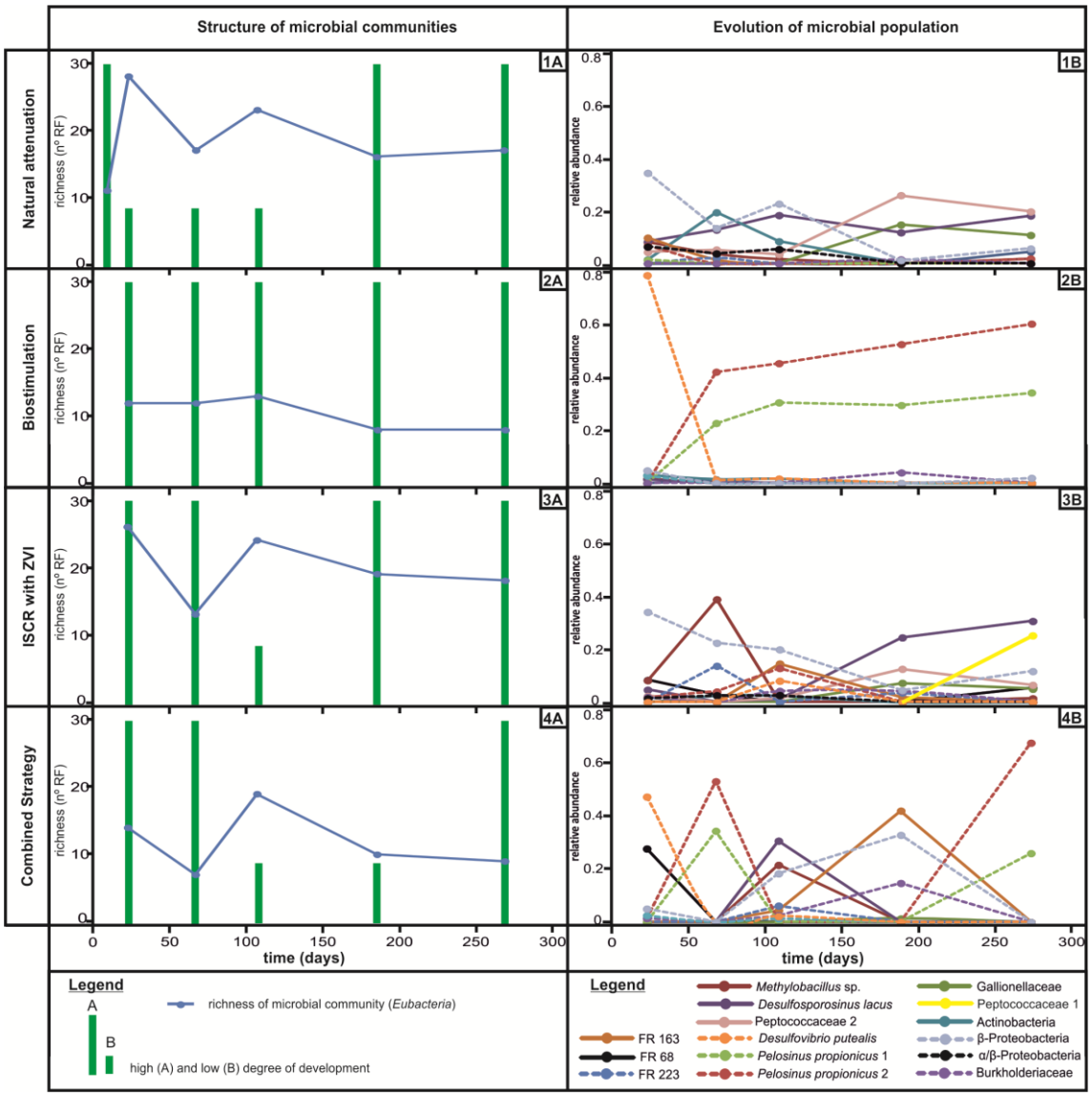


Figure 2.2B and 4B). This can be related to the fact that lactic acid is consumed more quickly during these experiments than that of the biostimulation experiments, and therefore, there is an exhaustion of lactic acid and a displacement of fermenting microorganisms. Moreover, there is the presence of the sulfite reducer and potential OHRB *Desulfitobacterium* sp. (Figure 4).

