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Dissertation thesis

Divergent organohalide-respiring consortia in PCB-contaminated sediments

Divergentní mikrobiální konsorcia respirující organohalidy v sedimentech kontaminovaných PCB

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I declare that I developed this doctoral thesis independently under the guidance of my supervisor and I used the only sources that I quote and mention in the reference list. I didn´t use this work or it´s essential part for gaining any of doctoral or other academic degree.

I declare that the results presented in section 4.2.2. were made with substantial contribution of Monika Čvančarová, Ph.D. and section 4.2.3 with contribution of Daniel Berdat, Ph.D.

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Content

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Abstract

This study is focused on one particular group of halogenated compounds known as polychlorinated biphenyls (PCBs) – synthetic organic substances, where the hydrogen atoms of the biphenyl core are replaced by chlorine atoms. Number of chlorine atoms in the PCB molecule may vary from 1 to 10. Depending on the position and number of chlorines, there are theoretically 209 individual PCB congeners, some of which are toxic.

Although PCBs production was brought to a halt thirty years ago, recalcitrance to degradation makes them a major environmental pollutant at a global scale. Large amounts of PCBs were produced in several countries, and former Czechoslovakia belonged to the ten major world producers. Despite the high chemical stability of PCB congeners, bacterial process of reductive dechlorination, named organohalide respiration (OHR), was shown to be efficient in the dechlorination of extensively chlorinated PCB congeners as a prerequisite step towards their subsequent complete mineralization by aerobic bacteria. In our study, reductive dechlorination of PCBs was assessed using long term anaerobic microcosms. The microcosms were inoculated with highly contaminated sediments with weathered Delor located in the efflux channel of the former PCB manufacturer Chemko Strazske. After 10 months of cultivation the chemical analysis showed a dechlorination of up to 36 % of the highly chlorinated congeners in favor of medium and low chlorinated ones. T-RFLP analysis of the bacterial communities demonstrated that the diverse microbial consortia evolved from the original sediment samples after inoculation. Analysis of the rRNA gene pools indicated the presence of numerous members of the phylum *Chloroflexi*, including "*Dehalococcoides*" sp. and "*Dehalococcoides*"-like group (DLG) known OHR bacteria, as well as secondary fermenters.

Second study aimed at exploring degradation contributions by other taxa using sediment-free microcosms (SFMs) supplemented with the commercial PCB mixture Delor 103. Our results showed high rates of congener dehalogenation (up to 95.5 %) in the long term (692 days). The bacterial communities were represented by twelve major phyla dominated by *Chloroflexi*, *Proteobacteria*, and *Firmicutes*. In the first batch of SFMs, *Dehalococcoides mccartyi* strains CG4 and CBDB1 were considered as the main congener degraders. However, addition of 2-bromoethanesulfonate (BES) in a second

batch of SFMs inhibited population of *Dehalococcoides* sp. From all retrieved partial 16S rRNA gene sequences from BES-treated microcosms only two sequences affiliated to genus *Dehalococcoides* were detected contributing to a mere 0.02 % and 0.04 % of the community (one sequence out of 5983 sequences from SFM 11 and one out of 2494 sequences from SFM13). The BES-treated SFMs showed different community structures, especially with contributions of organisms involved in syntrophic activities. Indirect evidence provided by both statistical and phylogenetic analyses validated the significant implication of a new cluster of actors, affiliated with family *Geobacteraceae* (phylum δ-*Proteobacteria*) in the dechlorination of PCBs. Members of this family are known already for the degradation capacity of chlorinated solvents. This study provides first evidence about the possible implication of the *Geobacteraceae* members in the PCB congener degradation.

Abstrakt

Tato práce je zaměřena skupinu halogenovaných sloučenin označovaných jako polychlorované bifenyly (PCB) – syntetické organické látky, u nichž jsou vodíkové atomy na bifenylovém skeletu nahrazeny v různé míře atomy chloru. Počet atomů chloru v molekule PCB může být v rozmezí 1-10 a dle poloh umístění těchto atomů může být vytvořeno až 209 různých kongenerů, z nichž některé jsou toxické.

Přestože byla produkce PCB směsí zastavena již před více než třiceti lety, odolnost těchto látek k degradaci je učinila jedním z hlavních polutantů životního prostředí v globálním měřítku. Velké množství PCB bylo produkováno několika zeměmi a bývalé Československo patřilo k deseti největším světovým producentům. Přestože jsou PCB kongenery velice chemicky stabilní, bylo zjištěno, že proces bakteriální reduktivní dechlorinace, nazvaný též jako respirace organohalidů (OHR) je velmi účinným způsobem dechlorinace vysoce chlorovaných molekul PCB. Tento proces je také nutným krokem ke kompletní mineralizaci aerobními bakteriemi.

V rámci této studie byla reduktivní dechlorinace PCB studována pomocí dlouhodobě kultivovaných anaerobních mikrokosmů. Mikrokosmy byly inokulovány sedimentem s vysokou kontaminací PCB. Vzorky byly odebrány z vypouštěcího kanálu bývalého výrobce PCB Chemko Strážske. Po 10 měsících kultivace byla provedena chemická analýza, která prokázala dechlorinaci vysoce chlorovaných PCB až o 36 % ve prospěch středně a méně chlorovaných sloučenin. T-RFLP analýza bakteriálních komunit prokázala, že došlo k vývoji odlišných mikrobiálních společenstev v různých vzorcích, přestože na počátku byly profily podobné. Analýza genu rRNA naznačila přítomnost početné skupiny zástupců kmene *Chloroflexi*, zahrnujícího také rod *Dehalococcoides* a skupinu bakterií blízce příbuzných *Dehalococcoides*, které jsou známými OHR bakteriemi a také skupinu sekundárních fermentujících bakterií.

Druhá studie v rámci této práce cílila na odhalení degradačního potenciálu dalšími taxony bakterií s použitím mikrokosmů bez přítomnosti sedimentu, tzv. sediment-free kultur (SFM) doplněných o komerční směs PCB (Delor 103). Naše výsledky prokázaly vysokou míru dehalogenace kongenerů (až o 95.5 %) v dlouhodobě kultivovaných mikrokosmech (692 dní). Bakteriální komunity byly reprezentovány především dvanácti kmeny, mezi nimiž dominovaly především bakterie taxonů *Chloroflexi, Proteobacteria* a *Firmicutes.* V prvním souboru kultur bez sedimentu byly detekovány kmeny

Dehalococcoides mccartyi CG4 a CBDB1 jako bakterie dechlorinující hlavní kongenery. V druhém souboru mikrokosmů byl přidán 2-bromoethansulfonát (BES), který růst *Dehalococcoides sp.* nepřímo inhiboval. Ze všech detekovaných částečných sekvencí genu rRNA v BESem ošetřených mikrokosmech byly získány pouze dvě sekvence vykazující příbuznost s rodem *Dehalococcoides*, tvořící 0.02 % a 0.04 % celkové komunity (jedna sekvence z celkových 5983 byla získána ze SFM11 a jedna z celkových 2494 sekvencí pocházela ze SFM13). BESem ošetřené kultury ukázaly odlišnou strukturu komunit, především s ohledem na organismy zapojené v syntrofních procesech. Nepřímé důkazy poskytnuté na základě statistických a fylogenetických analýz prokázaly významný vliv nového taxonomického shluku (patřícího do čeledi *Geobacteraceae*) na dechlorinaci PCB. Členové této čeledi jsou známí také svou kapacitou degradovat chlorovaná rozpouštědla. Tato studie tedy poskytuje první důkazy o možném zapojení taxonu *Geobacteraceae* do dechlorinace PCB kongenerů.

List of Abreviations

ANOVA	analysis of variance
BES	2-Bromoethanesulfonate
bp	base pair
CB	chlorobiphenyl
DCE	1,1-dichloroethene
DLG	Dehalococcoides-like group
DNA	deoxyribonucleic acid
GC-ECD	gas chromatography with electron capture detection
GC-MS	gas chromatography – mass spectrometry
MFA	multifactorial analysis
NGS	next generation sequencing
OHR	organohalide respiration
OHRB	organohalide-respiring bacteria
OTU	operational taxonomic unit
PAH	polyaromatic hydrocarbon
PCA	principal component analysis
PCB	polychlorinated biphenyl
PCE	tetrachloroethene
PCR	polymerase chain reaction
RDA	redundancy analysis
RDH	reductive dehalogenase
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SFM	sediment-free microcosm
TCE	trichloroethene
TOC	total organic carbon
TPH	total petroleum hydrocarbon
T-RFLP	terminal-restriction fragment lenght polymorfism
VC	vinyl chloride

1. Introduction

1.1. General aspects

Water is an essential component of all life forms and activities on Earth. The water used by human comprises only 1 % of all water resources. Rivers and lakes contain just ca. 0.0001 % of the total amount of this resource. They are vital carriers of water and nutrients, draining nearly 75 % of the Earth's land surface (Hebert and Kundell, 2008). For ages, rivers and lakes had a great importance biologically, historically and culturally. As an example, all European country capitals have been set on the shores of large rivers (Prague, Rome, London, etc). Nowadays, an increasing demand related to the growth of the world population, to agricultural, and to industrial practices leads to an increased pressure of the surface water resources.

Besides the quantitative aspects of dependence on water resource, additional pressure is exerted qualitatively equally. Numerous substances produced by human activities accumulate in surface water as a result of agricultural practices, industrial activities, and waste disposal, including careless handling and storage, spills and leakages (Danielopol et al., 2003). In Europe, about 250 000 contaminated sites were reported as endangering human and ecosystem health in 2006, and 3 million sites are potentially threatened by polluting anthropogenic activities (European Environment Agency, 2007).

The presented thesis is focused on a significant class of molecules which are considered nowadays as one of the main classes of water contaminants called organohalides. Organohalides, and mainly chlorinated ones, have been present naturally in the environment for ages as they are formed through biotic or abiotic processes (Asplund and Grimvall, 1991; Gribble, 2003; Keppler et al., 2002). High temperatures and pressure facilitate their formation and geothermal processes, such as volcano eruptions and forest fires, are providing such conditions (Häggblom and Bossert, 2003). These compounds are also naturally synthetized by seaweeds, corals, algae, jellyfish, sponges, fungi, bacteria, insects, and mammals (Asplund and Grimvall, 1991; van Pée and Unversucht, 2003). Thus, organohalides are present in a wide variety of environments. In 2003, Gribble et al. estimated the species number of naturally-occurring organohalides to be larger than 3800 (Gribble, 2003). In a more general way, biological agents play an essential role in cycling halogenated compounds in the environment (Häggblom and Bossert, 2003; van Pée and Unversucht, 2003).

Beside the naturally formed organohalide compounds, artificially synthetized halogenated compounds have been massively used in a widerange of industrial activities during the last century. Massive usage, along with careless handling and storage, spills and leakages have made organohalides one of the most abundant classes among the surface and groundwater contaminants. Due to their low solubility in water, these compounds can persist in the environment for long periods of time, causing plumes of dissolved material to remain at concentrations that are many orders of magnitude above the level of concern (http://www.env.gov.bc.ca/wat/wq/BCguidelines/pcbs/pcbs-02.htm).

1.2. Aim of this study

This study is focused on one particular group among the halogenated compounds called polychlorinated biphenyls (PCBs). These were produced in large amounts in several countries, and former Czechoslovakia belonged to the ten major world producers. A large quantity of PCBs generated during the manufacturing was released into the surrounding area by improper disposal, resulting in high environmental PCB levels in various matrices (i.e. soil, sediments, water, and wildlife) in the Michalovce district (Kočan, 2001). Nowadays, part of the Eastern Slovakia is still highly polluted by persisting chemicals from the former PCB producer – the factory Chemko Strazske (Chovancová et al., 2005; Jursa et al., 2006; Kočan, 2001; Petrík et al., 2006).

The main goals of the presented thesis were defined as i) characterization of the microbial communities structure present in the sediments at the Strazske efflux channel, ii) development of stable sediment-free cultures so as to study microbial communities without impact of the sediment, iii) identification of new putative organohalide-respiring bacteria.

2. Aromatic compounds

Aromatic compounds derive their names from their early discovery, as their scent made them a class of oily compounds with dedicated and characteristic fragrant odours. According to current definition, the chemical meaning of the "aromaticity" is associated with a remarkable stability resulting from their molecular structure. Aromaticity is a chemical property describing the way in which a conjugated ring of unsaturated bonds, lone pairs, or empty orbitals exhibits a stabilization stronger than would be expected by the stabilization of conjugation alone. Aromatic compounds group a large number of unsaturated chemical compounds with conjugated planar ring systems with delocalized π electron clouds. (Messmer and Schultz, 1987; Schleyer, 2001).

2.1. Organohalide compounds

Organohalides constitute a wide group of aromatic and non-aromatic organic chemicals with covalently bound halogen atoms on the hydrocarbon core. Although organohalide compounds are typically considered to be anthropogenic industrial compounds, several thousands of them have natural biogenic and geogenic origin making up a significant portion of the global organohalogen budget (Gribble, 1999). Various simple chlorinated hydrocarbons including dichloromethane, chloroform, and carbon tetrachloride have been isolated from marine algae, plants, bacteria, fungi, insects, corals and sponges recently. For instance, the dry-cleaning solvents trichloroethylene and tetrachloroethylene are produced by at least 27 species of marine algae (Abrahamsson et al., 1995). Also, numerous organochlorides, including dioxins, are produced under the high temperature during forest fires and as the consequence of geophysical and volcanic activities (Gribble, 2003; Jordan et al., 2000).

Besides naturally occurring organohalides, production of man-made compounds has increased throughout the last century and enormous amounts of these chemicals have been released into the environment (Stringer and Johnston, 2001). However, no accurate information on the quantity of leaked chlorinated organohalides is available. The degree of contamination is determined based on the information about polluted localities count only (The Interstate Technology and Regulatory Council, 2005).

2.2. Polychlorinated biphenyls (PCBs)

2.2.1. PCBs production

The term "polychlorinated biphenyls" is used as a general name for a group of chemical compounds containing one to ten atoms of chlorine bound on the biphenyl frame. PCBs were produced by the direct chloration of biphenyl in the presence of $FeCl_3$ as a catalyst (Bailey, 2002). Theoretically, it could be created up to 209 various congeners differing in the number and positions of the substituents in the molecule (Fig. 1). However, conventional production methods can produce only 102 isomers in significant amounts. Nevertheless, commercial mixtures produced by various chemical factories around the world contained ca. 30 to 60 congeners in average. These mixtures of PCB congeners were manufactured under different brand names depending on the country of the origin, e.g. Aroclor (USA), Clophen (Germany), Kaneclor (Japan), Fenclor (Italy) and Delor (former Czechoslovakia) (Taniyasu et al., 2003).

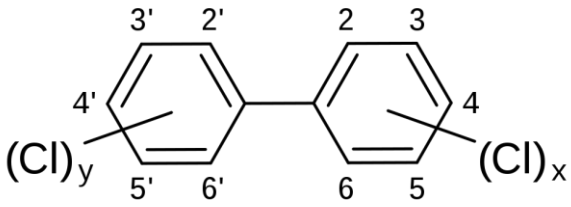

Figure 1. General chemical structure of polychlorinated biphenyl (PCB). Numbers denote potential locations of chlorine atoms in the molecule.

The massive production of PCBs in the world started in the late 1920s. In 1966, starting evidence showed their impact on living organisms, and 50 years after the beginning of their production, the use of PCBs was prohibited worldwide. Since then, congeners have been indentified in samples from very different environments on the whole planet (Kalantzi et al., 2001). Although no precise amounts are known, it was calculated that the worldwide production averaged ca. $2x10^6$ tons of PCBs, from which ca. 20 % were released into the environment (Hooper et al., 1990).

In Eastern Europe, PCBs were manufactured by Strazske plant in former Czechoslovakia from 1959 to 1984 under the commercial name Delor (Kočan, 2001). By this time, the factory belonged to the eight largest world manufacturers of commercial PCB mixtures. Total production reached approximately 22 000 tons, from which 46 % was exported to former East Germany (Taniyasu et al., 2003). Around 60 % of the whole production was based on the Delor 103 containing a majority of tri- and tetra-chlorobiphenyls. Mixture was equivalent to the Aroclor 1242 produced in USA (Grabic et al., 2006). Although the production was stopped almost 25 years ago, the immediate vicinity of the plant still belongs to one of the most PCB-contaminated hot spots in Europe (Brázová et al., 2012; Wimmerová et al., 2015).

Highest concentrations of PCBs were found in the efflux water channel leading from Chemko Strazske factory to Laborec River. Waterflow continues subsequently to the water reservoir and holliday area Zemplinska Sirava (Brázová et al., 2012; Dercová et al., 2008).

2.2.2. Properties and use of PCBs

Polychlorinated biphenyl (PCB) congeners constitute a very dangerous form of environmental pollution, due to their very chemically stable structures. They are strongly resistant to oxidation and hydrolysis by acids or alkali. They also have high thermal stability, are inflammable and have special dielectric properties which made them ideal for their use as industrial agents. In the electrotechnical industry, they were mainly used in condensators and transformators as electroinsulation liquids. In the mechanical engineering field, they were used as hydraulic and fluid liquids which were resistant to high temperatures. In chemical industry PCB congeners were heavily utilized as heat-carrying media. They were also added to paints, waxes and plastic materials (Borja et al., 2005). Their intrinsic chemical properties, mainly their high stability, and their inertness, induced a very high persistence in natural and man-made environments. Low solubility in water (from 0.0027 to 0.42 ng/l at 25 °C) and their hydrophobicity causes that PCBs are often associated with the solid fraction (e.g. dust particles, sediments etc.) of the aquatic and terrestrial environments (Luotamo et al., 1991). In general, sorption of PCBs increases with the degree of chlorination, the surface area and the organic carbon content of the sorbents (Mackay, 1992). Thus, it is extremely difficult to remove PCB congeners once they have been released into the environment.

2.2.3. Toxicity, food chain and health effect of PCBs

The manufacturing of PCBs has been running for 30 years until the first monitoring of their biological-toxicological effect was performed. Since then, a lot of studies showed that the toxicity of PCBs depends on the chloration degree (the more chlorine substituents molecule has, the more toxic is) and on the position of the substituents in the molecule of biphenyl. The most toxic PCB congeners are those with chlorines substituted in the *meta*- and *para*- positions. Toxicity is decreasing in this order: *para*- > *meta*- > *ortho*- (Mondello, 1989). The lipophilic nature of PCBs induces their accumulation in animal body tissues which has a dramatic impact on the food chain (Abramowicz, 1990). Higher organisms such as freshwater fishes European perch, wels catfish, common carp, freshwater bream, goldfish etc. in contaminated water reservoirs showed clear symptoms caused by the bioaccumulation of PCBs, particularly an increase of the mortality and a decrease of their reproductive abilities. The presence of PCBs was confirmed by the analysis of their adipose tissues, in which not less than 40 different congeners (from 3- to 8-chlorine substituted) were detected (Brázová et al., 2012).

A dramatic toxic effect on the human organism was established in 1968 in a collective poisoning in Japan caused by rice oil contaminated by Kaneclor 400. The so-called Yusho disease was described by exhaustion, vomiting and light hepatitis. In difficult cases, pneumonia, skin rash and hair loss were present (Marhold, 1986). It is known today that concentrations of PCB reaching 1 mg/m^3 and higher can be absorbed through the skin and being transported through the lymphatic and blood system, reaching spleen and impacting the metabolism (Tryphonas, 1995; Wang et al., 2013).

Although PCBs were not found to be active in point mutations, PCB exposure could lead to significant increases in the DNA strand breaks and chromosomal aberrations both *in vivo* and *in vitro*. Sargent et al. showed that planar PCB77 congener caused dose-related chromosome breakage in human lymphocytes exposed *in vitro* to concentrations $0.1–10^{-4}$ μg/ml (Sargent et al., 1989). Furthermore, some recent studies showed that some PCBs and their metabolites can covalently covalently bind to the DNA and proteins (Mörck et al., 2002). Thus, PCBs might increase the possibility for DNA damage (Zhu, 2011).

2.3. Degradation possibilities

Facing the immense challenge of cleaning up the large number of contaminated sites, several remediation technologies have been developed for the remediation of PCB congeners. PCB´s extreme resistance to physical and chemical influence is the main reason explaning why the (bio)-degradation of these compounds was considered as a difficult task. The degradation of PCB congeners occurs either through metabolic or non-metabolic reactions. Major non-metabolic reactions are hydrolysis, photolysis and thermal degradation, which require extreme physical conditions. However, these processes are expensive and time consuming (Kaštánek and Kaštánek, 2005). On the other hand, metabolic (so-called biological) methods used for environment cleaning have been receiving growing attention for the past few decades. Recent research has shown that microorganisms can provide an important natural tool for the gentle and environmental friendly detoxification of natural habitats (Anyasi and Atagana, 2011).

2.3.1. Thermal elimination processes

In general, thermal processes involve the destruction or removal of contaminants through exposure to high temperature in treatment reactors. The main advantage of such approaches is requiring of short period of time to eliminate the pollutants. However, the *ex-situ* processes need an excavation of the material (usually soil) and it increases the final expenses and engeneering equipment, permitting and material handling worker safety issues (http://www.clu-in.org).

Nowadays, several approaches are applied for *in situ* thermal PCB degradation. *Thermal desorption* involves an application of high temperature (320-560 °C) to volatilize water and organics to a treatment system (Uzgiris et al., 1995). *Hot gas decontamination* involves raising temperature of contaminated material to 260 °C for specified period of time and the gas effluent is then treated in an afterburner system so as to terminate all volatilized components (Hyman and Dupont, 2001). *Plasma systems*, based on the plasma arc, work under the conditions where the contaminated material is treated with 5000-15 000 °C and the vapors are afterburned as fuel (Heberlein and Murphy, 2008). *Pyrolysis* decomposes the contaminated material using high-temperature (more than 430 °C) with less than stochiometric quantities of oxygen (Pavel and Gavrilescu, 2008). Nowadays, one of the most applied remediation techniques is use of *thermal incineration* technology. This process allows for the elimination of a variety of contaminants using high temperature as well (870-1200 °C) in the presence of oxygen (Soesilo and Wilson, 1997).

Finally, the *vitrification technology* uses an electric current to melt contaminated matter at high temperatures (1600-2000 °C). Upon cooling, the product of vitrification is chemically stable and leaching-resistant (Pavel and Gavrilescu, 2008).

The advantage of these termal technologies consists in handling large volumes of contaminated material, achieving the destruction of up to 99.99 % of the organic compounds. The releasing material produced in this way is resistant to leaching. However, these high technology procedures require large and expensive devices to provide extreme conditions needed for safe PCB decontamination (Anyasi and Atagana, 2011) as well as high energy consumption. Also, some thermal technologies such as incineration or desorption can form highly toxic by-products when the temperature decreases below 700 °C, such as polychlorinated dibenzo-p-dioxins or polychlorinated dibenzo-furans (Altarawneh et al., 2009).

2.3.2. Chemical and physico-chemical PCB elimination

Compared to thermal processes, the combined physico-chemical methods avoid the risk of production of harmful by-products. The most common technology is the metallic *sodium dechlorination* of PCBs. Sodium is easily oxidized and has a strong affinity for certain elements, including chlorine. It reacts with the chlorine atoms on the PCB congener to give sodium chloride. A reaction is carried out at 98 °C (above the sodium melting point) (Miyoshi et al., 2000). Also a process called as *super critical water oxidation (SCWO)* could be used for dechlorination. Water at high temperature and pressure, above 374 °C and 221 bar, is a fluid that is neither a gas nor a liquid, in so-called supercritical status. Under these conditions, water gains unique properties that can be used for the destruction of hazardous waste (Veriansyah and Kim, 2007). *Solvated electron technology (SolV^{TM})* involves the exposition of the chlorinated molecules to free electrons (Getman, 1998). The *ECO LOGIC Process* involves the gas-phase chemical reduction of organic compounds by hydrogen at 900 °C or higher. Thus, the compounds are reduced to methane and hydrogen chloride (Fiedler, 2002). Chemical dehalogenation can include also an *alkaline polyethylene glycolate (APEG)* process or *base-catalyzed decomposition (BCD)* (Fiedler, 2002). Another technology - *Advanced oxidative processes (AOPs)* - uses O_2, H_2O_2, TiO_2, iron or other oxidizing compounds to degrade PCBs (Stasinakis, 2008). Finally, *photochemical degradation* uses short light wavelengths (295-400 nm) to generate direct and indirect photolytic processes that can degrade PCBs (Lodolo et al., 2001).

2.3.3. Bioprocesses for the elimination of the PCB

Biological processes, so-called bioremediation processes, have been used in many waste treatment procedures and are one of the most cost-effective means for environmental remediation. These methods are accomplished through the use of living organisms including fungi, bacteria, and algae (http://www.clu-in.org). For the same purposes, recent advances showed that also plants can participate in the PCB degradation in the process called phytoremediation (Gavrilescu, M., 2010). Several direct and indirect interactions and mutual cooperation of plants and growth promoting rhizosphere bacteria were studied (Macková et al., 2006). Mackova *et al.* 1997 used *in vitro* cell cultures of a variety of plants species (*Armoracia rusticana*, *Solanum aviculare*, *Atropa belladonna*, and *Solanum nigrum*) to characterize the metabolism of a commercial mixture (Delor 103) and observed that PCB transformation capability greatly differed from strain to strain (Macková et al., 1997). It was also shown, that plant roots release organic compounds, such as sugars, amino acids, and organic acids that can be used as electron donors to support aerobic co-metabolism or anaerobic dehalogenation of chlorinated compounds. In some instances, microbial aerobic metabolism will consume oxygen, resulting in anaerobic conditions favorable to PCB dehalogenation. Also, plants secrete extracellular enzymes that can initiate transformation of PCBs and facilitate further microbial metabolism (Aken et al., 2010). The word bioremediation appeared first time in a peer-reviewed scientific publication in 1987 (Sloan. R., 1987).

Due to the importance of this chapter for the present thesis, full details on the biological processes will be developed in the next chapters.

2.4. Bacterial metabolism and degradation of organohalides

Numerous bacterial species with an ability to degrade organic pollutants have been discovered until now. The number of newly discovered bacteria is growing with the advance of cultivation techniques and molecular research methodology. The biodegradation of PCBs is known to occur in two ways involving anaerobic and aerobic processes respectively. The selective pressure caused that very versatile and flexible bacteria were able to react to the presence of the pollutants and use them as a potential substrate or electron acceptor for their growth (Gribble, 1994, 1999, 2003). Thus, anaerobic degradation, so-called organohalide respiration, corresponds to a decrease of the number of chlorine substituents on the biphenyl core. On the other hand, aerobic way handles the PCBs in co-metabolism with biphenyl, which is served as a carbon and energy source and induces respective enzymes (Pieper, 2004).

2.4.1. Coupling anaerobic and aerobic degradation

Interaction between aerobic and anaerobic bacteria in terms of degradation of organohalide pollutants occurring in the environment was first time described in 1995 (Abramowicz, 1995). And, it was suggested to be a potential strategy for PCB removal from impacted sediments. Several examinations demonstrated that sequential treatment of the PCB contaminated sediment first by the anaerobic PCB organohalide-respiring consortia followed by transfer to aerobic culture with *Burkholderia xenovorans* LB400 effectively mineralize PCB mixture Aroclor by as much a 70 % (Master et al., 2002).

2.4.2. Anaerobic metabolism of aromatic compounds

The first documented observation of the anaerobic degradation bacteria-mediated process was made in Hudson River sediment samples in 1987 (Brown et al., 1987). Different pattern of PCBs relative to the original Aroclor composition within the sediment was characterized by a higher portion of mono- and di-chlorinated congeners, higher proportion of *ortho* chlorines and a selective depletion of tri- through penta-CBs (Brown et al., 1988; Furukawa, 2000). Since then dechlorination activity was demonstrated on many sites all around the world (Abramowicz, 1990; Baba et al., 2007; Fagervold et al., 2007; Fava et al., 2003; Kočan, 2001).

Bacteria conserving energy during reductive dechlorination have been discovered in the early 1990's. These bacteria were able to utilize organohalides as terminal electron acceptors in a respiration process referred as organohalide respiration (OHR). OHR is a microbial-mediated process that uses halogenated organic compounds (from natural or anthropogenic origin) as a terminal electron acceptor in the respiration chain (Bradley and Chapelle, 2010).

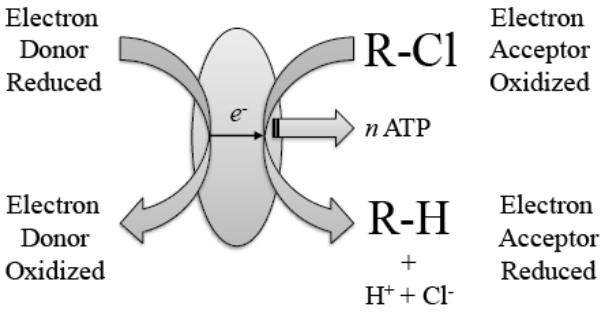

Figure 2. Biological mechanism involved in the organohalide respiration (Nunez, Andres Enrique, 2008).

In the specific case of PCB congeners, reductive dehalogenation is the exergonic process involving the transfer of two electrons and one proton to the molecule, which results in the release of chloride ion and a less chlorinated PCB congener (Figure 2) (Fetzner and Lingens, 1994; Häggblom and Bossert, 2003).

Table 1. Position of chlorines removed by each OHR process (Wiegel and Wu, 2000).

Dechlorination process	Susceptible chlorines
M	Flanked and unflanked *meta*
Q	Flanked and unflanked *para, meta* of 23-group
H´	Flanked *para, meta* of 23- and 234-groups
H´	Flanked *para,* doubly flanked *meta*
P	Flanked *para*
N	Flanked *meta*
LP	Flanked and unflanked *para*
T	Flanked *meta* of 2345-group, in hepta- and octachlorobiphenyls

The main benefit of OHR is the conversion of highly chlorinated congeners to less chlorinated ones, which are often less toxic and more prone to further biotransformation. Typically, less chlorinated and o*rtho*-chlorinated congeners are accumulated as highly chlorinated *meta*- and *para*-chlorinated congeners tend to be depleted (Quensen et al., 1988). Recently, several OHR processes have been described (Table 1). It is expected that a wide variety of bacterial metabolism types could be responsible for the various pathways (Bedard, 2008; Wiegel and Wu, 2000).

2.4.3. Enzymes involved in organohalide respiration

Large efforts have been made to identify and characterize key enzymes so as to understand the biochemical mechanisms of reductive dehalogenation. As mentioned above, OHR bacteria are able to grow while using chlorinated compounds as terminal electron acceptor. In this case, the reduction of chlorinated compounds by specific enzymes is coupled to energy conservation via electron transport phosphorylation (Futagami et al., 2008). Reductive dehalogenases (RDHs) are the final reductases of the respiration chain and the key catalytic enzymes in the respiratory chain of OHR microorganisms (Holliger et al., 1998).

All RDHs are typically composed of two subunits – a large catalytic subunit A (encoded by *rdh*A) and a small protein subunit B (encoded by *rdh*B) acting as a membrane anchor (Futagami et al., 2008). In some cases RDH operons contain also genes *rdh*C-H, whose function has yet to be determined (Kube et al., 2005) (Fig. 4). All reductive dehalogenases characterized so far are associated with the cytoplasmic membrane, reinforcing their role in membrane-associated electron transport-coupled phosphorylation. The N-terminal region of most reductive dehalogenases contains a TAT (twin arginin translocation) consensus sequence (usually “RRXFXK” but it can vary in some cases), which is involved in the translocation of the protein into or across the cytoplasmic membrane (Palmer et al., 2005). Additionaly, all enzymes contain in the C-terminal region a characteristic consensus sequences “CXXCXXCXXXCP” and “GXXCXXCXXXCS”. These two sequences encode two iron-sulphur clusters (Fe-S) present in the catalytic center of the α-subunit of the RDH enzyme (Fig. 3) (Suyama et al., 2002).

