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Bioremediation of PCB-contaminated marine sediments:
From identification of indigenous dehalorespirers to enhancement of microbial reductive dechlorination

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Abstract

Marine sediments are the main accumulation reservoir of organic recalcitrant pollutants such as polychlorinated biphenyls (PCBs). In the anoxic conditions typical of these sediments, anaerobic bacteria of the phylum *Chloroflexi* are able to attack these compounds in a process called microbial reductive dechlorination. Such activity and members of this phylum were detected in PCB-impacted sediments of the Venice Lagoon. The aim of this work was to investigate microbial reductive dechlorination and design bioremediation approaches for sediments of the area. Three out of six sediment cultures from different sampling areas exhibited dechlorination activities in the same conditions of the site and two phylotypes (VLD-1 and VLD-2) were detected and correlated to this metabolism. Biostimulation was tested on enriched dechlorinating sediment cultures from the same site using five different electron donors, of which lactate was the best biostimulating agent; complementation of microbial and chemical dechlorination catalyzed by biogenic Pd^0 nanoparticles was not effective due to sulfide poisoning of the catalyst. A new biosurfactant-producing strain of *S. frigidimarina* was concomitantly obtained from hydrocarbon-degrading marine cultures and selected because of the low toxicity of its product. All these findings were then exploited to develop bioremediation lab-scale tests in shaken reactors and static microcosms on real sediments and water of the Venice lagoon, testing i) a bioaugmentation approach, with a selected enriched sediment culture from the same area, ii) a biostimulation approach with lactate as electron donor, iii) a bioavailability enhancement with the supplementation of the newly-discovered biosurfactant, and iv) all possible combination of the afore-mentioned approaches. The best bioremediation approach resulted to be a combination of bioaugmentation and bioremediation and it could be a starting point to design bioremediation process for actual sediments of the Venice Lagoon area.

Keywords

Polychlorinated biphenyls (PCBs), Microbial reductive dechlorination, Marine sediments, Venice Lagoon, *Dehalococcoidia*, dechlorinating microorganism, biostimulation, electron donors, Bio-Pd NPs, bioaugmentation, biosurfactants, bioremediation.

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1 Introduction

1.1 Polychlorinated biphenyls

1.1.1 Chemical features

Polychlorinated biphenyls (PCBs) are a family of chlorinated non-polar hydrocarbons having a biphenyl molecule, which may carry from 1 to 10 chlorine substitutions (Figure 1.1). The different position and number of chlorine substitutions of the biphenyl ring, generate up to 209 different molecules, called congeners, each indicated with numbers from 1 to 209 according to the classical nomenclature (see Table 9.1 in the Appendix section).

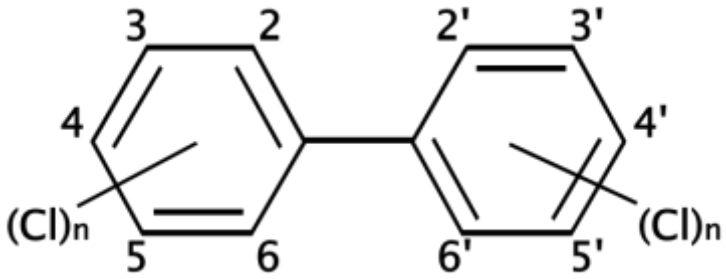

Figure 1.1: Molecular structure of PCBs. Chlorine substitutions may be in all position with $1 \leq n \leq 10$, leading to 209 possible combinations called congeners

Polychlorinated biphenyls do not exist as such in nature, and are artificially produced and sold either in solid, transparent crystalline form, or in commercial liquid mixtures, whose viscosity depends on the quantity of chlorine atoms. The chemical synthesis involves electrophilic chlorination using chlorine gas and $FeCl_3$ as Lewis acid catalyst: this procedure harbors mixtures of different congeners, which number of substitution depends on the time of reaction and on the quantity of chlorine gas used. The *degree of chlorination* indicates the average number of chlorine substitution per biphenyl ring, and in commercial mixtures it ranges between 21% and 68% w/w, usually indicated by the last two digits of the mixture brand (i.e.: Aroclor 1221, 1232, 1242, 1248, 1254, 1260, 1262 and 1268 had 21, 32, 42, 48, 54, 60, 62 and 68% weight of chlorine, respectively), with the exception of Aroclor 1016 which is a modified Aroclor 1242 mixture were penta- and hexachlorobiphenyls were removed (Ahlborg et al., 1992). Commercial mixtures were sold according to their physical properties, rather than chemical composition, containing also other contaminants such as polychlorinated dibenzofurans, polychlorinated naphtalenes and quarterphenyls (Voogt and Brinkman, 1989). Main characteristics of PCBs are: their very low water

solubility (0.0027 to 0.42 ng/l for Aroclor mixtures) (Fiedler, 1997) and vapor pressure (10^{-12} to 10^{-14} at 25 °C (atm)), both of which decrease as the degree of chlorination increases (Schwarzenbach et al., 2005); lypophilicity and melting point which increase with the increase of chlorination; the density range, between 1.182 and 1.566 kg/l, very high flash point (between 170 and 380 °C), high thermal stability, high thermal conductivity, low electric conductivity and non-explosives features (Fiedler, 1997).

1.1.2 Uses history

PCBs were first discovered and produced in Germany from 1881 to 1914, but the commercial production was taken over by the Monsanto Chemical Company in the U.S. in 1929 from Swann Chemical Company. Manufacturing level increased as with the need of safer, cooling and insulating fluid for transformers and capacitors, as well as stabilizing, flame-resistant additives of PVC electrical coatings. They were sold as complex mixtures carrying 60 to 90 congeners (Peters, 2001) with commercial brands known as Aroclor® in the US and the UK (Monsanto company), Clophen® in Germany (Bayer), Kanechlor® or Santotherm® in Japan (Kanegafuchi and Mitsubishi, respectively), Fenclor® in Italy (Caffaro/Monsanto).

PCBs were used as coolants and insulants fluids for transformers and capacitors, which represents more than half of all PCB uses, as well as plasticizers in paints and cements, stabilizant additives in PVC coatings of electrical components and wiring, pesticide extenders, cutting oils, lubricating oils, flame retardants, hydraulic fluids, sealants, adhesives, wood finishes, de-dusting agents, vacuum pump fluids, fixatives for microscopy, surgical implants and carbonless copy paper (UNEP, 1999).

Main application in industrial and home machineries can be subdivided, according to (UNEP, 1999), into:

- Closed applications, where there are hardly any leak into the environment of the dielectric fluids, such as alternators/transformers (77% of total)
- Partially closed applications, where leaks might occur frequently, such as dielectric fluid in hydraulic systems and radiators
- Open applications which lead to direct contact of the PCB with the environment, such as lubricants, pesticides, sealants etc.

The cumulative production until 1993 is estimated to be around 1.3 billion tons of PCBs, with a peak production in the 1970s (Breivik et al., 2007). PCBs were mainly emitted in the atmosphere during the utilization and production process, but other sources of emission include volatilization from reservoirs, from combustion and disposal of materials containing PCBs, as well as incorrect operations (Breivik et al., 2002a;b).

1.1.3 Environmental issue

Natural sources of PCBs include biological oxidation and abiotic processes, such as fire or volcanic eruptions (Gribble, 1994). However, illegal use and disposal, as well as accidental release during manufacture, transport and use, dispersed millions of kilograms of PCB into the environment, leading to persistent contamination of air, water, soil and food, representing the major source of contamination. In air, significant amount of PCB contamination is due to municipal, hazardous and medical waste incineration or combustion, as well as accidental leaks; in water, PCB concentrations are generally higher near the shoreline, where sediments act a reservoir which accumulates and slowly release PCBs in the upper phase; soil is another major source for this kind of contamination, as PCB characteristic lipophilicity is responsible for accumulation in organic matter (Borja et al., 2005); currently, another major source of PCB contamination is constituted by the e-waste landfills in poor countries, hosting tons of disposed electronic equipment containing PCBs (Wong et al., 2007). Redistribution cycle of PCBs involves volatilization from water into the atmosphere, transport in air and removal from the atmosphere via wet/dry deposition (Dobson and van Esch, 1993). Global distribution depends on the physical-chemical properties and on environmental parameters: transport goes along with phase change, such as partitioning to aerosols or absorption to organic matter in seawater (Wania and Daly, 2002). PCBs undergo multiple deposition and re-volatilization cycles (Wania and Mackay, 1993) and, under declining primary emissions, re-volatilization will soon gain major importance for PCB emission in the air.

Aquatic sediments still remain the final reservoir of the PCB cycle, as a condition of equilibrium between diffusion and adsorption is present; this condition, however, is prone for bioaccumulation: this process is defined as the increment of concentration in an organism compared to its surrounding environment from which it is transferred (Gobas and Morrison, 2000). This can happen through skin or respiratory surfaces (bioconcentration) or via ingestion (biomagnification); through the food chain, bioaccumulation might increase with each step, resulting in chemical concentrations higher in predators than in preys: since PCBs are lipophilic molecules, their concentration increases as the lipid content of the organism increases; particularly, the high-chlorinated congeners contribute more than the lower chlorinated congeners moving up the food chain (Borgå et al., 2001). This is due to the capability of higher organism to metabolize and/or secrete lower chlorinated congeners, rather than higher chlorinated ones, which are then transferred to higher level of the food chain(Oliver and Niimi, 1988). Further investigation on the mechanisms of bioaccumulation is needed, mostly regarding the differences of biomagnification factors between aquatic organisms and homeotherms (Kelly et al., 2004). Currently, it is also of relevance to understand the implication of global climate

change to forecast variation of impacts in organic contaminants (Manciocco et al., 2014): recent geochemical distribution models underline an increased volatilization emission and mobility of PCBs in a future scenario considerably impacted by climate change (Lamon et al., 2009); it is indeed true that temperature shifts may change distribution of chemicals (Sweetman et al., 2005) together with their toxicology, because of major changes in metabolism both on seasonal and year-basis (Buckman et al., 2007; James and Kleinow, 2014). Eventually, PCBs accumulate and biomagnify through the food chain up to humans, which is particularly vulnerable to biomagnified compounds (Bierman, 1990); in addition, current shifts in the food chain in consequence of global climate change, must take into considerations the strong linkage between organic contaminants and the carbon cycle (Teng et al., 2012), as the food chain is far more important than other environmental characteristics to determine the potential human exposure to chemicals (Undeman et al., 2010).

The chemical and physical characteristics of PCBs and their tendency to bioaccumulation lead to their persistency in the environment, from which they exert their toxic effects and spread all over the planet. PCBs contamination has been detected not only close to manufacturing sites, like Anniston, AL, US (Hermanson and Johnson, 2007; Hinck et al., 2009), the Hudson River (Nadeau and Davis, 1976), the Maulach area, Germany (Hagenmaier et al., 1992), the lake Shinji, Japan (Sugiura, 1992), the city of Brescia (Donato et al., 2006) and the Venice Lagoon, Italy (Moret et al., 2001; Moret et al., 2005): the contamination spread, including close areas, such as the Mediterranean Sea (Elder and Villeneuve, 1977), or far-away zones, up to the Artic and the Antarctic pole (Mulr et al., 1988; Risebrough et al., 1976). Ù

All the afore-mentioned issues led to the inclusion of PCBs in the Priority Organic Pollutants (POPs) lists of the Stockholm Convention, entered in force on May 17th 2004, which gives time to participating parties to phase out all products containing PCBs within 2025, with complete cleanup within 2028 (Stockholm Convention, 2004).

1.1.4 Toxicity

The first concerns about PCB hazards for environment and human health were first raised by Jensen (1966) when these substances were first discovered to be present among pesticide residues analysis in Sweden. Little after, PCBs were detected in a huge range of aquatic animals (Jensen et al., 1969; Mulr et al., 1988) an then in terrestrial mammals and humans (Tanabe et al., 1987).

PCB structure and position is crucial to toxicity mechanisms, as it has been shown that the higher the degree of chlorination the higher the resistance of the molecule to degradation *in vivo*, and that para- and meta substitution of the biphenyl ring with chlorine atoms yield the most toxic congeners, particularly when fully planar (Safe et al., 1985; Safe and Hutzinger, 1984). The toxicity

mechanisms of PCBs in animals is related to their binding to the *Aryl hydrocarbon receptor* AhR (Okey, 1983) which activates the detoxification cytochrome system and acts as a transcription factor activating the expression of heat-shock proteins and triggering events that are part of the cytotoxic responses pathways, including lymphocyte suppression and hormonal interference (Kerkvliet et al., 1990) which, in turn, induces erroneous growth differentiation signals. PCBs accumulate in tissues, particularly fat, liver, skin and nerves; during detoxification, the cytochrome detoxification system (P-450 and P-480) in the liver microsomes uses NADPH as a reducing agent (Huang and Gibson, 1991). The reductive dehalogenation catalysis encompasses a binding to the heme group of the CYP2 enzymes with stereoisomeric selectivity, which usually releases the PCB with one less chlorine group after catalytic reduction but, more often, hydroxylated PCBs. Hydroxylated PCBs inhibit the catabolic pathway (Pieper, 2005) and might bind to a thyroid hormone transporter (Purkey et al., 2004); when hydroxylated, PCBs become more soluble and can be excreted from the organism, even showing still a strong tendency to bioaccumulation (Braathen et al., 2009).

Several PCB mixture were not mutagenic on *Salmonella typhimurium*, (Schoeny et al., 1979), on rodents cell lines (Hattula, 1985), or in rats (Green et al., 1975), *Drosophila melanogaster* (Nilsson and Ramel, 1974) or chicken (Blazak and Marcum, 1975); it has been observed, though, that a PCB mixture was able to induce change in genic expression in fish (Stegeman et al., 2001) and some congeners can interact with others, yielding synergistic effects on human cell lines (Sargent et al., 1989). Carcinogenetic effects on laboratory animals was extensively studied and there is also evidence for immunotoxic effects in guinea pig and Sprague-Dawley male rats (Exon et al., 1985; Vos and de Roij, 1972)

PCBs were correlated to toxic effects in humans including: hormonal interference, together with sexual and reproductive disorders (McKinney and Waller, 1994; Winneke et al., 2014), immunitary system deficiencies (Weisglas-Kuperus et al., 2004), diabetes (Zeliger, 2013), neurological disorders, developmental and cognitive problems (Grandjean and Landrigan, 2006; Schantz et al., 2003), chloracne (James et al., 1993), metabolic problems (Baker et al., 1980), non-Hodgkin lymphoma and breast cancer (Freeman and Kohles, 2012; Høyer et al., 1998). Studies on human carcinogenicity were conducted to assess if tumor incidence (in guts, liver, nervous system or skin) was due to chronic exposure to PCBs: those investigations were conducted on workers in PCB production plants, workers who often made contact with PCBs because of their work or population eating food which resulted to be contaminated. Some studies revealed an increment in prostate cancer incidence (Prince et al., 2006), and a big cohort study evidenced correlation between PCB exposure and cancer (Loomis et al., 1997). Even though PCBs have long been considered toxic by

themselves, recent studies provide also convincing evidence that specific PCB congeners can be also biotransformed to genotoxic metabolites (Ludewig and Robertson, 2013).

In 1968, in Fukoka prefecture, north Japan, 1668 people were poisoned by rice oil contaminated from PCBs and pyrolysis products, leading to an increase in chloracne incidence, dark-skinned newborns and blood contamination in mothers (Kuratsune et al., 1972). A follow-up study to that incident after 40 years proved a general increase of liver, lung and all type of cancer mortality, compared to the rest of the population, even though these factors seemed to decrease for male population over time (Onozuka et al., 2009). A similar case in Taiwan, in 1979, led to the poisoning of more than 2000 people, and a 24-years follow-up study detected an increase in liver mortality more than 20-fold higher than the background rates (Tsai et al., 2007). Due to these reasons, and to the fact that more studies are still required to obtain consistent epidemiology on the matter, PCBs are classified as probably carcinogenic agents for human, according to the *Department of Health and Human Services* (DHHS) and the *US Environmental Protection Agency* (US EPA); however, in 2013, the *Agency for Research on Cancer* (IARC) reassessed the carcinogenicity of PCBs to group 1 (carcinogenic for humans) (Lauby-Secretan et al., 2013).

1.1.5 Exposure routes

PCB production is banned worldwide, thus significant releases of newly manufactured or imported materials to the environment do not occur. However, exposure to these substances still occurs: PCBs are in fact redistributed from one environmental compartment to another, or might be released from different sources such as incineration, leakage from older electrical equipment or uncontrolled landfills and hazardous waste sites, improper disposal of old PCB-containing liquids, leachate from contaminated sewage sludge, dispersion from contaminated soils and vehicular emissions (Agency for Toxic Substances and Disease Registry (US Department of Health and Human Services), 2000).

The main exposure route for humans is consumption of contaminated foods, where vegetables result to be contaminated particularly by lower-chlorinated congeners, whereas fish, meat, poultry and dairy products are contaminated mainly by higher chlorinated ones (Duarte-Davidson and Jones, 1994). Due to bioaccumulation properties of PCBs through the food chain up to higher predators (Leonards et al., 1998), and to the inherent technical difficulties of detection methodologies, there still is the need for a correct risk assessment in higher trophic levels of the food chain (Leonards et al., 2008). Direct tests on humans after contamination, show that PCBs can be detected in the blood, adipose tissue, and breast milk of non-occupationally exposed members of the general population (EPA, 1986). Luckily, general level of exposures have declined during the years (Agency for Toxic Substances and Disease Registry (US Department of Health and Human

Services), 2000), and strategies to reduce the presence of these molecules in the environment and in the food chain, including maximum levels for food and rapid alert systems for incidents, did sort a beneficial effect on dietary exposures, leading to decreases in percentage ranging from 16.6% to 79.3% (Malisch and Kotz, 2014).

Inhalation, together with drinking water, is the second major PCB exposure route; contamination values for air are generally higher in urban areas rather than in rural locales (Offenberg and Baker, 1999) and are usually characterized by lower chlorinated congeners, which are mole volatile and prone to re-volatilization from contaminated water and contaminated soil areas (Ockenden et al., 1998). Water and air contamination generally decreased in values over the years (Agency for Toxic Substances and Disease Registry (US Department of Health and Human Services), 2000), resulting in a general risk decrease for humans. However, soils and sediments are still a major reservoirs for PCBs and a possible source for re-emission (Fernández and Grimalt, 2003). Although adsorption can immobilize PCBs for relatively long periods of time in the aquatic environment, re-solution into the water column has been shown to often occur on an environmental level suggesting that the substantial quantities of PCBs contained in aquatic sediments can act as an environmental sink for environmental redistribution of PCBs (Qaker et al., 1985).

Additional exposure to PCBs may also occur in indoor environments, mainly due to finishes, paints and sealants (Frederiksen et al., 2012) and manufacturing plants where equipment might still contain PCB which might be leaked or release with age (Esser et al., 2015). These are, however, minor concern, as PCB-contaminated equipment and materials must be dismissed within 2025, as from the Stockholm convention. A major environmental and health risk is still constituted by soil and sediment contamination where accidental spills occurred and around landfills and dismissed manufacturing plants (Agency for Toxic Substances and Disease Registry (US Department of Health and Human Services), 2000).

1.1.6 Legislation

Banishment countermeasure for PCB production were first adopted in 1972 in Japan, followed by United States in 1977, Germany in 1983, Italy in 1984 (Fiedler, 2001). The European Community in 1996 abolished the previous regulations, stating that member states must take the necessary measure to ensure that used PCB are disposed as well as equipment contained PCB, following decontamination (Directive 96/59/EC). All PCB-containing equipment and fluids must be decontaminated and disposed by 2010 at the latest. Moreover, member states must prohibit separation of PCBs from other substances for reuse and take necessary measures to ensure that:

- PCBs, used PCBs and equipment containing PCBs which is subject to inventory are transferred to licensed undertakings, at the same time ensuring that all necessary precautions are taken to avoid the risk of fire;
- any incineration of PCBs or used PCBs on ships is prohibited;
- all undertakings engaged in the decontamination and/or the disposal of PCBs, used PCBs and/or equipment containing PCBs obtain permissions to operate;
- transformers containing more than 0.05% by weight of PCBs are decontaminated under the conditions specified by the Directive.

In Italy, the European directive has been actuated with the Dlgs. 209/99 n.20, setting incineration as major form of disposal for PCBs, for which only few plants are authorized, while all the others should limit to chemical-physical treatment, or regroupment, conditioning and preliminary deposit. Following modifications of law 62/05, art. 18 introduce the obligation to integrate communication of disposal with indication of disposal pathway and decontamination of equipment, setting as deadline the disposal of all equipment within Dec 31st, 2009; at the present time, no data are available on the respect of these deadlines. Environmental contamination and treatment for contaminated area depends on local guidelines, even though the European Directive 2008/105/CE, in conformity with its predecessor 2000/60/CE, sets standard for priority pollutants to achieve a "good chemical state" of water columns within 2015 (Directive 2000/60/EC and Directive 2008/105/EC). The US guidelines, instead, with the approval of Title 40: protection of Environment, 2007, set programs for purification of air, water, solid waste and sewage sludge (US GPO, 2007).

Maximum concentrations for dioxin-like PCBs are also regulated: those limits depend on products, but must not be higher than 12 ng/kg of fat for food and 24 ng/kg for feed, while no limits are yet set for the other PCBs (Regulation EC/1881/2006). The toxic weekly intake for dioxin-like PCBs has been set to 14 pg of World Health Organization toxic equivalent (WHO-TEQ) (kg body weight)$^{-1}$ (Directive 2006/13/EC). The United Nations set plans for disposal of hazardous substances (UNEP, 2003) and control of international black market of hazardous waste (UN Basel Convention, 2007).

1.1.7 Treatment of contaminated sites

The potential danger caused by complex organic mixtures, such as PCBs is widely recognized by governments and public, and there is the need for treatment strategies, which must be quick, feasible, and deployable in several physical settings and with the least impact for the environment.

Many technologies have been developed to treat soil and sediments, which still represent the major reservoirs for PCB contamination, where concentrations may be as high as 10^4 mg/kg (Vasilyeva and Strijakova, 2007). Treatment strategies include *in situ* and *ex situ* methods: the selection of these strategies is heavily depending on the site characteristics, regulatory requirements, costs, time and environmental constrains (Khan et al., 2004). Main strategies for PCB remediation are usually subdivided into classical treatment, which involves physical and/or chemical processes, and bioremediation technologies (Figure 1.2). Albeit extremely different approaches and techniques, it has been shown that, in case of soil contamination, best results come from integrated strategies (Reddy et al., 1999).

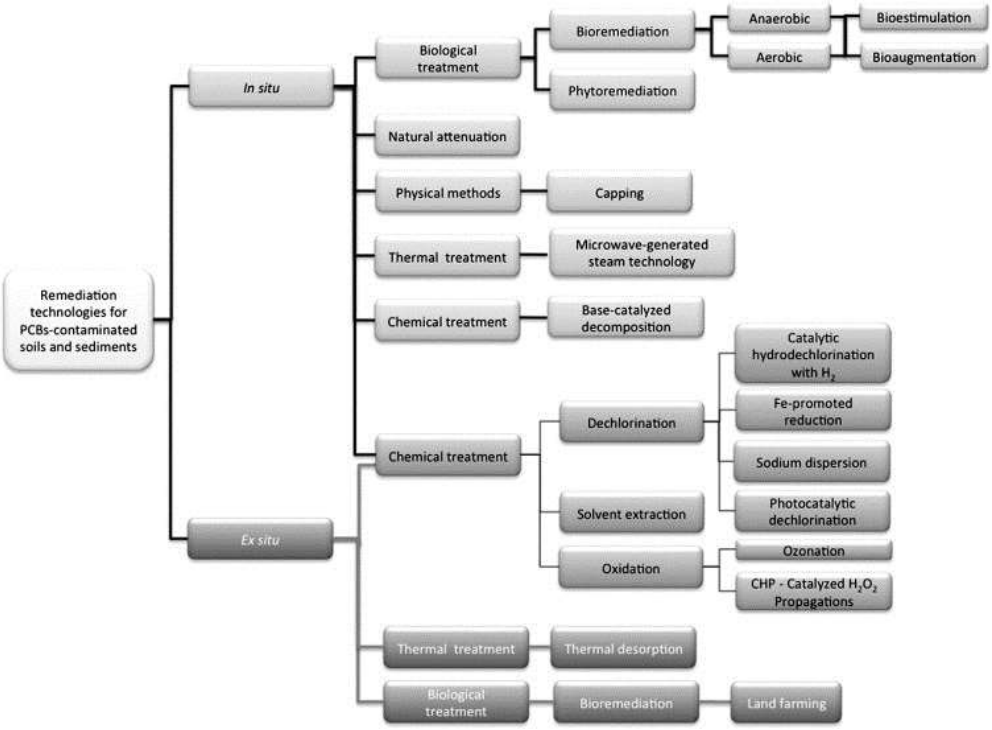

Figure 1.2 Quick overview of remediation technologies for PCB-contaminated soils and sediments as from Gomes et al. 2013.

1.1.7.1 Physical and chemical treatment

Classical treatment strategies are applied *in situ,* such as capping and microwaves, or, more often, they are applied *ex situ,* which requires dredging and transport of the sediment (with risk of re-suspension of the contaminant and accidental spills) but it is suitable for most technology which require separate plants such as thermal desorption, catalyzed decomposition, reductive dechlorination oxidation and solvent extraction.

Capping is a typical *in situ* physical method for PCB contaminated sediment treatment: it consists in the isolation of the contaminated area with a clean layer (mostly consisting of sand, gravel or

debris) which must physically separate the contamination from the aquatic environment, avoiding resuspension (Agarwal et al., 2007). Some of the capping strategies may involve the use of activated carbon for a better immobilization of the PCB molecules (Cho et al., 2009), or biochar (Rakowska et al., 2012) or more advanced materials such as zero-valent iron (Choi et al., 2009).

Microwave energy is also a promising remediation technology for sediment treatment, since most of its components are transparent to microwaves and it might also be implemented with absorbers allowing very high rate of removal of PCBs because of mechanisms as self-condensation, dechlorination, oxidation, reduction, hydration and fragmentation and increased solubility of the contaminant, as shown in lab-scale systems (Wu, 2008).

Thermal desorption, on the contrary, requires dredging of the sediment and successive deposition in rotary kilns heated at sufficient temperature to allow evaporation or combustion of the contaminant to desorb it effectively from the matrix. This process must be accurately tailored depending on the contamination profile, (which is almost never limited exclusively to PCBs) and even allowing removal of up to 70% of the original contamination, yields also the production of polychlorinated dibenzofurans (PCDFs) (Sato et al., 2010). In addition, the condensation liquid from this process must undergo further treatments, mostly separation of the organic phase and successive incineration. Incineration itself is also an extreme strategy for sediment treatment, requires higher temperatures (800-1000 °C) and contaminants may be also oxidized with oxygen supply; all products of the incineration process, including gases and particulate, must be treated, inerted and (if solid) disposed in isolated landfills. In addition, this process may yield a lot of toxic compounds, as heavy metals may react with sulfur and chlorine, and chlorinated organics such as PCBs may be converted in PCDFs and/or polychlorinated *p*-benzo dioxins (PCDDs) (Weber, 2007).

Chemical treatment strategies include solvent extraction, reduction and oxidation. Solvent extraction is one of the cheapest treatment methods, it is usually feasible for oil, fats and organic contaminants but it is not efficient with clay-rich soils or sediments. Solvent extraction usually consists in an extractor unit, where a blend of polar and non-polar solvents is mixed with the contaminated matrix, and a stage of separation of watery, organic and solid phases, followed by eventual treatment, such as chemical dechlorination, or water treatment systems. Organic phases are usually distilled, while water phases are treated in bioreactors; solvents can be recirculated and reused; (Murena and Gioia, 2009).

Soil washing, instead, separates the contaminant from the matrix through a process of washing in aqueous solution, containing surface-active agents (surfactants); after filtration, the contaminant can be removed by dissolution, suspension or precipitation in the aqueous solution. Since the matrix is often contaminated with more than one substance, this process might require different washing

cycles, with different reagents; moreover, the contaminated effluent, rich in surfactant, cannot undergo biological treatment because of the toxicity of the surfactants used, thus it is usually confined in landfill; recently, a 60% removal of PCB contamination from a 650 mg/kg contaminated sediment has been achieved using biologic surfactant such as soya lecithin (Occulti et al., 2008).

Reductive dechlorination is a process in which chlorine atoms are substituted with hydrogen, eventually leading to biphenyl, which is relatively non-toxic. Base-catalyzed chemical dechlorination utilizes a mixture of sodium bicarbonate, sodium hydroxide and high temperatures (200-400 °C); the process can remove up to 99.9% of PCB contamination (Taniguchi et al., 1998) but may result difficult in case of very wet material, and leads anyway to residual products in the effluent. Dechlorination can also be achieved using metal catalysts, such as Pd/Mg bimetals, which can completely dechlorinate historically contaminated soils, but catalysts can be inhibited by some sulfide compounds (Agarwal et al., 2009); nanoparticles of zero-valent iron (nZVI), which are consumed in participating to the reaction, unlike catalysts, are a promising reagent for dechlorination in liquid systems, having a minimum destruction yield of 95% of the original contamination, but may require high temperatures of exercise and other pretreatments or catalysts in the process (Varanasi et al., 2007). Sodium dispersion is a technique for solid state chemical dechlorination, which involves the reaction of sodium salt with the polychlorinated molecule, leading to sodium chlorine and organic salts; it is highly dependent on the contact surface, thus milling pretreatment is required, it can be applied at room temperature and might lead to high removal rates (2600 mg/kg total PCB contamination was lowered to <0.2 mg/kg (Aresta et al., 2003)); however, some salts which are produced can be toxic for *in situ* remediation. Another promising technique could be the photocatalytic dechlorination, which was efficient in dechlorinating up to 83% total PCB of an Aroclor 1254-contaminated sediment in a bench-scale system (Poster et al., 2003).

Alternatively, destruction of the contaminant can be achieved by oxidation: ozone and cycles of pressurization and depressurization can remove completely a 5 mg/kg total PCB contamination from a sediment (Hong et al., 2008); Fenton reagent is a laboratory procedure where diluted H_2O_2 is slowly added to a solution of excess iron (II) to generate hydroxyl radical (OH•), a strong, relatively nonspecific oxidant that can remove 98% of PCBs in an *ex situ* soil treatment process (Ahmad et al., 2011).

All these techniques have different costs and environmental impact and their effectiveness is also site-specific, since every technology depends on the contamination profile, on the type of soil, on the aging of the contamination and other environmental factor. Active caps may be generally more

suitable than passive caps, in terms of effectiveness and minimization of risk exposure, but changes in the capping material may lead to dissequestration of the contaminant into the environment. Thermal treatments usually imply high energy, thus high costs, and the transfer from the contaminant from one media to another, which must be further treated. Chemical treatments are usually faster, can treat higher contaminant concentration and are usually more effective, leading to almost complete destruction of the contaminant; however, they are very aggressive, expensive and lead to byproducts, some of which yet uncharacterized, which must follow successive treatment steps. Ultimately, the selection of the technology to use in each site will be based on its cost-effectiveness and social acceptability, as well as to the ecological and health risks associated with the site. Recently, the adoption of combined or sequential remediation technologies has been studied and progress can be made in this area. These technologies, however, result to be generally not sustainable, both for the energetic and material cost of exercise and for the generation of other toxic products.

1.1.7.2 Biological treatment

Life Cycle Assessment analyses show that bioremediation has less impact than incineration for PCB contaminated soils, with the lowest environmental footprint being for electric aeration, especially in terms of global warming and depletion of abiotic resources. At equal distances between the polluted sites and the treatment plant, bioremediation had lesser impact than incineration in eight out of 13 categories, the impact on global warming was nine times greater for incineration producing 6.5×10^5 and 7.2×10^4 kg eq. CO_2, respectively (Busset et al., 2012).

Natural attenuation is one of the most common methods of biological treatment for contaminated sediments *in situ*, which can take place through two primary pathways: burial of contaminated bed by clean sediment and successive transformation via biodegradation, immobilization, dilution or volatilization, which reduces bioavailability and, thus, the environmental impact. However, being a year-long process, it requires continuous monitoring and it's always at risk for redispersion of the contaminant in the environment and in the food chain (Magar et al., 2009). This process is site-specific, and so it is its efficiency, being lower usually in the most contaminated regions (Sivey and Lee, 2007).

Landfarming, on the other hand, is a bioremediation treatment performed off-site, where contaminated sediments are incorporated in the soil surface of a chosen site and periodically turned over to aerate the mixture. Complete biostabilization can be achieved and it is mostly caused by a combination of photolysis, volatilization and biodegradation rather than a single process (Tang and Myers, 2002).

Phytoremediation is based on the use of plants to extract, sequester and detoxify pollutants; PCBs are taken from the soil (phytoextraction) and accumulated in the tissues where enzymatic transformation occurs (phytodegradation); at the same time, the plant can enhance the microbial activity in its root zone due to various hormones and nutrients (rhizoremediation) (Van Aken et al., 2010; Mackova et al., 2009). A 50% degradation of 100 mg/kg Aroclor 1248 contamination can be achieved used spermatophites within 6 months (Dzantor et al., 2000). Plants are able to synthetize the same enzymes of aerobic dehalogenating microorganisms (Sylvestre et al., 2009). One of the main concerns regarding phytoremediation is crop disposal after phytoextraction and the issues associated with pollution transfer from the biomass disposal or the generation of toxic metabolites into the environment. For these reasons, genetically modified crops and/or genetically modified soil bacteria can be used (Abhilash et al., 2009), but the release of GMOs into the environment sparks yet a big debate.

Fungal remediation of PCB-contamination is a promising field, thanks to the ability of fungi to form extended mycelial networks, the low specificity of their catabolic enzymes and their independence from using pollutants as a growth substrate which make fungi well suited for bioremediation processes (Harms et al., 2011). So far, however, it has only been tested in soils, not in sediments, and results are controversial: even having most of the machinery required to metabolize PCBs, fungi tend not to survive in contaminated fields, mostly because of competition with the indigenous microbiota or because of the lack of adaptation to the environmental condition; in these case, the utilization of autochthonous strain can be a strategy worth pursuing (Mouhamadou et al., 2013).

The most important biodegradation process for PCB contamination is due to microbial activity, which can be either an aerobic degradation or an anaerobic degradation (Abraham et al., 2002). Oxidative degradation consists in the breakdown of lower chlorinated congeners in the catabolic biphenyl pathway down to chlorobenzoic acid (Figure 1.3), which is further degraded by other species (Borja et al., 2005). Biodegradability is highly dependent on the number of chlorines (decreases with increased number) and their positions, and also highly strain dependent. PCB congeners with chlorines on only one biphenyl ring are degraded more easily and PCB with chlorine at position 2,6- or 2,2'- (double ortho-substituted congeners) are poorly degraded (Furukawa and Fujihara, 2008). Aerobic bacteria which catalyze this process are from the genus *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Burkholderia*, *Comamonas*, *Sphingomonas*, *Ralstonia* and *Acinetobacter* and gram-positive genera such as *Rhodococcus*, *Corynebacterium*, and *Bacillus* (Unterman, 1996). This is the only biodegradation pathway leading to complete mineralization of the contaminant; on the other hand, aerobic bacteria cannot oxidate higher chlorinated congeners

since they tend to accumulate in cell membranes, exerting toxic effect on the cell and resulting inaccessible to oxidases, eventually leading to cell death (Ohtsubo et al., 2004).

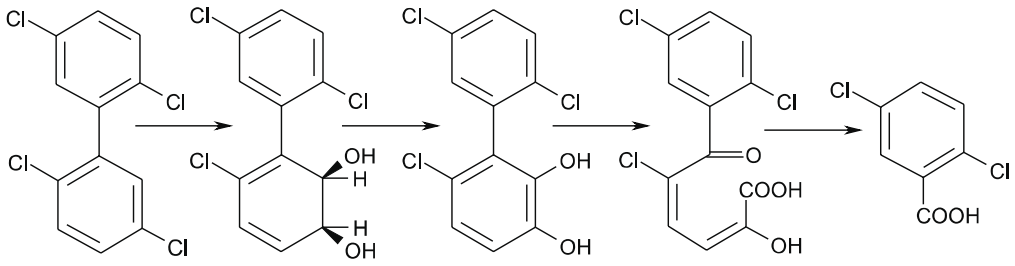

Figure 1.3 Metabolic pathway of 2,2,5,5-tetrachlorobiphenyl via 2,3-dioxygenase attack in *Pseudomonas sp.2* (Komancová et al., 2003).

Anaerobic dechlorination is the most important microbial activity in contaminated soils and sediments, which are usually anaerobic below 2-3 cm depth. These activities can attack highly chlorinated congeners and lead to an efficient detoxification of the matrix, and are described in detail in the following section 1.2.

1.2 Microbial dehalogenation

Several microorganisms can anaerobically dehalogenate chlorinated compounds. The dehalogenation metabolism was widespread even before the advent of chemical industry and the consequential pollution problems, because chlorinated organic compounds are ubiquitous in nature and have a role in many metabolisms: they are involved in catalysis and formation of structural components, they are oxidation products and they represent key-intermediate in the organic matter and chlorine cycles, which include both biological releases (algae, plants, etc.) and abiotic processes (volcanoes, fires) (Gribble, 1994). Thus, the ability to degrade these compounds is a competitive advantage, favored by evolution, and this is why dehalogenating microorganisms can be found even where no contamination had ever occurred (Lanthier et al., 2001). Biogenic sediments from various epochs, and representing different stages of coalification, contain considerable concentrations of organohalides [up to several hundred mg·(kg dry weight)$^{-1}$]. Natural halogenated compounds in sediments may comprise primary organohalides as well as structures synthesized de novo biotically and abiotically within the sediment (Müller et al., 1996). However, natural organic chlorinated species are far easier to biodegrade compared to industrial contaminants (Oberg, 2002) and there is a strong evidence that the impact of man-made chlorinated compounds over the environment acted as a driving force, specializing substrate specificity of some dehalogenating species (Hiraishi, 2008).

Some Gram-positive species (*Dehalobacterium* sp.) can utilize mono- and di-chloromethane, fermenting to acetate and formate (Holliger et al., 2003); members of the phylum *Proteobacteria* (such as *Ochrobacterium* sp. and *Pseudomonas* sp.) are able to oxidate chlorobenzoates in denitrifying conditions (Song et al., 2000) and some phototrophic bacteria (*Rhodospirillum* sp.) are able to dehalogenate chlorobenzoate via reductive dechlorination, using Coenzyme A (Smidt and de Vos, 2004). Those microorganisms tend to prevail in presence of these substances and not extremely negative redox potentials, since they don't need reducing equivalents and, therefore, are advantaged in the competition for these substrates (Dolfing, 2000). A number of bacterial strains belonging to *Pseudomonas*, *Arthrobacter*, *Acinetobacter*, *Alcaligenes*, *Nocardia* and *Corynebacterium* genera can perform hydrolytic dehalogenation (Dunaway-Mariano and Babbitt, 1994), another metabolism where the chlorine atom is substituted with an hydroxyl group involving alidohydrolases or glutathione (Fetzner and Lingens, 1994). Haloalkane dehalogenase Dh1A from *Xantobacter autotrophicus* are able to catalyze the dehalogenation of more than 24 aliphatic compounds (Janssen et al., 1994) and several more have been described in Gram-positive bacteria (Fetzner, 1998)

Chloromethane dehalogenases have been isolated in *Acetobacterium dehalogenans*, which then produces acetate and uses it as energy source (Traunecker et al., 1991): this enzyme transfers the methyl group of its substrate to tetrahydrofolate, eliminating the chlorine from the molecule.

Dehalogenation can happen also in cometabolism, when the microorganism incorporates the chlorinated compound, which is degraded by enzymes of the metabolic pathway, but without energy recovery from either haloaliphatic or haloaromatic compounds. In aerobic microorganisms, co-metabolic oxidation of halogenated alkenes, short-chain alkanes and some haloaromatic compounds is catalyzed by several mono- and dioxygenase systems, as the chlorinated hydrocarbon competes with the growing substrate for the active site of the enzyme (Fetzner and Lingens, 1994). Anaerobic microorganisms, such as many methanogenic, acetogenic, sulfate-reducing and iron-reducing bacteria, can perform co-metabolic dehalogenation (Holliger and Schraa, 1994) mediated by electron carriers of the respiratory electron transport chain, such as *c*-type cytochromes (Picardal et al., 1993) CO-dehydrogenases (Jablonski, 1992), cytochrome P-450_{CAM} (Logan et al., 1993) or vitamin B_{12} (Gantzer and Wackett, 1991). However, this metabolism does not lead to energy recovery.

1.2.1 Microbial reductive dechlorination

Microbial reductive dehalogenation, on the other hand, is a respiratory process and it is the main responsible for the dechlorination of highly chlorinated compounds such as PCDD/PCDFs and PCBs (Bedard et al., 1998; Bedard and Quensen, 1995; Wu et al., 1998a). Several microorganisms with this metabolism have been identified, and are mostly member of the *Chloroflexi, Firmicutes* and *Proteobacteria* phyla. There is no association between the phylogenetic affiliation of the strain and its substrate specificity.

Desulfomonile tiedjei was the first isolate to use formate or H_2 as electron donor and 3-chlorobenzoate as terminal electron acceptor, demonstrating chemiosmotic coupling with dechlorination and ATP synthesis (Mohn and Tiedje, 1991). Several species of the *Desulfitobacterium* genus are able to reductive dechlorinate chlorophenolic compounds for growth (Mackiewicz and Wiegel, 1998; Utkin et al., 1994), probably using reductive dehalogenases containing cobalamin, as proposed for the *Desulfitobacterium dehalogenans* (van de Pas et al., 1999).

The *Dehalococcoidia* class (Figure 1.4) was recently established to regroup various members of the phylum *Chloroflexi* which exhibit dehalogenating metabolisms, regrouping both the old *Dehalococcoides* and *Dehalococcoides*-like genus (Löffler et al., 2013). *Dehalococcoides mccarty* strain 195 (formerly *D. ethenogenes*) is designed as type strain, it was isolated in enrichment culture

using ampicillin and vancomycin and is able to completely dechlorinate PCE to ethylene, using hydrogen as electron donor (Maymo-Gatell et al., 1997). It can also dechlorinate in cometabolism 2,3,4,5,6-CB to 2,4,6-CB in presence of PCE (Fennell et al., 2004). Other *Dehalococcoides* sp. are similar in terms of 16s rRNA sequence (98% identity) and every specie has a different selectivity for chlorinated substrates. So far, the *Dehalococcoides* genus description is still incomplete, due to difficulties in isolation. Cells are similar to cocci, 0.5-1 µm large, with a cell membrane similar to *Archaea* and lacking a cell wall; the lipid fraction of some members contain furanicic acids and lots of ubiquinone, thought to preserve the cell from the dechlorination byproducts (Hiraishi, 2008). *Dehalococcoides* CBDB1, which has been shown to dechlorinate chlorobenzenes (Adrian et al., 2000) but also 2,3-dichloro-*p*-dibenzodioxin and the 'Seveso dioxin' 2,3,7,8-tetrachloro-*p*-dibenzodioxin (Bunge et al., 2003), is one of the most studied strain of this class and, after the discovery of 32 reductive dehalogenase homologous (*rdhA*) genes that are candidates for the chlorobenzene dehalogenase, it was eventually cultivated as pure isolate from which three PCB reductive dechlorinases genes were characterized (Wang et al., 2014).

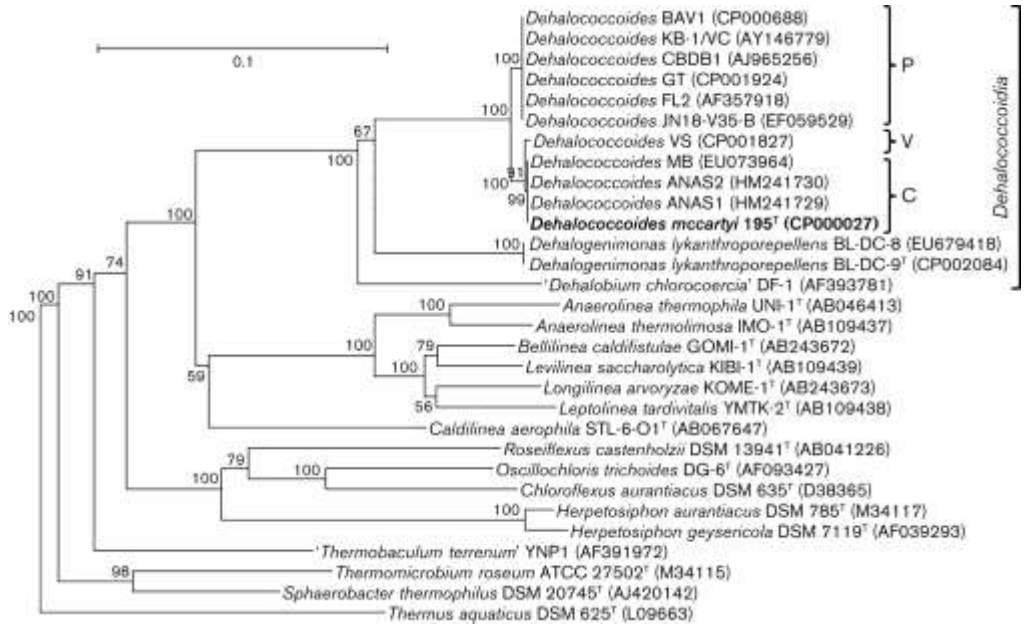

Figure 1.4: Phylogeny of *Dehalococcoides mccartyi* gen. nov., sp. nov. 195^{T} (in bold) and related *Dehalococcoides* isolates among the isolated members of *Chloroflexi* based on nearly complete 16S rRNA gene sequences (Löffler et al., 2013).

1.2.2 Biochemistry and characteristics of the process

Several pathways for reductive dechlorination have been detected and summarized. (Bedard and Quensen, 1995; Field and Sierra-Alvarez, 2008; Wiegel and Wu, 2000) (Figure 1.5 and Table 1.1). PCB congeners are attacked mostly in the *para* (3 and 5 positions on each ring) and *meta* positions (position 4 on each ring), leaving lower chlorinated congeners with the *ortho* position which is not

easily attacked (Quensen et al., 1990). These groups, however, are the major responsible for PCB toxicity (Quensen et al., 1998; Safe et al., 1985), thus, the microbial reductive dechlorination process is extremely advantageous. Chlorine in *ortho* positions (2 and 6 positions of each ring) can also be removed, but the occurrence of this process is less frequent both in sediment samples and in enrichment cultures (Bedard et al., 2005; Berkaw et al., 1996; Wiegel and Wu, 2000). This position is harder to attack because it cannot be accessed by common dehalogenases (the enzyme responsible for the dechlorination) and, being the ortho-chlorinated congeners chiral compounds, wo dehalogenases should exists for the respective two possible enantiomeric forms (Bedard et al., 2005). Reductive dechlorination usually yields mono- and di-chlorobiphenyl, and the occurrence of complete dechlorination to biphenyl has been demonstrated only in in sediment cultures spiked with single congeners (Natarajan et al., 1996).

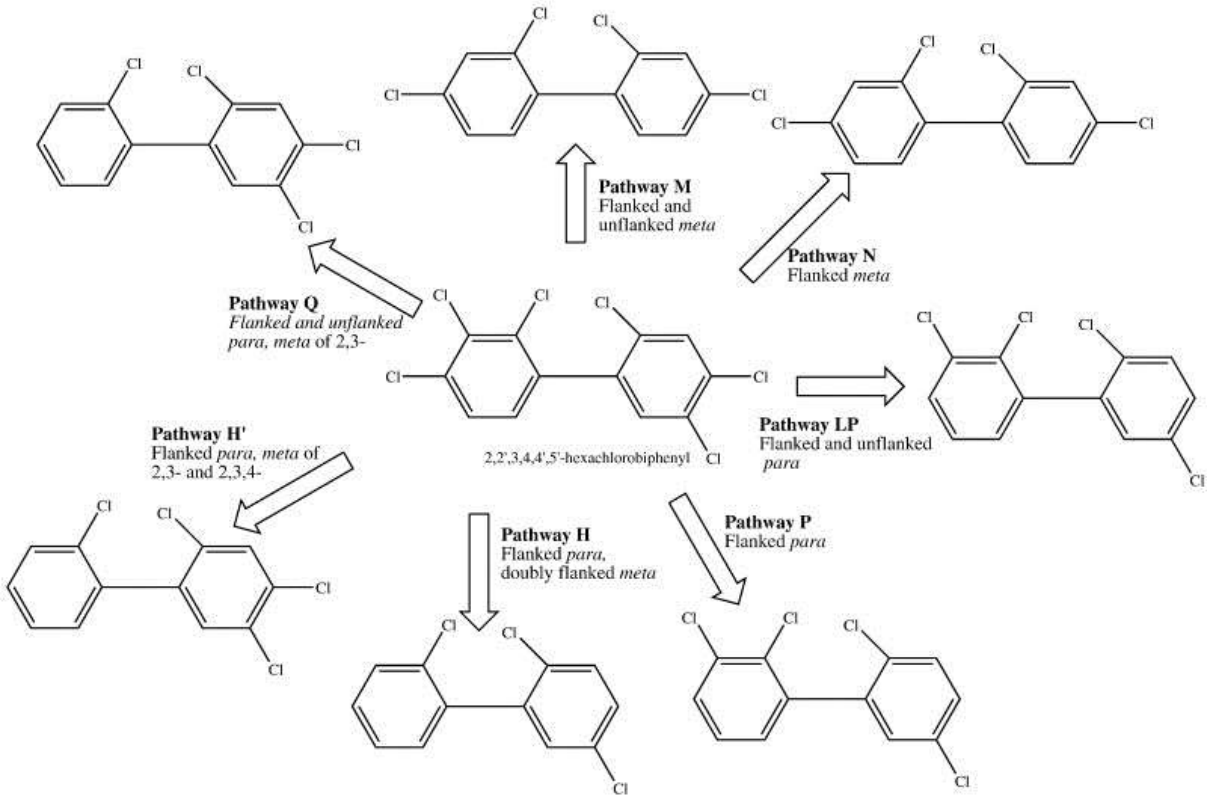

Figure 1.5: Overview of the microbial dechlorination pathways identified by Bedard and Quensen (1995) and Wiegel and Wu (2000) applied to PCB 138 (2,2,3,4,4'5'-hexachlorobiphenyl). "Flanked" means adjacent chlorine. Beside the pathways presented there is also the T pathway that removes flanked meta-chlorine of 2,3,4,5- in hepta- and octachlorobiphenyls (from Gomes et al., 2013).

Pattern	Targeted chlorine	Reactive chlorine group
P	Flanked *para*	3<u>4</u>, 23<u>4</u>, 2<u>4</u>5, 23<u>4</u>5, 23<u>4</u>56
H	Flanked *para* and also double flanked *meta*	3<u>4</u>, 23<u>4</u>, 2<u>4</u>5, 23<u>4</u>5
H'	Flanked *para* and also flanked and double flanked *meta*	2<u>3</u>, 3<u>4</u>, 23<u>4</u>, 2<u>4</u>5, 23<u>4</u>5
N	Flanked *meta*	23<u>4</u>, 236, 2<u>4</u>5, 23<u>4</u>5, 23<u>4</u>6, 23<u>4</u>56
M	Flanked and unflanked *meta*	<u>3</u>, 2<u>3</u>, 2<u>5</u>, <u>3</u>4, 23<u>4</u>, 2<u>3</u>6
Q	Flanked and unflanked *para* but also flanked and double flanked *meta*	<u>4</u>, 2<u>3</u>, 2<u>4</u>, 3<u>4</u>, 23<u>4</u>, 2<u>4</u>5, 2<u>4</u>6
LP	Flanked and unflanked *para*	2<u>4</u>, 2<u>4</u>5, 2<u>4</u>6
T	Double flanked *meta*	23<u>4</u>5

Table 1.1: Patterns of reductive dechlorination of PCBs adapted from Field and Sierra-Alvarez (2008). The attacked chlorine in each pattern is underlined in the reactive chlorine group column.

Microbial reductive dechlorination is an ATP-generating process: PCBs are used as a final electron acceptor by dehalogenating microorganisms, coupled with electron transport over the transport chain and the consequent phosphorilative oxidation (Brown et al., 1987; Quensen et al., 1988). Since the biphenyl ring is not cleaved in the process, microorganisms with this metabolism require other carbon sources for growth and other compounds as electron donor, of which H_2 is the principal (Zhang and Wiegel, 1990). The free Gibbs energy for the dechlorination can be estimated at around -130 to -180 kJ/mol of chlorine released, equivalent to redox potential between 260 and 480 mV (Dolfing and Harrison, 1992). These values are higher than sulfate reduction ($SO_4^{=}/H_2S$; E_0'=217mV) and similar to nitrate reduction (NO_3^-/NO_2^-; E_0'=433mV): this should lead to a competitive advantage of this species over other metabolisms, but in actual environment this is highly limited due to the low bioavailability and concentration of PCBs (Borja et al., 2005). Similarly it has been proposed that this activity could also be explicated in aerobic environment, but there is no experimental evidence, even if these species have been detected in microaerophilic environments (Bossert et al., 2003a; Hiraishi, 2008).

A major influence on the microbial reductive dechlorination efficiency is played, of course, by the bioavailability of the polychlorinated molecule, in terms of adsorption and desorption, the rates of which depend on the different PCB congeners and the different matrix and it is a complex site-specific issue (Lamoureux and Brownawell, 1999).

Temperature, on the other hand, plays a heavy role both on the bioavailability and on the growth and physiological activity including uptake and enzymatic dehalogenation. It has been shown that in a contaminated sediments, flanked *meta* dechlorination occurred in the ranges of 8-34 °C and 50-60 °C, while flanked *para* dechlorination was observed only between 18 and 34 °C, doubly unflanked *para* chlorines were attacked only in the range of 18-30 °C and *ortho* dechlorination of only two congeners was observed between 8 and 30 °C (Wu et al., 1996; Wu et al., 1997a;b); in

another experiment, samples incubated at 12, 25, 37, 45 and 60 °C exhibited reductive dechlorination of Aroclor 1242 at 12 °C and (roughly twice as fast) at 25 °C but not at 37 °C or above (Tiedje et al., 1994).

Even though sediments are often buffered systems, anaerobic microbial processes may lead to an increase in acidic compounds. Such pH changes influence both the adsorption equilibrium of PCB congeners and the interaction between bacterial populations. The optimal pH for overall removal is between 7.0-7.5, but the different dechlorination pathways varied sensibly: flanked *meta* dechlorination occurred at pH 5.0-8.0, unflanked *para* dechlorination at pH 6.0-8.0 and *ortho* dechlorination at pH 6.0-7.5 (Wiegel and Wu, 2000).

Basic kinetic information on the process are not yet available, due to the difficulties in obtaining pure cultures and the intrinsic syntrophic nature of the dehalogenation process itself. Specific activities of higher PCB dechlorination are very low, from $6.0 \cdot 10^{-6}$ to $2.7 \cdot 10^{-2}$ mgPCB·(g dry weight biofilm)$^{-1}$·day^{-1} (Field and Sierra-Alvarez, 2008). It has been estimated that the half-life of several PCB congeners can be as high as 9 or 16 years in the outside environment (Beurskens et al., 1993; Magar et al., 2005), which is 1-2 order of magnitude lower compared to laboratory experiments, were the higher concentration and availability play a stimulatory role (Magar et al., 2005).

1.3 PCB microbial reductive dechlorination in contaminated sediments

Highly chlorinated congeners represent relevant fraction of the commercial mixtures (Ahlborg et al., 1992), they are more prone to bioaccumulation and generally more toxic (Safe, 1994) and they are not metabolized by aerobic bacteria. In anaerobic condition, however, they can undergo a process called microbial reductive dechlorination, a hydrogenation process which sequentially removes chlorine atoms from the biphenyl ring, leading to lower chlorinated congeners which are generally less toxic and can be successively mineralized by aerobic bacteria (Boyle et al., 1992). The first indication for a natural degradation of PCB mixtures in anaerobic sediments were discovered by Brown et al. (1987), who discovered a different congeners distribution profile from the original mixture in six sediments where PCB spill had occurred: the higher chlorinated PCBs resulted depleted while, on the contrary, higher ratio of lower-chlorinated congeners were detected; in addition, the higher the deepness of sampling, the higher the PCB congener variation detected (Lake et al., 1992). Evidence of the biological nature of this process was then assessed in laboratory microcosms, where dechlorination of Aroclor 1242 mixtures congeners distribution changed during incubation only in active microcosms, whereas no change was detected in the sterile microcosms (Quensen et al., 1988).

1.3.1 Dechlorinating microorganisms

Sediment community is a complex microbial network where syntrophic relationship between different metabolism help microbes to degrade the complex organic matter: a typical case of this network is the degrading process for cellulose, which is degraded down to methane and carbon dioxide through several fermentation steps, operated by different microorganisms, many of which require hydrogen-scavenging metabolism to sink redox equivalents and favor equilibrium shift towards products which would be energetically disfavored. Recent studies have revealed that syntrophically-fermenting bacteria synthesize ATP by substrate-level phosphorylation and reinvest part of the ATP-bound energy into reversed electron transport processes, to release the electrons at a redox level accessible by the partner bacteria and to balance their energy budget (Schink, 1997).

Different species are able to catalyze different dechlorination activities (Alder et al., 1993; Bedard et al., 2005; Kjellerup et al., 2008; Ye and Quensen, 1992), which are only present in sediments where contamination occurred long ago, and absent where no contamination could be detected (Kim and Rhee, 2001).

Sulfate reducers and methanogens are crucial in this process of mineralization, because they constitute the main scavengers for acetogenic species (Laanbroek et al., 1982). It has been also hypothesized that these species could play a role in PCB degradation in sediments. A study

demonstrated that addition of antibiotics to dehalogenating culture inhibited both methanogenesis and dechlorination: this was probably due to an indirect inhibition of methanogens which was successively removed by adding selective substrates for methanogens and showing that also dechlorination activity was restored (Ye et al., 1995). The same group achieved selective inhibition of methanogens was achieved with 2-bromo-ethansulfonate (BES) and proved that dechlorination was still inhibited, thus confirming that methanogenic species played a role in reductive dechlorination of PCBs (Ye and Quensen, 1999). Other studies evidenced the implication of *Dehalobacter restrictus* and *Clostridium pascui* in the dechlorination of Aroclor® 1248 using ARDRA (amplified ribosomal DNA restriction analysis) and subsequent dot-plot hybridization: those are molecular techniques which avoid the obstacle of obtaining a pure culture, but gave hint on the number of dechlorinating microorganisms using serial dilution (similar to the most probable number technique); this study also evidenced a predominant *Methanosarcina barkeri*-related clone, which confirmed the implication of methanogens in the process (Oh et al., 2008).

On the other hand, the addition of Fe_2SO_4 to cultures in marine sediments (where usually sulfate concentrations are higher than freshwater sediments) induced dechlorination activities stimulating sulfate-reducing bacteria and avoiding the toxic effect of sulfide, which precipitated as FeS (Zwiernik et al., 1998). Similar experiments with selective inhibitors for sulfate reduction (sodium molibdate) or for eubacteria (cycloserin and penicillin G) also stopped dechlorination (Fava et al., 2003b; Fava et al., 2003c) In addition, *Desulfitobacterium* sp. Are able to dechlorinate a wide range of substituted aromatics, including hydroxylated PCBs (Holliger et al., 2003). It has been hypothesized that sulfate-reducing species attacked PCBs in absence of this electron acceptor (Zanaroli et al., 2006), but so far no conclusive association has been found between sulfate-reducers and dechlorinating activity. In fact, dechlorination can also be observed in absence of sulfate (Øfjord et al., 1994).

Dehalococcoidia and *Dehalococcoides* members of the phylum *Chloroflexi*, however, are considered to be the principal dechlorinating microorganisms in contaminated sediments. The first to be associated with *ortho* dechlorination of 2,3,4,5,6-CB to 3,4-CB was the o-17 bacterium isolated from the Baltimore Harbor (MD, USA), capable to dechlorinate flanked *ortho* and *meta* positions, without any *Archaea* required for dehalogenating activities (Cutter et al., 2001). This bacterium is closely related to *D. ethenogenes*-related clones which were also found to exhibit similar dechlorination pattern in the same area (Pulliam Holoman et al., 1998). Other *Dehalococcoides* not related to those previously described, but rather to the *D. mccarty* CBDB1 were detected in the Housatonic River sediments and dechlorinated Aroclor 1260 in sediment-free cultures (Bedard et al., 2007). A common *Dehalococcoides* DGGE band was also detected in three

different sites (freshwater, estuarine and marine) only in the presence on dechlorinating activities of 2,3,4,5-CB, which was not present in absence of dehalogenation or when methanogenesis was inhibited (Yan et al., 2006). However, differences in dechlorination activities were detected together with differences in site characteristics: in Kjellerup et al. (2008) *Dehalococcoides* were detected in the most active sediment, while *Dehalococcoidia* members were detected in the less active cultures.