The presence of ZVI has a positive and differential effect on the stimulation of the dehalogenating bacterial community. For example, the presence of *D. lacus* (

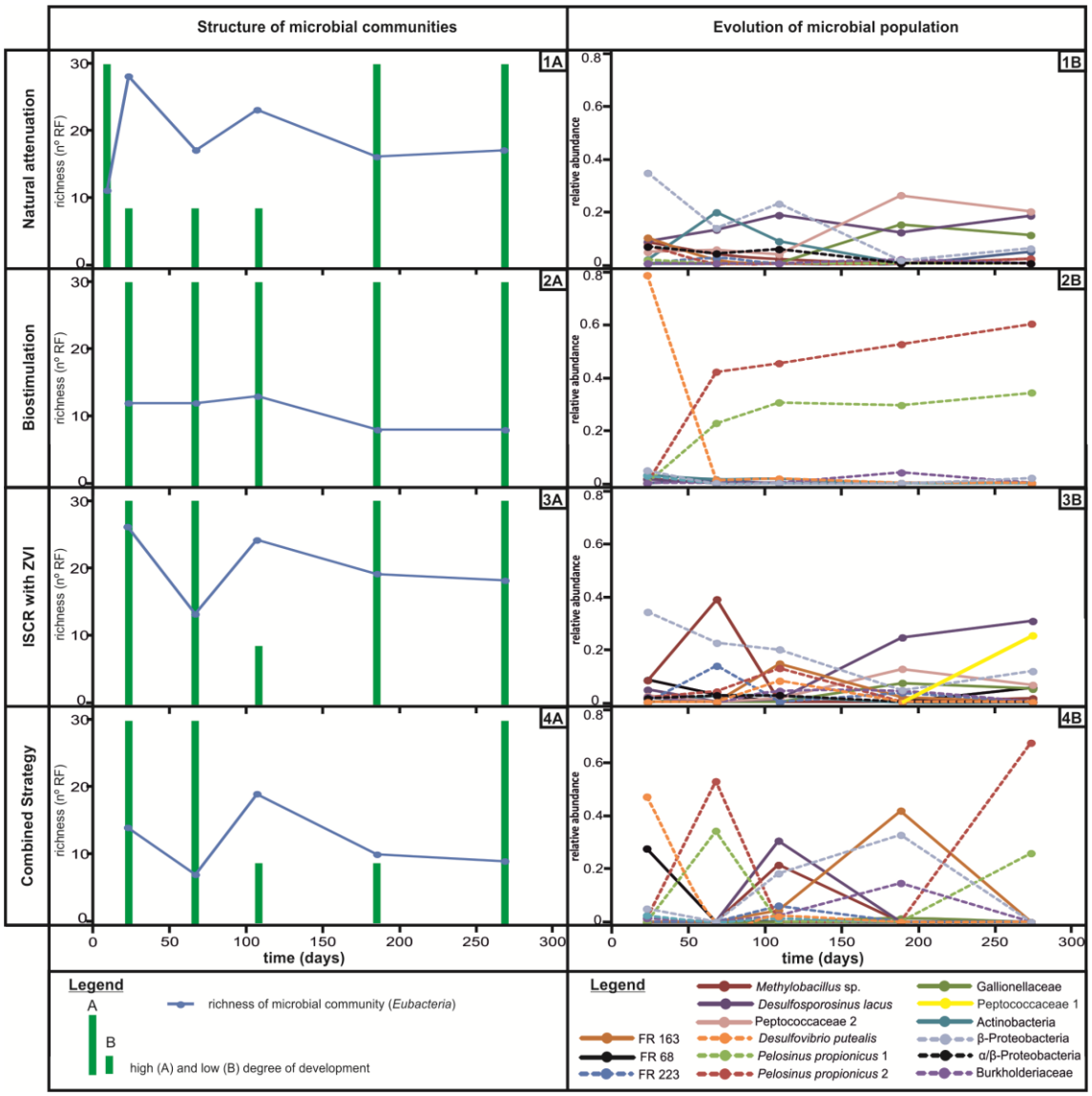


Figure 2.4B) highlights that the presence of ZVI modify the bacterial community, and these microorganisms perform a fermentative metabolism due to the absence of sulfate. However, lactic acid is the most important conditioning factor according to the degree of similarity between the bacterial communities of the biostimulation and combined strategy experiments. The results demonstrate that combined strategy of adding ZVI and lactic acid is the most efficient, as there is a fast reductive dehalogenation of PCE and TCE and substantial decrease in cDCE and increase of VC and ethene in comparison to the other set-ups. Limiting factors on dehalogenation processes

The main factors limiting reductive dehalogenation that have been characterized are competition on electron donors, lack of bioavailable electron donors, toxicity and displacement of a potentially dehalogenating bacterial community by a fermenting bacterial community.