RDHs are corrinoid- and B_{12}-dependent enzymes, which are now one of the three currently recognized classes of B_{12} enzymes (Banerjee and Ragsdale, 2003). Co(I) corrinoid in its free form has previously been shown to reductively dechlorinates tetrachloroethene (PCE) and other chlorinated ethenes in homogeneous aqueous solutions (Glod et al., 1997; John et al., 2006).

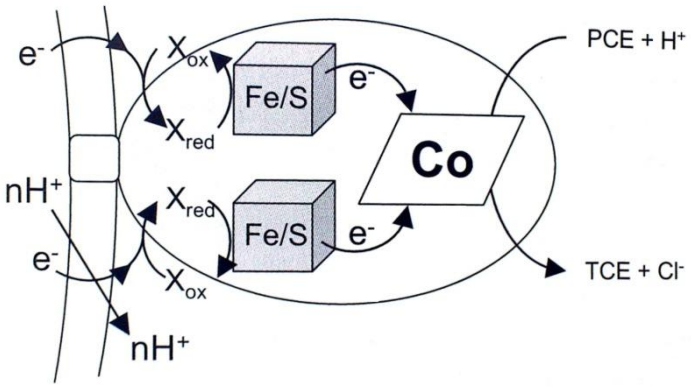

Figure 3. Presumed membrane attachment of the catalytic subunit of tetrachloroethylene dehalogenase (PceA) by a small membrane anchor protein (PceB), and postulated flow of electrons (Díaz, 2008).

The first purified RDH was a 3-chlorobenzoate-specific enzyme obtained from the membrane fraction of *Desulfomonile tiedjei* DCB-1 (Ni et al., 1995). Then, RDHs were identified and characterized, targeting trichloroethene (TCE) (*tce*A encoded trichloroethene RDH) and vinyl chloride (*vcr*A encoding vinyl chloride RDH) from a bacterium called *Dehalococcoides ethenogenes* strain 195 (Maymó-Gatell et al., 1997), reclassified in 2013 to *D.mccartyi* strain 195 (Löffler et al., 2013). Subsequently, other few reductive dehalogenases have been identified and biochemically characterized within the genera *Dehalococcoides*: Tetrachloroethylene reductive dehalogenase (encoded *pce*A) dechlorinating PCE to TCE (Magnuson et al., 1998), trichloroethylene reductive dehalogenase (encoded by *tce*A) dechlorinating TCE (Magnuson et al., 2000), and vinylchloride reductive dehalogenase (encoded by *vcr*A or *bvc*A), which both calatyze the dechlorination of vinyl chloride (VC) to ethane (Krajmalnik-Brown et al., 2004; Müller et al., 2004). Additionaly, it was shown that *vcr*A RDH is involved also in transformation of TCE and various DCE isomers (Hug, 2012; Müller et al., 2004).

Other reductive dehalogenases were isolated from *Desulfitobacterium hafniense* and *D.frappieri – crpA* and *crd*A – dechlorinating a variety of chlorophenols (Bisaillon et al., 2010; Boyer et al., 2003). Also, RDHs were isolated for instance from *Desulfitobacterium dehalogenans* (van de Pas et al., 1999) which was shown to be able to respire chlorophenol. Maillard et al. isolated a tetrachloroethene reductase (*pce*A) from *Dehalobacter restrictus* (Maillard et al., 2003). Variety of other different bacterial phyla were shown to harbor various RDHs (Hug, 2012; Picardal et al., 1995; Sung et al., 2006a).

Multiple presences of the RDH genes may be found in specific cases. An analysis of the genome of *Dehalococcoides mccartyi* strain 195 revealed 18 putative *rdh*A genes that appeared homologous to the genes encoding biochemically purified RDHs (Seshadri et al., 2005; Villemur et al., 2002). Based on these sequences, Krajmalnik-Brown et al. designed degenerate primers to amplify *Dehalococcoides*-specific homologous RDHs sequences (Krajmalnik-Brown et al., 2004). This allowed amplification of many RDH genes from different strains of *Dehalococcoides*, including strain BAV1 (7 homologues), strain FL2 (14 homologues), and strain CBDB1 (32 homologues) (Krajmalnik-Brown et al., 2004; Kube et al., 2005; Waller et al., 2005). Also, *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 harbor 19 *rdh* genes (Moe et al., 2009).

Interestingly, till now, only one archeal putative RDH gene (from *Ferroglobus* species) has been identified (Hafenbradl et al., 1996). However, the list of proven enzymes is still quite restricted mainly due to the difficulty to analyse the structure of these strictly anaerobic enzymes bound to the cytoplasmic membrane.

Three distinct *Dehalococcoides* strains CG1, CG4 and CG5 harbouring 35, 15 and 26 *rdh*As were found in 2014. It was found that the most highly transcribed *rdh*A genes were identical when cultures were fed with either PCBs or PCE, suggesting that the same dominant *rdh*As were involved in chlorine removal from both PCBs and PCE (Wang et al., 2014).

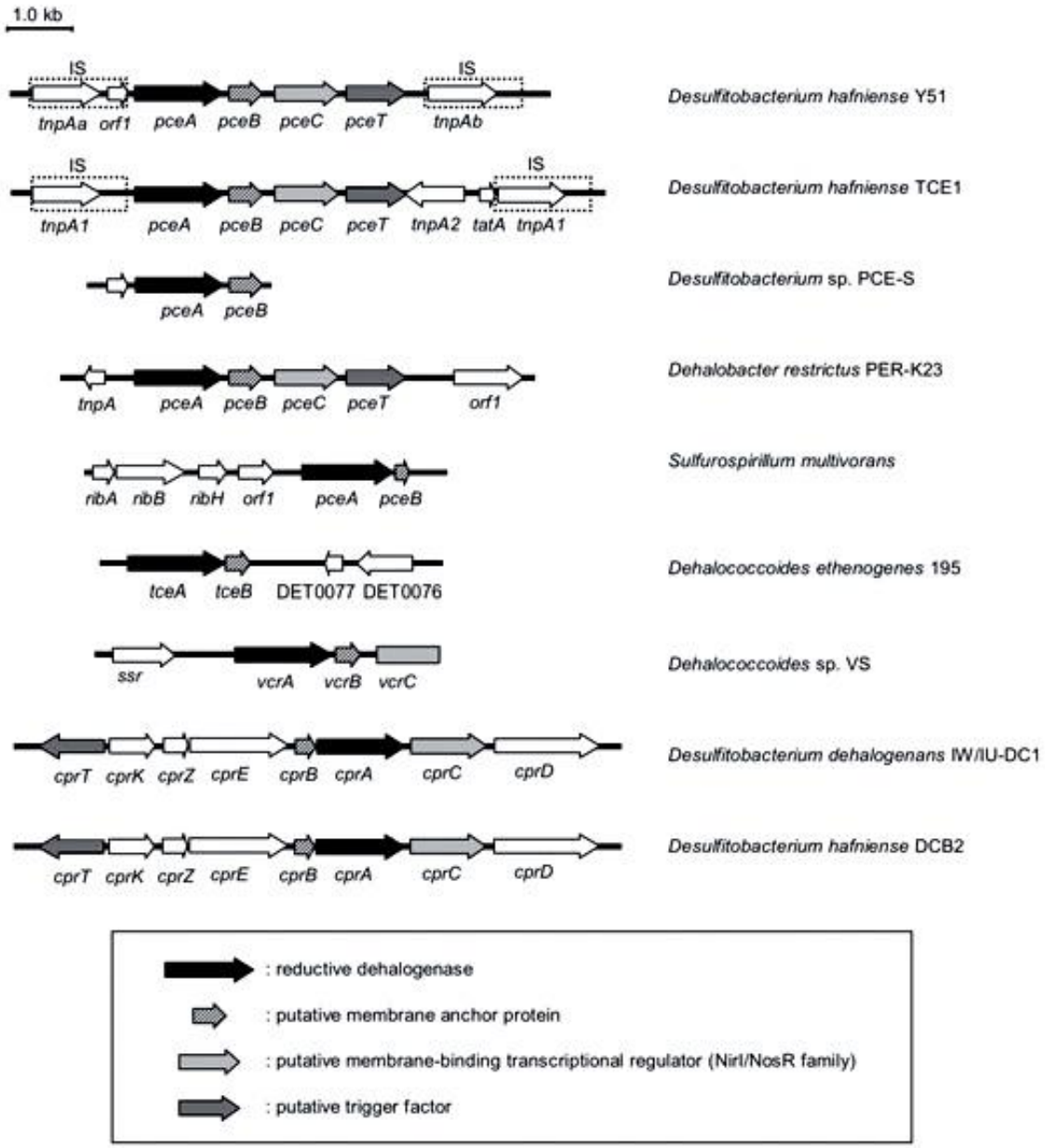

Figure 4. Organisation of the reductive dehalogenase genes in selected OHR bacteria (Futagami et al., 2008).

2.4.4. Diversity and phylogeny of dehalorespirers

Until now discovered organohalide respiring bacteria (OHRB) have been affiliated with several distinct bacterial phyla, such as δ-*Proteobacteria*, ε-*Proteobacteria*, *Chloroflexi* or *Firmicutes* (Fig. 5) (Taş et al., 2010). And, obviously there is no relation between organohalide specificity and phylogenetic affiliation of the OHRB, suggesting that this type of respiration process could be a common feature shared by prokaryotes (Shani, 2012; Smidt and de Vos, 2004).

Additionaly, frequent detection of phylotypes related to OHRB in environments without historical anthropogenic exposure and highly developed mechanisms of organohalide respiration indicates that the reductive dehalogenation is likely an ancient evolutionary process (Yoshida and Katayama, 2010). OHRB can be classified in two metabolic categories – facultatively and obligatory OHR bacteria. In general, the strict OHRB are represented by three bacterial genera – *Dehalobacter* (phylum *Firmicutes*), *Dehalococcoides* (phylum *Chloroflexi*) and *Dehalogenimonas* (phylum *Chloroflexi*). The most famous dehalorespiring bacterium is probably *Dehalococcoides mccartyi* strain 195 (described in 1997 as *Dehalococcoides ethenogenes* strain 195 (Maymó-Gatell et al., 1997), the first obligatory dehalorespiring bacterium capable reductively dechlorinate tetrachlorethene to VC and ethene, chloroethenes, chlorophenols and polychlorinated biphenyls/dioxins (PBDs) (Adrian et al., 2000, 2007; Bunge et al., 2003; Fennell et al., 2004; Hiraishi, 2008; Taş et al., 2010; Yoshida and Katayama, 2010). Since then, several of *Dehalococcoides* isolates were described (e.g. strains CBDB1, VS, BAV1, FL2, GT etc.). Recently, two *Dehalococcoides* strains CG4 and CG5 were described to specifically dechlorinate PCBs (Wang et al., 2014).

Interestingly, 16S rDNA gene in *Dehalococcoides spp.* is highly conserved throughout the entire genus (sharing >98 % similarity in nucleotide sequence and >85 % identity at the amino acid level) (Fig. 5). However, various studies showed that this group is functionally very diverse (Adrian et al., 2000; Duhamel et al., 2004; He et al., 2003; Hendrickson et al., 2002; Krajmalnik-Brown et al., 2004; Maymó-Gatell et al., 1997).

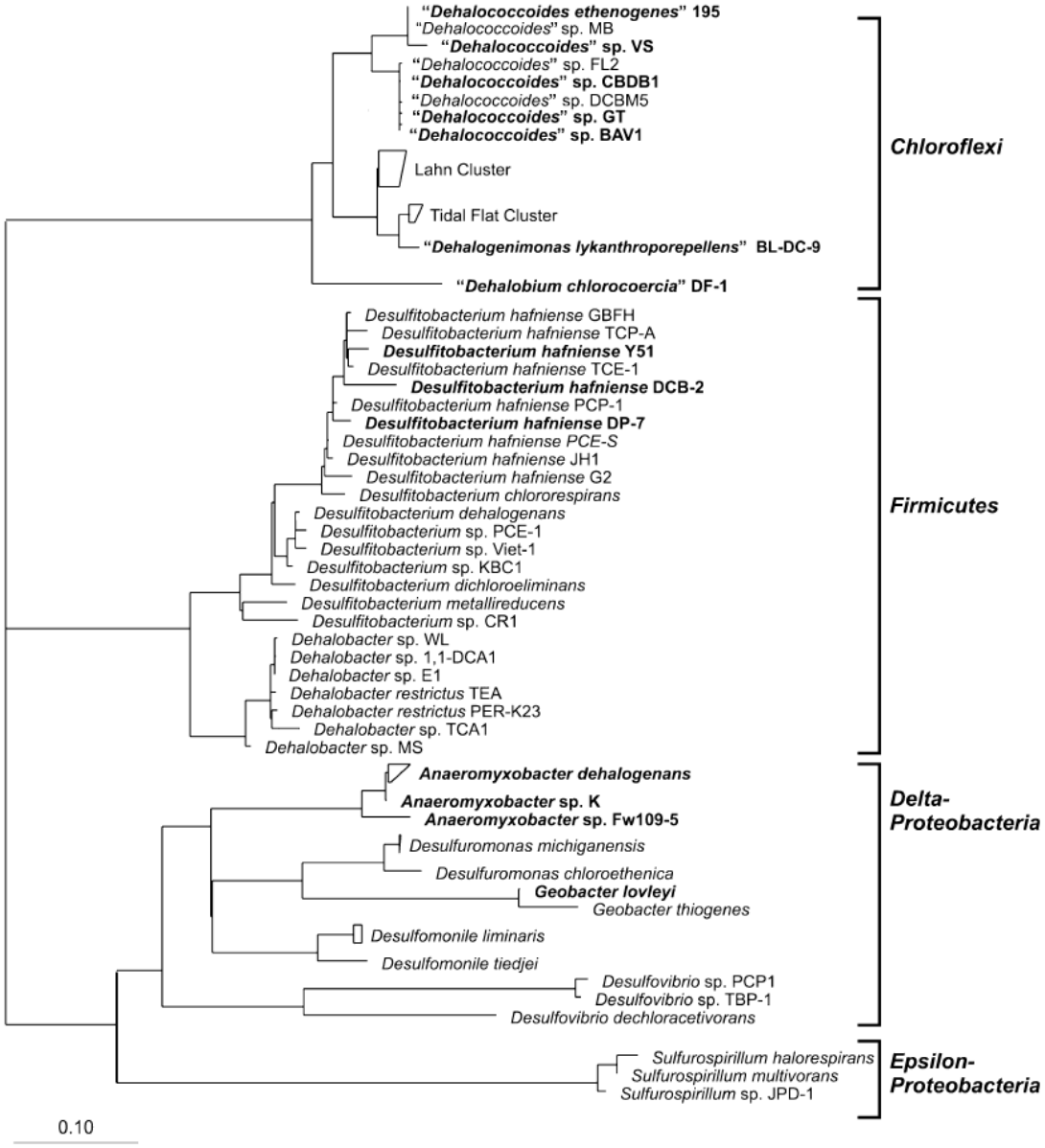

Figure 5. Phylogenetic tree of organohalide respiring bacteria based on 16S rRNA sequences (Taş et al., 2010).

Chemical compounds proved to be used were 1,1-dichloroethene (DCE), *cis*-DCE, *trans*-DCE and VC for strain BAV1 (He et al., 2003); TCE and *cis*-DCE for strain FL2 (He et al., 2005); TCE, 1,1-DCE, *cis*-DCE and VC for strain GT (Sung et al., 2006b); and *cis*-DCE and VC for strain VS (Rosner et al., 1997); PCBs for strains CG4 a CG5 (Wang et al., 2014). On the other hand, strain CBDB1 is able to grow on chlorobenzenes, chlorophenols and polychlorinated dibenzo-*p*-dioxins/furans (PCDD/Fs) (Adrian et al., 2000, 2007; Bunge et al., 2003; Fennell et al., 2004). *Dehalococcoides spp.* is difficult to grow in pure cultures. Conversely, members of this genus are easily maintained in microbial communities such as Cornell (Maymó-Gatell et al., 1997), Victoria (Hendrickson et al., 2002), Pinellas (Harkness et al., 1999), KB-1 (Duhamel et al., 2002) or ANAS cultures (Richardson et al., 2002).

A relatively diverse group of bacteria exists as a phylogenetic sister cluster within the phylum *Chloroflexi* designed as *o*-17/DF-1, sometimes called as *Dehalococcoides*-like group (DLG) (Fig. 5). Bacterium *o*-17, an organism responsible for *ortho*- OHR process of 2,3,5,6-chlorobiphenyl, was identified using comparative sequence analysis of 16S rDNA identical to unidentified eubacterium RFLP-17, which was first obtained from 2,3,5,6-CB *ortho*-dechlorinating enrichment culture (Holoman et al., 1998). Later on, this rDNA sequence was assigned to the same specific microorganism referred as bacterium *o*-17. This bacterium shares a highest 16S rDNA similarity (89 %) with *D.mccartyi* strain 195 (Cutter et al., 2001).

On the other hand, bacterium DF-1, isolated from DF cultures, belonging to the same deeply branching phylogenetic cluster as *D.mccartyi* strain 195, shares only 87 % of similarity of its 16S rDNA with the bacterium *o*-17. This bacterium was shown to reductively dechlorinate PCB congeners with doubly flanked chlorines when incubated with formate or H_2:CO_2 (80:20) (Wu et al., 2002).

Recently, two promising novel *Chloroflexi* clusters designated as Lahn and Tidal cluster were discovered. Members of both clusters showed a potential to use PCE as a terminal electron acceptor transforming it to *cis*-DCE (Lahn cluster) and *trans*-DCE (Tidal cluster). Lahn cluster members were detected in Lahn river sediment (Germany) and are remotely related to *Dehalococcoides spp*. sharing 92-94 % similarity of 16S rDNA (Kittelmann and Friedrich, 2008). On the other hand, population of Tidal cluster was studied in the tidal flat sediment from North Sea exposed to low concentrations of PCE (Kittelmann and Friedrich, 2008). Bacteria of this cluster are closely related to obligate OHR bacterium *Dehalogenimonas lykanthroporepellens* BL-DC-9 (97 % of 16S rDNA similarity) and to Lahn cluster bacteria (96 % of 16S rDNA sequence identity) (Fig. 5).

Another group of obligate OHR bacteria belonging to the genus *Dehalobacter* was established in 1998 (Holliger et al., 1998). At present, three type strains were identified *D. restrictus* strain PER-K23, strain TEA growing on PCE and TCE (Wild et al., 1996) as well as strain TCA1 growing on 1,1,2-trichloroethane and 1,1-dichloroethane as a terminal electron acceptor (Sun, 2002). Additionaly, it was proved that the PER-K23 strain can debrominate polybrominated diphenyl ethers (Robrock et al., 2008). It was reported also that cultures containing members of genus *Dehalobacter* (described as KFL culture) were able to remove chlorines substituted at the *para*-, *meta*- and *ortho*-positions of PCBs and 1,2,3-trichlorodibenzo-*p*-dioxin (1,2,3-CDD) (Yoshida et al., 2009).

The last genus composing obligatory OHR bacteria known up to date was established and described in 2009 as *Dehalogenimonas lykanthroporepellens* belonging to the phylum *Chloroflexi* (Moe et al., 2009). Two strains labeled as BL-DC-8 and BL-DC-9 isolated from chlorosolvent-contaminated groundwater were shown to be closely related, sharing 99.93 % of 16S rDNA sequence similarity. The closest environmental clone sequence is the sediment/soil slurry clone ErMS-91 (EU542454) with 93.7 % 16S rDNA gene sequence identity. The identity of their 16S rDNA gene sequences with cultured relatives of genus *Dehalococcoides* was proved to be about 90 %. However, the similarity with other published OHR species is less than 90 % (Moe et al., 2009; Yan et al., 2009a, 2009b). These bacteria were proved to grow on 1,2,3-trichloropropane (1,2,3-TCP) as electron acceptor (Yan et al., 2009b). Strain BL-DC-9 also couples cell growth to reductive dehaloelimination reactions involving a variety of other polychlorinated aliphatic alkanes. 1,2-dichloropropane (1,2-DCP) is transformed to propene, 1,2-dichloroethane (1,2-DCA) is transformed to ethene, 1,1,2-trichloroethane (1,1,2-TCA) is transformed to VC, 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA) is transformed to a mixture of *cis*- and *trans*-dichloroethene (DCE) (Moe et al., 2009; Yan et al., 2009b).

Facultative OHR bacteria are represented by multitude of bacteria present in the genera *Anaeromyxobacter, Desulfitobacterium, Sulfurospirillum, Desulfomonile, Desulfuromonas, Desulfovibrio,* and *Geobacter*. All members of these genera were shown to be metabolically versatile with respect to their spectrum of electron donors and acceptors. These OHRB are able to dehalogenate a wide range of halogenated compounds (Hiraishi, 2008). Additionaly, these genera include also species unable to carry out organohalide respiration process.

The first described facultative OHRB isolated in pure culture was *Desulfomonile tiedjei* DCB-1 (DeWeerd et al., 1990; Shelton and Tiedje, 1984). *D. tiedjei,* belonging to the phylum δ-*Proteobacteria* (Fig. 5), was found to dehalogenate a limited range of aromatic compounds, such as benzoates, benzamides, tetrachloroethene (Holliger et al., 1998; Mohn and Kennedy, 1992). The same phylum contains members of the genus *Geobacter* represented by one reported species *Geobacter lovleyi* strain SZ capable of coupling the oxidation of acetate and H_2 to the reduction of a variety of electron acceptors, including PCE, TCE, nitrate, uranium, iron or elementar sulphur (Sung et al., 2006a). As other examples from the same phylum could be mention the members of the genus *Desulfuromonas,* represented by *D. chloroethenica* strain TT4B and *D.michiganensis* strains BB1 and BRS1 utilizing PCE and TCE (Krumholz, 1997; Sung et al., 2003). Also, the representative of the genus *Anaeromyxobacter*, species *A. dehalogenans,* was established as aryl-halorespiring bacterium (Sanford et al., 2002).

Two important representatives of the phylum ε-*Proteobacteria* have been isolated and affiliated to the genus *Sulfurospirillum* (Luijten et al., 2003) (Fig. 5) – *S.halorespirans* reducing PCE to cDCE (Luijten et al., 2003; Scholz-Muramatsu et al., 1995) and *S.multivorans* has been shown to grow also in cultures debrominating polybrominated biphenyl ethers (He et al., 2006). One of the most characterized groups of facultative OHR bacteria is the genus *Desulfitobacterium* affiliated to the phylum *Firmicutes* (Fig. 5) containing several species able to reduce aliphatic or aromatic chlorides or both *D.dehalogenans* dechlorinating a broad range of chlorophenols (PCP, TeCP, 2,3,4-TCP, 2,3,6-TCP, 2,4,6-TCP, 2,3-DCP, 2,4-DCP and 2,6-DCP) but only at the *ortho* position (Utkin et al., 1994, 1995). Another member, *D. frappieri* strain PCP-1 is the only known pure isolate capable of dechlorinating pentachlorophenol (PCP) to monochlorophenol in metabolic pathway PCP -> 2,3,4,5-TeCP -> 3,4,5-TCP -> 3,5-DCP -> 3-CP (Bouchard et al., 1996). Several *Desulfitobacterium* strains are able to use humic substances, metals and metalloids as terminal electron acceptor, however, no strain was described to completely reduce PCE or TCE to ethene, neither PCBs (Hiraishi, 2008).

Additionaly, genomic studies have further expanded the knowledge on the genetic diversity among cultured isolates from different species, building on findings obtained through cultivation and physiological characterization (Kube et al., 2005; McMurdie et al., 2009; Nonaka et al., 2006; Thomas et al., 2008) (Fig. 6). Genomics has been a tool for the understanding of differences in the metabolism of the OHR bacteria, giving us clues on the adaptation and dedication of these bacteria to organohalide respiration.

The large genomes of *Anaeromyxobacter dehalogenans* (Thomas et al., 2008) and of two strains of *Desulfitobacterium hafniense* (Nonaka et al., 2006) with size of more than 5 Mbps confirm the versatile metabolism of these bacteria as compared to *Dehalococcoides spp.*, which have smaller genomes of 1.5 Mbps and which are strictly restricted to organohalide respiration (Fig. 6). *A. dehalogenans* 2CP-C genome has up to 68 putative c-type cytochrome genes (Thomas et al., 2008), most likely enabling this organism to occupy environments with variable redox conditions.

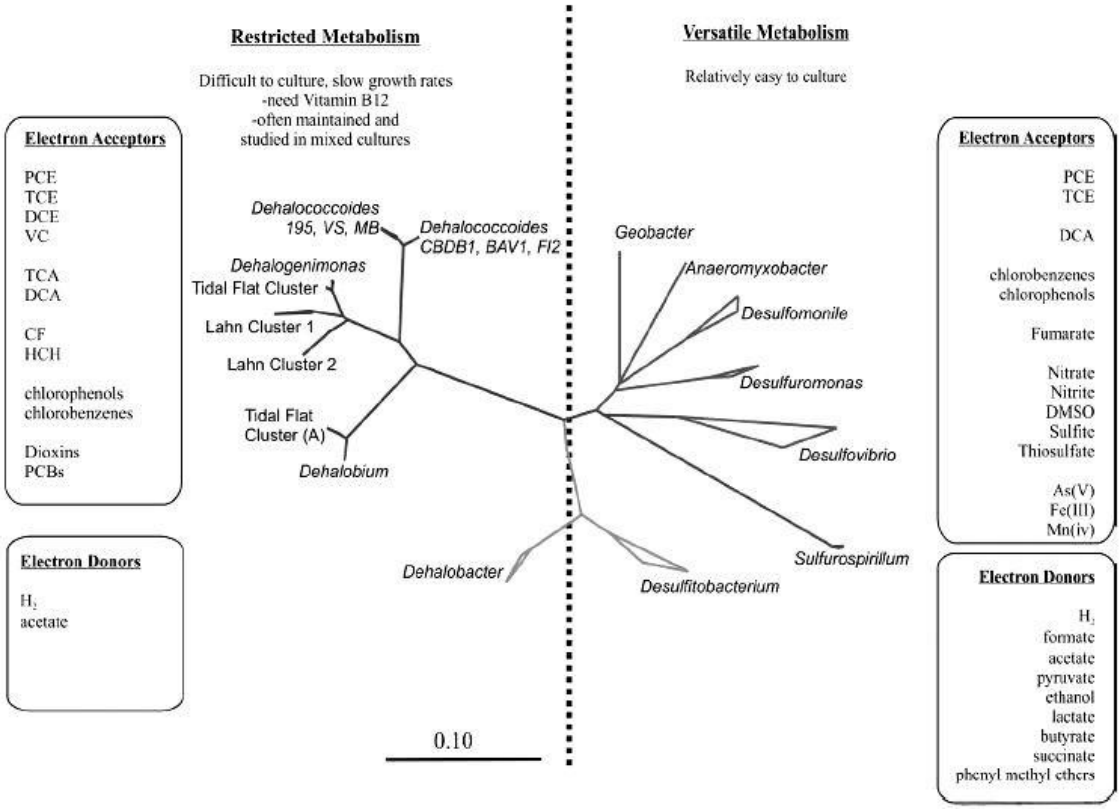

Figure 6. Phylogenetic tree of organohalide respiring bacteria based on 16S rRNA sequences (Maphosa, 2010).

2.5. Syntrophic interactions and competition for resources

Many types of microorganisms with different metabolic strategies exist over a broad redox spectrum. The dehalogenation capabilities of aerobic and anaerobic microorganisms often overlap, underscoring their complementary interactions in and with their environment (Häggblom and Bossert, 2003).

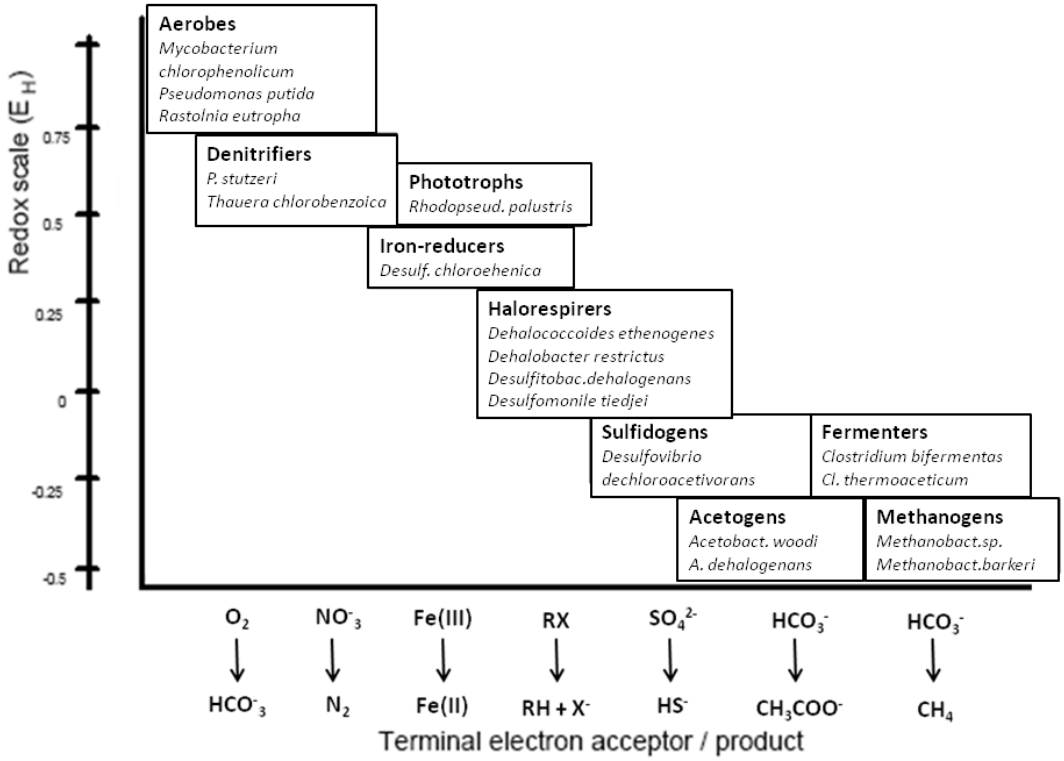

Figure 7. Relationships among redox microbial processes prevailing in the environment (Häggblom and Bossert, 2003).

Aerobic dehalogenating bacteria such as *Pseudomonas*, *Mycobacteria* and methylotrophs occupy the most oxidized zones, where the O_2 (as a reactant or as an electron acceptor) is present. Facultative, denitrifying dehalogenating microorganisms (e.g. *Pseudomonas stutzeri*, *Thauera chlorobenzoica*) may compete or overlap at the periphery of aerobic zones in presence of nitrate. Dehalorespirers, strict anaerobes, occupy a niche in the middle of the redox range (Fig. 7) and are flanked by iron- and sulphate-reducing bacteria. At the end of the redox spectrum acetogens and methanogens live in the most reduced anoxic sphere, often competing for the same electron donor (H_2) produced during secondary fermentation process (syntrophy). In this redox zone, a large number of microbial groups compete for the available H_2, and therefore production and utilization of H_2 is one of the most important factors that control the kinetics and thermodynamics of all running processes (Fig. 8).