The presence of different species seems needed to dechlorinate complex Aroclor mixtures, such as Aroclor 1260 (Bedard et al., 2007), supporting the theoretical associations between microorganism and patterns. Later, from the same cultures, isolation of *D. mccarty* JNA strain was performed, which was capable to dechlorinate Aroclor 1260 in flanked *meta* positions (LaRoe et al., 2014) and could be cultivated in sediment-free pure culture for the first time. Species of all three subgroups of *Dehalococcoides* and *Dehalogenimonas* dechlorinating mainly flanked *para* position but also double flanked *meta* and partially *ortho* positions of the Aroclor 1260 mixture were isolated in sediment-free cultures (Wang and He, 2013a). As previously cited, *D. mccarty* 195^{T} (formerly *D.ethenogenes* 195) was shown to dechlorinate in double flanked *meta* and flanked *para* position, in presence of chlorinated ethenes (Fennell et al., 2004).

It is worth noting that also *D. mccarty* CBDB1 was isolated in pure culture and, for the first time, showed a complete dechlorination pattern of Aroclor 1260 mixture (Adrian et al., 2009); however, similar strains of *D. mccarty* were not able to dechlorinate PCBs in sediment-derived cultures, where *Dehalococcoides* and *Dehalobacter* were detected, instead, removing flanked *para* and doubly flanked *meta* chlorines (Wang and He, 2013b). *Dehalobacter* sp. were found also to dechlorinate 2,3,4-CB to 4-CB with a novel flanked *ortho* flanked *meta* pathway in sediment-free culture (Yoshida et al., 2009).

Primers designed specifically to target dechlorinating species discovered several OTUs in the Baltimore Harbor, always inside the phylum *Chloroflexi* but not necessarily related to *Dehalococcoides* (Watts et al., 2005) two more phylotypes were detected: DEH10, closer to *Dehalococcoides* and SF-1, closer to *Dehalococcoidia* and were associated to different congeners dechlorination, showing peculiar stereoselectivity, both flanked *meta* and *para* positions, respectively (Fagervold et al., 2005) and later the opposite experiments was performed, starting from a mixed culture grown on Aroclor 1260 and selecting one phylotype over the other by supplementation of single congeners and analysis of the dechlorination products (Fagervold et al., 2007). The presence of a *Chloroflexi* member not related to *Dehalococcoides*, (now included in the *Dehalococcoidia* class) was also found to dechlorinate Aroclor 1254 in marine sediments of the Venice Lagoon (Italy), and its activity was inhibited by the growth of methanogens and sulfate-

reducers (Zanaroli et al., 2012a). Other phylotypes were detected in Er-Jen estuarine sediments (Taiwan, Taiwan), namely Cp-1 closer to the N5-12 subgroup and Cm-1 closer to the SF-1 subgroup of the *Dehalococcoidia* class and capable of dechlorinating singly and doubly flanked *para* chlorines and singly and unflanked *meta* chlorines, respectively (Ho and Liu, 2011).

A major importance to dechlorination activities in contaminated sediment was attributed also to *Dehalobium chlorocoercia* DF-1, a member of the phylum *Chloroflexi* separated from the *Dehalococcoides* family (but also from the recently constituted *Dehalococcoidia* class), which is able to dechlorinate double flanked *meta* and *para* chlorinated biphenyls in co-culture with *Desulfovibrio* sp. and supplementation with formate and H_2:CO_2 atmosphere (Wu et al., 2000; Wu et al., 2002a; Wu et al., 2002b). Recently, DF-1 was described as ultramicrobacterium and has a doubling time of 2 days on 2,3,4,5-CB as sole electron acceptor (May et al., 2008). In Venice Lagoon sediments, a phylotype having 100% similarity to *D. chlorocoercia* DF-1, removing double flanked *meta* chlorines from Aroclor 1254 PCBs was detected and dechlorination activities were induced by the addition of zero-valent nanoparticles of iron, probably because of contemporary inhibition of sulfate-reducers and hydrogen release (Zanaroli et al., 2012b).

1.3.2 Techniques for identification and isolation

First studies on the reductive dechlorination used the contaminated sediments resuspended in water or in mineral medium to follow PCB dechlorination; dechlorinating cultures were then transferred in the same condition and enriched. This approach is still widely used, as it allows the selective enrichment of dechlorinators and the use of selective inhibitors to block other bacterial population from growing, or the use of selective electron donors/electron acceptors or PCB congeners to assess the specificities of the different metabolisms. In particular, when the biogeochemical conditions that are created can replicate those occurring *in situ*, obtained data can be useful to estimate the real dechlorination potential of the contaminated site (Fava and Agathos, 2006).

In order to isolate dechlorinating microorganisms, the use of serial dilutions and selective media was proposed: this hugely simplifies the microbial community, thus differentiating from what happens *in situ*, and the cultivated biomass is scarce, because of the lack of the complex syntrophic interaction that regulate sediment communities (Amann et al., 1995). Several authors also utilized sediment-free cultures, using silica beads as carrier for organic pollutants (Bedard et al., 2006), and this helped to isolate *D. chlorocoercia* DF-1 (in co-culture with a sulfate reducer) (May et al., 2008) and *D. mccartyi* JNA (LaRoe et al., 2014).

These techniques are nowadays integrated with molecular biology techniques, which are able to reveal non-cultivable microorganisms such as dechlorinators. Genes for 16s rRNA are typically

used, since they are ubiquitous among microorganisms and host polymorphisms which account for the phylogenetic affiliation (Stackerbrandt and Goebel, 1994). The detection of close-related species, however, does not imply the same metabolic specificity, thus there is the requirement to investigate also on functional genes, such as dehalogenases, or assess the absence of the detected species in absence of the pollutant.

The isolation of the nucleic acid of interest (typically DNA), must ideally represent all organisms of the sample, complete and not degraded and clean from inhibitors which might bias PCR reactions. However, these conditions are seldom obtained, and this leads to preferential amplification of some targets over other or even false negatives. False negatives can be minimized using nested-PCR, a technique in which the amplicons obtained in the first cycles are template for a second primer pair which targets an inner sequence.

Most used techniques to characterize microbial communities are: Amplified rDNA Restriction Analysis (ARDRA), Terminal Restriction Fragment Length Polymorphism (TRFLP) and Denaturing Gradient Gel Electrophoresis (DGGE). ARDRA is based on the amplification of the conserved regions in 16s rRNA genes and subsequent enzymatic digestion with tetracutter restriction enzymes, followed by analysis of the restriction fragment patterns (Vaneechoutte et al., 1993). TRFLP is based, instead, on digesting a mixture of PCR amplified variants of the single gene (mostly 16s rRNA) using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments using a DNA sequencer; the result is a graph image where the X axis represents the sizes of the fragment and the Y axis represents their fluorescence intensity (Liu et al., 1997). DGGE (and its similar technique TGGE) is based on the electrophoresis in polyacrylamide gels where a denaturing agent gradient (or the temperature) cause denaturation of the PCR products until they do not migrate further in the gel (Muyzer et al., 1993).

Each of these techniques is peculiar, with its own advantages and drawbacks; when applying all three techniques on the same cultures, they give slightly different results because DGGE is faster but cannot detect sequence represented for less than 1%, TRFLP can integrate results with further information and ARDRA is able to reveal polymorphism in intragenic space of 16s (Watts et al., 2001).

DGGE is usually the elected technique for analyzing microbial communities because, even though the band intensity is not quantitative, and biases can occur because of 16s rRNA gene copy numbers and preferential amplification, it allows inferences based on the relative abundance of every member in the community. DGGE can be therefore associated to statistical analysis, including biodiversity indexes such as richness, community dynamics and evenness of the culture (Marzorati et al., 2008; Wittebolle et al., 2009).

To solve the quantification problems, recently qPCR protocols have been developed, both on the 16s rRNA gene and on the homologous dehalogenase genes. Real-time PCR (qPCR) is a technique where a fluorescence signal, generated by a fluorescent DNA-intercalating molecule (i.e. SYBR® Green) or by a fluorophore bound to a probe (i.e. Taq-Man® probes), measured every amplification cycle, is logarithmically proportional to the number of amplicons in that cycle (Heid et al., 1996). It has been recently shown that, in environmental sample, the choice of the target gene is crucial: the 16s rRNA gene, indeed, does not provide sufficient information to infer the range of chlororganic electron acceptors used by different dehalogenating microorganisms. For these reasons, several studies utilize mostly the reductive dehalogenase genes as targets (Ritalahti et al., 2006) when the community is unknown. Quantitative real-time PCR can also be used to correlate the dechlorination activities and the increase in cell number (Bedard et al., 2007) or to detect the increase of members carrying dehalogenase genes inside a *Chloroflexi* population during dechlorination (Chun et al., 2013).

1.4 Sediment bioremediation via PCB microbial reductive dechlorination

The remediation of polychlorinated biphenyls in soils and sediments remains a particularly difficult problem to solve, due to the contaminant characteristics (hydrophobicity, low availability, toxicity) and to the slow growth and low diffusion rates of PCB dechlorinating phylotypes (Bedard et al., 2007). The possibility of *in situ* degradation by microorganisms has been pursued for many years, since this approach has the potential to provide a cost-effective and environmentally sustainable alternative to dredging for treatment of PCB impacted sites. Being hydrophobic, PCBs partition into organic material and accumulate in anoxic environments well poised to support anaerobic dechlorination of highly chlorinated congeners, products of which are susceptible to complete aerobic degradation. Laboratory research over the past years is now leading to new microbial technologies that could soon be tested for treatment of PCB impacted sediments in the field.

1.4.1 Actual challenges

PCBs still contaminate a variety of sediments and soils, especially fine-grained and carbon-rich, where they tend to bind to the organic fraction, which acts as a reservoir for releases over time. The treatment of sediments is problematic, because of their limited accessibility and high water content, but it must also be considered that intervention on contaminated sites implies also impact assessment, evaluation of the benthic disruption, transport phenomena etc. The main challenges could be subdivided as follows:

- Scientific: total PCB concentration is often used as a site risk assessment, but, due to the intrinsic differences in terms of toxicity, bioavailability and biodegradability of the different congeners, this might not be a proper parameter; at the same time, toxicity studies are conducted on single congeners, ignoring all the synergistic effects that could arise because of a constant changing complex mixtures; knowledge of the microbial metabolism, species and implication on the sediment community, not to say on aquatic wildlife, is still highly limited, as almost all experiments have been conducted in controlled conditions.
- Technological: at the moment, there are few technologies available to deal with strongly adsorbed contaminant; also, due to the history of these contamination, PCBs are usually present together with heavy metals, pesticides and other organics, making decision and intervention processes, not to say bioremediation, extremely difficult
- Regulatory: at the moment, rules for the reclamation of sediments and repositioning into the environment are lacking, and the treatment strategies depicted in the legislations usually involve rather incineration and/or disposal

- Social: population has generally an aggravated risk perception of PCB-contaminated sites, mostly due to accidents in the past, which further delays the decision-making process rather than hasten it, especially where contamination is harder to remediate

1.4.2 Enhancement strategies

The first step to apply bioremediation approaches is the detection and *in situ* monitoring of PCB dechlorinating bacteria: this is a particularly difficult task, because of their slow growth rates and low yields, compared to other species. At the moment, specific primers have been designed for the 16s rRNA gene sequences of *Dehalococcoides* with dechlorinating activities (Hendrickson et al., 2002), as well as inside the non-*Dehalococcoides* groups (Watts et al., 2005) or both groups but with a specific focus on known dechlorinating targets (Fagervold et al., 2005). This approach might not be sufficient, as 16s rRNA gene will not differentiate strains within clades, misleading to distinguish between dechlorinating and non dechlorinating species: for this reason, primer pairs have also been designed for the putative reductive dehalogenase gene homologs of *D.mccartyi* 195 and/or *D.* CBDB1 (Hölscher et al., 2004; Park et al., 2011) and a further help will come with the discovery of reductive dehalogenase genes in known genomes such as *D. mccartyi* JNA (Wang et al., 2014) and (hopefully soon) *D. chlorocoercia* DF-1.

1.4.2.1 Halopriming

Successive to detection, these species need stimulation in the environment. It has been shown that *halopriming*, which is a supplementation of the indigenous culture with an halogenated compound acting as a "primer" to activate dechlorinating metabolism, could be a potential stimulation techniques in laboratory experiments (Bedard, 2003). Aroclor 1260 contaminated sediment cultures showed a sudden onset of dechlorination when added 2,5,3',4'-CB or polybrominated congeners, which are even more effective stimulants (Bedard et al., 1996; Bedard et al., 1998). 2,6-CB was shown to increase thousand-folds the concentration of dechlorinating bacteria in freshwater sediment culture contaminated with Aroclor 1260 (Wu et al., 1999), while 2,3,6-CB enriched dehalogenating members in communities showing hundred-fold higher dechlorination activities than control groups (Boyle et al., 1993). 2,4,6-CB was used to prime enriched cultures from Sandy Creek, but 2,3,4,6-CB was not active in cultures from Woods Pound, probably because microbial communities were different, as were the dechlorinating species (Wu and Wiegel, 1997). The deliberate addition of other contaminant molecules to the environment, even at low concentration such as 0.6-1 ppm, would be subject to scrutiny. Possible alternatives could be constituted by other

molecules such as fungicides or halobenzenes, or the use of enriched cultures together with priming molecules (Krumins et al., 2009; Park et al., 2011).

1.4.2.2 Biostimulation

Hydrogen is the main electron donor for the PCB dechlorination process (Bedard and Quensen, 1995; Zhang and Wiegel, 1990). Hydrogen has been shown to support the dechlorination of 2,3,4-CD in microcosms with freshwater sediments (Sokol et al., 1994) and to support anaerobic dechlorination of Aroclor in sediment containing mixed microbial cultures (Morris et al., 1992). However, the direct supplementation in sediment is technically controversial and most of the times, results in the stimulation of non-dechlorinating members of the microbial community, such as methanogens or sulfate reducers (Wiegel and Wu, 2000).

A possible way to overcome this problem is the use of a selective carbon source, which can be fermented to hydrogen by fermenting species and/or a direct nutrient for dehalorespiring microorganisms. Small fatty acids like pyruvate, acetate, lactate, etc. are usually components of synthetic media or amendments of indigenous site water in which sediment cultures experiments are performed (Alder et al., 1993; Bedard et al., 2006; Berkaw et al., 1996; Fagervold et al., 2007). Acetone, methanol and glucose stimulated the rate and extent of microbial dechlorination of Aroclor 1242 in freshwater sediments, while acetate was inefficient (Nies and Vogel, 1990). Conversely, bacterium o-17 was in fact shown to suffer from inhibition from molecular hydrogen while acetate is required for growth (Cutter et al., 2001). Malate accelerated the dechlorination of 2,5,3',4'-CB and 2,6-BB in sediments of the Woods Pond (Bedard et al., 1996). Lactate, pyruvate and yeast extract were shown to decrease the amount of *meta* and *para* substitutions in sediment-free dechlorinating culture (Dudkova et al., 2009). The choice of a selective carbon source, which stimulated dechlorination rather than other anaerobic metabolisms inside the microbial community, is a difficult process and deeply depends on the composition of the site-specific community and the organic matter present inside the sediments.

Biostimulation can also be achieved after addition of a slow release electron donor. This could be achieved with the use of hydrogen releasing compounds, molecular polymers (usually polylactate modified chains) that slowly release monomers in the contaminated sites, which are then converted by fermentation into hydrogen (Sandefur and Koenigsberg, 1999). These compounds and derivatives have been successfully used to remediate TCE and PCE in contaminated soils (Koenigsberg et al., 2000). The same idea has also been applied with the design of poly-β-hydroxybutyrate which stimulate TCE dechlorination, whereas acetate was the major sink and no acetotrophic dechlorination was detected (Aulenta et al., 2008).

The addition of Fe^0 as source of cathodic hydrogen stimulated the microbial dechlorination in PCB-impacted Baltimore Harbor sediments (Rysavy et al., 2005) and the application of nanoparticles of Fe^0 was efficient also in Venice Lagoon sediment contaminated by Aroclor 1254 (Zanaroli et al., 2012b). The addition of Fe^0 also stimulated *Dehalococcoides* in different sediments (Lake Hartwell, New Bedford Harbor, Rosanna Marsh): the key of this techniques is the low levels of hydrogen continuously released, which give a selective advantage to *Dehalococcoides* over competitors due to their higher affinity for this electron donor (Srinivasa Varadhan et al., 2011). This does not imply necessarily an increase in dechlorinating activity, though; indeed, other studies showed that if the population lacks dehalogenating microorganisms, the stimulation of *Dehalococcoides* itself is not sufficient to induce dechlorination (Winchell and Novak, 2008). A recent study showed also a potential inhibitory effect caused to soil bacterial community composition, which reduced the activity of chloroaromatic mineralizing microorganisms but not dechlorinating activities on Aroclor 1242 (Tilston et al., 2013).

1.4.2.3 Zerovalent metal-catalyzed complementation

More recently, nanoparticle-mediated remediation has become one of the leading alternatives to overcome such concerns (Zhang, 2003). Among metal nanoparticles, Palladium nanoparticles (Pd-NPs) have found to be an effective catalyst to remove a wide variety of common environmental contaminants, such as chlorinated organic solvents. Microbially-fabricated palladium nanoparticles (BioPd NPs) are also considered to be an effective and promising bio-based catalyst in removing a wide variety of common environmental contaminants, such as dechlorination of chlorinated organic compounds such as TCE (Hennebel et al., 2009) and PCBs (Baxter-Plant et al., 2003; Windt et al., 2005).

The bioreductive deposition of Pd^0 on the cell wall and in the periplasmic space of *S. oneidensis* has been described in presence of a series of electron donors (H_2, formate, lactate, pyruvate, ethanol) with H_2 and formate being the most efficient donors (Hennebel et al., 2009), BioPd nanoparticles can be separated from the biomass prior use, and catalyze reductive dechlorination of chemical by the formation of H^+ radicals from molecular hydrogen (Windt et al., 2005) (Figure 1.6); many microorganisms can bioprecipitate BioPd nanoparticles (De Corte et al., 2012) and recently the occurrence of this phenomenon was also detected in marine environments, where also catalytic action of BioPd nanoparticles could take place (Hosseinkhani et al., 2014).

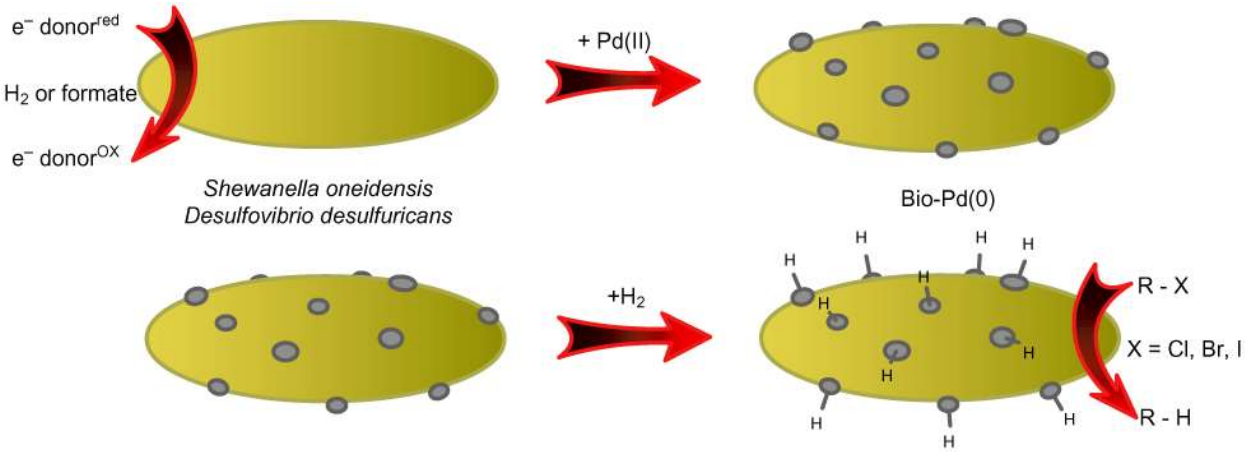

Figure 1.6: Two-step dehalogenation involving bioprecipitation of Pd^0 nanoparticles and further addition of an external hydrogen donor and a one-step dehalogenation (De Corte et al., 2012).

Hydrogen or electron equivalents can also be supplied directly in the sediment using electrochemical techniques. These techniques proved already to be promising and *in situ* feasible for bioremendiation enhancements in case of soils contaminated with organic solvents such as PCE, TCE, TeCA etc. (Aulenta et al., 2006). These methods allow controlling the redox oxygen or hydrogen supply to microorganisms in electrochemically reactive sediment caps. With regards to PCBs, so far only one study has been conducted in sediment, showing a total 40 to 60% decrease in mass of weathered contamination, with oxidation and reduction pathways stimulated more or less selectively depending on the voltage applied (Chun et al., 2013).

1.4.2.4 Bioaugmentation

Another approach for *in situ* bioremediation of PCBs is the supplementation of the microbial community, with a culture enriched in dehalogenating microorganisms, a process called bioaugmentation. Bioaugmentation is necessary, since a critical mass of dehalorespiring microorganisms may be necessary to start dechlorination activities on site; this has been detected in sediment cultures spiked with Aroclor 1260 (Bedard et al. 2007). A crucial point in effectiveness of bioaugmentation approach, which basically depends on the survival of the selected strains in the microbial community and in the real environment; bioaugmentation can be inhibited by a variety of factors, including pH and redox, the presence of other toxic contaminants, concentration and bioavailability of contaminants, or the absence of key co-substrates, thus the key factor to successful bioaugmentation is the selection of the appropriate bacterial strain (Thompson et al., 2005). The dechlorination of weathered Aroclor 1260 can also be stimulated through bioaugmentation with *D. chlorocoercia* DF-1-containing cultures in microcosms (May et al., 2008) but also in mesocosms, where the bioaugmentation was sustained within 120 days, meaning that

inoculated strain did not disappear in the microbial community (Payne et al., 2011). Cultures of *D. mccarty* together with pentachloronitrobenzene as haloprimer could stimulate the dechlorination of weathered Aroclors 1248, 1254, and 1260 in microcosms (Krumins et al., 2009) and enriched cultures from Baltimore Harbor sediment, containing phylotypes SF-1 and DEH-10 could be used in fresh sediment of the same site to stimulate spiked Aroclor 1260 dechlorination (Fagervold et al., 2011). An approach of bioaugmentation with a PCB-dechlorinating enrichment culture was able to further increase a biostimulated dechlorinating community amended with Fe^0, resulting in dechlorination of 2,3,4,5,-CB to 2-CB (Winchell and Novak, 2008).

1.4.2.5 Enhanced bioavailability

A general problem in biodegradation is the availability of a pollutant to the degrading organisms: the more complex and hydrophobic the substance, the harder the biodegradation process. Surfactants are a possible solution to this problem, enhancing solubility and removal of contaminants from the sediment matrix (West and Harwell, 1992). The use of surfactants, of biological and synthetic origin, has been investigated in several studies and the results vary between facilitating, retarding or not affecting the biodegradation at all (Volkering et al., 1997). Surfactants and emulsifiers are amphiphilic compounds containing both a hydrophilic and hydrophobic moiety, therefore able to display a variety of surface activities that allow solubilization of hydrophobic substrates; these compounds are subdivided by their hydrophilic group in cationic (mainly quaternary ammonium compounds), anionic (detergents and fatty acids), zwitterionic (many natural products such as lecithin and casein) and not ionic. Chemical surfactants have the advantage of low price, but are often toxic for biological systems and, hence, are pollutants in their own right. Biosurfactants can be grouped either as low molecular weight (namely glycolipids and lipopeptides) and high molecular weight biosurfactants (complex polymeric molecules) (Rosenberg and Ron, 1999) and generally exhibit higher interfacial tension reduction activities than chemical surfactants. They are non- (or less) toxic and readily biodegradable, properties which are important not only for the natural turnover of hydrophobic substrates by microbes but also for the bioremediation of polluted site (Banat et al., 2000); as such, biosurfactants are investigated for their potential in industrial and environmental applications, related to emulsification, foaming, detergency, wetting, dispersion and solubilization of hydrophobic compounds (Banat et al., 2000).

Marine microorganisms show a variety of metabolic and physiological capabilities, including the production of novel metabolites which are not present in microorganisms of terrestrial origin. (Desai and Banat, 1997). Moreover, the marine environment suffer from anthropogenic pollution with domestic and industrial waste, and large quantities of crude oil, hydrocarbons, oil products and halogenated compounds find their way in the marine ecosystem; under such conditions (but not

exclusively), marine microorganisms offer a rich source of novel surface active compounds. Biosurfactants are usually more interesting than synthetic surfactants as they are highly specific and biodegradable (Mulligan et al., 2001). Low molecular weight biosurfactants have low solubility, thus can be more persistent, while high molecular surfactants have low critical micelle concentration (CMC) and increase apparent hydrocarbon solubility by surrounding them in the hydrophobic cavities of micelles and by decreasing the surface tension between oil and water. Particularly, esopolysaccharides (EPSs) produced by marine microbes plays a crucial role in the removal of organic pollutants (Rosenberg et al., 1979; Zuckerberg et al., 1979), and are applicable for utilization in oil recovery and bioremediation (Ruiz et al., 2006). Surfactants might be toxic for bacteria because either they disrupt the cell membrane interacting with the lipid components, or by reactions with essential proteins of the cell itself; in addition, surfactant molecules can also be degraded as carbon sources, thus impeding their function as bioavailability enhancers. In landfill leachate, sequential anaerobic reductive dechlorination of 2,2',3,4,4',5'-CB was not stimulated by the use of rhamnolipids (Royal et al., 2003). The use of a glycolipid surfactant from marine bacterium *Alcanivorax borkumensis* showed in several tests a strong enhancing effect on microbial metabolization of Aroclor 1248 congeners (Golyshin et al., 1999). Randomly methylated-β-cyclodextrins (RAMEB) were also showed to increase dechlorination in Aroclor 1260-contaminated soils, further amended with biphenyl and inorganic nutrients (Fava et al., 2003a). A further approach is the use of genetically modified organisms for bioaugmentation, which are able to degrade both PCBs and surfactants, so to provide competitive advantage over other microorganisms in the environment and avoid the surfactant's toxic effects, an approach that has been shown to work in Aroclor 1242-contaminated soils (Singer et al., 2000).

1.4.2.6 Sequential anaerobic-aerobic dechlorination

It has been long known that, in nature, reductive dechlorination of highly chlorinated congeners occurs in the anaerobic layers and the low-chlorinated products are then further dechlorinated or even mineralized in the aerobic upper layers of the sediments (Abramowicz, 1995). Studies which combine aerobic and anaerobic dechlorination of PCBs have been conducted in lab scale: transferring a PCB impacted sediments first in PCB dehalorespiring enrichment and then in aerobic *Burkholderia xenovorans* LB400 cultures degraded Aroclors as much as 70 % (Master et al., 2002), and, when engineered to contain the oxygenolitic *ortho* dehalogenation operon (*orb*), the same strain could remove up to 57% of products obtained after an Aroclor 1242 anaerobic dechlorination in freshwater sediment from the Red Cedar River (Rodrigues and Kachel, 2006).

1.4.3 *In situ* challenges for bioremediation

The development of a bioremediation strategy for PCB-contaminated sites is crucial in terms of sustainability and reclamation of the contaminated sediment. The factors that mainly influence the extent and the route of a microbial dechlorination process in sediments are: the microbial community, the bioavailability of the contaminant, the availability of hydrogen or other electron donors, as well as other electron acceptor rather than PCBs (Furukawa and Fujihara, 2008; Wiegel and Wu, 2000). The higher the concentration of highly-chlorinated congener (i.e. degree of chlorination), the lower the bioavailability and bioactivity (Liu et al., 2007).

In situ treatments require sufficient scale-up prior to actual application. It has been estimated that, for bioaugmentation approaches, 1 km^2 of PCB impacted sediment would require 10^{15} cells grown in a culture volume of 10000 l and maximum cell density of 10^8 cells ml^{-1} (Sowers and May, 2013). Another problem is the delivery of dechlorinating bacteria in the field: effective bioaugmentation will require methods for inoculation either by direct injection or deployment on solid particles (Natarajan et al., 1998). Unlike more soluble organohalides such as chloroethenes, whose degradation can be bioaugmented by pumping microorganisms and nutrients into groundwater, PCBs are hydrophobic and tend to become immobilized by adsorption to sediment particles. Effective bioaugmentation of PCB impacted sediments will require a method for inoculating sediment either by direct injection or deployment of microbes onto solid particles. Dehalogenating microorganisms enriched in microbial granules have been proposed as a mean for deployment in sediment (Natarajan et al., 1997; Nollet and Verstraete, 2003).

So far, the only electron acceptors known to support growth of PCB-dechlorinating bacteria are mostly aliphatic and aromatic compounds, which are also persistent organic pollutants, and small fatty acids which, in turn, could stimulate competitors in the environment, resulting in ineffective approaches. Alternatively, substituting more readily biodegraded electron acceptors such as PPBs might be a viable approach for application in the field (Bedard et al., 1998).

Development of a tractable microbial in situ treatment system would provide a cost-effective, and environmentally sustainable alternative to dredging by reducing the health risks associated with sediment disruption, reducing overall energy use, effectively negating the requirement for extensive waste management and obviating the requirement for substantial habitat restoration. Novel means of supplying electron donor to the dechlorinators and electron acceptors, methods to mass culture and harvest PCB dechlorinators, design of molecular tools for monitoring the fate of inocula, and approaches for field deployment are currently under development. While much remains to be done to develop methods to advance degradation further, many of the critical components are in place to

begin field trials and optimize this biotechnology for effective in situ treatment in PCB-impacted environments

1.4.4 *Ex-situ* processes

Dredging is still the most diffused intervention in contaminated areas, especially regarding sediments. It must be conducted extremely slowly with such controls as slit screens and water surface covers to limit resuspension and volatilization (Magar, 2001). However, this seldom happens and sediment beds disturbed during dredging carry increased risk of exposure, destruction of benthic communities, residual risks because of fallbacks or incomplete removal and possible losses during contaminant transport. In addition, treatment techniques usually destroy the sediment, by incineration: these are the major reasons why dredging is not a sustainable technique anymore.

The excavated sediment could then be treated in a controlled bioreactor, where the slurry (i.e. the mixture of solid and water phases) is mixed to keep the solids suspended and the microorganisms in contact with the sediments. Those reactors usually achieve higher biodegradation rates and, after the treatment, the slurry is dewatered and disposed (Khan et al., 2004). Slurry bioreactors are one of the most important types of *ex situ* technique. Treatment of soils and sediments in slurry bioreactors has become one of the best options for the bioremediation of soils polluted by recalcitrant pollutants under controlled environmental conditions (Mueller et al., 1991), because the pollutant depletion depend mainly on the degradation activity of the microorganisms used. The huge advantage of using controlled bioreactors is the complete control over the process, which might also be tailored by application, other than the possibility to apply all of the possible biostimulation/bioaugmentation strategies discussed previously, in a safe and controlled vessel. For example, aerobic treatment combined with a chemical surfactant (quilaya saponin) has highly increased the mineralization of PCB contaminants in a soil slurry reactor (Fava and Gioia, 1998), and the same treatment could be further implemented through the bioaugmentation with *Pseudomonas* sp. CPE1 alone, capable of cometabolising low-chlorinated biphenyls into chlorobenzoic acids, or in co-culture with two chlorobenzoic acid degrading bacteria *Pseudomonas* sp. CPE2 strain and *Alcaligenes* sp. CPE3 (Fava and Bertin, 1999). Using limited aeration it is also possible to achieve a single process in which anaerobic and aerobic processes can proceed in parallel, catalyzing the removal of Aroclor 1242 in granular sludge of up to 81% (Tartakovsky et al., 2001). A completely anaerobic process can also be amended with Fe^0 to enhance dechlorination of spiked Aroclor 1260 in a non-contaminated soil, using anaerobic sludge as inoculum (Long et al., 2014). At the present time, no bioreactors containing PCB-contaminated sediment slurries have been reported.

1.5 Venice lagoon sediment

1.5.1 Contamination history and profile

Until the seventies Porto Marghera, (Venice, Italy) was one of the most important industrial areas in Italy and it has long been considered the main source of contaminants to the Venice Lagoon. The first Industrial Area, bordered by the industrial canals West and North, was built in the 1920s after World War I, through the infilling of 550 ha of marshes. It was followed, after World War II, by the 2nd Industrial Area, mainly located between the South and West industrial canals. Minor canals that cross the industrial area are the Lusore–Brentella, the Brentella and the Salso. This zone is bordered by the canals Vittorio Emanuele III and Malamocco–Marghera, which connect Porto Marghera with Venice and the Adriatic Sea, respectively (Figure 1.7).

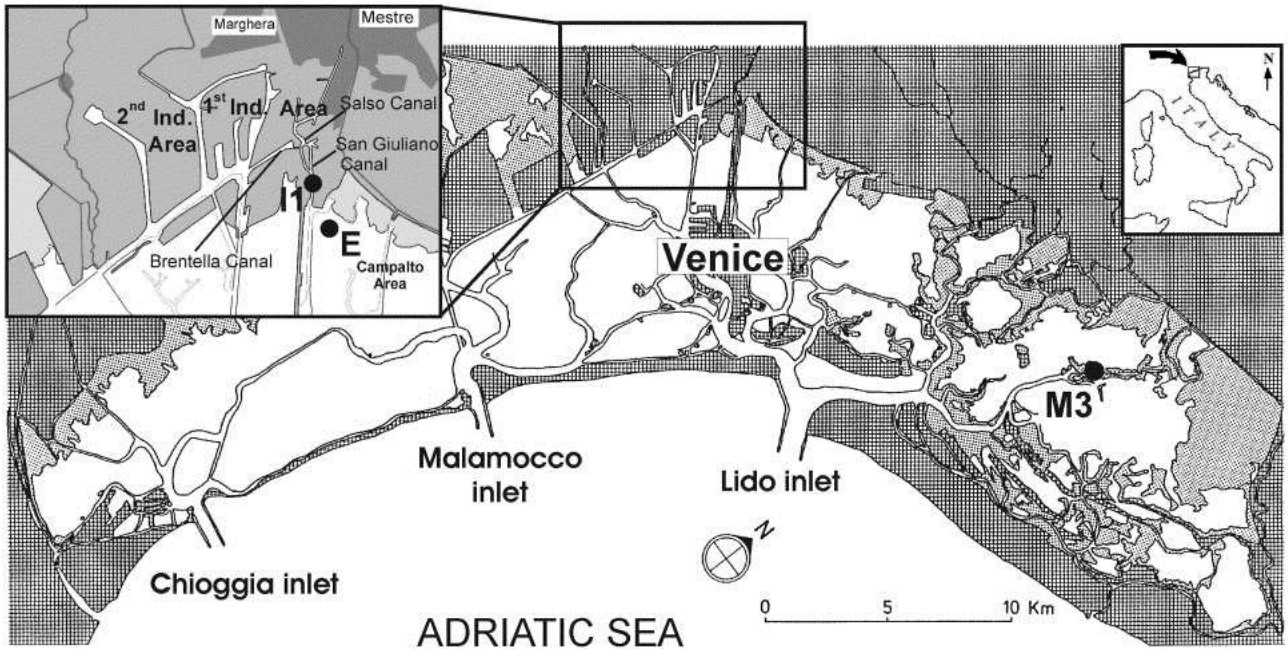

Figure 1.7: air-view of the Venice Lagoon area, with focus on the 1st and 2nd industrial areas of Porto Marghera (Frignani et al., 2005).

Sediments keep memory of the characteristics of the environment at the time of their accumulation and Venice Lagoon sediments can be used to reconstruct the history of pollutant delivery (Frignani et al., 2004). PCDDs and PCDFs were detected and, comparing their distributions and concentration between the Porto Marghera area and the rest of the Venice lagoon area, three sources of contamination can be distinguished: combustion, production of vinyl chlorine monomer and metallurgical industries, with highest values of 64130 ngI-TE (international toxic equivalents)/kg which are anyways decreasing over time thanks to the improvements in waste treatments and the dismissing of many pollutant plants (Bellucci et al., 2000; Frignani et al., 2001b). PCB

contamination was detected in 14 sediment samples from five sites, with a total weight of 4.05 to 239.15 ng (g dry weight)$^{-1}$, values that are mostly connected with the sand content of the sediments, but there is evidence that the pattern of contamination and the ratios between congeners are constant in the Venice Lagoon (Moret et al., 2001). In particular, the analysis of 24 surficial samples demonstrated that the concentration ranges from 44 to 2049 μg (kg dry weight)$^{-1}$; the comparison of the concentrations calculated as sum of congeners and as Aroclor (1254+1260 1:1) and the congener profiles suggest a certain homogeneity of contamination, except for the sediment of the Brentella Canal, where, instead, there is a peculiar concentration of lighter chlorinated PCBs. The main contamination is probably due to the discharge of industrial wastes into the Brentella Canal, within the first Industrial Area, being the rest of lagoon samples 11 to 205 times less contaminated, and influenced by pollutants stored in the canals only occasionally (Frignani et al., 2001a).

1.5.2 PCB dechlorinating microorganisms and activities

Studies on microbial reductive dechlorination in sediments have been conducted mainly in freshwater sediments (see paragraph 0). Among the dehalorespiring *Chloroflexi*, members of *Dehalococcoides* genus were predominantly associated to PCB dechlorination in freshwater systems (Bedard et al., 2006; Bedard et al., 2007; Dudkova et al., 2009; Yan et al., 2006); on the other side, *Dehalococcoidia* belonging to the o-17/DF-1 clade have been mainly linked to this activity in estuarine settings (Cutter et al., 2001; Fagervold et al., 2005; Fagervold et al., 2007; May et al., 2008; Wu et al., 2002b), where salinity and sulfate concentration may shift over time and space from freshwater to marine conditions (Capone and Kiene, 1988).

Conversely, microbial dechlorination has been seldom investigated in marine conditions (Alder et al., 1993; Øfjord et al., 1994), where remarkably higher salinity and sulfate concentrations distinctively drive the main respiration metabolisms, typically favoring sulfate reducers over methanogens (Wang et al., 2012).

It has been showed that PCB dechlorination can happen in PCB-contaminated Venice lagoon sediments, in anaerobic slurry microcosms, where contamination was between 1.5 and 2.5 mgPCBs (kg dry weight)$^{-1}$, showing also that the dechlorinating activities were higher when using site water rather than synthetic marine medium (Fava et al., 2003b). Dechlorination activities were not stimulated by 2,3,4,5,6-CB as haloprimer, were not affected by pasteurization and happened both in presence and in absence of methanogenic activities, but required sulfate-reducing activities, leading to the hypothesis that sulfate reducers were involved in dechlorinating activities in these sediments (Fava et al., 2003c).

Further experiments using biogeochemical conditions close to environmental site condition (which included resuspension of the sediment in site water and anaerobic atmosphere N_2:CO_2 80:20) detected dechlorination activities on dioxin-like PCBs, particularly 3,3',4,4'-CB, 3,3',4,4',5-CB, 2,3',4,4',5-CB, 3,3',4,4',5,5'-CB and 2,3,,3',4,4',5-CB, particularly toxic and recalcitrant to biodegradation, which were converted up to 90% into lower chlorinated congeners without affecting the rate and extension of weathered PCBs (Zanaroli et al., 2006).

These cultures were then transferred in sterile slurry microcosms (first subculturing step) and then further transferred in exponential phase, in sterile slurry microcosms with 6% and 12% w/v sediment, respectively. The analysis of the 16s rRNA genes of the Metagenomic community through Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient gel Electrophoresis (DGGE) revealed a decrease of the methanogenic component of the microbial community (mainly *Methanomicrobiales* and *Methanosarcinales*) through subculturing, with a contemporary enrichment of two sequence phylotypes related to the genus *Sulforovum* and the species *Desulfococcus multivorans* and two *Chloroflexi* phylotypes. Since the dechlorinating activities had the same specificity of the primary cultures but showed a 2-fold increase in dechlorination rate, the disappearance of lag-phase and the increase of sulfate reduction rates, these species are thought to be involved in dechlorination activities. In addition, the dechlorination activities continued after complete sulfate depletion, which poses questions on the effective role of sulfate-reducers in PCB dechlorination (Zanaroli et al., 2010).

Cultures enriched on coplanar dioxin-like biphenyls in 6% w/v sediment slurry cultures (M3C-6) were used as inoculum for a further experiment in which the dechlorination of freshly spiked Aroclor 1254 PCBs was assessed, in biogeochemical condition mimicking those occurring *in situ.* A first subculturing step was prepared, using Aroclor 1254 at a final concentration of 1 g (kg dry sediment)$^{-1}$ (M1), and a second subculturing step was then set up (M2), where i) electron donors were tested through the monthly addition of hydrogen and a mixture of fatty acids (formate, acetate, propionate and butyrate 20 mM each), ii) the contemporary presence of electron donors and antibiotics (vancomycin or ampicillin 100 μg ml^{-1} each) was tested; finally a third subculturing step (M3) was set up and the not amended active controls of the previous step were now tested in presence of the two antibiotics (alone or combined) and the absence of electron donors (Figure 1.8).

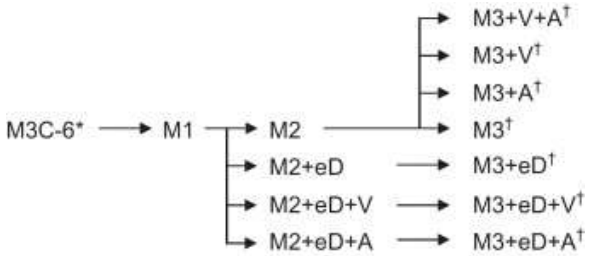

Figure 1.8: Cultures developed in (Zanaroli et al., 2012a). eD: H_2 (5 ml) + formate, acetate, propionate and butyrate (20 mM each); V: vancomycin (100 μg ml^{-1}); A: Ampicillin (100 μg ml^{-1}). (*) PCB dechlorinating culture enriched on coplanar PCBs (Zanaroli et al., 2010) used as inoculum in this study. (†) PCB-free cultures were also established under these conditions.

All the cultures resulting from sub-culturing steps carried out in this study displayed the ability to rapidly and extensively dechlorinate most of the highly chlorinated congeners of the mixture in *meta* and *para* positions, by exhibiting the same selectivity (pattern H') reported previously for the same culture during its enrichment on coplanar PCBs. Since this was observed under in situ-like conditions, it can be speculated that such a prominent activity towards a wide range of PCB congeners with different degree of chlorination and substitution can also be expressed by the culture *in situ* in the reductive dechlorination of weathered PCBs. The persistence of the same dechlorination pattern during the sequential sub-culturing steps on both coplanar PCBs and Aroclor 1254 suggests that the same PCB dechlorinators were involved in the biodegradation of both the synthetic and commercial mixture of PCBs.

Hydrogen and/or short fatty chain acids were employed but resulted in detrimental effects on the dechlorination rate and stimulation of the competing metabolisms, methanogenesis and sulfate reduction. This has suggested that neither sulfate-reducers nor methanogens were involved in PCB dechlorination and that the negative effects on the dechlorination rates depended on the stimulation of non-specialized members of the culture; when methanogenesis and sulfate-reduction activities were inhibited by antibiotics, the dechlorination activities were indeed higher.

A single *Chloroflexi* phylotype (VL-CHL1) having 100% identity with bacterium m-1 was detected under all culture conditions in the sub-cultures amended with PCBs and not in the corresponding PCB-free ones using DGGE analysis of the 16s rRNA genes amplified with specific primers for the dechlorinating members of the phylum *Chloroflexi* (Figure 1.9) (Zanaroli et al., 2012a). The identification of VL-CHL1 as PCB dechlorinator is consistent with the occurrence of PCB dechlorination in the presence of vancomycin and ampicillin, since the majority of the dechlorinating members of the *Chloroflexi* phylum described so far are resistant to such antibiotics. Bacterium VL-CHL1 was the first dechlorinator identified so far in marine sediments and it displays a dechlorination activity and specificity much wider than those of the other *Dehalococcoides*-like bacteria described so far in the literature.

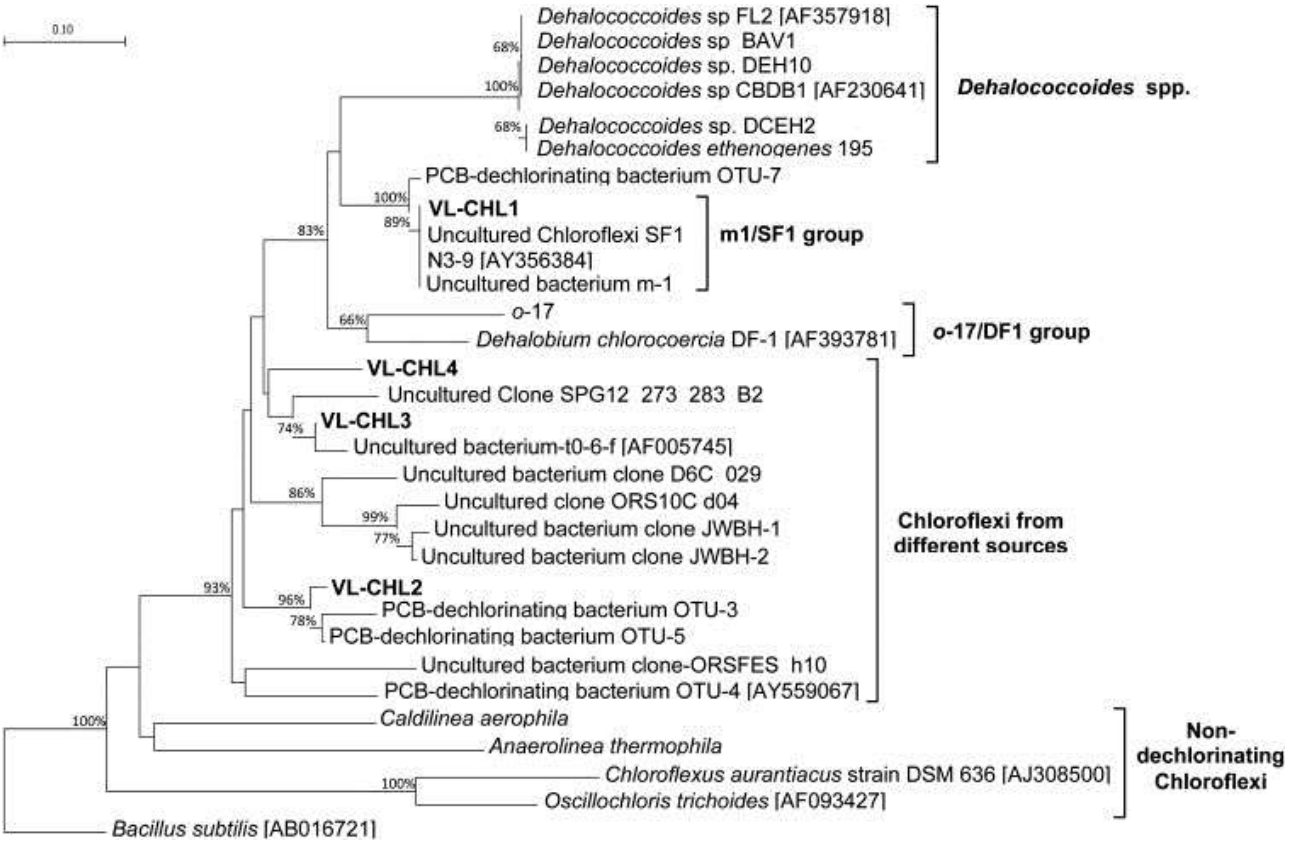

Figure 1.9: Phylogenetic placement of the *Chloroflexi* members of the enriched microbial communities in Zanaroli et al., 2012a.

Primary cultures from the same sediment showed a very long lag-phase, where dechlorination did not occur (ca. 30 weeks). The use of nanoparticles of Fe^0 (nZVI), instead, was shown to stimulate the onset of dechlorination reducing the lag phase by 10 weeks (Zanaroli et al., 2012b). The dechlorination of Aroclor 1254 was modest in active control with a double flanked *meta* and *para* chlorine removal, which did not correspond to the dechlorination process observed previously. Since coplanar congeners are rich in single flanked meta and single or double flanked para chlorines and almost lack double flanked meta chlorines, this might be due to the different mixtures of PCBs that were spiked in the sediment, which might have favored the selection of PCB dechlorinating indigenous bacteria with different dechlorinating specificities.

In the nZVI amended microcosms, instead, the depletion rate was 2-fold higher and lowered the degree of chlorination from 5.04±0.02 to 4.74±0.02 chlorines per biphenyl molecule. PCB dechlorination was directed (at a lower extent) also towards single flanked *meta* and *para* chlorines. The occurrence of different PCB dechlorination processes in sediments has frequently been ascribed to the activity of different indigenous dechlorinating bacteria. NZVI might thus have favored the growth of additional dehalorespiring microbes with different dehalogenation specificities. However, given the very limited dechlorination of Aroclor 1254 PCBs observed in the

nZVI-free microcosms, the broader activity observed in the presence of nZVI might be simply the result of the intensification of already existing PCB dechlorination specificities, which became evident only after a certain dechlorination extent occurred.

The microbial communities evolved with and without nZVI resulted peculiar: nZVI promoted the simultaneous enrichment of sulfate-reducing *Deltaproteobacteria* and *Dehalobium chlorocoercia* DF-1; the latter was also found in the nZVI-free cultures, and its dechlorination activity (May et al., 2008) support the hypothesis that it was the main dechlorinating microorganism in this sediment. Different *Chloroflexi* phylotypes were previously detected in enrichment cultures developed with the same sediment on coplanar PCB congeners and exhibiting a different dechlorination specificity, suggesting that a wider dechlorination of Aroclor 1254 PCBs could be obtained with a proper stimulation of all the sediment indigenous dehalorespiring bacteria.

The information provided was obtained under laboratory biogeochemical conditions close to those occurring in situ, and this allows to speculate that a similar enhancement of PCB dechlorination and effects on the sediment indigenous microbial community might be obtained in situ upon nZVI supplementation of marine sediments of the Venice lagoon. Thus, taken together, these results indicate that supplementation of nZVI particles might be a sustainable effective strategy to intensify PCB reductive dechlorination processes in marine sediments.

2 Aim of this work

Polychlorinated biphenyls (PCBs) are toxic and persistent pollutants and represent a threat for the ecosystem and human health. PCB contamination is widespread in soils and both freshwater and marine sediments. Difficulties in treatment of contaminates sites require the design of more eco-compatible processes based on the microbial degradation, which is the most promising strategy.

Microbial reductive dechlorination processes are known and several members of the phylum *Chloroflexi* were identified for their capability of dehalorespiring PCBs, some of which were also isolated; some PCB reductive dehalogenase genes were also identified. Information on *in situ* dechlorination activities is limited and isolation methods for these strains are extremely demanding. In addition, much less is known about PCB dechlorination in marine sediments, where the different condition and the different microbial communities cause different metabolic dynamics from the freshwater sediments, usually target of such studies.

Bioremediation approaches for PCB-contaminated sediments are difficult because of the dearth of dehalorespiring bacteria in the environment, due to their low growth rates; additionally, dehalorespiring members of the community face strong competition for hydrogen sources against other species, which in marine environments are mainly methanogens and sulfate-reducers; also, due to their hydrophobic nature, bioavailability of PCBs is extremely low as they tend to adhere to the sediment matrix where the dehalorespiring species have no access for degradation; finally, low-chlorinated congeners, result of the microbial reductive dechlorination process of highly chlorinated mixtures, are often toxic for the dehalorespiring species itself and need to be further degraded to biphenyl or completely mineralized.

This work was totally supported by the EU through the project ULIXES ‘‘Unravelling and exploiting Mediterranean sea microbial diversity and ecology for xenobiotics’ and pollutants’ clean up’’ (GA266473) (Daffonchio et al., 2012; Daffonchio et al., 2013). The rationale of this project considers that a plethora of microbial consortia inhabits the diverse metabolic niches of the Mediterranean Sea, that are naturally or accidentally exposed to organic pollutants or heavy metals: from the characterization and recovery of this diversity, the exploitation of these microorganisms and/or their enzymes could spring novel biotechnological processes for bioremediation purposes. ULIXES is using different integrated strategies for recovering novel microbial resources for bioremediation, which include establishment of collections of microbes capable of degradation of selected pollutants, characterization of the microbial communities at the metagenomic, proteomic and metabolomics level, including molecular culture-independent techniques. The identified

microbial resources could then be tested for enhancing and tailoring bioremediation techniques, in stepwise applications from lab to pilot and field scale. The sites considered in the project include the Elefsina Gulf (Greece), oil-polluted sites in Egypt, Tunisia (Zrafi-Nouira et al., 2009) and Morocco, the seashore of Aqaba Gulf (Jordan) as a model of a global warming-impacted Mediterranean Sea and two contaminated sited in Italy: Milazzo harbor, contaminated by hydrocarbons (Yakimov et al., 2005) and the Venice Lagoon, contaminated by PCBs (Frignani et al., 2001a).

Previous studies detected microbial reductive dechlorination activities in marine sediments of the Venice Lagoon, historically contaminated by PCBs, and phylotypes carrying out these activities were detected and dechlorination activities were stimulated (Fava et al., 2003b;c; Zanaroli et al., 2006;2010;2012a;b).

The aim of this work was to determine how widespread the dechlorination potential is in the Venice Lagoon area, with the detection of dechlorinating phylotypes and the design of bioremediation enhancement strategies for the sediments of the area. For this reason, six sediments dredged from different areas of the Venice Lagoon were resuspended in biogeochemical conditions mimicking those occurring *in situ* (same site water, anaerobic atmosphere, 28 °C, static incubation in the dark) and dechlorination activities were monitored after spiking the sediment with a commercial PCB mixture, Aroclor 1254. Successively, microorganisms carrying out these microbial activities were detected through PCR-DGGE and the association with dechlorination processes was confirmed by qPCR assays. After the obtainment of enriched dechlorinating cultures, this work aimed to enhance the dechlorination activities through the selection of selective electron donor(s) able to stimulate the dehalogenating population in enriched slurry sediment cultures, and complementation with a nanometallic zerovalent catalyst, to remove lower-chlorinated congeners and/or accelerate the microbial dechlorination process. In addition, biosurfactants from marine oil-degrading mixed cultures and isolates were obtained. The results of these experiments were then further exploited in bioremediation lab-scale test, where the aim was to solve the major problems of PCB microbial reductive dechlorination activities, namely lack of dehalorespirers in the microbial community, lack of hydrogen sources and low bioavailability of the pollutant, through bioaugmentation, biostimulation and bioavailability enhancement approaches, respectively, and combination thereof. The bioremediation lab-scale tests were performed in real sediments of the Venice Lagoon with its indigenous microbial community, in order to simulate at best real bioremediation processes.

The knowledge of this work could then be used to further implement future research for designing real scale-up bioremediation processes and develop tools for the detection of the microbial dechlorination potential in the contaminated area.

3 Materials and Methods

3.1 Venice lagoon sediment samples

Six samples of dredged surface sediment collected from different locations of the first industrial area of Porto Marghera (Venice lagoon, Italy), where widespread PCB contamination was previously reported to occur (Moret et al., 2005) were used in this study, along with the seawater collected from the same area. Sediments and water samples were anaerobically stored in sealed jars at 4°C until use. Sediments were preliminarily characterized in terms of dry weight, density, PCB contamination, and total organic carbon, according to the procedures described below.

The six sediments (A, B1, B2, C, D and E) where characterized by PCB contamination in the range 0.2 (sediment B2) - 3.3 (sediment D) mg PCBs (kg dry sediment)$^{-1}$ (Table 3.1). PCB contamination could not be attributed to any specific commercial mixture of PCBs, in particular in the less contaminated sediments (namely A, B2, C and E), where a very limited number of PCB congeners (7 to 9) potentially deriving from different mixtures was detected (Figure 9.1). However, sediment A was contaminated mainly by low and medium chlorinated congeners, while sediments B2, C and E mainly by highly chlorinated and to less extent by medium chlorinated ones. Conversely, sediments B1 and D were contaminated by a higher number of different congeners (13 and 19, respectively) that were chlorinated mainly on 6 to 8 positions of the biphenyl ring similarly to Aroclor 1260 PCBs (Figure 9.1). However, some of the most abundant PCBs in Aroclor 1260, such as 2,2',3,4',5',6-CB, 2,2',4,4',5,5'-CB and 2,2',3,4,4',5'-CB (which account for approximately 25 wt% of the commercial mixture) were not detected, suggesting that Aroclor 1260 was not the main source of the contamination. The sediment total organic carbon ranged from 1.6 (sediment B2) to 6.1 (sediment D) gC (kg dry sediment)$^{-1}$ (Table 3.1).

Sediments	**PCB contamination**					**Total organic carbon**
	Total concentration *mg PCBs (kg dry sediment)$^{-1}$*	Di- and Tri-chlorinated congeners *% wt*	Tetra- and Penta-chlorinated congeners *% wt*	Hexa- and Hepta-chlorinated congeners *% wt*	Octa- and Nona-chlorinated congeners *% wt*	*gC (kg dry sediment)$^{-1}$*
A	0.4±0.1	0.26%	28.08%	5.02%	66.64%	-
B1	1.5±0.8	0.00%	0.75%	5.96%	93.29%	1.85±0.2
B2	0.2±0.0	0.00%	5.11%	10.36%	84.54%	1.58±0.2
C	1.3±0.2	0.02%	1.52%	3.13%	95.33%	3.31±0.0
D	3.3±2.8	0.00%	0.91%	27.27%	71.81%	6.12±0.0
E	0.6±0.1	0.00%	3.00%	6.66%	90.34%	3.79±0.4

Table 3.1 Sediment characterization in terms of PCB total concentration, congeners distribution and total organic carbon. Values are average of replicate analysis and errors represent standard deviation.

Sediments were also contaminated by heavy metals. Metal analysis was conducted by Dr. Guerra Roberta at the Centro Interdipartimentale di Ricerca per le Scienze Ambientali (CIRSA) of Ravenna, University of Bologna, Italy. The values of chromium and nickel measured in sediments varied from 33 to 58 mg/kg and from 19.3 to 33.5 mg/kg respectively (Table 3.2); these values are similar to the typical sediments of the Adriatic sea and the Venice Lagoon. Values of zinc, lead, cadmium and copper were instead higher than common background values, but in accordance to other contamination reports of the industrial area of Porto Marghera.(Bellucci et al., 2002; Zonta et al., 2007)

Sediments	**Cd**	**Cu**	**Cr**	**Ni**	**Pb**	**Zn**
A	2.2	118	35	19	88	401
B1	3.3	391	33	22	249	827
B2	2.2	128	58	35	148	459
C	5.2	330	44	29	179	1043
D	5.4	285	50	23	161	842
E	3.7	184	46	24	121	605
Err %	*7.7*	*5.5*	*6.4*	*6.7*	*6.3*	*3.3*

Table 3.2: Measured concentrations of cadmium, copper, chromium, nickel, lead and zinc (mg /kg) in six sediments of the Venice lagoon. Errors are calculated using a standard sample in a reference material MESS-3 (Marine Sediment, "sewage sludge IAEA-CU-2010-02)

Two marine water aliquots were used in this study is from the water column overlying the dredged sediments in and were collected at the same moment, stored in glass jars at 4°C after degassing with nitrogen with an Hungate apparatus (Hungate, 1969). Water contained no PCBs and its main characteristics are reported in Table 3.3.

Water	**pH**	**Anions concentration (g/l)**				
		Cl^-	**NO_2^-**	**NO_3^-**	**$PO_4^=$**	**$SO_4^=$**
C	8.03±0.01	28.97±2.16	0.00±0.0	0.05±0.04	0.02±0.03	4.34±0.78
E	7.91±0.02	21.39±1.80	0.00±0.00	0.07±0.05	0.01±0.02	3.00±0.63

Table 3.3: Water characterization in terms of pH and anion concentrations. Values arte average of replicate analysis and errors represents standard deviations.

3.2 Sediment cultures preparation and sampling

3.2.1 Primary microcosms

A set of four 100 ml anaerobic slurry microcosms (2 biologically active and 2 sterile controls) was prepared for each sediment as follows: 500 ml of seawater were purged with filter-sterilized O_2-free N_2:CO_2 (70:30) for 2 h under vigorous magnetic stirring in a 1 liter Erlenmeyer flask. Sediment was added to obtain a 20% (dry w/v) suspension and the resulting slurry was mixed and purged as described above for 2 additional hours. Four 100 ml aliquots of slurry were then withdrawn, while mixing and purging, and transferred into 120 ml serum bottles equipped with a magnetic bar; slurries were subjected to magnetic stirring and purging with filter-sterilized O_2-free N_2:CO_2 (70:30) for 15 min before being sealed with Teflon-coated butyl stoppers and aluminum crimp sealers. Two bottles were used to prepare sterile controls by autoclave sterilization at 121 °C for 1 h on 3 consecutive days with static incubation at 28 °C between each autoclaving treatment. Given the limited number and low concentration of PCB congeners occurring in each sediment, microcosms were spiked with Aroclor 1254 (20 g/l stock solution in acetone) at a final concentration of 1 g of PCBs (kg dry sediment)$^{-1}$, in order to better assess the PCB dechlorination potential of the sediment indigenous microbial communities. All microcosms were incubated statically in the dark at 28 °C and periodically sampled at weeks 0, 6, 11, 14, 18, 23, 27 and 31 according to the procedure described in (Fava et al., 2003c) to analyze: i) the volume and composition of the biogas produced in the microcosm headspace, ii) the pH and redox potential of the slurry, iii) the type and concentrations of PCBs in the sediment, iv) the concentration of sulfates in the water phase, v) the structure and composition of the microbial community. Aliquots of 2 ml sediment slurry were withdrawn at each sampling point under O_2-free N_2:CO_2 (70:30) filter-sterilized gas purging and vigorous stirring; of these, two aliquots of 0.3 ml were used for PCB extraction and analysis (immediately), while the remaining was frozen at -20 °C for DNA extraction and anion analysis until use.