459 The OHRBs characterized in natural attenuation and biostimulation experiments act under sulfate-
460 reducing conditions; although the processes of denitrification and Fe and Mn reduction are energetically
461 more favorable than reductive dehalogenation and sulfate reduction, as has been seen in other site
462 studies, such as those of Bouwer (1994), Bradley (2003) and Bradley & Chapelle (1996). Although this
463 statement is generally true, if the amount of electron acceptors is small, the available energy will
464 decrease.

465 Another limiting factor is the lack of organic substrate. This limitation means that denitrification; Mn, Fe
466 and sulfate reduction; and PCE reductive dehalogenation are slower and start later in the natural
467 attenuation experiments than in the biostimulation experiments.

468 Injection of lactic acid in biostimulation experiments resulted in substantial dehalogenation of PCE/TCE
469 but with an accumulation of cDCE and a bacterial community with exclusive acetogenic and fermenting
470 metabolism (s. 1.1.2). This is a common problem for dehalogenating bacterial communities (Maymó-
471 Gatell et al., 1997; Sung et al., 2003; Yoshida et al., 2007). The absence of reductive dehalogenation of
472 cDCE may be either to toxicity of high concentration of cDCE, although it is possible that there is strong
473 competition between acetogenic microorganisms and OHRBs for the use of H_2 or the absence of cDCE-
474 degraders, e.g. *Dehalococcoides* spp..

475 One possible limiting factor that occurs in many dehalogenating bacterial communities is the absence of
476 OHRBs capable of the complete dehalogenation of PCE to non-chlorinated products (Dowideit et al.,
477 2010). This seems not to be the case here because *Dehalococcoides* and complete reductive
478 dehalogenation (based on the presence of VC) have been detected in the pollutant source of the study
479 area (Puigserver et al., 2016a).

480

481 **4. Conclusions**

482 Natural attenuation is not an efficient strategy. In the presented study, microcosm experiments showed
483 that the main limiting factors are the lack of electron donors and toxicity of PCE in the source area.
484 However, OHRBs capable of complete dehalogenating PCE seems to be present.

D. putealis is an OHRB capable of reductively dehalogenating PCE and TCE in high concentrations when electron donors are supplied to the environment. However, no OHRB have been detected capable of dehalogenate cDCE. Therefore, there is a need to use a second strategy to reduce the total amount of chloroethene. Under stable reductive conditions, there was an increase in the proportion of fermenting bacteria, and it was higher in the experiments in which lactic acid was injected. These fermenting bacteria have a key role in supporting reductive dehalogenation. ZVI is a reducing reagent that effectively reduces all chloroethenes. Biotic and abiotic reductive dehalogenation processes were coupled, producing TCE, cDCE, ethene, ethane and methane. The addition of ZVI demonstrated that, under a lower total amount of chloroethenes, OHRBs can dehalogenate reductively all chloroethenes more efficiently. This finding is in line with evidence of dehalogenation in the source area, where, in areas with lower concentrations due to heterogeneities, OHRBs can degrade cDCE and VC. However, this approach is not optimal, due to the difficulty to monitor products of abiotic dehalogenation, the potential inhibition of microbial communities by ZVI and the complexity to deliver ZVI in aquifers.

A combined strategy of biostimulation with lactic acid and ISCR with ZVI is proposed to be the most efficient strategy to completely remediate the source area. In this strategy, *D. putealis* rapidly dehalogenates PCE and TCE to cDCE, and ZVI slowly reduces the total amount of chloroethenes, reducing the toxicity and allowing other OHRBs to dehalogenate the rest of chloroethenes. Additionally, the injection of lactic acid promotes the reach of methanogenic conditions and the addition of lower amount of ZVI does not inhibit microbial communities.