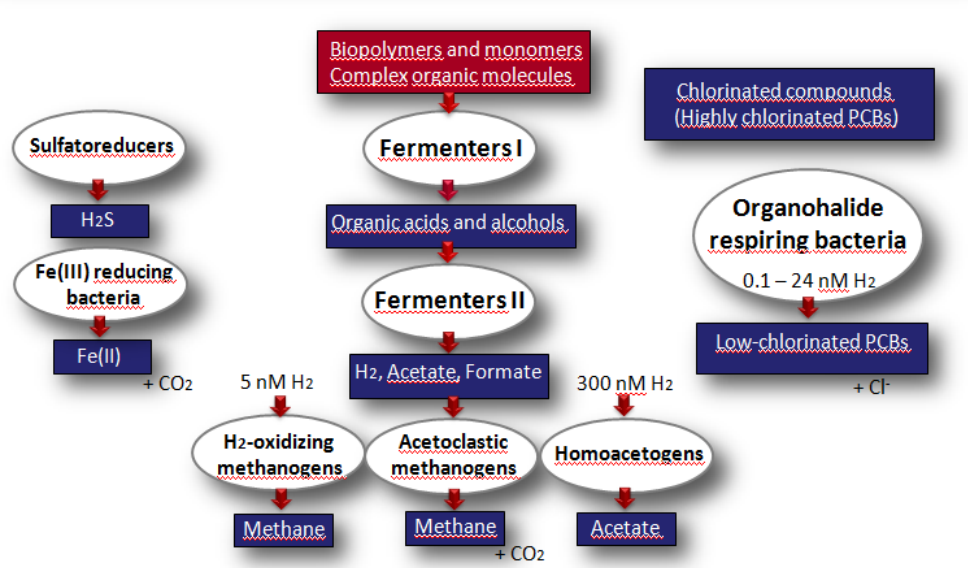

Figure 8. Carbon and energy flux as well as hydrogen demand along the anaerobic degradation chain (Shani, 2012).

It has been predicted that hydrogenotrophic halogen-respiring bacteria should out-compete other hydrogenotrophic processes such as sulfate reduction, acetogenesis and methanogenesis in environments where the slow release of H_2 by obligate syntrophic fermenting populations is supplied (He et al., 2002; Smidt and de Vos, 2004). Slowly growing filamentous bacteria from *Chloroflexi*-Subphylum I (*Levilinea*, *Belilinea*, *Leptolinea*, *Longilinea* and *Anaerolinea*) are known as primary and secondary fermenters that utilize saccharides or peptides, producing H_2 as the end product and thus, participate in the OHR process (Yamada and Sekiguchi, 2009). One of the biotechnologic strategies favoring OHR microorganisms over other hydrogenotrophic populations involves addition of certain fermentable substrates such as butyrate or propionate (Fennell et al., 1997) enabling slow release of H_2 by syntrophic fermentation (Tab. 2) (Becker et al., 2005; Löffler et al.,1999).

Table 2. Role of H_2 in determining predominant electron accepting process (modified from (Löffler et al., 1999).

Electron Acceptor Process	Hydrogen Concentration (nM)
Aerobic (O_2) respiration	< 0.1
Denitrification	< 0.1
Iron(III) reduction	0.2 – 0.6
Dehalorespiration	< 0.3
Sulfate reduction	1 -4
Methanogenesis	> 5
Acetogenesis	> 336

3. Material and methods

3.1. Material

3.1.1. Chemicals

$(NH4)_2SO_4$	Lachema, Czech Republic
Agar bacteriological	ThermoFisherScientific, USA
Agar purified	Difco, BD Diagnostics, USA
Agarose	Lonza, Switzerland
Bacteriological Peptone	Scharlau, Spain
$CaCl_2$	Sigma-Aldrich, USA
$CaSO_4$	Lachema, Czech Republic
$CuSO_4$	Lachema, Czech Republic
dNTP Mix (2mM, 10mM)	Fermentas, USA
EDTA	Sigma-Aldrich, USA
Ethanol (for molecular biology)	Lachema, Czech Republic
Ethidium bromide (solution, 10mg/ml)	Sigma-Aldrich, USA
Phenol	Serva, Germany
$FeSO_4 . 7H_2O$	Lachema, Czech Republic
GelRed	Biotium, USA
Glucose	Lachema, Czech Republic
Glycerol	Serva, Germany
GuHCl (guanidin-hydrochlorid)	Sigma-Aldrich, USA
Chloramphenicol	Sigma-Aldrich, USA
IPTG (isopropylthiogalaktosid)	Fermentas, USA
Isopropanol	Lachema, Czech Republic
$K_2HPO_4 . 3H_2O$	Lachema, Czech Republic
KCl	Lachema, Czech Republic
$MgCl_2 . 6H_2O$	Merck, Germany
$MgSO_4 . 7H_2O$	Lachema, Czech Republic

$MnSO_4 . H_2O$	Lachema, Czech Republic
$Na_2HPO_4 . 2H_2O$	Lachema, Czech Republic
NaCl	Lachema, Czech Republic
$NaMoO_4 . 2H_2O$	Lachema, Czech Republic
Orange G	Sigma-Aldrich, USA
SDS (dodecylsulfát sodný)	Scharlau, Spain
TEMED (tetrametylendiamin)	Sigma-Aldrich, USA
Tripton	ThermoFisherScientific, USA
Tris-hydroxymetyl-aminometan	ICN Biomedicals, USA
Triton X-100	Sigma-Aldrich, USA
Tween 20	Calbiochem, Merck, Germany
Water (molecular biology reagent)	5Prime, Germany
Yeast extract	Fluka, Sigma-Aldrich, USA

3.1.2. Commercial kits

BigDye® Terminator v3.1 Cycle Sequencing	ABI, USA
MSB® Spin PCRapace	Invitek, Germany
NucleoBond AXG-100	Macherey-Nagel, Germany
pGEM®-T Easy Vector Systems	Promega, USA
PowerClean® DNA Clean-Up Kit	MoBio, USA
PowerSoil™ DNA Isolation Kit	MoBio, USA
Quant-iT™ PicoGreen® dsDNA Assay Kit	Invitrogen, USA
RNA PowerSoil® Total RNA Isolation Kit	MoBio, USA
SV Total RNA Isolation system	Promega, USA
Tissue SEV kit Maxwell	Promega, USA
Wizard® SV Gel and PCR Clean-Up System	Promega, USA

The kits were used according to the manufacturers´ recommendations unless otherwise stated.

3.1.3. Laboratory equipment

Autoclave Sanyo MAC235EX	Sanyo, USA
Automatic pipets	Eppendorf, Germany
Equipment for the gas exchange	Home-made system
Hettich Micro 20 microfuge	Hettich instruments, USA
Centrifuge Eppendorf 5408	Eppendorf, Germany
Camera Kodak EDAS290	Kodak, USA
Anaerobic glove box	COY Laboratory
Horizontal electrophoresis Easy Cast B1	Thermo Scientific, USA
Icemaker	Zanussi, Italy
In-flow box Clean Air	CleanAir Tech, Netherlands
Freezer (-20°C)	Liebherr, Germany
Freezer (-80°C)	Sanyo, USA
Mastercycler ep	Eppendorf, Germany
Mastercycler ep realplex	Eppendorf, Germany
Maxwell® 16 Research System	Promega, USA
MiniCentrifuge	Eppendorf, Germany
NanoDrop ND-1000 UV-VIS Spectrophotometer	Thermo Scientific, USA
Nikon microscope – Eclipse E800	Nikon, Japan
pH meter with ISE electrode Orion	Orion Research Inc., USA
Power supply Owl OSP-250L	Thermo Scientific, USA
Refrigerator (+4 °C)	Liebherr, Germany
ABI 3130xl DNA capillary sequencer	ABI, USA
Thermo Electron FastPrep FP120 Cell Disrupter	Thermo Electron, USA
Thermomixer Comfort	Eppendorf, Germany
UV transiluminator UVT-20M	Herolab, Germany
Vortex Mixer MS2	IKA Labortechnik, Germany
XL-410 D electronic balance	Denver Instrument, USA

3.1.4. Cultivation media and solutions

LB (Luria Broth) medium and LB agar

Triptone	5 g
Yeast extract	2.5 g
NaCl	5 g
Distilled water	up to 500 ml

pH was adjusted at 7 and autoclave-sterilized at 121 °C for 20 min.

In order to prepare solid media, bacteriological agar was added (final concentration 15 g/l). For selective growth, ampicillin was added (final concentration 100 mg/l).

***Dehalobacter* cultivation medium**

An anaerobic cultivation medium designed specifically for growth of *Dehalobacter sp.* developed by Holliger et al. 1998 was used for the cultivation of sediment and sediment-free microcosms (Holliger et al., 1998). The medium was composed of four separately prepared solutions (A, B, C and D).

Solution A was made up with demineralized water, boiled at 100 °C and cooled at room temperature under stream of N_2:CO_2 (80 %:20 %; v/v). The solution was dispersed into serum bottles (VWR international AG, Merck, Switzerland) and the bottles were closed with viton stoppers (Maag Technic AG, Switzerland). The gas atmosphere was exchanged with N_2:CO_2 mixture (80 %:20 %; v/v) with 1.0 bar overpressure using a gas exchange system (Druva Sonderventie GmbH, Germany). The bottles were then autoclaved at 121 °C for 20 min.

Solution A	0.958 g K_2HPO_4 . $3H_2O$
	0.218 g NaH_2PO_4 . $2H_2O$
	0.01 % yeast extract
	1 ml resazurin solution 0.5 g/l

Solution B was composed of five solutions (IV to VIII). Solution IV was prepared by dissolution of EDTA in the 900 ml of demineralized water with HCl. The trace elements were added each one by one and the volume was then adjusted to 1000 ml and stored at 4 °C. The salts of solutions V, VI, VII and VIII were dissolved in appropriate amounts in demineralized water and sterilized in portions of 50-60 ml through 0.2 μm filter (Sarstedt AG, Switzerland) into anaerobic sterile bottles with N_2 atmosphere, covered with aluminium foil to avoid photolysis and stored at 4 °C.

Solution B was prepared aseptically adding of 1 ml of each stock solution of the trace element IV-VIII by syringe to 20 ml anaerobic sterile demineralized water in a 100-ml bottle. The distilled water was added up to 25 ml.

Solution B	1 ml solution IV
	1 ml solution V
	1 ml solution VI
	1 ml solution VII
	1 ml solution VIII
	distilled water up to 25 ml
Solution IV	500 mg/l EDTA
	2 000 mg/l $FeCl_2 .4H_2O$
	100 mg/l $MnCl_2 . 4H_2O$
	190 mg/l $CoCl_2 . 6H_2O$
	70 mg/l $ZnCl_2$
	2.55 mg/l $CuCl_2 . 2H_2O$
	5.52 mg/l $AlCl_3$
	6 mg/l H_3BO_3
	41.4 mg/l $Na_2MoO_4 . 2H_2O$
	24 mg/l $NiCl_2 . 6H_2O$
	1ml HCl (concentrated)

Solution V	50 mg/l Biotin (vitamine H)
	250 mg/l P-amino benzoate (Na-salt)
	50 mg/l Pantothenate (Na-salt)
	20 mg/l Folic acid (dihydrate)
	50 mg/l Lipoic acid (thioctic acid)
	100 mg/l Pyridoxine (vitamine B6)
	550 mg/l Nicotinamide
Solution VI	100 mg/l Thiamine HCl (vitamine B1)
Solution VII	50 mg/l Riboflavine (vitamine B2)
Solution VIII	50mg/l Cyanocobalamin (vitamine B12)

Solution C was composed of two solutions - IX and X. The appropriate amounts of salts for solution IX were dissolved in demineralized water. 49 ml of the solution were dispensed into 100-ml bottles. The bottles were closed and gas phase was exchanged with N_2 and autoclaved at 121°C for 20 min. Solution X was composed of Na_2S . $9H_2O$ dissolved in 100 ml of water and sterilized through 0.2 µm filter in anaerobic sterile bottles with N_2 atmosphere. The solution was stored at 4 °C until use. The final solution C was prepared from 49 ml of the solution IX and 1ml solution X which was transfered to solution IX anaerobically using sterile syringe.

Solution C	49 ml solution IX
	1 ml solution X
Solution IX	114 mM NH_4HCO_3
	906 mM $NaHCO_3$
Solution X	24.02 g Na_2S . $9H_2O$ (1M)
	MilliQ water up to 100 ml

The mineral salts of solution D were dissolved in demineralized water and the solution was dispensed in 100-ml bottles, which were closed with gum ruber, gas phase was exchanged with N_2 and bottles were autoclaved at 121 °C for 20 min.

Solution D	30 mM $CaCl_2 . 2H_2O$
	20 mM $MgCl_2 . 6H_2O$

The final medium was then prepared by compilation of the following solutions:

90 % Solution A, 2.5 % Solution B, 5.0 % Solution C and 2.5 % Solution D. Solutions B, C and D were added aseptically using syringe in the bottle containing Solution A.

SOC medium

Solution was prepared by dissolution of:

Trypton	20 g
Yeast extracts	5 g
NaCl	0.6 g
KCl	0.2 g
Glucose (50 %)	7 ml
Distilled water	up to 1 000 ml

After dissolution of chemicals in distilled water and sterilized by autoclaving 10 ml of sterile Mg^{2+} solution was added (1 M $MgCl_2$ + 1 M $MgSO_4$).

3.1.5. DNA-markers

For evaluation of DNA electropherograms were used the following DNA markers:

pBR322/*Alu*I – Fermentas, USA

Lambda DNA/*Hind*III – Fermentas, USA

100bp DNA Ladder – Promega, USA

1kb DNA Ladder – Promega, USA

3.1.6. Bacterial strains

Bacterial strains used in this study:

***Escherichia coli* strain JM109**: F´ (traD36, *pro*AB+ *lac*Iq, *lac*ZDM15*) end*A1 *rec*A1 *hsd*R17(rk$^-$, mk$^+$) *mcr*A *sup*E44 l- *gyr*A96 *rel*A1 D*(lacproAB)*

***Escherichia coli* XL1-Blue Supercompetent Cells:** *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lac*IqZΔ*M15* Tn*10* (Tetr)]

3.1.7. Primers

Table 3. Primers used in this study

Name	Sequences 5‘-3‘	Targets	(Reference)
8f-FAM	FAM-GAGTTTGATCMTGGCTCAG	*Eubacteria*	(Lane, D.J., 1991)
518r	ATTACCGCGGCTGCTGG		(Muyzer et al., 1993)
28f	GAGTTTGATCNTGGCTCAG	*Eubacteria*	
519r	GTNTTACNGCGGCKGCTG		
SP6	ATTTAGGTGACACTATAG	sequencing inserts cloned	Promega, USA
T7	TAATACGACTCACTATAGGG	into the pGEM® vector	
Dre441f	GTTAGGGAAGAACGGCATCTGT	*Dehalobacter sp.*	(Smits et al., 2004)
Dre645r	CCTCTCCTGTCCTCAAGCCATA		
DHC1f	GATGAACGCTAGCGGCG	*Dehalococcoides* Victoria & Pinellas	(Hendrickson et al., 2002)
DHC1377r	GGTTGGCACATCGACTTCAA	communities & Pinellas groups	
DHC883f	GGAGCGTGTGGTTTAATTCGATGC	*Dehalococcoides sp.*	(Adrian et al., 2007)
DHC1152r	GCCCAAGATATAAAGGCCATGCTG		
Dsb406f	GTACGACGAAGGCCTTCGGGT	*Desulfitobacterium* sp.	(Smits et al., 2004)
Dsb619r	CCCAGGGTTGAGCCCTAGGT		
Desulfo494f	AGGAAGCACCGGCTAACTCC	*Desulfuromonas sp*.	(Bond et al., 2002)
Desulfo1050r	CGATCCAGCCGAACTGACC		
Geo73f	CTTGCTCTTTCATTTAGTGG	*Geobacter lovleyi* strain SZ	(Duhamel et al., 2002)
Geo485r	AAGAAAACCGGGTATTAACC		
DHSPM576f	GCTCTCGAAACTGGTTACCTA	*Sulfurospirillum sp.multivorans* group	(Daprato et al., 2007)
DHSPM1210r	GTATCGCGTCTCTTTGTCCTA		
vcrA880f	CCCTCCAGATGCTCCCTTTA	*vcr*A	(Behrens et al., 2008)
vcrA1018r	ATCCCCTCTCCCGTGTAACC		
bvcA277f	TGGGGACCTGTACCTGAAAA	*bvc*A	(Behrens et al., 2008)
bvcA523r	CAAGACGCATTGTGGACATC		
PceA-b3f	ATIACICMNRARTWYGGICC	*pce*A group 1&2	This study (unpublished)
PceA-b4r-g1	TANCCRAACCAYTCRTCRAA	*pce*A group 1	
PceA-b4r-g2	TANCGNARIGCRTCRTCCAT	*pce*A group 2	
TceA-sp-b3f	TGGGARGGNACICCIGARGARAA	*tce*A	this study (unpublished)
TceA-sp2-b7r	CCNGCRTCDATIGGYTTIGT		
GNSB941f	AGCGGAGCGTGTGGTTT	*Chloroflexi*	(Gich et al., 2001)
GNSB1340r	GTTGCTAGTAACCGCG		
Arch349f-FAM	6FAM-GYGCASCAGKCGMGAAW	Archaeal 16S rDNA	(Takai and Horikoshi, 2000)
Arch806r	GGACTACVSGGGTATCTAAT		

3.1.8. Solutions for DNA extraction

Buffers for extraction method described by Selenska *et al.* using the Macherey-Nagel columns were prepared according to the following formulas (Selenská and Klingmüller, 1991):

G4 buffer	GuHCl	14.329 g
	Tween20	10 ml
	H_2O	to 50 ml

pH was adjusted to 5.5, sterilized through 0.22 µm filter and stored at 4 ºC.

N2 buffer	Tris	1.211 g
	Ethanol (99 %)	15 ml
	KCl	6.709 g
	Triton X-100	150 µl
	H_2O	to 100 ml

pH was adjusted using H_3PO_4 to 6.3 and solution was autoclaved (121 °C, 20 min).

N3 buffer	Tris	1.211 g
	Ethanol (99 %)	15 ml
	KCl	8.573 g
	H_2O	to 100 ml

pH was adjusted using H_3PO_4 to 6.3 and solution was autoclaved (121 °C, 20 min).

N5 buffer	Tris	1.211 g
	Ethanol (99 %)	15 ml
	KCl	7.455 g
	H_2O	to 100 ml

pH was adjusted using H_3PO_4 to 8.5 and solution was autoclaved (121 °C, 20 min).

TE buffer	TRIS (1 M), pH=8	500 µl
	EDTA (0.5 M), pH=8	100 µl
	H_2O	to 50 ml

Solution was sterilized by autoclaving (121 °C, 20 min).

3.1.9. Computer software

Computer software used in this study:

1. **DNAStar LaserGene 99** - analysis of nucleotide sequences (http://www.dnastar.com)
2. **BLAST -** Comparison of nucleotide sequences with known sequences from the internet database (http://www.ncbi.nlm.nih.gov/BLAST)
3. **Chromas Lite 2.01** - Analysis of sequencing chromatograms (http://www.technelysium.com.au)
4. **ClustalX –** a tool for multiple sequence alignments (Larkin et al., 2007)
5. **GeneDoc -** a tool for editing and annotating multiple sequence alignments (Nicholas et al., 1997)
6. **MEGA -** Molecular Evolutionary Genetics Analysis (Tamura et al., 2011)
7. **GeneMapper** – T-RFLP fragment analysis (ABI, USA)
8. **R** – statistical software with Vegan package (Oksanen et al., 2013; Team, 2014)
9. **CLC Viewer -** a software for bioinformatic analyses (http://www.clcbio.com)
10. **Mothur** - open-source, expandable software for the bioinformatics needs of the microbial ecology community (Schloss et al., 2009)
11. **ESPRIT-Tree** - hierarchical clustering analysis software (Cai and Sun, 2011)
12. **Zotero -** free bibliographic software (http://www.zotero.org)
13. **KODAK 1D Image Analysis Software**: Shoot and editation of DNA electrophoregrams.

3.1.10. Research sites

The river sediment samples were taken from an old efflux water channel leading from the former chemical PCB plant Chemko Strazske to the Laborec River (Michalovce district, Slovakia). A total of eight locations were selected along this channel (Fig. 9). The sampling points were located on the GPS coordinates: 48°52'12.597"N, 21°49'35.240"E (sample M1), 48°52'6.425"N, 21°49'37.640"E (sample M2), 48°51'32.087"N, 21°50'37.109"E (sample M3), 48°51'23.554"N, 21°50'43.211"E (sample M4), 48°51'18.037"N, 21°50'46.581"E (sample M5), 48°51'0.749"N, 21°51'0.324"E (sample M6), 48°51'27.599"N, 21°50'40.621"E (sample S2) and 48°51'2.151"N, 21°50'56.390"E (sample S3).

Figure 9. Location of the sampling sites along the derivation channel issued from the Chemko Strazske factory.

3.2. Methods

3.2.1. Sampling

Eight sediment samples (M1 to M6, and S2, S3) were collected on August 13th, 2009 from the channel at depths of 5-30 cm below the water level using sampling rod with closing valve from five places within the sampling point. The samples were collected in plastic sterile pots and manually homogenized. Subsamples were distributed into sterile glass jars, tightly closed and transferred into anaerobox (Difco, USA) with AnaeroGen bag (Oxoid, Thermo Fisher Scientific, USA) to keep anaerobic environment. The samples were kept cold (max. 12 °C) during transportation. Second sampling campaign was carried out on August 25th, 2010 from the localities M1, M4, S2 and S3 and processed the same way as the first sampling.

3.2.2. Anaerobic sediment microcosm cultivation

Anaerobic microcosms were set up using the sediment samples so as to simulate the growth conditions present in the original river sediment conditions. All manipulations were carried out in an anaerobic glove box. Anaerobic microcosms were set up on August 25th, 2009, using one liter glass bottles, filled with 600 ml of *Dehalobacter* sp. liquid growth medium (Holliger et al., 1998) and 50 g of sediments (Fig**Chyba! Nenalezen zdroj odkazů.**. 10). Eight ml of an electron donor solution (1:1:1:1 mixture of 100 mM ethanol:propionate:butyrate:acetate) were added. The gaseous phase was exchanged aseptically, so as to remove all possible traces of oxygen, and replaced by a mixture of N_2:CO_2 (80 %:20 %; v/v), keeping overpressure 0.6 bar insuring total anaerobic conditions. The bottles were stored in the dark at 30 °C without agitation. Inner pressure was measured on a regular basis using a homemade system (pressure transductor). Electron donors were added so as to maintain microbial activities and the inner pressure was kept between 0.2 and 0.6 bar during the whole duration of the operation. Control microcosms were run without the sediment. Samplings of the microcosms were carried out in the anaerobic glove box (Fig. 10). About 5 g of the sediment were sampled aseptically for DNA and RNA extraction, as well as for PCB congener measurements.


Figure 10. Sediment microcosms and handling glove box chamber.

3.2.3. Anaerobic sediment-free microcosms

Anaerobic sediment-free microcosms (Fig. 11) were set up according to the technique developed by (Bedard et al., 2006). PCB congeners present in the Delor 103 mixture were added first as a concentrated acetone solution to 300 mg of silica particles (ca. 240 mesh). The acetone was evaporated and 200 ml of *Dehalobacter* sp. liquid growth medium (Holliger et al., 1998) was added to the bottles. The final PCBs congener concentration was set up at 20 µg/ml. 2,6-dibromo-biphenyl (final concentration 350 µM) was added to prime the reduction of PCB congeners (Bedard et al., 2006). Six of the microcosms received 2-Bromoethanesulfonate (BES) (final concentration 5 mM), a selective inhibitor of methanogenesis. Two ml of an electron donor solution (a 1:1:1:1 mixture of 100 mM ethanol:propionate:butyrate:acetate) was added. The gaseous phase was exchanged aseptically by a mixture of N_2:CO_2 (80 %:20 %) keeping overpressure insuring total anaerobic conditions (Fig. 11). The microcosms were inoculated with 20 ml of the supernatant of the microcosm M1, a sediment-based microcosm. Sediment-free microcosms were stored in the dark at 30 °C without agitation. Inner pressure was measured on a regular basis using a homemade system (pressure transductor). Electron donors were added so as to maintain microbial activities and the inner pressure was kept between 0.2 and 0.6 bar during the cultivation (up to 692 days). Control microcosms were composed of autoclaved (killed) microcosms and non-inoculated microcosms.


Figure 11. Sediment-free microcosms and apparatus for gas exchange.

3.2.4. Pressure measurement

Pressure measurement was performed with home-made transductor connected to the microcosm system with a sterile needle. Measurement was carried out in regular intervals always 1st, 2nd, 3rd and 7th day after electron donor mixture addition until the significant increase of the inner pressure was detected.

3.2.5. DAPI staining

DAPI staining was performed on 25 mm, 0.22 μm polycarbonate filter (GE water&process technology). The filter was equilibrated with 1 ml of PBS buffer. 1 ml of the sample was applied on the equilibrated filter and let go through by gravity flow. The filter was placed on the slide and 30 μl of Vectashield mounting medium for fluorescence with DAPI H-1200 (Vector Laboratories, USA) was added on the top of the filter. After 2 min incubation at room temperature the stained bacterial cells were vizualized using a Nikon microscope (Eclipse E800, Nikon).

3.2.6. DNA extraction

Total DNA extraction from environmental sample is the first and key step for the various downstream applications in molecular ecology. Three methods were used for the extraction: manual extraction using silica columns, extraction kit using silica technology and automated solution using magnetic bead technology (see below).

3.2.6.1. DNA extraction from river sediment

For the purpose of soil high-molecular-weight DNA extraction the procedure previously described by Selenská *et al.* was used (Selenská and Klingmüller, 1991). 10 g of the original sediment samples taken from Strážské channel were separately homogenized and suspended in 30 ml of lysis buffer (0.12 M Na_2HPO_4, 0.02 % Tween 20, 10 % SDS; pH=8.0) at room temperature and agitated at 300 rpm for 30 min, at 70 °C. RNA was removed using RNAse A (final concentration 20 μg/ml) and incubated for 30 min at 37 °C with shaking at 300 rpm. Subsequently, proteins were digested by proteinase K (final concentration 100 μg/ml) in incubation conditions 30 min at 65 °C, 300 rpm. Sediment particles were collected by centrifugation (15 min, 8000 rpm, 10 °C) and the supernatant was transferred intoa new falcon tube and incubated on ice for 1.5 h. Precipitate was removed by centrifugation (30 min, 11000 rpm, 4 °C). The supernatant was incubated with 0.1volume of 50 % PEG6000 and 0.4 volume of NaCl (5 M) overnight at 4 °C. Precipitated nucleic acids were harvested by centrifugation (30 min, 11000 rpm, 4 °C). The resulting pellet was resuspended in 5 ml of TE buffer (pH=8.0) and treated by 1.5 ml of G4 buffer at 50 °C for 30 min without agitation. Buffer N2 was added to the sample in ratio 1:1 and the whole volume was transferred onto a buffer N2-equilibrated NucleoBond AXG-100 cartridges (Macherey-Nagel, Germany). The column with trapped DNA was washed with 12 ml N3 buffer. DNA was eluted using 7 ml of N5 buffer and collected in 850 μl aliquots in 1.5-ml microcentrifuge tubes. DNA was precipitated with 0.7 volume of 100 % isopropanol and visualized in 0.7 % agarose gel stained with GelRed (Biotium, USA).

3.2.6.2. DNA extraction from sediment microcosms

Total DNA from sediment microcosms was recovered using PowerSoil™ DNA Isolation Kit (MoBio, USA). The kit was used according to the manufacturer recommendation.

3.2.6.3. DNA extraction using Maxwell 16

For the purpose of DNA extraction from sediment-free microcosms the procedure using the automated Maxwell® 16 Research System was used. 15 ml of the sediment-free microcosm were shaken to homogenize and silica particles with bound bacterial biomass were harvested by centrifugation (4000 rpm, 10 min, room temperature). Pelet was resuspended in 400 µl of TE buffer and 100 µl of lysozyme (final concentration 5 mg/ml) was added to disrupt the cell walls. Sample was incubated for 1 h at 37 °C. One ml of extraction buffer (5 M Guanidine thiocyanate, 100 mM EDTA, 1 % Sodium lauroyl sarcosinate, 1 % PVP K30, 150 mM Sodium Phosphate buffer pH=8.0) was added to the sample. Mixture was transferred into 1.5-ml screw-cap microcentrifuge tube with 0.5 g of zirconia-silica beads (Fisher Thermoscientific, USA). Finally, 5 µl of a 1M DTT solution were added and tubes were processed in FastPrep FP120 Cell Disrupter (Thermo Electron, USA) (30 s, 4.5 m/s). The tube was incubated at 60 °C for 5 min. And then, tubes were centrifuged at 10000 rpm for 2 min. Supernatant was added to the well #1 of the SEV DNA Tissue extraction kit cartridge (Promega, USA). Cartridges were placed in the cartridge holder on the platform of the automated Maxwell® 16 Research System (Promega, USA) and the program for DNA extraction from tissue was run. DNA was eluted into 250 µl of the elution buffer provided within the kit.

3.2.7. RNA extraction and reverse transcription

RNA was extracted from both the sediment and sediment-free microcosm.

In the case of the sediment microcosms 2.5 ml of the homogenized suspension was mixed up with 2.5 ml of the Bead Solution from the commercial kit RNA PowerSoil® Total RNA Isolation Kit (MoBio, USA) designed especially for the RNA extraction from the soil. Samples were immediately frozen in the liquid nitrogen and stored at -80 ºC until their use. Samples were processed using the above mentioned commercial kit according to the manufacturers´ recommendations.

For the purpose of RNA extraction from sediment-free microcosms the commercial kit SV Total RNA Isolation System (Promega, USA) designed primarily for RNA isolation from the tissues, cultured cells and white blood cells was used. 50 ml of the culture (homogenized biomass fixed on the surface of silica particles with cultivation medium) were centrifuged (8000 rpm, 4 °C, 2 min). Cell pellets were resuspended in 1 ml of Stop RNAse Solution (Qiagen, USA), transfered to the 1.5-ml microcentrifuge tube and centrifuged again (13000 rpm, room temperature, 1 min). Supernatant was removed and pellets were immediately frozen in liquid nitrogen and stored at –80 ºC or used immediately. Pellet was resuspended in 100 µl of DNAse and RNAse-free TE buffer with lysozyme (final concentration 3 mg/ml) and incubated for 10 min at room temperature. Further steps were used with respect to the manufacturers´ instructions.

Additional DNA digestion using DNAse was used for all RNA extracts. 28 µl of the RNA solution were incubated with 2 µl of DNAse and RNAse-free water (5Prime, Germany), 3.75 µl of RQ RNAse-free DNAse 10x reaction buffer (Promega, USA) and 3.75 µl of RQ RNAse-free DNAse (1 U/µl) (Promega, USA) for 30 min at 37 °C. Subsequently, DNAse was inactivated by addition of 3.75 µl DNAse Stop Solution (Promega, USA), incubated for 10 min at 65 °C and kept on ice until the hexanucleotides were added.

2 µl of random hexanucleotides (0.5 µg/µl) (Microsynth, Switzerland) were incubated with 18 µl of DNAse-free RNA for 5 min at 70 °C and kept on ice until further processing. cDNA was obtained in reverse transcriptase reaction prepared according to the Tab. 4. Negative control reaction was performed to prove an absence of DNA contamination present in the sample.

Table 4. Preparation of RT reaction mix and negative control mix.