3.2.2 Secondary cultures stimulated with electron donors

A set of 70 ml anaerobic slurry microcosms was prepared as follows: 26 g of sediment D were aliquoted in 17 serum bottles (125 ml) and 19 g of sediment D were aliquoted in other two serum bottles (75 ml). All bottles contained a magnetic bar and were flushed with filter-sterilized O_2-free N_2:CO_2 (70:30) for 15 minutes before being sealed with Teflon-coated butyl stoppers and aluminum crimp sealers. All bottles were autoclave sterilized at 121 °C for 1 h on 3 consecutive days with static incubation at 28 °C between each autoclaving treatment. 2 l of seawater were centrifuged for 10', 7000 rpm to remove particulate matter, and then filter-sterilized and degassed with filter-

sterilized O_2-free N_2:CO_2 (70:30) for 2 h under vigorous magnetic stirring in a 5 l Erlenmeyer flask. All 125 ml serum bottles were then filled with filter-sterilized degassed marine water to a total volume of 70 ml, while the remaining 75 ml serum bottles were filled up to a total volume of 50 ml. Successively, only 125 ml serum bottles were inoculated with 3,5 ml slurry (5% v/v) from the slurry cultures of Sediment D at the end of dechlorination activity (see section 4.1.1). All bottles were purged with filter-sterilized O_2-free N_2:CO_2 (70:30) for 15 minutes. Microcosms were then spiked with Aroclor 1254 (20 g/l stock solution in acetone) at a final concentration of 1 g of PCBs (kg dry sediment)$^{-1}$, in order to better assess the PCB dechlorination potential of the sediment indigenous microbial communities.

The following amendment conditions were then set up:

- Unamended controls: active controls, no amendment (triplicate microcosms)
- Pyruvate: active, amended every 2 weeks with sodium pyruvate filter-sterilized stock solution (200 g/l) to a final concentration of 2.5 mM (triplicate microcosms)
- Lactate: active, amended every 2 weeks with sodium L-lactate filter-sterilized stock solution (200 g/l) to a final concentration of 2.5 mM (triplicate microcosms)
- Molasses: active, amended every 2 weeks with molasses filter-sterilized diluted stock solution (500 g/l) to a final concentration of 460 mg/l (triplicate microcosms)
- Cheese whey: active, amended every 2 weeks with cheese whey filter-sterilized stock solution to a final concentration of 2.7% v/v (triplicate microcosms)
- Polylactate: active, amended at the beginning of incubation with polylactate PLA 4042D to a final concentration of 900 mg/l (duplicate microcosms)
- Sterile: sterile controls, no amendment (duplicate microcosms)

The characteristics of molasses, cheese whey and polylactate are listed in Table 3.4.

Cheese whey		**Molasses**		**PLA 4042D**	
pH	4.63	Sucrose (% p/p)	45.9	L Isomer (%)	95,8
COD	50.63±0.81	Lactic acid (% p/p)	4.94	D Isomer (%)	4,2
Carbohydrates (g/l)	8.61±0.54	Acetic acid (% p/p)	1.5	Mw (kDa)	$2{,}0 \cdot 10^5$
Proteins (g/l)	2.78±0.22			Polydispersity index	1,5
Acetic acid (g/l)	1.35			Tg (°C)	60
Butyric acid (g/l)	191.77				
Caproic acid (g/l)	156.29				

Table 3.4: Characteristics of Molasses, Cheese Whey and Polylactate used in this work

All microcosms were incubated statically in the dark at 28°C and periodically sampled at weeks 2, 4, 6, 8, 10, 13, 16 and 19 according to the procedure described in (Fava et al., 2003c) to analyze: i)

the volume and composition of the biogas produced in the microcosm headspace, ii) the pH and redox potential of the slurry, iii) the type and concentrations of PCBs in the sediment, iv) the concentration of sulfates in the water phase. Aliquots of 2 ml sediment slurry were withdrawn at each sampling point under O_2-free N_2:CO_2 (70:30) filter-sterilized gas purging and vigorous stirring; of these, two aliquots of 0.3 ml were used for PCB extraction and analysis (immediately), while the remaining was frozen at -20 °C for anion analysis until use.

3.2.3 Evaluation of catalytic dechlorination activity of BioPd in marine water and marine sediment slurries

BioPd nanoparticles (BioPd NPs) were provided as dry pellet from the LabMET Laboratory of Microbial Ecology and Biotechnology, University of Ghent, Gent, Belgium, partner of the project. A time-series of sacrificial batch experiments were conducted to test the dechlorination activity of BioPd towards the commercial mixture of PCBs Aroclor 1254 under different marine conditions including marine water and slurries of sandy and muddy sediment, suspended in marine water. Experiments on marine water were performed in 15 ml serum bottles filled with 50 mg/l of BioPd NPs suspended in 5 ml of sterile marine water. 100% v/v of the headspace was replaced with hydrogen gas after three repeated cycles of N_2 overpressure and vacuum, followed by spiking with 100 µl of an Aroclor 1254 stock solution in methanol (100 mg/l) to obtain a final concentration of 2 mgPCBs/l.

A marine sandy sediment collected from the Westerschelde from the harbor of Antwerp, Belgium (sandy sediment), and a marine sediment collected from the first industrial area Porto Marghera, Venice lagoon, Italy (muddy sediment) were used. Sediment slurries were carried out in 25 ml serum bottles containing 5 ml of a 20% w/v sediment suspension in marine water amended with BioPd NPs at the final concentration of 50 mg (kg dry sediment)$^{-1}$. 100% v/v of the headspace was replaced with hydrogen gas after repeated cycles of N_2 overpressure and vacuum. Bottles were then spiked with 500 µl of an Aroclor 1254 stock solution in methanol (100 mg L^{-1} of PCBs) to obtain a final PCB concentration of 50 mg (kg dry sediment)$^{-1}$. BioPd NPs free controls with added H_2 were set up under all conditions tested. Bottles were incubated for 48 h at 28 °C with mild shaking (120 rpm). Two bottles of the marine water set were sacrificed after 0, 1, 3 and 8 h of incubation and two bottles of the sediment slurry sets after 0, 1, 3, 8, 24, 48 and 72 h of incubation to extract and analyze Aroclor 1254 PCBs.

3.2.4 Secondary cultures coupled with Pd^0-catalyzed chemical dechlorination

A set of anaerobic slurry microcosms was prepared as follows: 26 g of sediment E were aliquoted in 12 serum bottles (125 ml) and 19 g of sediment E in other 12 serum bottles (125 ml). All bottles contained a magnetic bar and were flushed with filter-sterilized O_2-free N_2:CO_2 (70:30) for 15 minutes before being sealed with Teflon-coated butyl stoppers and aluminum crimp sealers. All bottles were autoclave sterilized at 121 °C for 1 h on 3 consecutive days with static incubation at 28 °C between each autoclaving treatment. 2 l of seawater were centrifuged for 10', 7000 rpm to remove particulate matter, and then filter-sterilized and degassed with filter-sterilized O_2-free N_2:CO_2 (70:30) for 2 h under vigorous magnetic stirring in a 5 l Erlenmeyer flask. All bottles were then willed with filter-sterilized degassed marine water to achieve a dilution of the sediment in water of 20 % (kg dry weight)/l. Successively, 18 serum bottles were inoculated with 5% v/v sediment slurry from mixed slurry cultures of Sediment E at the end of dechlorination activity (see section 4.1.1). All bottles were purged with filter-sterilized O_2-free N_2:CO_2 (70:30) for 15 minutes. All 70 ml microcosms and six 50 ml microcosms were then spiked with Aroclor 1254 (20 g/l stock solution in acetone) at a final concentration of 1 g of PCBs (kg dry sediment)$^{-1}$, in order to better assess the PCB dechlorination potential of the sediment indigenous microbial communities.

The following conditions were tested in active secondary slurry cultures, spiked with Aroclor 1254 at the final concentration of 1 g/kg dry sediment^{-1}

- Unamended controls: active controls, with PCBs, no amendment (triplicate 70 ml microcosms)
- Hydrogen: active, with PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking (triplicate 70 ml microcosms)
- Hydrogen + BioPd 5 mg/kgdw: active, with PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 5 mg/kg dry sediment^{-1} (triplicate 70 ml microcosms)
- Hydrogen + BioPd 50 mg/kgdw: active, with PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 50 mg/kg dry sediment^{-1} (triplicate 70 ml microcosms)

The following conditions were tested in non-spiked secondary slurry cultures

- Hydrogen: active, without PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking (duplicate 50 ml microcosms)

- Hydrogen + BioPd 5 mg/kgdw: active, without PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 5 mg/kg dry sediment^{-1} (duplicate 50 ml microcosms)
- Hydrogen + BioPd 50 mg/kgdw: active, without PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 50 mg/kg dry sediment^{-1} (duplicate 50 ml microcosms)

The following conditions were tested in sterile slurries, spiked with Aroclor 1254 at the final concentration of 1 g/kg dry sediment^{-1}

- Unamended controls: sterile controls, with PCBs, no amendment (duplicate 50 ml microcosms)
- Hydrogen: sterile, with PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking (duplicate 50 ml microcosms)
- Hydrogen + BioPd 5 mg/kgdw: sterile, with PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 5 mg/kg dry sediment^{-1} (duplicate 50 ml microcosms)
- Hydrogen + BioPd 50 mg/kgdw: sterile, with PCBs, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 50 mg/kg dry sediment^{-1} (duplicate 50 ml microcosms)

All microcosms were incubated statically in the dark at 28°C and periodically sampled at weeks 0, 6, 9, 14, and 18 according to the procedure described in (Fava et al., 2003c) to analyze: i) the volume and composition of the biogas produced in the microcosm headspace, ii) the pH and redox potential of the slurry, iii) the type and concentrations of PCBs in the sediment, iv) the concentration of sulfates in the water phase, v) the structure and composition of the microbial community. Aliquots of 2 ml sediment slurry were withdrawn at each sampling point under O_2-free N_2:CO_2 (70:30) filter-sterilized gas purging and vigorous stirring; of these, two aliquots of 0.3 ml were used for PCB extraction and analysis (immediately), while the remaining was frozen at -20 °C for DNA extraction and anion analysis until use.

A parallel set of anaerobic slurry microcosms was prepared as follows: 8 ml of sediment slurry from mixed slurry cultures of Sediment at the end of dechlorination activity, where the average degree of chlorination was 4.1 and 8 ml of sediment slurry from sediment E sterile controls where

dechlorination activity didn't happen (see section 4.1.1), were aliquoted in pre-autoclaved serum bottles (50 ml), with a magnetic bar, degassed filter-sterilized O_2-free N_2:CO_2 (70:30) for 15 minutes before being sealed with Teflon-coated butyl stoppers and aluminum crimp sealers. The following amendment conditions were then set up:

- Hydrogen: active, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking (duplicate microcosms)
- Hydrogen + BioPd 5 mg/kgdw: active, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 5 mg/kg dry sediment^{-1} (duplicate microcosms)
- Hydrogen + BioPd 50 mg/kgdw: active, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 50 mg/kg dry sediment^{-1} (duplicate microcosms)
- Sterile + hydrogen: sterile, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking (duplicate microcosms)
- Sterile + hydrogen + BioPd 5 mg/kgdw: sterile, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 5 mg/kg dry sediment^{-1} (duplicate microcosms)
- Sterile + Hydrogen + BioPd 50 mg/kgdw: sterile, atmosphere replaced with H_2:CO_2 80:20 through three cycles of vacuum and 1 bar pressure, under vigorous shaking, amended at the beginning of incubation with BioPd NPs resuspended in marine water (500 mg/l) to a final concentration of 50 mg/kg dry sediment^{-1} (duplicate microcosms)

All microcosms were incubated statically in the dark at 28°C and periodically sampled at weeks 0, 6, 9, 14, and 18 according to the procedure described in (Fava et al., 2003c) to analyze: i) the volume and composition of the biogas produced in the microcosm headspace, ii) the type and concentrations of PCBs in the sediment. Aliquots of 0.3 ml were used for PCB extraction and analysis (immediately).

3.2.5 Scale-up secondary cultures

To scale up the inoculum culture, a set of five anaerobic slurry microcosms (four active and one killed control) was prepared as follows: 37 g of sediment D were aliquoted in serum bottles (125

ml). All bottles contained a magnetic bar and were flushed with filter-sterilized O_2-free N_2:CO_2 (70:30) for 15 minutes before being sealed with Teflon-coated butyl stoppers and aluminum crimp sealers. All bottles were autoclave sterilized at 121 °C for 1 h on 3 consecutive days with static incubation at 28 °C between each autoclaving treatment. 500 ml of seawater were centrifuged for 10', 7000 rpm to remove particulate matter, and then filter-sterilized and degassed with filter-sterilized O_2-free N_2:CO_2 (70:30) for 2 h under vigorous magnetic stirring in a 1 liter Erlenmeyer flask. All 125 ml serum bottles were then filled with filter-sterilized degassed marine water to a total volume of 100 ml. Successively, serum bottles were inoculated with 5 ml slurry (5% v/v) from the slurry cultures of at the end of dechlorination activity (see section 4.1.1). All bottles were purged with filter-sterilized O_2-free N_2:CO_2 (70:30) for 15 minutes. Microcosms were then spiked with Aroclor 1254 (20 g/l stock solution in acetone) at a final concentration of 1 g of PCBs (kg dry sediment)$^{-1}$. All microcosms were incubated statically in the dark at 28°C and periodically sampled at weeks 0, 4, 10, 13, 18, 22, 26 according to the procedure described in (Fava et al., 2003c) to analyze: i) the volume and composition of the biogas produced in the microcosm headspace, ii) the pH and redox potential of the slurry, iii) the type and concentrations of PCBs in the sediment. Aliquots of 2 ml sediment slurry were withdrawn at each sampling point under O_2-free N_2:CO_2 (70:30) filter-sterilized gas purging and vigorous stirring; of these, two aliquots of 0.3 ml were used for PCB extraction and analysis (immediately), while the remaining was frozen at -20 °C for anion analysis until use.

3.2.6 Reactors and microcosms for lab-scale bioremediation tests

A set of eight 400 ml anaerobic slurry reactors and 18 anaerobic slurry microcosms was prepared as follows: 157 g of Sediment E were aliquoted in 4 baffled bottles (1 l volume) and 142 g in other 4 baffled bottles of the same size, while 19 g were aliquoted in 10 serum bottles (75 ml) and 35 g in other 8 serum bottles of the same size. 4 l of seawater were centrifuged for 10', 7000 rpm to remove particulate matter and then filter-sterilized and degassed with filter-sterilized O_2-free N_2:CO_2 (70:30) for 2 h under vigorous magnetic stirring in two 5 l Erlenmeyer flasks. All bottles were then filled with filter-sterilized degassed marine water to achieve a dilution of the sediment in water of 20 % (kg dry weight)/l. Baffled flasks were equipped with GL45 screw-caps, with one port equipped with GL14 screw-cap with Teflon-coated silicon septum for biogas sampling and a second port equipped with GL14 screw-cap with Teflon-coated tubing (~5 ml volume) and three-way glass valves with Teflon tap for slurry sampling; serum bottles were equipped with Teflon-coated butyl stoppers and aluminum crimp sealers. All bottles were purged with filter-sterilized O_2-free N_2:CO_2 (70:30) for 1 h and two serum bottles were autoclave sterilized at 121 °C for 1 h on 3 consecutive days with static incubation at 28 °C between each autoclaving treatment. All bottles were then

spiked with Aroclor 1254 (20 g/l stock solution in acetone) at a final concentration of 500 mg of PCBs (kg dry sediment)$^{-1}$. The following conditions were then set up:

- Active control: actual sediment of the Venice lagoon in site water with no amendments in one 400 ml reactor and two replicate 50 ml microcosms
- Bioaugmentation: sediment of the Venice lagoon in site water amended with 10% v/v inoculum from sediment slurry scale-up cultures at the end of incubation, (see section 4.5.1), in one 400 ml reactor and two replicate 50 ml microcosms
- Biostimulation: sediment of the Venice lagoon in site water amended with sodium L-lactate filter-sterilized stock solution (200 g/l) to a final concentration of 5 mM every two weeks, in one 400 ml reactor and two replicate 50 ml microcosms.
- Bioavailability enhancement: sediment of the Venice lagoon in site water amended with 5% v/v with a dialyzed crude supernatant containing biosurfactants (see sections 3.5 and 4.4.2), in one 400 ml reactor and two replicate 50 ml microcosms
- Bioaugmentation + biostimulation (combined approach): sediment of the Venice lagoon in site water amended at the beginning of incubation with 10% v/v inoculum from sediment slurry scale-up cultures the end of incubation (see section 4.5.1), and with sodium L-lactate filter-sterilized stock solution (200 g/l) to a final concentration of 5 mM every two weeks, in one 400 ml reactor and two replicate 50 ml microcosms
- Bioaugmentation + bioavailability (combined approach): sediment of the Venice lagoon in site water amended at the beginning of incubation with 10% v/v inoculum from sediment slurry scale-up cultures the end of incubation (see section 4.5.1), together with 5% v/v of a dialyzed crude supernatant containing biosurfactants molecules (see sections 3.5 and 4.4.2), in one 400 ml reactor and two replicate 50 ml microcosms
- Biostimulation + bioavailability (combined approach): sediment of the Venice lagoon in site water amended 5% v/v with a dialyzed crude supernatant containing biosurfactants molecules (see sections 0 and 4.4.2) at the beginning of incubation and with sodium L-lactate filter-sterilized stock solution (200 g/l) to a final concentration of 5 mM every two weeks, in one 400 ml reactor and two replicate 50 ml microcosms
- Bioaugmentation + biostimulation + bioavailability (combined approach): sediment of the Venice lagoon in site water amended at the beginning of incubation with 10% v/v inoculum from sediment slurry scale-up cultures the end of incubation (see section 4.5.1), together with 5% v/v of a dialyzed crude supernatant containing biosurfactants molecules (see sections 3.5 and 4.4.2) and with sodium L-lactate filter-sterilized stock solution (200 g/l) to

a final concentration of 5 mM every two weeks, in one 400 ml reactor and two replicate 50 ml microcosms

- Sterile controls: sediment of the Venice lagoon in site water, autoclave-sterilized with no amendments, in two replicate 50 ml microcosms.

All approaches were tested at room temperature (between 20 °C and 25 °C); all 400 ml reactors were mechanically shaken at 150 rpm, while microcosms were incubated statically. All cultures were periodically sampled at weeks 0, 2, 4, 7, 11, and 14 according to the procedure described in (Fava et al., 2003c) to analyze: i) the volume and composition of the biogas produced in the microcosm headspace, ii) the pH and redox potential of the slurry, iii) the type and concentrations of PCBs in the sediment, iv) the concentration of sulfates in the water phase, v) the structure and composition of the microbial community. Aliquots of 10 ml sediment slurry were withdrawn from the reactors at each sampling point under O_2-free N_2:CO_2 (70:30) filter-sterilized gas purging and vigorous stirring, of which the first 5 ml were discarded; of these, two aliquots of 0.3 ml were used for PCB extraction and analysis (immediately), while the remaining was frozen at -20 °C for DNA extraction and anion analysis until use. Aliquots of 2 ml sediment slurry were withdrawn from microcosms at each sampling point under O_2-free N_2:CO_2 (70:30) filter-sterilized gas purging and vigorous stirring; of these, two aliquots of 0.3 ml were used for PCB extraction and analysis (immediately), while the remaining was frozen at -20 °C for DNA extraction and anion analysis until use.

3.3 PCB extraction and analytical procedures

PCB extraction was performed in duplicate from each replicate culture. PCBs were batch extracted from 0.3 ml aliquots of sediment slurry with 3 volumes (0.9 ml) of hexane:acetone (9:1), 0.150 ml of elemental mercury and 10 µl of a 40 mg L^{-1} stock solution of octachloronaphtalene (OCN) in hexane as internal standard according to Fava et al. (2003). The qualitative and quantitative analyses of the extracted PCBs were performed with a gas chromatograph (Agilent 6890 N) equipped with a HP-5 capillary column (30 m by 0.25 mm), a ^{63}Ni electron capture detector and a 6890 series II automatic sampler (Agilent Technologies, Milan, Italy) under the following analytical conditions (Fava et al., 2003): initial temperature 60 °C; isotherm for 1'; first temperature ramp at 40 °c/min up to 140 °C; isotherm for 2'; second temperature ramp of 1.5 °C/min up to 185 °C; third temperature ramp at 4.5 °C/min up to 275 °C; isotherm for 5'; splitless injection; injector temp 250 °C; detector temp (ECD) 320 °C, carrier gas nitrogen, constant flow at 1.5 ml/min; injected volume 3 µl. Qualitative analysis of the freshly spiked PCBs and of their possible dechlorination products was performed by comparing the retention time (relative to OCN) of the peaks obtained from the analyses of the sediment organic extracts with those of PCBs occurring in Aroclor 1242 and Aroclor 1254 PCB standard mixtures analyzed under identical conditions. Quantitative analyses of each PCB congener were performed by using the GC-ECD response factor of each target PCB obtained through linear five-points calibration curves of Aroclors (in the range 1.0 to 100.0 mg l^{-1}). Standards were identified with confrontation between the analytical peaks and the chromatograph profiles reported by (Schulz et al., 1989). Response factors were calculated for each peak, which was identified using the relative retention time to OCN as internal standards, and were verified monthly. Coeluted congeners were considered present at equal quantities.

The degree of chlorination of the PCB mixture (N) expresses the average number of chlorine substitution in the biphenyl molecule ad it is calculated as from the Equation 3.1:

$$N = \frac{\sum C_i \cdot n_i}{\sum C_i}$$

Equation 3.1: calculation of the degree of chlorination (N); C_i is the concentration of the single congener in µmol (kg dry weight)$^{-1}$ and n_i is the number of the chlorine substitutions.

The dechlorination rate (v) is expressed in µmoles of chlorine released (kg dry sediment)$^{-1}$ (week)$^{-1}$ and it is calculated following Equation 3.2:

$$v = \frac{(\sum C_i \cdot n_i)_{initial} - (\sum C_i \cdot n_i)_{final}}{t}$$

Equation 3.2: calculation of the dechlorination rate (v); C_i is the concentration of the single congener in µmol (kg dry weight)$^{-1}$, n_i is the number of the chlorine substitutions, t is the time of incubation (weeks).

The redox potential and pH of the sediment slurry were measured with a Hamilton Liq-Glass™ ORP probe calibrated with 475 mV Standard Hamilton solution and with a Hamilton Polilyte Lab™ probe calibrated with pH=4.01 and pH=7.00 solutions, respectively.

Biogas production was measured at each sampling before opening of the microcosms with an air tight glass syringe, while its composition was determined via a µGC-TCD model 3000A (Agilent Technologies, Milano, Italy) equipped with a 10m x 0.32mm MolSieve 5A column under the following conditions: injector temperature 90 °C; column temperature 60 °C; sampling time 30 s; injection time 50 ms; column pressure 25 psi; run time 44 s, carrier gas nitrogen.

Anions concentration in the water phase was determined, after slurry centrifugation at 10000*x g* for 10mins and 1:100 dilution of the supernatant, with a Dionex DX-120 ion chromatograph equipped with an IonPac AS14 4x250 mm column and a conductivity detector combined to an ASRS-II Ultra conductivity suppressor system (Dionex, Sunnyvale, CA, USA), as described elsewhere (Fava et al., 2003b). Linear 4-point calibration curves (1.0–25.0 mg l^{-1} range) for SO_4^{2-}, Cl^-, NO_3^- and NO_2^- were obtained by using mixtures of these compounds.

Total organic carbon (TOC) was measured using the wet oxidation method followed by titration of ferrous ammonium sulfate (Springer and Klee, 1954). The sediments were exsiccated overnight at RT; successively, aliquots of 0.5 g, 1 g and 2 g were weighted in a 250 ml glass tube; 16.2 ml of a 0.25 N solution of potassium dichromate ($K_2Cr_2O_7$) were added and the content was mixed gently before slow addition of 2.6 ml H_2SO_4 95% w/w and slow heating to 150 °C and isotherm 30', using a DKL-8 heating digester (VELP Scientifica, Usmate, MB, Italy). The reaction goes as follows:

$$2Cr_2O_7^{2-} + 3C^0 + 16H^+ = 4Cr^{3+} + 3CO_2 + 8H_2O$$

2 ml of water were then added to stop the reaction. 4 ml of liquid phase was then transferred in an Erlenmeyer flask and diluted to 50 ml with distilled water; after the addition of 0.8 ml of H_3PO_4 85% w/w, and 50 µl of a oxred indicator(sodium 4-diphenylamminosulfonate, $C_{12}H_{10}NaNO_3S$) 4 g/l in sulfuric acids. The titration solution was prepared dissolving 0.02 M $FeSO_4 \cdot 7H_2O$ with the addition of 20 ml H_2SO_4. The titration was executed for a blank sample (prepared in the same way

with no sediment added) and, as the color turned from dark violet to green and the total organic carbon (C) was calculated using Equation 3.3.

$$C = \frac{3}{2} \times \frac{B - A}{1000} \times \frac{MFe(II)}{6} \times \frac{V_d}{V_s} \times 12 \times \frac{1000}{W_s}$$

Equation 3.3: Calculation of the total organic carbon (C) expressed as gC (kg dry sediment)$^{-1}$; 3/2 is the molar ratio between dichromate and carbon; B is the volume of titrant used in the blank sample (ml), A is the volume of titrant used in the sample, MFe(II) is the title of the titrant solution (0.02 M); V_d is the total volume (ml); V_s is the dichromate solution volume used for titration after digestion (ml); 12 is the atomic weight of C (g/mol); W_s is the weight of the sample (g).

3.4 Community analysis

3.4.1 DNA extraction

Metagenomic DNA was extracted from the wet sediment (approximately 250 mg) recovered from the centrifugation of 2 ml slurry samples at 10,000 ×g for 10 minutes with the UltraClean Soil DNA kit (MoBio Laboratories, Carlsbad, CA, USA) according to the protocol "for maximum yield" provided by the manufacturer. Sediment samples suspended in the bead solution supplied with the kit were treated with 4.5 µl of a 100 mg ml^{-1} Proteinase K solution from *Streptomices Griseus* and 8.2 µl of a 100 mg ml^{-1} of chicken egg lysozyme solution at 37°C under shaking at 150 rpm for 30 min, prior to cell lysis steps described in the provided protocol. The quality of extracted genomic DNA was checked through electrophoresis on 1% agarose gel and staining in 0.5 µg/ml ethidium bromide solution. DNA samples were quantified using nanophotometer P-330 (Implen GmbH, Münich, Germany).

3.4.2 PCR-DGGE of the 16s rRNA gene

For DGGE analysis, 16S rRNA genes of the bacterial community were PCR amplified from the metagenomic DNA with the GC-clamped forward primer GC-357f (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTACGGGAGGCAGCAG-3') and the reverse primer 907r (5'-CCGTCAATTCCTTTGAGTTT-3') (Sass et al., 2001) and the following PCR conditions: hot-start at 60 °C, initial temp 94 °C for 10' and then 29 cycles of denaturation at 94 °C for 30", annealing at 55 °C for 1', extension at 72 °C for 1', followed by a last final extension step at 72 °C for 10' (Zanaroli et al., 2012b). 16S rRNA genes of putative dechlorinating *Chloroflexi* were PCR amplified with the GC-clamped forward primer GC-348f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGAGGCAGCAGCAAGGA A-3'), the reverse primer Dehal844r (5'-GGCGGGACACTTAAAGCG-3') (Fagervold et al., 2005)

and the following PCR conditions: hot-start at 60 °C, initial temp 94 °C for 10' and then 39 cycles of denaturation at 94 °C for 45", annealing at 52 °C for 1'25", extension at 72 °C for 1', followed by a last final extension step at 72 °C for 10'. DGGE of bacterial amplicons (approximately 400 ng DNA per lane) were performed with a D-Code apparatus (Bio-Rad, Milan, Italy) in 7% (w/v) polyacrylamide gels (acrylamide-N,N'-methylenebisacrylamide, 37:1) in 1× TAE buffer with a denaturing gradient from 40% to 60% denaturant, at 55 V for 16 h, 60 °C (Zanaroli et al., 2010). A denaturing gradient from 45% to 55% denaturant was used to resolve *Chloroflexi* amplicons. Gels were stained with SYBR Green I 1x in TAE 1x for 20' and their image captured in UV transilluminator with a digital camera supported by a Gel Doc apparatus (Bio-Rad, Milan, Italy).

Richness, dynamics and functional organization indexes were calculated as described in literature (Marzorati et al., 2008; Wittebolle et al., 2009). The range-weighted richness (Rr) value is the total number of bands multiplied by the percentage of denaturing gradient needed to describe the total diversity of the sample analyzed. This is done according to the formula $Rr = (N^2 \times Dg)$, where N represents the total number of bands in the pattern, and Dg the denaturing gradient comprised between the first and the last band of the pattern. The dynamics (Dy) value is based on moving window analysis, and the relative the rate of change (Δt) parameter averages the degree of change between consecutive DGGE profiles of the same community over a fixed time interval (one week). The community organization concept is derived from the graphical representation of the structure of a bacterial community (species distribution = evenness) according to Pareto-Lorenz (PL) evenness curves (Lorenz, 1969) and the respective Gini coefficient (Wittebolle et al, 2009).

For sequencing, the most prominent DGGE bands were cut from the polyacrylamide gel with a sterile scalpel and eluted overnight at 4 °C in 50 µl sterile water. Eluted bands were then re-amplified and resolved again in DGGE electrophoresis under the conditions described above. After elution from the second polyacrylamide gel, bands were amplified as described above with non GC–clamped primers and PCR products purified in the presence of 10 U of ExoI and 1 U of FastAP enzymes at 37°C for 15' before sequencing with the corresponding forward primer. Sequencing was performed by BMR Genomics (Padova, Italy). Each 16S rRNA gene sequence obtained was aligned to the bacterial 16S rDNA database of the Ribosomal Database Project (RDP, release 11, http://rdp.cme.msu.edu) and the closest relative and closest cultured relative retrieved with the Seqmatch tool. The phylogenetic affiliation of each sequence was obtained from the same website with the Classifier tool.

3.4.3 Relative quantification of target *Chloroflexi* by qPCR

Primer pairs were designed for the specific amplification of two partial 16S rRNA gene sequences that were detected in the PCB dechlorinating cultures by DGGE with the bacterial primers pair 357f/907r (see paragraph 3.4.2). Primers were designed with the Primer-BLAST website http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Ye et al., 2012) using the region from *E. coli* position 357 to position 907 of each target sequence as PCR template, a primer melting temperature from 58 to 61, the Refseq RNA database for specificity checking, PCR product Tm between 80 and 90 °C, optimum at 80. The specificity of candidate primer pairs was checked in silico with the ProbeMatch tool available at the RDP II web site; primer pairs having the highest in silico specificity were checked for cross specificity in vitro using the two DGGE bands from which the sequences were obtained, in end-point PCR and qPCR assays (see details below). Selected primers and their main features are reported in Table 3.5.

Target Microorganism	**Primer name**	**Primer sequence**	**Annealing position** ***(referred to E.coli)***	**Primer Tm** ***(°C)***	**Amplicon length** ***(bp)***	**Number of non-target sequences probed** ***(per allowed mismatches)***		
						0	***1***	***2***
Uncultured *Chloroflexi* VLD-1	682f 814r	AGGCGAAAGCGGTTTCCAA ACTTAAGCGTTAGCTTCGGCA	682 814	60.5 60.3	153	0	4	26
Uncultured Chloroflexi VLD-2	585f 697r	TCAACTGGGAGGAGTCATTCG GAAACAGCCTAGAAAACCGCC	585 697	59.5 59.8	133	0	6	11

Table 3.5: Characteristics of the specific primer pairs designed for uncultured *Chloroflexi* VLD-1 and VLD-2 16s rRNA gene sequence as described in this work (see also section 4.1.3.1).

The gene copies of the target dehalogenating species and the total bacterial 16s rRNA genes were quantified through qPCR using a StepOne™ Real-Time PCR System (Applied Biosystems, Fil. Life Technologies Europe BV, Monza, Italy) using StepOne software v 2.0 according to the manufacturer instructions. PCR cycles used for evaluating 16s rRNA gene copies of the dechlorinating species were as follows: 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 30 sec, annealing at 56 °C, 59° or 54°C for 30 sec (for Uncultured *Chloroflexi* VLD-1 and VLD-2 specific primer pairs or total bacterial 16s rRNA gene primer pair respectively), elongation at 72 °C for 30 sec and a melt curve stage of 95 °C for 15 sec followed by a melting step from 60 to 95 °C and fluorescence measure every 0.3 °C. The following primers were used: 682F (5'-AGGCGAAAGCGGTTTCCAA-3') and 814r (5'-ACTTAAAGCGTTAGCTTCGGCA-3') (obtained as described previously) for Uncultured *Chloroflexi* VLD-1 specific 16s rRNA gene; 920F (5'-AAACTCAAATGAATTGACGG-3') (Yamada et al., 2000) and 1044r (5'-

GACARCCATGCASCACCTG-3') (Bach et al., 2002) for total 16s rRNA gene; 585F (5'-TCAACTGGGAGGAGTCATTCG-3') and 697r (5'-GAAACAGCCTAGAAAACCGCC-3') for Uncultured *Chloroflexi* VLD-2 specific 16s rRNA genes. The qPCR reactions (25 µl) were set-up as follows: 1 x Power SYBR® Green PCR Master Mix, forward and reverse primers at 350 nM each and 2.5 µl of DNA template. Standard curves were included in each plate using 10^{-2} to 10^{-6} ng/µl of PCR products obtained by amplification of DGGE gel-purified bands (for specific pairs) or *E. coli* 16s rRNA gene (for total bacteria) and purified with Wizard® SV Gel and PCR Clean-Up System according to the manufacturer protocol. Amplification efficiencies ranged from 85 to 106%, with R^2=96-100 %; all samples and standards were set up in triplicate reactions.

3.5 Novel biosurfactant production

3.5.1 Isolation of biosurfactant-producing strains from marine cultures

Five mixed marine cultures and eight isolates were provided from the Technical University of Crete, Biochemical Engineering and Environmental Biotechnology Laboratory, Division II: Environmental Process Design and Analysis, partner of the project. Cultures were then cultivated on ONR7a mineral medium which was containing (per liter of total volume): solution i) NaCl 22.79 g, Na_2SO_4 3.98 g, KCl 0.72 g, NaBr 83 mg, $NaHCO_3$ 31 mg, H_3BO_3 27 mg, NaF 2.60 mg, NH4Cl 0.27 g, $Na_2HPO_4 \cdot 7H_2O$ 89 mg, TAPSO (N-[Tris(hydroxymethyl)methyl]-3-amino-2-hydroxypropanesulfonic acid) 1.30 g, final pH=7.6 (optionally with 15 g Agar for solid medium); solution ii) $MgCl_2 \cdot 6H_2O$ 11.18 g, $CaCl_2 \cdot 2H_2O$ 1.46 g and $SrCl_2 \cdot 6H_2O$ 24 mg; solution iii) $FeCl_2 \cdot 4H_2O$ 2 mg. The three solutions were autoclaved separately and mixed after autoclave in sterile conditions when the temperature reached ~ 60 °C (Dyksterhouse et al., 1995). ONR7a was then supplemented with 0.5% w/v crude oil as unique carbon source (ONR7a + C). Cultures were cultivated in 20 ml ONR7a + C, 15 °C, 125 rpm.

Colonies were obtained through dilution and plating over different media: when the OD of the culture was above 1.2 (1 to 2 weeks of growth) serial dilutions were prepared in ONR7a. 100 µl of each dilution were then plated either on i) ONR7a + C, ii) ONR7a amended with 1 g/l glucose, iii) ONR7a amended with 0.5 g/l yeast extract, iv) HLB, v) HLB amended with 0.5% w/v crude oil or vi) Zobell marine broth. HLB is a rich medium, modified from the common LB medium, containing (per liter) NaCl 30 g, yeast extract 5 g and tryptone 10 g. Zobell marine broth is a medium recommended for cultivation and isolation of marine bacteria, full of nutrients and vitamins, containing (per liter of total volume): solution i) peptone 5 g, yeast extract 1 g, NaCl 19.45 g, H_3BO_3 0.022 g, Na_2SO_4 3.24 g, Na_2HPO_4 8 mg, Na_2O_3Si 4 mg, NaF 2.4 mg, KCl 0.55 g, NH_4NO_3 1.6 mg, $NaHCO_3$ 0.16g KBr 0.08 g (optionally with 15 g Agar for solid medium); solution ii) $MgCl_2 \cdot 6H_2O$ 18.79 g, $CaCl_2 \cdot 2H_2O$ 2.38 g, $SrCl_2 \cdot 6H_2O$ 0.057 g; solution iii) Fe(III)citrate 0.1 g (ZoBell, 1941). After colony development, separate colonies were striked again on the same solid media for purification. Pure colonies were then resuspended in 20 ml of the corresponding liquid medium. Growth was measured by optical density (OD_{600}) and purity of the colonies was verified through microscope observation. Cultures were then sampled for EI_{24} measurements to assess biosurfactant production. After a first phase of isolation and screening, 2 ml of the best-producing cultures were frozen for DNA extraction at -20 °C until use, as described previously (see section 3.4.2). Genomic DNA was amplified with primers 27f (5'-AGAGTTTGATCCTGGCTCAG- 3') and 1525r (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991) with the PCR conditions described previously. PCR products purified in the presence of 10 U of ExoI and 1 U of FastAP enzymes at

37°C for 15 min before sequencing with the corresponding forward primer. Sequencing was performed by BMR Genomics (Padova, Italy). Each 16S rRNA gene sequence obtained was aligned to the bacterial 16S rDNA database of the Ribosomal Database Project (RDP, release 11, http://rdp.cme.msu.edu) and the closest relative and closest cultured relative retrieved with the Seqmatch tool. The phylogenetic affiliation of each sequence was obtained from the same website with the Classifier tool.

Glycerol stocks of the best-producing isolates were prepared aliquoting cultures in sterile glycerol 20% v/v and immediately stored at -80 °C.

3.5.2 Biosurfactant characterization and production

To screen biosurfactant production, emulsification index assays (EI_{24}) were performed on the supernatant. 2 ml cell culture were centrifuged at 14'000 rpm, for 10' and pellets were discarded. 1 ml of supernatant was added 2 ml toluene in a glass tube and vortexed at maximum speed for 2'. After 24 h of settling, at room temperature, the EI_{24} is the ratio between emulsion phase and total height of the sample (Cooper and Goldenberg, 1987).

IFT (interfacial tension) is measured with the pendant drop method (Stauffer, 1965) using a Drop Shape Analyzer DSA30 (KRÜSS GmbH, Hamburg, Germany), using a 500 µl glass syringe with a Ø=2.098 cm. The IFT is calculated when the biggest pendant drop possible is formed (Figure 3.1), using image analysis following Young-Laplace equation (Equation 3.4)

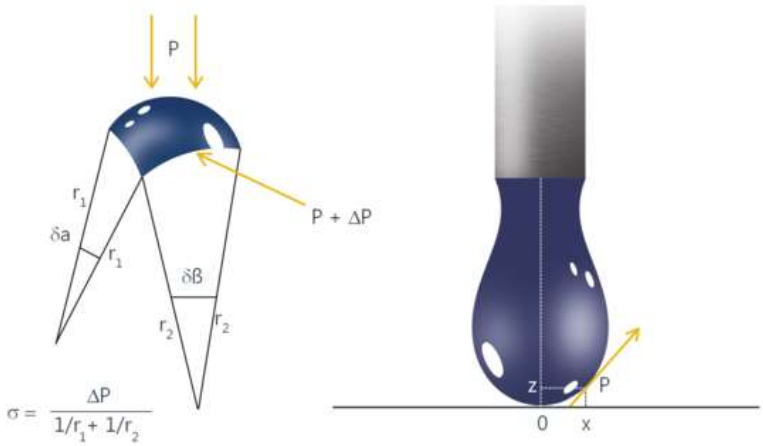

Figure 3.1: Shape of the pendant drop image and definition of the main parameters of the image analysis

$$\Delta p = \sigma\left(\frac{1}{r_1} + \frac{1}{r_2}\right)$$

Equation 3.4: Young-Laplace equation where which describes the correlation between pressure difference Δp, the radii of curvature of the surface r_1 and r_2 and the interfacial tension σ.

After a first phase of isolation and screening, the best-producing isolates transferred successively at 10% v/v up to a volume of 500 ml each. Supernatants of the cultures, obtained via centrifugation at 14'000 rpm, 4°C for 10', were precipitated in 3 v of glacial acetone and incubated overnight at 4 °C. After precipitation, the solution was centrifuged at 12'000 rpm, 4 °C, 30' and the pellet was exsiccated overnight at RT and resuspended in 10 ml PBS (phosphate buffer solution I containing, per liter, NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, $CaCl_2 \cdot 2H_2O$ 0.133 g, $MgCl_2 \cdot 6H2O$ 0.10 g). The solution was the dialyzed against distilled water, at 4 °C overnight, using a dialysis membrane with a 12 kDa cut-off.

Acetone crude extracts and dialyzed extracts were then analyzed ecotoxicity against *Vibrio fischeri* (strain NRRL B-11177), a marine-living bacterium very commonly used for ecotoxicity testing. The growth inhibition of *V. fischeri* was measured using Microtox™ 500 analyzer (Modern Water plc, UK) following the manufacturer's instruction. The light emission of this bacterium is directly proportional to the metabolic activity of the bacterial population (Blaise, 1991). The bacterial suspension was incubated for 30' in presence of 4 serial dilution of a 81.9% v/v biosurfactant solution in osmotic solution. The bioluminescence signal is measured at 5', 15'. and 30' and it is directly related to the growth of the *V.fischeri:* after analysis of the response curve, the EC_{50} is measured as the concentration of biosurfactant which causes a decrease in bioluminescence of 50% versus positive control; the lower the EC_{50}, the higher the toxicity towards the bacterium.

3.6 Chemicals

Aroclor 1221, 1242, 1254 and 1260 were supplied by ULTRA Scientific Italia S.r.l, Bologna Italy.

Sodium L-lactate, sodium pyruvate, Proteinase K from *S. griseus*, Lysozyme from chicken egg, urea, formamide, acrylamide-bisacrylamide solution, SYBR® Green I, medium components, and organic solvents acetone were supplied by Sigma Aldrich Italia S.r.l, Milan, Italy.

FastAP and ExoI enzyme stock solutions were provided by Thermo Scientific, part of Thermo Fisher Scientific Italia S.r.l, Milan, Italy.

PowerSYBR® Green PCR master mix were provided by Life Technologies, part of Thermo Fisher Scientific Italia S.r.l, Milan, Italy.

Wizard® SV Gel and PCR Clean-Up System was provided by Promega Italia S.r.l, Milano,Italy

Molasses was provided by Eridania Sadam S.r.l, Treccasali, Parma, Italy .

Cheese whey was supplied by Granarolo, Granarolo, Bo, Italy.

Polylactate PLA 4042D was supplied by NatureWorks LLC Minnetonka, USA.

4 Results and discussion

4.1 Assessment of PCB microbial reductive dechlorination potential in sediments of the Venice Lagoon and detection of dehalorespiring phylotypes

In order to evaluate the PCB dechlorination potential of the indigenous microbial communities of contaminated marine sediments of the first Industrial area of Porto Marghera (Venice lagoon), slurry cultures of six sediments suspended in the site water were incubated under anaerobic conditions at 28 °C for 31 weeks. Sediments A and B1 looked brownish and sandy, while B2, C, D and E looked muddy and black. The contamination was not amenable to any of the commercial mixtures described in literature (Schulz et al., 1989; Frame, 1997a;b). Since sediments contamination was low and characterized by the occurrence of a limited number of PCB congeners (Table 3.1), cultures were spiked with the commercial PCBs mixture Aroclor 1254 at the final concentration of 1 g PCBs (kg dry sediment)$^{-1}$ in order to favor the enrichment of sediment indigenous PCB dehalorespiring bacteria and to better assess and characterize their PCB dechlorination capabilities (Alder et al. 1993; Oh et al. 2008; Zanaroli et al. 2010).

The six sediment cultures were prepared in slurry microcosms, suspending the sediment in site water at 20% w/v and with the addition of Aroclor 1254 PCB mixture at a final concentration of 1000 mg (kg dry sediment)$^{-1}$. Every microcosm was sampled at 0, 6, 11, 14, 18, 23, 27 and 31 weeks of incubation.

Cultures were anaerobically cultivated in atmosphere of N_2:CO_2 70:30, where the presence of carbon dioxide stimulate mineralization of organic matter, representing carbon and energy source for chemiolytotrophic autotrophic microorganisms, including methanogens and sulfate-reducers. The temperature was chosen at around 28 °C to mimic a summer average temperature: in the Porto Marghera area of the Venice Lagoon, indeed, temperatures shift from an average of 10-15 °C in winter to 25-32 °C in summer. The cultures were incubated statically in the dark, simulating the dearth of benthic movement and illumination of the actual sediment. Atmosphere, temperature, site water and incubation conditions were chosen to represent *bioegeochemical* condition of the sediment occurring *in situ*, which were shown to be more effective in promoting dehalogenating activities in these sediments, rather than other conditions (Fava et al., 2003b).

4.1.1 PCB dechlorination

No PCB dechlorination occurred in all autoclaved controls nor did it in the biologically active cultures of sediment A, B1 and B2 throughout incubation (31 weeks). Conversely, PCB dechlorination started on after 11 weeks of incubation in sediment D cultures and after 14 weeks of incubation in sediment C and E cultures as showed by the trend of degree of chlorination (i.e. the average number of chlorine per PCB molecule) (Figure 4.1: Time –course dechlorination of Aroclor 1254 in active sediment cultures. Values are average of duplicate microcosms.).

It is worth noticing that the onset of dechlorination in sediments C, D and E cultures rather than in A, B1 and B2, seems to be independent from sediment characteristics: while it is true that sediment A and B1 looked brownish and sandy, suggesting a higher redox state than the other, it is also true that sediment B2 cultures showed no evidence of dechlorination. The only parameter, so far, which seems to be related to the occurrence of dehalogenation could be the TOC, which is at least twice higher in sediment C, D and E, independently of the PCB total contamination (Table 3.1); this could suggest the presence of higher quantity of nutrients or a higher quantity of biomass, thus a more flourishing bacterial community, but so far no data support this speculation. The occurrence of Aroclor 1254 dechlorination was already shown in freshwater sediments (Chen et al., 2001; Pakdeesusuk et al., 2005) and in marine sediments (Øfjord et al., 1994); more recently, dechlorination of Aroclor 1254 was shown in one sediment of the Venice Lagoon (Zanaroli et al., 2010) where a *Chloroflexi* bacterium was associated with this activity (Zanaroli et al., 2012a) and these latest findings confirm the presence of such in the area.

Dechlorination proceeded at maximum rates of 379.5±43.7 and 199.6±30.6 μmoles of chlorine removed (kg dry sediment)$^{-1}$ per week (average of replicate cultures which behaved comparably) in sediment D and C cultures, respectively. Surprisingly, each replicate of sediment E cultures, exhibited different PCB dechlorination activities, being dechlorination rate in replicate 1 (herein after referred to as sediment E1 culture) comparable to that of sediment D cultures (325.4±21.0 μmoles of chlorine removed (kg dry sediment)$^{-1}$ per week) and dechlorination rate in replicate 2 (herein after referred to as sediment E2 culture) comparable to that of sediment C cultures (149.7±6.5 μmoles of chlorine removed (kg dry sediment)$^{-1}$ week^{-1}). Sediment D and E1 on the one side and sediment C and E2 on the other behave comparably pairwise also in terms of penta- through epta-chlorinated congeners percentage depletion: more extensive depletions initially occurring in the spiked PCB mixture were observed after 31 weeks in sediment D and E1 cultures (84 mol% and 77 mol%, respectively), compared to sediment C and E2 cultures (62 mol% and 51

mol%, respectively). The stoichiometric accumulation of several tetra- and tri-chlorinated congeners was observed in all PCB dechlorinating cultures, leading to the reduction of the initial average number of chlorines per biphenyl ring (5.1) to 4.0 in sediment D and E1 cultures and to 4.4 and 4.5 in sediment C and E2, respectively (Figure 4.1: Time –course dechlorination of Aroclor 1254 in active sediment cultures. Values are average of duplicate microcosms.)

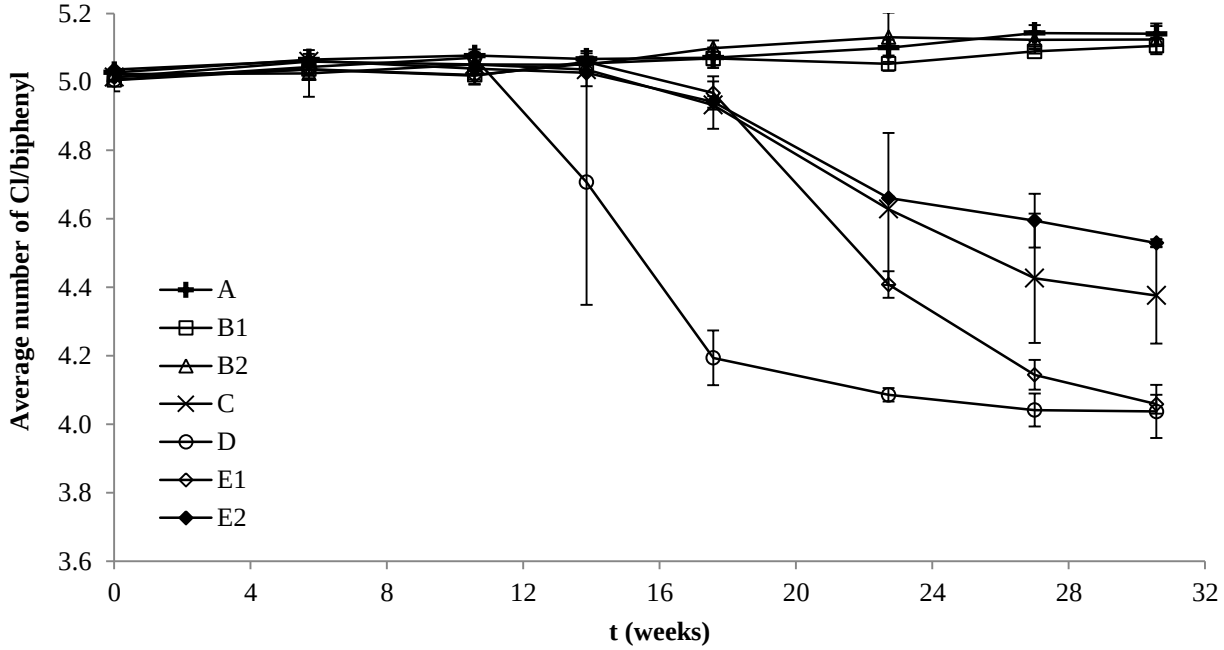

Figure 4.1: Time –course dechlorination of Aroclor 1254 in active sediment cultures. Values are average of duplicate microcosms.

The main dechlorination products that accumulated at the end of the incubation in sediment D were, in decreasing order of mol% abundance on total congeners accumulated, 2,2',4,5'-CB (27 mol%) > 2,2',4,4'-/2,2',4,5-/2,4,4',6-CB (12 mol%) > 2,2',5,5'-CB (10 mol%) > 2,2',4,4',6-/2,3',4,5-CB (9 mol%) > 2,4',5-/2,4,4'-CB (8 mol%) > 2,2',3,4-/2,3,4',6-CB (7 mol%) > 2,3',4,4'-/2,2',3,5',6-CB (5 mol%) > 2,2',5-/2,2',4-/4,4'-CB (5 mol%) (Figure 4.2). Dechlorination pattern in sediment D cultures resembles the process N (see Table 1.1), thus attacking unflanked *meta* positions of the biphenyl ring, and accumulating congeners with *ortho* chlorine substitutions.

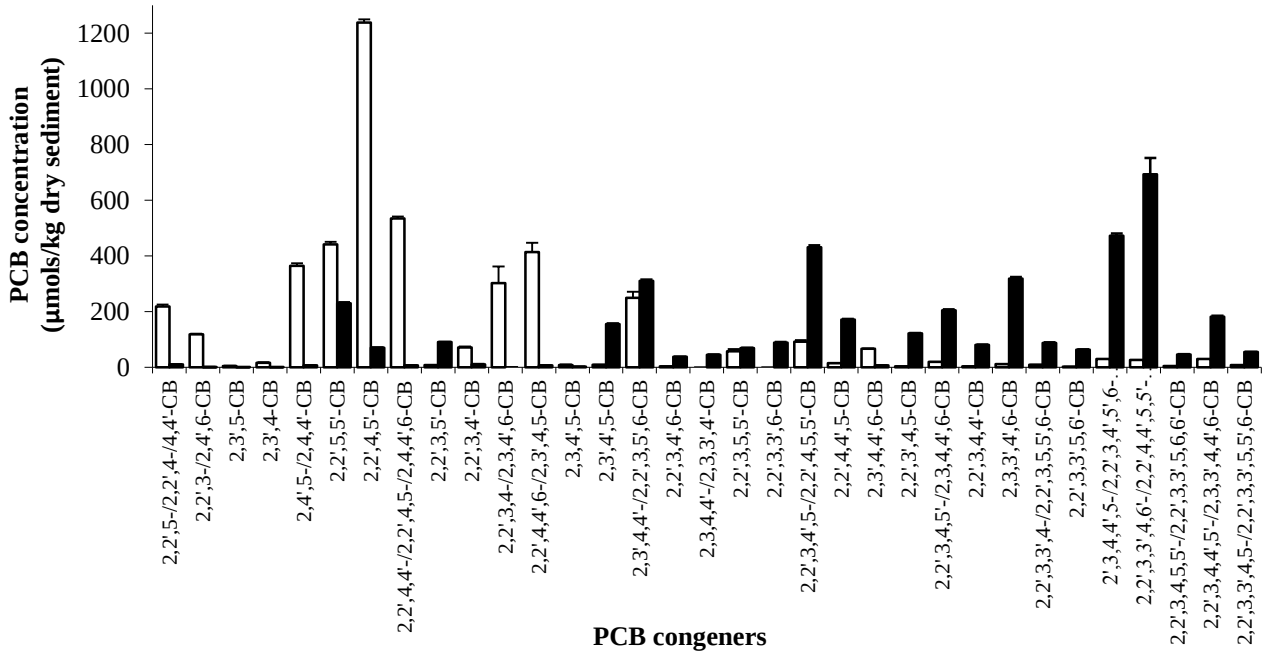

Figure 4.2: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active cultures (empty bars), and sterile microcosms (black bars) after 31 weeks of incubation of sediment D cultures. Values are average of duplicate microcosms and error bars represent standard deviations.

2,2',4,5'-CB was the most abundant dechlorination product (19 mol%) also in sediment C. However, a more prominent accumulation of 2,2',5,5'-CB (18 mol% *vs* 10 mol%), 2,3',5-CB (5 mol% *vs* 0.1 mol%) and 2,3',4-CB (2 mol% *vs* 0.4 %), as well as a much lower accumulation of 2,2',4,4',6-/2,3',4,5-CB (3 mol% *vs* 9 mol%), 2,2',4,4'-/2,2',4,5-/2,4,4',6-CB (2 mol% *vs* 12 mol%), 2,2',3,4-/2,3,4',6-CB (2 mol% *vs* 7 mol%) and 2,4',5-/2,4,4'-CB (1 mol% *vs* 8 mol%) was observed in sediment C as compared to sediment D (Figure 4.3), indicating that process P seems to be predominant in Sediment C cultures, attacking flanked *para* and also single/double flanked *meta* positions, thus PCBs were dechlorinated in different sediments through specific pathways with different selectivity.

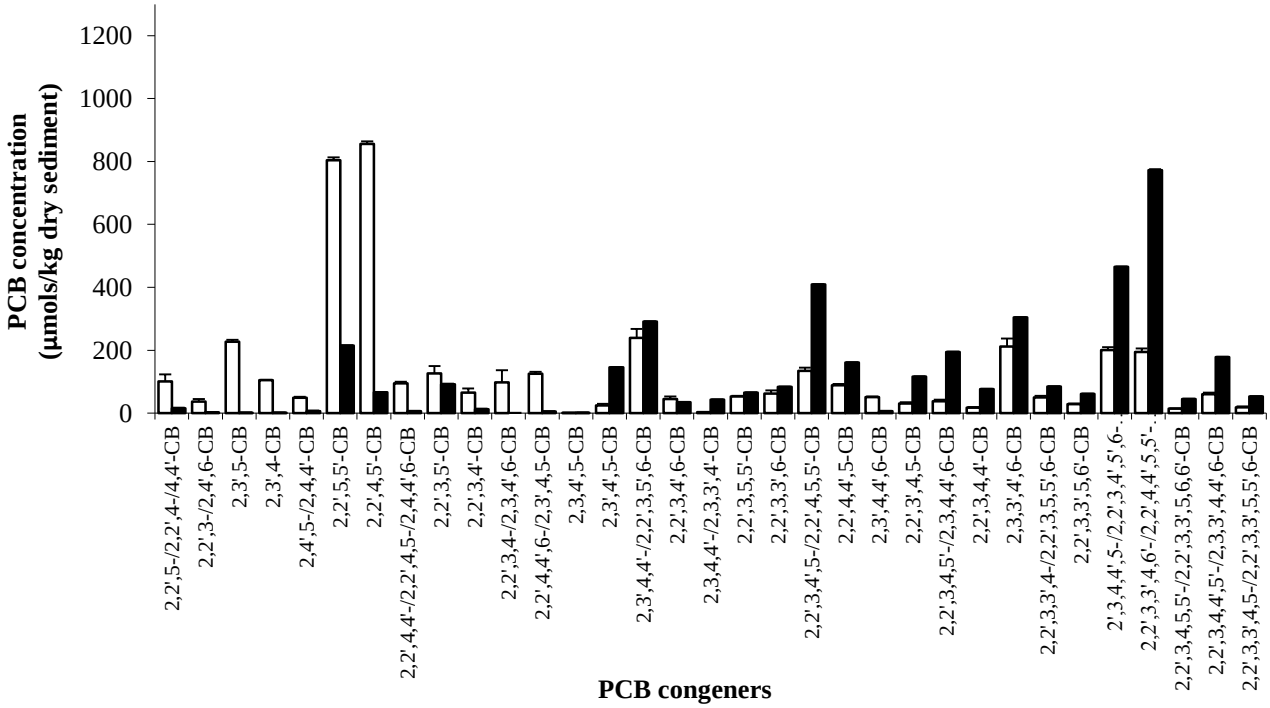

Figure 4.3: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active cultures (empty bars), and sterile microcosms (black bars) after 31 weeks of incubation of sediment C cultures. Values are average of duplicate microcosms and error bars represent standard deviations

Sediment E1 culture exhibited a dechlorination pattern more similar to that of sediment D, being 2,2',4,5'-CB (25 mol%), 2,2'5,5'-CB (11 mol%), 2,2',3,4-/2,3,4',6-CB (7 mol%) and 2,2',4,4'-/2,2',4,5-/2,4,4',6-CB (7 mol%) the main dechlorination products accumulated at the end of incubation (Figure 4.4a); however, some common feature to sediment C culture were also observed, such as the low accumulation of 2,4',5-/2,4,4'-CB (4 mol%) and 2,2',4,4',6-/2,3',4,5-CB (4 mol%) and the detection of 2,3',5-CB (2 mol%) (Figure 4.4a); the accumulation of 2,3',5-CB, 2,3',4-CB and 2,2',5,5'-CB is higher than in sediment D cultures, suggesting that process P was also concomitant to process N, and specifically more efficient than in sediment C. A dechlorination pattern similar to that of sediment D culture was apparently observed also in sediment E2 culture; however, the less extensive dechlorination occurring in this microcosm does not allow identifying the dechlorination specificity of the culture (Figure 4.4b). The different dechlorination patterns observed in sediment C as compared to sediment D, E1 and, to some extent, sediment E2, suggest that dechlorinating species enriching therein might be peculiar (Fagervold et al. 2005; Fagervold et al.2007; Bedard 2008).