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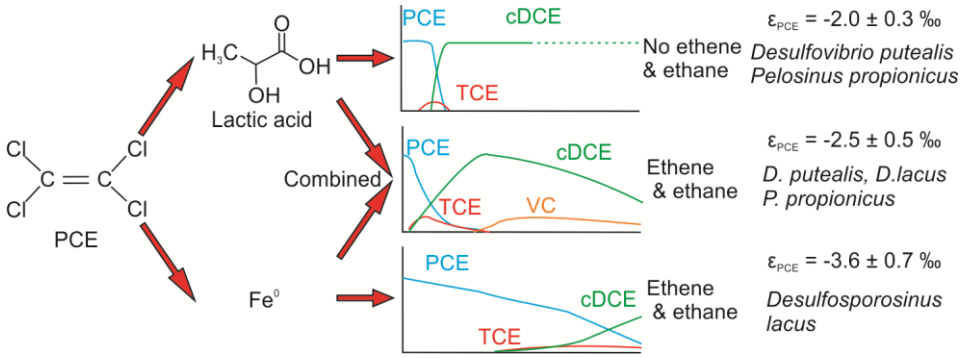
753 Graphical abstract



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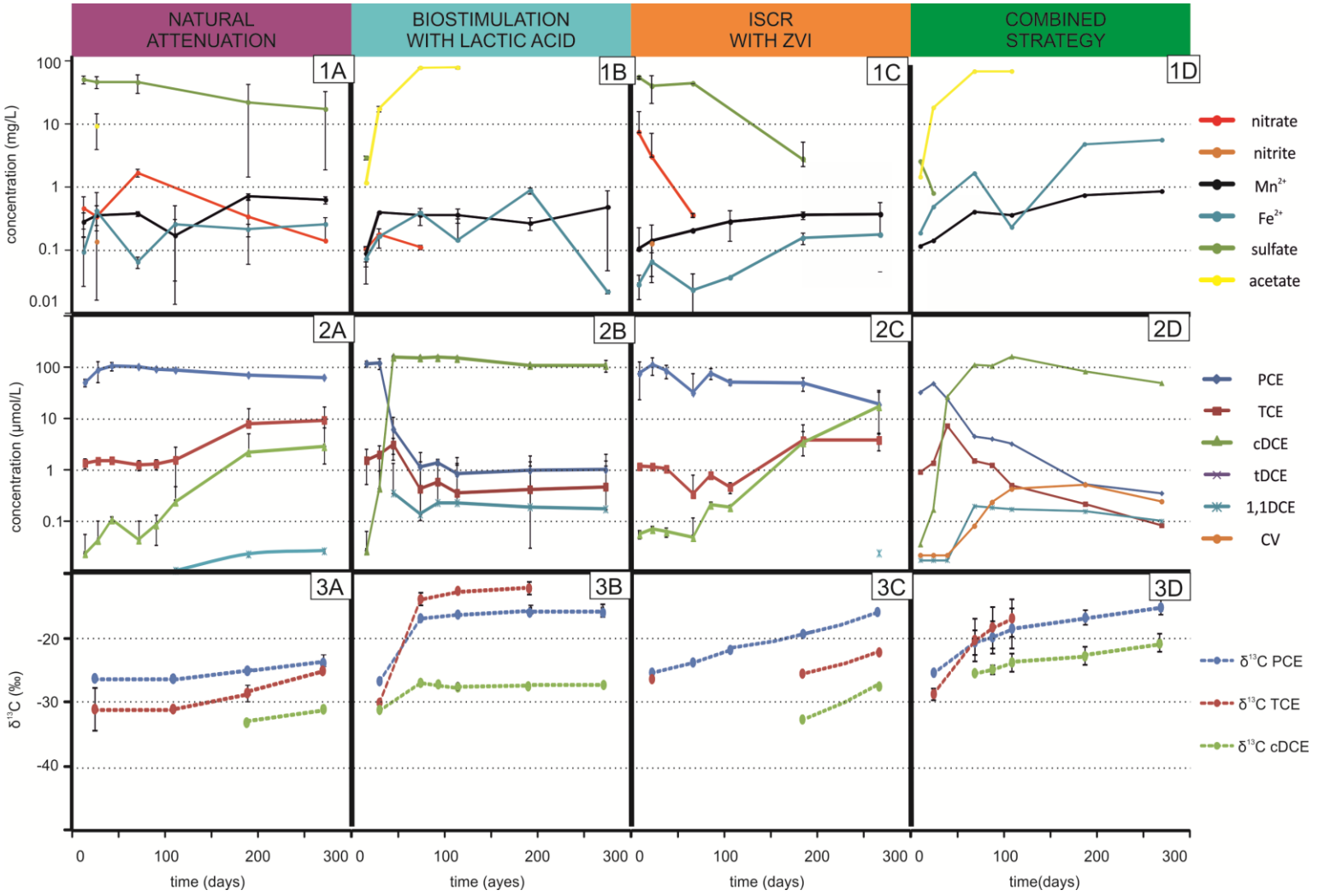
NATURAL ATTENUATION
BIOSTIMULATION WITH LACTIC ACID
ISCR WITH ZVI
COMBINED STRATEGY
1A
1B
1C
1D
2A
2B
2C
2D
3A
3B
3C
3D
concentration (mg/L)
concentration (µmol/L)
δ13C (‰)
time (days)
time (ayes)
time (days)
time(days)
nitrate
nitrite
Mn2+
Fe2+
sulfate
acetate
PCE
TCE
cDCE
tDCE
1,1DCE
CV
δ13C PCE
δ13C TCE
δ13C cDCE