	RT reaction	**Negative control**
Nuclease-free water (MoBio, USA)	9.04 µl	3.56 µl
Im-PromII 5x reaction buffer (Promega, USA)	9.6 µl	2 µl
$MgCl_2$ (25 mM) (Promega, USA)	5.76 µl	1.4 µl
dNTPmix(10 mM each) (Promega, USA)	4 µl	1 µl
RNAsin (40 U/µl) (Promega, USA)	1.2 µl	-
Im-PromII RTase (Promega, USA)	2.4 µl	-
RNA sample	16 µl	4 µl

RT reaction was conducted in a Biometra T3000 thermocycler (Biometra, Germany) as follows: annealing step at 25 °C for 5 min, elongation 42 °C for 90 min and inactivation 70 °C for 15 min.

Presence of cDNA was prooved by PCR reaction with universal 16S primers 8f a 518r in reaction volume 10 µl containing 5 µl 10x PCR buffer (Promega, USA), 1.2 µl of both primer at 10 µM, 4 µl of 10 mM dNTPs, 2.5 U of GoTaq™ DNA polymerase (Promega, USA) and 0.1 ng/µl template DNA (final concentration), completed with MilliQ water (5Prime, Germany). PCR amplification was performed in a Biometra T3 thermal cycler (Biometra, Germany) as follows: initial denaturing step at 94 °C for 4.5 min, followed by 30 cycles of 0.5 min denaturation at 94 °C, annealing at 56 °C for 90 s, 1 min of elongation at 72 °C and a final elongation step of 10 min at 72 °C. PCR products were visualized in 1.5 % agarose gel stained with GelRed (Biotium, USA).

3.2.8. DNA concentration measurement

Various approaches were used for detection of DNA concentration. In common, concentration of the DNA solution was established using spectrophotometer NanoDrop ND-1000 (ThermoScientific, USA).

Some of the downstream applications required precious quantification (i.e. next-generation sequencing). For this purpose a Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, USA) was used. It allowed selectively detect as little as 25 pg/ml of dsDNA and quantify DNA using standard dilution curve. DNA quantification measurement of the samples and the standards were done in triplicates. Measurement was conducted using the Infinite M200 (Tecan, Switzerland) equiped by the software Magelan 06 (version 6.3).

Preparation of the standards and samples for DNA concentration measurement was carried out according to the manufacturers´ recommendations.

3.2.9. Preparation of competent cells

A single colony of the required *E.coli* strain was picked and grown overnight in 2 ml LB media at 37 °C. 500 µl of the overnight culture was subcultured into 40 ml of LB and culture was grown to OD_{600} ~ 0.6. The culture was left on ice during the OD measurement and centrifuged (4000 rpm , 4 °C, 5 min). The cell pellet was resuspended in 30 ml of ice-cold 50 mM calcium chloride ($CaCl_2$) and further incubated for 20 min on ice. The centrifugation step was repeated and cells resuspended in solution containing 8 ml ice-cold $CaCl_2$ (50 mM), 15 % glycerol and 10 mM Tris and pipetted in 200 µl aliquots into 1.5-ml microcentrifuge tubes and immediately frozen in liquid nitrogen and stored at -80 °C.

3.2.10. 16S rRNA gene cloning and sequencing

The PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA), concentration of DNA was established by NanoDrop ND-1000 (ThermoScientific, USA) and PCR products were ligated into pGEM®-T Easy Vector (Promega, USA) according to the manufacturers´ recommendations. Transformation into *E.coli* XL1-Blue chemically competent cells was carried out using the heat shock. Competent cells were taken from -80 °C freezer and thaw on ice for 5 min. 2 µl of circular DNA were added to 50 µl aliquots of competent cells and gently stirred with a tip, incubated on ice for 20 min. Tubes were incubated for 45 s at 42 °C without shaking and placed on ice for 2 min to reduce damage to the cells. 950 µl of pre-warmed LB media (without antibiotics) was added. Tubes were incubated for 1 h at 37 °C with shaking (300 rpm). 100-150 µl of the resulting culture were spread on the LB plates with the appropriate antibiotic added (ampicillin, tetracycline) and grow overnight at 37 °C.

The 16S rRNA gene was targeted in the plasmid using a PCR amplification with primers T7 and SP6 (Promega, USA) and PCR product was purified as described above. Sequencing reaction was carried out on each purified PCR product as follows: 10 µl reaction mixtures contained 2 µl of Terminator mix V3.1 (ABI, USA), 2 µl of 5x BigDye V3.1 Sequencing buffer (ABI, USA), 1.6 µl of primer T7 at 1 µM, 200 ng DNA template and completed Milli-Q water (MoBio, USA). The sequencing reaction was carried out with the following program: 30 cycles of 10 s denaturation at 94 °C, 5 s of primer annealing at 50 °C, and 4 min of elongation at 60 °C. The products were sequenced using ABI 3130xl DNA capillary sequencer (ABI, USA) equipped with 50 cm long capillaries (80 µm inner diameter) and POP7 electrophoresis matrix with dye set G5 (ABI, USA). Sequences were tested for chimeras using Bellerophon (Huber et al., 2004) and aligned with ClustalX 2.1 (Larkin et al., 2007). The sequences were compared with those available in GenBank® (Benson et al., 2010) by BLAST searches (Altschul et al., 1997) to find the nearest relatives and percentage of gene sequence identity. Phylogenetic trees were built with GeneDoc software (Nicholas et al., 1997) and Mega5 (Tamura et al., 2011).

3.2.11. End-point polymerase chain reaction (PCR)

50 µl reactions were composed of 5 µl 10x PCR buffer (Promega, USA), 1.2 µl of both primer at 10 µM, 4 µl of 10 mM dNTPs, 2.5 U of GoTaq™ DNA polymerase (Promega, USA) and 0.1 ng/µl template DNA (final concentration), completed with MilliQ water (5Prime, Germany). Typical PCR amplifications were conducted as follows: initial denaturing step at 94 °C (4.5 min), followed by 25 to 35 cycles of 0.5 min denaturation at 94 °C, 1- 2 min annealing at the required temperature, 1 – 2 min elongation at 72 °C and a final elongation step of 10 min at 72 °C. PCR products were examined in GelRed stained 1.5 % agarose gel to confirm the specificity of the resulting amplicons.

3.2.12. Community profiling

T-RFLP analysis of bacterial communities from sediment and sediment-free microcosms as well as their numerical treatment were modified from the protocol described by Rossi et al. (Rossi et al., 2009). Templates were amplified by PCR in triplicates using universal primer labeled on the 5'-end with 6-carboxyfluorescein (FAM) 8f-FAM and universal primer 518r for bacterial 16S and Arch349f-FAM and Arch806r for archaeal 16S. PCR amplification was conducted as following: PCR reaction was composed of 3 µl of 10x Y buffer, 2.4 µl of 10 mM dNTPs, 1.5 µl of each primer (10 µM), 6 µl of 5x Enhancer P, 1.5 U PeqGold Taq polymerase and 0.2 ng/µl template DNA (final concentration), completed with Milli-Q water (5Prime, Germany) to 30 µl. PCR amplifications were conducted in a T3000 Thermocycler (Biometra, Germany) as follows: initial denaturation at 94 °C for 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, a final elongation step of 10 min at 72 °C and cooling to 10 °C. Specificity of amplicons was examined in a 1.5 % agarose gel. PCR products were purified with MSB® Spin PCRapace (Invitek, Germany) according to the manufacturer's instructions. Bacterial communities were analyzed with adaptation of the T-RFLP procedure described by Ebrahimi et al. (Ebrahimi et al., 2010). 200 ng of the purified amplicons were digested at 37 °C for 3 h with 0.5 U of the *Hae*III endonuclease (Promega, USA) having 5'-GG^CC-3' recognition site (eubacterial 16S rRNA) or 0.5 U *Hha*I endonuclease (Promega, USA) having 5´-GCG^C-3´ recognition site (archaeal 16S rRNA). The T-RFLP analytes were prepared by mixing 1 µl of digested PCR product with 8.5 µl of HiDi formamide (ABI, USA) and 0.5 µl of GeneScan 600-LIZ internal size standard (ABI, USA) and denaturated for 2 min at 95 °C. Individual T-RFs were separated by capillary gel electrophoresis in ABI 3130xl DNA capillary sequencer (ABI, USA) equipped with 50 cm long capillary filled with a fluid POP7 gel matrix. Resulting T-RFLP profiles were generated between 50 and 500 bp according to Rossi et al. (Rossi et al., 2009). T-RFLP profiles were aligned using Treeflap (http://www.sci.monash.edu.au/wsc/staff/walsh/treeflap.xls) and expressed as relative contributions of OTUs.

3.2.13. Statistical analysis

Numerical treatment and analysis of the data were carried out with R (Team, 2014) and the Vegan library (Oksanen et al., 2013). Principal component analysis (PCA) was used for a graphic representation of the evolution of the communities with time. Redundancy analysis (RDA) was used so as to define possible correlations between apparent microbial diversity provided by T-RFLP analysis and the environmental variables. Multifactorial analysis (MFA) was used for detailed taxonomical analysis and finding of the putative taxa involved in the PCB congener dehalogenation.

3.2.14. Next-generation sequencing

DNA from sediment microcosm M1, 4 DNA samples from the sediment-free microcosms first generation, 14 cDNA samples from sediment-free microcosms first generation and 4 cDNA samples from sediment-free microcosms second generation were examined using next-generation sequencing method performed in the Research and Testing Laboratory (http://www.researchandtesting.com/).

Sample preparation

Samples were amplified using KAPA HiFi HotStart PCR Kit (KAPA Biosystems, USA) under the following conditions: 10 µl of 5x KAPA HiFi buffer (containing 2 mM $MgCl_2$), 1.5 µl of 10 mM dNTPs, 1.5 µl of each primer (10 µM), 1 µl KAPA HiFi HotStart DNA polymerase (1 U/µl) (KAPA Bio, USA) and 0.5 ng/µl template DNA (final concentration), completed with Milli-Q water (5Prime, Gemany) to 50 µl of reaction volume. PCR products were generated using eubacterial primer set 28f and 519r or archaeal primer set Arch349f and Arch806r under following conditions: initial denaturation at 95 °C for 30 min, followed by 27 cycles of 98 °C for 20 s, 57 °C for 15 s, 72 °C for 20 s, and final extension at 72 °C for 3 min. The PCR amplicons were loaded into the 1.5 % agarose gel stained with GelRed (Biotium, USA). Gel slices containing DNA were quickly excised using UV Transilluminator UVT-20M (Herolab, Germany). DNA was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and re-purified additionaly by Agencourt AMPure XP beads (Beckman Coulter, USA) according to the manufacturers´ recommendations. The purified samples were quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, USA) according to manufacturer´s recommendation. Measurement was

performed using the Infinite M200 (Tecan, Switzerland) equiped by the software Magelan 06 (version 6.3). The final DNA concentration was adjusted using nuclease-free water to be in range 40 – 100 ng/µl.

3.2.1. Taxonomic assignment and diversity estimation

Obtained high-throughput data in .sff formats were processed using Mothur workflow (Schloss et al., 2009) on a Biolinux 7 platform as described in Diaby et al. (Diaby et al., 2015). Clean sequence data sets obtained from mother workflow were consequently visualized using Excel (MS Windows). Numerical ecology computations were carried out on R (Team, 2014) and species-accumulation curves were created using ESPRIT-tree tool (Cai and Sun, 2011).

3.2.2. GC-MS chromatography

PCB extraction: Extraction of PCBs was performed with the ASE 200 System (Dionex, France). One gram of the sediment samples was placed in an extraction cell (11 ml) and subsequently extracted with hexane–acetone (1:1, v/v) mixture. Static heating was applied to the vessel (100 °C, 5 min) and subsequent extraction was performed at 100 °C under 2000 psi for 6 min. The cell was then flushed with fresh solvent (60 % of total cell volume) and the solvent was finally purged from the cell by nitrogen for 60 s. For each sample, the extraction cycle was performed four times. The resulting organic extracts were evaporated under vacuum at room temperature and dissolved in hexane.

PCB analysis: The analyses of PCBs were performed using GC chromatograph Varian 450-GC (Varian, USA) equipped with Varian 240-MS mass detector. The PCB congeners were separated on DB-5MS capillary column (30 m, 0.25 mm i.d.×0.25 µm film thickness). Helium was used as carrier gas with a constant flow of 1.2 ml/min. One microliter of each sample was splitless injected at 240 °C. After 1 min, the spliter was opened with ratio 1:50. The GC oven temperature program started at 60 °C, was held for 1 min and the increased with 25 °C/min to 120 °C. Further heating followed with 2.5 °C/min to 240 °C finally kept for 10 min. The solvent delay time and transfer line temperature were set at 6 min and 240 °C, respectively. Mass spectra were recorded at 3 scan/s under electron impact at 70 eV and mass range 140-410 amu. The PCB congeners were identified using their GC retentions and mass spectral characteristics compared with their respective standards.

3.2.3. GC-ECD chromatography

PCB extraction: All the glassware was washed with acetone and hexane distillated 2x. And, a "glassware-blank" with pure hexane was prepared. Amout of 0.3 ml of standard PCB 189 at concentration 35 µg/ml (dissolved in acetone) was weighted in a vial and added to the sample before extraction. Vial was washed twice with approximately 0.5 ml of acetone. Sample was stirred and poured in a separating funnel. The funnel was decanted for 2-3 min and water phase was collected in the sample bottle. The organic phase was collected (poured from the top of the funnel) in a heart-shaped vessel of 250 ml after drying on a funnel with sodium sulphate. Extraction was repeated twice with 50 ml of pure hexane. With the last extraction the separating funnel was decant for 15 min. The extracts were collected and the volume was reduced to 2-3 ml by rotating evaporator (T° = 40 °C at 330 mbar). Extracts were transferred in a brown tared 20 ml–bottle. The balloon was rinced twice with 1 ml of hexane. The sample was diluted 8.5x in isooctane (0.2 ml in 1.7 ml).

PCB analysis: The PCBs were determined using GC-ECD. The system consisted of an Agilent 6890 equipped with an Agilent automatic injector and ECD detector. Separation was carried out using a DB-5 ms type capillary column (60 m x 0.25 mm I.D., df: 0.25 µm). Helium was used as carrier gas at a constant pressure of 35 psi. Injections were performed in on-column mode with the temperature program as follows: 85 °C, 0.2 min hold and 70 °C/min to 250 °C. The injection volume was 1 µl. The GC oven was programmed as follows: 80 °C, 0.5 min hold; 50 °C/min to 150 °C, 1min hold; 2.5 °C/min to 285 °C, 30 min hold (total time: 86.9min). The ECD was set at 300 °C.

3.2.4. Heavy metals analysis

Heavy metal analysis was carried out by the accredited laboratory ALS Czech Republic, Ltd. Content of heavy metals in samples was determined using ICP-optical emission spectrometry (ICP-OES) according to the method EPA 200.7, ISO 11885. The method for mercury detection differed and was done using EPA 245.7 and EPA 1631 standard.

3.2.5. Granulometric analysis

Granulometric analysis was performed by the laboratory ALS Czech Republic, Ltd. Grain size distribution was carried out using the sieving method for soil particles from > 2 to 0.063 mm size. Other fractions (less than 0.063 mm) were identified by laser particle size analyzer using the liquid dispersion mode (test method CZ_SOP_D06_07_N11).

4. Results

4.1. Characterization of sediment samples

Sediment samples located downstream of the PCB factory contained large amounts of PCB congeners. Independently of their locations, they displayed concentrations ranging from 16 mg/kg (sampling site S2) to 136 mg/kg (sampling site M2) (Tab. 5). General laboratory GC-MS analysis of the seven most abundant PCB congeners used to be analyzed in commercial mixtures showed that the the fraction of highly chlorinated hexa- and hepta-chlorobiphenyls (CBs) ranged between 2.7 mg/kg (sediment M5) and 62.0 mg/kg (sediment M2). The sum of middle chlorinated tetra- and penta-CB was detected at a minimum of 3.1 mg/kg (sediment M5) and maximum of 65.9 mg/kg (sediment M2). The sum of low chlorinated di- and tri-CB varied from 1.0 mg/kg (sediment M5) to 16.1 mg/kg (sediment M4) (Tab. 5).

The sediment samples were also massively contaminated by organic substances derived from petrol. Total sum of petroleum hydrocarbons (PHs) ranged between 336 mg/kg (sediment M5) to 4390 mg/kg (sediment M1). The main fraction of petroleum hydrocarbons was represented by the carbon range of C16-C35. Polyaromatic hydrocarbons (PAHs) were detected inconcentrations up to 7.7 mg/kg (sediment M3) except the highly contaminated sample sediment M2 where PAHs were measured at 290 mg/kg.

Table 5. PCB and total petroleum hydrocarbon (TPH) concentrations measured in the original river sediment sampled in 2009.

			M1	**M2**	**M3**	**M4**	**M5**	**M6**	**S2**	**S3**
PCBs	PCB28	<mg/kg>	3.7	8.5	6.0	16.1	1.0	2.7	2.5	5.6
	PCB52	<mg/kg>	8.7	41.6	10.2	39.3	2.0	4.0	4.8	7.8
	PCB101	<mg/kg>	4.5	17.3	4.6	11.4	0.8	3.4	1.8	5.9
	PCB118	<mg/kg>	1.8	7.0	1.3	2.7	0.3	1.3	0.5	2.0
	PCB138	<mg/kg>	6.0	23.5	5.2	11.9	1.0	4.7	2.4	7.7
	PCB153	<mg/kg>	5.8	21.7	4.8	9.2	0.9	4.7	2.1	7.7
	PCB180	<mg/kg>	4.3	16.8	3.9	8.4	0.8	3.7	1.9	5.8
	Sum of PCBs	<mg/kg>	**34.8**	**136.0**	**36.0**	**99.0**	**6.8**	**24.4**	**16.0**	**42.4**
TPH	C10-C12 Fraction	<mg/kg>	<2	<2	<2	4	<2	<2	<2	<2
	C12-C16 Fraction	<mg/kg>	18	25	25	39	5	17	14	31
	C16-C35 Fraction	<mg/kg>	3640	2010	2900	2530	258	3490	1070	6070
	C35-C40 Fraction	<mg/kg>	735	480	561	559	73	736	216	1220
	Sum of PHs	<mg/kg>	**4390**	**2520**	**3490**	**3140**	**336**	**4250**	**1300**	**7330**
	PAH	<mg/kg>	**3.1**	**290**	**7.7**	**7.0**	**0.3**	**1.7**	**4.1**	**4.5**

Second sampling campaign in 2010 showed constrasting results (Tab. 6). High water flow regime (including flood event in spring 2010) modified the river bed sediment composition and changed contaminant concentrations. Increase in PCB content was observed mainly in samples from 2010 taken from site S3 where the concentration increased nearly three-times (from 42.4 mg/kg in 2009 to 125.5 mg/kg in 2010). On the other hand, decrease in PCB content was detected in sample M4 where the concentration dropped rapidly (from 99.0 mg/kg to 30.9 mg/kg). In sampling points M1 and S2 concentration of the tested PCB congeners increased approximately 1.5-times compared to the previous year.

Content of petroleum hydrocarbons remained at the significant amounts in the second sampling campaign, as well. The PHs concentration increased two-times in sediment S2 (from 1300 mg/kg to 2565 mg/kg). Contrarily, the massive decrease was observed in sediment S3 (the PHs content decreased 2.7-times - from 7330 mg/kg to 2735 mg/kg).

Concentration of PAHs reached in all tested samples up to 9.1 mg/kg.

Table 6. PCB and total petroleum hydrocarbon (TPH) concentrations measured in the original river sediment sampled in 2010.

			M1	**M4**	**S2**	**S3**
PCBs	PCB28	<mg/kg>	5.7	7.2	5.3	10.1
	PCB52	<mg/kg>	18.7	5.1	10.0	19.7
	PCB101	<mg/kg>	2.3	2.1	1.0	11.9
	PCB118	<mg/kg>	1.6	1.1	0.6	7.5
	PCB138	<mg/kg>	11.3	6.8	3.7	28.8
	PCB153	<mg/kg>	7.3	4.6	2.6	25.7
	PCB180	<mg/kg>	7.1	4.1	2.7	21.9
	Sum of PCBs	<mg/kg>	**53.9**	**30.9**	**25.9**	**125.5**
TPH	C10-C12 Fraction	<mg/kg>	<2	<2	<2	<2
	C12-C16 Fraction	<mg/kg>	94	51	49	63
	C16-C35 Fraction	<mg/kg>	3270	2665	2130	2235
	C35-C40 Fraction	<mg/kg>	661	504	384	439
	Sum of PHs	<mg/kg>	**4030**	**3215**	**2565**	**2735**
	PAH	<mg/kg>	**8.5**	**7.7**	**9.1**	**7.4**

Heavy metals were detected also at high concentrations (Tab. 7). Concentrations of zinc reached especially alarming values, as they ranged between 180 mg/kg (sampling site M5) to 2050 mg/kg (sampling site M3) in samples from 2009. Also, a significant amount of other heavy metals such as chromium was found. Situation after one year showed that the content of chromium increased two-times in the sediment at sampling site S2 (from 56 mg/kg to 114 mg/kg). And, the same trend was observed in the sample M4 in the case of copper (concentration increased 2.5-times from 67 mg/kg to 172 mg/kg). Contrarily, copper level decreased in the S3 sediment 2-times (from 112 mg/kg to 55 mg/kg).

Table 7. Extractable heavy metals in the efflux channel sediment samples. Concentrations are given in mg/kg.

	Sediment samples 2009								Sediment samples 2010			
	M1	**M2**	**M3**	**M4**	**M5**	**M6**	**S2**	**S3**	**M1**	**M4**	**S2**	**S3**
Cadmium	1.2	1.0	3.9	2.8	>0.4	1.9	2.1	3.8	0.5	4.4	1.5	1.7
Arsenic	16.5	18.3	26.4	21.2	6.1	13.2	10.5	19.5	21.9	20.7	14.2	12.3
Chromium	69	102	95	113	33.8	68.5	56	103	71,7	103.1	114.2	83.5
Nickel	49.6	52.9	44.4	48.3	41.1	34.6	39.3	43.1	42.0	45.2	48.9	41.4
Lead	59.6	70.8	55.6	53.3	18.9	38.6	33.2	53.4	76.5	53.6	48.9	56.2
Copper	88.1	75.2	96	66.7	30.3	72.5	48	112	86.9	172.3	56.1	54.9
Zinc	1080	991	2050	1020	180	1230	694	1900	841.5	1645	463.2	706.5

Monitored pH values measured in the sediment samples stayed relatively constant in a range varying between 6.8 and 7.8 (Tab. 8). Total organic carbon (TOC) showed a significant difference among samples in 2009. The lowest value was detected in the sediment M5 (1.4 %) which was 7 times less than the TOC detected in sediment S3 (10.0 %). The sampling in 2010 showed more similar results with values between 7.0 % and 8.9 %, respectively (Tab. 8).

Table 8. pH and total organic carbon and other physical parameters present in the sediment samples taken from the Strazske efflux channel (2009 and 2010). DW: dry weight.

		Sediment samples 2009								Sediment samples 2010			
		M1	**M2**	**M3**	**M4**	**M5**	**M6**	**S2**	**S3**	**M1**	**M4**	**S2**	**S3**
TOC	% DW	8.7	7.3	6.8	6.7	1.4	5.0	2.2	10.0	8.9	8.1	7.7	7.0
pH		7.1	6.8	6.9	7.1	7.0	6.8	7.2	7.0	7.6	7.8	7.8	7.4
Moisture	% DW	80.4	68.8	77.2	66.9	41.2	73.6	57.1	85.8	78.4	70.8	52.8	69.5
Dry matter	% DW	19.6	31.2	22.8	33.1	58.8	26.4	42.9	14.2	21.6	29.2	47.2	30.5
Density	g/cm^3	2.1	1.9	2.3	2.1	2.7	2.2	2.1	2.1	2.7	2.2	1.9	2.1

Particle size distribution analysis showed that the main composition of the Strazske channel sediments was based on sand particles in 2009. However, grain size analysis revealed substantial changes between years 2009 and 2010 in the river bed geological habitat (Fig. 12). Significant increase in silt particles was found in samples M1, S2 and S3. Also, the portion of the clay decreased within a year significantly. Additionaly, samples S2 and S3 also showed a strong concomitant increase in the PCB concentration. Contrariwise, sample M4 was enriched in sand and showed a strong decrease in its PCB concentration.

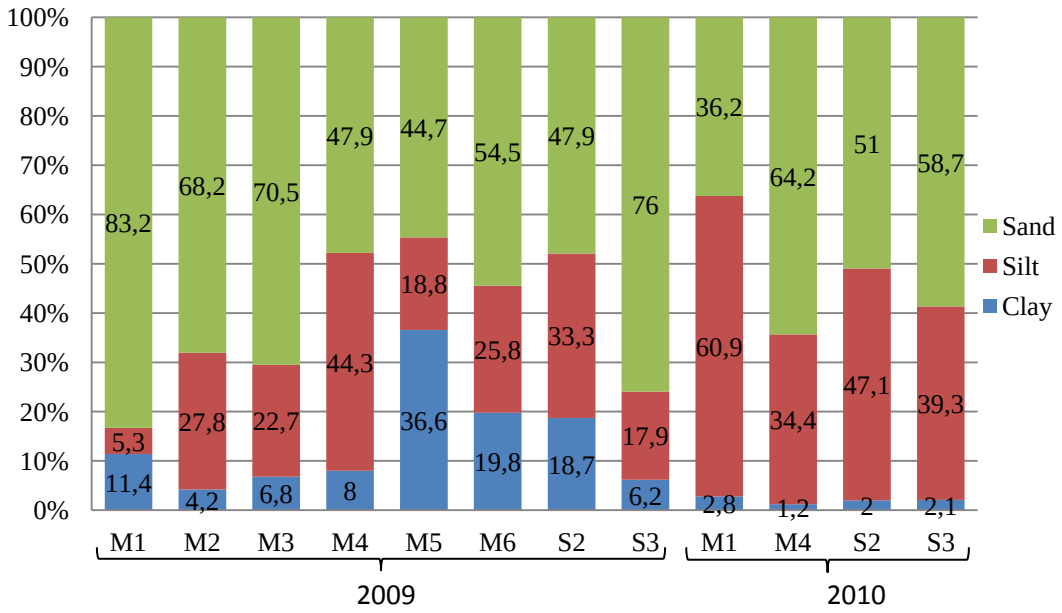

Figure 12. Particle size distribution showed as fraction percentage part (in %) in sediment samples from the Strazske channel in years 2009 and 2010.

4.2. Sediment microcosm experiments

Sites with old ecological burden are known as a good source of bacteria with required degradation potential for particular pollutant (Domínguez, 2008). In order to study bacterial communities involved in organohalide respiration processes, sediments from the efflux channel from Strazske locality were used as inoculum for anaerobic laboratory scale microcosms. Long-term microcosm experiment was established from the sediment samples collected from eight different locations (described as M1-M6 and S2-S3) along derivation channel taken in 2009 (Fig. 9).

4.2.1. Microcosm follow-up and pressure evolution

The addition of electron donors (ethanol, butyrate, acetone and propionate) was rapidly followed by an increase of inner pressure in the bottle induced predominantly by methane formation (data not shown). The pressure was controlled on a regular basis after each electron donor addition. Production of methane in response to the injection of the electron donors was therefore used as an indicator for growth and evolution of the microbial communities in the culture. The pressure in the non-inoculated control microcosms remained stable during the time of cultivation, which could be taken as a proof that the gas production was dependent on the biological processes only. The best results in the pressure evolution were achieved at the microcosm samples M6, S3 and M1 (Fig. 13). On the other hand, samples M3, M5 and S2 showed the slowest pressure production.

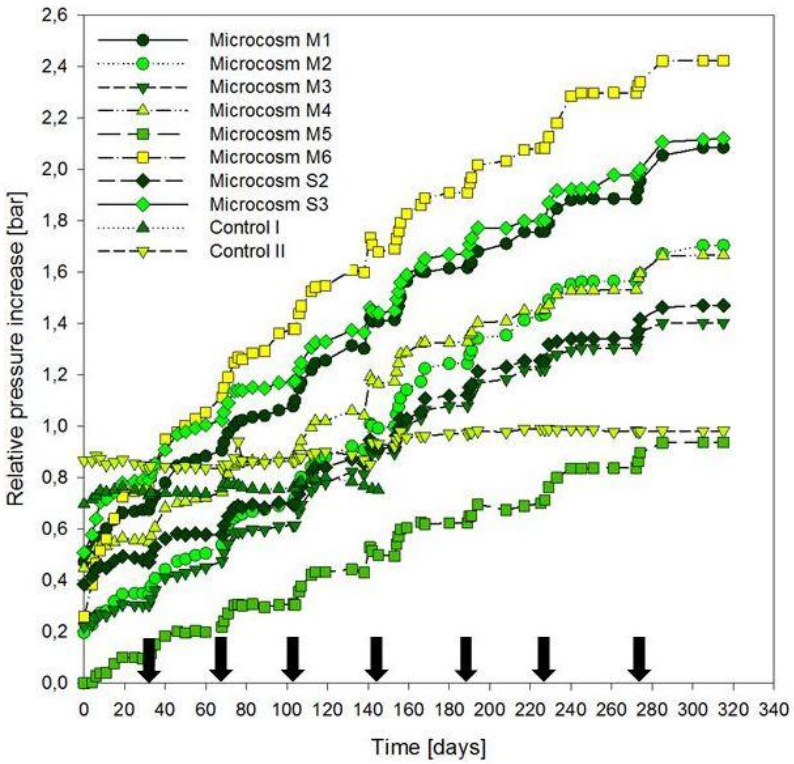

Figure 13. Cumulative relative pressure increase measured in the microcosms. Injections of electron donors are displayed with black arrows.

4.2.2. PCB congener analysis

High resolution GC-MS analysis of 35 dominant PCB congeners after 10 months of continuous cultivation revealed large discrepancies among all microcosms (Fig. 14). In general, all congeners were impacted by the dechlorination process and divergent degradation patterns were observed. The enriched bacterial consortia degraded the PCB congeners at different extent regardless to the sampling location. Lowest values in total PCB congener elimination were detected in microcosms M6 (5.11 %), M1 (6.26 %) and M5 (6.82 %). On the other hand, microcosms M2, M3 and S2 showed 10.2 %, 15.62 % and 18.48 % decreases in the total PCB congener concentration, respectively. The largest decreases were observed for M4 and S3 – 24.81 % and 35.92 %, respectively. Three microcosms (M1, S2 and S3) showed a high capacity in the degradation of highly chlorinated PCB congeners.

The microcosm S2 with the highest degree of degradation was also the only able to completely remove hepta-CB showing also an unusual strong capacity in the elimination of hexa- and penta-chlorinated PCB congeners (decrease of 19.4 %). Contrariwise, microcosms M1, M2, M5 and M6 showed categories which increased their relative contributions as a result of the dechlorination of higher congeners.

Finally, microcosm M1 was able to degrade significantly highly chlorinated congeners. In this case however, these congeners were converted into middle-chlorinated ones (mainly PCB47, PCB48, PCB49, PCB52 and PCB74), which was also the case to in microcosm M2. Additionaly to microcosm M1, microcosms M2 and M5 were able to degrade low-chlorinated congeners (2- and 3-CB) efficiently. Microcosm M4 was able to degrade middle-chlorinated congeners principally, whereas M3 and M5 handled almost exclusively low-chlorinated ones, mainly 2-CB. Microcosm M6 showed almost no degradation activity and focused on low-chlorinated PCB congeners only.

Interestingly, based on the PCB degradation data (Fig. 14), there was no correlation between the degradation abilities of each particular sample and pressure evolution (Fig. 13).