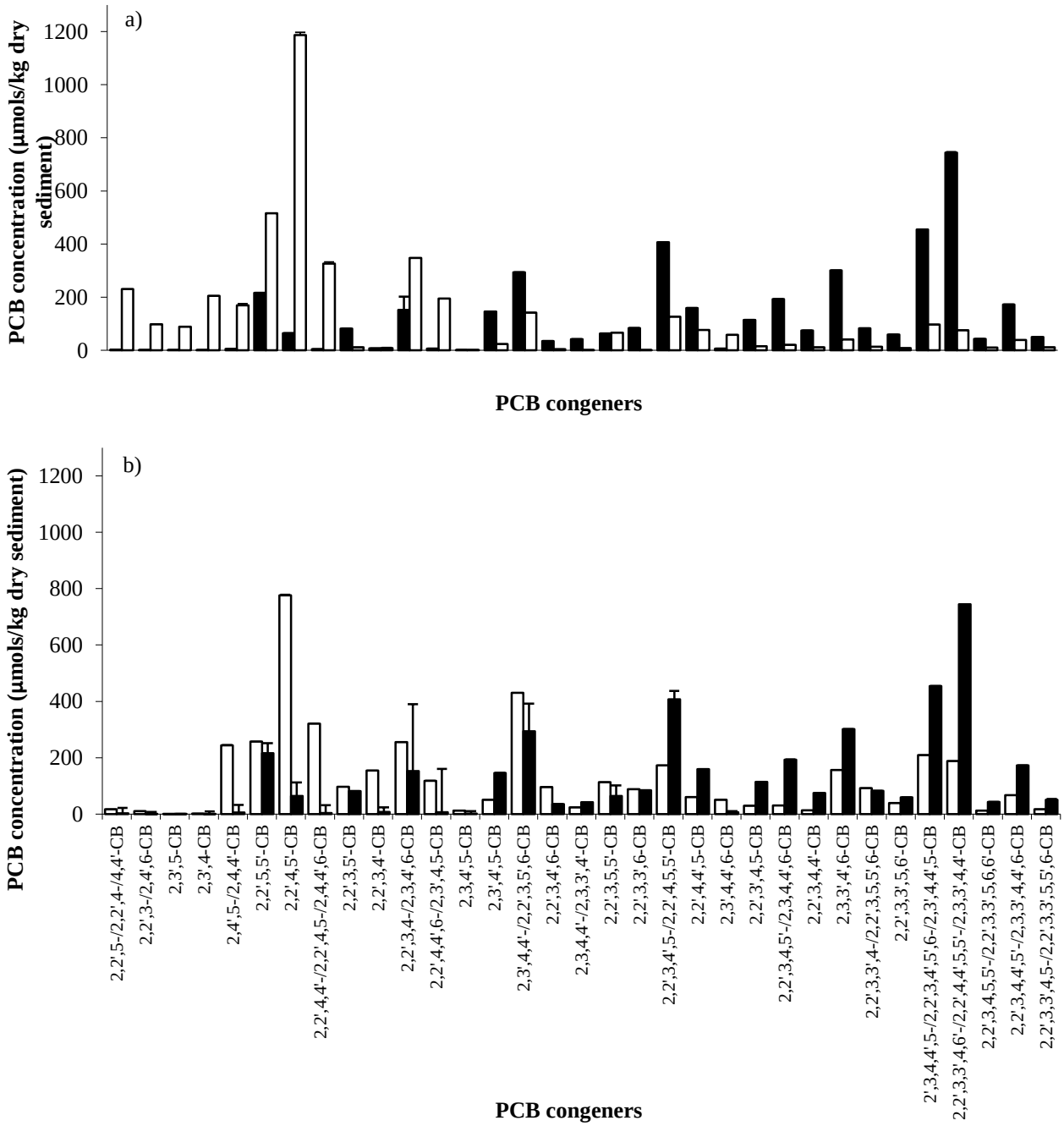

Figure 4.4: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active cultures (empty bars), and sterile microcosms (black bars) after 31 weeks of incubation of sediment E1 and E2 cultures (a and b, respectively). Values are average of duplicate microcosms and error bars represent standard deviations

4.1.2 Sulfate reduction redox potential and methanogenic activities

Sulfate concentrations ranging from 2.6±0.1 g/l (in sediment D culture) to 3.7±0.1 g/l (in sediment B1 culture) were detected in the cultures at the beginning of the incubation. With the exception of sediment C, where sulfate reduction set on since the beginning and consumed more than 40% of the initially occurring sulfate during the first 6 weeks, sulfate consumption stared to be detected after a

lag phase of 6 to 11 weeks of incubation (Figure 4.5a). Remarkably, complete sulfate consumption was achieved in sediment D and sediment C cultures after 11 weeks of incubation, i.e. before the onset of the dechlorination process, same as in sediment A and B2 cultures, where no PCB dechlorination ever occurred (Figure 4.5). Slower sulfate reduction took place in the non PCB-dechlorinating culture B1, where sulfates were completely reduced to sulfur before week 23, and in both sediment E culture replicates, where sulfate depletion occurred at weeks 14 and 23 in replicates 1 and 2, respectively (Figure 4.5). No correlation was therefore observed between the extent and rate of sulfate reduction and the occurrence, extent and rate of PCB dechlorination.

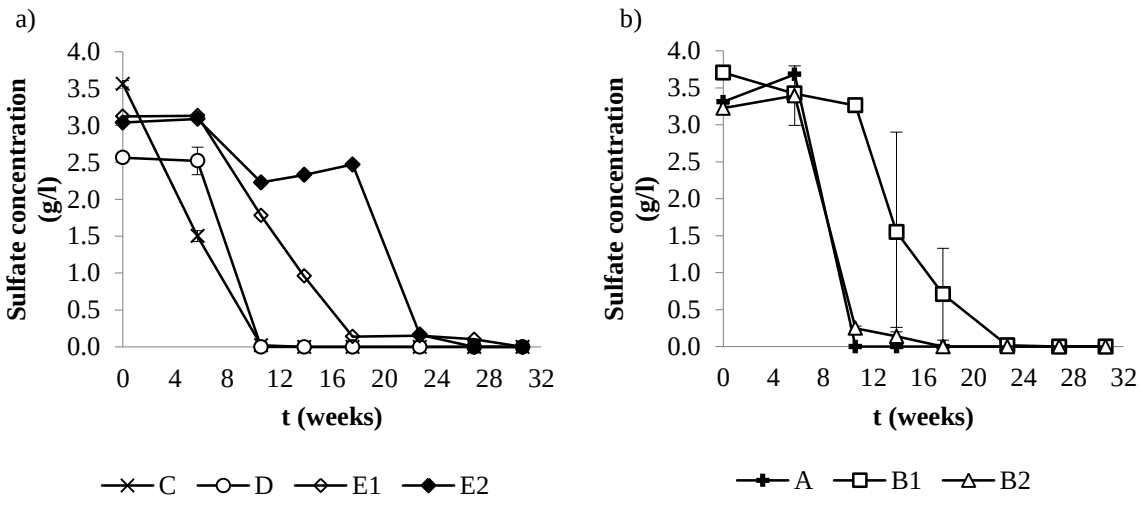

Figure 4.5: Sulfate concentration in active dechlorinating cultures (A) and active non-dechlorinating cultures (B) throughout incubation. Values are average of duplicate microcosms and error bars represent standard deviations

Redox potential measurements varied according to the sulfate reduction activities. At the beginning of incubation, sediment A and B1, which looked brownish, had a higher potential (-131±63 and +59±1 mV respectively), compared to the other sediments which were below -200 mV (Figure 4.6). However, this was not a sufficient indication for the start of dechlorination activities, since sediment B2 did not show any dechlorination activity, in spite of its lower potential. Each sediment culture showed decrease in potential during the incubation, with the lowest points reached at the end of the sulfate reduction, with the exception of sediment E cultures, where redox potentials were similar between replicates whereas sulfate reduction activities varied noticeably. The correlation between redox potential and sulfate-reduction is well known in marine sediments (Jørgensen, 1977) and it is a key factor in mineralization of several toxic compounds, mainly heavy metals (Compeau and Bartha, 1985; Park and Jaffé, 1996); however, as shown in previous experiments on another sediment of the Venice Lagoon, there are no data to hypothesize a role of sulfate-reducers in dechlorination activities (Zanaroli et al., 2006; Zanaroli et al., 2010).

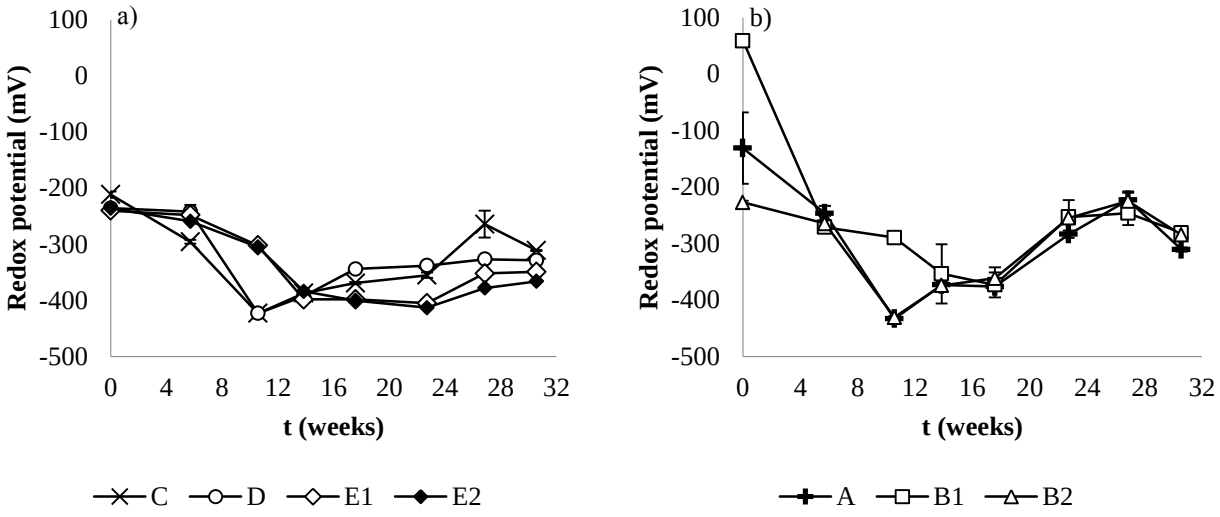

Figure 4.6: Redox potentials in active dechlorinating cultures (A) and active non-dechlorinating cultures (B) throughout incubation. Values are average of duplicate microcosms and error bars represent standard deviations

Negligible methane production (lower than 4 ml over the 31 weeks of incubation) was observed in all sediment cultures, with the exception of the non PCB-dechlorinating cultures of sediment A, where 55.7±3.6 mL of methane were produced (Figure 4.7). As with sulfate-reduction activities, no correlation between the onset of dechlorination and methanogenic activities could be found. It has been shown previously that the inhibition of methane production increased dechlorination (Zanaroli et al., 2012a), as methanogens compete against dechlorinators for H_2 in the microbial community (Bossert et al., 2003b). However, the establishment of methanogenic conditions, lower than -250 mV in terms of redox potential, is a key factor for the onset of dechlorination activities (Alder et al., 1993; Kim and Rhee, 1999), and these data confirm that it's the low redox potential, rather than the presence of methanogenic activities itself, which might favor the onset of dechlorination activities. Neither methane production nor any evidence of sulfate reduction was ever observed in sterile controls, as expected (data not shown).

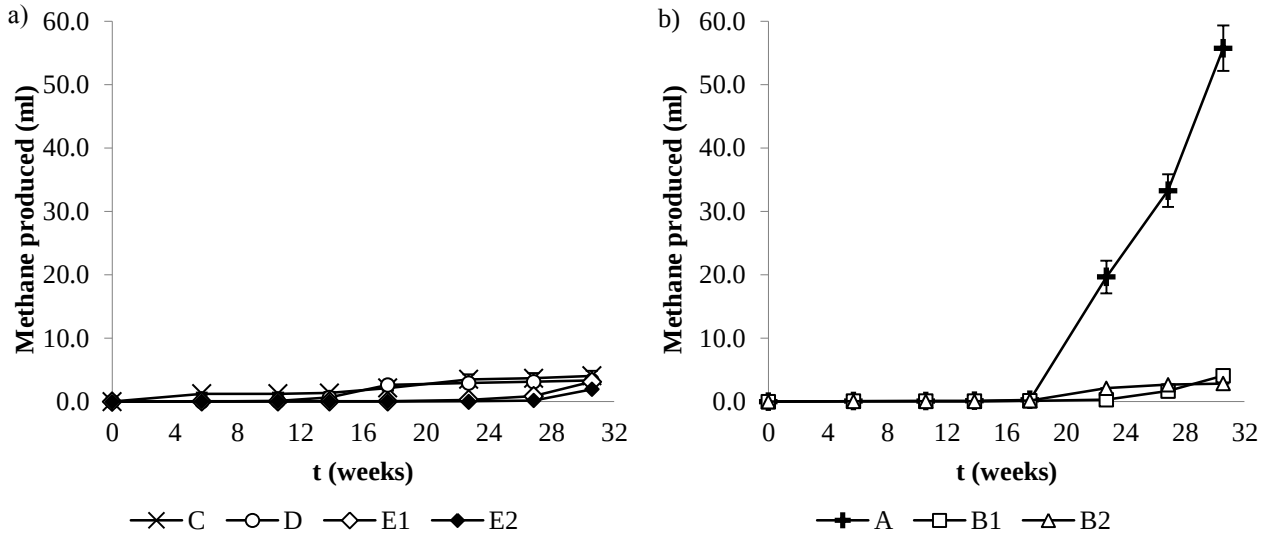

Figure 4.7: methane production in active dechlorinating cultures (A) and active non-dechlorinating cultures (B) throughout incubation. Values are average of duplicate microcosms and error bars represent standard deviations

4.1.3 Microbial community analysis

4.1.3.1 Identification of PCB-dehalogenating phylotypes

DGGE profiles for total bacterial community evidenced the presence of many different phylotypes, which mutual organization evolved with the time course of dechlorination (Figure 4.). Phylotypes showing relative band intensities increasing during incubation were excised and sequenced after a purification step. Two main phylotypes were detected: VLD-1 (bands 1, 4, 5 and 8 in Figure 4.) resulted to have high similarity with the known PCB dechlorinating *Chloroflexi* bacterium *Dehalobium chlorocoercia* DF-1 (99.8% sequence identity with GenBank accession number AF393781) and was found to enrich throughout incubation in sediment D, in both sediment E culture replicates and in sediment C. Phylotype VLD-2 associated with the band which becomes predominant in sediment C and in sediment E2 cultures (bands 2, 3, 7 and 9 in Figure 4.), resulted to have 100% sequence identity to the uncultured *Chloroflexi* bacterium N3-9 (GenBank accession number AY356384) and 93% sequence identity with *Dehalococcoides* sp. JN18_A30_B (GenBank accession number DQ168646).

DGGE analysis of the bacterial community enriched in sediment A, B1 and B2, where no PCB dechlorination occurred, showed no band ascribable to any of the two *Chloroflexi* phylotypes detected in the PCB dechlorinating cultures (data not shown). Therefore, phylotype VLD-1 is the candidate PCB dehalorespiring bacterium responsible for PCB dechlorination in sediment D and sediment E1 cultures, where very similar PCB dechlorination patterns were observed, while the

dechlorination activities seem to be ascribed for sediment C and E2 cultures to the contemporary presence of both phylotypes. Due to the differences in dechlorination specificity, as described in section 4.1.1, the association between phylotype and dechlorination could be done only through quantification of the candidate dechlorinators, via qPCR assays.

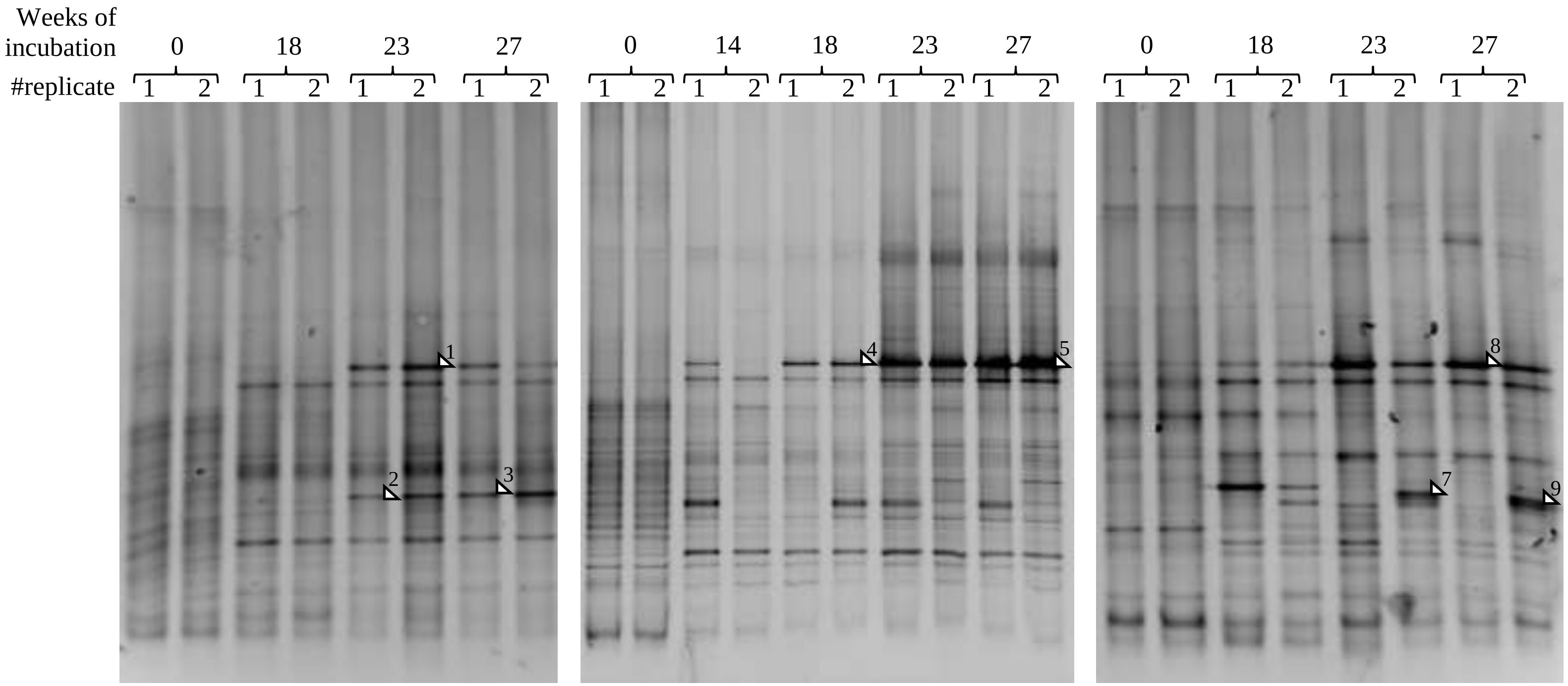

Figure 4.8: DGGE analysis of the total bacterial community of the dechlorinating cultures obtained from Sediment C, D and E (left, center and right, respectively). Arrows indicate excised bands, and band numbers are indicated. Phylotype VLD-1 (bands 1, 4, 5, and 8) enriched mainly in sediment D and E1 cultures, while phylotype VLD-2 (bands 2, 3, 7 and 9) enriched mainly in sediment C and E2 cultures. The analysis was performed independently on the duplicate cultures for each dechlorinating sediment**.**

PCR-DGGE analysis carried out with primers specific for *Chloroflexi* individuated again two phylotypes enriched at week 27; phylotype VLD-1c sequence (bands 1c, 3c and 4c) had 100% sequence identity with the phylotype VLD-1 and phylotype VLD-2c (bands 2c and 5c in Fig. 6)showed

100% identity to phylotype VLD-2. This confirmed that no other *Chloroflexi* came to relevance in any of the PCB dechlorinating cultures when the dechlorination process was almost completed (week 27).

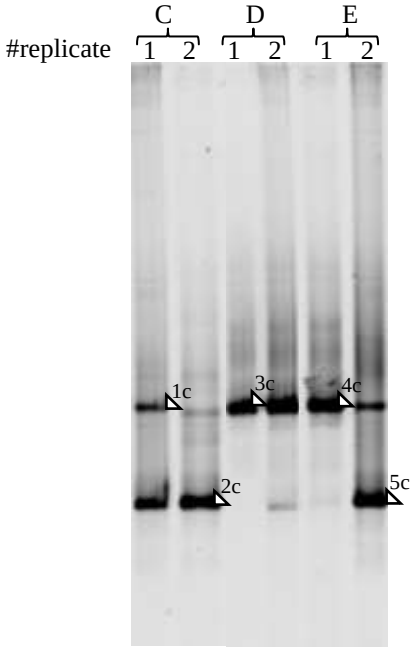

Figure 4.9: DGGE analysis of the Chloroflexi bacterial community after 27 weeks of incubation in the dechlorinating cultures from sediment C, D and E. Phylotype VLD-1c (bands 1c, 3c and 4c) phylotype VLD-2c (bands 2c and 5c) have 100% sequence identity to phylotype VLD-1 and with phylotype VLD-2 described previously. The analysis was performed independently on the duplicate cultures for each dechlorinating sediment.

4.1.3.2 Evolution of sediment indigenous microbial communities during PCB dechlorination

In order to investigate the evolution of the indigenous microbial community in the PCB dechlorinating cultures, PCR-DGGE analyses of the 16S rRNA genes were carried out on sediment C, D and E cultures at the beginning of the incubation, at the onset of dechlorination and throughout the incubation. In particular, samples were taken from sediment D after 0, 14, 18, 23 and 27 weeks of incubation, i.e., when the degree of chlorination was 5.1, 4.7, 4.2, 4.1 and 4.0, respectively; sediment C and E cultures were sampled after 0, 18, 23 and 27 weeks of incubation, when average number of chlorines per biphenyl molecule was 5.1, 5.0, 4.6, 4.4 (C), 5.1, 5.0, 4.4, 4.0 (E1) and 5.1, 4.9, 4.7, 4.5 (E2), respectively. Since a very low methanogenic activity occurred in all PCB dechlorinating cultures, the community analysis was limited to Bacteria only, so excluding the investigation of Archaea.

Variation of community richness (Rr) and organization (Co) grasped from the PCR-DGGE profiles were first used to analyses changes occurring at the community level in the cultures. In all the cultures the major changes occurred in the community structure of all cultures between the

beginning of incubation and the onset of PCB dechlorination. The parameters obtained from such DGGE analyses are reported in Figure 4.10. Original sediments differ in terms of species richness and community evenness, but can be grouped in pairs: sediment D and C host most diverse and even resident bacterial populations, as compared to sediment E; in the latter there is no relevant difference between replicates at the beginning of incubation. In sediments D and C, the initial Richness was above 20% and Gini significantly below 40%. On the contrary, sediment E1 and E2 populations were composed of 11 species more unevenly organized (Gini about 50%). At the end of incubation, sediment C population had the number of species it is composed of almost halved (Rr at the end of incubation =11) and became a bit more evenly organized (final Gini 45%), both modifications occurring utterly i.e. between T3 and T4. On the contrary, Sediment D community keeps almost constant the number of species throughout incubation but since week 18 it rearranges into a much more dominated population (final Gini =63%). As compared to the beginning of incubation, both sediment E1 and E2 communities evolve towards a more diverse population (Rr values are 13 and 17, respectively) and to a less even organization (final Gini 63% in both replicates). Community evenness gets to the top value at the end of incubation in both sediment E1 and E2, but it does it almost all at a sudden before 18th week of incubation in sediment E1, while it more gradually increases throughout incubation in sediment E2. In addition, while in sediment E1 species richness gets to its maximum value after 18 weeks of incubation before decreasing, in sediment E2 it steadily increases before topping at the 23rd week, not to be modified further on.

From the comparison between the trends of PCB dechlorination and evolution of bacterial communities taking place in different sediment cultures, it was possible to infer some interesting observations. The results are reported in Figure 4.10.

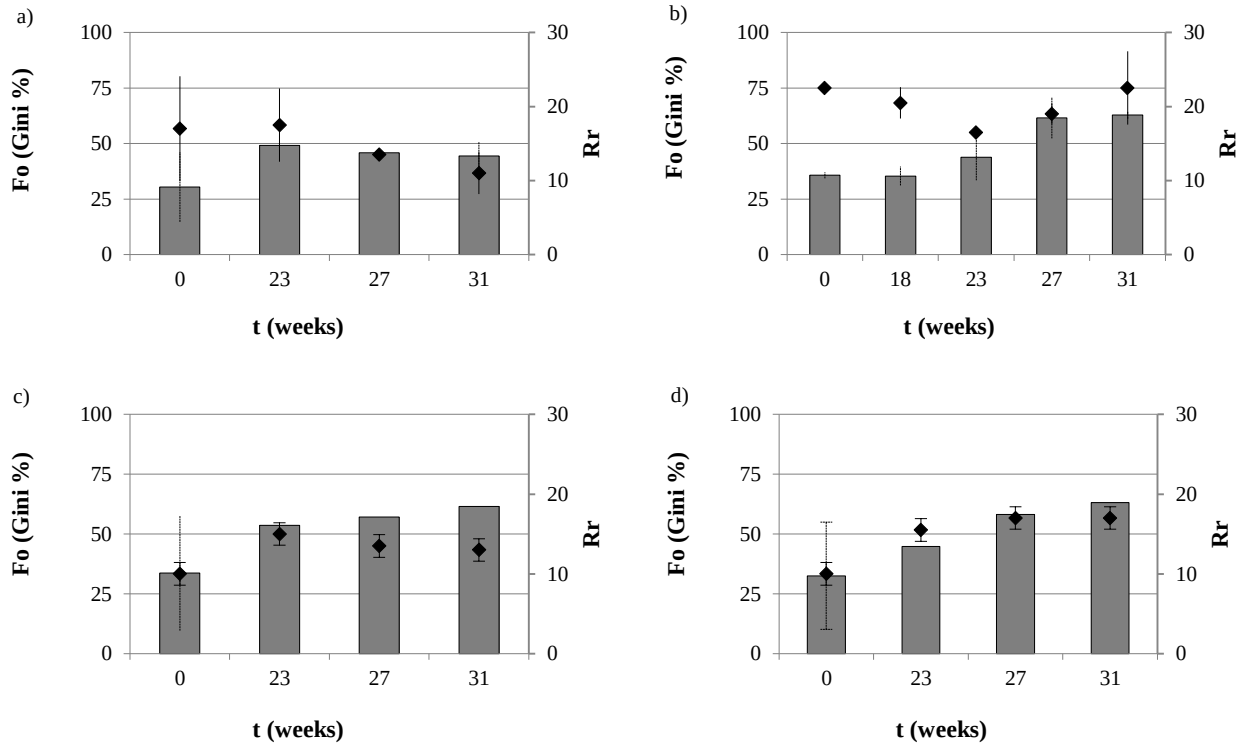

Figure 4.10: Fo (i.e. evenness reported as Gini coefficient percentage) and Rr in each sediment C, D, E1 and E2 cultures (A, B, C and D respectively) throughout incubation. Gini coefficients are represented by bars and Rr by diamonds. Standard error bars are reported and, where not evident, are either included in the symbol or not present because there were no replicates in DGGE analysis (sediments E).

The presence of even and highly diverse bacterial community in sediment D might be an effect of the PCB contamination occurring in that sediment; furthermore, it can possibly explain the remarkable rate of dechlorination and its early onset observed, since higher richness and evenness are connected to better functionality (Bell, 2005; Wittebolle et al., 2009). Indeed, in sediment D, alongside a slightly higher total amount of PCB as compared to that detected in other sediments, much more congeners were represented: this might have been either a consequence and a support for developing a diverse actively dechlorinating species, although a stimulation of other species, able to concur to or take advantage of dechlorination, cannot be excluded. The differences between the populations of sediment D on one side and of sediment C and E on the other are reflected by the differences in their initial richness and evenness. Nonetheless, a similarity can be grasped between diversity among microbial populations and differences in PCB dechlorination. During the exponential phase of dechlorination both communities' evenness and richness decrease. In particular, at the end of exponential phase the degree of chlorination reaches the lowest values in all cultures: interestingly the Gini is about 50% and the richness about 15 in all the cultures. Such approximate values might be as assumed to be the dechlorination threshold values which indicates the completion of dechlorination in the sediments studied here. While the F_o in sediment C is

constant, in sediment D, E1 and E2 the community becomes progressively less even. This trend of community evolution in sediments D and E fits with the progressively relevant selective advantage which the dechlorinating members gain over other possible competitors, thus coming to dominance. When dechlorination is actually over, during the stationary phase observed in sediment D for instance, the dechlorinators have no more advantage and other bacteria which on the contrary are released from possible competitors for carbon and energy sources and regain relevance among the whole. Nonetheless, in sediment D the community slowly shifts towards a more uneven organization after the completion of dechlorination: this might possibly not having been observed in other cultures because the exhaustion of the slurry cultures impeded further samplings.

As far as richness in concerned, it seems almost out of reach to identify any identity between different sediments. In sediment D and C the decline of richness until the completion of dechlorination is in line with the progressive selection of dechlorinators. Nonetheless, in sediments E the increase in number of the species detected during the progress of dechlorination apparently suggests a more cohesive system. It is interesting to notice that in sediment E the sulfate reduction is the slowest (Figure 4.5). The progression of sulfate reduction might have brought top dominance some species which benefit from dechlorination or which even support it somehow (May et al., 2008). It is also possible that in these sediments such microorganisms are initially inhibited than in sediment D or C, or less represented, the latter possibility being supported by the very low initial Rr in both sediments E. In addition, in replicate E2, in which the dechlorination does not proceed as much as it does in replicate E1, the richness does not decrease; it is possible that the dechlorinating species are either fewer or more entangled with competitors or by a somehow harsher environment than in sediment E1, where the dechlorination and consequently the ecological parameters might follow the same trend observed in other replicates.

Since the sudden spike of a considerable amount of PCB in sediments in which the initial contamination was negligible represents a stress, the sediment D which hosts the most diverse (i.e., having the highest initial Rr) reacts more promptly and efficiently than others. In fact, the presence of a species (the dechlorinator) which outcompetes the others can be resembled to the presence of an invader which has been proved to supports the functionality of the host community, as suggested by De Roy et al. (2013).

4.1.3.3 Quantification of PCB-dehalogenating phylotypes throughout incubation

Copy numbers of 16s rDNA genes of the specific phylotypes were quantified through qPCR and normalized versus total bacterial 16s rDNA copy numbers (Figure 4.11). Sediment D and E1 cultures showed that phylotype VLD-1 became dominant after 27 weeks (17±1% and 57±2% relative abundance, respectively) with concomitant negligible increase of phylotype VLD-2 (2%

and 10% relative abundance, respectively). On the contrary, in Sediment C Phylotype VLD-2 represented the major share of *Chloroflexi* populations (17±1% of total bacteria) after 27 weeks of incubation. Finally, in sediment E2 cultures the relative abundance of both VLD-1 and VLD-2 phylotypes increased (16±4% for phylotype VLD-1 and 11±0% for phylotype VLD-2) accounting together for 27% of total bacteria.

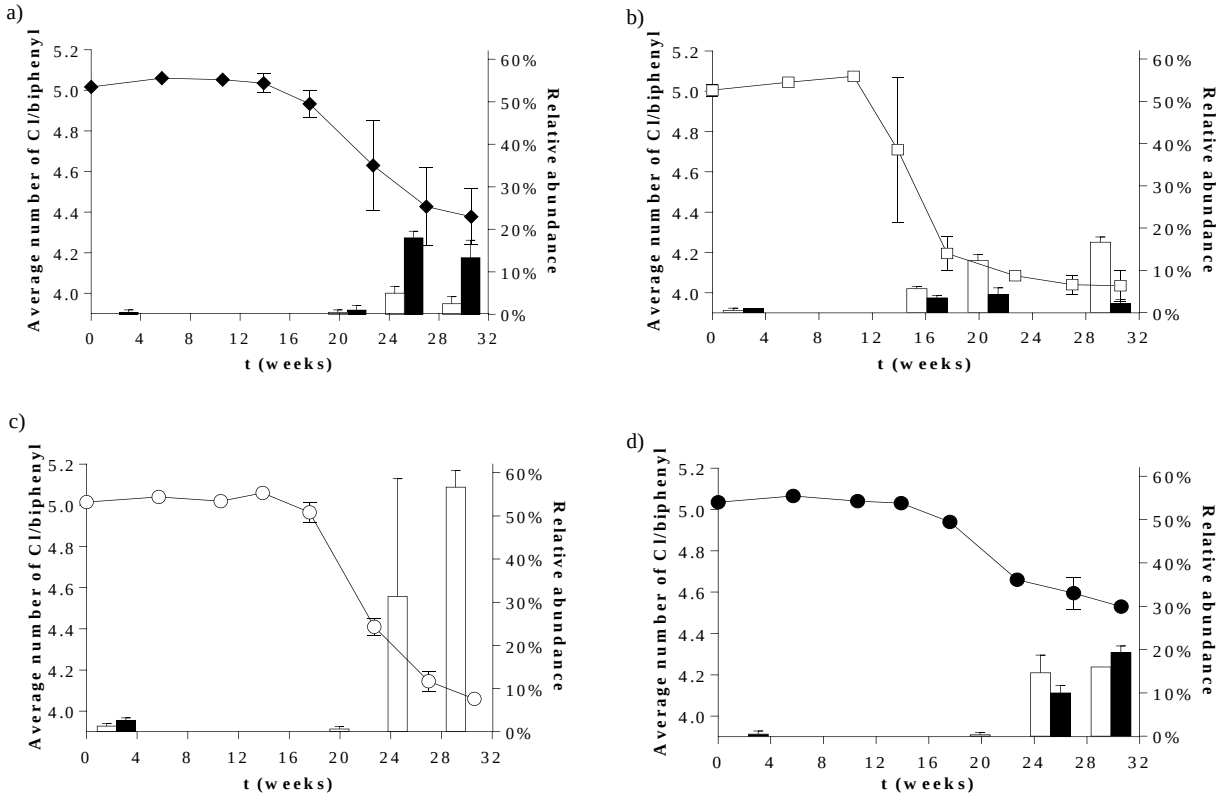

Figure 4.11: Time-course dechlorination of sediment C, D, E1 and E2 cultures (A, B, C and D respectively) expressed as decrease of degree of chlorination and the increase of phylotype VLD-1 (white bars) and phylotype VLD-2 (black bars) in the respective cultures expressed as percent relative abundance (16s rDNA gene copy numbers vs total 16s rDNA genes). Values are average of triplicate analysis performed on DNA extracted from each replicate culture and error bars represent standard errors.

Pearson similarity index analyses measured the correlation between VLD-1, VLD-2 and PCB dechlorination observed. A clear correlation between the relative abundance of phylotype VLD-1 and dechlorination was observed (Pearson index +0.98) in sediment D, E1 and E2 cultures; the increase of phylotype VLD-2 showed a moderate correlation (Pearson index of +0.68) in sediment C, E1 and E2 cultures. Based on this results, it can be concluded that VLD-1 is likely to be the main responsible for dechlorination activities in sediment D and E cultures, in spite of the differences in efficiency in the replicate E2, while the association between the presence of VLD-2 and the occurrence of dechlorination activity seem to be less consistent.

D. chlorocoercia DF-1 was first identified from estuarine sediment, in a bacterial culture capable to dechlorinate PCBs (Wu et al., 2002b), and successfully isolated only in co-culture with a *Desulfovibrio* sp. (May et al., 2008). To our knowledge, this is the first time that such *Chloroflexi* has been detected in a marine environment, but this is consistent with the ultramicrobacterium's high tolerance to salt conditions, which can be as high as 43.5 g/l (May et al., 2008), whereas salt concentration in the marine water of the dredging site were between 21 and 29 g/l (Table 3.3). On the other hand, the uncultured *Chloroflexi* N3-9 was found in the Baltimore Harbor sediments enriched with PCBs (Watts et al., 2005), a marine condition not far from those occurring in the Venice Lagoon.

The dechlorination pattern in sediment D, however, does not seem to be the same as reported in literature for *D.chlorocoercia* DF-1 (May et al., 2008; Watts et al., 2005): whereas the *D.chlorocoercia* DF-1 selectively dechlorinates double-flanked positions, the more prominent dechlorination in single-flanked *meta* chlorinated congeners detected in sediment D suggests that the phylotype VLD-1 is a different dechlorinator belonging to the same clade. This supposition is also sustained by the sequence similarity of the 16s rRNA gene, which is slightly below 100%. It is important to notice that VLD-1 sequence showed very high similarity to four clones isolated from a tidal sediment (namely Uncultured bacterium clone TfC20H76, TfC20H31, TfC20H77 and TfC20H32) which were shown to dechlorinated PCE and trans-DCE (Kittelmann and Friedrich, 2008). It is unsure whether these phylotypes and VLD-1 band could be ascribed to the same bacterium and further studies are required; however, the occurrence of dechlorination capabilities for both chlorinated solvents and PCBs in the same isolate has been already shown for other *Dehalococcoidia*, such as *D. mccartyi* CBDB1 (Adrian et al., 2009; Löffler et al., 2013) or *D. mccartyi* strain 195^T (Fennell et al., 2004; Yoshida et al., 2009). On the contrary, dechlorination pattern in sediment E1 showed a marked dechlorination in double flanked position, which is consistent with a contemporary presence of *D.chlorocoercia* DF-1 and the same phylotype detected in sediment D: these assumption might suggest that the amplified regions of the 16s rRNA gene used for this study might be too similar between the two to make distinction using our PCR-DGGE conditions.

While it is true that phylotype VLD-2 did not enrich exclusively in any of the sediment cultures, it is worth noticing that sediment C cultures showed a prominence of dechlorination process P, which targets also single and double-flanked *meta* positions, as it has been observed in the mixed cultures from the Baltimore Harbor sediment, from which uncultured bacterium N3-9 was isolated, which showed single *ortho* and double-flanked *meta* attack (Watts et al., 2005). Due to the complete

similarity of the 16s rRNA gene sequences and the overlapping dechlorination patterns detected, it can be speculated that phylotype VLD-2 could be uncultured *Chloroflexi* N3-9.

The finding of these two phylotypes is consistent with the detection of similar dechlorinating members from the *Dehalococcoidia* in other sediments of the Venice Lagoon: in particular, clone VL-CHL1 sequence had 100% similarity with phylotype VLD-2 (Zanaroli et al., 2012a), but exhibited dechlorination pattern H', which is considered to be very similar to process P (Bedard and Quensen, 1995). On the contrary, phylotype VLD-1 shows a different pattern compared to *D. chlorocoercia* DF-1 detected in a sediment dredged from the same area, whose activity was also stimulated by amendment with nanoparticles of Fe^0 (Zanaroli et al., 2012b). The detection of different phylotypes in different sediments may be dependent upon the sediment characteristics or the contamination history of the different sites (Kjellerup et al., 2008). It was already observed that the physical characteristics of the sediment did not influence the microbial dechlorinating population (Kim and Rhee, 2001). It is also true that TOC could be related to the development of dechlorinating species (Kjellerup et al., 2008) but, in the case of the Venice Lagoon, it seems that higher concentration of TOC only stimulate the onset of the dechlorination while no data so far support the hypothesis that TOC could be responsible for the differentiation of the dechlorinating community, as sediment C and E were similar in TOC but different in dechlorination pattern and in community evolution (Table 3.1. and Figure 4.10, Figure 4. and Figure 4.11). Unfortunately there are no data of TOC in sediments of the Venice Lagoon were PCB dechlorination was detected in previous studies, to determine whether it could have been a key factor in the enrichment of *D.chlorocoercia* DF-1 and clone VL-CHL1 in the other sediments.

There was also no information on the contamination history to determine if this was the main cause for the diversification of different dechlorinating phylotypes in different sediment. The contamination profile of the sediment D is also different from sediment C and E and, in particular, the presence of double-flanked congeners (2,2',3,3',4,5'-CB, 2,2',3,3',4,5-/2,2',3,3',5,5',6-CB, 2,2',3,3',4,4',6-/2,3,3',4,4',5-CB, 2,2',3,3',4,5,6-/2,2',3,3',4,5',6,6'-CB and 2,3,3',4,4',5,5',6-CB) (data not shown) which were not present in sediment C and E contamination, suggests a lack of activity against these congeners, coherently to what detected in sediment D cultures were only phylotype VLD-1 enriched.

Eventually, three out of six sediments used in the study showed PCB-dechlorination activities, suggesting that a potential for dehalogenation subsists in the Porto Marghera area of the Venice Lagoon. The detection of two new dechlorinating phylotypes which were linked through quantitative analysis to PCB dechlorination activities, is also a promising finding for the application of bioremediation approaches, which backs up the previous findings in the same area (Fava et al.,

2003b; c; Zanaroli et al., 2006; 2010; 2012a; b). Despite the fact that some of the conclusions might be verified further, for example by analyzing rRNA and rDNA levels to discriminate between actively growing and not-growing species (Amann et al., 1995; Marzorati et al., 2013), the evidences collected by q-PCR analyses and the investigation of bacterial communities evolution offered the chance to highlight some interesting features of the dechlorination carried out by bacterial populations of the sediments under study and allowed to draw strengthen the conclusions drawn and the discussion of the data presented here might represent a case of study for proving some recently proposed ecological theories.

Cultures from sediment D and E and their original sediments were used for successive experiments, where i) a set of electron donors was tested to biostimulate the enriched dechlorinating population (section 4.2), ii) a possible strategy of complementation with chemical dechlorination catalyzed by biogenic nanoparticles of Pd^0 was tested both on freshly-spiked Aroclor 1254 and on products of the dechlorination activities (section 4.3) and iii) scale-up cultures were evolved for bioremediation tests in actual sediments of the Venice Lagoon (section 4.5).

4.2 Selection of suitable electron donors for the biostimulation of enriched PCB-dechlorinating cultures

Different electron donors were tested on an enriched PCB-dehalogenating culture which was selected to minimize the complexity of the microbial community and elucidate effects of the selected compounds over the dehalogenating species. Sediment D was then aliquoted in different serum bottles, degassed with N_2:CO_2 70:30 and sterilized for three consecutive days at 121 °C for 1 h, with incubation at 30 °C in the dark between sterilization cycles to kill any spore-forming species. Marine water from the same site was then degassed with N_2:CO_2 70:30 and filter-sterilized prior mixing with sterilized sediment aliquots. Slurries were then inoculated 5% v/v with the active dechlorinating culture from sediment D at the end of the dechlorination activity (see section 4.1.1) and spiked with Aroclor 1254 at the final concentration of 1 g/l for a better assessment of the dechlorination activities. Secondary cultures were then incubated in anaerobic conditions, 28 °C statically in the dark.

Five electron donors were tested:

- Pure compounds: sodium pyruvate and sodium L-lactate. The positive effect of these short-chain fatty acids has been already detected in different freshwater sediments contaminated by PCBs (Chang et al., 2001; Dudkova et al., 2009; Azizian et al., 2010).
- Agro-food waste: cheese whey and molasses. The positive effects of these wastes were elucidated on the dechlorination of chlorinated solvents (PCE and TCE) in contaminated aquifers (Wu et al., 1998b; Buchner et al., 2001; DiStefano et al., 2001). The use of agro-food waste in biostimulation processes is a recent biovalorization strategy for waste, both economically advantageous and technically feasible. In particular, cheese whey was rich in fatty acids (butyric and caproic) while molasses were rich in sucrose, but also lactic acid (Table 3.4).
- Model for hydrogen release compound: polylactate PLA 4042D. The hydrolysis of this polymer is supposed to release electron donor (lactate) at low concentration and constant rate. Its beneficial effects were assessed in TCE dechlorination (Sandefur and Koenigsberg, 1999).

All electron donors were added periodically as filter-sterilized stocks, except polylactate which was added only at the beginning of incubation in form of grinded pellets. The concentration of each electron donor (see section 3.2.4) was chosen to be equivalent in terms of carbon to 2.5 mM of pyruvate, a concentration which is normally used to stimulate PCB-dechlorinating

activities in sediments resuspended with synthetic media (Bedard et al., 2006; Fagervold et al., 2007).

4.2.1 PCB dechlorination

No dechlorination was observed in sterile microcosm throughout incubation. Dechlorination activities were, instead, detected in every active culture.

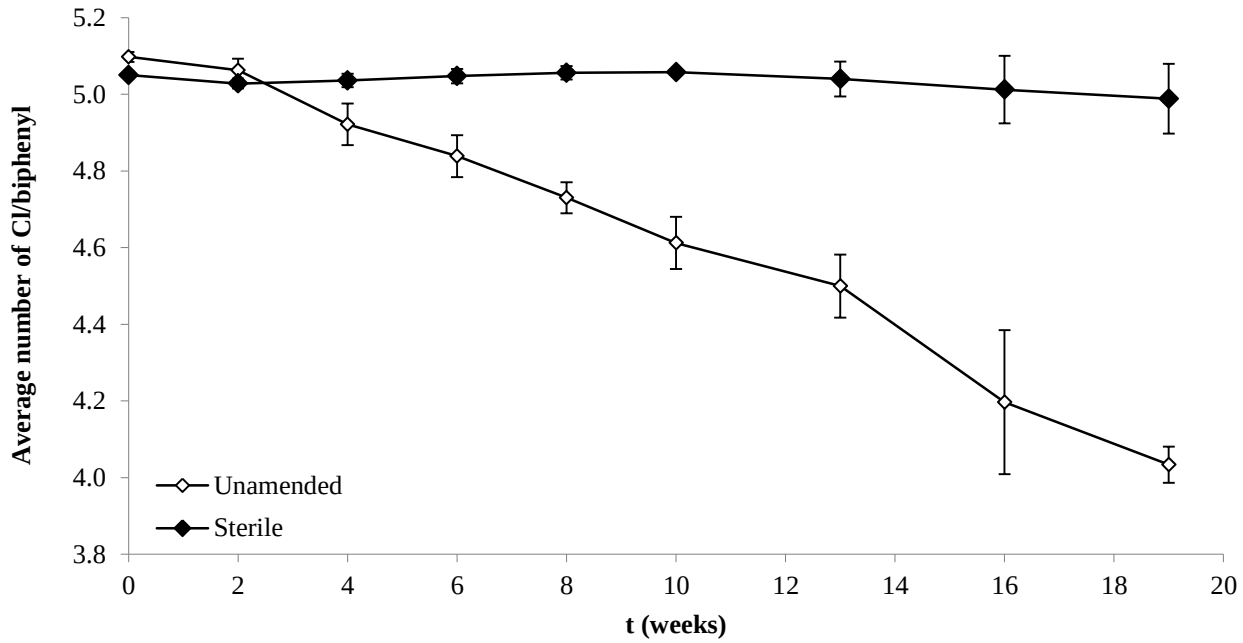

Figure 4.12: Time-course dechlorination of Aroclor 1254 mixture in not-amended secondary cultures and sterile controls. Values are average of triplicate microcosms.

The active unamended cultures exhibited dechlorination activity after 2 weeks of lag phase and the average number of chlorine atoms per biphenyl molecule was decreased in the PCB mixture from 5.1 to 4.0, with a maximum rate of dechlorination of 250±33 μmoles of chlorine removed (kg dry sediment)$^{-1}$ per week (Figure 4.12), a rate lower than the original culture from sediment D. At the end of dechlorination phase, after 19 weeks of incubation, 76±2% of penta- through heptachlorinated congeners were removed and bioconverted into tri- to tetra-chlorinated ones, of which the most accumulated congeners were 2,2',4,5'-CB (19 mol%) > 2,2'5,5'-CB (10 mol%) > 2,3',4-CB (6 mol%) > 2,2',4,4'-/2,2',4,5-/2,4,4',6-CB (6 mol%) > 2,3,3'-/2',3,4-/2,2',5,6'-CB (6 mol%) > 2,2',3,4-/2,3,4',6-CB (5 mol%) > 2,2',4,4',6-/2,3',4,5-CB (4 mol%) (Figure 4.13). This dechlorination pattern appears to be a combination of pattern P and N, which differs from pattern depicted in sediment D (Figure 4.2).

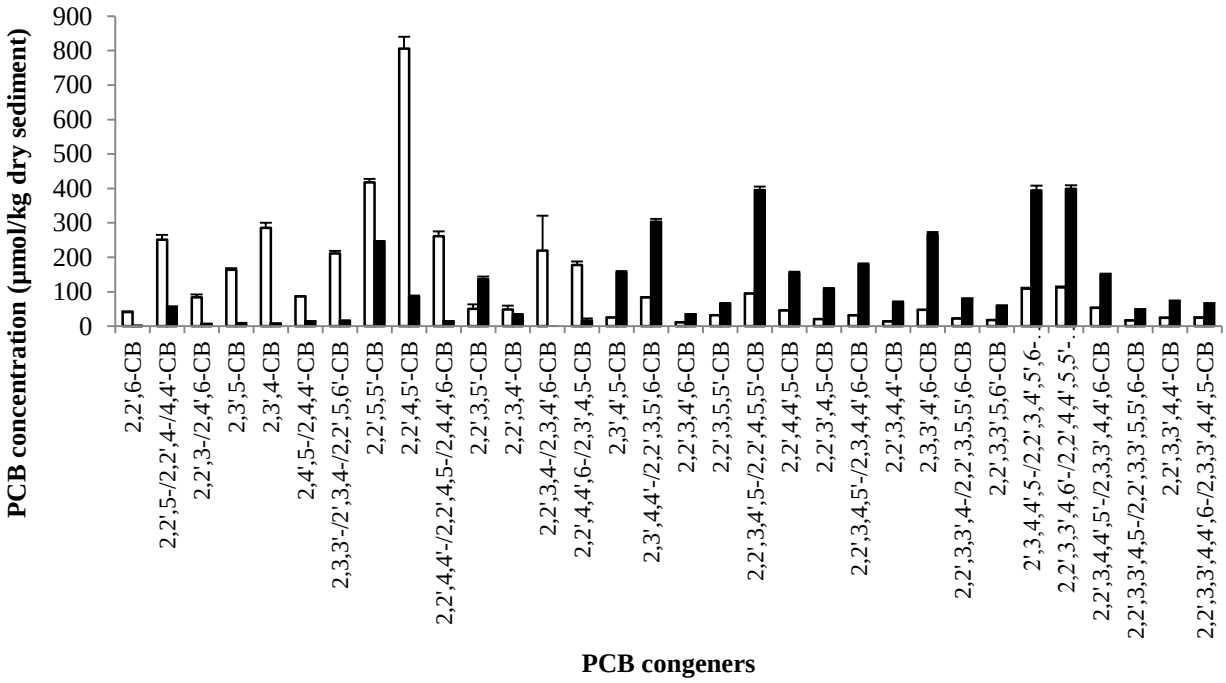

Figure 4.13: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active unamended cultures (empty bars), and sterile microcosms (black bars) after 19 weeks of incubation in secondary slurry cultures. Values are average of triplicate microcosms and error bars represent standard deviations.

In active microcosms amended with pyruvate as electron donor, the dechlorination process started also after 2 weeks and lowered the degree of chlorination from 5.0 to 4.0 at the rate of 252±23 µmoles of chlorine removed (kg dry sediment)$^{-1}$ per week (Figure 4.14) and the concentration of penta- through –heptachlorinated congeners was lowered by 75±5%.

Lactate-amended microcosms exhibited microbial reductive dechlorination after 4 weeks of incubation, instead of 2, but after 19 weeks the degree of chlorination was lowered to 3.9 with a dechlorination rate of 305±49 µmoles of chlorine removed (kg dry sediment)$^{-1}$ per week (Figure 4.14) and a depletion of 80±5% of penta- through octachlorinated congeners. In spite of the longer lag phase, dechlorination rate in lactate-amended culture is higher compared to unamended and pyruvate-amended cultures, the degrees of chlorination differs significantly (p <0.05, tested with Bonferroni method) after 12 and 16 weeks of incubation (Figure 4.14), and the removal of highly-chlorinated congeners was also more relevant. Pyruvate, on the other hand, does not show a significant difference with unamended cultures either in degree of chlorination, or in the percentage of bioconverted highly-chlorinated congeners.

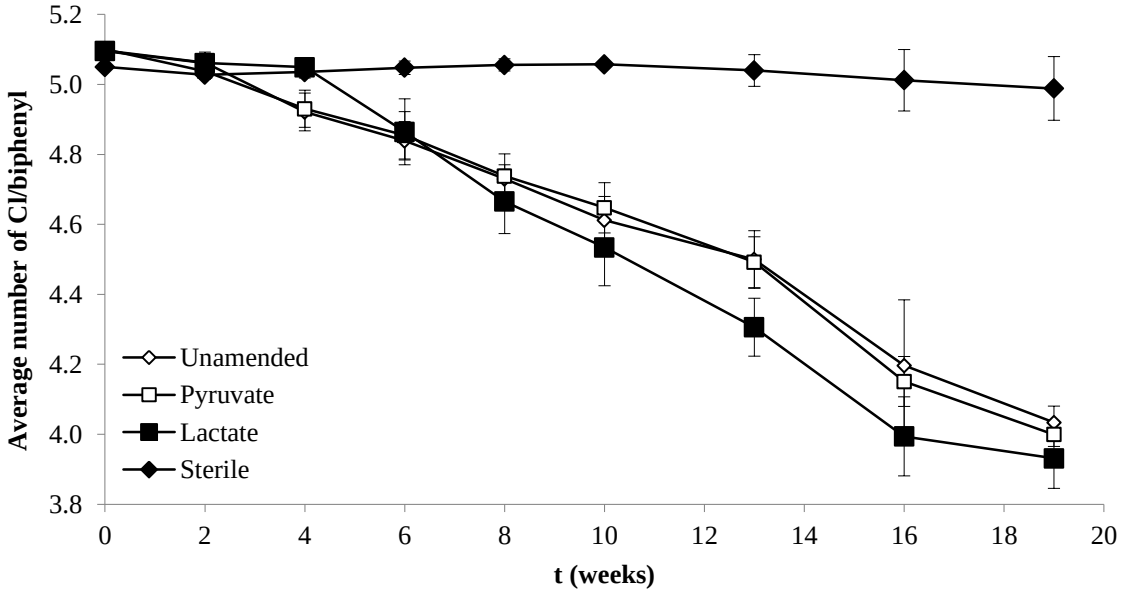

Figure 4.14: Time-course dechlorination of Aroclor 1254 mixture in unamended, pyruvate- and lactate-amended secondary cultures and sterile controls. Values are average of triplicate microcosms.

Pyruvate and lactate did not influence the dechlorination pattern, which results to be the same as unamended cultures, with only minor differences in concentration between the low-chlorinated congeners accumulated (Figure 4.15 and Figure 4.16).

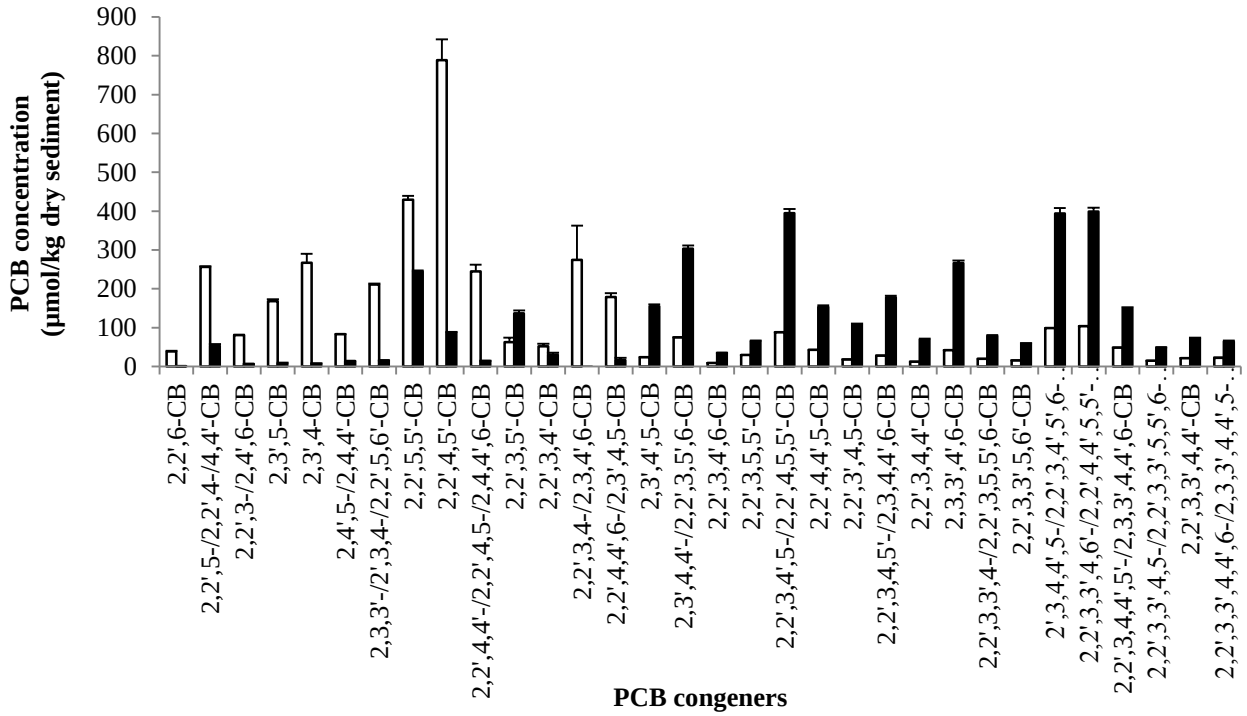

Figure 4.15: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active lactate-amended secondary cultures (empty bars), and sterile microcosms (black bars) after 19 weeks of incubation. Values are average of triplicate microcosms and error bars represent standard deviations.

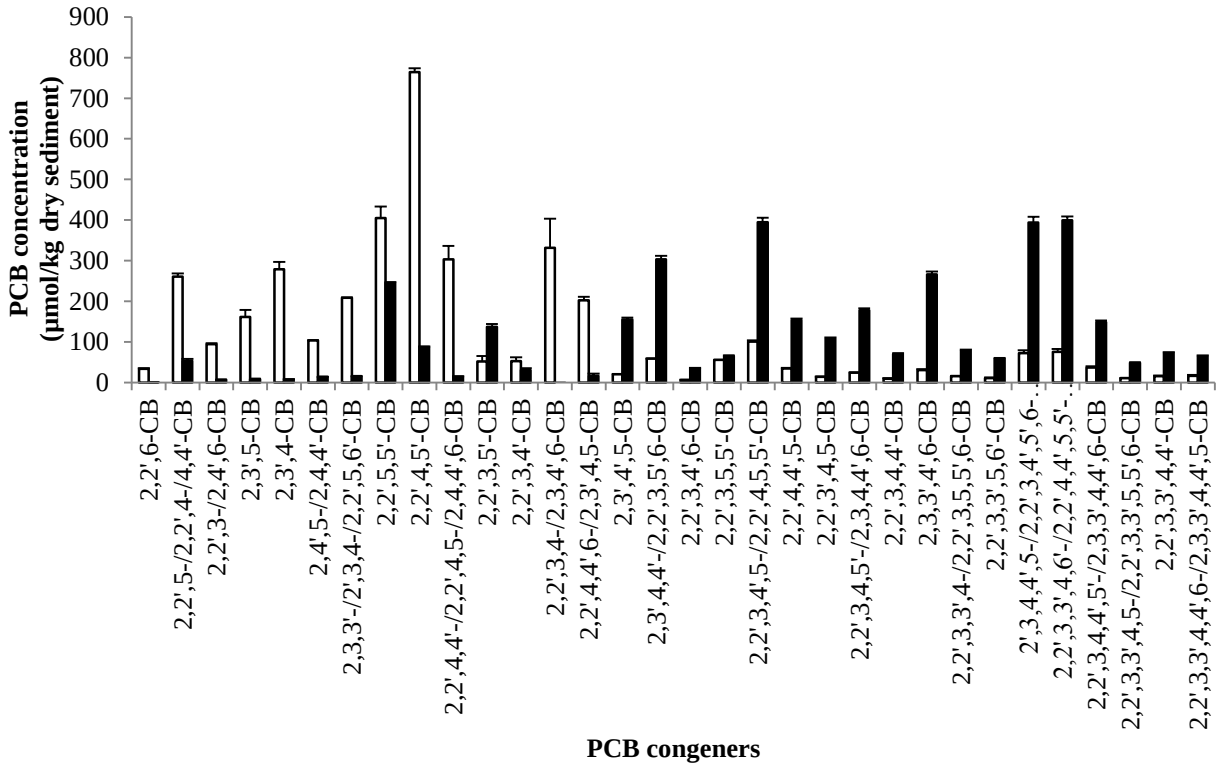

Figure 4.16: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active pyruvate-amended secondary cultures (empty bars), and sterile microcosms (black bars) after 19 weeks of incubation. Values are average of triplicate microcosms and error bars represent standard deviations.

In molasses-amended microcosms, dechlorination activities started from the second week of incubation, reducing the degree of chlorination from 5.0 to 4.0 in 19 weeks of incubation, with a maximum dechlorination rate of 252±25 µmoles of chlorine removed (kg dry sediment)$^{-1}$ per week (Figure 4.17) leading to a depletion of 75±35 of highly-chlorinated congeners.