757

Figure 1: Shifts of concentration of NO_3^-, NO_2^-, Mn^{2+}, Fe^{2+}, SO_4^{2-}, acetate (1) and chloroethenes (2) and isotopic composition (in $\delta^{13}C$) of chloroethenes (3) during incubation of the four microcosm experiments of natural attenuation (A), biostimulation with lactic acid (B), ISCR with ZVI (C) and combined strategy (D) for 267 days. Error bars represent standard deviation of replicate microcosms.

	Natural attenuation		Biostimulation		ISCR with ZVI		Biostimulation and ISCR	
	biotic	abiotic	biotic	abiotic	biotic	abiotic	biotic	Abiotic
Total degraded PCE mass (%)	34.5	11.3	99.2	21.0	83.1	48.0	97.8	50.6
$\delta^{13}C_{\sum(CEs)}$ (‰) t=267 days	-25.9± 0.3	-25.9± 0.1	-26.4± 0.1	-26.2± 0.3	-23.1± 0.7	-24.7± 0.2	-21.3± 0.6	-24.4± 0.6
ε_{PCE} (‰)	<-1	-	-2.0 ± 0.3	-	-3.6 ± 0.7	-3.2 ± 0.5	-2.5 ± 0.5	-3.1 ± 0.6
Methane	+	-	+	-	+	-	+	+
Ethane	-	-	-	-	+	+	+	+
Ethene	-	-	-	-	+	-	+	-
Ethine	-	+	-	+	-	+	-	+

Table 1: Synthesis of evidence related to dehalogenation processes. +: presence. -: absence. Initial $\delta^{13}C_{\sum(CEs)}$ is -26.2 ‰. $\delta^{13}C_{\sum(CEs)}$ of time 265 days given as average ± standard deviation of two experiments. ε given with ± Interval of confidence of 95%.