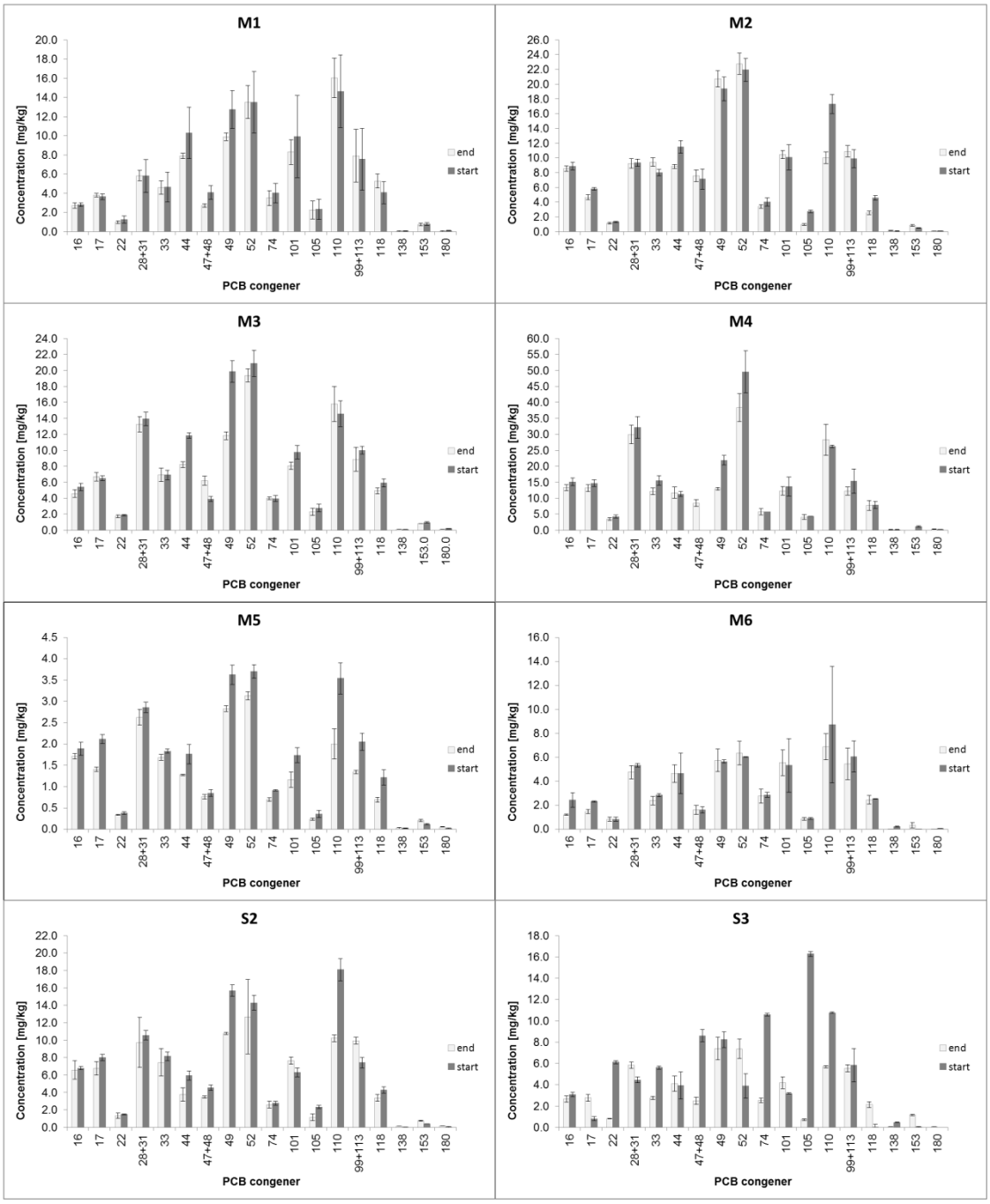

Figure 14. Comparison of PCB congener concentrations in 10-month-old microcosms (white bars) with the respective sediment samples used for the microcosm setup (black bars). The GC-MS analyses were performed in triplets, the SD values ranged from 0.001 to 6.5.

4.2.3. Specific PCR detection of 16S rDNA of known OHR bacteria

Primers for the end-point PCR detection of the genera *Dehalococcoides sp., Desulfitobacterium sp., Sulfurospirillum multivorans, Geobacter sp., Desulfuromonas sp.* and *Dehalobacter sp.* were used. DNA was extracted from both the original sediment samples (Tab. 9) and microcosm samples (Tab. 10). Additionally, total RNA was extracted from all sediment micrococosms (Tab. 11).

The targeted obligate and non-obligate organorespiring bacterial species were found in all samples, showing that the microbial net with organohalide respiration abitity was present at the locality Strazske (Tab. 9).

Table 9. Specific PCR amplification signals obtained from DNA extracted from the original sediment samples.

	M1	**M2**	**M3**	**M4**	**M5**	**M6**	**S2**	**S3**
Dehalococcoides sp. **(DHC1f+DHC1377r)**	-	+	+	+	+	-	+	+
Desulfitobacterium sp. **(Dsb406f+Dsb619r)**	+	+	+	+	+	+	+	+
Sulfurospirillum multivorans group **(DHSPM576f+DHSPM1210r)**	+	+	+	+	+	+	+	+
Geobacter sp. **(Geo73f+Geo485r)**	+	+	+	+	+	+	+	+
Desulfuromonas sp. **(Dsm494f+Dsm1050r)**	+	+	+	+	+	+	+	+
Dehalobacter sp. **(Dre441f+Dre654r)**	-	+	+	+	+	-	+	-

Results from the PCR detection of 16S rRNA in microcosm DNA samples after six months of continuous cultivation showed that the fraction of obligate organorespirers, *Dehalococcoides* and *Dehalobacter,* was enriched in all samples (except *Sulfurospirillum multivorans* in microcosm M2) (Tab. 10).

Table 10. Specific PCR amplification signals obtained from DNA extracted from the microcosm samples after six months of cultivation.

	M1	M2	M3	M4	M5	M6	S2	S3
***Dehalococcoides sp.* (DHC1f+DHC1377r)**	+	+	+	+	+	+	+	+
***Desulfitobacterium sp.* (Dsb406f+Dsb619r)**	+	+	+	+	+	+	+	+
***Sulfurospirillum multivorans group* (DHSPM576f+DHSPM1210r)**	+	-	+	+	+	+	+	+
***Geobacter sp.* (Geo73f+Geo485r)**	+	+	+	+	+	+	+	+
***Desulfuromonas sp.* (Dsm494f+Dsm1050r)**	+	+	+	+	+	+	+	+
***Dehalobacter sp.* (Dre441f+Dre654r)**	+	+	+	+	+	+	+	+

In order to study the active guild composition of the microcosm communities the total RNA was extracted from the microcosms after 6 months of cultivation. RNA, resp. cDNA-based PCR offers potentially a more realistic outline of the active population.

The detected composition was quite different from the tested DNA samples. Members of *Dehalococcoides* species were detected in microcosms M4, M6, S2 and S3. The absence of the band in the M3 corresponded with no PCB degradation in this microcosm. Species *Geobacter sp.*was found in almost all microcosms (except M6). *Desulfuromonas sp.* and *Desulfitobacterium sp.* were found in selected microcosms only. However, no amplification products were obtained for members of *Sulfurospirillum multivorans* and *Dehalobacter sp.* (Tab. 11).

Table 11. Specific PCR amplification signals obtained from cDNA extracted from the microcosm samples after eight months of cultivation. Results for sample M5 are missing, as the RNA extraction from this microcosm failed.

	M1	M2	M3	M4	M5	M6	S2	S3
***Dehalococcoides sp.* (883f+1152r)**	-	-	-	+	x	+	+	+
***Desulfitobacterium sp.* (Dsb406f+Dsb619r)**	-	+	+	+	x	-	-	-
***Sulfurospirillum multivorans group* (DHSPM576f+DHSPM1210r)**	-	-	-	-	x	-	-	-
***Geobacter sp.* (Geo73f+Geo485r)**	+	+	+	+	x	-	+	+
***Desulfuromonas sp.* (Dsm494f+Dsm1050r)**	-	-	-	-	x	+	-	+
***Dehalobacter sp.* (Dre441f+Dre654r)**	-	-	-	-	x	-	-	-

4.2.4. Detection of known catabolic genes involved in OHR process

PCR examination using primers targeting known catabolic genes involved in the OHR process was done on the DNA extracted from original sediment, DNA and cDNA from microcosms after six months of cultivation. Six primer pairs were used for the screening of the presence of tetrachlorethene reductase-encoding genes (*pce*A group 1 and 2), trichlorethene reductase-encoding genes (*tce*A), and vinylchloride reductase-encoding genes (*bvc*A and *vcr*A), and *o*-chlorophenol reductase-encoding genes (*cpr*A).

All genes were showed to be present in the DNA samples from both original sediment and microcosms. The only exception was tetrachlorethene reductase-encoding genes of group 1, which was found to be negative in all samples and *vcr*A in DNA from microcosms M4 and M5 (Tab. 12).

Table 12. PCR amplification signals obtained from total DNA extracted from the original sediment (orig DNA) and after 6 months of cultivation (6m DNA and cDNA).

	M1			M2			M3			M4		
	orig DNA	6m DNA	6m cDNA	orig DNA	6m DNA	6m cDNA	orig DNA	6m DNA	6m cDNA	orig DNA	6m DNA	6m cDNA
***pce*A-g1**	-	-	-	-	-	-	-	-	-	-	-	-
***pce*A-g2**	+	+	-	+	+	-	+	+	-	+	+	-
***tce*A**	+	+	-	+	+	-	+	+	-	+	+	-
***bvc*A**	+	+	-	+	+	-	+	+	-	+	+	-
***vcr*A**	+	+	-	+	+	+	+	+	+	+	-	-
***cpr*A**	+	+	-	+	+	-	+	+	-	+	+	-

	M5			M6			S2			S3		
	orig DNA	6m DNA	6m cDNA	orig DNA	6m DNA	6m cDNA	orig DNA	6m DNA	6m cDNA	orig DNA	6m DNA	6m cDNA
***pce*A-g1**	-	-	x	-	-	-	-	-	-	-	-	-
***pce*A-g2**	+	+	x	+	+	-	+	+	-	+	+	-
***tce*A**	+	+	x	+	+	-	+	+	-	+	+	-
***bvc*A**	+	+	x	+	+	-	+	+	-	+	+	-
***vcr*A**	+	-	x	+	+	+	+	+	+	+	+	+
***cpr*A**	+	+	x	+	+	-	+	+	-	+	+	-

On cDNA from the microcosm samples none of the *pce*A, *tce*A, *bvc*A, and *cprA* genes were detected, and were then presumably not expressed within these samples. The only positive signal detected on cDNA was in the microcosms M2, M3, M6, S2 and S3 using primer set targeting *vcr*A genes known to be present in *Dehalococcoides* species exclusively (Tab. 12). This result was confirmed by the cloning-sequencing strategy (data not shown). Positive amplification of the *vcr*A gene confirmed an implication of members of the genus *Dehalococcoides* (or close relatives from the phylum *Chloroflexi*) in the degradation of the PCB congeners in Strazske sediments.

4.2.5. Temporal evolution of the bacterial communities

T-RFLP analyses were carried out for the purpose of determining the community composition differences within the eight microcosms. Moreover, T-RFLP enables monitoring the evolution in time in order to establish correlation of the obtained structures with detected PCB degradation and variety of environmental factors.

The impact of abiotic factors on bacterial communities present in the Laborec River sediment samples was studied using a redundancy analysis (RDA) of T-RFLP and abiotic data sets (including measurements of 7 reference PCB congeners, PAHs, TPHs, grain-size distribution, heavy metals, pH and TOC) for both sampling campaigns.

Separation of the samples from 2009 and 2010 demonstrated a diverse evolution of the bacterial communities influenced by redistribution of contaminants and subsequent efflux channel bed changes (Fig. 15).

The analysis showed a significant impact of selected variables, namely TPHs, PAH, PCB congeners 118 and 138 and two grain size categories (clay and sand). These variables expressed 18 % of the variance of the system. Data collected from the cultivation technique (expressed as the number of microorganisms able to degrade biphenyl vapors) also contributed to the explained variance. The first axis of the plot expressed the impact of the particle sizes present in the sediment. Second axis of the ordination can be interpreted as the evolution of the TPH and PAH concentrations, interesting sources of carbon and energy for anaerobic and aerobic bacterial degraders.

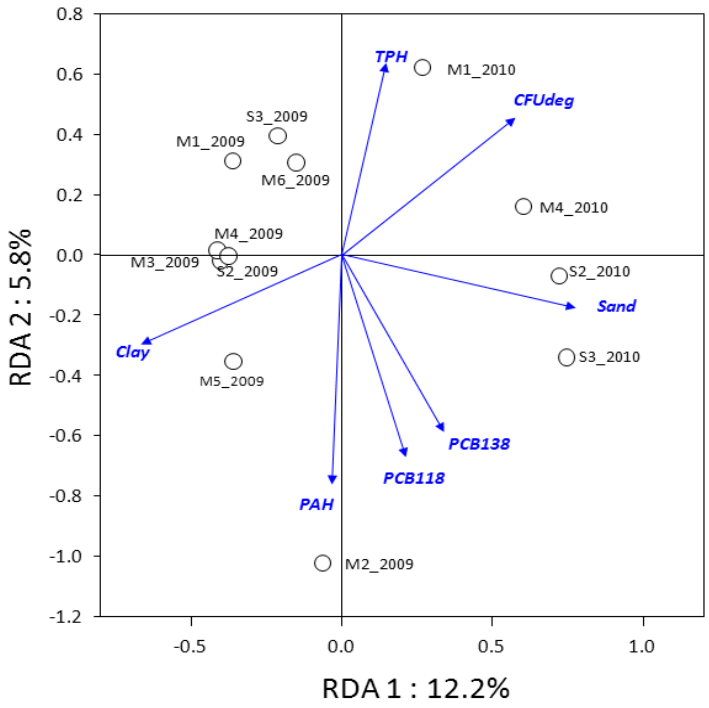

Figure 15. Redundancy analysis conducted on T-RFLP data obtained from bacterial communities present in Strazske efflux channel sediments sampled in 2009 and 2010. Environmental variables significantly correlated ($p<0.05$*) with the communities are displayed with arrows.

Interestingly, only two highly chlorinated PCB congener families (5- and 6-CBs) were found to be significantly correlated with the bacterial communities present in the corresponding river sediment samples. Despite the very high PCB concentrations (Tab. 5), no other congeners could be related to the bacterial community structures.

Surprinsingly, heavy metals, which were contaminating the samples massively (Tab. 7) did not show any significant impact on bacterial communities. This absence of correlation can be interpreted as the consequence of an indistinct relationship between both communities and metal concentrations.

T-RFLP profiles of the microbial populations in the original river sediments and their derived microcosms were generated after 6, 9, 10 and 13 months of continuous cultivation. And, a Hellinger distance-based principal component analysis was used to show possible changes in microbial communities over time (Fig. 16).

The original river sediment samples (grouped in cluster CZ in the Fig. 16) displayed large similarity in their bacterial community structures. However, even though the *in-vitro* microcosm cultivation conditions were unified, the communities' patterns diversified in a large extend. Microcosm-specific shift in the bacterial community structures was detected after six months of cultivation. The Hellinger distance-based principal component analysis distributed the T-RFs of the sample in a pattern that could be related to the PCB congener degradation and the stability of the communities within the cultivation time. The microcosms with higher degradation efficiency – S2, S3, M3 and M4 showed more homogeneous communities development and were aggregated closer to the center of the PCA diagram (Fig. 16). An exception was microcosm M6, which was revealing high methane production and lowest degradation rate within the cultivation period suggesting absence of the OHRB, but still really stable community structure.

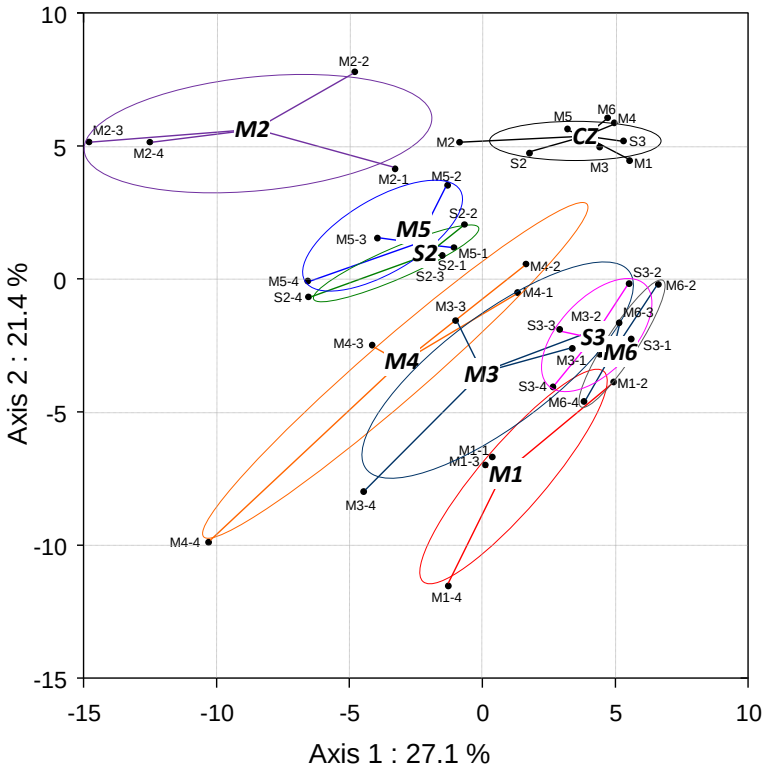

Figure 16. Hellinger distance-based PCA of the T-RFLP profiles performed on 16S rRNA gene pools present in the original sediment samples (CZ cluster) and the microcosms (coloured ovals) analyzed after 6 (x-1), 9 (x-2), 10 (x-3) and 13 (x-4) months of cultivation.

4.2.6. Detection of OHR active members in bacterial consortium

In order to study the diversity of bacterial consortia, clone libraries of cDNA were obtained from 6-month-old S3 and M4 microcosms, where the highest dechlorination activities for the assayed congeners were detected, reaching a total of 24.81 % in M4 and 35.93 % in S3 (Fig. 14). Specifically, two taxonomic levels – phylum and class - were chosen for assessment of active OHR community guilds. Primer pairs targeting V6 hypervariable region of 16S rRNA for phylum *Chloroflexi* and class *Dehalococcoidia* containing bacterial strains supposedly involved in PCB degradation allowed the retrieval of 145 non-*Dehalococcoides* sequences within the *Chloroflexi* group and 32 *Dehalococcoides* and *Dehalococcoides*-like group (DLG) related sequences of about 400 bp and 270 bp, respectively.

Phylogenetic analysis of the predominant bacterial representatives in the S3 and M4 microcosm communities showed that significant amount of uncultured bacteria was observed in the both studied microcosms. The retrieved phylogenetically distinct 16S rRNA gene sequences showed 88–99 % similarity with their closest relatives having annotation in the NCBI database.

Diverse groups of bacteria within phylum *Chloroflexi* distinct from class *Dehalococcoidia* were collected and displayed in Fig. 17. Retrieved clones were assigned to classes *Anaerolineae*, *Caldilineae* and *Ktedonobacteria*. In detail, class *Anaerolineae* was represented by 91 % (61 % S3 and 30 % M4) of all sequences affiliating with genera *Bellilinea, Leptolinea*, *Levilinea, Anaerolinea, Longilinea*, and *Thermanaerothrix (*Fig. 17). The most abundant groups were affiliated to genera *Leptolinea* and *Thermanaerothrix* accounting for 24.1 % and 22.8 % of all clones, respectively. Methanogens (Imachi et al., 2011), PCB-dechlorinating consortia (EU542512, EF393104, GU180177) and 1,2-dichloropropane-dechlorinating mixed cultures (Schlötelburg et al., 2000) were find to be closest relatives to our retrieved sequences.

Figure 17. Neighbor-joining phylogenetic tree of non-*Dehalococcoides* partial 16S rRNA gene sequences of M4 and S3 microcosms using GNSB and DHC primer sets. Tree was rooted with the 16S rRNA gene sequence of *Aquifex aeolicus* VF5.

Group of clones related to genus *Anaerolinea* originated mainly from microcosm S3 and showed 99 % similarity with members of bacterial community in PAH-contaminated soil (Martin et al., 2012) and 94 % similarity with TCE-degrading consortium (von Wintzingerode et al., 1999). The rest of *Anaerolinea* clones isolated from M4 exhibited the 99 % sequence identity with uncultured microorganisms in wastewater sludge, and with members of enrichment cultures capable to reductively dechlorinate 1,2-dichloropropane (AJ306749).

Genus *Longilinea*, entirely represented by the S3 clones phylogenetically related (97-98 % similarity) to the filamentous *Longilinea arvoryzae* KOME-1^T isolated from methanogenic propionate-degrading consortia, comprised 13.1 % of all retrieved non-*Dehalococcoides* sequences. Genus *Bellilinea* was represented by the fraction of 16S rRNA clones counting 9.7 % of all the sequences and shared 96 % similarity with filamentous bacterium, *Bellilinea caldifistulae* strain GOMI-1^T, isolated from methanogenic propionate-degrading consortia (Yamada et al., 2007). Only 8.3 % of the all retrieved sequences were affiliated to genus *Levilinea*. And, only one clone (from microcosm M4) was detected with 99 % similarity with an uncultured bacterium involved in anaerobic digestion of wastewater sludge belonging to class *Caldilineae* (Rivière et al., 2009). Also, 8.3 % of the identified clones were affiliated with class *Ktedonobacteria* sharing 93-94 % sequence similarity with uncultured bacteria (EF515582), reported for a full-scale anaerobic sludge bioreactor, and a hypersaline microbial mat (EU245384).

The *Dehalococcoides*-related 16S rRNA gene sequences present in cDNA from both S3 and M4 microcosms, representing 18 % of all retrieved sequences, formed distinct phylogenetic clusters displayed in the Fig. 18. Most of the sequences shared a high degree of similarity with sequences isolated previously from PCB- or chloroethene-contaminated environments. However, *Dehalococcoides*-related bacterial sequences isolated from both microcosms shared a low similarity degree. Only a single cluster composed of five sequences originated from both microcosms.

DLG-related sequences isolated from the microcosm M4 clustered in two main homogenous groups, sharing a high degree of similarity with JWBH clone series isolated from marine sediments (Baltimore Harbour) from which PCB dechlorinators *Dehalobium chlorocoercia* strain DF-1 and clone *o*-17 were isolated equally (Watts et al., 2005). A third cluster was related to clones isolated from CE-contaminated ethane aquifers (Kleikemper et al. 2005, unpublished data) and clones found in oil sand tailings (Siddique et al., 2011), in which bacterial communities were capable of utilizing long chains of *n*-alkanes under methanogenic conditions. Interestingly, the clones retrieved from the microcosm M4 did not show taxonomic association with known cultivated obligate OHR bacteria from genus *Dehalococcoides* or *Dehalogenimonas*.

Obligate OHR bacteria, closely related to "*Dehalococcoides*" sp. subgroup Pinellas, were found in S3 microcosm only, and they comprised 3.4 % of all S3-derived sequences (Fig. 18).

Traditional clusters found in PCB contaminated habitats, such as the Tidal flat cluster and Lahn cluster (Kittelmann and Friedrich, 2008a, 2008b) were not found in the observed clone diversity. Furthermore, no clone was found to be closely related to the *Dehalobium chlorocoercia* DF-1, clone m-1, and the series of OTU clones (Watts et al., 2005), indicating the presence in both cases of a different *Chloroflexi* diversity which was not yet encountered in former PCB degradation studies.

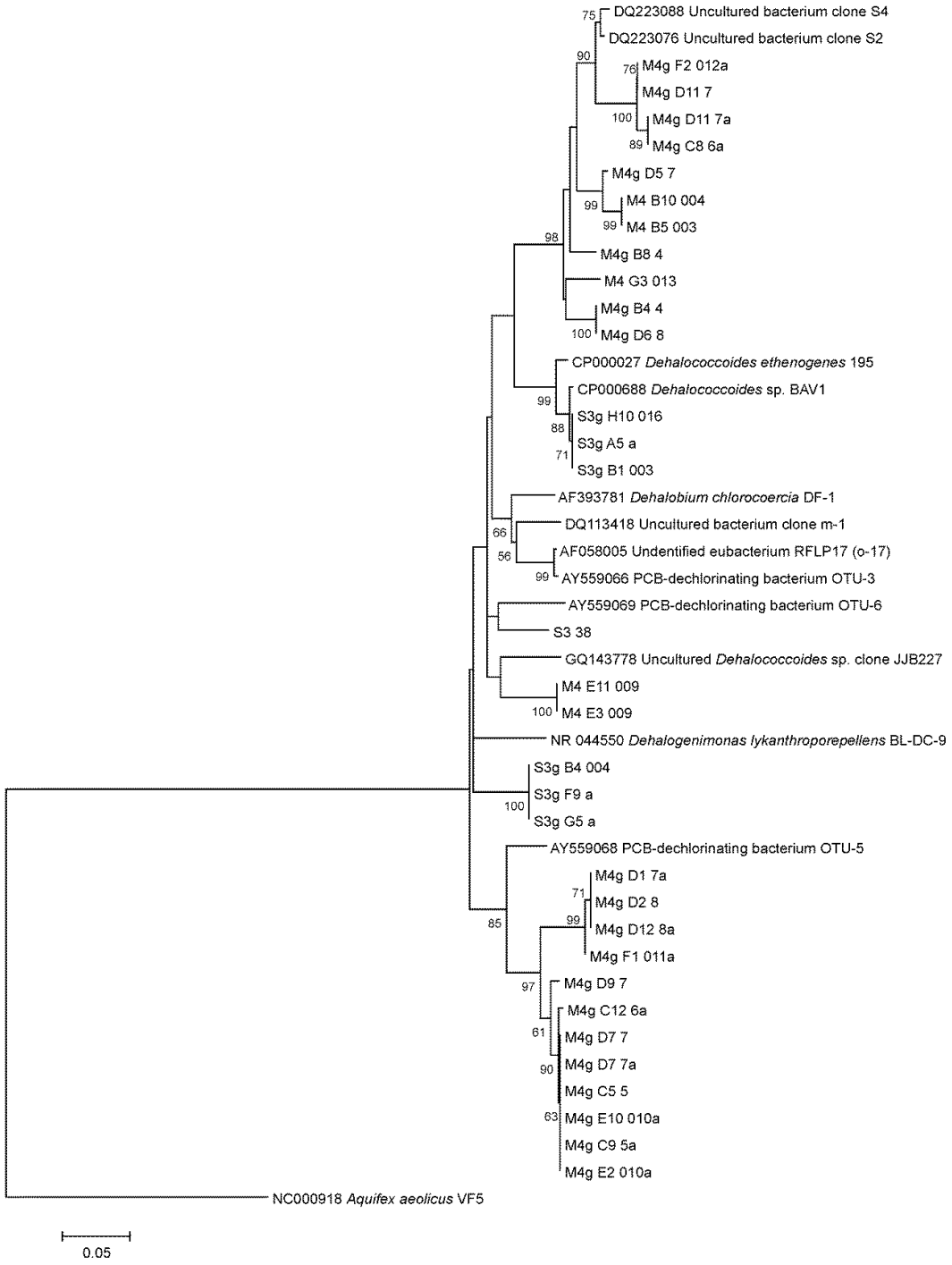

Figure 18. Neighbor-joining phylogenetic tree of class Dehalococcoides-related 16S rRNA gene sequences present in cDNA from the anaerobic microcosms S3 and M4. Tree was rooted with the 16S rRNA gene sequence of Aquifex aeolicus VF5.

4.3. Sediment-free microcosm experiments

Fourteen sediment-free microcosms supplemented with 20 ppm of the Strazske manufactured PCB mixture Delor 103 were established in order to study microbial communities in chemically defined environment. For that purpose we used a modified protocol developed by Bedard et al. (2006). Sites with the historical PCB contamination are known as good sources of dechlorinators (Domínguez, 2008), thus, 20 ml of the supernatant from the sediment-based microcosm M1 from Strazske channel was used as the inoculum. Six microcosms were supplemented with the 2-bromoethane sulfonate (BES), inhibitor of the methanogenesis. First generation of the sediment-free microcosms (samples SFM1 – SFM14) was maintained from 98 to 692 days.

Additionaly, four sediment-free microcosms of the second generation were set up under the same conditions except the inoculum, which was issued from the four selected first generation SFMs (SFM7, SFM8, SFM13 and SFM14) after 176 days of cultivation. Two of the SFMs were supplemented by BES as well. Second generation (samples SFM7A, SFM8A, SFM13A and SFM14A) was cultivated for 503 days.

Fluorescent light microscopy technique using 4′,6-diamidino-2-phenylindole (DAPI) stain enabled evaluation of the viability of the sediment-free cultures of the first generation. The presence of numerous tiny round-shaped bacterial cells (Fig. 19) was detected after 98 days of the continuous cultivation of the first generation microcosms.

Interestingly, almost no visual difference could be observed between no BES and BES-treated microcosms. The only distinction detected in SFM6 (Fig. 19, left) is the presence of bacteria creating small chains (labeled with white arrow in Fig. 19, left).

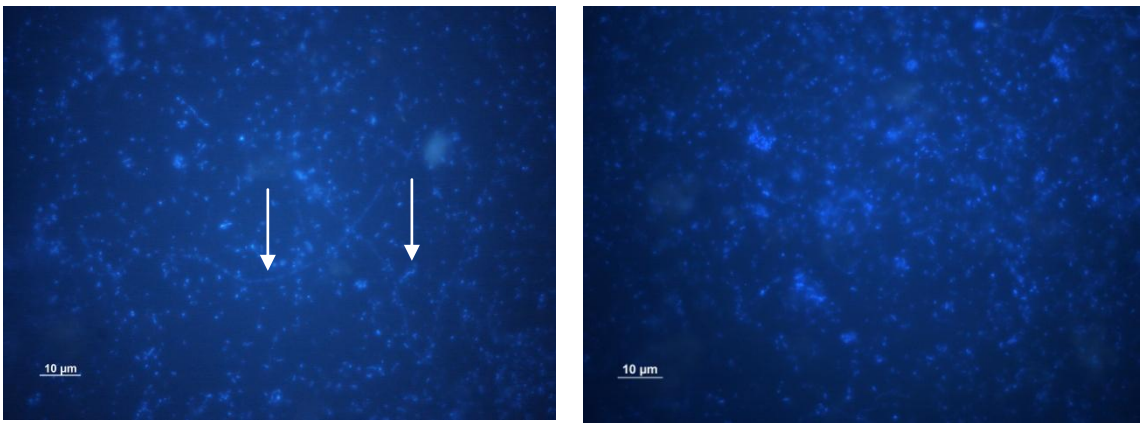

Figure 19. Fluorescent light microscopy of a DAPI stained bacterial PCB-degrading consortia. Three months old sediment-free consortia – SFM6 (without BES treatment) and SFM9 (with BES treatment). White arrow: bacteria creating small chains.

4.3.1. PCB congener analysis

GC-ECD analysis detected transformation of the PCB congeners in the first and second generation of sediment-free microcosms. Surprisingly, no effect of BES on the PCB dechlorination was observed. And, no dechlorination was observed in the negative and autoclaved controls (data not shown).