The addition of cheese whey, instead, delayed the onset of the lag phase to 4 weeks of incubation, but the extension of the dechlorination process resulted the same as in molasses-amended cultures (minimum degree of chlorination reached 4.0; 78±5% of highly-chlorinated congeners removed at 245±48 µmoles of chlorine removed (kg dry sediment)$^{-1}$ per week average) (Figure 4.17).

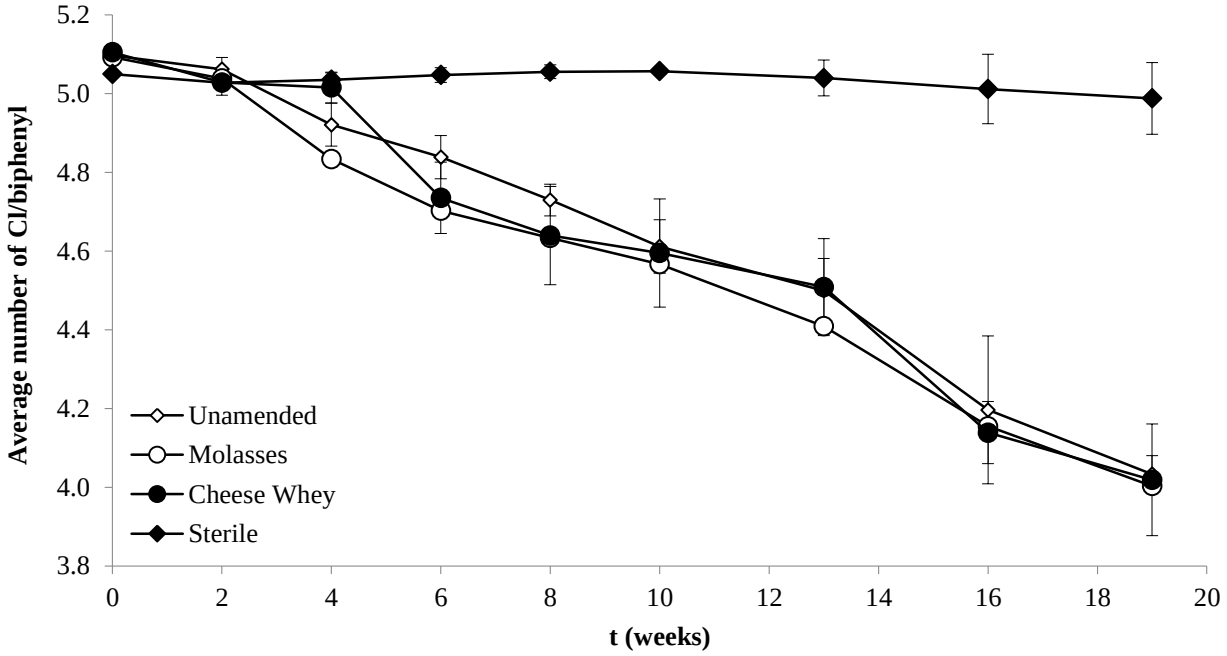

Figure 4.17: Time-course dechlorination of Aroclor 1254 mixture in unamended, molasses- and cheese whey-amended secondary cultures and sterile controls. Values are average of triplicate microcosms.

Dechlorination specificity was again the same in molasses- and cheese whey-amended cultures, as it was in the unamended cultures (Figure 4.18 and Figure 4.19). Both electron donors, then, did not have any significant influence on the dechlorination process.

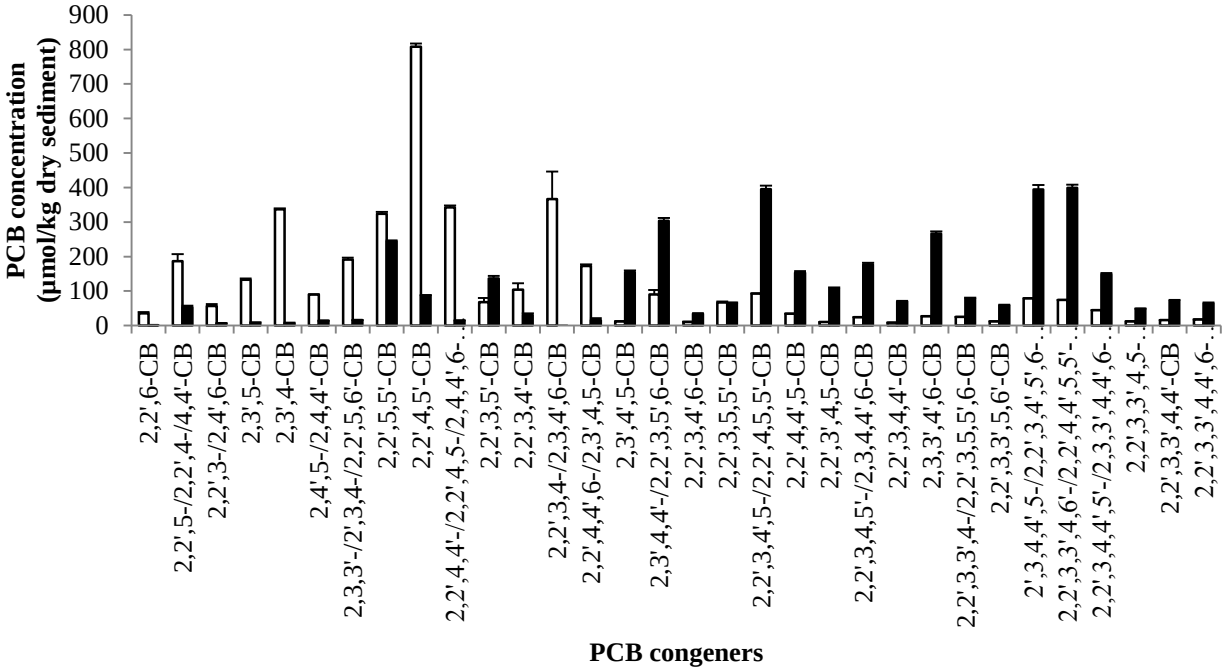

Figure 4.18: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active molasses-amended secondary cultures (empty bars), and sterile microcosms (black bars) after 19 weeks of incubation. Values are average of triplicate microcosms and error bars represent standard deviations.

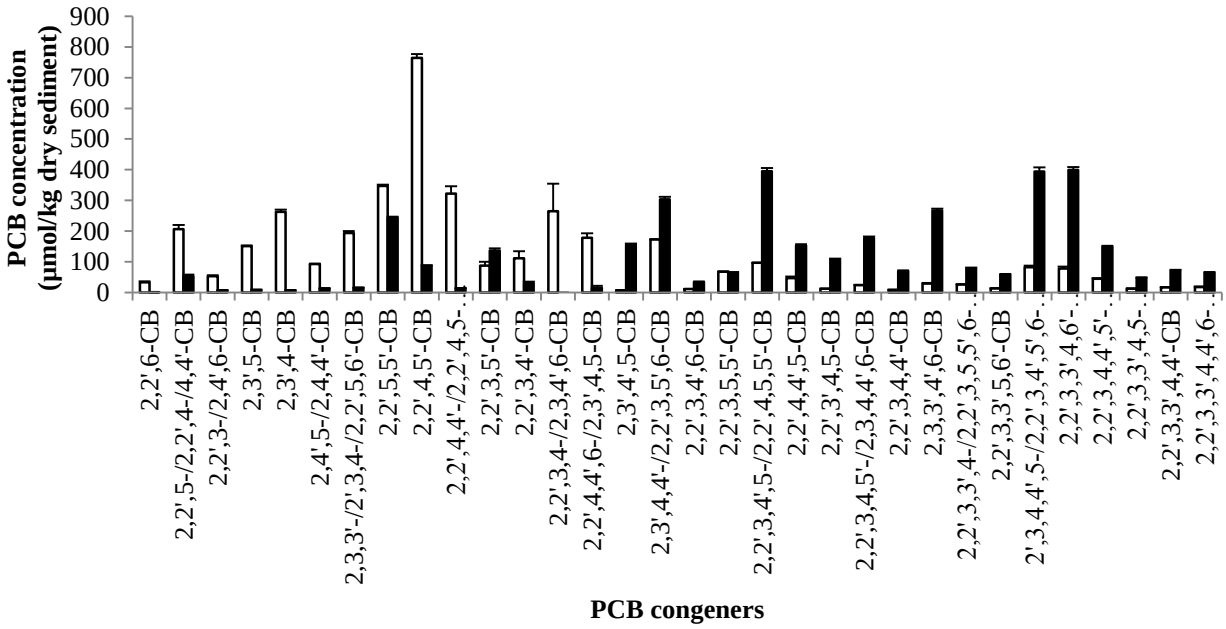

Figure 4.19: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active cheese whey-amended secondary cultures (empty bars), and sterile microcosms (black bars) after 19 weeks of incubation. Values are average of triplicate microcosms and error bars represent standard deviations.

Finally, the polylactate-amended cultures exhibited onset of dechlorination after 4 weeks of incubation, but this time the degree of chlorination was reduced to 4.1 after 16 weeks of incubation, when the dehalogenating process stopped (Figure 4.20). Even being the maximum dechlorination rate quite high (261±38 µmoles of chlorine removed (kg dry sediment)$^{-1}$), depletion of highly chlorinated congeners was lower compared to all other conditions (58±10%), with the same dechlorination process.

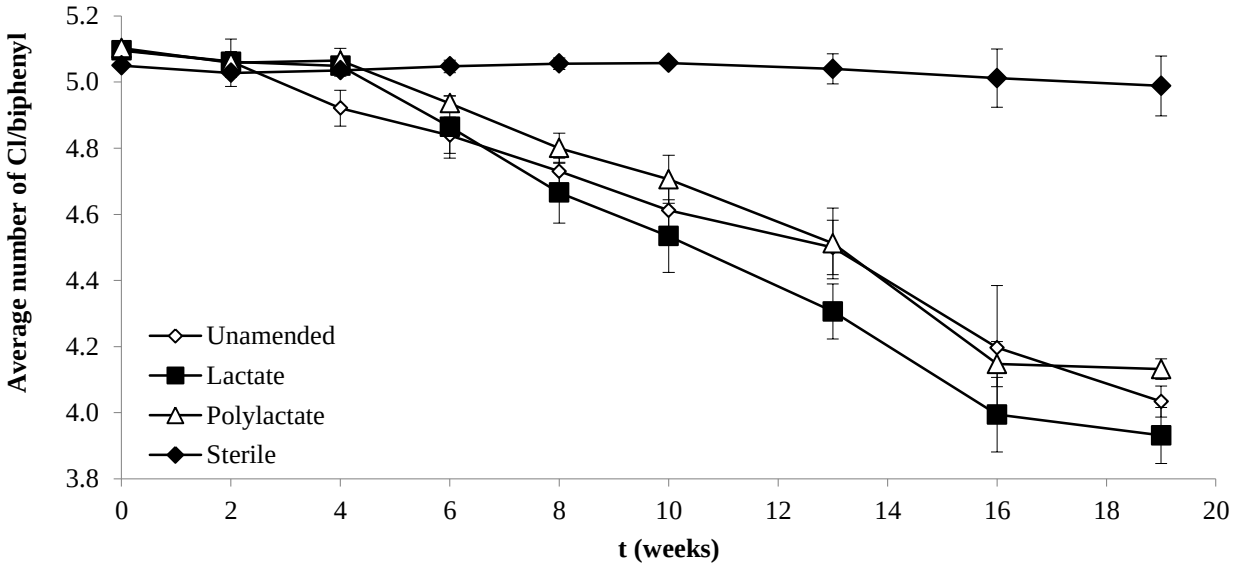

Figure 4.20: Time-course dechlorination of Aroclor 1254 mixture in unamended, lactate-, polylactate-amended secondary cultures and sterile controls. Values are average of triplicate microcosms.

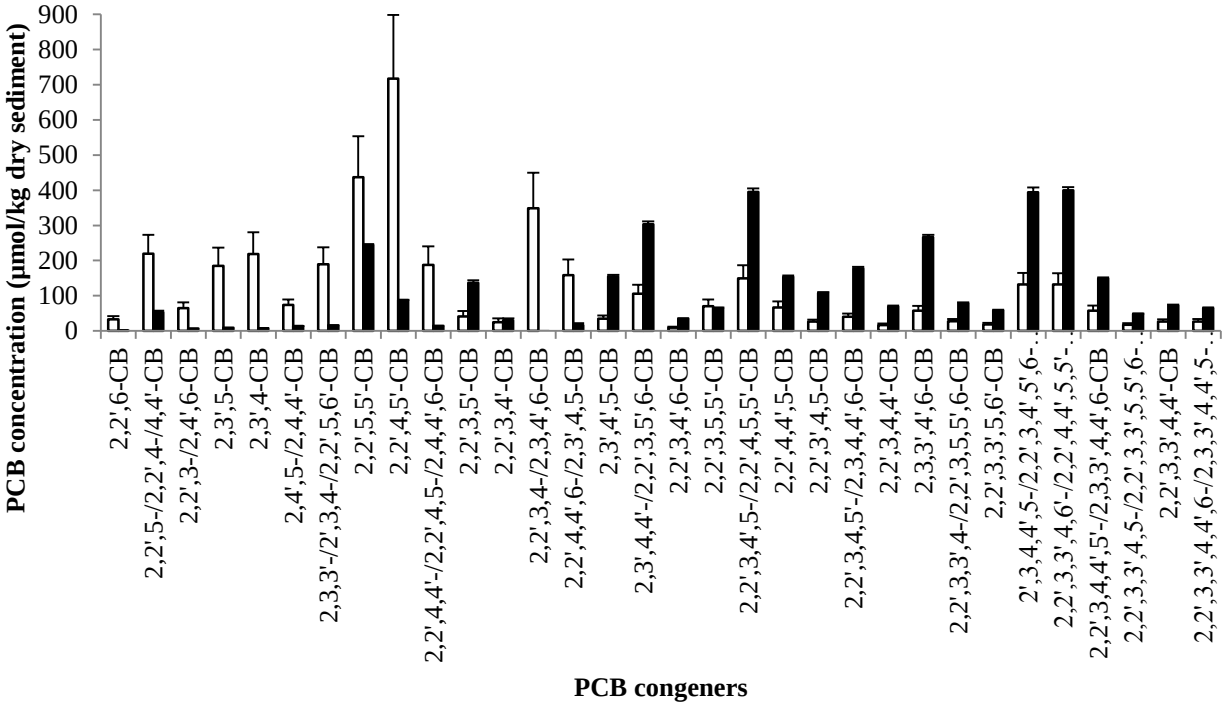

Figure 4.21: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active polylactate-amended secondary cultures (empty bars), and sterile microcosms (black bars) after 19 weeks of incubation. Values are average of triplicate microcosms and error bars represent standard deviations.

Eventually, dechlorination activities were detected in all secondary cultures, regardless of added electron donors. Pyruvate did not show any beneficial effect on dechlorination activities, contrary to what reported in literature (Chang et al., 2001; Dudkova et al., 2009; Klasson et al., 1996; Morris et al., 1992). This could either depend on the lower concentration used, compared to what reported elsewhere (2.5 mM vs 20 mM) (Morris et al., 1992) where the population was also enriched through different subculturing passages, contrary to the approach here described. Moreover, differences in microbial community and especially in the dehalogenating population might have been relevant.

Molasses was a suitable electron donor in TCE/PCE dechlorination by freshwater sediment cultures (Buchner et al., 2011; Suthersan and Payne, 2004); in marine sediments, however, it was ineffective in stimulating PCB dechlorination activities, and in this case the reason could be again the lower concentration that was used (2940 mg/l *vs* 6000 mg/l reported in Suthersan and Payne (2004)) as well as the differences in microbial population but also in the contamination itself.

On the other hand, cheese whey resulted to be ineffective in stimulating dechlorination activities, but the reason could possibly depend on a too high concentration: concentrations higher than 0.1 % v/v show inhibition of dechlorinating activities on PCE (Ibbinia et al., 2010); however, no inhibition of the PCB dechlorination process was detected, probably because of the different nature of the contaminant and of the microbial community.

Lactate was the only electron donor to stimulate dechlorination, slightly extending the process compared to unamended cultures. Moreover, the dechlorination rate and the percentage of highly-chlorinated congeners removed were significantly higher compared to all other electron donors, and this confirm data from freshwater sediment cultures (Chang et al., 2001; Dudkova et al., 2009).
Conversely, the addition of polylactate, backbone of the commercial HRC® used to stimulate TCE/PCE dechlorination in freshwater sediments (Semkiw et al., 2009) resulted to be inhibitory towards PCB dechlorination activities. It was considered that polylactate hydrolysis should have supplied the dehalogenating culture with a constant-rate low quantity of lactate, mimicking the periodic addition and limiting the use of hydrogen by competitors. The results, however, suggest a possible toxic effect of the polymer over the dehalogenating members of the community, which were less performing compared to unamended cultures. To our knowledge this is the first time that such effect is reported on a dechlorinating community.

4.2.2 Sulfate reduction and methanogenic activities

No sulfate reduction or methanogenic activities were detected in sterile microcosms throughout incubation. Unamended active cultures exhibited sulfate reduction after two weeks of incubation, and all sulfates were depleted within four weeks; methanogenic activities were limited and, starting at week 2 of incubation, produced totally 16.5±4.5 ml of methane in 19 weeks of incubation (Figure 4.22).
In pyruvate-amended cultures, sulfate depletion started at the beginning of incubation and all sulfates were depleted within 4 weeks of incubation, whereas methanogenic activities were slightly higher than unamended cultures, with a total production of 20.8±3.0 ml methane in 19 weeks of incubation (Figure 4.22). Conversely, lactate-amended cultures showed a slower depletion of sulfates which started at the beginning of incubation and ended by week 6; methanogenic activities produced 19.8 ±2.8 ml methane in 19 weeks (Figure 4.22).

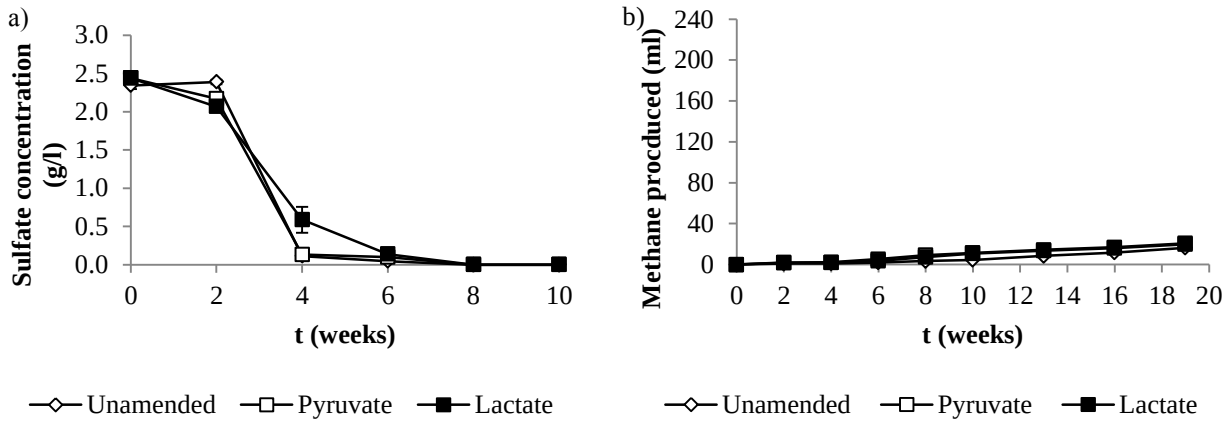

Figure 4.22: Sulfate concentration (A) and methanogenesis (B) in active unamended, pyruvate- and lactate-amended secondary cultures throughout incubation. Values are average of duplicate microcosms and error bars represent standard deviations

In molasses-amended cultures, the sulfate depletion also started at week 2 and ended at week 6; methanogenic activities were slightly higher than unamended microcosms (28.5±2.2 ml methane in 19 weeks of incubation) (Figure 4.23). Cheese whey stimulated sulfate reduction activities at the beginning of incubation, with complete depletion at week 4; most importantly it highly stimulated methanogenesis, up to a total production of 214±21 ml methane in 19 weeks of incubation (Figure 4.23).

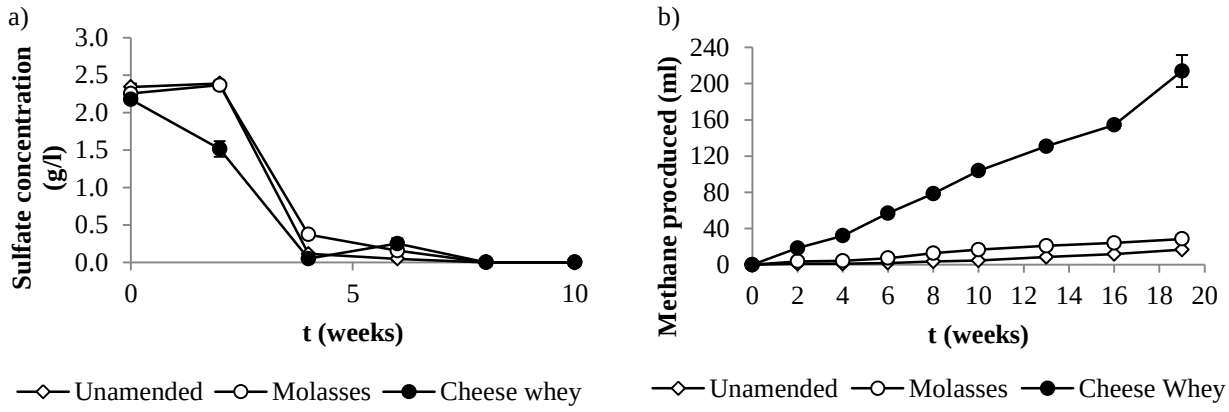

Figure 4.23: Sulfate concentration (A) and methanogenesis (B) in active unamended, molasses- and cheese whey-amended secondary cultures throughout incubation. Values are average of duplicate microcosms and error bars represent standard deviations

Polylactate-amended cultures exhibited sulfate reduction after 2 weeks of incubation, but at a slower rates compared to all other active cultures, as sulfates were depleted after 8 weeks of incubation; methanogenic activities were also partially inhibited, leading to a total production of 6.4±0.0 ml methane in 19 weeks of incubation, less than unamended cultures' total methane production (Figure 4.24).

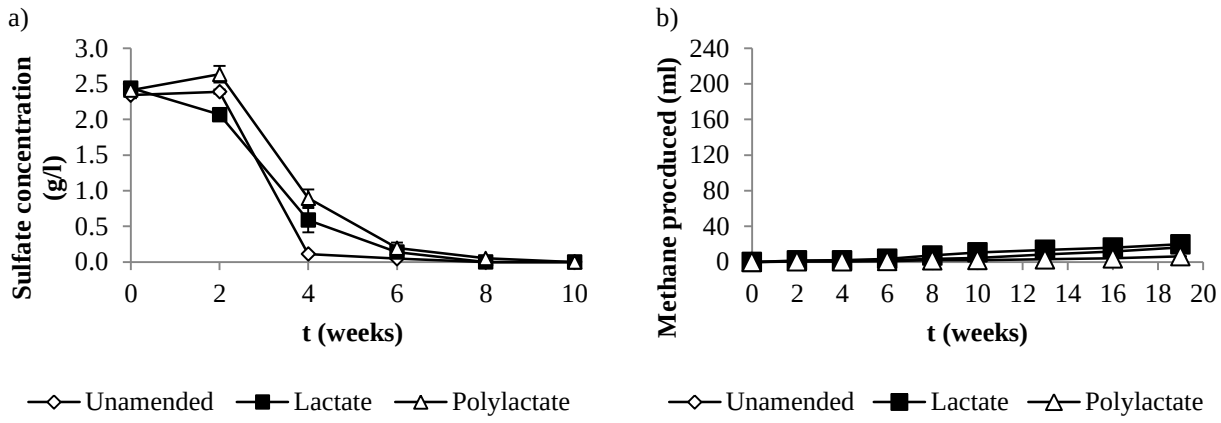

Figure 4.24: Sulfate concentration (A) and methanogenesis (B) in active unamended, lactate- and polylactate-amended secondary cultures throughout incubation. Values are average of duplicate microcosms and error bars represent standard deviations.

The effects of electron donors resulted to be, on the whole, more relevant on other anaerobic metabolisms rather than dechlorination. This was expected and it is actually the main obstacle to the application of biostimulation approaches in PCB-contaminated sediments, where competitors may profit of the hydrogen generated from these compounds, outcompeting the dechlorinating microorganisms (Wiegel and Wu, 2000).

Pyruvate, which did not stimulate dechlorinating activities, had also a weak impact on sulfate reduction and methanogenesis, confirming the hypothesis that the concentration employed was too low to measure any effect. Molasses, instead, were not able to stimulate dechlorination but slightly inhibited sulfate reduction and stimulated methanogenesis: this effect could be due to the high presence of sugars, which can be degraded down to methane through several different fermentation steps, in a syntrophic pathway (Stams, 1994). It is known that sulfate-reducers could outcompete methanogens for hydrogen and acetate, due to their higher affinity to these electron donors, particularly with high sulfate concentration, such as in marine conditions (Lovley et al., 1982; Robinson and Tiedje, 1984). Many methanogens, mostly in the marine environments, evolved mechanisms to outcompete sulfate reducers, for example using "non-competitive" substrates, such as methylamines (Ferry and Lessner, 2008). The high stimulating effect of cheese whey on methanogenesis over sulfate reduction could be due to the metabolization of the high proteic and acidogenic content into such substrates, suitable for methanogenic activities. It is also not to be excluded a possible positive effect due to slight acidification of the culture by the constant addition of cheese whey, as the average pH values of these cultures were constantly 0.3 lower than unamended cultures, which could have favored methanogens over sulfate reducers but not so much to inhibit dechlorination (data not shown).

Lactate, which was the only electron donor which stimulated dechlorination, exhibited a weak inhibition of sulfate reduction with a slight stimulating effect on methanogenesis; this is not

surprising, since syntrophic association of methanogens and sulfate reducers can degrade lactate (McInerney and Bryant, 1981), but the most important effect was the stimulation of the dechlorination activity, which was so far reported only in freshwater sediment cultures (Dudkova et al., 2009). Polylactate, instead, inhibited sulfate reduction and methanogenesis: this confirm the speculation of toxicity of the used polymer against the microbial community of the sediment. To our knowledge, this is the first time that such compounds are reported to be toxic in marine environments, but more studies need to be conducted to verify such hypothesis.

In conclusion, lactate is the best electron donor to stimulate PCB dechlorination in enriched cultures from sediments of the Venice Lagoon: for this reason, it was successively used as biostimulating agent in real sediments of the Venice Lagoon in lab-scale bioremediation tests (see section 4.5).

4.3 Complementation of microbial reductive dechlorination activities with chemical dechlorination catalyzed by biogenic Pd^0 nanoparticles

The possible use of biogenic nano-Pd catalysts in the remediation of PCBs was investigated in contaminated sediments of the Venice Lagoon.

BioPd nanoparticles were tested i) in conjunction with the activity of dechlorination in an active enriched culture, in order to complement microbial and chemical dechlorination (section 4.3.1, 4.3.2 and 4.3.3); ii) in addition to a sediment culture where all dechlorination activities were completed, in order to remove lower-chlorinated congeners (section 4.3.4); iii) in marine water and in slurry with a different sediment to assess dechlorination effectiveness (section 4.3.5).

4.3.1 PCB dechlorination activities in secondary cultures and sterile microcosms

Sediment E was aliquoted in different serum bottles, degassed with N_2:CO_2 70:30 and sterilized for three consecutive days at 121 °C for 1 h, with incubation at 30 °C in the dark between sterilization cycles to kill any spore-forming species. Marine water from the same site was then degassed with N_2:CO_2 70:30 and filter-sterilized prior mixing with sterilized sediment aliquots. Slurries were then inoculated 5% v/v with the active dechlorinating culture from sediment E at the end of the dechlorination activity (see section 4.1.1). Secondary cultures were then incubated in anaerobic conditions, 28 °C statically in the dark.

The following conditions were tested:

- Unamended active controls: secondary cultures enriched in dechlorinating species and spiked with Aroclor 1254 at 1 g/kg dry sediment, to assess dechlorination activities.
- Hydrogen-amended cultures: secondary dechlorinating cultures in H_2:CO_2 70:30 atmosphere, to test hydrogen effect on microbial community both in absence and in presence of PCBs (the latter cultures spiked with Aroclor 1254 at 1 g/kg dry sediment)
- Hydrogen+BioPd-amended cultures: secondary dechlorinating cultures in H_2:CO_2 70:30 atmosphere, to test BioPd complementation on dechlorination activities and its effects on microbial community both in absence and in presence of PCBs (the latter cultures spiked with Aroclor 1254 at 1 g/kg dry sediment). Two different concentrations of BioPd were tested for each approach, namely 5 and 50 mg/kg dry sediment.

The results showed that the active control, with no hydrogen and no BioPd nanoparticles, showed the highest dechlorination activity, with the reduction of the average number of chlorine atoms per biphenyl molecule from 5.1 to 4.3 in 18 weeks of incubation, leading to the depletion of 54±3% of

penta- to octachlorinated congeners at the maximum rate of 243±16 μmol Cl removed week^{-1} (kg dry sediment)$^{-1}$ (Figure 4.25). This rate is coherent with what reported in sediment E primary cultures (section 4.1.1), considered an average value of the two cultures E1 and E2, which were mixed before inoculation in secondary cultures. The addition of hydrogen headspace showed a strong inhibitory effect on the dechlorination rates, all of which were less than half than unamended cultures (107±37, 118±75 and 97±27 μmoles of chlorine removed (kg dry sediment)$^{-1}$ week^{-1} for hydrogen-amended and hydrogen + BioPd-amended cultures at 5 mg/kgdw and 50 mg/kgdw, respectively). The final degree of chlorination in all cultures after 18 weeks of incubation resulted to be 4.7 for the hydrogen-amended cultures and 4.7 and 4.9 for the hydrogen-amended cultures where BioPd was added at 5 mg/kgdw and 50 mg/kgdw, respectively (Figure 4.25); the percentages of depletion of highly chlorinated congeners were as low as 25%, 24% and 21%, for the afore-mentioned conditions, respectively. The differences between the dechlorination activities were not statistically significant at any time-point, meaning that the major impact on dechlorination was due to the presence of hydrogen. Unfortunately, this was a known risk, since it was already reported that the presence of high hydrogen concentration would inhibit dechlorination in sediments of the Venice Lagoon (Zanaroli et al., 2012a).

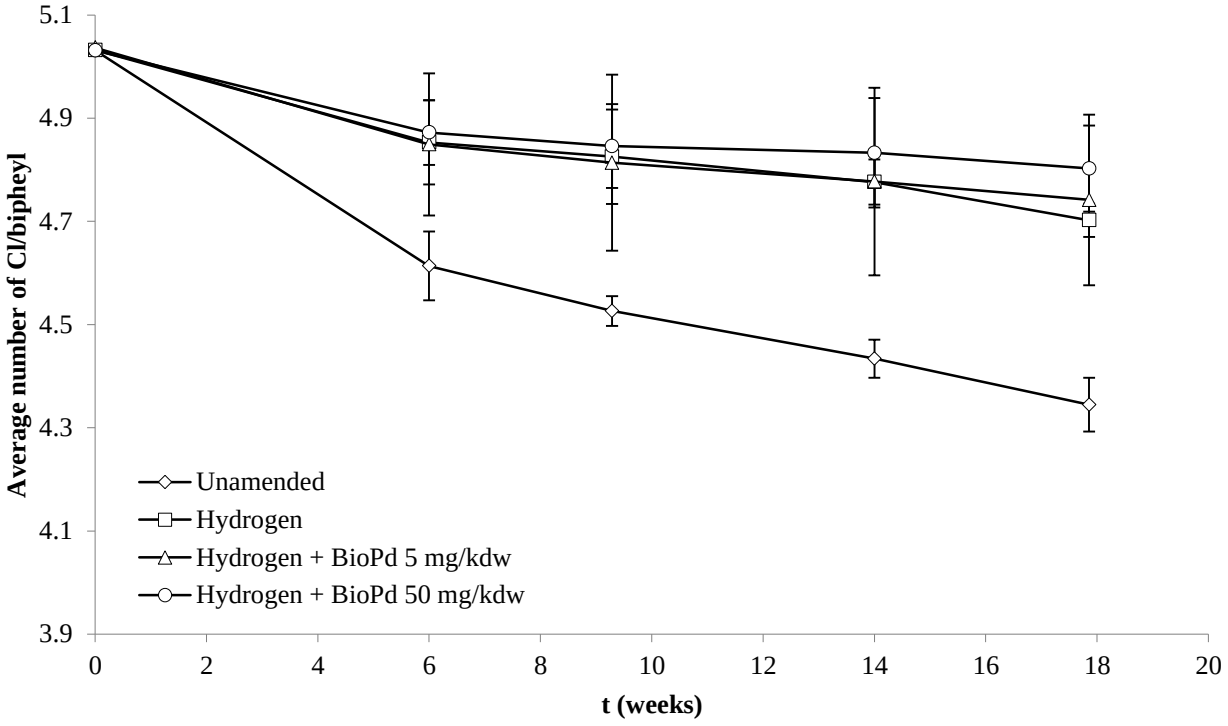

Figure 4.25: Time-course dechlorination of Aroclor 1254 mixture in not-amended, hydrogen-amended and hydrogen+BioPd-amended secondary cultures. Values are average of triplicate microcosms.

The dechlorination specificity in unamended cultures is similar to sediment E cultures, particularly replicate 1, with a predominance of process N rather than P and the accumulation of 2,2',4,5'-CB (14 mol %) > 2,3',4,4'-/2,2',3,5',6-CB (14 mol %) > 2,2',5,5'-CB (9 mol%) > 2,2',4,4'-/2,2',4,5-/2,4,4',6-CB (5 mol%) > 2,2',3,4-/2,3,4',6-CB (4 mol%) > 2,3,3'-/2',3,4-/2,2',5,6'-CB (3 mol%) > 2,2',5-/2,2',4-/4,4'-CB (3 mol%) after 18 weeks of incubation (Figure 4.26). The presence of penta-chlorinated biphenyls in high percentage (such as 2,2',3,5',6-CB, 2,2',3,4',5-/2,2',4,5,5'-CB and 2,2',4,4',5-CB) which are also relevant congeners in Aroclor 1254, indicates that the dechlorination process was still incomplete when the incubation stopped.

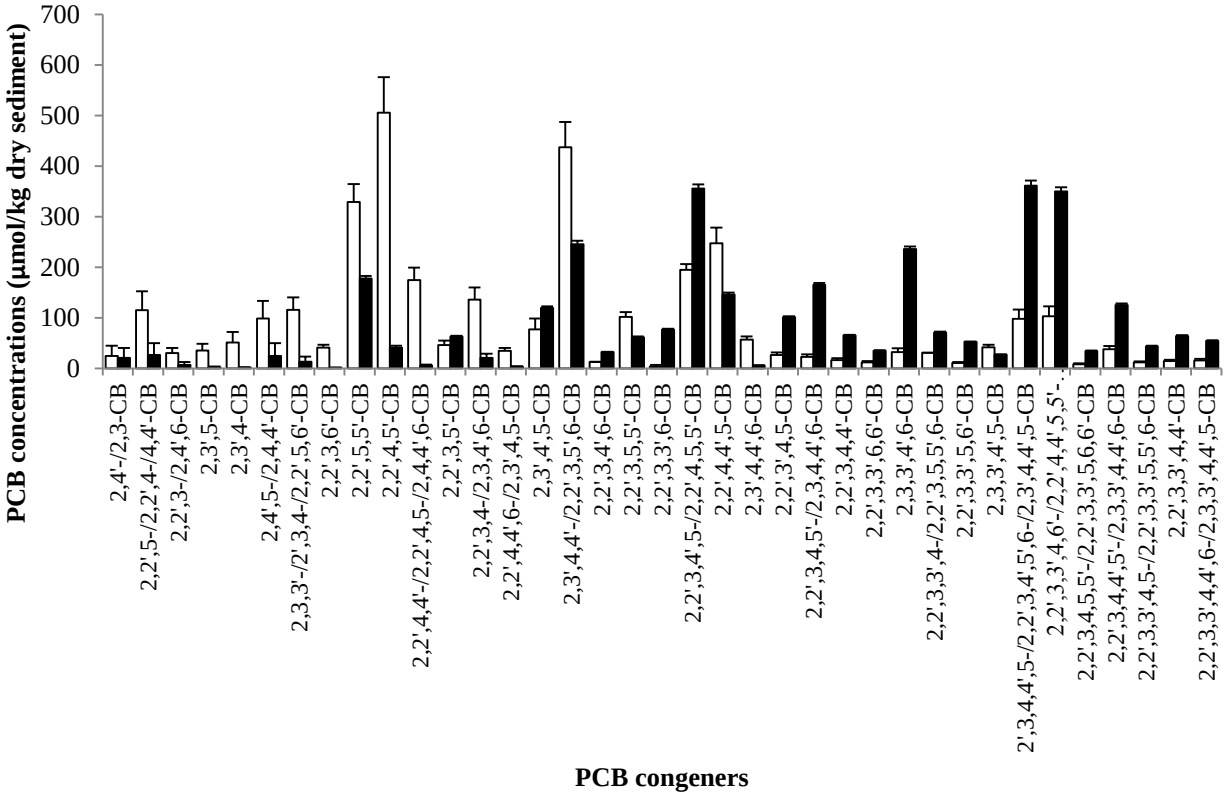

Figure 4.26: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the active unamended cultures (empty bars), and sterile unamended microcosms (black bars) after 18 weeks of incubation in secondary slurry cultures. Values are average of triplicate microcosms and error bars represent standard deviations.

Coherently with the lower activities in the hydrogen-amended microcosms, lower percentages of tetra- and tri-chlorinated congeners were detected at the end of incubation (Figure 4.27). Interestingly, hydrogen-amended microcosms showed a more prominent accumulation of 2,2',3,5'-CB detected in these cultures suggest a different dechlorination pattern, probably to process P (Figure 4.27). This is also true for cultures amended with hydrogen and BioPd, regardless of the

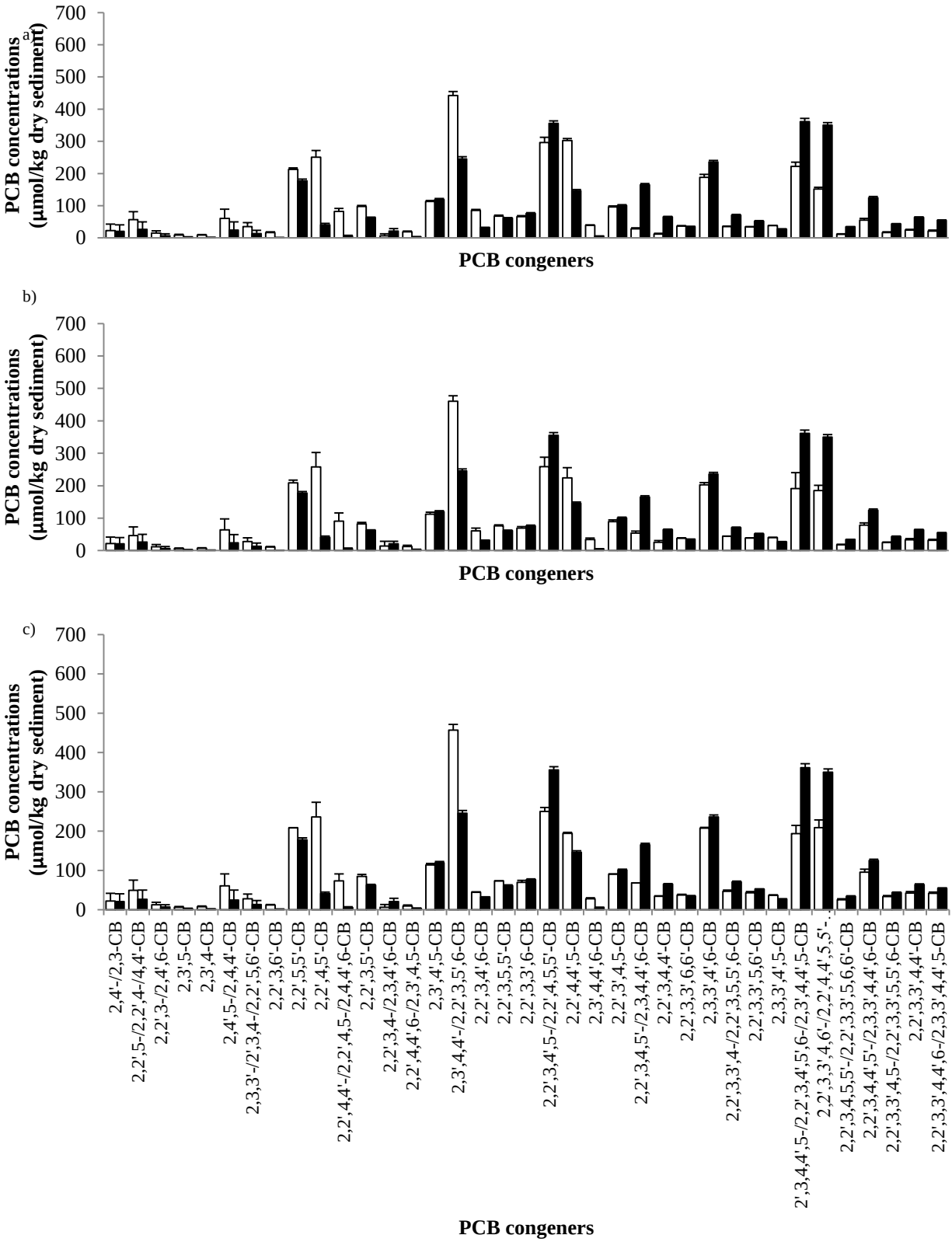

Figure 4.27: PCB congeners constituting more than 1% w/w of the contaminating mixtures in the hydrogen-amended (a) cultures and cultures with hydrogen+BioPd 5mg/kgdw (b) and 50 mg/kgdw (c) after 18 weeks of incubation in secondary slurry active cultures (empty bars) and sterile unamended controls (black bars). Values are average of triplicate microcosms and error bars represent standard deviations.

concentration of the latter, suggesting that this difference may depend only on the presence of hydrogen. These assumptions could not be verified further, due to the too low concentrations of tri-chlorinated congeners detected at the end of incubation in these cultures

Dechlorination activities were not monitored in non-Aroclor-spiked microcosms amended with either hydrogen or hydrogen with BioPd nanoparticles. Parallel to active secondary cultures, sterile microcosms were set-up with the same conditions described previously (unamended, hydrogen-amended and hydrogen+BioPd amended at 5 mg/kgdw and 50 mg/kgdw) and spiked with Aroclor 1254 at 1 g/kgdw. No dechlorination was detected in any of the sterile microcosms, regardless of hydrogen atmosphere and BioPd amendments (Figure 4.28). This suggests that BioPd nanoparticles were somehow inhibited in the conditions that were used, probably because of some characteristics of the sediment itself.

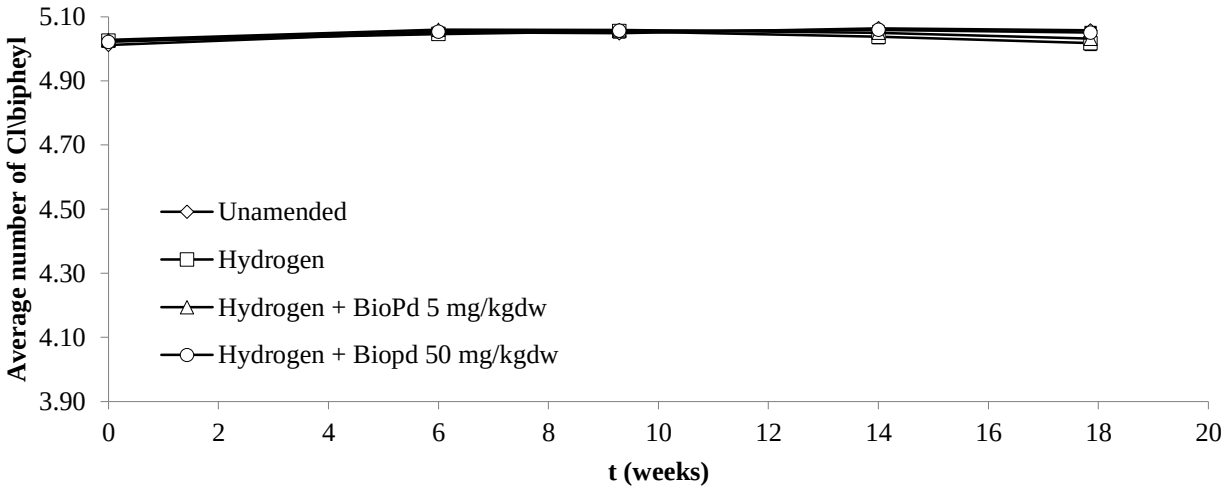

Figure 4.28: Time-course dechlorination of Aroclor 1254 mixture in not-amended, hydrogen-amended and hydrogen+BioPd-amended sterile microcosms. Values are average of triplicate microcosms.

4.3.2 Sulfate reduction and methanogenic activities

Sulfate-reduction activities were monitored both in spiked and in non-spiked secondary cultures. No sulfate consumption was detected in the sterile microcosms throughout the incubation. Conversely, the initially occurring sulfates (2.5 g/l average) were completely consumed in the not amended biologically active microcosms after 6 weeks of incubation. A similar sulfate consumption was detected in the biologically active control with hydrogen, while sulfate was consumed only after 9 weeks of incubation in the biologically active microcosms supplemented with BioPd at 5 mg/kg, and at the end of incubation in the biologically active microcosms supplemented with 50 mg/kg of

BioPd (Figure 4.29a). Thus, a dose-depended inhibition of BioPd on sulfate reduction was observed.

No methane accumulation was observed in the sterile microcosms throughout incubation. In all biologically active microcosms further contaminated with PCBs, hydrogen atmosphere stimulated methanogenesis compared to the not amended control, leading to a production of 27 ml of methane in the BioPd-free microcosms versus 6 ml in the control after 18 weeks on incubation. It has been reported that hydrogenotrophic methanogens typically compete for hydrogen with dehalorespirers (Wiegel and Wu, 2000; Kassenga and Pardue, 2006) and the positive effects of hydrogen were already observed on methanogens to the detriment of dechlorinators in sediments of the Venice Lagoon (Zanaroli et al., 2012a). In addition to the positive effect of hydrogen, a negative effect of BioPd nanoparticles was again noticed in biologically active microcosms, where 17±1 and 13±1 ml of methane were produced respectively for the 5 mg (kg dry sediment)$^{-1}$ and the 50 (kg dry sediment)$^{-1}$ BioPd amendments, corresponding to 62% and 48%, respectively, of the methane produced in the corresponding BioPd-free control (Figure 4.29b).

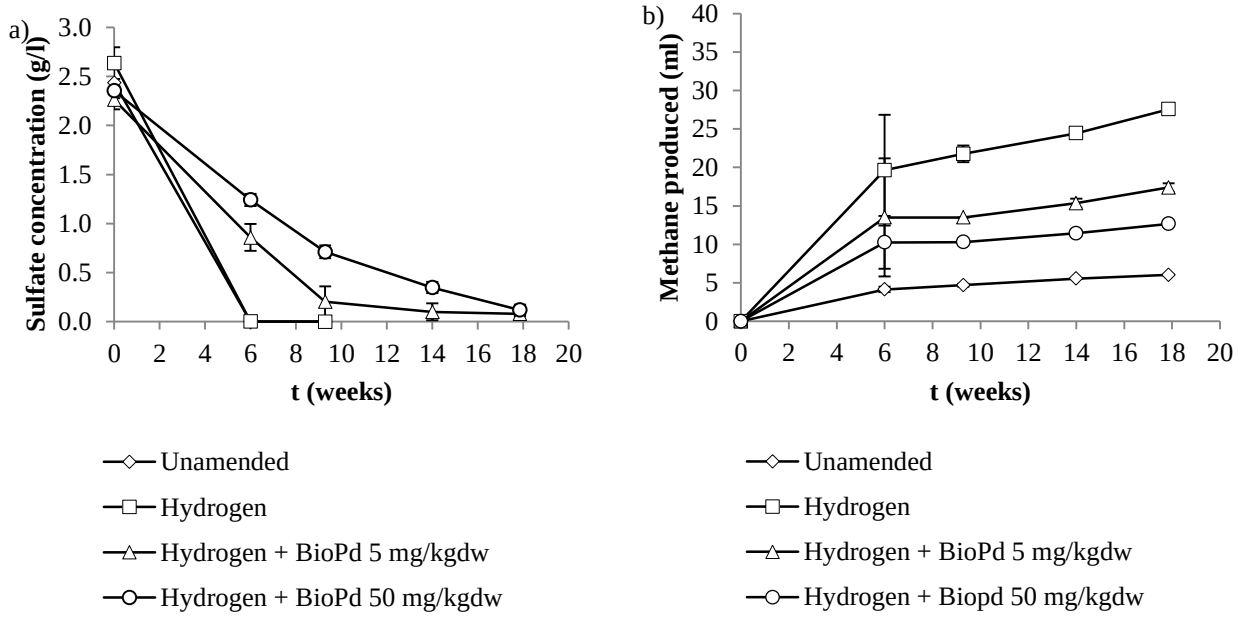

Figure 4.29: Sulfate concentration (A) and methanogenesis (B) in active unamended, pyruvate- and lactate-amended secondary cultures spiked with Aroclor 1254 at 1 g/kg dry sediment throughout incubation. Values are average of triplicate microcosms and error bars represent standard deviations

Similar dose-dependent effect of BioPd was noticed in the PCB-free active microcosms where sulfates were almost completely removed in 6 weeks of incubation only in the absence of BioPd, whereas complete sulfate depletion occurred at week 14 and at the end of incubation for BioPd-amended cultures with 5 mg/kgdw and 50 mg/kgdw, respectively (Figure 4.30a). This indicates the

occurrence of some inhibitory effect of Bio-Pd on the indigenous sulfate-reducing microorganisms. Methanogenic activities were observed in the corresponding microcosms not spiked with PCBs as 56% and 41% of the methane produced in the BioPd-free control were observed in the presence of 5 mg/kg and 50 mg/kg Bio-Pd, respectively (Figure 4.30b); these data suggest some inhibitory effect of BioPd on the methanogenic microorganisms, as well. To our knowledge, this is the first time that such effects are hypothesized in environmental applications on palladium nanoparticles; the environmental toxicity of nanometallic catalysts is currently under debate (Sánchez et al., 2011) and, depending on the nature of the particles, it has been shown they can exert toxic effects on mammal cell lines, too (Schrand et al., 2010); thus long-term evaluations are required in case of the application of such nanoparticles into the environment (Grieger et al., 2010).

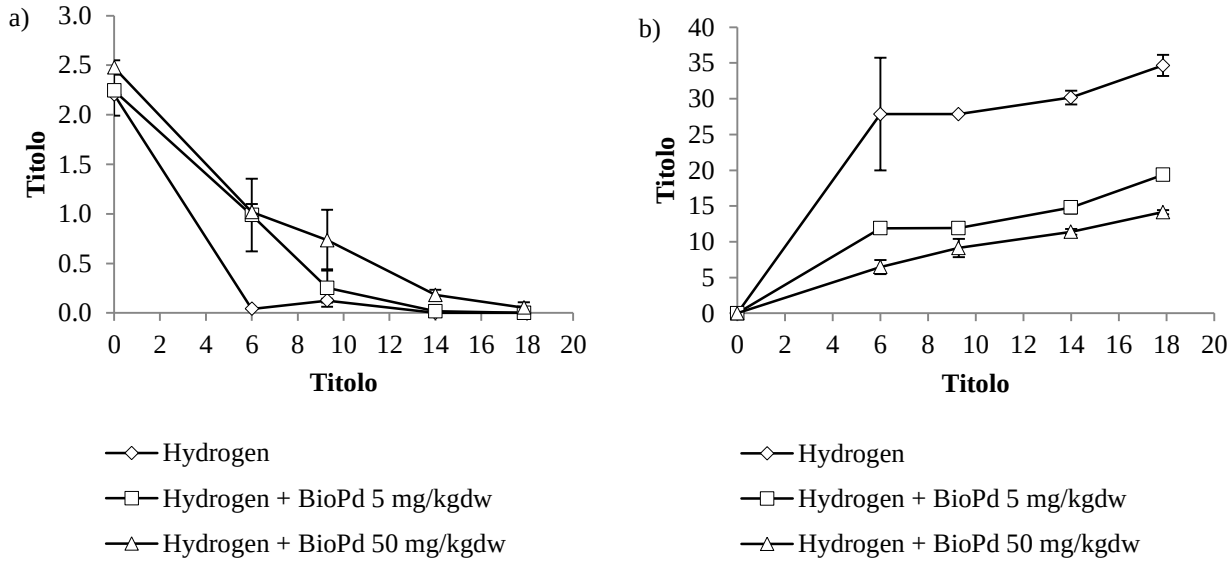

Figure 4.30: Sulfate concentration (A) and methanogenesis (B) in active unamended, pyruvate- and lactate-amended secondary cultures not spiked with PCBs throughout incubation. Values are average of duplicate microcosms and error bars represent standard deviations

4.3.3 Influence of BioPd nanoparticles on the microbial community

In order to investigate the evolution of the indigenous microbial community in BioPd-amended secondary cultures, PCR-DGGE analyses were carried out on the bacterial communities; samples were collected at the beginning, at the half and at the end of incubation (0, 9 and 18 weeks, respectively) and replicates of each condition were mixed before starting the electrophoretic run (Figure 4.31). All cultures showed a marked increase in community richness (Rr) at the end of incubation except for unamended cultures and it is reasonable to argue that the increase in the number of species could be due to a general stimulatory effect of hydrogen in all cultures (Figure 4.31).

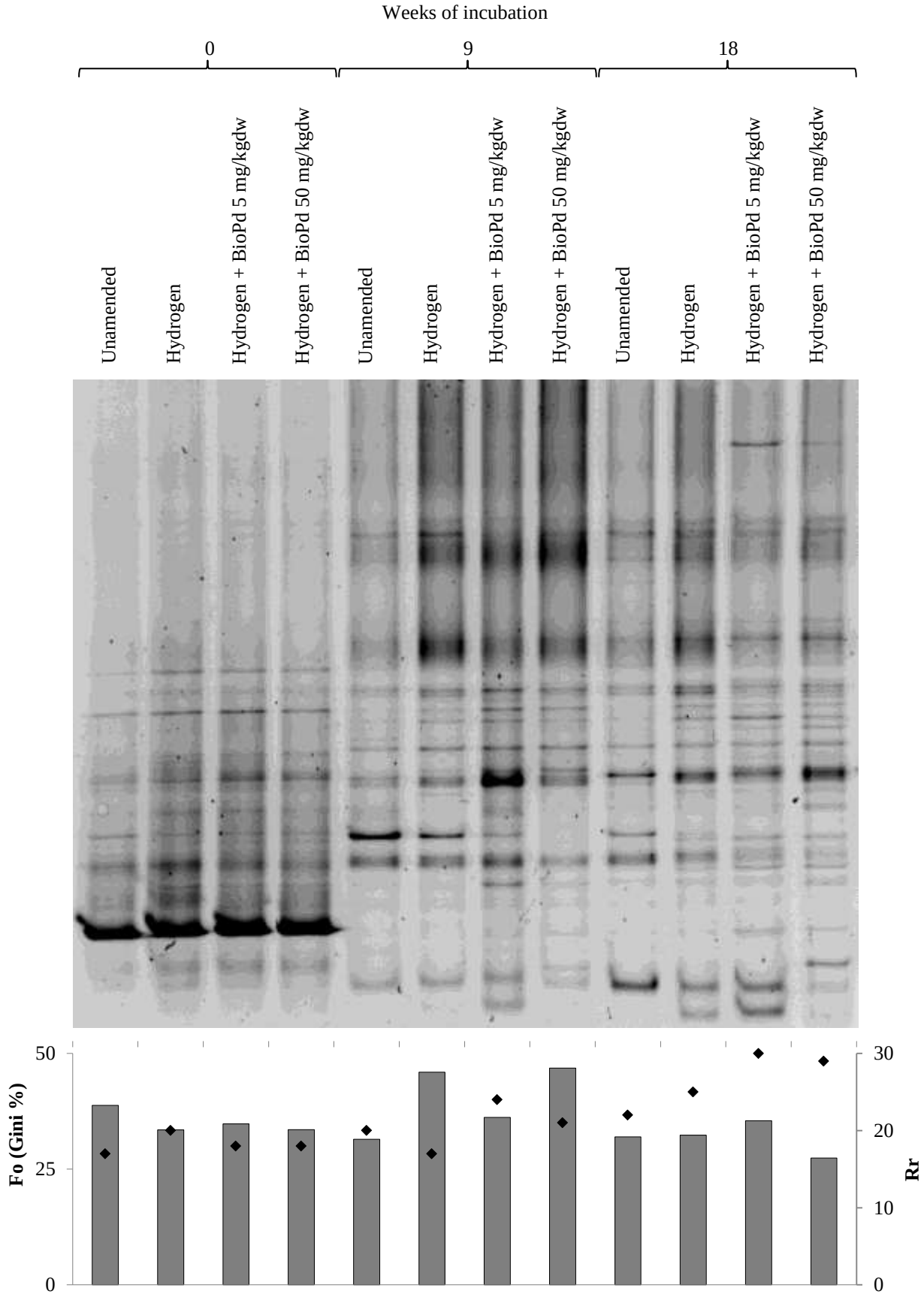

Figure 4.31: DGGE and evenness/richness analysis of the total bacterial community of the dechlorinating unamended, hydrogen-, and hydrogen + BioPd-amended cultures (5 and 50 mg/kgdw) at week 0, 9 and 18 of incubation. Each lane is a mixture of three replicate microcosms. Evenness is represented as Fo (i.e. Gini percentage) in bars and richness is represented in diamonds, for each lane.

In unamended PCB-dechlorinating cultures, conversely, the community organization increased during the incubation as the Gini decreased from 40% to 31%, suggesting that, even if the inoculum was enriched in dehalorespiring species, the community re-organized successively, probably because of the organic matter in the sediment which was source of nutrients for other species than PCB-dechlorinators.

On the contrary, all dechlorinating cultures amended with hydrogen showed a transient decrease in organization, followed by a successive re-organization, to reach final Gini similar to values measured in the unamended dechlorinating culture (Figure 4.31). The BioPd did not exhibit any effect on the microbial community, while apparently stimulating the evolution of a richer community: no significant differences can be observed between the hydrogen-amended and the hydrogen + BioPd 50 mg/kgdw-amended cultures, while final richness (Rr) values were 23, 25, and 27 for the no BioPd, 5 mg/kgdw and 50 mg/kgdw, respectively.

The same analysis has been conducted also on secondary cultures without PCB spiking (Figure 4.32). In this case, BioPd show again a dose-dependent increase effect on the richness of the bacterial community, whereas all bacterial communities showed a decrease in organization comparable to the unamended dechlorinating cultures: this suggests that the community itself is able to reorganize itself as the dechlorinating species decay, whereas PCBs are a major selecting factor for the community, as noted in previous experiments (section 4.1.3).

From these analyses it is not possible to determine an inhibitory effect of the BioPd nanoparticles on the microbial community as a whole. Even though experimental data show a dose-dependent inhibitory effect on sulfate-reduction and methanogenesis activities, this seems to be limited only to those species while others seem to be profiting from the abundance of hydrogen and maybe also from the catalytic activity of BioPd, which is still to be confirmed in sediments of the Venice Lagoon. It is unknown whether BioPd might exert inhibition on other anaerobic metabolisms (i.e. fermentation, nitrate-reduction, etc.) and the question transcends the approach of this work.