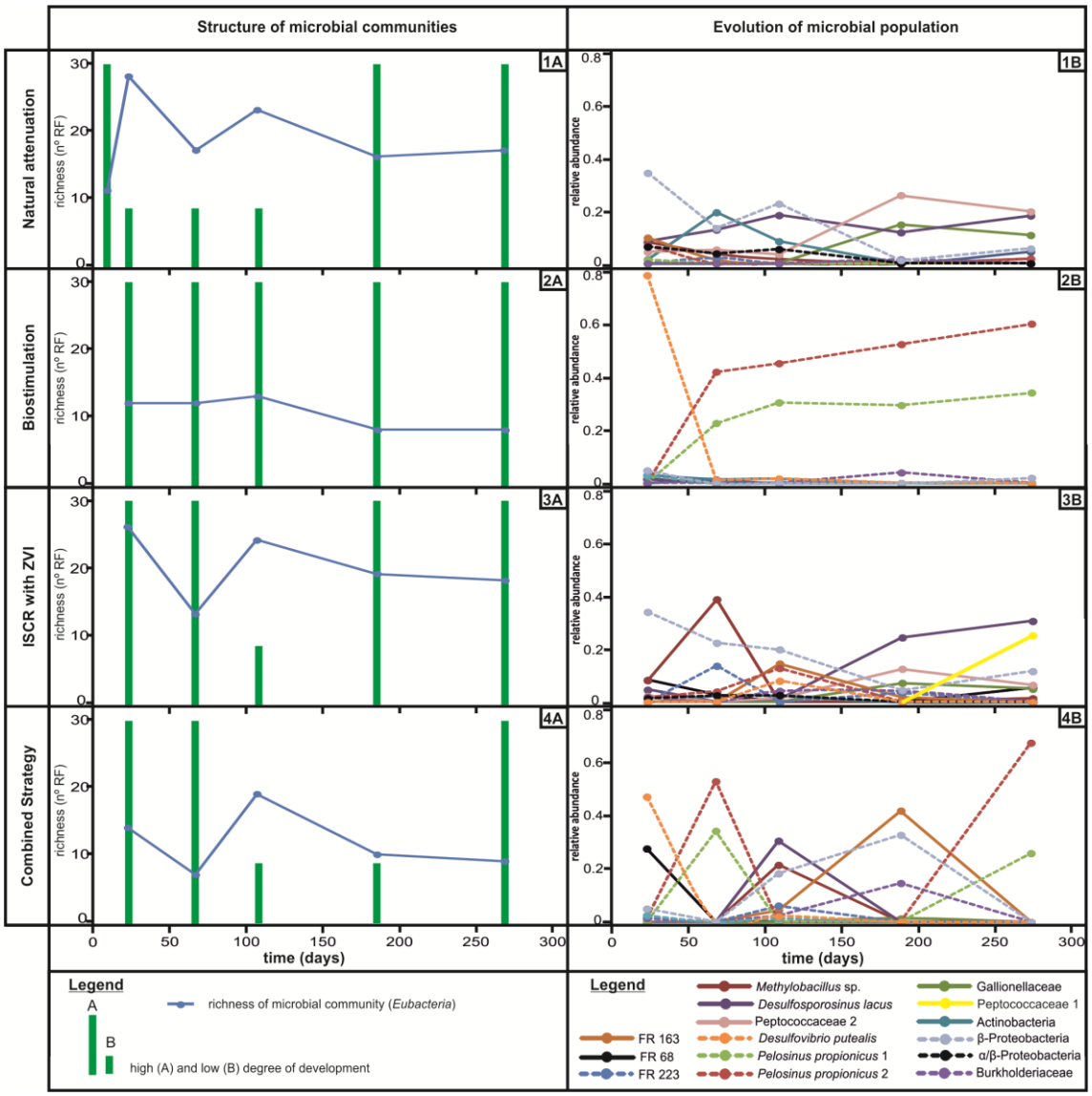

Figure 2: Evolution of the degree of development and richness of the microbial communities (A) and evolution of the different microbial populations detected using T-RFLP and identified with clone library (B) for experiments of microcosm of natural attenuation (1), biostimulation (2), ISCR with ZVI (3) and combined strategy (4).