4.3.1.1. First generation of sediment-free microcosms

Significant changes in PCB congener distribution were successfully detected using GC-ECD analysis of first generation of sediment-free microcosms. Chemical analysis of 74 major congeners showed that the dehalogenation occured in all SFMs. Highest dechlorination rate was observed in the long-term cultivated microcosms – SFM7, SFM8 and SFM13, SFM14, up to 95.5 % of all congeners were removed (Tab. 13). The PCB congeners were depleted within all groups of congeners with the significant decrease of 5- and 6-chlorinated PCB congeners (Tab. 13). Microcosms SFM1, 6 and 10 revealed an activity in dechlorination, however, total amount of congeners was kept at the constant level (Tab. 13). Very low total dehalogenation values (up to 14.9 % only) were detected in case of the microcosms which were maintained for less than 200 days in microcosms without BES addition.

Table 13. Comparison of PCB congener concentrations [in mg/l] in sediment-free microcosms with the control sediment-free microcosm setup. 1: number of days of continuous cultivation; 2: killed (autoclaved) control, expressed both in mg/l and, in parenthesis, in mole %; 3: Reference – composition of original Delor103 mixture in mole % according to Grabic et al. (Grabic et al., 2006).

	No BES								**BES added**							
SFM	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**CTRL[2]**	**REF[3]**
Days[1]	**98**	**126**	**126**	**155**	**173**	**173**	**569**	**692**	**98**	**121**	**126**	**126**	**569**	**692**	**503**	
Di-CB	1.6	2.3	0.8	1.1	2.5	3.8	0.0	1.3	0.7	3.3	0.4	0.2	0.0	0.0	1.7 (11.2%)	10.4%
Tri-CB	11.8	9.7	9.9	8.8	7.8	10.3	0.4	5.0	9.1	12.0	7.5	10.4	0.4	2.3	9.9 (58.0%)	53.8%
Tetra-CB	5.5	4.0	4.2	5.1	4.5	5.0	0.4	2.7	4.6	4.2	3.1	4.8	0.3	2.2	5.6 (28.8%)	32.3%
Penta-CB	0.3	0.2	0.2	0.3	0.2	0.3	0.0	0.3	0.3	0.2	0.1	0.3	0.1	0.3	0.4 (1.7%)	2.6%
Hexa-CB	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1 (0.3%)	0.3%
Hepta-CB	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0 (0.1%)	0.1%
TOTAL	**0.0**	**7.7**	**14.5**	**12.8**	**14.9**	**0.0**	**95.5**	**47.3**	**17.2**	**0.0**	**36.9**	**10.7**	**94.9**	**72.5**		

No cultivation transfer was needed for retaining of the development of stable active microbial communities with degradation ability. Although all the SFMs were inoculated from the same source of PCB dechlorinating consortia (sediment microcosm M1), SFMs showed different degradation patterns and different degree of congener elimination (Tab. 14).

Apparently all classes of PCB congeners were eliminated. Group of Hepta-CB showed from 3 % (SFM8) to 100 % (SFM7) of elimination, reaching the average of 48 % depletion. Group of Hexa-CB revealed from 9 % (SFM8) to 97 % (SFM7) of total dehalogenation. Penta-CB showed decrease of PCB congeners from 25 % (SFM4 and SFM8) to 90 % (SFM7). Tetra-CBs were reaching the total depletion from 5 % (SFM1) to 93 % (SFM7 and SFM13). The SFM1 was showing also a significant accumulation of the PCB42 (+17 %) and PCB40 (+20 %). In case of Tri-CB and Di-CB an accumulation of the PCB congeners was mainly occurring. The only SFMs where no accumulation of PCB congeners was found, were long term microcosms (SFM7, 8, 13 and 14) and SFM11 (Tab. 14).

It was shown that addition of the BES had no apparent impact of the elimination rate of the PCB congeners as well as on the degradation patterns.

Interestingly, despite the inoculation of all SFMs with the identical inoculum, discrepancies could be observed for the duplicate SFMs analyzed on the same day of cultivation, as for instance for SFM5 and SFM6 (day 173, without BES), and SFM11 and SFM12 (day 126, BES-treated).

Table 14. Detailed PCB congener's analysis - variations in concentration [in ppm] in SFMs during cultivation compared with control microcosm. Blue – accumulation; red –dehalogenation; the darker colour shades, the higher is the increase/decrease of concentrations as compared with the control SFM.

Congener IUPAC	Number of Cl	SFM1	SFM2	SFM3	SFM4	SFM5	SFM6	SFM7	SFM8	SFM9	SFM10	SFM11	SFM12	SFM13	SFM14	Control
4_10	**2**	0.7905	0.6255	0.2790	0.3810	0.7310	0.8698	0.0000	0.3593	0.6286	0.8631	0.3896	0.1988	0.0000	0.0000	0.3620
9_7	**2**	0.0291	0.0303	0.1307	0.0777	0.0267	0.0533	0.0000	0.0149	0.0289	0.0357	0.0000	0.0000	0.0000	0.0000	0.0260
6	**2**	0.2125	0.1101	0.1305	0.1874	0.2360	0.2055	0.0000	0.0401	0.0000	0.1222	0.0000	0.0000	0.0000	0.0000	0.0880
8_5	**2**	0.5983	1.5463	0.2504	0.4531	1.5068	2.6453	0.0000	0.8727	0.0000	2.2738	0.0000	0.0000	0.0000	0.0000	1.1920
19	**3**	0.1381	0.1757	0.0858	0.0728	0.1804	0.2239	0.0000	0.1017	0.1639	0.1838	0.0825	0.0727	0.0257	0.0373	0.1140
18	**3**	1.3183	1.9471	1.3842	0.9186	1.0710	1.7203	0.0331	1.0117	1.3963	1.8336	1.0255	1.2077	0.0710	0.0000	1.5200
16_32	**3**	1.5193	0.0000	1.2810	0.9642	0.0000	1.4742	0.0356	0.0000	1.3110	1.8050	1.0494	1.3360	0.0812	0.2522	1.2100
28_31	**3**	5.3855	4.6904	4.5543	4.3255	4.3254	4.2740	0.1890	2.3975	3.7408	5.0391	3.2245	4.7143	0.1204	1.1540	4.3160
33_53_20_21	**3**	2.6654	2.3439	1.9824	1.7925	1.5254	1.9442	0.0744	1.1443	1.8759	2.5326	1.6691	2.4201	0.0660	0.6094	2.0360
37	**3**	0.7723	0.5385	0.5874	0.7501	0.6491	0.6424	0.0484	0.3059	0.6004	0.6085	0.4446	0.6829	0.0193	0.2331	0.7440
51	**4**	0.0459	0.0379	0.0385	0.0388	0.0407	0.0384	0.0015	0.0221	0.0324	0.0388	0.0266	0.0387	0.0012	0.0105	0.0700
52	**4**	0.4375	0.3321	0.3467	0.4103	0.3720	0.3600	0.0286	0.2015	0.3275	0.3486	0.2518	0.3751	0.0142	0.1390	0.4320
49	**4**	0.4475	0.3362	0.3635	0.4203	0.4070	0.3799	0.0289	0.2047	0.3343	0.3455	0.2514	0.3780	0.0146	0.1418	0.4560
47	**4**	0.2422	0.1790	0.1914	0.2308	0.2268	0.2154	0.0145	0.1120	0.1805	0.1804	0.1310	0.2034	0.0089	0.0788	0.2580
44	**4**	0.4794	0.3556	0.3775	0.4614	0.4169	0.4000	0.0345	0.2055	0.3735	0.3831	0.2849	0.4206	0.0149	0.1508	0.4380
42	**4**	0.8303	0.5890	0.6502	0.7497	0.6948	0.7131	0.0487	0.3103	0.6360	0.6221	0.4203	0.6928	0.0207	0.2282	0.6920
41_71	**4**	0.6870	0.4898	0.5312	0.6685	0.5968	0.5941	0.0483	0.2875	0.5455	0.5247	0.3896	0.5927	0.0214	0.2230	0.6500
40	**4**	0.1755	0.1235	0.1375	0.1738	0.1542	0.1494	0.0128	0.0681	0.1404	0.1409	0.1041	0.1576	0.0045	0.0527	0.1500
67	**4**	0.0205	0.0149	0.0150	0.0191	0.0168	0.0200	0.0011	0.0107	0.0176	0.0146	0.0106	0.0178	0.0013	0.0084	0.0220
74_94_61	**4**	0.3175	0.2231	0.2285	0.2871	0.2507	0.3073	0.0206	0.1730	0.2725	0.2223	0.1660	0.2688	0.0265	0.1421	0.3460
70_76	**4**	0.5921	0.4260	0.4173	0.5202	0.4141	0.5712	0.0413	0.3326	0.5244	0.4276	0.3240	0.5154	0.0501	0.2674	0.6320
66_95	**4**	0.7805	0.5616	0.5378	0.6992	0.5941	0.7536	0.0560	0.4448	0.6743	0.5560	0.4171	0.6758	0.0717	0.3647	0.8480
59_60	**4**	0.4826	0.3766	0.3375	0.4381	0.3110	0.4498	0.0373	0.3219	0.5062	0.3962	0.2954	0.4911	0.0538	0.3875	0.5920
77	**4**	0.0013	0.0009	0.0010	0.0015	0.0012	0.0014	0.0003	0.0013	0.0012	0.0009	0.0007	0.0012	0.0006	0.0012	0.0020

Table 14. Continuation.

Congener IUPAC	Number of Cl	SFM1	SFM2	SFM3	SFM4	SFM5	SFM6	SFM7	SFM8	SFM9	SFM10	SFM11	SFM12	SFM13	SFM14	Control
91	**5**	0.0642	0.0423	0.0534	0.0732	0.0779	0.0680	0.0106	0.0365	0.0490	0.0403	0.0313	0.0501	0.0075	0.0310	0.0700
84_89_92	**5**	0.0328	0.0225	0.0236	0.0343	0.0288	0.0320	0.0038	0.0225	0.0299	0.0251	0.0203	0.0323	0.0280	0.0248	0.0420
99	**5**	0.0602	0.0415	0.0415	0.0589	0.0591	0.0659	0.0052	0.0653	0.0540	0.0413	0.0315	0.0592	0.0443	0.1102	0.0800
83	**5**	0.0061	0.0040	0.0046	0.0073	0.0062	0.0065	0.0009	0.0053	0.0053	0.0039	0.0031	0.0055	0.0015	0.0040	0.0080
97_86_81	**5**	0.0172	0.0117	0.0122	0.0163	0.0142	0.0181	0.0014	0.0151	0.0155	0.0115	0.0092	0.0147	0.0053	0.0126	0.0220
87_115_111	**5**	0.0227	0.0192	0.0162	0.0225	0.0085	0.0190	0.0021	0.0251	0.0261	0.0190	0.0150	0.0244	0.0088	0.0212	0.0380
82_110	**5**	0.0187	0.0132	0.0144	0.0211	0.0173	0.0200	0.0043	0.0196	0.0181	0.0133	0.0103	0.0167	0.0077	0.0159	0.0260
118	**5**	0.0333	0.0230	0.0241	0.0321	0.0273	0.0372	0.0090	0.0451	0.0306	0.0226	0.0166	0.0283	0.0251	0.0394	0.0460
114	**5**	0.0011	0.0009	0.0008	0.0011	0.0000	0.0010	0.0000	0.0015	0.0013	0.0009	0.0007	0.0012	0.0007	0.0012	0.0020
105	**5**	0.0177	0.0145	0.0132	0.0173	0.0000	0.0180	0.0015	0.0345	0.0206	0.0140	0.0103	0.0181	0.0201	0.0298	0.0300
151	**6**	0.0014	0.0009	0.0011	0.0019	0.0019	0.0016	0.0004	0.0016	0.0011	0.0008	0.0007	0.0011	0.0010	0.0016	0.0020
149_123	**6**	0.0067	0.0046	0.0052	0.0074	0.0064	0.0073	0.0010	0.0084	0.0059	0.0045	0.0037	0.0058	0.0053	0.0077	0.0100
134	**6**	0.0068	0.0043	0.0052	0.0081	0.0000	0.0077	0.0000	0.0082	0.0058	0.0043	0.0034	0.0056	0.0049	0.0078	0.0100
153	**6**	0.0114	0.0078	0.0083	0.0111	0.0000	0.0125	0.0000	0.0165	0.0099	0.0076	0.0059	0.0097	0.0128	0.0163	0.0180
132	**6**	0.0011	0.0009	0.0008	0.0010	0.0000	0.0010	0.0001	0.0017	0.0012	0.0009	0.0007	0.0011	0.0012	0.0017	0.0020
141	**6**	0.0011	0.0008	0.0008	0.0010	0.0005	0.0010	0.0000	0.0015	0.0011	0.0008	0.0006	0.0010	0.0010	0.0012	0.0020
137	**6**	0.0033	0.0024	0.0022	0.0033	0.0015	0.0033	0.0000	0.0052	0.0031	0.0028	0.0021	0.0037	0.0039	0.0040	0.0060
130_176	**6**	0.0011	0.0008	0.0008	0.0010	0.0006	0.0012	0.0000	0.0022	0.0010	0.0008	0.0006	0.0011	0.0022	0.0026	0.0020
138	**6**	0.0105	0.0076	0.0077	0.0107	0.0067	0.0110	0.0000	0.0179	0.0100	0.0074	0.0059	0.0096	0.0149	0.0179	0.0180
129_178	**6**	0.0012	0.0008	0.0009	0.0014	0.0012	0.0013	0.0000	0.0018	0.0010	0.0008	0.0006	0.0010	0.0016	0.0018	0.0020
128	**6**	0.0021	0.0016	0.0015	0.0021	0.0011	0.0022	0.0000	0.0045	0.0022	0.0016	0.0012	0.0021	0.0039	0.0044	0.0040
183	**7**	0.0011	0.0008	0.0008	0.0010	0.0007	0.0012	0.0000	0.0020	0.0011	0.0008	0.0006	0.0010	0.0019	0.0021	0.0020
185	**7**	0.0010	0.0007	0.0007	0.0010	0.0006	0.0011	0.0000	0.0017	0.0010	0.0007	0.0006	0.0010	0.0014	0.0015	0.0020
177	**7**	0.0011	0.0008	0.0008	0.0010	0.0007	0.0012	0.0000	0.0020	0.0010	0.0008	0.0006	0.0010	0.0020	0.0022	0.0020
180	**7**	0.0021	0.0015	0.0016	0.0021	0.0013	0.0024	0.0000	0.0039	0.0021	0.0015	0.0012	0.0020	0.0034	0.0036	0.0040
170	**7**	0.0011	0.0007	0.0008	0.0010	0.0007	0.0012	0.0000	0.0020	0.0010	0.0007	0.0005	0.0010	0.0019	0.0019	0.0020

4.3.1.2. Second generation of sediment-free microcosms

PCB congener analysis of four SFMs from the second generation revealed almost complete depletion of the Delor 103 mixture after 503 days. Using the same analytical procedure, which was used for the measurement of the first generation SFMs, all the microcosms from the second generation were under detection limit of the instrument. Thus, complete extracts without any dilution were used for repeating semi-quantitative analysis of the PCBs present in the microcosms. As a result, comparing only the peak height of the reference (original Delor103 mixture) with the peak heights of the samples after normalization, we detected following decreases in the PCB concentrations: 99.3 % (SFM7A), 99.3 % (SFM8A), 97.6 % (SFM13A) and 99.1 % (SFM14A).

4.3.2. Microbial diversity estimation

The massive sequencing data from the genetic pool of the 16S rRNA gene amplicons of 23 samples (DNA from slurry microcosm M1, cDNA from 1st and 2nd generation SFMs and DNA from four selected 1st generation SFMs) resulted in average of 6294 sequence reads in the range from 2567 to 9695 sequences. After quality control steps (denoising and removing chimeras) a total of 77487 sequences with the average of 3369 sequences per sample in the range 1193 to 6282 sequences were obtained. Estimated species-accumulation curves created at 97 % similarity level showed that the coverage of the OTUs was not fully exhausted (Fig. 20, 21). Curves for SFMs showed low diversity of the microbial communities that was confirmed by the richness estimators based on the total amount of high quality reads at 0.03 OTU cut-off level (Tab. 15). Comparing samples of DNA in the species-accumulation curves analysis showed that the coverage of slurry microcosm M1 was not fully exhausted, while almost horizontal asymptote was detected for sample SFM3 signalizing complete coverage in terms of OTUs (Fig. 21, left).

Within the cDNA extracted from SFMs, there was no effect of BES addition on the apparent richness, diversity and evenness of the communities of the first generation of SFMs (Fig. 20). SFM11, in general the most abundant, diverse and even sample according to diversity indexes (Tab.15) was showing the steepest asymptote (Fig.20, right).

Species-accumulation curves of SFMs 1st and 2nd generation showed the similar number of species recorded as a function of sampling effort in terms of OTUs (Fig. 20 and 21 right).

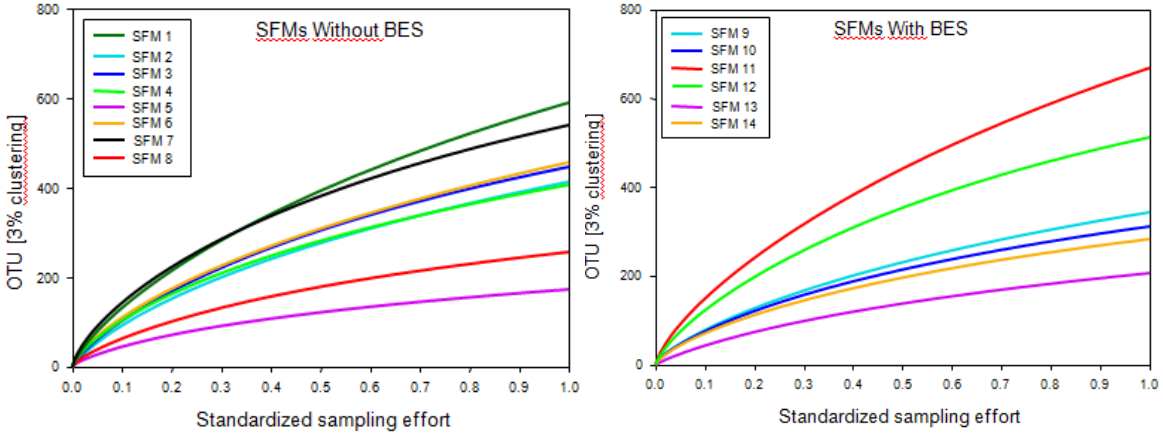

Figure 20. Species-accumulation curves analysis carried out on high-quality sequences from the first generation sediment-free microcosms. Samples without BES (left), samples with BES (right).

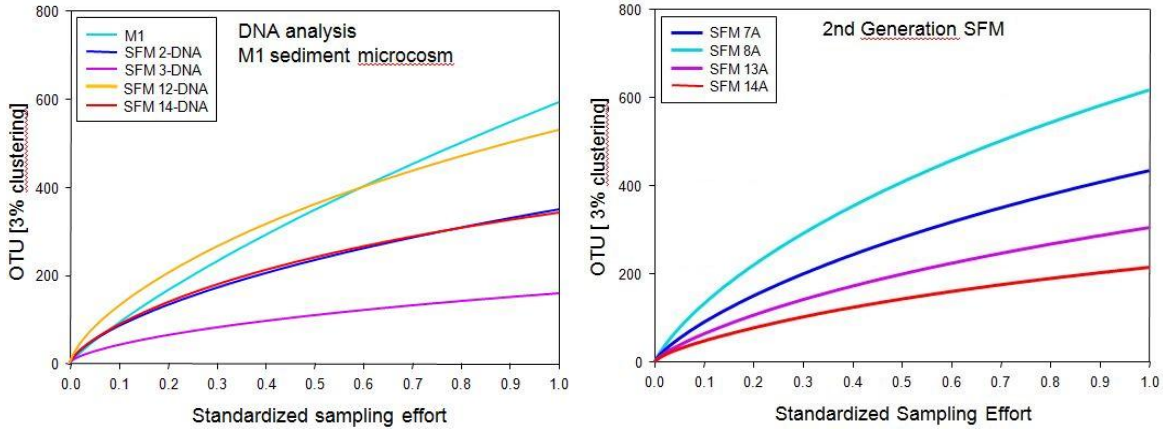

Figure 21. Species-accumulation curves analysis carried out on high-quality sequences from the DNA extracted from first generation SFMs and from the sediment microcosmM1 (left) and second generation of SFMs (right).

Several diversity indices (Tab. 15) were used to evaluate richness, diversity and evenness among all tested samples. In general, data showed a massive decrease in terms of diversity and evenness in the sediment-free microcosms in comparison with the original river sediment (Tab. 15). There was also the difference between the samples of DNA and cDNA. DNA samples had a higher diversity than the cDNA samples showing the hidden potential of the bacterial communities. Interestingly, higher values were observed (within DNA samples) in microcosms with BES addition (Tab. 15). ACE estimator of species richness showed that the richest communities were in SMF11 (1366) and SFM1 (1154), while the poorest microcosm was established SFM5 (293)

within the first generation of sediment-free microcosms. In general, in terms of the ACE estimator microcosms without BES addition were poorer than the BES-treated communities. However, second generation of sediment-free microcosms showed the opposite. Chao1 indexes confirmed the data measured by ACE method.

Shannon H´ diversity index showed that the highest diversity measured in first generation of SFMs was in SFM3 (2.44) and SFM11 (2.46). On the other hand, significantly lower diversity was detected in SFM13 (1.29). In terms of the evenness, Shannon and Pielou evenness indexes showed that SFMs communities were not even. Measures were fluctuating between 0.07, resp. 0.43 (SFM1) to 0.21, resp.0.61 (SFM11) in first generation of SFMs, and between 0.16, resp. 0.56 (SFM7A) to 0.30, resp. 0.67 (SFM14A). From the point of view of Fisher´s alpha sample size independent coefficient, the most abundant microcosm was SFM1 (16.97) while the lowest value was calculated for SFM5 (5.56) (Tab. 15). Second generation of the SFMs revealed higher diversity in no BES microcosms. In general, the most abundant, diverse and even microcososm of the first generation was SFM11 and lowest values were calculated for SFM5 and SFM13. No impact of BES addition on the richness, diversity and evenness of the communities was detected.

Table 15. Diversity indices for original microcosm M1 and sediment-free microcosms.

SFMs	Days	BES added ?	PCB [%]*	Reads	Genus (Sobs)	OTUs**	ACE**	CHAO1**	Shannon *H'*	Shannon Evenness	Pielou Evenness	Fisher's alpha
M1	169	No	6,3	1193	152	593	2090	1952	3.94	0.34	0.78	46.23
SFM1	98	No	2	4845	96	589	1154	1056	1.94	0.07	0.43	16.97
SFM2	126	No	26	2881	49	413	819	746	1.81	0.13	0.47	8.39
SFM2-DNA	126	No	26	2162	88	348	725	712	2.67	0.16	0.59	18.44
SFM3	126	No	25	3803	59	446	774	736	2.44	0.19	0.59	9.91
SFM3-DNA	126	No	25	1891	52	159	308	270	2.26	0.19	0.57	9.89
SFM4	155	No	13	5831	50	407	738	681	2.2	0.15	0.52	7.49
SFM5	173	No	26	1757	32	173	293	280	1.48	0.14	0.43	5.56
SFM6	173	No	13	5318	60	457	878	807	1.98	0.12	0.48	9.48
SFM7	569	No	94	6282	52	539	867	801	2.36	0.2	0.6	7.76
SFM8	692	No	43	2477	40	255	411	419	2.1	0.19	0.54	6.77
SFM9	98	Yes	21	2947	50	342	646	551	2.11	0.17	0.54	8.55
SFM10	121	Yes	20	2719	53	312	559	524	2.39	0.21	0.6	9.33
SFM11	126	Yes	44	5983	57	669	1366	1236	2.46	0.21	0.61	8.73
SFM12	126	Yes	14	5041	61	511	848	746	1.95	0.12	0.48	9.76
SFM12-DNA	126	Yes	14	3468	91	530	980	979	2.95	0.21	0.65	17.12
SFM13	569	Yes	85	2496	50	207	391	360	1.29	0.07	0.33	8.86
SFM14	692	Yes	55	3895	52	282	480	428	2.3	0.15	0.51	8.48
SFM14-DNA	692	Yes	55	3728	61	341	502	552	2.95	0.21	0.65	9.55
SFM7-2nd	503	No	99.3	3531	67	617	1214	1183	2.36	0.16	0.56	11.73
SFM8-2nd	503	No	99.3	2146	61	434	936	872	2.81	0.27	0.69	11.69
SFM13-2nd	503	Yes	97.6	1779	53	306	633	634	2.19	0.17	0.55	10.27
SFM14-2nd	503	Yes	99.1	1314	38	215	375	397	2.45	0.30	0.67	7.31

*: Cumulated congener degradation, total in %; **: 3% clustering level

4.3.3. Detection of OHR active members in bacterial consortium

For the better understanding of the microbial communities in sediment-free PCB organohalide respiring cultures, high throughput sequencing analysis was conducted to uncover the phylogenetic composition of the active taxa present in the microcosms.

Results showed that bacterial communities were essentially driven by 12 major phyla only, contributing for 99 % to 100 % of the communities present in the SFMs and about 97 % in the initial M1 sediment microcosm (Tab. 16). Phyla with a major presence within the SFMs of both generations were *Chloroflexi*, *Proteobacteria, Firmicutes*, *Synergistetes* and *Thermotogae*. In addition, second generation SFMs revealed high proportion of the phylum *Actinobacteria*.

Comparison between RNA- and DNA-based analyses revealed strong discrepancies in the relative proportions of the phyla *Chloroflexi* and *Firmicutes*.

Table 16. Relative contributions [in %] of the main phyla present in the all SFMs, obtained from the analysis of total RNA. Samples shaded in grey indicate that total DNA from the same SFM was analyzed in addition. The original sediment microcosm M1 is provided as a reference.

		1st generation SFMs																		2nd generation SFMs			
		No BES										BES added								No BES		BES added	
Phylum	M1-DNA	SFM1	SFM2	2-DNA	SMF3	3-DNA	SFM4	SFM5	SFM6	SFM7	SFM8	SFM9	SFM10	SFM11	SFM12	12-DNA	SFM13	SFM14	14-DNA	SFM7A	SFM8A	SFM13A	SFM14A
Actinobacteria	1,26	0,06	0,17	0,74	0,03	0,05	0,02	0	0,11	0	0,12	1,05	0	0,12	0,22	7,41	0,52	0,26	6,28	9,83	26,56	20,46	16,67
Bacteroidetes	0,76	0,60	1,11	2,78	3,08	2,70	5,75	0,28	0,58	0,10	0,16	1,59	5,08	1,10	2,66	8,39	0,72	1,87	1,05	1,03	1,36	1,52	0,15
Chloroflexi	0,76	67,06	35,30	17,48	42,55	5,29	74,06	2,90	22,66	6,92	7,87	48,18	17,32	33,65	57,67	28,20	8,49	4,88	18,16	35,27	46,13	44,91	30,67
Firmicutes	18,81	3,45	1,67	45,70	9,12	52,78	1,27	46,96	8,61	30,47	45,90	0,88	3,68	2,42	2,84	22,90	1,32	8,04	32,99	24,56	9,86	20,74	18,34
OP3	0,08	0	0	2,22	12,73	10,84	0,02	0	0,21	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planctomycetes	0	0,56	0,10	0,51	0,21	0	0,03	0,06	0,30	10,62	2,54	0,41	0,37	0,02	0,04	0,12	0,32	5,29	0,13	1,03	0,65	0,00	0,38
Proteobacteria	57,18	15,81	58,76	9,62	2,76	1,69	10,39	35,23	64,08	33,21	38,03	18,97	49,25	58,21	29,89	15,11	85,30	70,24	37,55	11,46	1,98	6,01	27,70
Spirochaetes	0,59	0,89	0,07	0,46	0,39	0,26	0,03	0,06	0,06	0,05	0	2,41	1,88	0,17	0,58	0,72	0,40	0,13	0,13	0	0	0	0,08
Synergistetes	0	3,84	0,52	13,92	3,87	7,72	0,27	9,33	0,64	13,51	1,53	5,50	13,35	0,72	0,85	12,57	1,04	1,85	1,31	9,74	6,68	0,39	2,82
TM7	7,39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thermotogae	0	3,47	0,56	0,74	2,45	0,58	1,80	1,14	0,68	0,16	0,20	12,45	3,27	0,90	1,31	0,46	0,84	5,60	0,43	0,37	0,17	0,34	0,53
WWE1	0	0,12	0	0,09	14,46	4,60	5,45	0,11	0,13	0	0,04	5,02	0,15	0,65	1,39	0,69	0	0,33	0	0	0	0	0
unclassified	9,91	3,74	1,67	4,90	8,31	13,33	0,88	3,93	1,90	4,97	3,51	3,19	5,52	2,01	1,94	2,97	0,88	1,51	1,90	6,48	6,57	5,56	2,51
TOTAL	96,73	99,59	99,93	99,17	99,95	99,84	99,98	100	99,96	100	99,92	99,66	99,85	99,97	99,39	99,54	99,84	100	99,95	99,77	99,97	99,94	99,85

4.3.4. Detailed taxonomical analysis

Principal component analysis (PCA) carried out on the NGS data at the family level. Results showed strong disparities among all SFMs despite the fact that the inoculation was done using the same microbial community (Fig. 22). For both analyses only families containing a minimum of 300 sequences among all SFMs were retained for the computation.

In the PCA, sediment-free microcosms showed a clear separation within the diagram based on the BES addition. BES-treated microcosms (white circles) clustered in the upper and upper left pane. Samples without BES treatment (black circles) were clustered in the lower part of the PCA diagram (Fig. 22). Interestingly, up to 155 days, communities present within all SFMs were composed of significant proportion of the class *Anaerolineaceae* (up to 73.84 % in SFM4), which is also depicted on the diagram.

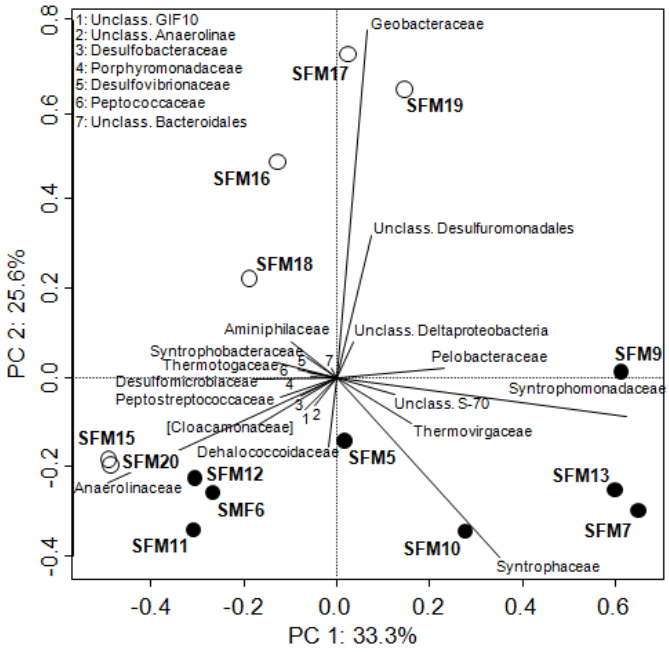

Figure 22. Principal component analysis based on the NGS data set at the family level on the data set from the first generation of SFMs. White circles: BES-treated SFMs; black circles: SFMs without BES.

Late microcosms with BES addition were mainly composed of family *Geobacteraceae* (up to 69.31 % in SFM13 and 48.34 % in SFM14), which was also showed on the *Geobacteraceae* vector (Fig. 22).