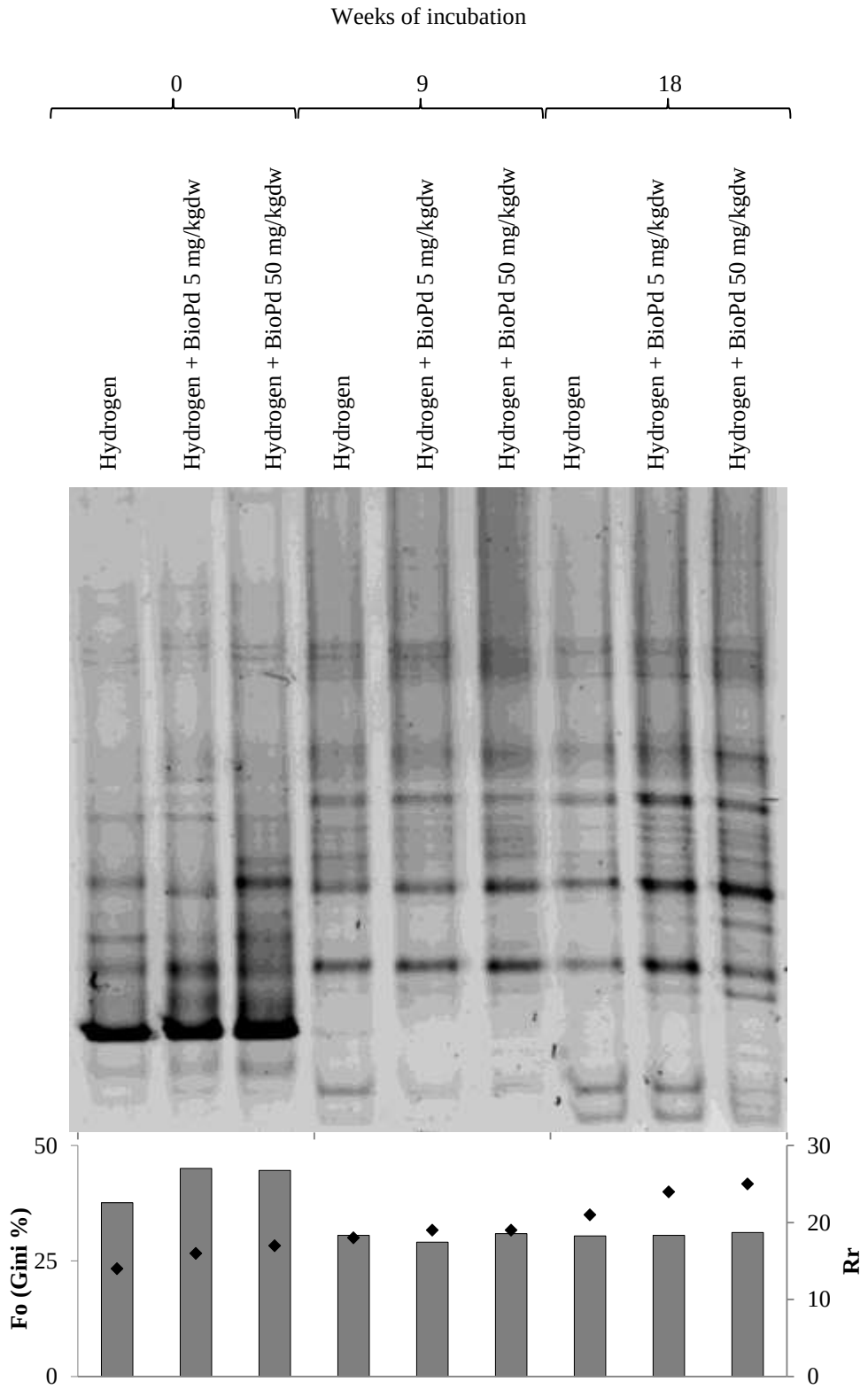

Figure 4.32: DGGE and evenness/richness analysis of the total bacterial community of the non-dechlorinating unamended, hydrogen-, and hydrogen + BioPd-amended cultures (5 and 50 mg/kgdw) at week 0, 9 and 18 of incubation. Each lane is a mixture of two replicate microcosms. Evenness is represented as Fo (i.e. Gini percentage) in bars and richness is represented in diamonds, for each lane.

4.3.4 PCB dechlorination activities in cultures at the end of dechlorination process

To test the possibility of chemically reduce PCB microbial reductive dechlorination end-products, sediment E cultures at the end of the dechlorination activity (see section 4.1.1) were mixed N_2:CO_2 70:30 atmosphere and aliquoted in serum bottles. No water or further PCBs were added; cultures were then incubated in anaerobic conditions, 28 °C statically in the dark.

The following conditions were tested:

- Hydrogen-amended cultures: cultures at the end of the dechlorination process in H_2:CO_2 70:30 atmosphere, to test hydrogen effect on possible further anaerobic degradation of lower-chlorinated congeners
- Hydrogen+BioPd-amended cultures: cultures at the end of the dechlorination process in H_2:CO_2 70:30 atmosphere, with two different concentrations of BioPd tested, namely 5 and 50 mg/kg dry sediment.

No dechlorination was detected in any of the conditions employed throughout 18 weeks of incubation (Figure 4.33), suggesting a possible inactivation of the BioPd nanoparticles by the sediment.

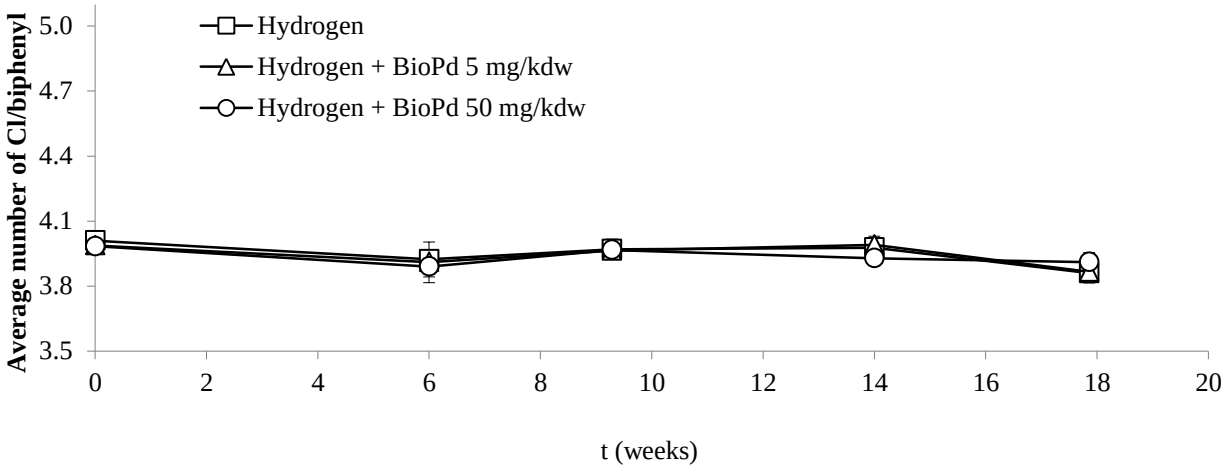

Figure 4.33: Time-course dechlorination of PCB dechlorination end products from sediment E cultures in hydrogen-amended and hydrogen+BioPd-amended slurries. Values are average of duplicate microcosms.

4.3.5 Evaluation of BioPd effectiveness in marine conditions

In order to assess the effectiveness of BioPd nanoparticles as dechlorination catalyst, sacrificial batches with i) marine water from the Venice Lagoon and ii) marine water with two different sediments at 20% w/v (namely sediment E from Venice Lagoon and a marine sediment from the Westerschelde Harbor of Antwerp, Belgium) were prepared. Due to the limited solubility of PCBs, batches with the sole marine water were conducted at 2 mg/l of Aroclor 1254 dissolved in methanol

(which has a higher solubility in water compared to acetone) and BioPd at 50 mg/l, while batches with sediment slurries were contaminated with Aroclor 1254 at the final concentration of 50 mg (kg dry sediment)$^{-1}$ and BioPd nanoparticles at 50 mg (kg dry sediment)$^{-1}$. All batches were incubated with 100% hydrogen atmosphere, at 28 °C, 120 rpm.

The sediment from the Venice Lagoon was a black and silty mud containing 13.8 mg/kg sulfide, probably due to the microbial sulfate reduction activities. Whereas, collected sediment from the Antwerp Harbor was a sandy sediment with a remarkable lower content of sulfide (Table 4.1).

Samples	**pH**	**TOC** *gC (kg dry sediment)$^{-1}$*	**Sulfide** *mgS (kg dry sediment)$^{-1}$*	**Chlorides** *gCl (kg dry sediment)$^{-1}$*
Antwerp Harbor	7.24	0.36 ± 0.02	0.09	1.86 ± 1.33
Venice Lagoon	7.60	1.64 ± 0.05	13.8	7.04 ± 0.11

Table 4.1: Principal physico-chemical characteristics of collected marine sediments from the Antwerp Harbor the Venice Lagoon

Dechlorination activities were detected in marine water and in marine slurry with Antwerp Harbor sediments, whereas no dechlorination was detected in the marine slurries with the Venice Lagoon sediment and in the BioPd-free controls, coherently to what was previously reported (see section 4.3.1) (Figure 4.34). Aroclor 1254 mixture was dechlorinated in marine water batches down to a minimum degree of chlorination of 0.65 after 3 hours of incubation (Figure 4.34) at a maximum dechlorination rate of 17.5 ± 6.6 μmoles of chlorine removed l^{-1} hours^{-1} (these rates cannot be compared to microbial dechlorination in sediments slurries, due to the different hydrophobicity of the system and the consequently unknown partition coefficients of the different congeners). While no dechlorination was observed in the Venice Lagoon sediment, BioPd catalyzed dechlorination of Aroclor 1254 in the Antwerp Harbor sediment to a minimum degree of chlorination of 2.7 after 48 h of incubation, where no further dechlorination was observed (Figure 4.34) at a maximum dechlorination rate of 51.7 ± 0.0 μmoles of chlorine removed (kg dry sediment)$^{-1}$ hours^{-1}, which is one order of magnitude higher compared to what happens in sediment cultures exhibiting dechlorination activities (which have week timespans instead of hours). To our knowledge, such extensive and fast dechlorination has never been observed before in marine sediments.

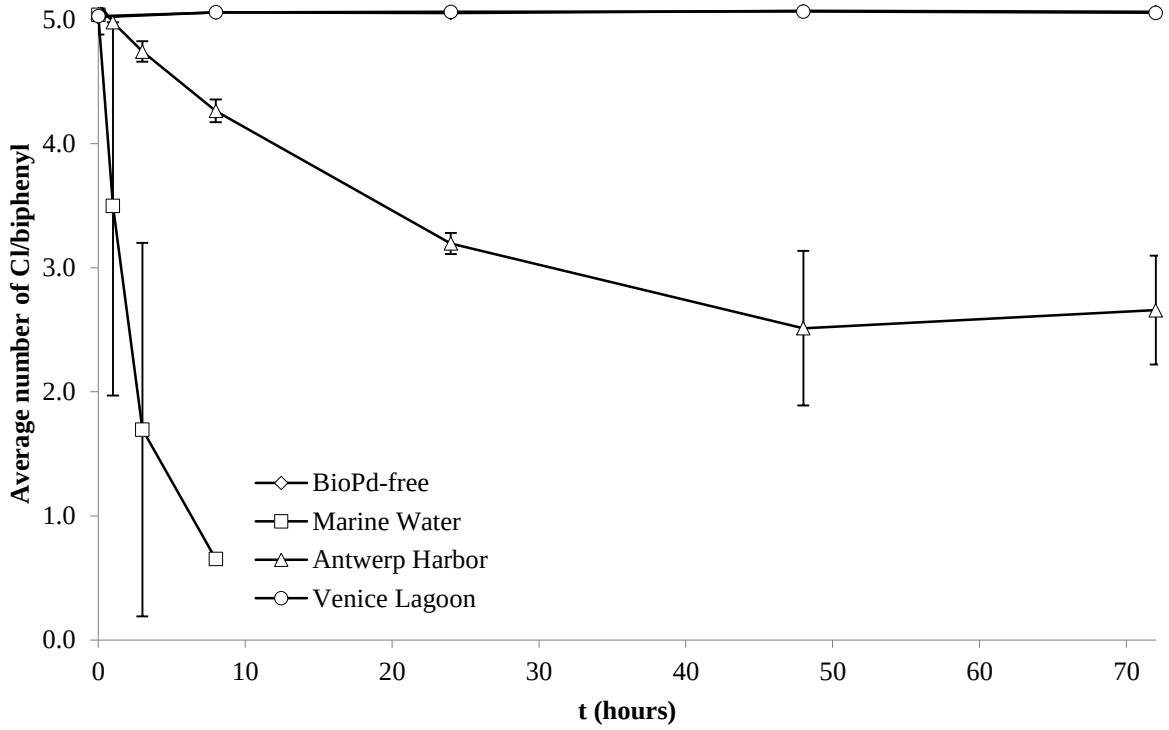

Figure 4.34: Time course of BioPd-mediated dechlorination of Aroclor 1254 PCBs in marine water (PCBs 2 mg/l, BioPd 50 mg/l) and in marine slurries of sediment A and B suspended in marine water (PCBs and BioPd 50 mg (kg dry sediment)$^{-1}$ each). Values are average of duplicate batches and error bars represent standard deviations.

After 3 h incubation in marine water with activated bio-Pd, Aroclor 1254 PCBs were extensively dechlorinated mainly to mono-chlorinated congeners, that represented 61 mol% of residual PCBs, and to biphenyl, that represented 33% of total molar mass (Figure 4.35), with a further accumulation of biphenyl after 8 h, where 5.8 ± 2.1 μmoles of PCB/l (ca. 85% of spiked PCBs) were fully dechlorinated to biphenyl (Figure 4.35). The accumulation of mono-chlorinated congeners or biphenyl from Aroclor 1254 has never been observed in marine sediments exhibiting microbial reductive dechlorination.

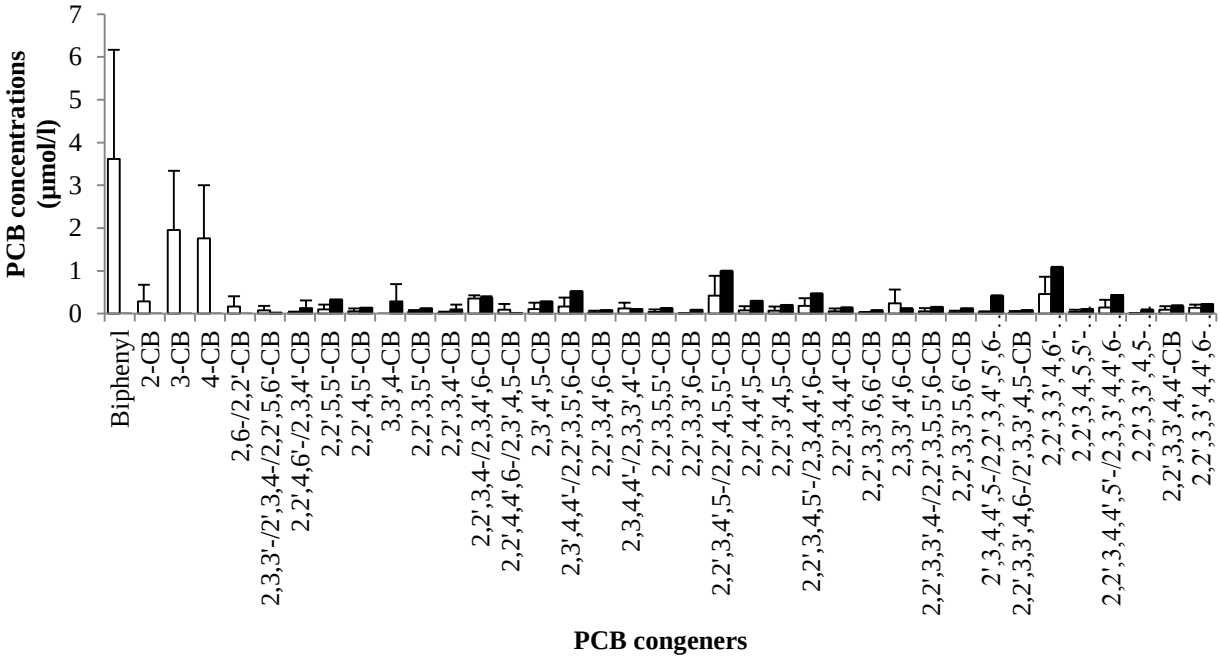

Figure 4.35: PCB congeners constituting more than 1% w/w of the spiked Aroclor 1254 (2 mg/l) in the marine water amended with hydrogen atmosphere and BioPd nanoparticles (50 mg/l) at the beginning (black bars) and after 3 h of incubation (empty bars). Values are average of triplicate microcosms and error bars represent standard deviations.

Such extensive dechlorination and conversion to mono-chlorinated congeners has been observed also in the Antwerp harbor sediment slurries amended with BioPd where, after 48 h of incubation, PCB congeners were depleted by 93% and converted into mono- and di-chlorinated congeners (Figure 4.36). In addition, the total concentration of PCBs decreased from 186 ± 0.5 to 64 ± 9 µmoles/kg dry sediment at the end of the incubation period, indicating that a remarkable fraction of the original PCB congeners was probably fully dechlorinated to biphenyl. However, no biphenyl was detected in the organic extracts. This was probably due either to the low concentration of the accumulated biphenyl (close to the detection limit), and/or to a lower biphenyl extraction yields from the sediment slurry in comparison with the sediment-free system.

The PCB dechlorination activity observed in the Antwerp Harbor sediment slurries was remarkably slower and more limited than sediment-free marine water. This might be due to the consequence of a less intimate contact between PCBs adsorbed onto the sediment and BioPd, as well as a different ratio of BioPd/PCB. In slurries of the Venice Lagoon sediment amended with BioPd, instead, no PCB dechlorination was observed throughout incubation. This clearly suggests that sediment characteristics, in particular the content of sulfide, may strongly affect the catalytic activity of BioPd, as discussed above.

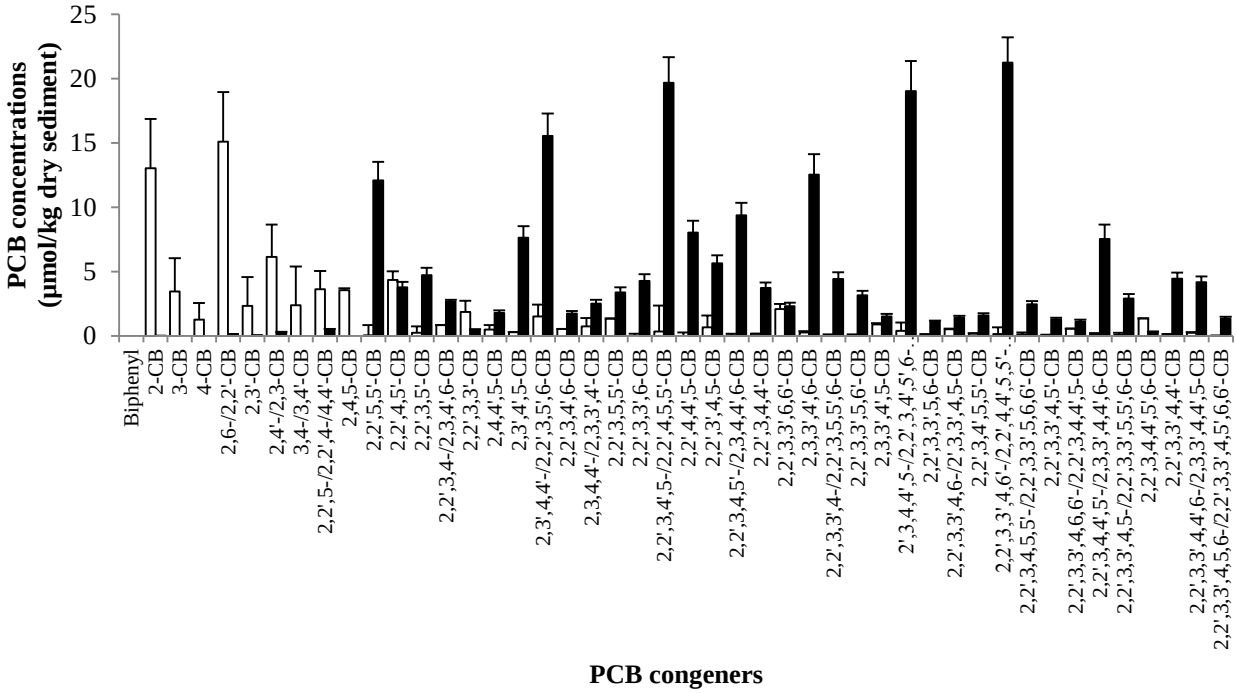

Figure 4.36: PCB congeners constituting more than 5% w/w of the spiked Aroclor 1254 (50 mg/kg dry sediment) in the marine slurries of Antwerp Harbor amended with hydrogen atmosphere and BioPd nanoparticles (50 mg/kg dry sediment) at the beginning (black bars) and after 48 h of incubation (empty bars). Values are average of triplicate microcosms and error bars represent standard deviations

These data were complemented with TCE dechlorination tests in the same conditions, confirming that the sediment of the Venice Lagoon inhibits the catalytic capability of BioPd (Hosseinkhani et al., 2015). In conclusion, the use of BioPd nanoparticles may be useful for catalyzing dechlorination of PCB and TCE in contaminated sediments, but the characteristics of the contaminated matrix have to be evaluated before application; from the data described in this work, the use of BioPd nanoparticles appears to be unfit for the remediation of PCB-contaminated sediments in the Venice Lagoon, and was discarded from successive experiments.

4.4 Novel biosurfactant-producing strains from marine mixed cultures

The possibility of isolating new biosurfactant-producing strains from oil-degrading mixed marine cultures was investigated. Before application in bioremediation system in order to increase PCB-bioavailability in contaminated sediments, preliminary characterization of the emulsion properties and ecotoxicity of the new biosurfactant molecules were performed.

4.4.1 Isolation and identification of biosurfactant-producing strains

Five mixed cultures (namely E2, E4, E5, E8 and E9) and 8 isolates (E4D, E4F, E8Y, XP8, ESP-A, ESP-C, ESPI-G, and RS) were provided by the Biochemical Engineering and Environmental Biotechnology Laboratory, Division II: Environmental Process Design and Analysis, Technical University of Crete, partner of the project. Mixed cultures and isolates were collected from the Elefsina Gulf, Greece, and were pre-screened for their biosurfactant production with the drop-collapse test. Mixed cultures E4 and E8 were analyzed by the partner of the project with pyrotag sequencing of the V4 16S rDNA hypervariable region, showing the predominance of seven families of the β- and γ-*Proteobacteria*, and further examination revealed that within-family evenness was very low, and the majority of reads of each family could be assigned to a single OTU (personal communication from Prof. Kalogerakis and Dr. Fodelianakis who performed this study, unpublished data). Thus, the number of possible isolates that could be obtained from this kind of consortia is limited, as they are dominated by a handful of strains, disfavoring the chance of isolating other strains than those.

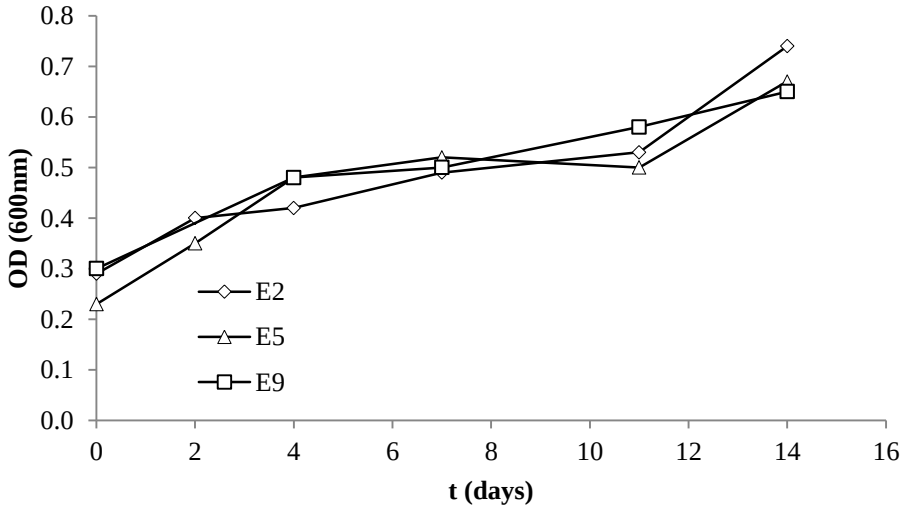

Figure 4.37: preliminary growth curves of mixed cultures E2, E4, and E8, in ONR7a mineral medium with 0.5% w/v crude oil as a sole carbon source.

Cultures E2, E5 and E9 were cultivated in aerobic shaken flasks, 120 rpm, 15 °C to simulate the marine conditions occurring in the Elefsina Gulf; all cultures were cultivated in ONR7a marine mineral medium amended with crude oil 0.5% w/v as a sole carbon source. Cultures showed an increase in OD_{600} in 8-16 days of incubation, when the crude oil was partially dissolved (Figure 4.37).

From these mixed cultures, serial dilutions were plated on several media to attempt the isolation of different strains from the mixed consortia. The used media involved rich marine media (Zobell Marine Broth, HLB) and mineral media such as ONR7a; optionally, these media were amended with 0.5% w/v crude oil to stimulate biosurfactant production, or with yeast extract 0.1 g/l, as source of vitamins and nutrients for pure cultures. Through this approach, 21 isolates were obtained from culture E2, 16 from culture E5 and 16 from culture E9. Isolates were then resuspended in liquid media and EI_{24} assays were performed to assess their biosurfactant production (Table 9.2). The best-producing isolates were classified through 16s rRNA gene sequencing, and resulted to be mainly *alfa-Proteobacteria*, especially of the *Thalassospira* genus (Table 4.2).

Significant differences were observed in the different *Thalassospira sp.* Strains. Isolates related to *Thalassospira sp.* M65-3N from E2 cultures behaved similarly in terms of OD_{600} but had different activities in terms of EI_{24} as isolate N2 E2 2B showed a peak value of 44% after 8 days of incubation, with a successive decrease to 12% at day 16, isolate N2 E2 2C produced biosurfactants only after 8 days of incubation and isolate N2 E2 3B did not show any decrease in EI_{24} after reaching 44% (Figure 9.2). This could mean that all these isolates represent the same strain which might have different metabolic states. Conversely, isolates related to the same *Thalassospira sp.* obtained from culture E5 and E9 exhibited different behaviors reaching higher OD_{600} (up to 3.0 in isolate N2 E9 5N), higher EI_{24} (up to 80% in isolate N2 E5 4L), and different production kinetics, ranging from immediate production and successive decrease in emulsion activity (isolate N2 E9 4F), to intensive emulsification activity only during the cell decay phase (isolate N2 E5 4L) (Figure 9.3 and Figure 9.4). These differences could depend on the different media used to isolate and cultivate the cultures, but also to differences between subspecific strains which could not be detected by the analysis of the whole 16s rRNA gene, but could be detected through the analysis of different *loci*, i.e. the intergenic sequence between 16s and 23s rRNA (Barry et al., 1991).

Isolate	Media	Maximum values				Closest relative	Identity	Closes described relative	Identity
		OD_{600}	*in (days)*	EI_{24}	*in (days)*	[GenBank accession number]	%	[GenBank accession number]	%
N2 E2 2B	Zobell	1.88	8	0.44	8	uncultured alpha proteobacterium [AY922191]	99.9	*Thalassospira sp*. M65-3N [KJ669369]	99.9
N2 E2 2C	Zobell	1.68	8	0.46	16	uncultured alpha proteobacterium [AY922191]	99.9	*Thalassospira sp*. M65-3N [KJ669369]	99.9
N2 E2 2F	ONR7+CA+YE	1.33	8	0.44	8	*Thalassospira lucentensis* [KC534321]	100	*Thalassospira lucentensis* [KC534321]	100
N2 E2 3A	HLB	1.01	8	0.56	8	*Thalassospira lucentensis* [KC534321]	100	*Thalassospira lucentensis* [KC534321]	100
N2 E2 3B	Zobell	1.68	8	0.46	8	uncultured alpha proteobacterium [AY922191]	99.7	*Thalassospira sp*. M65-3N [KJ669369]	99.7
N2 E5 1G	ONR7+CA+YE	1.68	14	0.53	10	uncultured alpha proteobacterium [AY922191]	99.9	*Thalassospira lucentensis* [KC534321]	100
N2 E5 1H	ONR7+CA+YE	1.66	10	0.51	14	uncultured alpha proteobacterium [AY922191]	99.9	*Thalassospira sp*. M65-3N [KJ669369]	99.9
N2 E5 3A	Zobell	2.24	8	0.24	8	-	-	-	-
N2 E5 3D	HLB	2.04	8	0.73	14	-	-	-	-
N2 E5 3F	HLB	0.9	14	0.72	14	-	-	-	-
N2 E5 4L	ONR7+CA+YE	2.48	3	0.75	14	*Thalassospira sp*. M65-3N [KJ669369]	99.9	*Thalassospira sp*. M65-3N [KJ669369]	99.9
N2 E5 TQ N	HLB/10 + CA	0.92	3	0.22	14	uncultured alpha proteobacterium [AY922191]	99.9	*Thalassospira sp*. M65-3N [KJ669369]	99.9
N2 E9 3F	HLB/10 + CA	1.77	14	0.54	10	Thalassospira lucentensis [KC534321]	100	*Thalassospira lucentensis* [KC534321]	100
N2 E9 4F	HLB/10	1.81	10	0.44	3	uncultured alpha proteobacterium [AY922191]	99.9	*Thalassospira sp*. M65-3N [KJ669369]	99.9
N2 E9 4G	ONR7+CA+YE	1.13	8	0.49	11	-	-	-	-
N2 E9 5N	Zobell	2.92	8	0.38	16	*Thalassospira sp*. M65-3N [KJ669369]	99.2	*Thalassospira sp*. M65-3N [KJ669369]	99.2
N2 E9 5P	Zobell	2.72	8	0.57	16	-	-	-	-
N2 E9 5S	Zobell	1.64	8	0.27	16	-	-	-	-
N2 E9 6G	ONR7+CA+YE	1.8	14	0.29	10	uncultured alpha proteobacterium [AY922191]	99.7	*Thalassospira sp*. M65-3N [KJ669369]	99.7

Table 4.2: Maximum values in terms of OD_{600} and EI_{24} of the best biosurfactant-producing isolates obtained from mixed cultures E2, E5 and E9, and phylogenetic classification (when possible).

A similar situation was detected in the isolates related to *Thalassospira lucentensis*, as isolate N2 E2 2F reached a maximum OD_{600} of 4.4 in 12 days of incubation, whereas N2 E2 3a, N2 E5 1G and N2 E9 3F showed lower OD_{600} but similar EI_{24} values (Figure 9.5). For these reason, no speculation could be made on the isolates whose phylogenic affiliation is still unknown, based on the sole growth curves and biosurfactant estimation (Figure 9.6)

In parallel, eight marine isolates (E4D, E4F, E8Y, XP8, ESP-A, ESP-C, ESPI-G, and RS) were provided directly by the partner of the project and were cultivated following their isolation conditions (Table 4.3).

Isolate	Media	Maximum values				Closest relative	Identity
		OD_{600}	*(days)*	*EI_{24}*	*(days)*	[GenBank accession number]	%
E4D	ONR7+CA	1.84	2	0.49	2	*Alcanivorax borkumensis* SK2 [NR_074890]	96
E4F	ONR7+CA	1.84	2	0.52	2	*Alcanivorax borkumensis* SK2 [NR_074890]	97
E8Y	ONR7+CA	1.84	2	0.46	2	*Alcanivorax borkumensis* SK2 [NR_074890]	99
XP8	Zobell	1.8	2	0.55	6	*Shewanella frigidimarina*	-
ESP-A	Zobell	1.82	2	0.43	6	*Paracoccus marcusii [NR_044922]*	97
ESP-C	Zobell	1.81	2	0.07	6	*Sulfitobacter pontiacus* ChLG-10 [AB617621]	99
ESPI-G	Zobell	1.91	2	0.47	6	*Pseudoalteromonas agarivorans* KMM255 [NR_025509]	99
RS	Zobell	1.78	2	0.54	2	*Roseovarius sp*	-

Table 4.3: Maximum values in terms of OD_{600} and EI_{24} of the eight biosurfactant-producing isolates provided by the partner of the project.

No significant differences were observed in the growth rate of all *Alkanivorax borkumensis* strains. Accumulation of surface-active molecules in the culture supernatants was observed during the exponential growth and the early stationary phase, reaching a maximum value of EI_{24} of approximately 50% after 48 hours of growth. Then, EI_{24} decreased, probably due to partial degradation of the surface-active molecules produced (Figure 9.7). Among strains growing on marine rich medium, ESP-A and ESP-C exhibited the lower growth rate, reaching the end of the exponential growth phase after two days, and accumulated only transiently surface-active molecules in the supernatant, being the maximum EI_{24} reached for only a short time interval lower than 40% (Figure 9.8). Strains XP8 and RS-A, conversely, grew faster (end of growth phase after 24 hours) and accumulated highly stable surface active compounds in the supernatant, being EI_{24} values of approximately 50% obtained after 24 hours and of approximately 55% after 6 days (Figure 9.8). A slower growth and a slower accumulation of surface active compounds in the supernatant was observed for strain ESPI-G, where however EI_{24} values of approximately 45% were obtained after 2 days as well as after 6 days of incubation (Figure 9.8).

The limited numbers of significantly different isolates obtained from mixed cultures E2, E5 and E9 confirm the predominance of the *Proteobacteria* phylum over other microorganisms in the sampling area due to the overwhelming presence of a particular specie (or OTU), as much as was detected in cultures E4 and E9 from the same area. Nevertheless, the isolation of supposedly new strains which show biosurfactant production is of strong importance for bioremediation purposes. *Thalassospira sp.* have long been known to be hydrocarbon degraders (López-López et al., 2002; Gertler et al., 2009) and were recently studied for their biosurfactant production and engineering (Mishra and Singh, 2012). *Alkanivorax borkumensis* (isolates E4D, E4F, E8Y) is a cosmopolitan marine bacterium that uses hydrocarbons as its exclusive source of carbon and energy and has a plethora of genes for biofilm formation at the oil-water interface and, of course, biosurfactants (Yakimov et al., 1998; Schneiker et al., 2006); the finding of these two strains dominating the mixed marine culture is not surprising and it is an indication for successive approaches of bioremediation in the area. On the contrary, less is known about other isolates: *P. marcusii* (isolate ESP-A) is a known hydrocarbon degrader (Harker et al., 1998); *S. pontiacus* (isolate ESP-C) was detected in enrichment microcosms amended with hydrocarbons (Yakimov et al., 2004); *S. frigidimarina* (isolate XP8) is capable of producing eicosapentanoic acid, a biosurfactant precursor (Bowman et al., 1997); *P. agarivorans* (isolate ESPIG-C) is known to have agarolytic activity (Romanenko et al., 2003) but many *Pseudoalteromonas* species produce surface-active compounds (Kalinovskaya et al., 2004) and *Roseovarius sp.* (isolate RS) increased with the depletion of high-molecular weight PAHs in microbial oil-degrading consortia (Vila et al., 2010); in conclusion, none of these strains was characterized in terms of biosurfactant productions and this is the first time that such activity is reported.

Isolates growing on crude oil were not further used in successive experiments, due to the technical difficulties in establishing big volume cultures with hydrocarbons; among the isolates growing in rich media, the isolates showing the higher productivity with shorter incubation, namely ESPI-G, RS and XP8 (*S. pontiacus*, *Roseovarius sp.* and *S. frigidimarina*), were used to scale up the culture volumes and characterize the biosurfactant production, before purification of the crude biosurfactant which was then used in bioremediation lab-scale tests.

4.4.2 Evaluation of biosurfactants properties and extraction procedures

Culture supernatants were collected after 3 to 5 days of incubation. Drop-collapse and pendant-drop tests were performed, to assess the presence of surface-active molecules and test a preliminary characterization. Drop-collapse tests is a qualitative method to assess the presence or the absence of surface-active agents in a liquid droplet, in comparison to a control (Bodour and Miller-Maier,

1998). *S. frigidimarina, Roseovarius sp.* and *S. pontiacus* showed a marked collapse of the droplet on parafilm foil, compared to the sterile medium control (data not shown).

Interfacial tension (IFT) is defined as the elastic tendency of liquids which makes it acquire the least surface area possible, and it is decreased by the presence of surfactants. IFT can be measured using pendant-drop test, where it can be calculated from the shape of a droplet of liquid hanging from a needle (Song and Springer, 1996a;b). However, in spite of the positive results obtained by drop-collapse tests, supernatants from the three isolates did not show any decrease in IFT, compared to sterile medium, being the measured values 71.2±0.3, 71.3±0.1 and 68.8±0.4 mN/m for *S. frigidimarina, Roseovarius sp.* and *S. pontiacus*, respectively (water control is 73.8±0.6 mN/m and a tween 1% v/v solution as a positive control is 34.7±0.2 mN/m).

Several extraction methods were tested and the EI_{24} was measured again after extraction, to assess the possible loss of biosurfactants in the extraction procedures. Ammonium sulfate precipitation (Rosenberg et al., 1979) or hydrolization with HCl (Haba et al., 2000) were not effective in separating biosurfactants from the supernatant, with more than 80% loss in the EI_{24} values (data not shown), while ethyl acetate solvent extraction (Desai and Banat, 1997) was not possible due to the elevate and persistent foaming (data not shown). Acetone precipitation (Rosenberg et al., 1979) yielded the best results and was used to partially separate biosurfactants from the culture supernatants, which were then concentrated in PBS. Due to the absence of IFT decrease but the contemporary presence of high emulsification activities (EI_{24}) it was hypothesized that the surface-active biocomponents in the culture supernatant were ascribable to high molecular weight bioemuslifiers, rather than small molecular weight biosurfactants (Desai and Banat, 1997); for this reason a further dialysis purification against synthetic marine water was added, using a membrane with a cut-off of 12 kDa.

Crude supernatant, acetone extract and dialyzed extract were then characterized in terms of EI_{24} and ecotoxicity (EC_{50}) using the *Vibrio fischeri* assay (Blaise, 1991). While all crude supernatant showed hormesis in the test, probably because of the carryover of carbon sources of the Zobell medium, toxicity decreased greatly in dialyzed extracts compared to acetone extracts (Table 4.4). Extracts from EPIG-C (*S. pontiacus*) cultures resulted to be toxic even after dialysis, while extracts from RS (*Roseovarius sp*) were not toxic; dialyzed extract from XP8 strain (*S. frigidimarina*) was the least toxic of all samples. Due to this reason and to the higher recovery ratio in terms of EI_{24} of XP8 biosurfactants rather than other strains, *S. frigidimarina* dialyzed extract was chosen to be produced in high volumes and used as a bioavailability enhancer in the successive bioremediation lab-scale tests.

Strains	Assay	Crude extract (3d incubation) (1:5)	Acetone extract (1:5)	Dialyzed extract (1:5)
S. frigidimarina (XP8)	EI_{24}	43.2%	35.7%	25.6%
	EC_{50}	Hormesis	22,57 ± 5,6 % (4,49 g/l)	457,0 ± 1,6%
Roseovarius sp.(RS)	EI_{24}	20%	30.8%	17.9%
	EC_{50}	Hormesis	Hormesis	111,81 ± 93,2%
S. pontiacus (ESPI-G)	EI_{24}	47.2%	37.5%	10.3%
	EC_{50}	Hormesis	19.34% (3.42 g/l)	7.20%

Table 4.4: Characterization of the culture crude supernatant, acetone and dialyzed extracts from cultures of *S. frigidimarina*, *Roseovarius sp.* and *S. pontiacus* isolates after 3 days of incubation. Values are average of duplicate analysis. Concentrations in terms of EC_{50} are reported in terms of g of dry acetone precipitate per liter, when possible, or in terms of volumetric percentage of the original liquid sample. Percentages of EC_{50} higher than 100% mean that the sample was not toxic but stimulated the growth of *V. fischeri.*

4.5 Lab-scale bioremediation tests

4.5.1 Preparation of inoculum cultures for *bioaugmentation*

Inocula for bioaugmentation approaches were prepared through subculturing of the sediment D culture in sterile sediment slurries of the Venice Lagoon. Sediment D was then aliquoted in 4 different serum bottles, degassed with N_2:CO_2 70:30 and sterilized for three consecutive days at 121 °C for 1 h, with incubation at 30 °C in the dark between sterilization cycles to kill any spore-forming species. Marine water from the same site was then degassed with N_2:CO_2 70:30 and filter-sterilized prior mixing with sterilized sediment aliquots. Slurries were then inoculated 5% v/v with the active dechlorinating culture from sediment D at the end of the dechlorination activity (see section 4.1.1) and spiked with Aroclor 1254 at the final concentration of 1 g/l for a better assessment of the dechlorination activities. Secondary cultures were then incubated in anaerobic conditions, 28 °C statically in the dark.

Dechlorination activities were as prominent as in sediment D primary cultures, with a maximum dechlorination rate of 482±162 μmoles of chlorine removed (kg dry sediment)$^{-1}$ per week, with a minimum degree of chlorination of 3.8 reached after 38 weeks of incubation (Figure 4.38).

The congeners which accumulated the most at week 38 were 2,2',4,4'-/2,2',4,5-/2,4,4',6-CB (16 mol%) > 2,2',4,5'-CB (16 mol%) > 2,3',4-CB (12 mol%) > 2,2',5-/2,2',4-/4,4'-CB (8 mol%) > 2,3',5-CB (6 mol%) > 2,2',5,5'-CB (6 mol%) > 2,2',3,4-/2,3,4',6-CB, 2,4',5-/2,4,4'-CB and 2,2',4,4',6-/2,3',4,5-CB (5 mol%), (Figure 4.39) defining a dechlorination specificity which looks different from sediment D primary cultures (Figure 4.3) and closer to sediment E replicates (Figure 4.4) and seems more related to pattern N than pattern P, as observed in the other secondary cultures obtained from the same inoculum (see section 4.2.1). It is unclear, though, the reason why the unamended controls described in section 4.2.1 exhibited lower rates compared to cultures here described, which were obtained at the same time and in the same way.

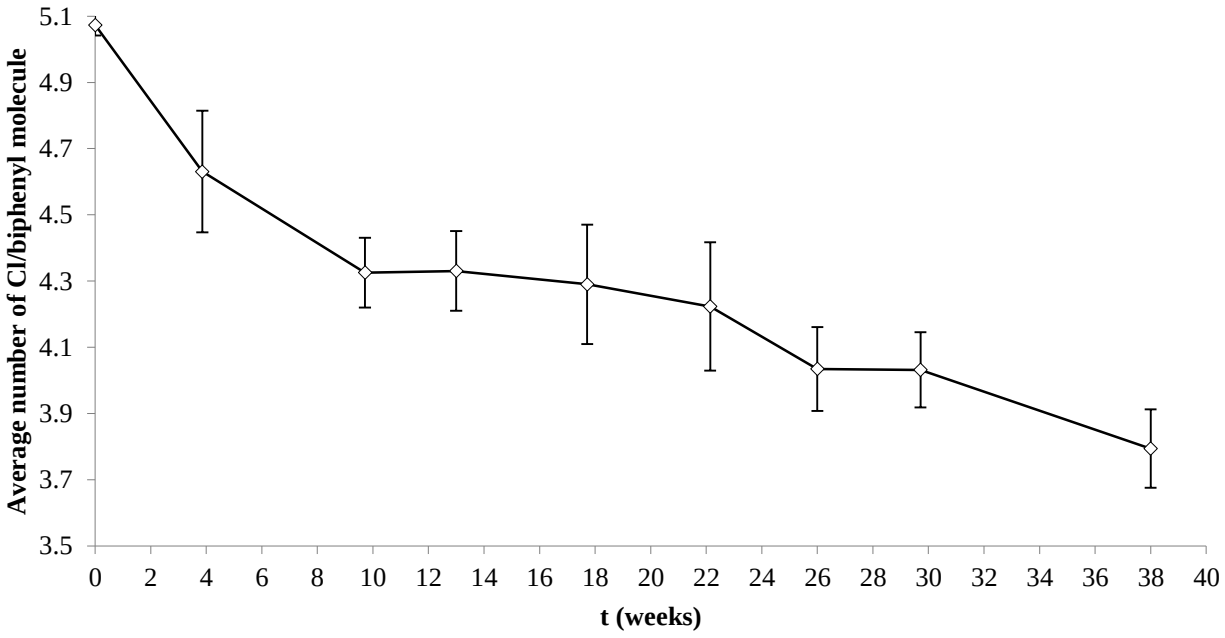

Figure 4.38: Time-course dechlorination of Aroclor 1254 mixture in sediment D secondary cultures. Values are average of quadruplicate microcosms.

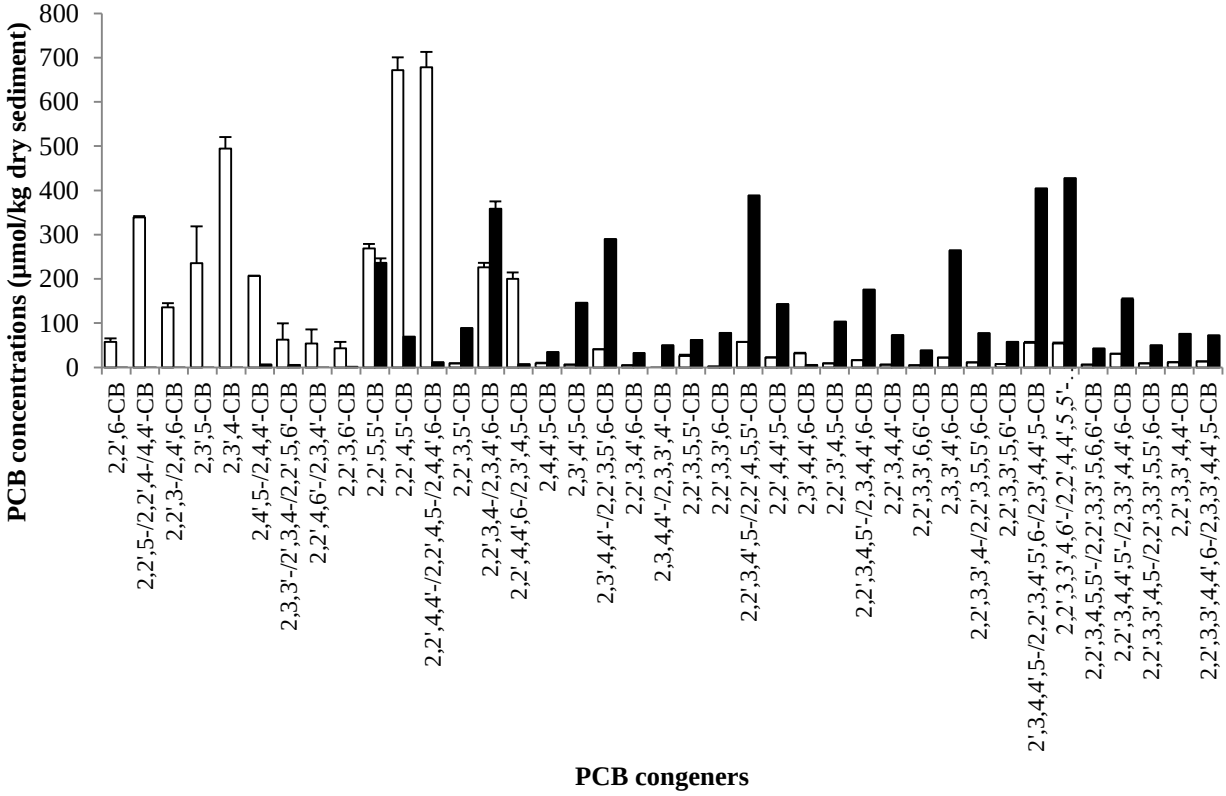

Figure 4.39: PCB congeners constituting more than 5% w/w of the contaminating mixtures in the active secondary cultures of sediment D (empty bars), and sterile microcosms (black bars) after 38 weeks of incubation in scale-up secondary cultures. Values are average of quadruplicate microcosms and error bars represent standard deviations.

4.5.2 Reactors and microcosms configuration

Sediment D was used to test different lab-scale bioremediation tests, as its indigenous microbial population exhibited the fastest dechlorination rates in primary microcosms (see section 4.1.1). The sediment was resuspended in the Venice Lagoon site water, degassed with N_2:CO_2 70:30 and spiked with Aroclor 1254 at the final concentration of 0.5 g/l for a better assessment of the dechlorination activities. No sterilization methods were used either on site water or on the sediment, so that the tested conditions would be as close as possible to the actual sediment condition, especially regarding the microbial community.

The following conditions were tested:

- Unamended controls
- Bioaugmentation: sediment slurries inoculated with a selected secondary enriched dechlorinating culture (from sediment D, see section 4.5.1)
- Biostimulation: sediment slurries amended with an electron donor, selective for enriched dechlorinating cultures (sodium-L-lactate, see section 4.2)
- Bioavailability enhancement: sediment slurries amended with a selected dialyzed biosurfactant extract (from *S.frigidimarina* cultures, see section 4.4)
- Bioaugmentation + biostimulation: sediment slurries inoculated with a selected secondary enriched dechlorinating culture (from sediment D, see section 4.5.1) and amended with an electron donor, selective for enriched dechlorinating cultures (sodium-L-lactate, see section 4.2)
- Bioaugmentation + bioavailability: sediment slurries inoculated with a selected secondary enriched dechlorinating culture (from sediment D, see section 4.5.1) and amended with a selected dialyzed biosurfactant extract (from *S.frigidimarina* cultures, see section 4.4)
- Biostimulation + bioavailability: sediment slurries amended with an electron donor, selective for enriched dechlorinating cultures (sodium-L-lactate, see section 4.2) and with a selected dialyzed biosurfactant extract (from *S.frigidimarina* cultures, see section 4.4)
- Bioaugmentation + biostimulation + bioavailability: sediment slurries inoculated with a selected secondary enriched dechlorinating culture (from sediment D, see section 4.5.1) and amended with a selected dialyzed biosurfactant extract (from *S.frigidimarina* cultures, see section 4.4) and with a selected dialyzed biosurfactant extract (from *S.frigidimarina* cultures, see section 4.4)

All tests were conducted in single baffled bottles shaken at 150 rpm and duplicate static microcosms to assess differences between a mechanically mixed systems, such as slurry reactors, and static systems such as bioremediation in confined sediment tanks.

4.5.3 PCB dechlorination activities

Unamended control and bioavailability enhanced reactors did not show any dechlorination activities throughout incubation, while biostimulated reactors exhibited an onset of dechlorination only after 14 weeks of incubation. Bioaugmentation, on the other hand, was sufficient to stimulate the onset of dechlorination at week 4, with a final degree of chlorination of 4.1 after 14 weeks of incubation (Figure 4.40a) at the rate of 226±56 μmoles of chlorine removed (kg dry sediment)$^{-1}$, a rate which is lower than sediment D secondary cultures used as inoculum (see section 4.1.1) probably due to the competition between the enriched dechlorinating population and the indigenous community of the sediments. The combination of bioaugmentation and biostimulation, in reactors, led to a sudden onset of dechlorination activity at the beginning of incubation, to a final degree of chlorination of 4.0 reached already after 10 weeks of incubation, with a maximum rate of 181±55 μmoles of chlorine removed (kg dry sediment)$^{-1}$. The presence of biosurfactants, unfortunately, seem to have an inhibiting effect on the dechlorination activity: bioaugmentation + bioavailability reactor showed a rate of 164±15 μmoles of chlorine removed (kg dry sediment)$^{-1}$ and reached a final degree of chlorination of 4.4, while bioaugmentation+biostimulation+bioavailability reactor was even slower (118±25μmoles of chlorine removed (kg dry sediment)$^{-1}$) and biostimulation+bioavailability reactor did not exhibit dechlorination (Figure 4.40b).

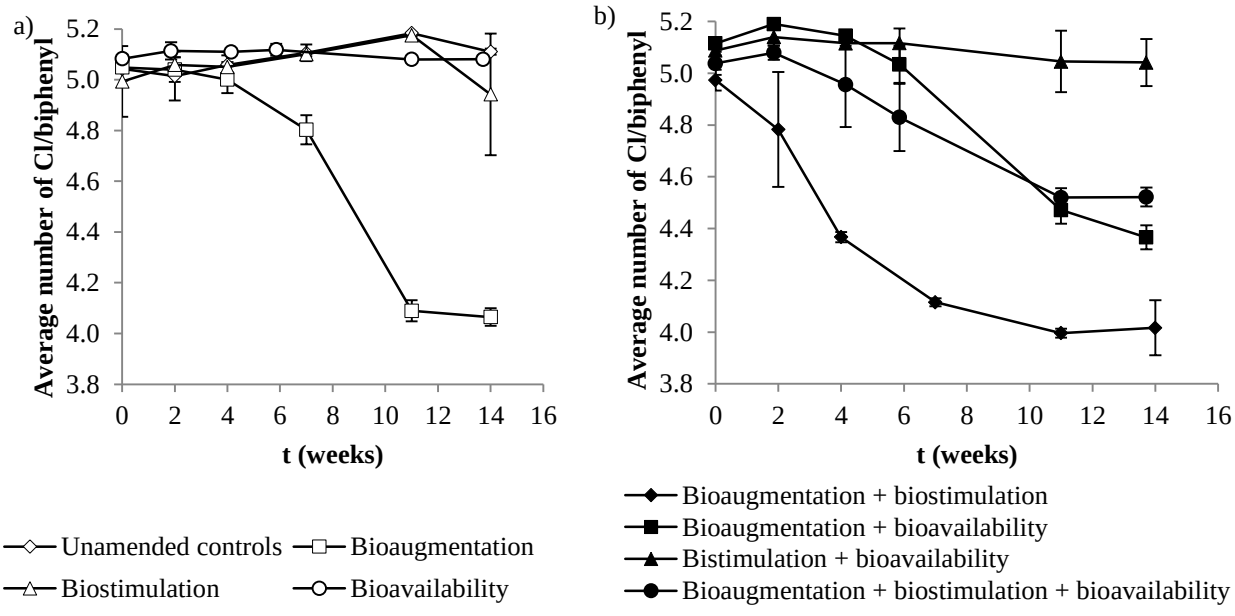

Figure 4.40: Time-course dechlorination of Aroclor 1254 mixture in lab-scale shaken reactors. Values are average of duplicate analysis and errors represent standard deviations.

The PCB congeners profile at the end of incubation is very similar for both the bioaugmentation and the bioaugmentation+biostimulation reactors, with the accumulation of the following congeners: 2,2',4,5'-CB (29 and 28 mol%, respectively) > 2,2',4,4',6-/2,3',4,5-CB (11 mol % in both reactors) > 2,2',4,4'-/2,2',4,5-/2,4,4',6-CB (5 mol% in both reactors) > 2,3',4-CB (4 and 5 mol%, respectively), with remarkable differences in the accumulation of 2,3',4,4'-/2,2',3,5',6-CB (10 and 4 mol%, respectively) and 2,2',5,5'-CB (9 and 12 mol% respectively) (Figure 4.41). These differences do not denote different dechlorination specificity, but rather a different efficiency in dechlorination activity. Dechlorination patterns observed in these two reactors denote a predominance of process P over process N, an effect that seems to increase in evidence during the subculturing process of sediment D cultures (compare the dechlorination patterns in section 4.1.1 and 4.5.1) and might depend on the predominance of one of the two detected phylotypes (section 4.1.3.1).

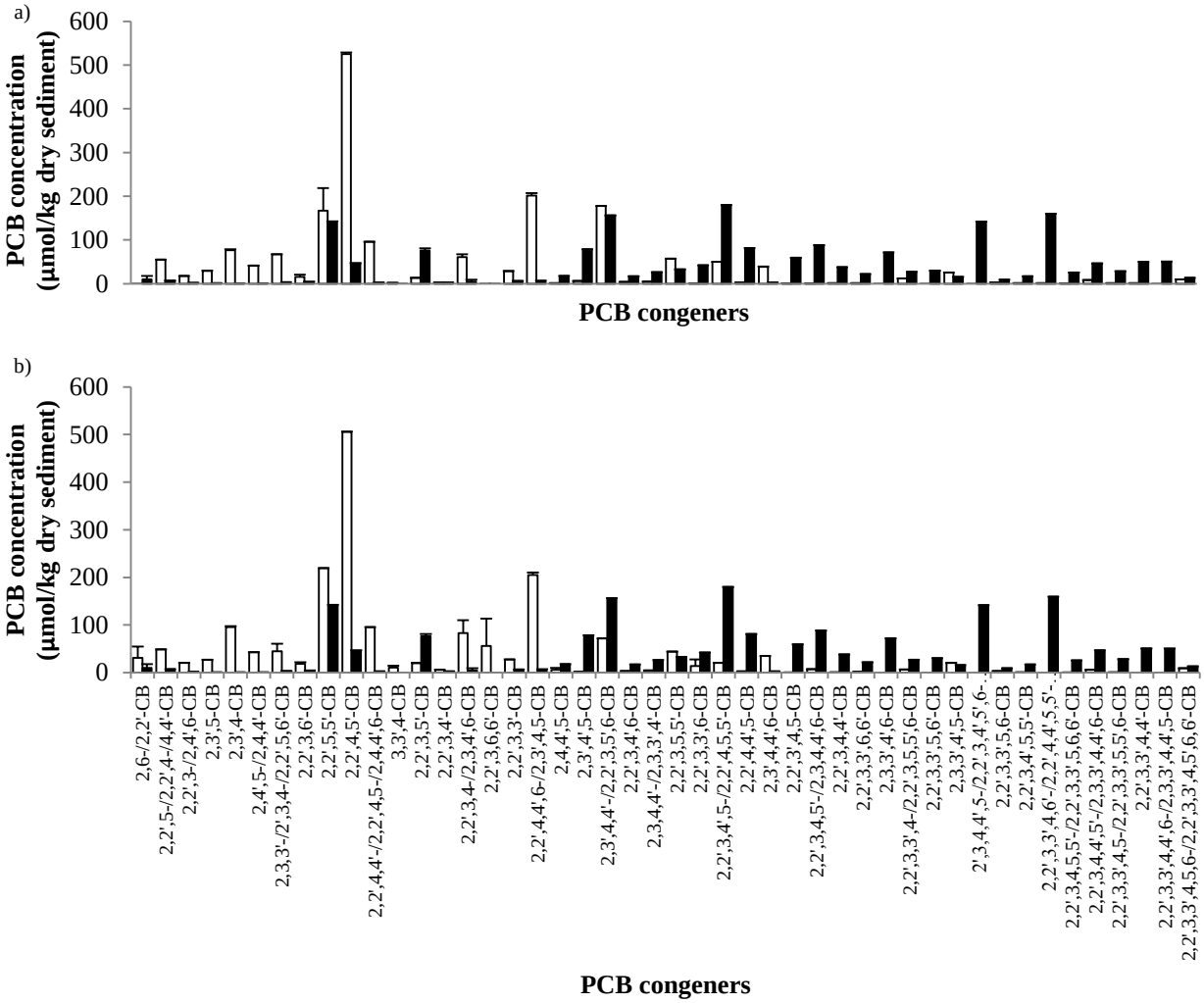

Figure 4.41: PCB congeners constituting more than 5% w/w of the contaminating mixtures in the bioaugmentation reactor (a, empty bars) and bioaugmentation+biostimulation reactor (b, empty bars) compared with sterile microcosms (black bars) after 14 weeks of incubation. Values are average of duplicate analysis and error bars represent standard deviations.

Biosurfactants had inhibitory effect in terms of rates in the bioaugmentation+bioavailability and bioaugmentation+biostimulation+bioavailability reactors, but the dechlorination profile at the end of incubation was similar to the respective counterparts not amended with biosurfactants. Again, the mostly accumulated congeners remarked a predominance of process P over process N in both cases, being the those congeners identical to those emerged previously, with the only exception of 2,2',3,4-/2,3,4',6-CB which accumulated up to 6 mol% in the bioaugmentation+biostimulation+bioavailability reactor (Figure 4.42).