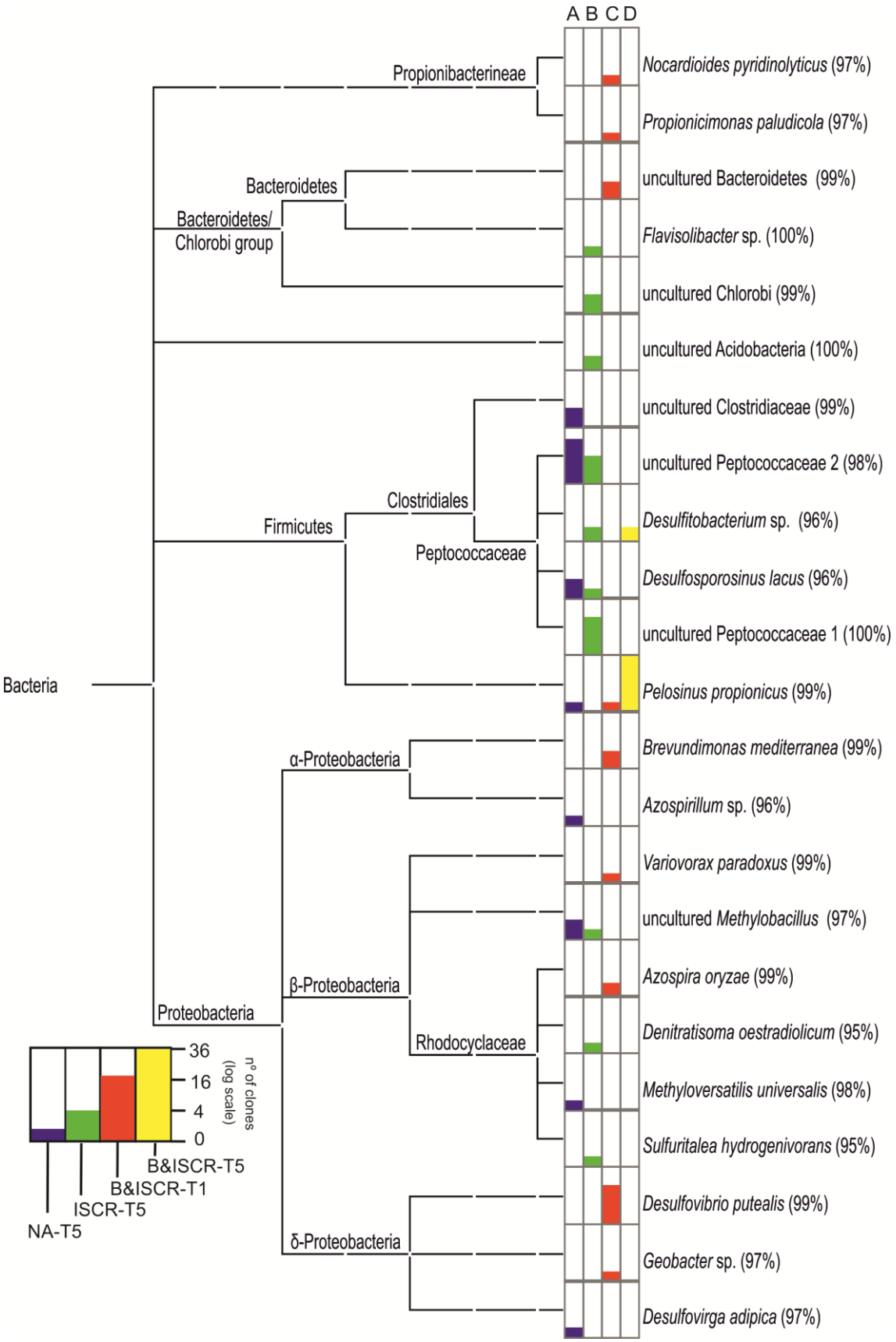

Figure 3: Phyla fraction for each of the sequenced samples. A: sample from natural attenuation experiment at 267 days (T5). B: sample from ISCR experiment at 267 days (T5). C: sample from combined strategy experiment at 22 days (T1). D: sample from combined strategy experiment at 267 days (T5).

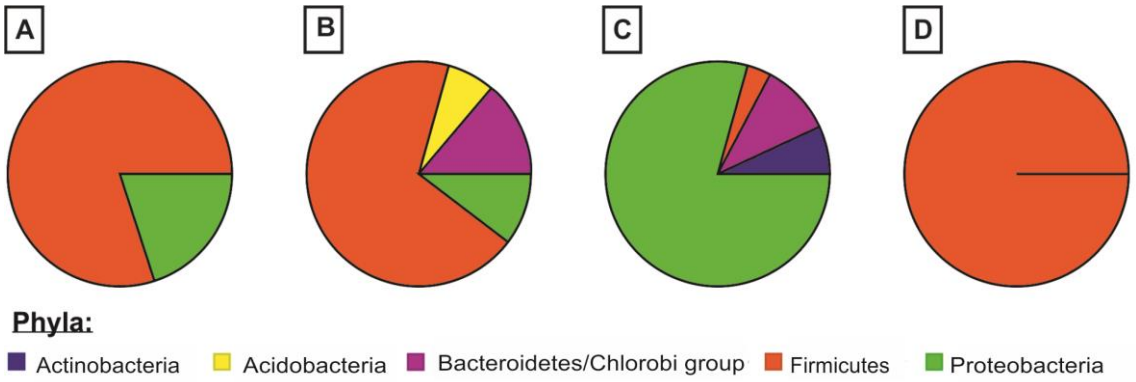

Figure 4: Phylogenetic tree of the sequenced samples. Percentage refers to the similitude degree with database sequences. NA-T5 (A): sample from natural attenuation experiment at 267 days (T5). ISCR-T5 (B): sample from ISCR experiment at 267 days (T5). B&ISCR-T1 (C): sample from combined strategy experiment at 22 days (T1). B&ISCR-T5 (D): sample from combined strategy experiment at 267 days (T5).