Multifactorial analysis combining NGS data with PCB congener dehalogenation data were used for detailed taxonomical analysis and finding of the putative taxa involved in the PCB congener dehalogenation (Fig. 23). ANOVA test carried out on the MFA output showed that the congener classes could explain significantly (p=0.012*) the distribution of the Families (data not shown).

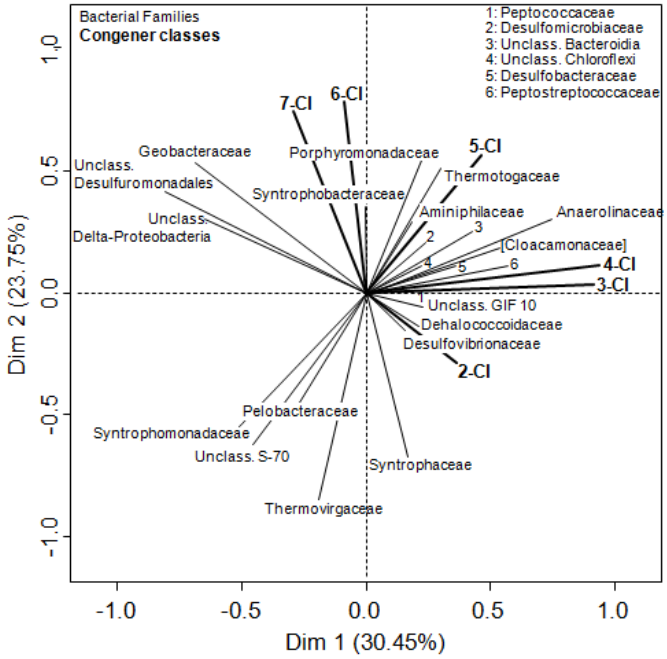

Figure 23. Multifactorial analysis using both the NGS data set at the family level and congener classes, grouping congeners sharing the same degree of chlorination.

Vectors of the Hepta-CB and Hexa-CB were collinear, as well the vectors of Tetra-CB and Tri-CB. This result showed a different dehalogenation behavior with time according to the degree of chlorination. The vector representing the family *Dehalococcoidaceae* was associated with vectors of taxa known for their sulfate-reducing and syntrophic activities, such as *Desulfovibrionaceae* (genus *Desulfovibrio*) and *Peptococcaceae* (genus *Desulfosporosinus*). This vector was opposed to the vectors of the highly chlorinated congeners (6-CBs and 7-CBs) and was collinear with the 2-CBs. This result can be understood as an indication of the implication of this particular taxon in the dechlorination of highly chlorinated congeners. Importantly, the vector of the family *Dehalococcoidaceae* was opposite to the vector of the family *Geobacteraceae*. Data collected from the cDNA of selected SFMs showed a significant impact of the BES addition on the metagenomic profiles within the microcosms.

Bacterial members of the class *Dehalococcoidia* were totally suspended in the microcosms with BES showing the negative effect on the activity of the syntrophic bacteria. In the microcosms without BES they created only small portion (from 0.2 % in SFM7 to 2.1 % in SFM1) of the microbial communities. The only exceptionally high occurrence of the members of *Dehalococcoidia* clade was detected in the microcosm SFM3 - 26.2 % (Fig. 24).

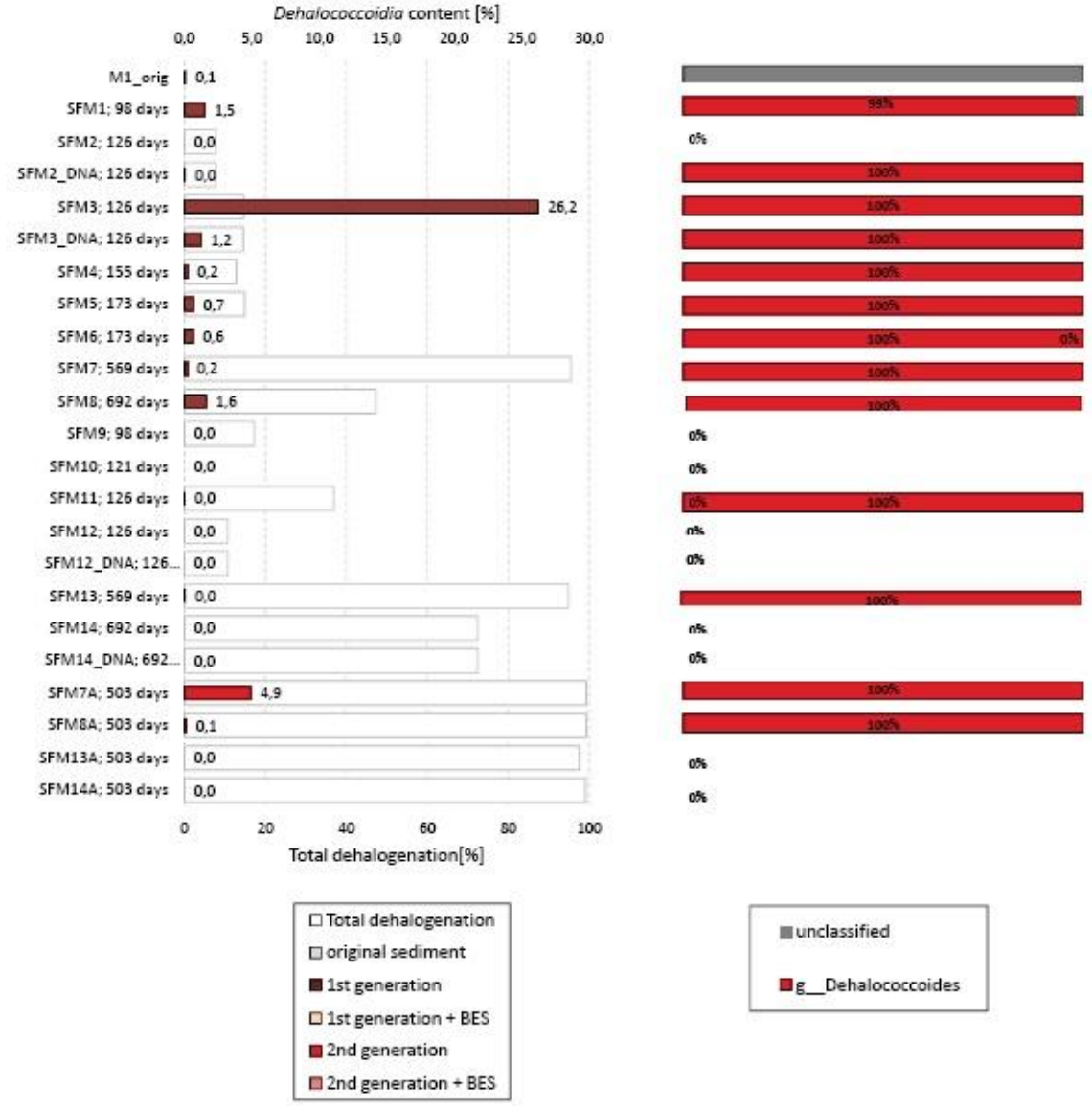

Figure 24. Relative proportions of the *Dehalococcoidia* among all detected classes in the microcosms with their respective time of cultivation (right graph). Representation of genera within the class *Dehalococcoidia* (left graphs).

Enriched was also the fraction of the *Dehalococcoides* members in the second generation of SFM7A (to 4.9 %) in comparison to the first generation of the same microcosm (SFM7) with 0.2 % only.

Deeper phylogenetic analysis based on the construction of the phylogenetic tree from first generation of sediment-free microcosms showed the affiliation to the three major *Dehalococcoides* strains – *D.mccartyi* strains 195, CG5 and CBDB1 (Fig. 25).

Figure 25. Neighbour-joining phylogenetic tree (Maximum composite likehood) of sequences affiliated with the class *Dehalococcoidia*. All sequences (1191) obtained from all first generation SFMs (red: BES-treated SFMs; blue: no BES) are displayed.

The presence of bacteria from the class *Anaerolineae* within the phylum *Chloroflexi* was found in all microcosms in the significant amount (up to 73.2 % in SFM4) (Fig. 26). Presence of the *Anaerolineae* was not influenced by the BES treatment; however, the time of cultivation had a significant impact on the presence of *Anaerolineae* in the microcosms.

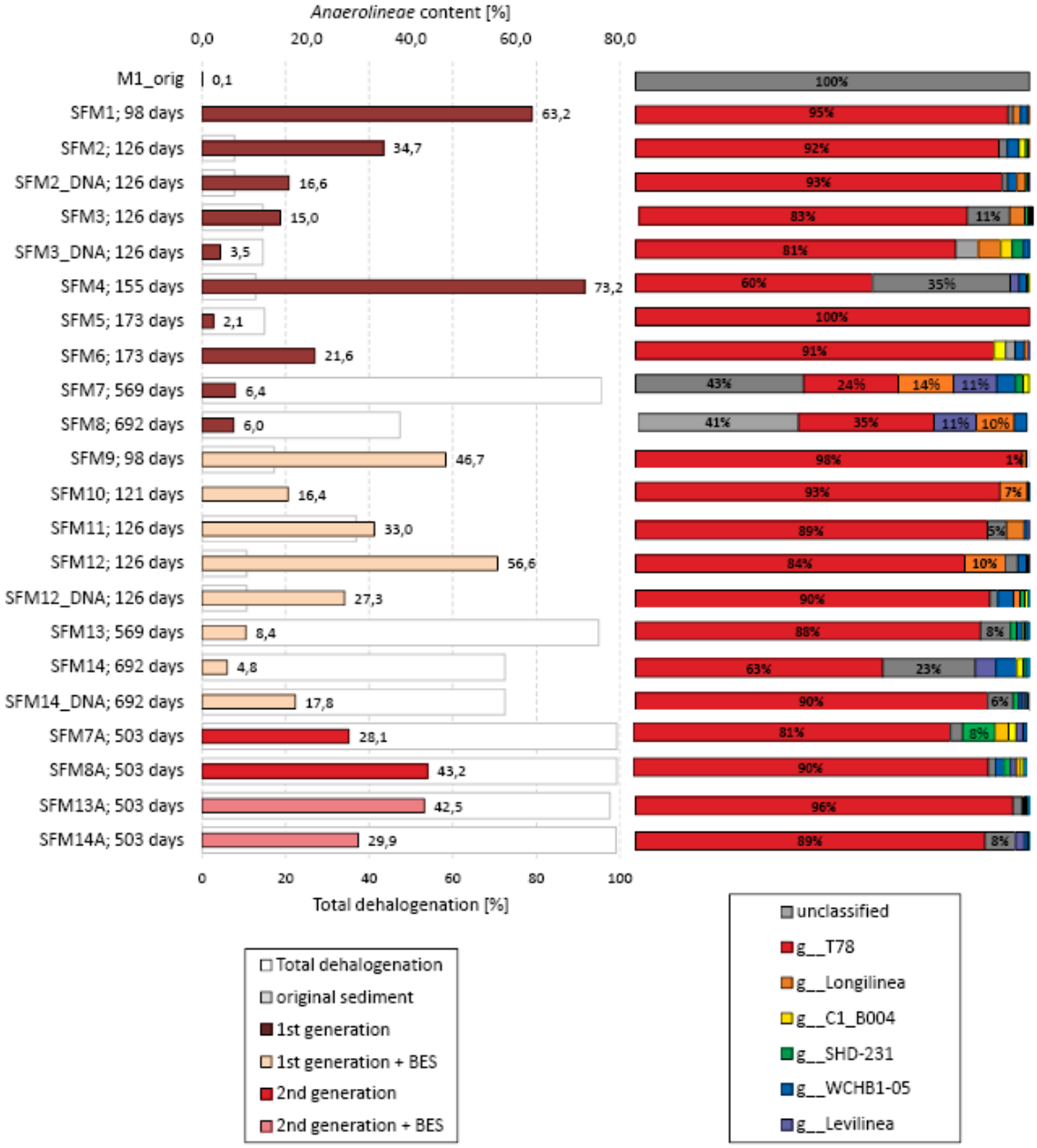

Figure 26. Relative proportions of the class *Anaerolineae* among all detected classes in the microcosms with their respective time of cultivation (right graph). Representation of genera within the class *Anaerolineae* (left graphs).

In general, bacterial consortia in long-term microcosms (569 and 692 days) revealed considerably lower amount of *Anaerolineae* members (ca. 6 % only). Interestingly, the content of *Anaerolineae* in the original sediment DNA was very low (0.1 %) and was mainly composed of the unclassified *Anaerolineae*. Contrarywise, the content of *Anaerolineae* in long-term second generation SFMs was reaching up to 43.2 % (SFM7A). In the deep analysis, the community of the *Anaerolineae* was created only by the members of the family *Anaerolineaceae*. The main portion of the *Anaerolineaceae* was created by the uncultivable genus *T78* (up to 98 % in SFM9). Significant change in the microbial pattern was detected in the late first generation SFMs without BES – proportion of the microbial community was shifted to 41-43% of unclassified bacteria, 24-35 % of *T78*, 10-14 % of *Longilinea* and 11 % of *Levilinea*, respectively (Fig. 26).

In terms of class *Clostridia* the impact of BES was observed in the first generation of SFMs. The prevalent genus was either *Syntrophomonas* (SFMs 2, 5, 6, 7, 8, 12, 13 and 14) or *Desulfosporosinus* (SFM3, 9 and 10) (Fig. 27). Interestingly, bacteria from genus *Clostridium* were present in significant amounts only in earlier microcosms (up to 126 days).

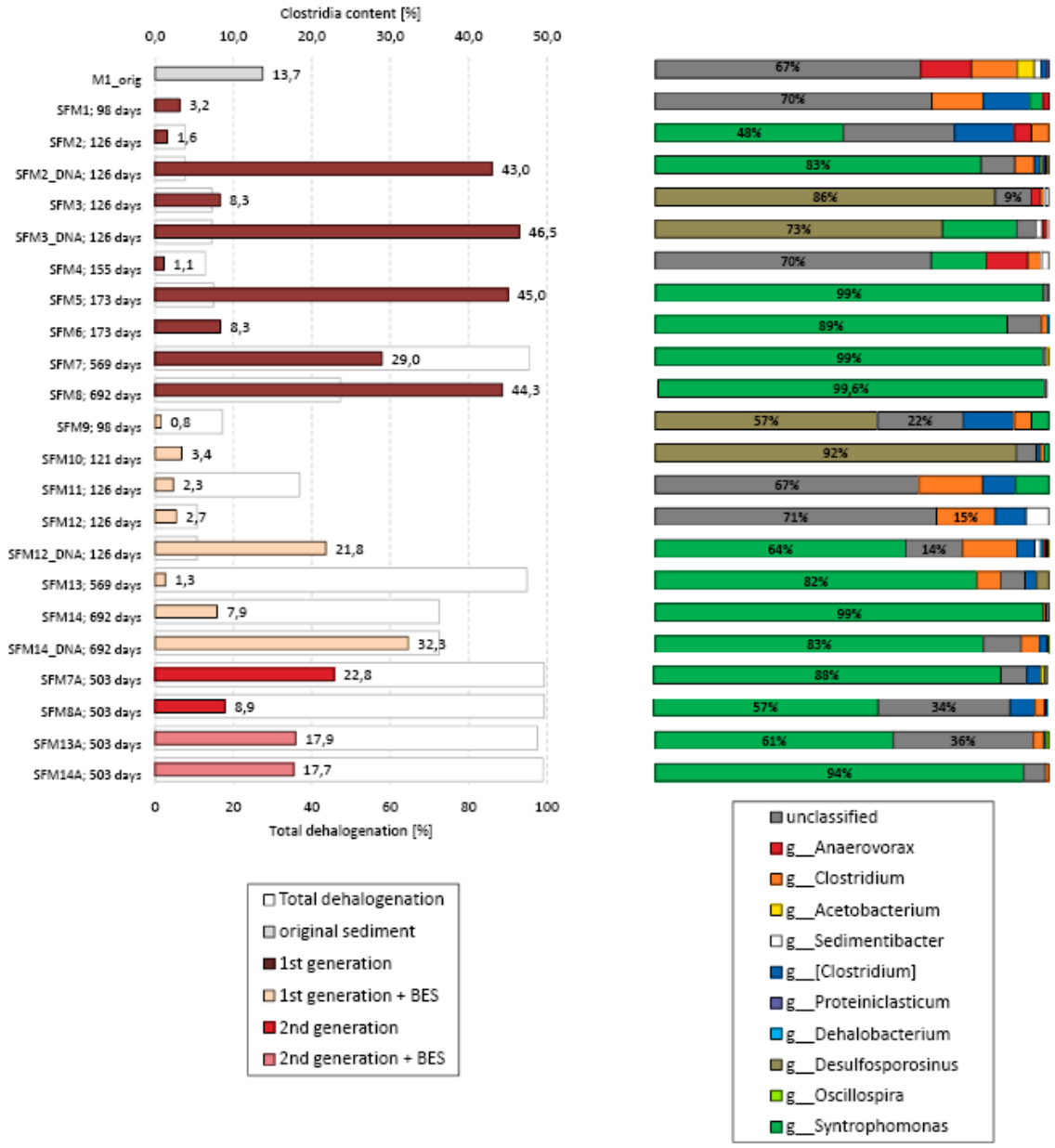

Figure 27. Relative proportions of the *Clostridia* among all detected classes in the microcosms with their respective time of cultivation (right graph). Representation of genera within the class *Clostridia* (left graphs).

Class δ-*Proteobacteria* showed a dependency on the presence of BES, significant amount of this class was found in the long-term first generation microcosms (up to 84.2 % in SFM13 and up to 68.8 % in SFM14). Composition of the genuses within class δ-*Proteobacteria* was heterogeneous with the prevalence of genus *Syntrophus* in the first generation microcosms without BES (Fig. 28).

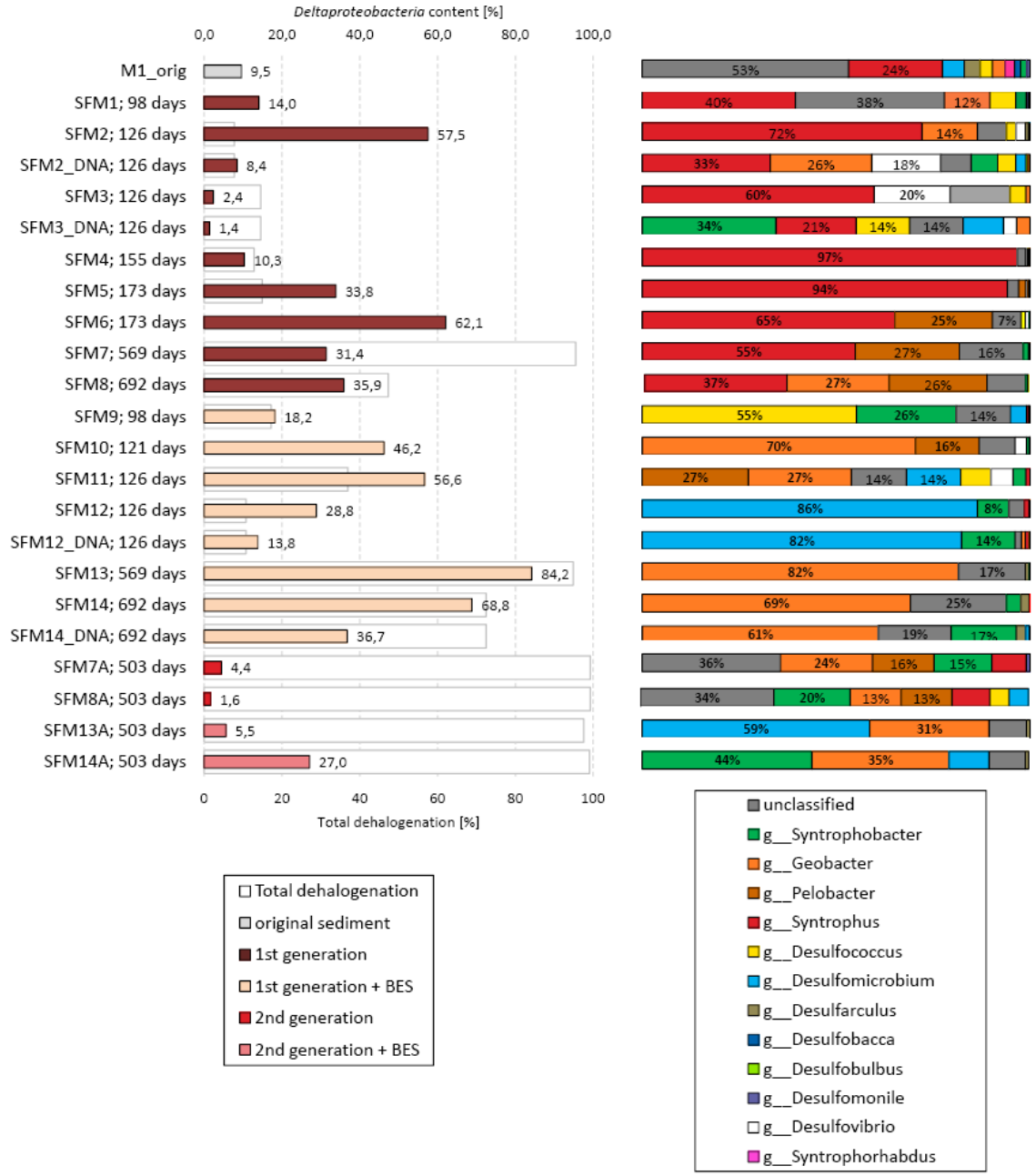

Figure 28. Relative proportions of the δ-*Proteobacteria* among all detected classes in the microcosms with their respective time of cultivation (right graph). Representation of genera within the class δ-*Proteobacteria* (left graphs).

Genuses *Geobacter and Desulfomicrobium* were dominant in microcosms in which BES was added. High occurrence of the genus *Geobacter* (family *Geobacteraceae*) in the first generation microcosms SFM9 – 14 and second generation SFM13A and SFM14A suggested the important role of these bacteria in the community function when typical OHR members were suppressed. The occurrence of the genus *Desulfomicrobium* (family *Desulfomicrobiaceae* within the class δ-*Proteobacteria*) was confirmed for microcosm SFM12 (creating ca. 25 % of the whole community) and SFM11 (showing ca. 8 % of the whole community).

By contrast, syntrophic bacteria of the genus *Syntrophus* (family *Syntrophobacteraceae*) were found in the major proportion in microcosms without BES and also in original DNA from sediment showing the effect of BES compound. As well the members of families *Pelobateraceae*, *Desulfomicrobiaceae*, *Desulfovibrionaceae* and *Desulfobacteraceae* were proved to occur in significant portion in the microcosms with BES addition (Fig. 28).

Interestingly, analysis done on the NGS data and congener degradation data set showed that there is a strong impact of the class *Geobacteraceae* and unclassified *Desulfuromonadales* on the degradation of 5-CB. (Tab. 17). This correlation was not showed in the no-BES samples (data not shown).

Table 17. Pearson correlations computed between bacteria families and congener classes measured in the BES-SFMs. Significant correlations (p<0.01*) are indicated with black bordering.

	2 Cl	3 Cl	4 Cl	5 Cl	6 Cl	7 Cl
Unclass. Geobacteraceae	-0.140	-0.224	-0.455	-0.929	-0.437	-0.195
Unclass. Desulfuromonadales	-0.491	-0.476	-0.573	-0.729	-0.111	0.118
Unclass. Delta-Proteobacteria	-0.458	-0.634	-0.763	-0.714	0.108	0.358
Syntrophomonas	-0.373	-0.674	-0.820	-0.625	0.257	0.502
Unclass. Anaerolinaceae	-0.654	-0.574	-0.276	0.494	0.814	0.730
Kosmotoga	0.077	-0.007	-0.025	0.170	0.167	0.182
Desulfovibrio	-0.069	0.376	0.532	0.328	-0.129	-0.232
Pelobacter	-0.035	0.399	0.545	0.324	-0.145	-0.248
Desulfomicrobium	-0.439	-0.401	-0.103	0.647	0.756	0.604
Syntrophobacter	-0.490	-0.337	-0.052	0.669	0.724	0.627
Desulfococcus	-0.045	0.450	0.664	0.615	-0.074	-0.230
Unclass. Aminiphilaceae	0.987	0.707	0.463	0.048	-0.377	-0.430
T78	-0.290	-0.041	0.317	0.947	0.618	0.382
Unclass. Bacteria	0.605	0.529	0.529	0.559	0.121	-0.031

Also, the neighbour-joining phylogenetic tree constructed from randomly selected 50 out of ca. 6000 sequences affiliated with the family *Geobacteraceae* showed only 91 – 98 % similarity with known *Geobacteraceae* members. All sequences formed a distant and tight cluster, named "Strazske cluster" hereafter (Fig. 29). The same procedure of the contruction of the phylogenetic tree based on the *Geobacteraceae* sequences from second generation SFMs was applied with the same result (data not shown).

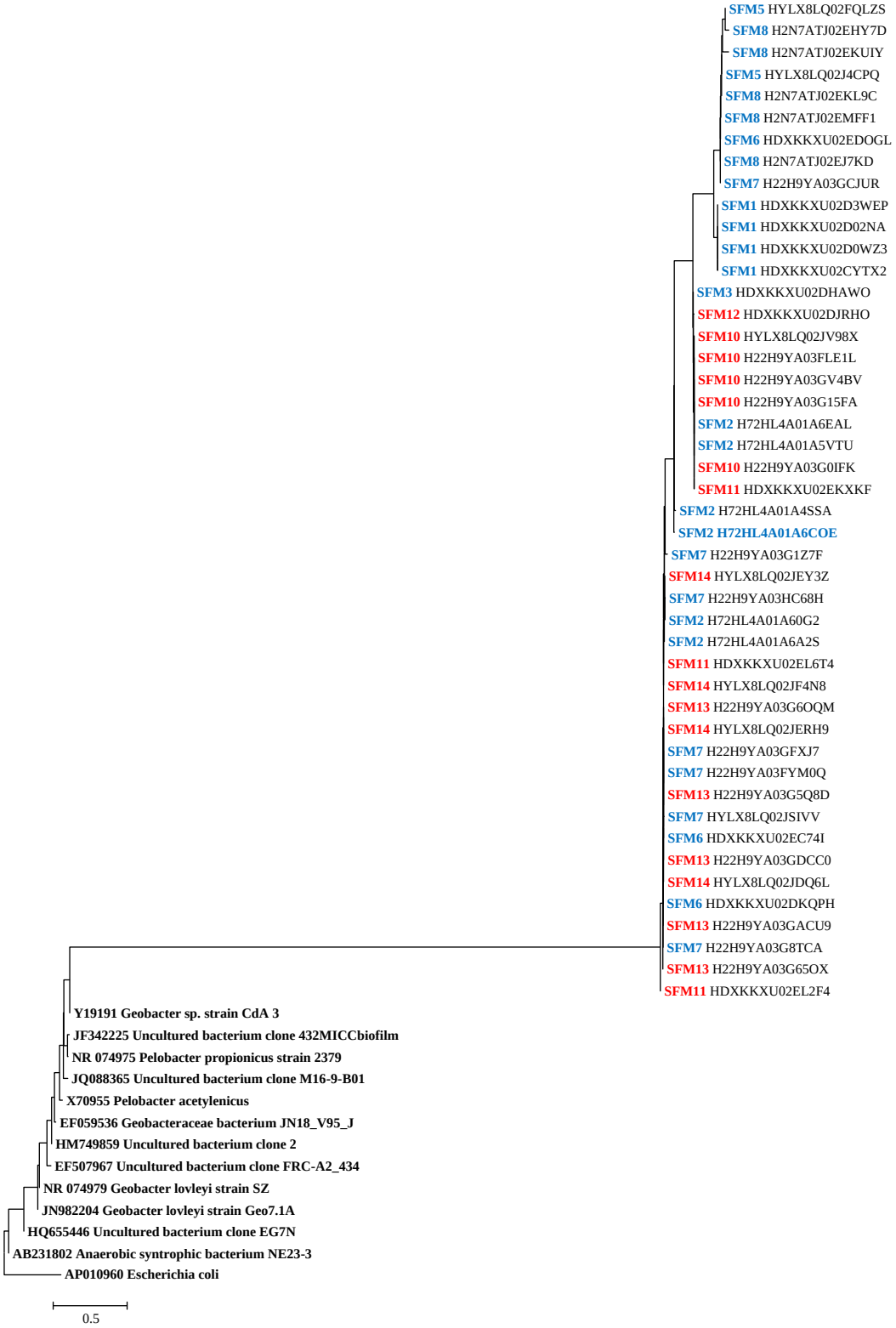

Figure 29. Neighbour-joining phylogenetic tree (Maximum composite likehood) of sequences affiliated with the family *Geobacteraceae* showing sequences randomly selected among more than 6000 sequences obtained from all first generation SFMs (red: BES-treated SFMs; blue: no BES).

The high occurrence of class *Actinobacteria* was quite surprising in the second generation of the sediment-free microcosms. These bacteria were found in the amount >1 % (except of DNA samples SFM12 and SFM14) in first generation of SFMs (Fig. 30), neither in original sediment microcosm M1.

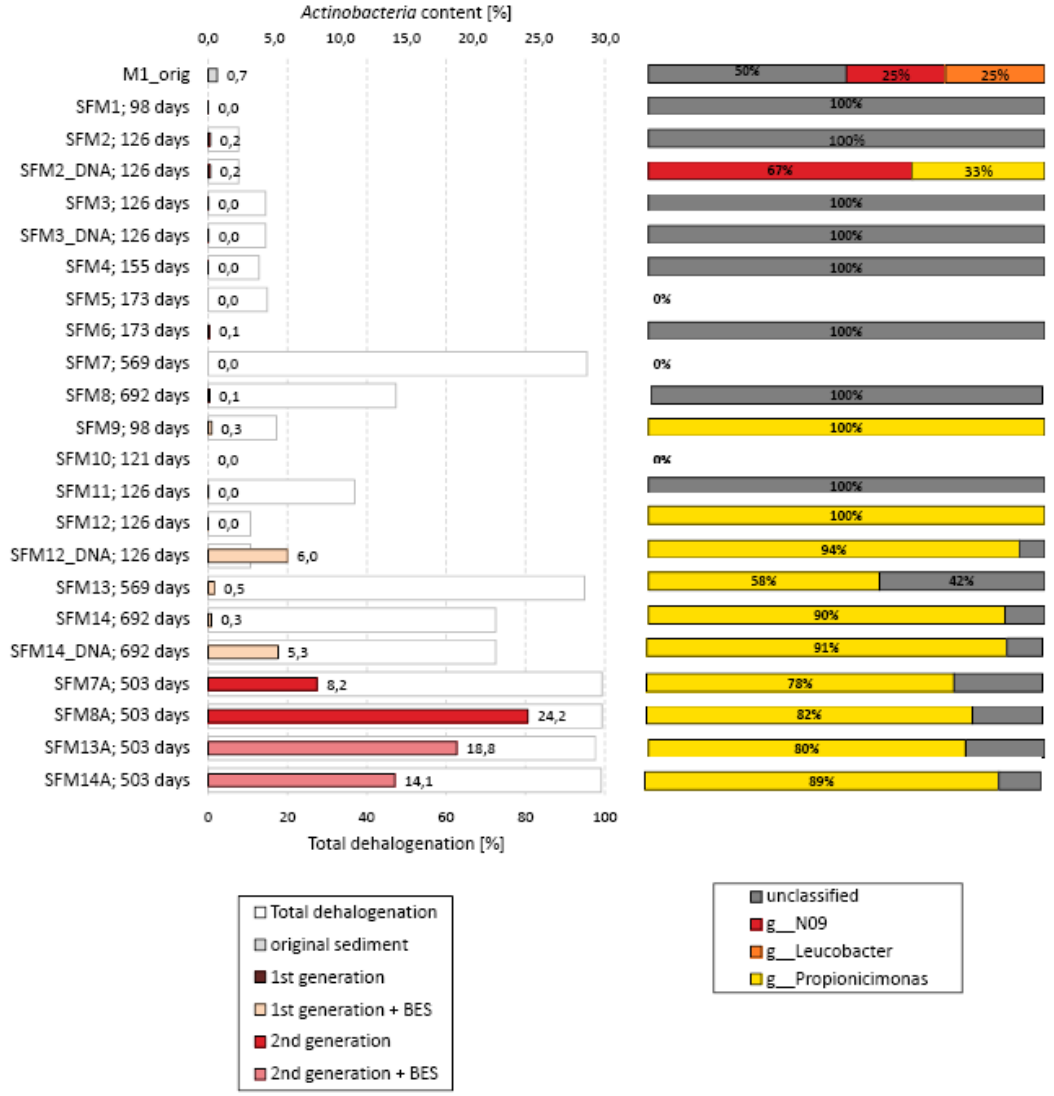

Figure 30. Relative proportions of the *Actinobacteria* among all detected classes in the microcosms with their respective time of cultivation (right graph). Representation of genera within the class *Actinobacteria* (left graphs).