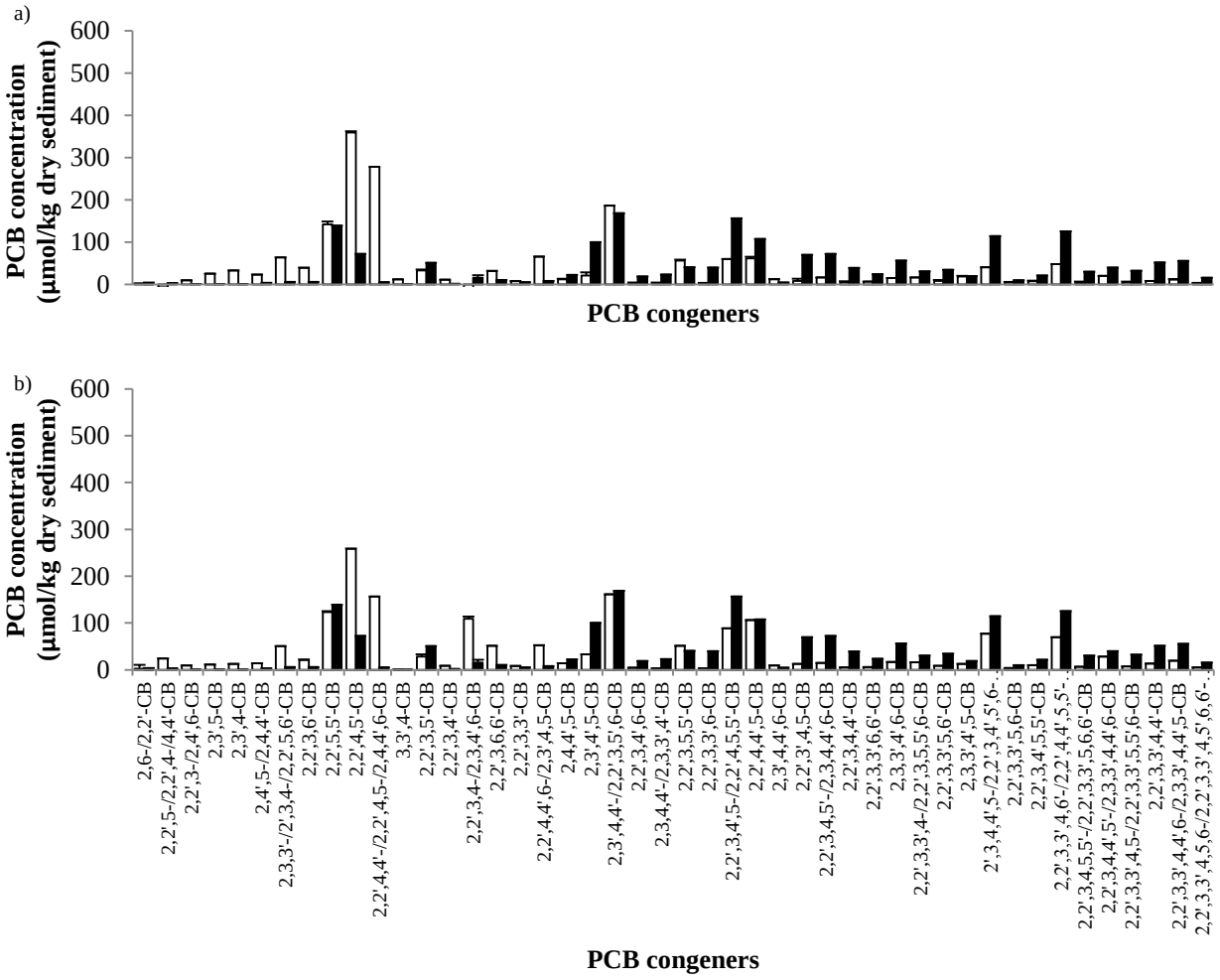

Figure 4.42: PCB congeners constituting more than 5% w/w of the contaminating mixtures in the bioaugmentation+bioavailability reactor (a, empty bars) and bioaugmentation+biostimulation+bioavailability reactor (b, empty bars) compared with sterile microcosms (black bars) after 14 weeks of incubation. Values are average of duplicate analysis and error bars represent standard deviations.

Results were comparable in the bioremediation test microcosms, as dechlorination activities were detected on week 4 with bioaugmentation and bioaugmentation+bioavailability conditions, and at the beginning of incubation in bioaugmentation+biostimulation and bioaugmentation+biostimulation+bioavailability conditions (Figure 4.43). However, rates are

generally lower compared to reactor counterparts (206±14, 118±41, 178±117 and 146±24 μmoles of chlorine removed (kg dry sediment)$^{-1}$, respectively).

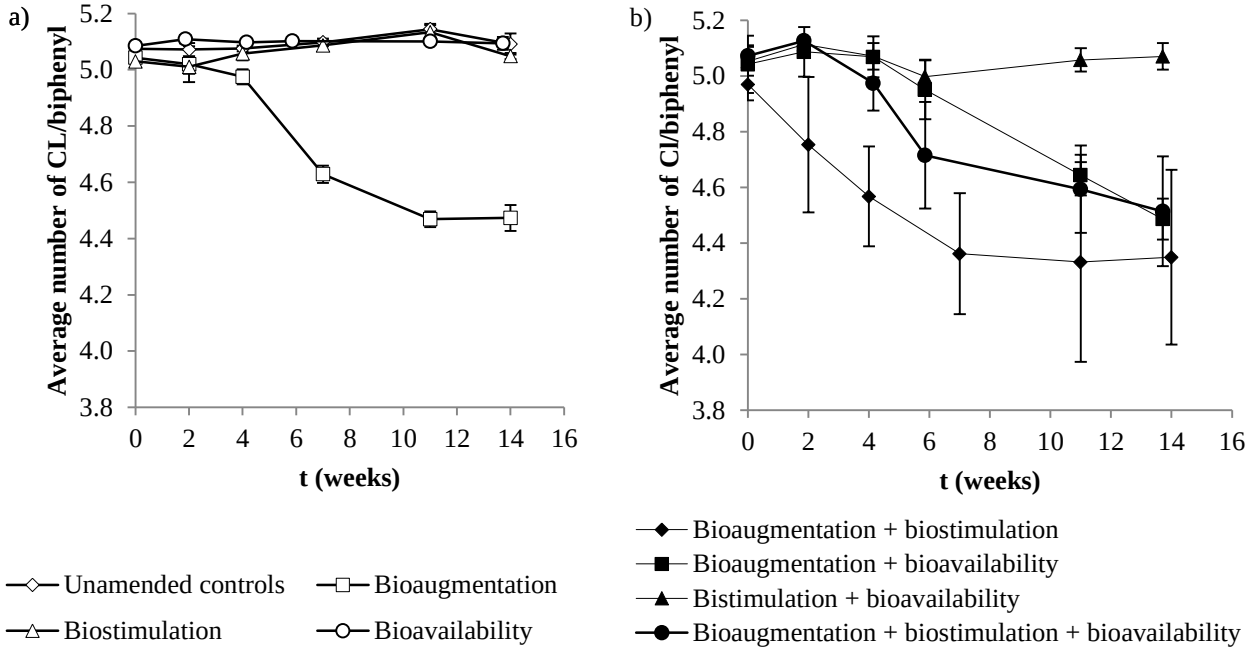

Figure 4.43: Time-course dechlorination of Aroclor 1254 mixture in bioremediation test microcosms. Values are average of duplicate microcosms and errors represent standard deviations.

PCB congeners profile was similar in all four conditions and congeners showing major accumulation were 2,2',5,5'-CB (16, 14, 11 and 9 mol%, respectively) > 2,2',4,5'-CB (15, 19, 14 and 17 mol%, respectively) > 2,2',4,4',6-/2,3',4,5-CB (6, 6, 4 and 4 mol%, respectively) > 2,3,3'-/2',3,4-/2,2',5,6'-CB (4, 3, 3 and 3 mol%, respectively) (Figure 4.44a and b). Accumulation of congeners 2,2',4,4'-/2,2',4,5-/2,4,4',6-CB were detected only in bioaugmentation+bioavailability and bioaugmentation+biostimulation+bioavailability conditions (6 and 10 mol%, respectively) (Figure 4.44c and d). These data indicate that a slightly different pattern develops in microcosms, which is mostly closer to process N than to process P, especially for the inoculated microcosms in presence of biosurfactants. These observations may indicate a difference between shaken and static treatment, which could have favored one or the other phylotype in the competition. Differences in dechlorination rates between inoculated microcosms in presence or in absence biosurfactants could again be due to a possible inhibitory effect of the used biosurfactant.

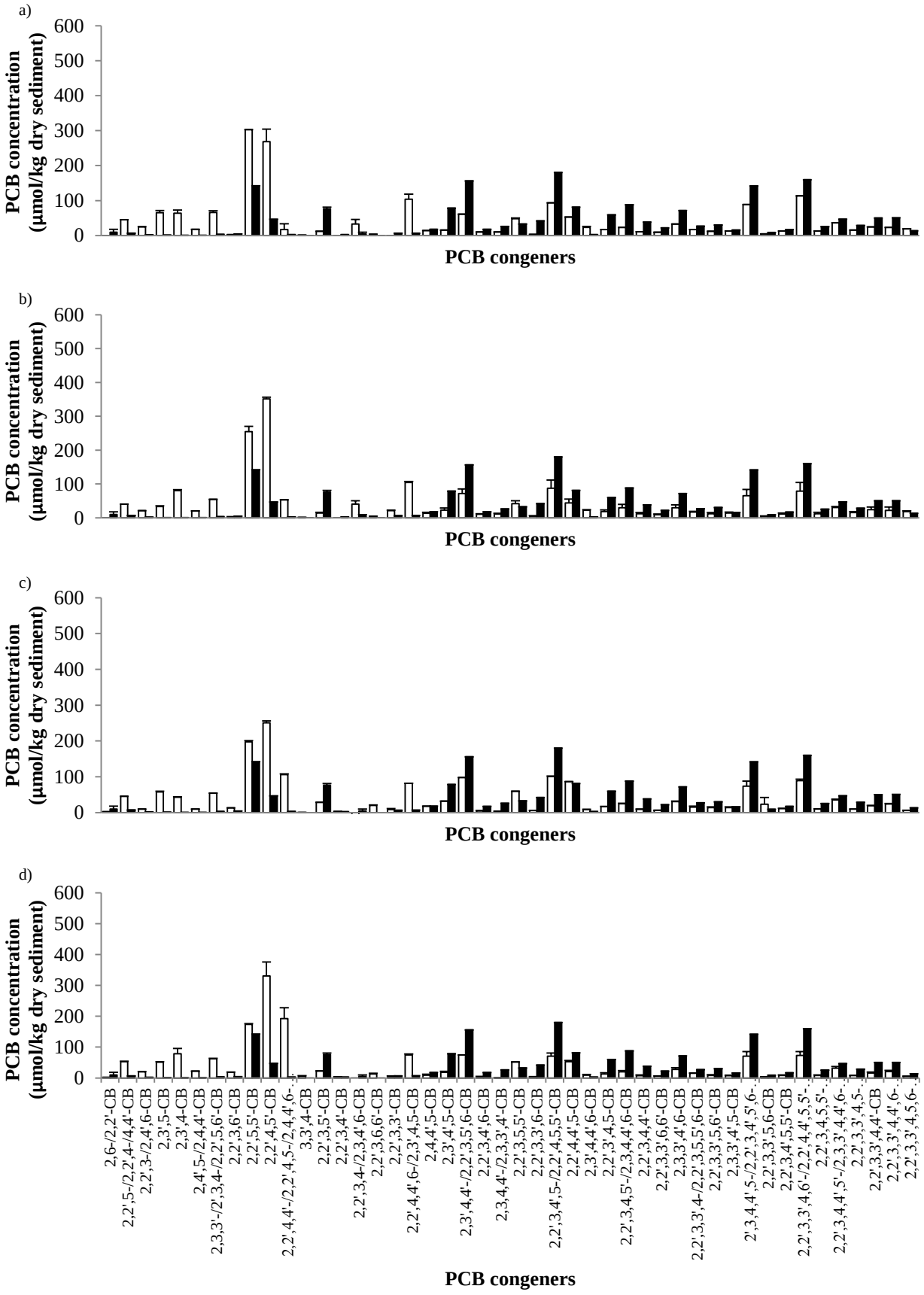

Figure 4.44: PCB congeners constituting more than 5% w/w of the contaminating mixtures in the bioaugmentation (a, empty bars) and bioaugmentation+biostimulation (b, empty bars) the bioaugmentation+bioavailability (c, empty bars) and bioaugmentation+biostimulation+bioavailability (d, empty bars) compared with sterile microcosms (black bars) after 14 weeks of incubation. Values are average of duplicate microcosms and error bars represent standard deviations.

4.5.4 Sulfate reduction and methanogenic activities

No sulfate reduction or methanogenic activities were detected in sterile controls (data not shown). Sulfate reduction activities in reactors started at week 4 for unamended, bioaugmentation and biostimulation condition, leading to complete sulfate depletion at week 11, 11 and 14, respectively, whereas no sulfate depletion was observed until week 11 in bioavailability enhancement reactors (Figure 4.45a). An inhibitory effect of the biosurfactant was also visible in the bioaugmentation+bioavailability reactor, where the sulfate depletion activities started at week 8, whereas in all other combined conditions sulfates reduction started right at the beginning of incubation, leading to a complete depletion within week 7 for biostimulation+bioavailability and bioaugmentation+biostimulation reactor, and within week 11 for bioaugmentation+biostimulation+bioavailability reactor (Figure 4.45b). Sulfate activities were stimulated by the inoculation and/or the amendment with lactate, while partially inhibited by the presence of biosurfactants: these three effects can be explained respectively by i) the carryover of sulfate-reducing species in the inoculum, ii) the higher concentration of lactate used, compared to secondary enriched cultures (5 mM vs 2.5 mM as described in section 4.2.2) and iii) a possible toxic effect of the biosurfactant over the sulfate-reducing microorganisms

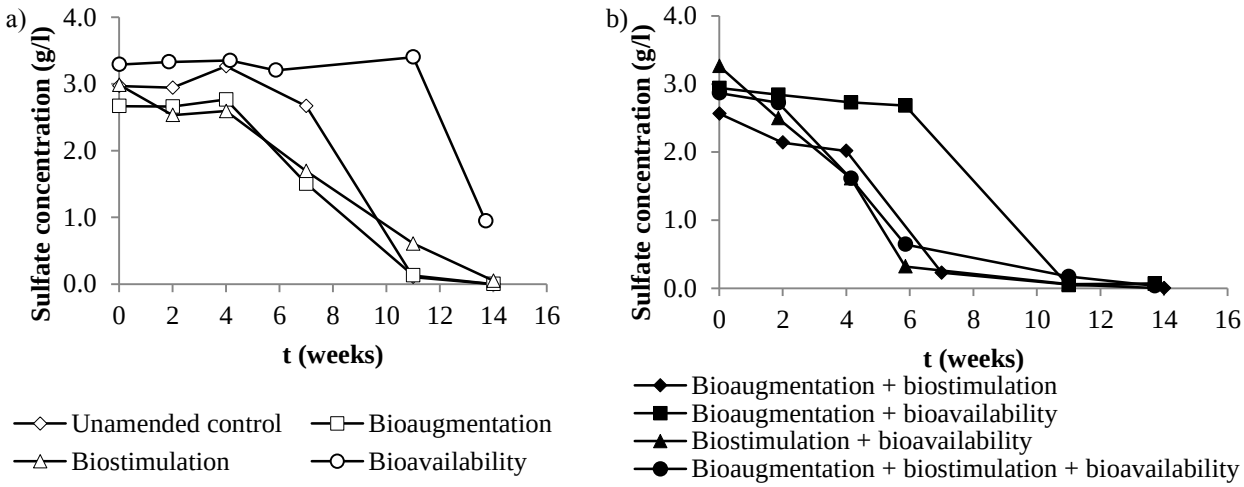

Figure 4.45: Sulfate concentration in bioremediation lab-scale test reactors, throughout incubation.

Methanogenic activities were stimulated by the inoculum in the bioaugmentation reactor, leading to a total production of 120 ml methane per l of slurry, whereas unamended control and bioavailability-enhanced reactors produced 16 and 27 ml methane per l of slurry, respectively; an even higher stimulation was observed in biostimulation reactors at week 11, where almost all sulfates were depleted, leading to a total production of 208 ml methane per l of slurry in 14 weeks (Figure 4.46a). The combined stimulation of inoculum and lactate yielded the highest methane

production in the bioaugmentation+biostimulation reactor (295 ml methane per l of slurry in 14 weeks), while biosurfactants stimulated the methanogenic population of the inoculum in the bioaugmentation+bioavailability reactors (263 ml methane per l of slurry in 14 weeks), and had a general inhibitory effect in presence of lactate, lowering the bioaugmentation+biostimulation+bioavailability methane production to 208 ml methane per l of slurry in 14 weeks of incubation, and neutralizing the positive effect of lactate in the biostimulation+bioavailability reactor which produced only 42 ml methane per l of slurry in 14 weeks (Figure 4.46b).

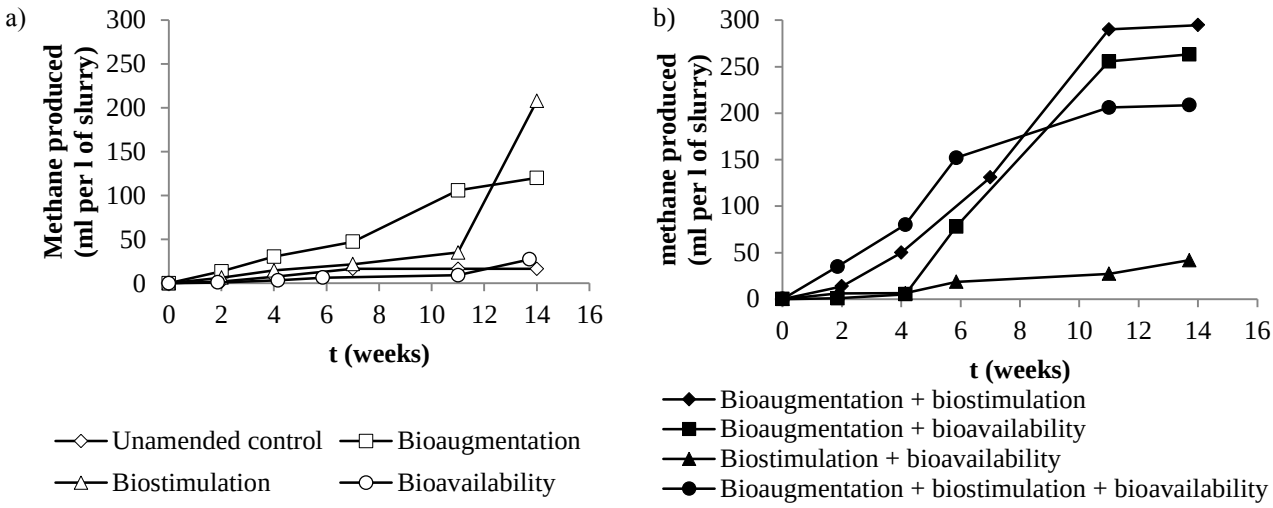

Figure 4.46: Methanogenic activities normalized per l of slurry in bioremediation lab-scale test reactors, throughout incubation.

Sulfate reduction activities were slower in bioremediation test microcosms, compared to reactor counterparts, and were highly stimulated by lactate addition in the biostimulation microcosms, where almost complete sulfate depletion was detected at week 6, and less stimulated by the inoculum in the bioaugmentation microcosms, where complete sulfate depletion was detected at week 11; on the other hand, biosurfactant exhibited an inhibitory effect in bioavailability microcosms, where sulfate depletion activities started only at week 11 and were not completed at the end of incubation, while in unamended control, only one of the replicates depleted sulfates within week 11 (Figure 4.47a). In combined approaches, the stimulatory effect of inoculum and lactate was evident in the bioaugmentation+biostimulation microcosms, which depleted sulfates at week 11, while all other conditions were inhibited by the presence of biosurfactants, delaying sulfate depletion in biostimulation+bioavailability and bioaugmentation+biostimulation+bioavailability microcosms (11 weeks) and blocking sulfate depletion activities until week 11 in bioaugmentation+bioavailability microcosms (Figure 4.47b).

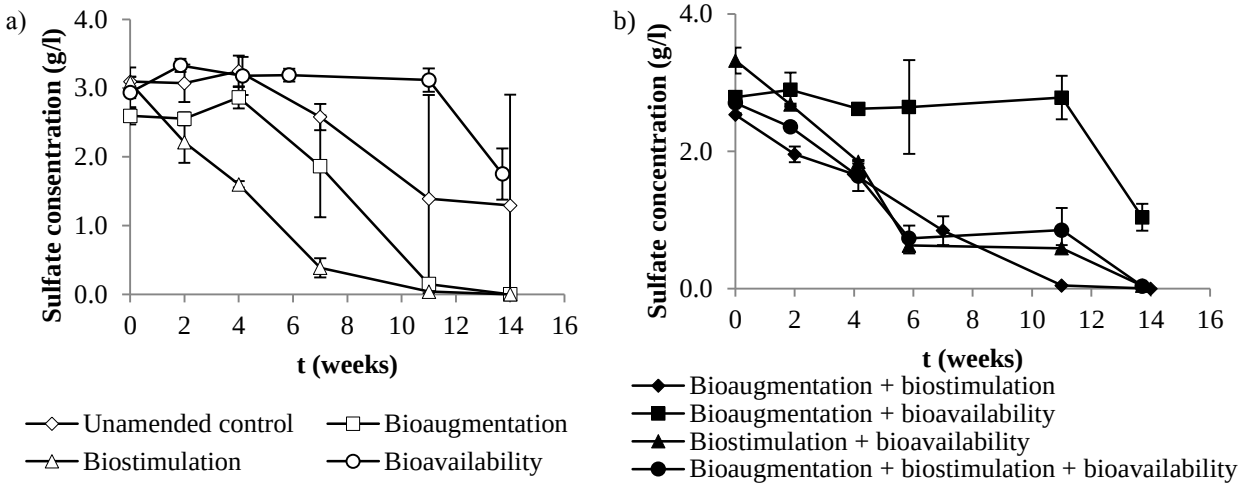

Figure 4.47: Sulfate concentration in bioremediation lab-scale test microcosms, throughout incubation. Values are average of replicate microcosms and error bars represent standard deviations.

Methanogenic activities were, again, stimulated only by lactate in the biostimulation microcosms, when the sulfates were almost completely depleted at week 8, yielding a total 165±1 ml methane per l of slurry in 14 weeks of incubation (Figure 4.48a). On the other hand, combined inoculum and lactate had the most stimulating effect on methanogenesis when combined, and not inhibited by biosurfactants, leading to a total methane production of 281±4 and 282±4 ml methane per l of slurry in 14 weeks of incubation (bioaugmentation+biostimulation and bioaugmentation+biostimulation+bioavailability microcosms, respectively); stimulating effect of biosurfactants could be noticed in the bioaugmentation+bioavailability microcosms (total production of 97±6 ml methane per l of slurry) while inhibitory effect were noted on the lactate-stimulated indigenous community in the biostimulation+bioavailability microcosms (total production 42±1 ml methane per l of slurry)(Figure 4.48b).

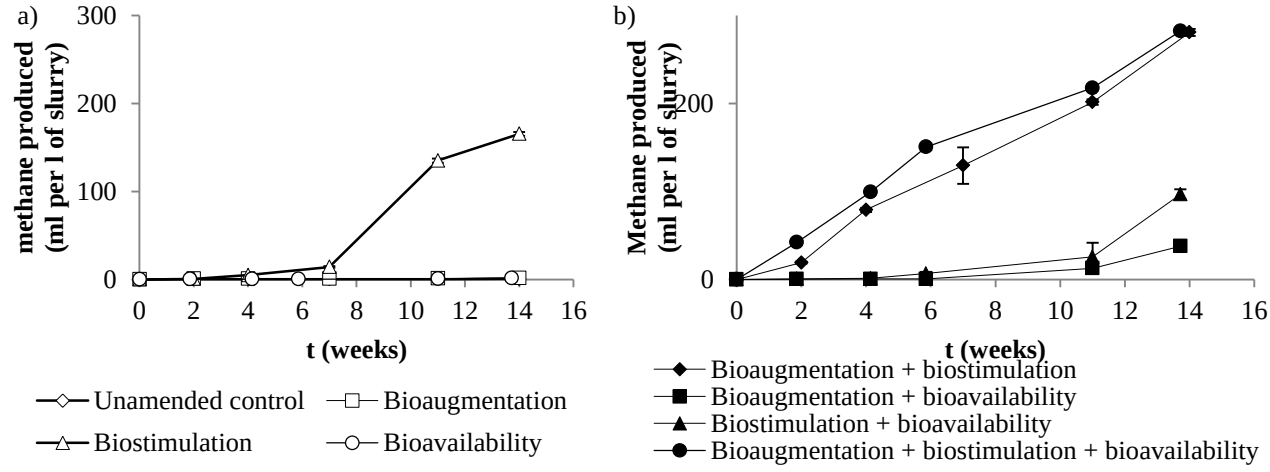

Figure 4.48: Methanogenic activities normalized per l of slurry in bioremediation lab-scale test microcosms, throughout incubation. Values are average of replicate microcosms and error bars represent standard deviations.

4.5.5 Microbial community analysis and quantification of dechlorinating isolates

DGGE profiles using specific primers specific for the phylum *Chloroflexi* were obtained from sampled week 0, 2, 7 and 14 of incubation. The results showed that unamended control and bioavailability reactors, where dechlorination activity did not occur, did not show the enrichment of any specific band throughout incubation, while in bioaugmentation reactors, where dechlorination activities occurred after 4 weeks of incubation, two strong band at the same height of the main phylotypes of the inoculum, referable to VLD-1 and VLD-2, were constantly present throughout incubation (Figure 4.49). Interestingly, the same bands were not present at the beginning of incubation but were evident from week 7 in biostimulation reactor, which exhibited dechlorination activity only at week 11 (Figure 4.49).

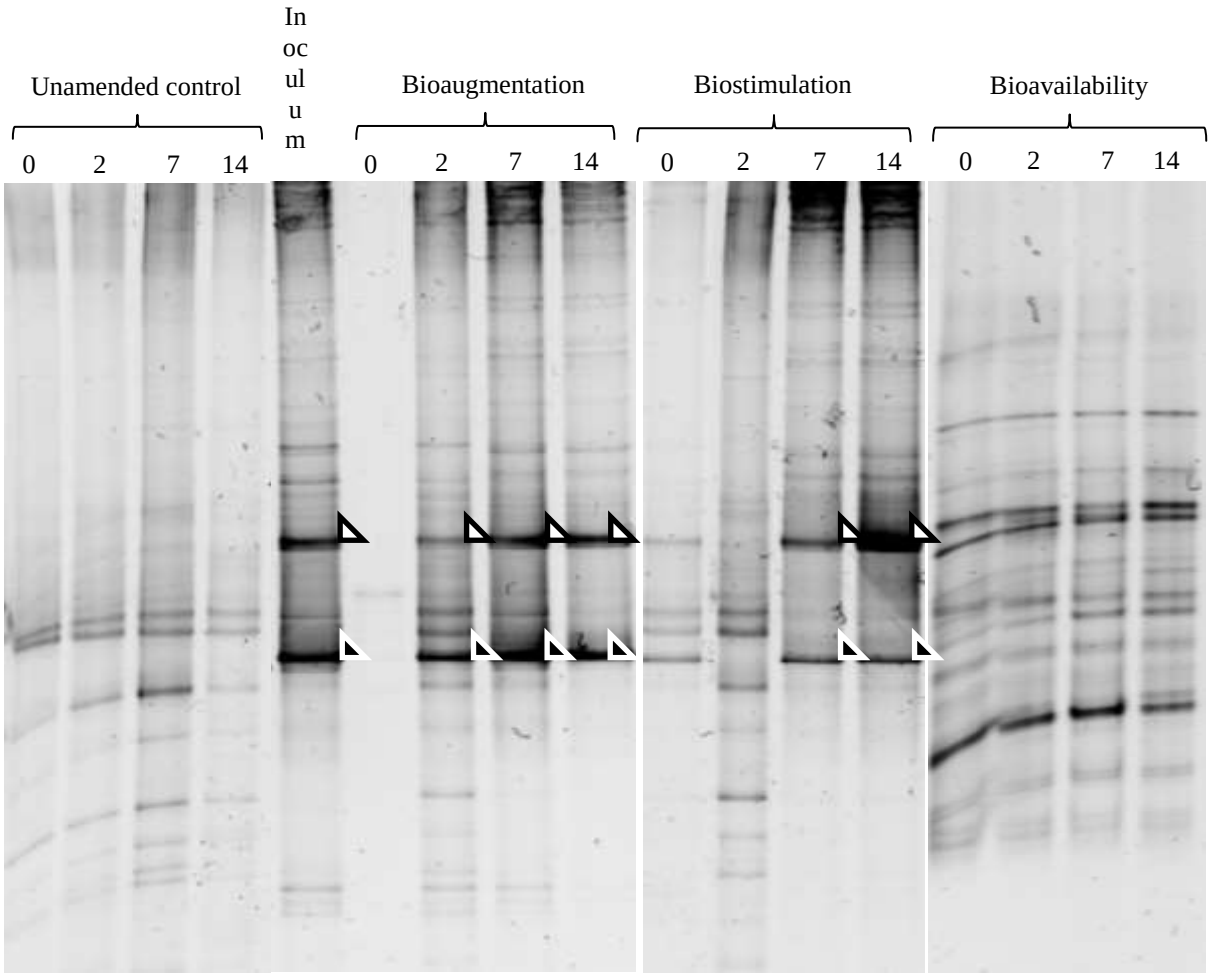

Figure 4.49: DGGE analysis of the *Chloroflexi* bacterial community throughout incubation of the unamended control, bioaugmentation, biostimulation and bioavailability reactors. Bands at the same height of phylotypes VLD-1 and VLD-2 are marked in empty and black triangles, respectively.

To confirm the association of the two phylotypes with the dechlorination activities, qPCR assays were performed as described previously. The results evidenced an increase in relative abundance of both phylotypes, which reached 4.8±0.8% and 7.4±0.6% of the total bacteria (for VLD-1 and VLD-

2 respectively) in the bioaugmentation reactor, which is strictly correlated to the dechlorination activity (p <0.001). Very low percentages of the two phylotypes could be detected also in other reactors where dechlorination activities did not occur (Figure 4.50).

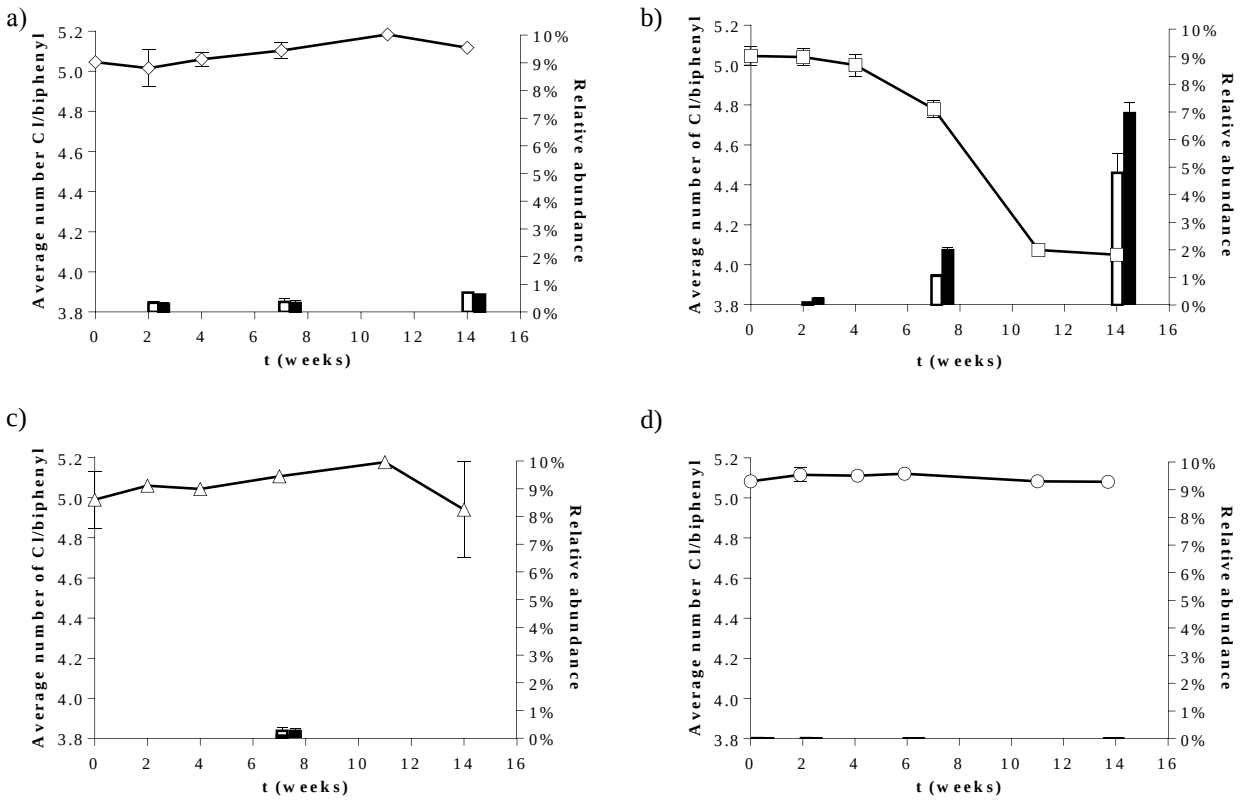

Figure 4.50: Time-course dechlorination of unamended control, bioaugmentation, biostimulation and bioavailability lab-scale test reactors (A, B, C and D respectively) expressed as decrease of degree of chlorination and the increase of phylotype VLD-1 (white bars) and phylotype VLD-2 (black bars) in the respective cultures expressed as percent relative abundance (16s rDNA gene copy numbers vs total 16s rDNA genes). Values are average of triplicate analysis performed on DNA error bars represent standard errors.

At the same time, the two dechlorinating phylotypes were detected also in all combined reactors whenever inoculated (bioaugmentation+biostimulation, bioaugmentation+bioavailability and bioaugmentation+biostimulation+bioavailability) (Figure 4.51). Additionally, a third unknown *Chloroflexi* phylotype was detected in the inoculum, but its band intensity seem to decrease throughout incubation thus was ignored from further analysis. It is important to notice that only inoculated reactors exhibited dechlorination activities, whereas the biostimulation+bioavailability reactor, where the two dechlorinating phylotypes could not be detected, did not show any dechlorination throughout incubation (Figure 4.40b).

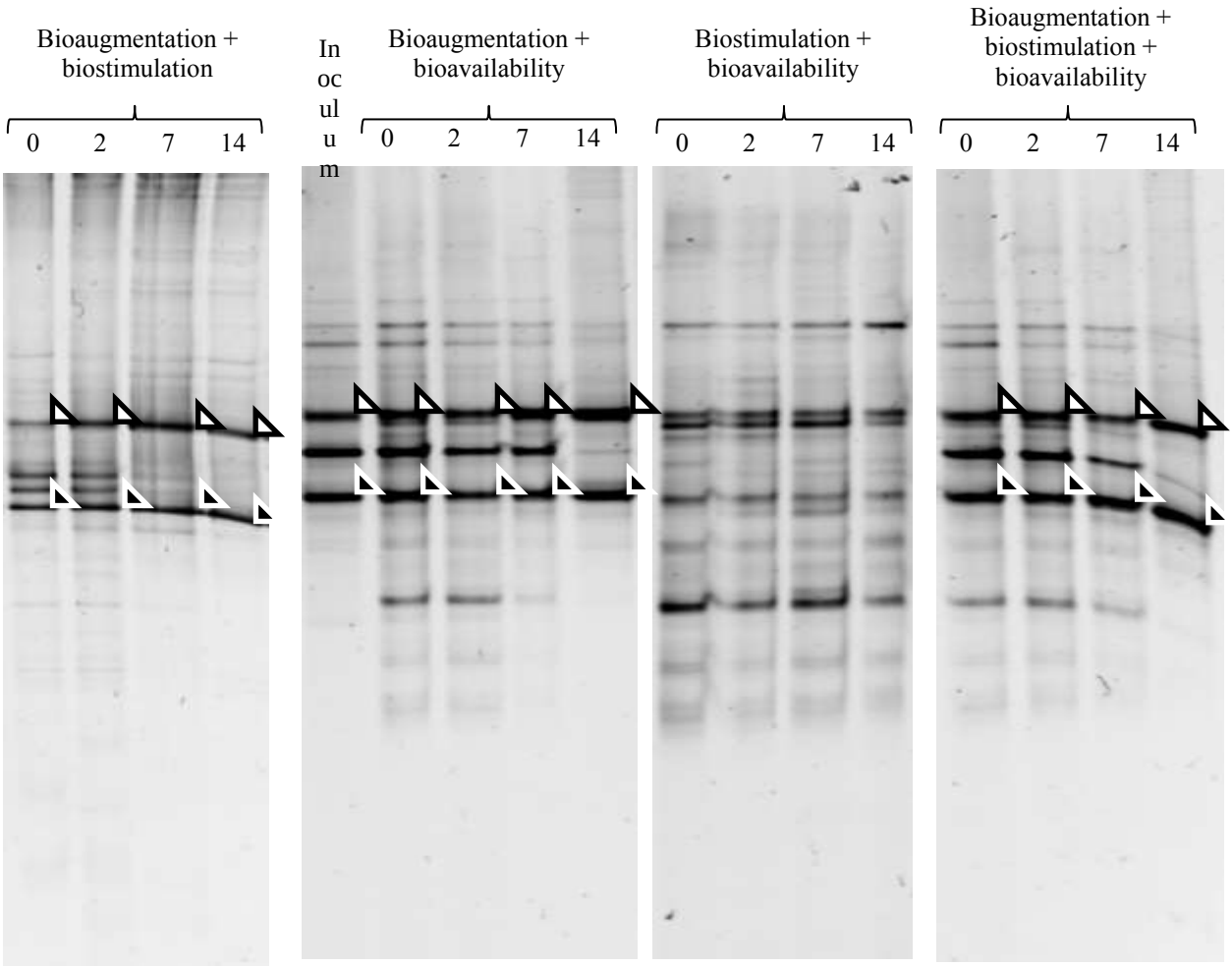

Figure 4.51: DGGE analysis of the *Chloroflexi* bacterial community throughout incubation of the bioaugmentation+biostimulation, bioaugmentation+bioavailability, biostimulation+bioavailability and bioaugmentation+biostimulation+bioavailability reactors. Bands at the same height of phylotypes VLD-1 and VLD-2 are marked in empty and black triangles, respectively.

In spite of the constant presence of two DGGE band relative to the two dechlorinating phylotypes, increase in relative abundance was detected in the bioaugnmentation+biostimulation reactor but with lower values reached at the end of incubation compared to the bioaugmentation reactor (2.6±0.3 and 3.9±0.1% of the total bacteria for VLD-1 and VLD-2, respectively) (Figure 4.52). Correlation coefficients with dechlorination activities were very high for the increase of both phylotypes in the bioaugmentation+bioavailability reactors, but in the bioaugmentation+biostimulation reactor they were stronger for the increase in VLD-1 relative abundance, albeit with lower statistical significance, whereas resulted to be stronger with the increase of VLD-2 relative abundance in the bioaugmentation+biostimulation+bioavailability reactors.

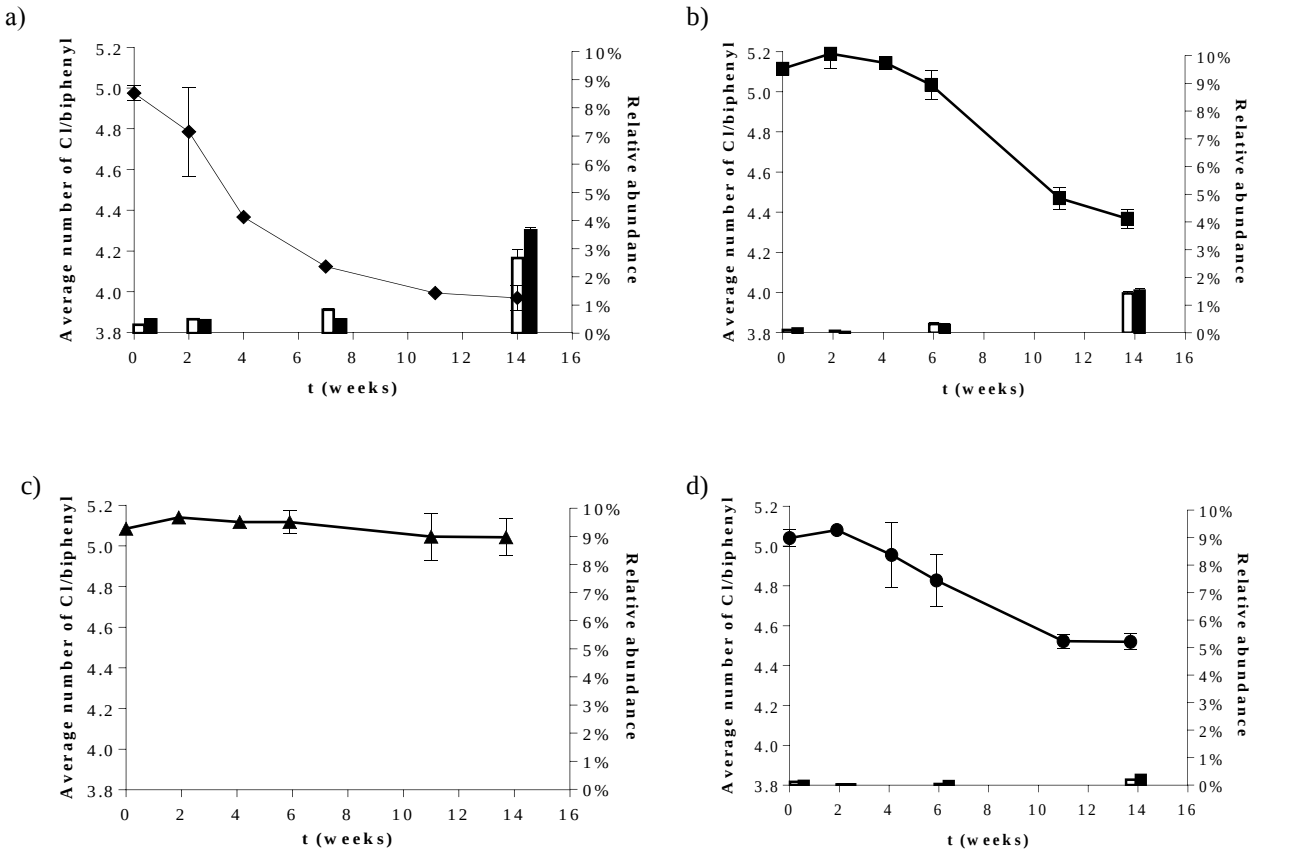

Figure 4.52: Time-course dechlorination of bioaugmentation+biostimulation, bioaugmentation+bioavailability, biostimulation+bioavailability and bioaugmentation+biostimulation+bioavailability lab-scale test reactors (A, B, C and D respectively) expressed as decrease of degree of chlorination and the increase of phylotype VLD-1 (white bars) and phylotype VLD-2 (black bars) in the respective cultures expressed as percent relative abundance (16s rDNA gene copy numbers vs total 16s rDNA genes). Values are average of triplicate analysis performed on DNA error bars represent standard errors.

Similar considerations were drawn from the bioremediation test microcosms. DGGE profiles of the *Chloroflexi* microbial community showed the presence of the two dechlorinating phylotypes only in bioaugmentation microcosms, where the dechlorination activities were detected and phylotypes were carried from the inoculum (Figure 4.53). Conversely to what happened in the reactors, however, phylotype VLD-1 band intensity decreased over the dechlorination activity and the band was not detectable anymore at week 7. Quantifications obtained through qPCR assays as described previously showed indeed very low relative abundance for phylotype VLD-1, whereas phylotype VLD-2 relative abundance increased throughout the incubation, up to 9.8±2.4% of the total bacteria in the bioaugmentation microcosms (Figure 4.54b), with very high correlation coefficients with the dechlorination activities (p<0.05). Very low abundance of the two phylotypes was detected in the other conditions, where dechlorination activities were not present.

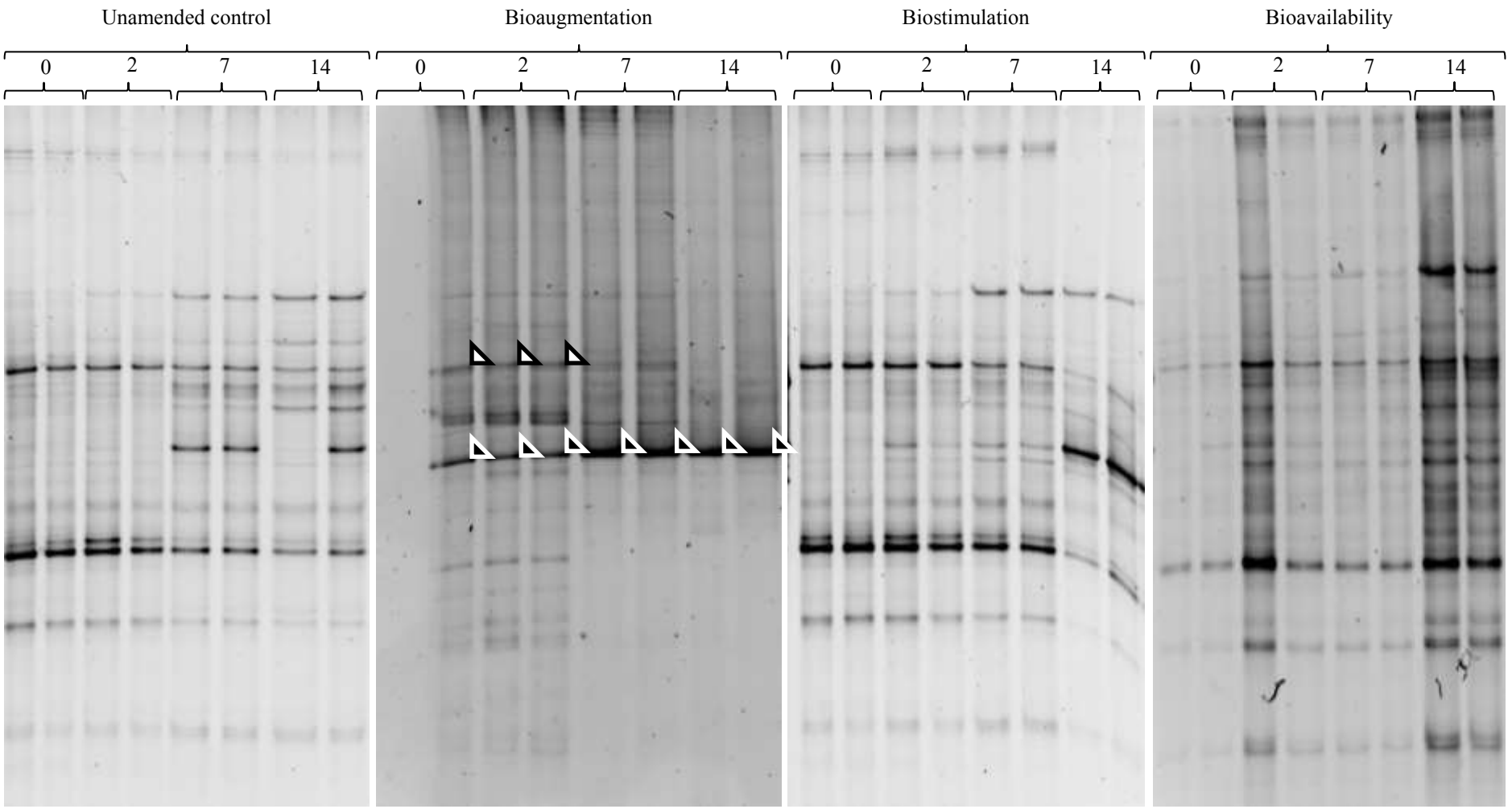

Figure 4.53: DGGE analysis of the *Chloroflexi* bacterial community throughout incubation of the unamended control, bioaugmentation, biostimulation and bioavailability microcosms. Bands at the same height of phylotypes VLD-1 and VLD-2 are marked in empty and black triangles, respectively. The analysis was performed independently on the duplicate cultures for each condition.

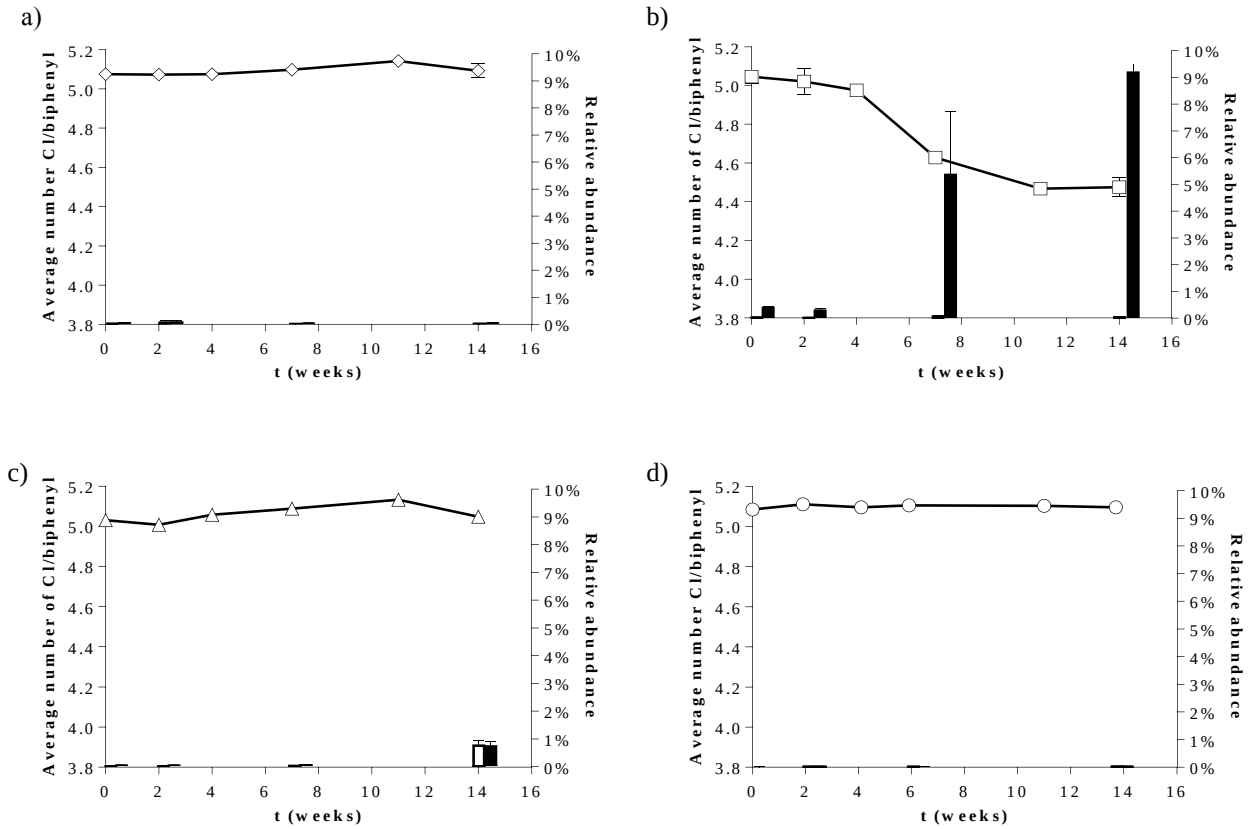

Figure 4.54: Time-course dechlorination of unamended control, bioaugmentation, biostimulation and bioavailability microcosms (A, B, C and D respectively) expressed as decrease of degree of chlorination and the increase of phylotype VLD-1 (white bars) and phylotype VLD-2 (black bars) in the respective cultures expressed as percent relative abundance (16s rDNA gene copy numbers vs total 16s rDNA genes). Values are average of duplicate microcosms and triplicate analysis performed on DNA; error bars represent standard errors.

Similarly to the corresponding conditions in shaken reactors, phylotypes VLD-1 and VLD-2 were carried by the inoculum in all bioaugmented combined approaches, and were absent where dechlorination activity did not occur, such as the biostimulation+bioavailability microcosms (Figure 4.55). Except for the bioaugmentation+biostimulation microcosms, which showed the highest dechlorination activities in the set, phylotype VLD-1 band intensity decreases over dechlorination process. The qPCR assays, revealed a total predominance of VLD-2 over VLD-1 in terms of relative abundance increase, which reached 4.6±1.3% of the total bacteria in the bioaugmentation+biostimulation microcosms and 4.8±1% in the bioaugmentation+bioavailability microcosms (Figure 4.56). Phylotype VLD-2 increase also showed stronger correlation coefficients with the dechlorination activities compared to phylotype VLD-1 increase, especially in the bioaugmentation+bioavailability microcosms.

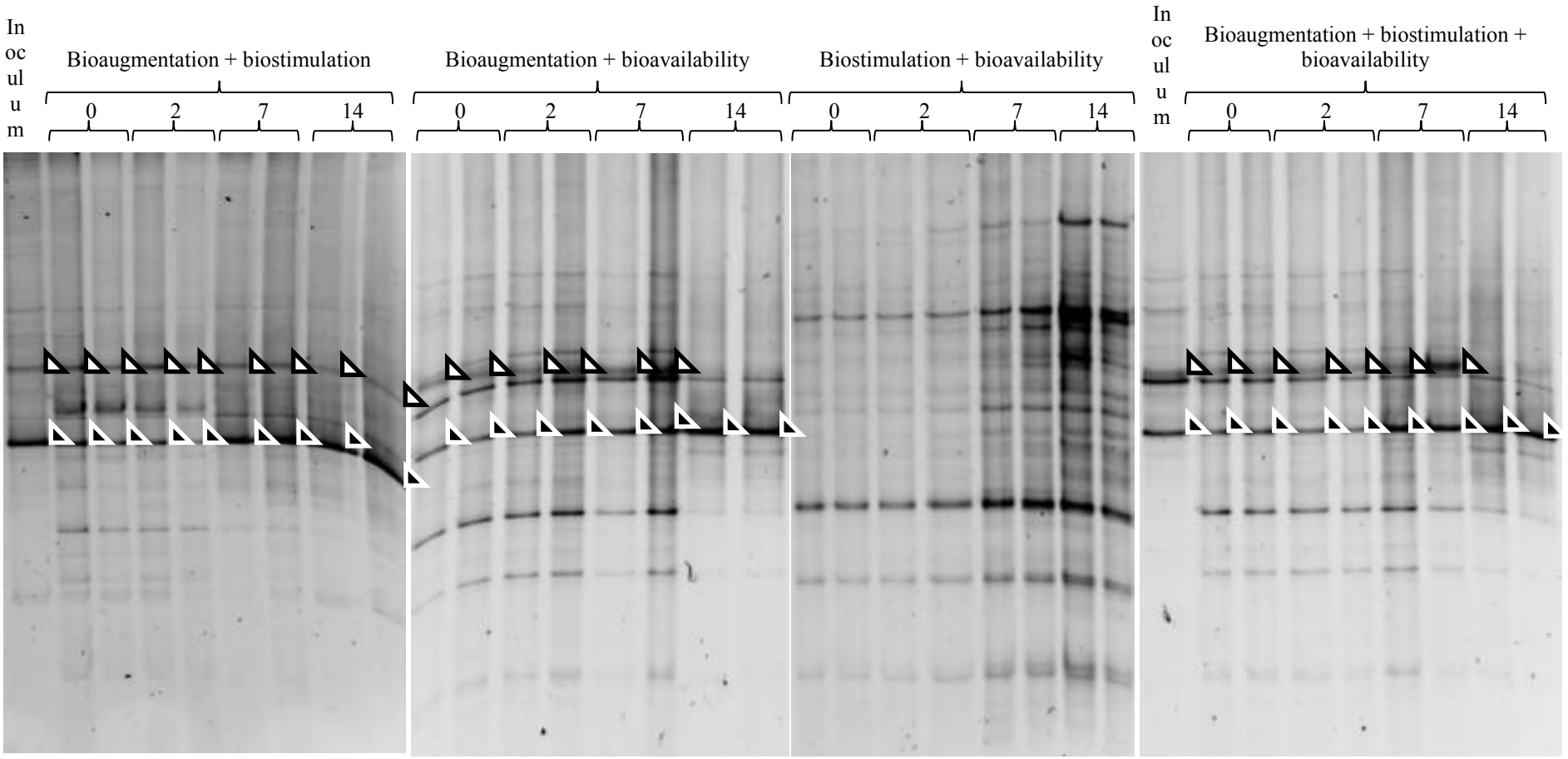

Figure 4.55: DGGE analysis of the *Chloroflexi* bacterial community throughout incubation of the bioaugmentation+biostimulation, bioaugmentation+bioavailability, biostimulation+bioavailability and bioaugmentation+biostimulation+bioavailability microcosms. Bands at the same height of phylotypes VLD-1 and VLD-2 are marked in empty and black triangles, respectively. The analysis was performed independently on the duplicate cultures for each condition.

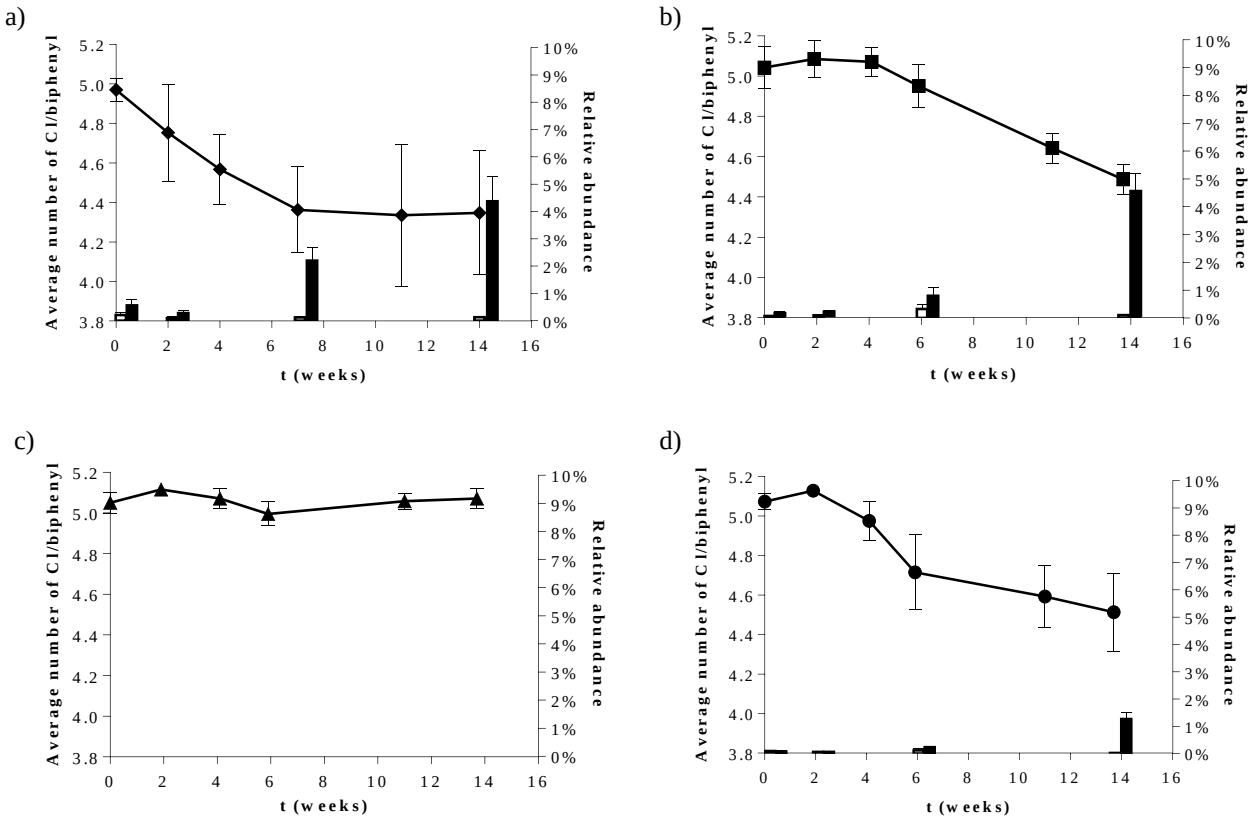

Figure 4.56: Time-course dechlorination of bioaugmentation+biostimulation, bioaugmentation+bioavailability, biostimulation+bioavailability and bioaugmentation+biostimulation+bioavailability microcosms (A, B, C and D respectively) expressed as decrease of degree of chlorination and the increase of phylotype VLD-1 (white bars) and phylotype VLD-2 (black bars) in the respective cultures expressed as percent relative abundance (16s rDNA gene copy numbers vs total 16s rDNA genes). Values are average of duplicate microcosms and triplicate analysis performed on DNA; error bars represent standard errors.

4.5.6 Final remarks

Biostimulation and bioavailability enhancement alone were not able to stimulate dechlorination activities in PCB-contaminated sediments of the Venice Lagoon while bioaugmentation itself was efficient. The stimulation effects of sodium lactate used in the biostimulation approach were evident mostly on the methanogenesis and on the sulfate reduction, and particularly in the static microcosms rather than in reactors: these findings are contradictory to what was detected in the enriched dechlorinating subculture (see section 4.2.1), where lactate had minor stimulating effect on methanogens while having inhibitory effect on sulfate reducers, and also different, to some extent, to findings reported elsewhere in different sediments (Dudkova et al., 2009); this is a strong evidence that the effectiveness of any biostimulation approach strongly depend on the structure of the microbial community itself, and on its own competition mechanisms for nutrients.

On the other hand, the dialyzed biosurfactant extract obtained from cultures of *S. frigidimarina* was not efficient in promoting any dechlorination activity, probably because of the presence of different hydrophobic substances in the sediment matrix which might have saturated the active sites of the

biosurfactant molecule; however, some previous studies have also denoted detrimental effects of biosurfactants on the microbial degradation activities of pollutants in complex microbial communities as well (Colores et al., 2000; Stelmack et al., 1999) and such effects are not to be excluded in this case.

Bioaugmentation with the selected enriched microbial population obtained previously (section 4.1) and further subcultures (section 4.5.1) was, instead, an efficient approach in promoting dechlorinating activities, and similar cases were also reported in literature (Winchell and Novak, 2008; Fagervold et al., 2011; Payne et al., 2013); the persistence of the two strains in the bioaugmentation cultures and their increase in relative abundance over total bacteria confirm that the used approach was successful, solving one of the major problem in bioaugmentation strategies which is usually the competition between the selected strain(s) and the indigenous microbial community, which often ends in a failure (Thompson et al., 2005).