Other important classes creating up to 15 % of bacterial classes (*Synergistia*, *Thermotogae*, *Cloacamonae* and *Bacteroidia*) showed no correlation with presence of BES (data not shown).

4.3.5. Archeal community profiling analysis

Deep phylogenetical analysis was applied also for uncovering of archaeal diversity present in sediment-free microcosms. Four samples (SFM3, 5, 11 and 12) were examined on the presence of *Archaea* using NGS. Interestingly, addition of BES had a significant inhibition effect on the methanogens population and *Archaea*. However, the impact of BES on the methanogenic *Archaea* was not absolute. We couldn't reach the complete removal of *Archaea* from microcosms where BES was added, which was probably caused by the combination of factors including a too low initial concentration and the degradation and dehalogenation of the molecule. Measurement of the gas phase revealed the presence of ethene after 60 days of continuous cultivation (data not shown), which could most probably came from the degradation of BES by methanogenic *Archaea* (Holliger et al., 1992). Data from NGS showed that *Archaea* were present in BES-treated microcosms in 10-times less amount in comparison with no BES microcosms.

Among archaeal population, prevalence of phyla *Euryarchaeota* was detected in all microcosms. Interestingly, in microcosms without BES were detected almost exclusively members of *Euryarchaeota* (99.8 % in SFM5 and 100 % in SFM3), while in microcosms with addition of BES also *Crenarchaeota* were occurring (3.0 % in SFM11 and 10 % in SFM12). Three main classes within *Euryarchaeota* were found: *Methanomicrobia*, *Methanobacteria* and *Thermoplasmata*. Major portion of *Euryarchaeota* was created by *Methanomicrobia* (over 74.2 %) represented by the genus *Methanosaeta* (Fig. 31).

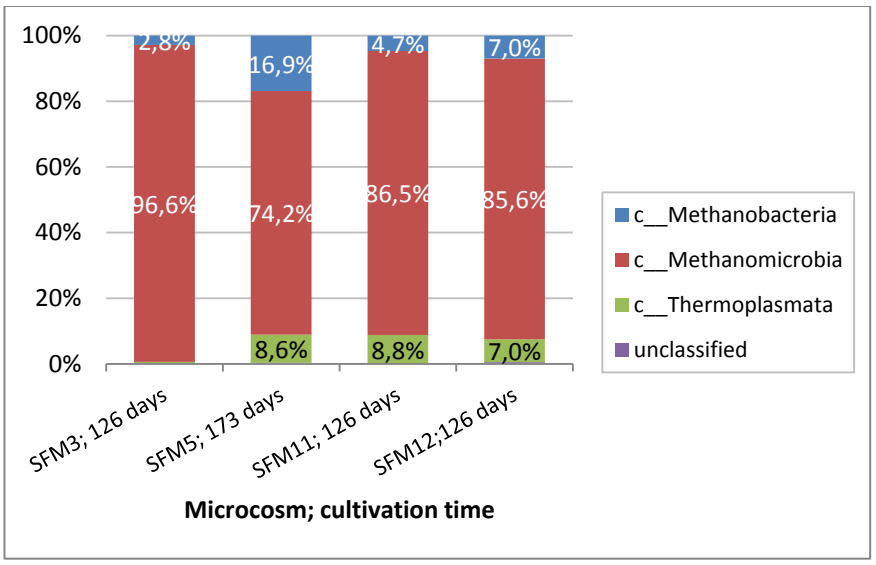

Figure 31. Composition of archaeal *Euryarchaeota* community for four selected SFMs.

5. Discussion

The main objective of this work was to explore both the phylogenetic diversity and degradation capacity of anaerobic microbial communities thriving in highly PCB-polluted sediments. As a target locality the efflux channel of the former major supplier of PCBs for Eastern communist bloc - the Chemko Strazske factory, situated in the Slovak Republic, was chosen. This study provides the first insight into the microbial complexity of the PCB-transforming organohalide-respiring bacteria in the region of Eastern Europe highly contaminated with weathered PCB congeners.

The Chemko Strazske manufactured ca. 60 % of the whole production in Eastern Europe (Taniyasu et al., 2003) corresponding to ca. 21482 tons of PCBs between 1959 and 1984 under several brand names such as “Delor”, “Hydelor”, and “Delotherm” (Dercová et al., 2008). An estimated amount of 1600 tons of PCB waste under the form of distillation residues was equally produced and a large fraction of this waste was released into the environment via the Strazske efflux channel (Kočan, 2001). Contaminants accumulated in the sediments of the efflux channel were further released in the Laborec River and downstream in the Zemplinska Sirava water reservoir – a popular recreating area in Eastern Slovakia (Kočan, 2001). Chemical residues were detected principally in the immediate vicinity of the manufacturer´s landfill and storage sites. The Chemko Strazske factory waste was composed mainly of highly chlorinated biphenyls, terphenyls and polychlorinated dibenzofurans. Thus, the Zemplin area located downstream is nowadays still highly contaminated, posing a serious environmental problem. Concentration of PCBs present in the lipid tissues of the water organisms in the Zemplinska Sirava water reservoir was found to be alarming - up to 375 ppm in predator fishes (Kočan, 2001)

As shown by several research groups (Domínguez, 2008; Sobolev and Begonia, 2008), long-term presence of chemicals (various chemical pollutants and heavy metals) in the environment leads to the shift in the composition of the microbial communities. For instance, presence of heavy metals imposes strong pressure to the autochthonous organisms in regard to the tolerance to higher concentrations (Lima de Silva et al., 2012). Changes in the cytoplasmic membrane and gene expression in presence of PCBs were showed by Murínová et al. (Murínová and Dercová, 2014).

Several studies were conducted on Strazske polluted locality with purpose to monitor the PCB contamination or to assess the pollution impact on human health (Langer et al., 2007; Wimmerová et al., 2015). Dudková et al. focused their investigation on the chemical aspect of PCB degradation within Strazske locality by use of sediment-free cultures amended with Aroclor 1242 (a PCB mixture similar to Delor 103). Their study showed that chlorines were removed from flanked *meta*- and *para*- positions of the PCB congeners in nearly equal proportions. No *ortho*- dechlorination was observed and haloprimers did not influence the specificity of dechlorination. Thus, the congener degradation pattern observed in Strazske did not match to any known dechlorination processes (Dudková et al., 2012). Little is known about the OHR bacteria involved in the dechlorination of the PCBs in Strazske channel sediment. Since microbial communities are a fundamental part of the water ecosystem and play a significant role in the PCB detoxification, understanding of their functions is a priority task, supporting the remediation of PCBs in the nature. In our study we aimed toward collecting missing information regarding the identification of the taxonomic structure of the active microbial guilds during anaerobic dechlorination of PCBs in sediments from Strazske locality.

The strategy of our research was based on examining of three sequential microcosm cultivations. The first cultivation set was composed of eight sediment-based microcosms where using of original sediments taken along the efflux channel were used. Both chemical composition and bacterial communities were characterized. The second microcosm set was represented by fourteen sediment-free cultures issued from one sediment microcosm (M1). And, the third microcosm set - four second-generation of sediment-free microcosms were based on the inoculum of sediment-free microcosms of the first generation. Both generations of sediment-free microcosms were supplemented with Delor and used for deep examination of the structure and temporal evolution of organohalide-respiring bacterial communities without any influence caused by the presence of sediment.

All microcosms' experiments were run using a complex cultivation medium. Since the organohalide respiration is thought to be a complex biological process and bacterial species involved in this process originate presumably from a very diverse phylogenetic horizon, the need of growth requirements could be very different. So as to satisfy the best possible support of the majority of bacterial spectra, the complex growing media developed by Holliger et al. (1998) proved to be effective for many organohalide

respiring bacteria was used (Holliger et al., 1998). Furthermore, this medium provided also a way to standardize the habitat of the consortia in the micrococosms which were initially inoculated using eight different river sediment samples. Electron donors (ethanol, propionate, butyrate and acetate) were injected on a regular basis so as to sustain correct development of bacterial communities due to the active interspecies H_2 transfer (Achtnich et al., 1995; Heimann et al., 2006). Additionally, vitamin B12 present in the cultivation medium helped to build-up a corrinoid co-factor, an essential part of the Fe-S active site of the reductive dehalogenase enzymes (Rupakula et al., 2015).

The design of the sediment microcosms differed from the conventional anaerobic culturing techniques classically applied to the dechlorination of PCBs and dioxins. Cultivation was conducted in 10 to 60 times higher volume than reported in the literature (Bedard et al., 2006, 2007a; D'Angelo and Nunez, 2010; Watts et al., 2005; Wu et al., 1998; Zanaroli et al., 2010). This volume allowed for multiple samplings of total DNA and RNA and could equilibrate the possible impact of the elimination of chloride ions during the OHR process, as pH has been shown to be of critical importance (Lacroix et al., 2014).

Sediment-free microcosms were used for the analyses of the degradation patterns and diversity of developed OHR bacterial guild members (Bedard et al., 2007b; Dudkova et al., 2012; Fagervold et al., 2007b; Yan et al., 2006a). The volume of these microcosms was kept at 200 ml due to the fact that only 300mg of solid silica particles were used. The format of sediment-free microcosms was developed and used for the first time by Cutter et al. (Cutter et al., 1998). Later, Bedard et al. improved this cultivation technique by introducing inert fine silica particles to the mineral medium so as to provide a large surface area for PCBs adsorption and desorption (Bedard et al., 2006, 2007). The advantages of such cultivation format are multiple. They include the standardization of the cultures and avoiding the undesired organic components of the sediment (Bedard et al., 2006, 2007). The reported study demonstrates sediment-free set-ups, which advantage is based on the development of stable dechlorinating consortia without any need of consecutive culture transfers (Bedard et al., 2006). Some of the sediment-free studies were shown to face problem with sustained PCB-dechlorinating activities due to the lack of the sediment supplying specific nutrients important for the growth of the PCB dechlorinators. The complex medium used in our study helped overcoming the above mentioned nutrient problems and allowed bacteria to grow in the environment of the sediment-free silica based culture.

The first chemical analyses of the sediments sampled in Strazske locality confirmed that high levels of PCBs were still present in the efflux channel. The concentration values ranged from 6.8 to 136 mg/kg. Thus, the PCB concentration was comparable with those found in the sediments in 1997/1998 by the Kocan et al. (Kočan, 2001). Highly chlorinated PCB congeners (with more than 6 chlorines) were significantly lower than the medium/low-chlorinated molecules. This observation could be explained by the fact that only 20 % of the total Chemko Strazske production was mainly based on the highly chlorinated PCB mixture Delor 106 (Holoubek, 2000). In addition, different content of the PCBs within the sediment despite expected values based on the composition of Delor 103 and 106 could be interpreted as a result of the natural degradation increasing the fraction of weathered congeners. Additionally, high amounts of heavy metals were also found in the sediments as a consequence of other chemical activities of Chemko Strazske after the ban of PCB production. These activities included production of explosives and various by-products for the army and civilian sectors (http://www.chemko.sk/index.php?cms=en). Interestingly, in our study, no correlation of heavy metals with composition of bacterial communities was found using the redundancy analysis of the T-RFLP data set and environmental factors (Fig. 15). This observation can be consequence of the long-term effect of heavy metals and adaptability of the bacterial communities (Sobolev and Begonia, 2008). The efflux channel is nowadays also used as collecting channel for sludge and other waste from the households in the Strazske city (as shown by the presence of an old television device and other exogenous objects in the river bed at the time of sampling). Grain size distribution of the sediment samples revealed that the structure of the geological habitat varied greatly among all sampling locations, although all samples were predominantly composed of fine sand (0.125–0.063 mm in diameter) (Fig. 12). Due to the hydrophobic nature, organic contaminants are more strongly adsorbed on fine sediment particles (Mehler et al., 2010). This adsorption mechanism was shown to have a strong impact on the bioavailability of the PCB congeners and consequently on the composition of the bacterial communities linked to sediment particle sizes (Jackson and Weeks, 2008; Santmire and Leff, 2007).

In the present study, the degradation potential and the composition of the microbial communities were initially examined using sediment microcosms. The results of dechlorination of the weathered PCB congeners after 10 months of continuous cultivation showed large discrepancies among all microcosms in terms of total amounts

of congener removal. The total PCB congener elimination ranged from a 5.11 % (M6) to about 35.9 % (S3) (Fig. 14). These numbers were equal or slightly lower than the degradation rates found in other studies, although the latter tended to focus on a restraint variety of congeners (Bedard et al., 1996; Fagervold et al., 2007). Interestingly, despite the fact that the sediment samples were taken from the same water channel, bacterial consortia handled the weathered PCB congeners in very different ways. Only three microcosms (M1, S2 and S3) showed a high dehalogenation capacity of the highly chlorinated PCB congeners (Fig. 14). M1 was able to remove highly chlorinated congeners, but accumulating 4-CBs. On the other hand, S2 was the only microcosm able to completely eliminate 7-CB showing also an unusual strong capacity in the removal of all PCB congener groups. S3-consortium eliminated highly-chlorinated congeners, but was not able to handle low-chlorinated ones to a large extend. Complete removal of PCBs often requires cooperation of various bacteria and all of them may have different preferences on their living environment (Borja et al., 2005). PCB-contaminated sediments were shown to host bacteria affiliated to several phylotypes, such as β-, δ-, and γ-*Proteobacteria*, as well as members of the *Clostridiales*, *Bacteroidetes,* low G+C Gram positive bacteria and *Chloroflexi* (Bedard et al., 2006, 2007; Yan et al., 2006). In this study, the structures of the whole bacterial communities present in the slurry microcosms were studied in time by T-RFLP analysis. The original sediment samples showed regularity in their structures, which could be related to the relative small distances between the sampling locations along the water channel (Fig. 9). However, after six months of continuous cultivation, shift in the bacterial community structures was detected, even though the cultivation conditions were unified. Similar situation was already described by Bedard et al. showing that even triplicate slurry microcosms could evolve differently and could develop different PCB dehalogenating capacities (Bedard et al., 2005). Bacterial strains known already for their capacity to reduce chlorinated compounds were detected in cDNA samples extracted from all microcosms (Tab. 11). The results confirmed the presence of "*Dehalococcoides*" *sp.* and *Dehalobacter sp.*, which are both known as obligate OHR bacteria. Known non-obligate OHR were detected equally, such as *Desulfitobacterium sp.*, *Desulfuromonas sp.*, *Geobacter lovleyi* and *Sulfurospirillum multivorans.* Thus, two samples (namely M4 and S3) were selected for the phylogenetic analysis based on their capacity to degrade either highly-chlorinated PCBs (sample S3) or low-chlorinated ones (sample M4) (Fig. 17, 18). Specifically, two groups of bacteria (class *Chloroflexi* and

"*Dehalococcoides*" sp. genus) containing bacterial strains supposedly involved in PCB dehalogenation were targeted. Early findings published by Watts et al. presented the phylum *Chloroflexi* as a minor contributor in natural habitats (Watts et al., 2005). Nowadays, increasing amounts of metagenomic studies demonstrated that members of this phylum contributed in a large extend to a high number of anaerobic microbial habitats (Krzmarzick et al., 2011). However, and despite their abundance, knowledge about *Chloroflexi* physiology is scarce. In our study, the both M4 and S3 bacterial communities displayed completely different bacterial structures. The diversity detected in the microcosm M4 was nearly two-times richer than in the microcosm S3 in terms of clone sequences (Fig. 17, 18). However, traditional obligate OHR bacteria closely related to "*Dehalococcoides"* sp. subgroup Pinellas were detected in microcosm S3 only, representing 2 % of all clone sequences (Fig. 18). These findings corresponded to the dehalogenation pattern where S3 microcosm handled highly-chlorinated congeners and M4 middle-chlorinated ones. A single cluster composed of five sequences was isolated from both microcosms. The related sequences demonstrated a low level of similarity with clone sequences isolated from PCB-contaminated sediment slurries (Ho and Liu 2008, unpublished data). *Dehalococcoides*-like sequences, isolated from microcosm M4, clustered in two main homogenous groups, sharing a high degree of similarity with clone series isolated from marine sediments (Baltimore Harbour) from which PCB dechlorinators *Dehalobium chlorocoercia* strain DF-1 and clone *o*-17 were isolated (Watts et al., 2005). A third cluster was related to clones isolated from CE-contaminated ethane aquifers (Kleikemper et al. 2005, unpublished data) and clones found in oil sand tailings (Siddique et al., 2011). The bacterial communities in these localities were capable of utilizing long chains of *n*-alkanes under methanogenic conditions (Fig. 18).

Interestingly, clones belonging to *Chloroflexi* - Subphylum I from the genera *Levilinea*, *Bellilinea*, *Leptolinea*, *Longilinea* and *Anaerolinea* (Yamada et al., 2007; Yamada and Sekiguchi, 2009) were found in significant number making up to 61 % and 34 % of all clones in S3 and M4, respectively. Slowly growing filamentous bacteria belonging to the *Chloroflexi* - Subphylum I are known already to participate in the production of H_2 as the end product of the fermentation of saccharides and peptides (Yamada and Sekiguchi, 2009). The high prevalence of clones belonging to this subphylum strongly suggested the role played by these members in the PCB degradation process, possibly fuelling the OHR bacteria with hydrogen molecules (Watts et al., 2005). Since bacteria

living in mixed cultures are creating invisible net of partners, the inter-population relations are over the importance of the each bacterial taxon alone. It was already described that OHR bacteria live in syntrophic interaction with hydrogen-producing populations (Smidt and de Vos, 2004). The interdependence between OHRB and their syntrophic partners has an impact on the dehalogenation process (Becker et al., 2005). Favoring OHR microorganisms over other hydrogenotrophic populations involves release of H_2 at partial pressures of $10^{-3.5}$ atm or less (Becker et al., 2005).

Main enzymes driving OHR processes in *Dehalococcoides* bacteria are the so-called reductive dehalogenases. The presence or absence of reductive dehalogenase operons reflects the main difference in gene content among the *Dehalococcoides* strains (Löffler et al., 2013). The functional diversity of the reductive dehalogenase gene family has still not been explored sufficiently (Maphosa et al., 2012). Reductive dehalogenase genes directly involved in the PCB dechlorination have been reported for first time in 2014. Wang et al. characterized three new *Dehalococcoides* strains CG1, CG4 and CG5 revealing 35, 15 and 26 *rdh*A genes. They showed that the PCB dechlorination was driven by common RDases showing specificity also for PCE (Wang et al., 2014).

Presence/absence of *bvcA* and *vcrA* genes for vinyl chloride reductases was targeted in the microcosms by using PCR (Behrens et al., 2008). Notably, no *bvcA*, which is specific for *D. mccartyi* strain BAV1, was found in this study in both S3 and M4 microcosms. On the other hand, *vcrA*-type genes reported for *D. mccartyi* strain VS were detected in microcosms M2, M3, M6, S2 and S3 (Tab. 11). Sequence analysis revealed the closest similarity with the *vcrA* gene present in *D. mccartyi* strain BTF08 (Pöritz et al., 2013).

For the purpose of a detail study of the microbial communities' structure and identification of bacterial species involved in the dehalogenation of the PCB congeners, sediment-free cultures with one sequential transfer were set up. Suspension originating from sediment microcosm M1 was used as an inoculum for fourteen first generation sediment-free cultures. Microcosm M1 was containing sediment taken in closest location to the Chemko Strazske factory (Fig. 9) and revealed dechlorination capacity for highly-chlorinated PCB congeners. Results of Dudková et al. analysis indicated that also other bacterial species aside to known OHR bacteria could be involved in the PCB dehalogenation at the Strazske locality (Dudková et al., 2012). For that reason, part of the microcosms was treated by 2-Bromoethanesulfonate (BES), a structural analog and competitive inhibitor of coenzyme M (CoM) of the methanogens. BES was proved

as an efficient inhibitor of methanogenesis (Zhou et al., 2011). Methanogens were described to be directly or indirectly involved in the organohalide respiration processes (Löffler et al., 1997). Interestingly, dechlorination rates and patterns were observed in all microcosms without any influence of BES suggesting the role of other microbial members, which are not strongly dependent on the interspecies H_2 transfer. All microcosms depleted mostly 5-CB and 6-CB congeners. And, as a result, the long-term maintained SFMs showed high rates of congener removal in total – up to 95 % in SFM7 and SFM13 (Tab. 13).

In addition, the second generation sediment-free microcosms issued from the first SFMs revealed more than 99 % of the total PCB removal after 503 days of cultivation in all monitored microcosms.

The phylogenetic data, obtained by using bioinformatic tools from next-generation sequencing of cDNA and DNA, proved the presence of several bacterial classes representing all the parts of the microbial interaction network required for OHR. Certain bacterial classes were found to be abundant in our sediment-free microcosms – *Anaerolineae*, *Clostridia*, *Dehalococcoidia*, *Synergistia*, *Actinobacteria*, δ-*Proteobacteria*, *Bacteroidia*, *Cloacamonae* and *Thermotogae*. All the bacterial guilds - primary and secondary fermenters as well as acetogens and dechlorinating bacteria – were found as a whole bacterial apparatus for successful cultivation and function of the community. Since the environment of the sediment-free microcosm is restricted to a few carbon and energy sources, it was possible to link the observed bacterial species to the specific function within the bacterial community.

Dehalococcoides were described as main actors in anaerobic organohalide respiration process (Bedard, 2008; Bedard et al., 2006; Wang and He, 2013). In this project, NGS analysis showed that there was a significant effect on the *Dehalococcoidia* group in microcosms with and without BES addition. In the microcosms supplemented with BES, only two sequences affiliated to *Dehalococcoidia* were found. In the microcosms cultivated without BES, *Dehalococcoides* sequences were found. They were affiliated at 99 % similarity to *D. mccartyi* CG5 from Pinellas subgroup, which is closely related to strains CBDB1, GT and BAV1 (Wang et al., 2014) (Fig. 25). BES had no impact on the abundance of class *Anaerolineae* belonging to phylum *Chloroflexi*. Inside *Anaerolineae* prevalence of 16S rRNA gene sequences related to *T78*, a candidate genus with potential to degrade carbohydrates was detected (Sekiguchi et al., 2001). This observation was made also in the second generation of SFMs, where *Anaerolineae* were

highly enriched (accounting for up to 56 % of total community composition). In the long-term microcosms (569 and 692 days of continuous cultivation) sequences affiliated to genus *Levilinea*, a carbohydrates degrading taxa, were found as well (Yamada et al., 2006) (Fig. 26). Additionaly, in sediment microcosms this taxon comprised the majority of the *Chloroflexi* sequences (up to 91 %). Thus, the role of these bacteria in the microcosms can be explained as primary fermenters. Abundant sequences were affiliated with the class *Clostridia*. Clear prevalence of bacteria from genus *Syntrophomonas* was detected in the late microcosms both first and second generation (Fig. 27). The role of these bacteria is a syntrophic fatty acid-oxidation. Syntrophic partnership is required for their growth (McInerney et al., 1981). It was thought that *Clostridia* can play some role in the OHR process due to some putative reductive dehalogenase genes, however, until now, no OHR activity was proven. In general, role of genus *Clostridia* is fermentation of organic substrates to hydrogen and acetate. High transcription level in the microcosms only demonstrates the importance of non-*Dehalococcoides* bacteria in the community structure (Maphosa et al., 2012).

Interestingly, high occurrence (up to 80 % of total community composition) of the members of *Actinobacteria* was found in the second generation SFMs with prevalence of genus *Propionicimonas*, a propionate producing bacterium (Akasaka, 2003).

Heterogeneous and abundant group of bacteria present in the microcosms was affiliated to the class δ-*Proteobacteria*. These bacteria are known to be metabolically versatile in their metabolism from sulfate-reducers to organohalide respirers. Several genera are involved in the phenol or benzoates degradation in association with H_2-scavenging methanogenic partner (Qiu et al., 2008; Schöcke and Schink, 1999). Among δ-*Proteobacteria*, genus *Geobacter* was showed to use a broad range of electron acceptors including tetrachloroethene, trichloroethene, nitrate, fumarate, Fe(III), malate, Mn(IV), U(VI) and elemental sulfur (Sung et al., 2006a). It was proved that genus *Geobacter* is capable of *de novo* cobalamin synthesis – the essential component of reductive dehalogenases, catalyzing the OHR process (Hug, 2012). *Geobacter thiogenes* strain K and *Geobacter lovleyi* strain SZ are the unique representatives of the family *Geobacteraceae* capable of coupling the reduction of organohalides to energy conservation and growth. Additionally, significant number of sequences that were associated with the class *Geobacteraceae* formed the „Strazske cluster“, sharing only ca. 94 % similarity with *G.lovleyi* strain SZ (Fig. 29). Interestingly, presence of members of this cluster in the microcosms where BES was added or in microcosms in

the late cultivation time suggest the probability that when the *Dehalococcoidia* are not active, this taxon takes place and successfully dehalogenated PCB congeners. Since 5 % level difference of unknown and known sequences is a differentiating criteria at the genus level, and 10 % at the family/class level, we can assume that the newly identified phyla in this study belongs to *Geobacteraceae*, and formed a new *Geobacter*-like group sharing 81 – 98 % of similarity among its members inside the V3 hypervariable region (Schloss and Handelsman, 2004). So as to define new genus within *Geobacteraceae*, the longer sequences of 16S rRNA gene would be needed.

Taking in account the high metabolic plasticity of *Geobacteraceae*, we can expect faster functional gene adaptability compared to the divergency of 16S rRNA gene. This result proved the presence of unknown taxa related to *Geobacter*. Our data from the multifactorial analysis (MFA) and the statistically significant computations using pairwise Pearson correlation coefficients provides evidence about the highly significant correlation of the Strazske cluster with PCB removal (Tab. 17). The NGS data indicated the persisting presence of this Geobacteraceae-like taxon in most of the sediment-free microcosms of second generation SFMs (Fig. 28). Recently, a detailed analysis of the genome of *G. lovleyi* revealed that lateral gene acquisition was the source of the capability to use chlorinated compounds as electron acceptors (Wagner et al., 2012). Genes related to PCE reduction showed the highest homology with reductases present in the *Firmicutes* - *Dehalobacter restrictus* and *Desulfitobacterium hafniense*. Lateral gene acquisition of new respiratory capacities could have been the source of the dehalogenating activity. The acquisition of genes encoding reductive dehalogenases catalyzing chlorine removal from low chlorinated congeners could make this *Geobacteraceae* cluster a putative candidate for the dehalogenation of PCB congeners. This hypothesis is reinforced further by the absence of any other facultative OHRB in BES-treated SFMs, such as the genera *Dehalobacter, Desulfitobacterium* (Firmicutes) and *Sulfurospirillum* (ε-*Proteobacteria*).

Lateral acquisition of the functional reductive dehalogenase genes could lead to evolving of the new "Strazske" cluster belonging to *Geobacteraceae* as a new putative candidate for effective PCB removal. The horizontal gene transfer of reductive dehalogenase genes could serve as mechanism of adaptation of *Geobacter* in the PCB contaminated environment.

Additionally, archaeal community was screened. The impact of BES on the methanogenic *Archaea* was not absolute. We couldn't reach the complete removal of

these *Archaea*, possibly due to a combination of factors including a too low initial concentration and the degradation and dehalogenation of the molecule. Additionally, ethene was measured in the gas phase after 60 days of cultivation as a possible indicator of the degradation of BES by methanogenic *Archaea*, as shown by Holliger et al. (1992), ethene is a classical degradation product of BES.

Sequences targeting archaeal community showed presence of the aceticlastic methanogens *Methanosaeta* (Fig. 30). It was reported in the previous studies that the role of this genus is not significant in terms of the interspecies hydrogen transfer to the OHR bacteria (Heimann et al., 2006). However, in co-culture with *Geobacter*, *Methanosaeta* was proved to support the growth of *Geobacter* via direct interspecies electron transfer (DIET) process (Rotaru et al., 2015). Thus, it can be inferred that a similar relationship can occur in the sediment-free cultures from Strazske sediment.

6. Conclusion

This study provided the first insight into the complexity of the microbial communities at the highly PCB-contaminated region located in the Eastern Europe. The contribution of the work is based on the understanding of the role of anaerobic bacteria in PCB dechlorination process. Thus, the main goals were defined as i) the characterization of the microbial community structures present in the sediments of the Strazske efflux channel; ii) the development of stable sediment-free cultures enabling deep phylogenetic analysis of communities using NGS technology and the possible link to the dehalogenation patterns, and finally iii) the identification of new putative taxa belonging to the organohalide-respiring bacterial guild. The main results obtained in this work were as follows:

i) Construction of slurry microcosms with the subsequent nucleic acids extraction and its examination allowed the detection of widespread PCB-dehalorespiring microbial community and its dehalogenation potential in Strazske channel sediments. In addition, multivariate analysis showed both temporal and spatial heterogeneity in the distribution of the microbial populations

ii) Sediment and sediment-free cultures demonstrated the evolution of sustainable microbial communities showing efficient PCB dechlorination function even after first cultivation transfer from slurry microcosms. Interestingly, the second generation of the sediment-free microcosms revealed even better dehalogenation capacities.

iii) Direct and indirect evidence obtained from this study strongly suggested that besides the known organohalide-respiring bacteria, a new Geobacteraceae cluster, the "Strazske cluster" may be involved in PCBs dechlorination.

The results in the presented thesis should encourage further research for exploring the full potential of the indigenous PCB dechlorinating populations in Strazske. Former lines of research were abandoned by the late '90s, when organohalide-respiring bacteria from the *Chloroflexi* were found and studied in details. Among these lines, presence and study of bacteria from other taxa, such as the *Firmicutes* (*Dehalobacter*) and the *Desulfuromonadales*, were discontinued. Evidences shown in the present work

demonstrated that the diversity of organisms able to dechlorinate complex organic molecules is possibly far higher than expected. This has also a notable impact on the capacity of nature to remediate other forms of anthropomorphic halogenated molecules.

Since the anaerobic dechlorination is a prerequisite step for the complete mineralization of the PCBs by aerobic processes, it is a good future perspective to understand also this part of the ecosystem. Thus, more effort is needed to get better insight into the PCB-contaminated sediment ecosystems, which represent valuable sources of knowledge about important processes with a global impact. This goal could be reached using the metagenomics and metatranscriptomic approaches based on the modern technologies, such as NGS in conjunction with bioinformatics and statistical computation tools. The work done here contributes to a better understanding of these mechanisms and illustrates the microbial potential that remains to be discovered.

7. References

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