The combination of bioaugmentation with the selected culture and biostimulation with the selected electron donor, resulted in even better dechlorination activities, albeit a general stimulation of methanogenesis and sulfate-reduction was also present: this combined approach showed a constant presence and increase of the two dechlorinating phylotypes throughout incubation, and confirms the previous findings on the effectiveness of lactate as electron donor for enriched dechlorinating marine sediment cultures; the finding that final relative abundances of the two phylotypes versus total bacteria were lower than bioaugmentation approaches could be justified by the nature of the qPCR assays: the quantification against total bacteria, in fact, may have caused an underestimation of the absolute increase of the two phylotypes because, at the same time, all the other members of the microbial community were stimulated by the treatment; in addition, the electron acceptors in the bioremediation lab-scale tests (PCBs) for those two phylotypes were half-concentrated compared to the sediment cultures described in section 4.1 (500 vs 1000 mgPCBs (kg dry sediment)$^{-1}$).

On the other hand, the presence of biosurfactants had inhibitory effects on the dechlorination activities in the combined approaches, which seemed to have stimulated competitors for hydrogen sources such as methanogens: in particular, VLD-1 phylotype suffered the most the toxic effects from the biosurfactants: it is known that some dehalorespiring *Dehalococcoidia* have weaker cell membranes, compared to other species (Löffler et al., 2013) and a membrane disruption (Banat et al., 2000) could be the cause of such toxicity against VLD-1, but it is unclear why the VLD-2 phylotype was not as much affected.

Selective increase of VLD-2 can be detected in all dechlorinating microcosms, whereas dechlorinating reactors showed a contemporary increase in both phylotypes: this could justify the faster dechlorination rates detected in the reactors and the occurrence of differences in the

dechlorination patterns between reactors and microcosms: phylotype VLD-1 was indeed proven to have more extensive and faster dechlorination processes than phylotype VLD-2 (see section 4.1.3.1). It is unsure whether the mechanical mixing could have favored the contact between the contaminant and the dechlorinating species, thus enhancing all stimulating approaches and helping phylotype VLD-1 in surviving the competition for nutrient and/or hydrogen sources, whereas in the absence of mechanical mixing phylotype VLD-2 was favored. The stimulating effect of mechanically mixed bioreactors over PCB microbial dechlorination activity is also known in literature, mainly regarding soil bioremediation (Fava et al., 2003a; Robles-González et al., 2008; Long et al., 2014). It is also worth noticing that phylotype VLD-2 had higher relative abundance versus total bacteria since the beginning of incubation, probably because it had outcompeted phylotype VLD-1 for nutrient and/or hydrogen sources already during the subculturing process.

In conclusion, the combination of bioaugmentation and biostimulation in a mechanically mixed bioreactor was the best lab-scale bioremediation enhancement for PCB-impacted marine sediments of the Venice Lagoon and could be a promising method for scale-up processes and an extensive *ex-situ* bioremediation of the area.

5 Conclusions

Polychlorinated biphenyls (PCBs) are highly recalcitrant toxic pollutants, which are present in soils and both freshwater and marine sediments (Ahlborg et al., 1992). Current technologies for sediment remediation involve dredging and further chemical or physical treatment, which involve high energetic and environmental costs, with the consequent destruction of the sediment and the disposal in landfill (Gomes et al., 2013). The individuation of bioremediation alternative strategies could lead the way for more sustainable processes.

Microbial reductive dechlorination is an anaerobic biological process in which highly chlorinated PCB congeners are sequentially reduced to low chlorinated ones, which are usually less toxic, less recalcitrant to further mineralization when diffused in aerobic environments and less prone to bioaccumulation (Wiegel and Wu, 2000). Such process has been studied in enrichment cultures, mainly from freshwater sediment in marine synthetic media, which allowed to identify members of the phylum *Chloroflexi* belonging to the *Dehalococcoides* genus or to the *Dehalococcoidia* class which possess PCB dechlorinating capabilities (Bedard et al., 2007; May et al., 2008; Löffler et al., 2013). On the other hand, there is scarce information on PCB dechlorination activities in marine sediments, where the high salinity and high sulfate concentration influence different microbial community evolution (Capone and Kiene, 1988). PCB dechlorination activities were observed in some Venice Lagoon sediments, both on freshly-spiked and weathered congeners (Fava et al., 2003b,c; Zanaroli et al., 2006) and members of the phylum *Chloroflexi* such as *Dehalobium chlorocoercia* DF-1 and uncultured bacterium m1 were identified to be the dechlorinating species (Zanaroli et al., 2012a,b).

In this work, the dechlorination potential of different sediments of the Venice lagoon was assessed and dehalorespiring microorganisms were identified and associated with dechlorination processes having different extension and specificities. To stimulate and complement such activities, different electron donors were tested on the enriched dechlorinating population and a test for complementation with chemical dechlorination catalyzed via nanometallic particles was performed. The best performing electron donor, the best performing dechlorinating culture and the best biosurfactant extract, obtained from a new marine oil-degrading isolates, were then tested for bioaugmentation, biostimulation and bioavailability enhancement in lab-scale bioremediation tests on real sediments and site water of the Venice Lagoon.

At first, six sediments dredged from the first industrial area of Porto Marghera, Venice Lagoon, were incubated in biogeochemical conditions mimicking those occurring on site (resuspended in

real site water 20% dry weigh/volume, in anaerobic atmosphere, static incubation in the dark, 28 °C) and contaminated with a commercial PCB mixture (Aroclor 1254) to better assess dechlorination activities. Three out of six samples, namely sediment C, D and E, exhibited dechlorination activities (Figure 4.1). PCR-DGGE approach identified two dechlorinating phylotypes, VLD-1 and VLD-2, and association with different dechlorination activities could be done through qPCR approach with specific primers designed on the 16s rRNA gene of the two species (Figure 4.11).

Microbial reductive dechlorination processes often lack of electron donors, mainly hydrogen (Bedard and Quensen, 1995), and a strategy to overcome this limitation could be the supplementation of small chain fatty acids. Five electron donors were tested on the dechlorinating population from sediment D cultures, subcultured in sterile slurries of the Venice Lagoon, spiked with Aroclor 1254 and cultivated in the same conditions described previously. The five electron donors were: pyruvate and lactate, already known to stimulate dechlorination activities (Chang et al., 2001; Azizian et al., 2010; Dudkova et al., 2009), cheese whey and molasses, agro-food waste used elsewhere for biostimulation approaches (Wu et al., 1998b; Buchner et al., 2001; DiStefano et al., 2001) and polylactate, as a model of the hydrogen releasing compounds, used as commercial bioremediation enhancers (Sandefur and Koenigsberg, 1999). The results showed that only lactate was able to stimulate selectively the dechlorination process (Figure 4.14) whereas other electron donors had either no stimulating or inhibiting effects on the dechlorination process (Figure 4.17 and Figure 4.20) or stimulated competitors (Figure 4.23).

To complement microbial reductive dechlorination activities, nanometallic catalysts can be used (Zhang, 2003). In particular, Pd^0 nanoparticles (BioPd), obtained through bioprecipitation with *S. oneidensis* M1 were shown to catalyze chemical dechlorination of PCBs in presence of hydrogen (Windt et al., 2005). Thus, BioPd nanoparticles were used to amend enriched dechlorinating cultures from sediment E: the complementation of chemical and microbial dechlorination was studied on enriched cultures which were subcultured in sterile slurries of the Venice Lagoon and sterile non inoculates slurries, while the possible chemical removal of low chlorinated congeners, product of the microbial dechlorination activities, was tested on the same enriched culture from sediment E at the end of the dechlorination phase; additionally, the impact of the BioPd nanoparticles was studied on enriched dechlorinating secondary cultures in presence and in absence of PCBs. The results, however, showed that dechlorination activities were inhibited by the high presence of hydrogen in the headspace, which was required for chemical catalysis (Figure 4.25), chemical dechlorination was not detected in any of the condition studied (Figure 4.28 and Figure 4.33) and some inhibitory effect of BioPd was noticed on sulfate-reducing and methanogenic

activities (Figure 4.29 and Figure 4.30) although the bacterial community did not suffer toxic effects as a whole (Figure 4.31 and Figure 4.32). Eventually, the lack of activity of BioPd nanoparticles could depend on the characteristics of the sediment of the Venice Lagoon itself , particularly the high sulfide concentration (Hosseinkhani et al., 2015), and, as such, this strategy was not further investigated in successive bioremediation approaches.

Biosurfactants and bioemuslifiers allow solubilization of hydrophobic compounds and can be obtained from microbial sources and used in bioremediation approaches (Banat et al., 2000). With this purpose, isolation procedures were performed from mixed oil-degrading marine cultures, to select and characterize new biosurfactant-producing strains. More than 20 isolates showing biosurfactant production were obtained, three of which showing the best productivity rates in rich medium with no oil. Extracts from the supernatant of these three strains were also characterized in terms of ecotoxicity, via the *V. fischeri* method. Eventually, dialyzed extract from XP8 strain (*S. frigidimarina*) was the least toxic of all samples and was selected to be produced in high volumes and used as a bioavailability enhancer in the successive bioremediation lab-scale tests.

Finally, all knowledge collected in the previous steps of this work was implemented in bioremediation lab-scale tests, aimed to solve major problems of PCB microbial reductive dechlorination activities in real impacted sediments of the Venice Lagoon: i) the dearth of dechlorinating species was addressed via *bioaugmentation*, i.e. the inoculation with an enriched dehalogenating culture obtained from sediment D; ii) the lack of electron donors was addressed via *biostimulation*, i.e. the amendment with lactate which was the best electron donor for enriched dechlorinating cultures; iii) the low solubility, thus *bioavailability*, of PCBs was addressed by the use of dialyzed extracts from *S. frigidimarina* culture supernatants; in addition, iv) all possible combinations of the afore-mentioned approaches were also tested. All tests were performed in mechanically mixed reactors and static microcosms, each of them set up with real sediments of the Venice Lagoon with its indigenous microbial community suspended in real water of the site, spiked with Aroclor 1254 and incubated at room temperature. The results showed that bioaugmentation approach was efficient in promoting dechlorination activities and its beneficial effects were enhanced by the combination of bioaugmentation and biostimulation, whereas biostimulation alone was not efficient and biosurfactants showed a general inhibitory effect over the dechlorination activities (Figure 4.40 and Figure 4.43). The PCR-DGGE analysis of the *Chloroflexi* bacterial community confirmed that the bioaugmentation strategy with the selected enriched culture efficiently supplemented the microbial community with the two dechlorinating phylotypes VLD-1 and VLD-2 (Figure 4.49, Figure 4.51, Figure 4.53 and Figure 4.55) and that their increase in relative abundance versus the total bacterial community can be related to the onset and extent of the

dechlorination activities. The survival of the selected degrading strain is usually a major problem in bioaugmentation (Thompson et al., 2005) and the selected procedure was efficient in overcoming it, resulting even more interesting when contemporarily biostimulated by the addition of lactate. Finally, best results were obtained in mixed systems, compared to their static counterparts, which is a key information for the further design of bioremediation slurry bioreactors (Robles-González et al., 2008).

In conclusion, the dechlorination potential in Venice Lagoon PCB-contaminated sediments was proven to be widespread, and two more dechlorinating phylotypes were detected and associated with different dechlorination activities, in biogeochemical conditions mimicking those occurring *in situ*. From this ground, different enhancement strategies of the dechlorination activities were tested and, eventually, eight different bioremediation approaches were assessed in lab-scale tests. The combination of bioaugmentation and biostimulation (with a selected enriched culture from the same site and with a suitable selectively stimulating electron donor, respectively) resulted to be the best bioremediation approach, particularly in slurry bioreactors. This is the first time that such extensive studies are performed on the impacted sediments of the Venice Lagoon and future implementation of this work lay in the development of novel bioremediation processes and tool for detection of the microbial dechlorination potential in the contaminated area.

6 International exchange research period: RWTH-Aachen

6.1 Bioelectrochemical systems (BESs)

Electromicrobiology deals with the interactions between microorganisms and electronic devices and with the novel electrical properties of microorganisms. Some microorganisms can perform biochemical oxidation of organic substances using an anode as metabolic electron acceptor (Bond, 2010), while other microorganisms are able to accept electrons from a cathode and utilize them for metabolic reactions such as simple redox transformations or to generate metabolic reducing equivalents (Rosenbaum et al., 2011). Bioelectrochemical systems are usually categorized depending on energy requirements: Microbial Fuel Cells (MFCs) produce electricity via the microbial consumption of organic matter (Logan, 2009), whereas Microbial Electrosynthesis Systems (MESs) require an energy input for operation (Rabaey and Rozendal, 2010). Cathodic and anodic chambers are often separated with a semipermeable barrier which allows counter ion exchange but separates redox conditions and prevent short circuits (Figure 6.1)

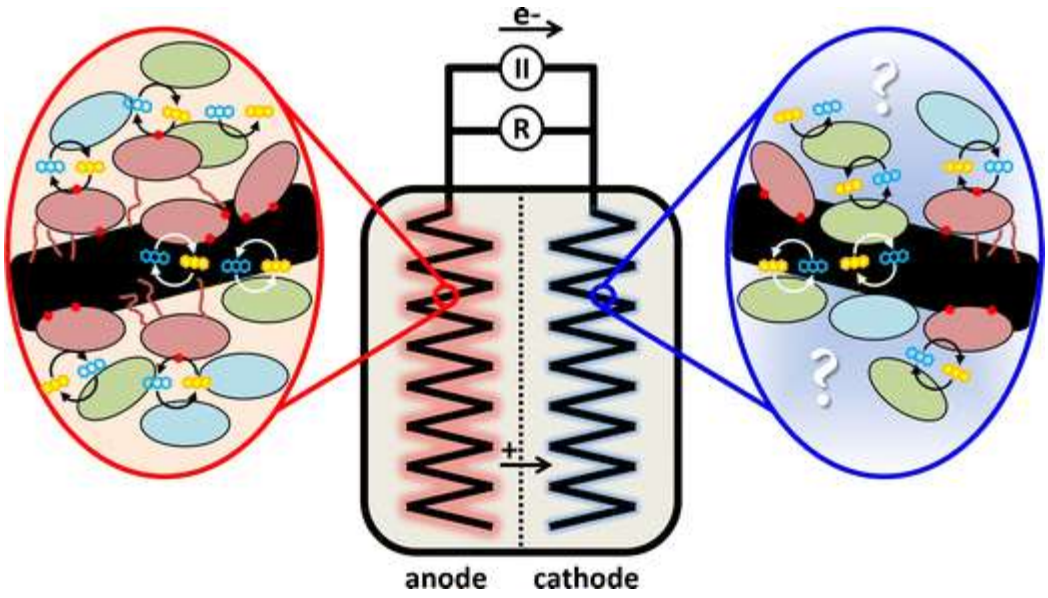

Figure 6.1: Schematic overview of the important components and physioelectrochemical principles that govern a bioelectrochemical system with insight into the anodic biofilm on the *left* and the cathodic biofilm on the *right*. Each compartment houses a biofilm covered electrode, which are separated from each other by an ion exchange membrane that facilitates charge balancing between the compartments. Anode and cathode can be connected externally via an electrical load (R) in case of an MFC or a power supply (II) in case of an MEC. Microorganisms might interact with anode or cathode directly via redox proteins (*red dots*) or protein nanowires or via soluble redox mediators (*yellow*, reduced; *blue*, oxidized redox mediator); mechanisms of cathodic electron transfer are hypothetical — indicated by *question marks* (Rosenbaum and Franks, 2014).

The mechanisms of electron transfers between microorganisms and the electrodes are different; it has been suggested that microbe-electrode exchange is a result of the development of evolutionary-effective strategies for extracellular electron exchange with insoluble minerals and related natural extracellular electron acceptors or donors (Lovley, 2006). Several gram-positive microorganisms can produce electron shuttles to promote electron transfer (Rabaey et al., 2007)(Figure 6.2b), particularly *Shewanella oneidensis* (Marsili et al., 2008) and *Pseudomonas aeruginosa* (Venkataraman et al., 2011). *Geobacter sulfurreducens* has been shown to conduct electricity via a wide diversity of c-type cytochromes (Bond and Lovley, 2003) (Figure 6.2a) or through nanowires in biofilm structures (Malvankar and Lovley, 2012) (Figure 6.2c).

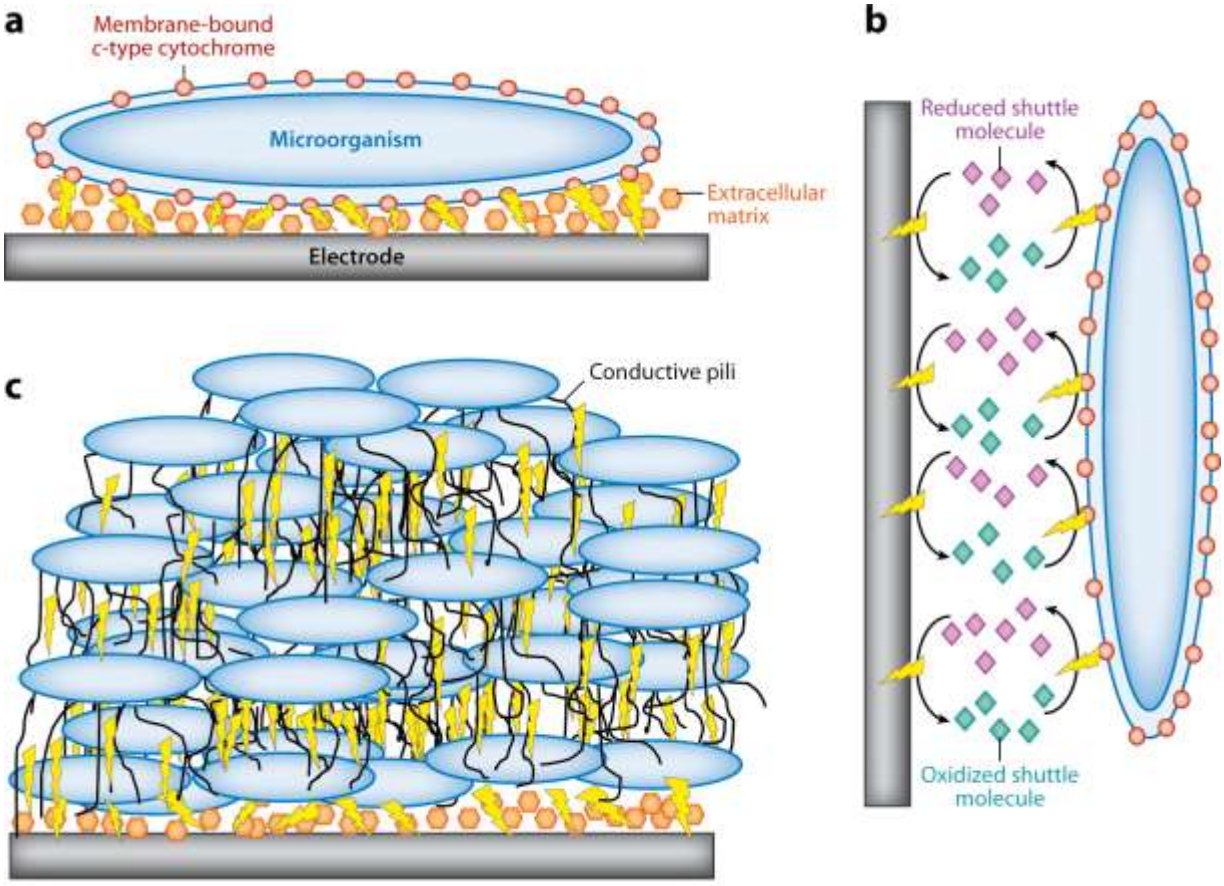

Figure 6.2: Potential mechanisms for microorganisms to transfer electrons to electrodes. (*a*) Short-range electron transfer by microorganisms in close association with the electrode surface through redox-active proteins, such as *c*-type cytochromes associated with the outer cell surface or in the extracellular matrix. (*b*) Electron transfer via reduction of soluble electron shuttles released by the cell. Oxidized shuttle molecules are reduced at the outer cell surface, and the reduced shuttle molecules donate electrons to the electrode. (*c*) Long-range electron transport through a conductive biofilm via electrically conductive pili, accompanied by short-range electron transfer from the biofilm to the electron mediated by extracellular cytochromes as in panel *a*. (Lovley, 2012)

Applications for BESs can involve biosensors for environmental monitoring (Friedman et al., 2012) sediment and wastewater Microbial Fuel Cells for electricity production (Franks and Nevin, 2010)

or chemical production (Rabaey and Rozendal, 2010) and bioremediation. BESs for bioremediation are another promising application. BES concepts have been proposed for the clean-up of various types of contamination and depending on the size and severity of the contamination, experimental applications of BES might be in reach right now. In commercial bioremediation, provision of sufficient electron donors to the intrinsic microbial remediation catalyst (biostimulation) is a common approach. A BES cathode could directly provide these lacking reducing equivalents (Lovley, 2011a). But currently most bioremediation reports focus on the remediation principles in laboratory systems. Microbial degradation activities were shown to be enhanced by electrochemical systems both in aquifers (Aulenta et al., 2009) and sediments (Zhang et al., 2010); particularly, PCB degradation activities were recently enhanced in freshwater sediments using a bioelectrical system (Chun et al., 2013).

To gain insights of bioelectrochemical techniques, a research period was spent in the laboratories of Prof. Miriam Angler-Rosenbaum, at the iAMB Institut für Angewandte Mikrobiologie (Bio IV) at the RWTH (Rheinisch-Westfälische Technische Hochschule) in Aken, Germany.

6.2 Direct interspecies electron transfer (DIET) in defined methanogenic co-cultures

6.2.1 Introduction and aim of the work

Microbial communities evolve syntrophic networks in nature to degrade organic matter, often with methane production in the anaerobic environments (Schink, 1997; Stams, 1994). The process usually involves four steps: 1) hydrolytic bacteria break down complex compounds to monomeric substances; 2) primary fermenters convert monomers into small organic molecules, H_2/formate and CO_2; 3) syntrophic bacteria carry out secondary fermentation to produce acetate, formate H_2 and CO_2 and 4) methanogenic *Archaea* uses electrons from H_2/formate/shuttles or directly to reduce CO_2 to methane (Schink and Stams, 2006). In this last step, interspecies electron transfer could occur (Shrestha and Rotaru, 2014) via i) the use of H_2 or formate as an electron transfer molecule (Bryant et al., 1967; Rotaru et al., 2012), ii) magnetite or other conducing minerals (Liu et al., 2014) or iii) direct interspecies electron transfer (DIET) (Summers et al., 2010). The latter is an interesting new mechanism for syntrophic association in the microbial community (Nagarajan et al., 2013; Shrestha et al., 2013) and, recently, it has been observed that DIET could be obtained between *Geobacter metallireducens*, a non-producing H_2 bacterium, and *Methanosaeta harundinacea*, an acetoclastic Archaea (Rotaru et al., 2014).

The main target of this work was to investigate direct interspecies electron transfer (DIET) mechanisms in anaerobic bioelectrochemical systems. To do so, co-cultures of *Geobacter metallireducens* and *Methanococcus maripaludis* have been investigated, in batch cultures and in bioelectrochemical reactors. *G.metallireducens* is a gram-positive bacterium known to take electrons from the cathode (Gregory et al., 2004) and to perform DIET in syntrophic cultures (Summers et al., 2010; Shrestha et al., 2013) but it is unable to use protons as final electron acceptors (Lovley et al., 1993). *M.maripaludis* is an hydrogenotrophic marine methanogen (Jones et al., 1983). Side-experiments were also run to test *M.maripaludis* alone in a bioelectrochemical system, as well as co-cultures of *G.metallireducens* and *Methanosaeta harundinacea* (Rotaru et al., 2014) were also performed.

6.2.2 Materials and methods

All strains are strictly anaerobic and cultivated into either Hungate tubes or serum bottles, equipped with gas-tight butyl septum and aluminum crimp sealers, as described elsewhere (Hungate, 1969). *G.metallireducens* was cultivated in its optimal medium (DSMZ 579) or medium MixLMgNO_3, described below. Either acetate or ethanol (20 mM) were used as electron donor, and Fe(III) or

nitrate as electron acceptor, and N_2:CO_2 (80:20) atmosphere; transfers were performed at 20% v/v. *M.maripaludis* was cultivated in its optimal medium (DSMZ 141 and modifications thereof) or MixLMg medium, described below, in H_2:CO_2 (80:20) overpressure of 2 bar; transfers were performed at 20% v/v. *G. sulfurreducens* was cultivated in its optimal medium (ATCC 2260) in N_2:CO_2 (80:20) atmosphere; transfers were performed at 2% v/v. *M.harundinacea* was cultivated in its optimal medium (DSMZ 334b and modifications thereof) and N_2:CO_2 (80:20) atmosphere; transfers were performed at 20% v/v. Co-culture of *G.metallireducens* and *M.maripaludis* were performed only in MixLMg medium, described below. Different ratios of the two strains were tested. *G.metallireducens* cultures were washed three times with physiological NaCl 0.9% sterile solution before inoculation in the MixLMg medium, to get rid of iron which would form black precipitates in the medium. MixLMg medium contains (g per liter of solution): $MgCl_2 \cdot 6H_2O$ 4.00, KCl 0.1, $MgSO_4 \cdot 7H_2O$ 3.45, NH_4Cl 0.6, NaH_2PO_4 0.6, Trace elements and vitamin solution from DSMZ141 10ml each, Na-acetate$\cdot 3H_2O$ 4.12, $NaHCO_3$ 2.5, Cysteine-HCl$\cdot H_2O$ 0.18, Na_2S x 9 H_2O, 0.12, Resazurin 0.001. This medium has problem with precipitates. Add the components in the described order and adjust pH to 7.00 (usually not needed) with KOH or HCl. Flush with N_2:CO_2 mixture and autoclave. After cooling to room temperature, flushing again will dissolve the precipitates. Mix$LMgNO_3$ medium contains (g per liter of solution $MgCl_2 \cdot 6H_2O$ 4.00, KCl 0.1, $MgSO_4 \cdot 7H_2O$ 3.45, NH_4Cl 0.6, NaH_2PO_4 0.6, Trace elements and vitamin solution from DSMZ141 10ml each, Na-acetate$\cdot 3H_2O$ 4.12, $NaHCO_3$ 2.5, $NaNO_3$ 20 mM.

DNA extraction was conducted using CTAB protocol adapted for bacteria which goes as follows: 2 ml of cell culture were centrifuged at 14'000 rpm, 4 °C for 10'; 0.5 g of glass beads ø = 0.1 mm and 700 µl of CTAB buffer freshly prepared were added to the pellet; samples were shaken at maximum speed in the Beadbeater™ for 30" and incubated at 65 °C, 350 rpm for 30'; 750 µl chlorophorm-isoamylachohol mixture (24:1) were added and sample were gently inverted for 2' and centrifuged at 14'000 rpm for 10'; 600 µl of the aqueous phase were then transferred in a new tube and 600 µl of isopropanol were added and gently mixed; samples were centrifuged at 22'000 g, 4 °C for 15', the supernatant was discarded, the pellet dried at room temperature and resuspended in 25 µl TE pH=8. DNA samples were quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Darmstadt, Germany) and a Biotek Synergy™ Mx Microplate Reader (BioTek, Winooski, United States).

Mastermixes for qPCR assays were prepared as follows (final concentrations per single reaction) 2x SYBR® Green Supermix (Bio-Rad, Munich) 10 µl, Primer Fw 10 mM 0.6 µl, Primer Rev 10 mM 0.6 µl, Template (diluted to contain $\approx 10^6$ copies of the 16s rRNA gene) 1 µl, Water 7.8 µl, and the

following protocol: denaturation at 95.0 °C for 10', then 45 cycles of 95.0 °C for 15", 60.0 °C for 30" and 72.0 °C for 30" + Plate Read, followed by a melt Curve 56.0 to 95.0 °C, increment 0.5 °C + Plate Read, and 4 °C for 30' on the CFX96 Real-Time PCR detection system (Bio-Rad, Munich). Selective primers for *G.metallireducens* and *M.maripaludis* were designed using Primer-BLAST website (www.ncbi.nlm.nih.gov/tools/primer-blast) with the complete genome of the two strains and the same parameters described in section 3.4.3. Sequences are represented in Table 6.1.

Target	Primer name	Primer sequence (5'-3')	Tm (°C)	Product length (bp)
G.metallireducens	Gmet_360f	TGGGGAATTTTGCGCAATGG	60.04	131
	Gmet_490r	GTACCGTCAAGACCAAGGGG	60.04	
M.maripaludis	Mmar_585f	TGAGACCGGGAGAGGACAAG	60.61	142
	Mmar_726r	TATCTAATCCGGTTCGCGCC	60.04	

Table 6.1: Characteristics of the specific primer pairs designed for uncultured *Chloroflexi* VLD-1 and VLD-2 16s rRNA gene sequence as described in this work (see also section 6.2.3).

Glass bioelectrochemical reactor were one-chambered and two chambered, with the anodic chamber separated by an anion exchange membrane (Membranes International, Ringwood, NJ) either between the two chambers. A three electrode system consisting of working, counter, and reference electrodes was used to maintain a consistent and defined electrochemical environment in the bioreactors. The working electrode consisted of a 9x0.5x5 cm piece of graphite affixed to a carbon rod with carbon cement (CCC Carbon Adhesive, EMS, Hatfield, PA). The counter electrode consisted of a 9x0.5x2 cm piece of graphite affixed to a carbon rod (Poco Graphite, Decatur, TX) with conductive carbon cement. Before any operation, the electrodes were cleaned with successive boiling in H_2O_2 3% v/v, distilled water, H_2SO_4 0.5 M and distilled water again for 2 h each. Both chambers were filled with the same medium, either DSMZ 141 or MixLMg, both without reducing agents and resazurin, to a final volume of 500 ml in the one-chambered reactor and 2z450 ml in the two-chambered reactors.. The working electrode was poised at - 0.8 V versus Ag/AgCl/sat'd KCl (-0.6 VSHE) using a potentiostat (VSP, BioLogic USA, Knoxville, TN). Cyclic voltammetry analysis was performed weekly, and on all inoculated reactors and daily prior to inoculation. Background subtraction was not used in analysis of cyclic voltammograms because no appreciable catalytic activity was observed in abiotic cyclic voltammetry. The entire system was sterilized by autoclaving at 121 °C for 1 h. The working chamber was maintained at 37 °C using a water jacket and a recirculating water heater (Model 1104, VWR Scientific, Radnor, PA) and the working chamber was stirred by a magnetic stir bar at ca. 150-200 rpm. Anaerobic reactors were constantly sparged with sterile N_2:CO_2 (80:20) gas. The reactors were sampled for headspace analyses, pH, $OD_{600,}$ TSS and DNA extraction.

Gas chromatography analyses were performed with a GC-TCD (model 310 SRI Instruments, Torrance, CA; with a 3 ft Molsieve 5A column, a TCD detector, and helium carrier gas at 5 psi) using a constant temperature of 50 °C, default event table lists and calibrations were performed using standards of H2 and N2 from 80% to 10% v/v, and CH4, O_2 and CO_2 from 1% to 60% v/v.
Acetate concentrations in the samples were measured by HPLC (600 HPLC, Waters, Milford, MA). We used an Aminex HPX-87H column (Bio-Rad, Hercules, CA) at a temperature of 65 °C, and a 5 mM sulfuric acid eluent at a flow rate of 0.6 mL/min. Metabolites were detected via an RI (refractive index) detector (410 Differential Refractometer, Waters) using calibration curves for acetate from 1 to 50 g/l.
Coulombic efficiency were calculated using the Faraday's law (Equation 6.1)

$$Q = nzF$$

Equation 6.1: Faraday's law of chemical electrolysis. Q is the quantity of current in coulombs (or I/dt in mA/s), n is the number of moles, z is the valence and F is the Faraday's constant (96485 C mol^{-1}).

And then calculating the following (Equation 6.2)

$$Y = \frac{Q_{obs}}{Q_t}$$

Equation 6.2: calculation of the coulombic efficiency, where Q_{obs} is the quantity of current consumed in the system and Q_t is the theoretical amount of current consumed by the moles of products in the system (hydrogen and methane in this work)

6.2.3 Results and Discussion

Reactor configuration included a separation between a small anodic chamber inside the whole reactor, to avoid the growth of *Geobacter* around the anode in unfavorable conditions. For this purpose, 50 ml PTTE tubes were equipped 0.22 µm cellulose acetate filter or anion exchange membranes. A preliminary test was conducted on the system described medium DSMZ141 (which also contains yeast extract and trypticase). Electrodes were poised at -900 mV vs AgCl, so to have a consistent hydrogen evolution. Sterile reactors were then inoculated with culture of *M.maripaludis*. After inoculation, a sudden current drop was noticed, probably due to reducing agents and resazurin traces still present in the inoculum (data not shown). Hydrogen evolution was consistent and H_2 moles present in the system could be almost entirely justified by the coulombic consumption (3%, 97% and 99% of H_2 moles justified respectively), except for reactor 1, where probably a flawed gas analysis falsified the data. However, the complete absence of methane produced (data not shown), as well as the absence of acetate consumption, OD and TSS increase leaded to the conclusion that the inoculum was possibly not active (Figure 6.3).

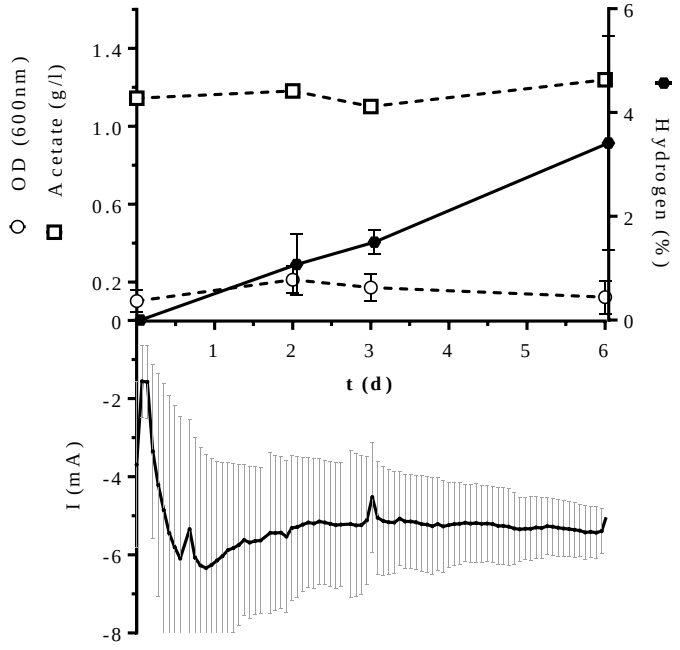

Figure 6.3: Preliminary results in terms of acetate and current consumption, OD_{600} and hydrogen increase in the bioelectrochemical reactors inoculated with *M. maripaludis*. No methane production was observed throughout incubation. Values are average of three replicate independent reactors and error bars represent standard deviations.

In the hypothesis of living cell culture, the first reactor was killed after 6 days (being the less active) and cleaned completely. To assess the presence of biofilm growing around the electrodes, an approach adapted from (Marsili et al., 2008) was used: the first reactor was killed and cleaned: this allowed to separate planktonic cells present in the medium (which were transferred anaerobically in the first sterile reactor) and an eventually grown biofilm on the electrodes of the second reactor (which was then supplemented with fresh medium).

The results showed that electro active compounds, possibly biofilm was present on the electrodes, as the same current drop was noticed in the clean reactor filled with the medium from the previous batch (red vs green line, respectively, in Figure 6.4) while current was sensibly weaker with fresh sterile medium compared to the previous CA (blue vs green line, respectively, in Figure 6.4). However, the absence of methane production or differences in the CV profiles after other 4 days of incubation (data not shown) still confirmed the assumption that inoculum was not active.

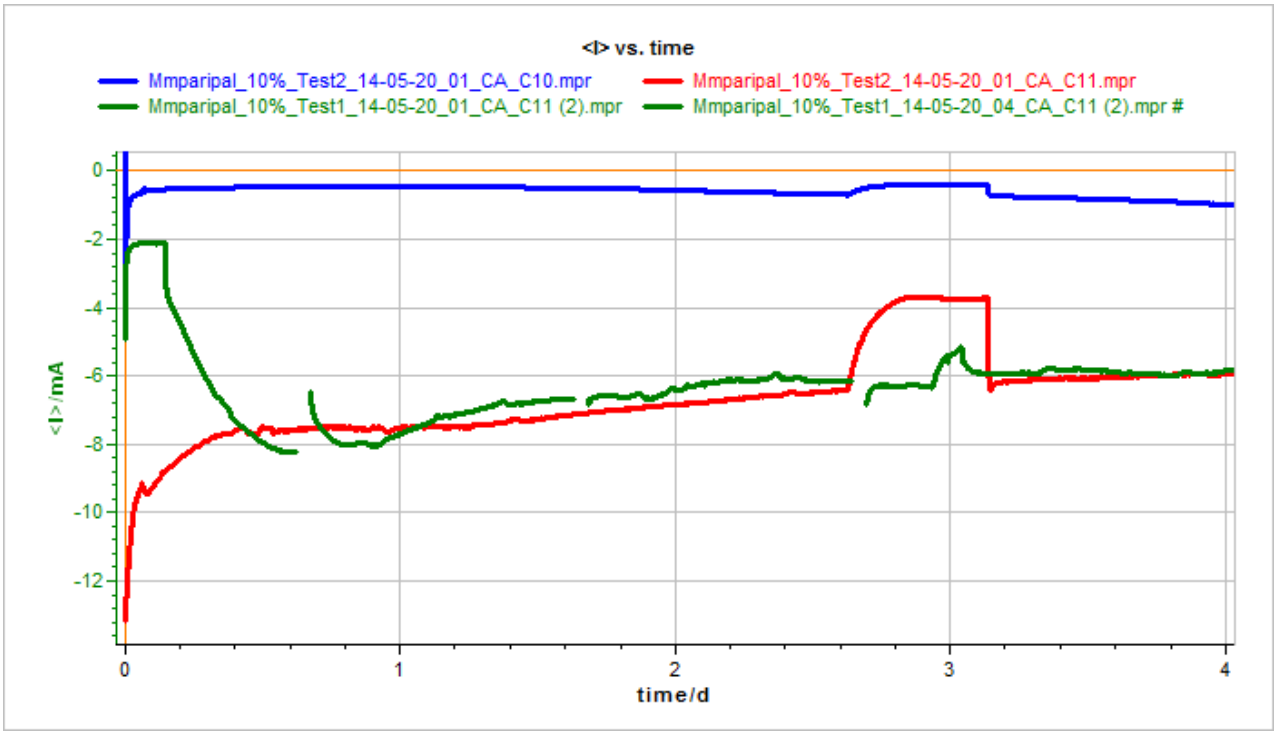

Figure 6.4: CA profile of second preliminary test where the medium coming from the second reactor from previous batch (green line) was transferred in a clean reactor, showing weaker currents (blue line), while the electrodes were soaked in fresh sterile medium (red line) showing same CA profile of the previous batch. Shifts in current at day 2.5 were due to a failure of the temperature control system.

A new set of reactors was built and was inoculated with an *M.maripaludis* culture after one week of incubation which was killed with three cycles of freezing at -20 °C O.N. and thawed at 37 °C for 2 h. The same culture was also inoculated in 5 ml medium 141 and incubated in H_2:CO_2 atmosphere (2 atm) to assess the eventual presence of residual living cells. This batch was intended to confirm the presence of electro active compounds which are present in the culture (either cell walls, or some released chemical, or some cytochrome etc.) and may cause increase in current but no conversion of the evolved hydrogen into methane. The results showed clearly a drop of current after inoculation, resembling the one obtained in the previous experiments, but no methane production as expected, as well as no consumption in acetate (Figure 6.5a). However, OD increases were noticed first in the control cultures, as well as methane production (Figure 6.5b) and later in the reactors themselves, suggesting the hypothesis that cells were not killed after freeze-thawing. Indeed, by closing the gas flow for 6h and after analysis of the coulombic current, only 58%; 90% and 62% of the H_2 moles produced could be justified with the current; unfortunately, methane was not detected, leading to the hypothesis that its absence could be due to stripping phenomena because of the N_2:CO_2 continuous flow (concentration might be so low that evolved methane would not be detected by the system).

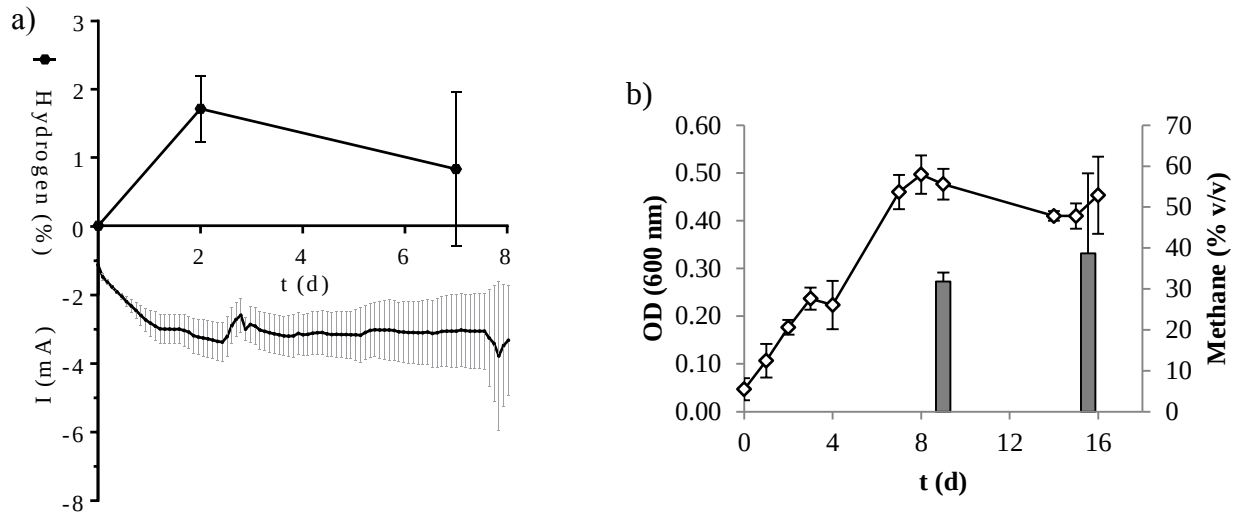

Figure 6.5: results in terms of current consumption and hydrogen increase in the bioelectrochemical reactors inoculated with *M. maripaludis* killed culture (a) and methane production (bars) and OD_{600} increase (lines) in batch controls (b). No methane production was observed throughout incubation in the reactors. Values are average of three independent replicates and error bars represent standard deviations.

G.metallireducens and *M.harundinacea* were cultivated in their own media, but the latter did not show any growth or methanogenic activity, thus the possibility of performing a co-culture replicating experiments found in literature (Rotaru et al., 2014) was discarded (data not shown).

In parallel, different media were tested to develop a suitable medium for the co-culture development Different compositions of medium DSMZ579 and medium DSMZ141 were tested to allow the growth of *G.metallireducens* and *M.maripaludis* separately. *M. maripaludis* is strongly inhibited by the presence of iron in the medium DSMZ579 whereas the high saline concentration of the medium DSMZ141 resulted to be toxic for *G.metallireducens* (data not shown). Eventually, a medium based on the work from Rotaru et al., (2014) was formulated and indicated as MixLMg. Such medium was adapted for *G.metallireducens* with the addition of $NaNO_3$ as an electron acceptor instead of Fe(III), and for *M.maripaludis* with the addition of reducing agents and H_2:CO_2 atmosphere at 2 bar pressure. *G.metallireducens* showed slower growth rate and maximum OD reached, but survived during successive transfer in Mixl$MgNO_3$ medium (Figure 6.6a and b), whereas methane production and growth rates of *M.maripaludis* in MixLMg medium were comparable to growth rates and methane production in DSMZ141 medium (Figure 6.6c and d).

Selective primers were then designed to perform qPCR assays on the co-cultures to show selective increase of one or the other species during incubation in the co-culture experiments.

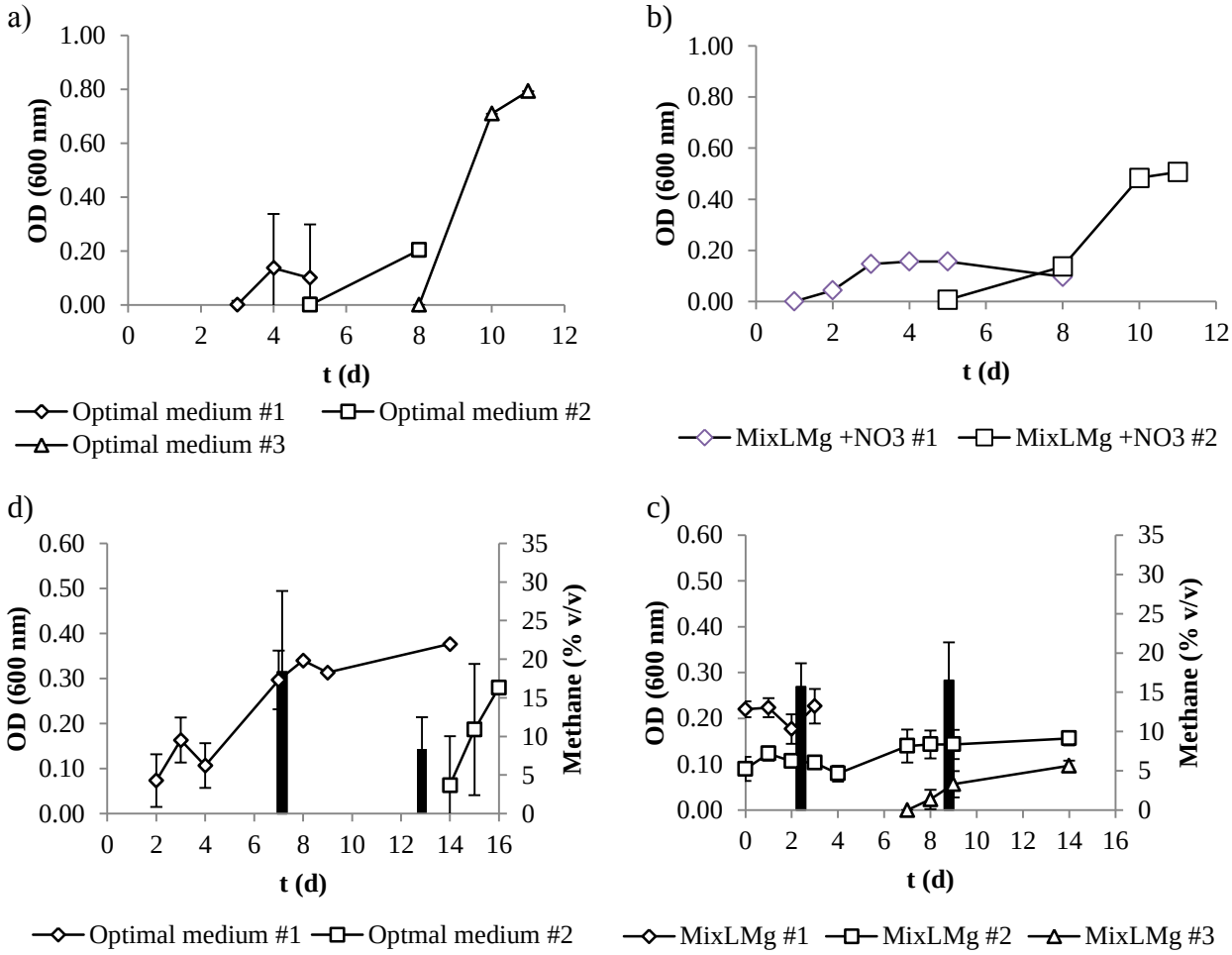

Figure 6.6: Growth curves of *G.metallireducens* successive transfers in DSMZ579 (a) and MixLMg adapted medium (b) and growth curves (lines) and methane production (bars) in *M.maripaludis* successive transfer in medium DSMZ141 (c) and MixLMg adapted medium (d). Values are average of triplicate cultures and error bars represent standard deviations.

Co-culture experiments have been conducted in MixLMg medium. This medium contains acetate (2.5 g/l), which is a suitable electron donor for *G.metallireducens* but no electron acceptor. This approach was supposed to force *G.metallireducens* to discharge its electrons through DIET with *M.maripaludis*: to achieve this condition, a pre-culture of *M.maripaludis* in medium MixLMg were all hydrogen was already depleted, was inoculated (10% v/v) with a culture of *G.metallireducens* in medium MixLMgNO_3 and amended with ethanol as electron donor (20 mM). Before ethanol addition, aliquots of this mixture were transferred in fresh medium and set up in presence of H_2:CO_2 mixture headspace (80:20, 2 bar overpressure) or in presence of N_2:CO_2 mixture headspace (80:20, atmospheric pressure). The results showed that no methanogenesis occurred in the culture amended with ethanol, neither in the one without hydrogen (data not shown). This was an indication of no DIET occurring between the two strains (Figure 6.7). Since the carryover of nitrate and ammonia from the *G.metallireducens* culture might be inhibitory for *M.maripaludis*, the fresh culture test in presence of hydrogen was repeated adding a preliminary washing of the *G.metallireducens* culture

with NaCl 10 g/l sterile solution, for three times (13'400 rpm for 15' each) before inoculation of the co-culture; to optimize this step, *G.metallireducens*. inoculum was actually raised to 20% v/v. The results did not show particular improving of growth and lower methane production.

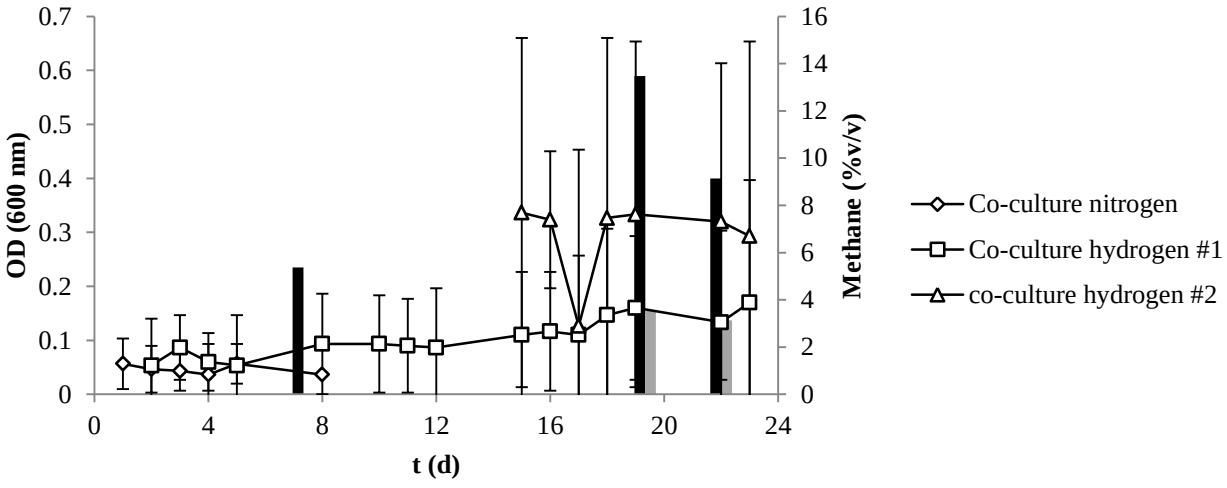

Figure 6.7: Growth curves (lines) and methane production (bars) in co-cultures of *G.metallireducens* (5% v/v) and *M.maripaludis* (20% v/v) without washing step of *G.metallireducens* culture before inoculation (#1) and with washing step (#1b) in presence and absence of hydrogen headspace. No methane production was detected in absence of hydrogen. Values are average of triplicate cultures and error bars represent standard deviations.

Endpoint qPCR quantification test proved that the first hydrogen culture showed a predominance of *M.maripaludis*, which accounted for 80±6% of the cells, while the second co-culture had a strong predominance in *G.metallireducens*, which accounted for 89±10% of the cells in the culture (Figure 6.8). The samples were analyzed using gDNA dilutions which theoretically were corresponding to 10^6 and 10^5 16s rRNA gene copy numbers, and spiked samples were also prepared to check for cross-amplification and absence of inhibitors. This result was probably due to the inoculum/washing procedure, but is compatible with methane production. However, no evidence of aggregation (or DIET) seemed to occur in both cultures, regardless of their composition, when observed under the phase contrast microscope (data not shown).

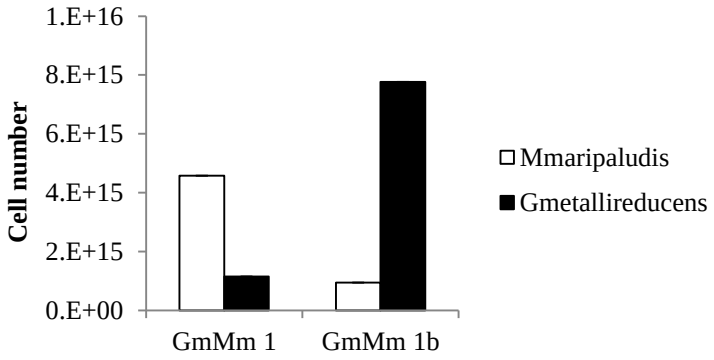

Figure 6.8: cell number calculated from 16s rRNA genes copy number of *M.maripaludis* (empty bars) and *G.metallireducens* (black bars) for co-cultures in MixLMg medium in hydrogen overpressure headspace, without washing step of G.metallireducens culture before inoculation (#1) and with washing step (#1b). Values are averages of three replicates in two different dilutions, and error bars represent standard deviations.

Inoculum with co-cultures of *G.metallireducens* and *M.maripaludis* were set up as described before in the same medium. To get rid of the reducing agents, inocula of the co-cultures washed and concentrated 3x before being used as inoculum in the reactor. Cultures were checked on the microscope before and after washing: the original co-cultures seemed not to show any aggregation between *M.maripaludis* and *G.metallireducens* (supporting the absence of DIET as described previously) (data not shown). Nevertheless, reactors were set-up again and abiotical tests were performed at -800 mV vs AgCl, so to follow the same conditions described in (Lohner et al., 2014). The results showed almost no current and almost absent hydrogen evolution, as expected (data not shown). The co-culture was inoculated in the reactor after washing, and aliquots of the inoculum were transferred in the MixLMgNR medium, and cultivated in N_2:CO_2 mixture headspace or in H_2:CO_2 mixture overpressure headspace. The reactors electrodes were poised this time The results showed very low current, no methane production and no acetate consumption (Figure 6.9a). Moreover, oxygen concentrations were unexpectedly high after inoculation, starting from 1.31±1.73% before incubation to 4.01±3.66 after 24h of incubation, with N_2:CO_2 running, and up to 12.17±0.13% after 4h of no flushing and closed valves for methane quantification. CV tests confirmed that there was actually no difference between the active co-culture and the blank medium (Figure 6.9b), thus leading to the conclusion that the co-culture might be dead. However, control batch cultures showed turbidity increases and methane production compatible to the previous inoculum, when in presence of hydrogen overpressure (no methane was noticed in the cultures with nitrogen mixture headspace) (data not shown). This could either mean that the inoculum was damaged during inoculation/washing procedure, and that these reactors are not suitable for these experiments, or could as well be dependent of the lack of reducing agents inside the reactor medium which might be not completely reduced by the only cathode.

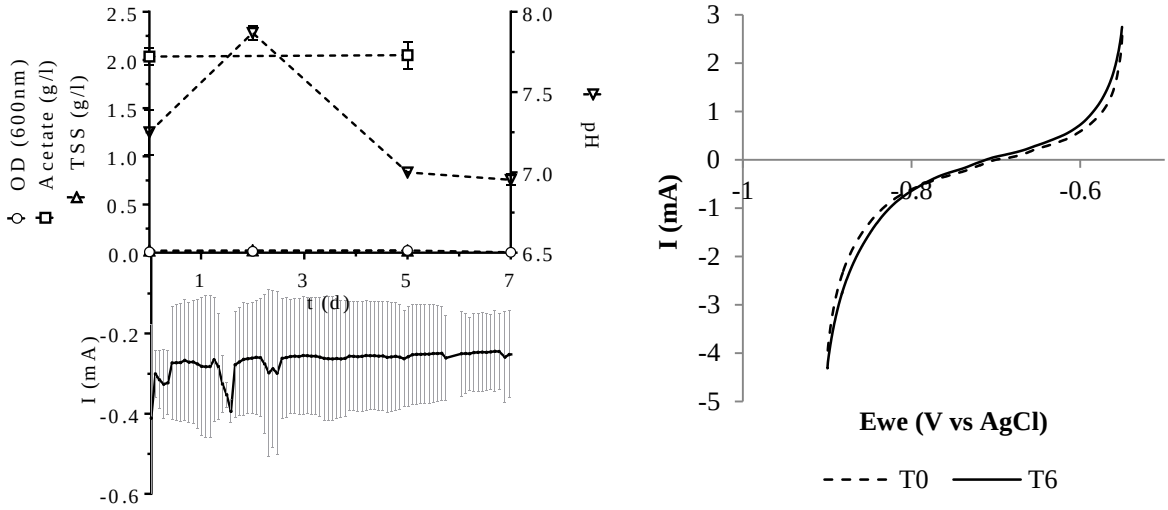

Figure 6.9: a) current, OD, Acetate, pH and TSS measurements in the reactors with MixLMg Salt Solution medium and co-cultures of *G.metallireducens* and *M.maripaludis*. Currents were very low, and no depletion of acetate was noticed, as well as any increase of TSS and OD. Increase in current at day 2 is associated with a problem in the N_2:CO_2 flushing. Values are average of duplicate reactors and error bars represent standard deviation b) Cyclic voltammetry of the reactors with MixLMg Salt Solution medium and co-cultures of *G.metallireducens* and *M.maripaludis*. Values are average of reduction and oxidation cycles, repeated 4 times each. No detectable difference subsists between the beginning and the end of incubation.

Medium MixLMg seems to be suitable for cultivation of *G.metallireducens* and *M.maripaludis* strains, with adjustments for the specific strains (such as the addition of the NO_3^- as electron acceptor for *G.metallireducens* or the hydrogen headspace overpressure and the reducing agents for *M.maripaludis*). However, when combined, the two strains seem not to interact with each other: this could be due either to the presence of carryover contaminants form the *G.metallireducens* cultures (such as nitrate or ammonia), to the presence of reducing agents (which is required for *M.maripaludis* growth but seems inhibitory for *G.metallireducens*) or to the low concentration of the two strains. These problems can be solved by further testing, but, so far, no evidence of DIET was assessed. Reactor experiments are still affected by issues regarding the separation between the anodic chamber, which might diffuse oxygen in the cathodic space, and the tightness of the reactors themselves. To run these experiments eventually, new gas-tight configurations have to be designed, as the chances of leakages also heavily affect the detection limit of methane production.

6.2.4 Conclusion

Unfortunately, *M.harundinacea* strain did not grow throughout the experimental period and co-cultures with either *G.metallireducens* or *G.sulfurreducens* could not be tested. The cultivation of *G.metallireducens* and *M.maripaludis* was first tested in their optimal medium and in different intermediate compositions, which might be suitable for both strains. After different tests, a final composition was determined, which required some adjustments for the single strain, co-culture or

reactor experiment. The suitable medium was then used for co-culture tests, which exhibited no methane production in the absence of hydrogen (electron donor for *M.maripaludis)* and no visible aggregation of the two strains, suggesting that DIET mechanisms did not occur under the experimental conditions tested. In parallel, primers were designed for the specific strains and qPCR tests were also run on endpoint co-culture experiments.

Bioelectrochemical systems used were mainly flawed by the non-complete tightness of the different reactors tested, which affected methane measurements as well as the anaerobic growth. Moreover, efficient separation of the anodic chamber from the rest of the liquid phase has still to be achieved.

Results in bioelectrochemical tests showed that *M.maripaludis* alone, in its optimal medium, was able to exhibit increase in currents and hydrogen evolution and, probably, by direct contact with the cathode. However, these assumptions still need to be verified by methane production, which was not possible to assess due to the gas leakages of the system, or by microbiological analysis on the electrodes after incubation, even though, direct electron uptake of *M.maripaludis* from the electrode has been described in literature recently (Lohner et al., 2014).

Bioelectrochemical systems inoculated with co-cultures showed no methane production and no growth of any of the two strains. This could be due either to the reactor configuration problems described previously, or to the absence of DIET mechanisms in the first place.

DIET mechanisms could be evidenced by forcing the cultures to exhibit this behavior. To do so, a convenient strategy could be the utilization of knock-out strains of *M.maripaludis* which carry no hydrogenases, thus requiring other sources of electrons to survive. Furthermore, a fine-tuning of the medium could be required: reducing agents are necessary for *M.maripaludis* growth and might be necessary in the reactors too, but they also seem to be slightly inhibitory for the *G.metallireducens*. It could also be a good idea to use ethanol instead of acetate as electron donor for *G.metallireducens,* due to its higher energy state.

Further investigations have to be conducted on the standard potential required for coupling of the two reactions: *G.metallireducens* can take up electrons at -400 mV vs SHE (Lovley, 2011b), while *M.maripaludis* needs at least -600 mV vs SHE (Lohner et al., 2014): this difference in electron uptake potential can be due to the need for the methanogen to reduce its ferredoxin groups. Thus, a DIET will occur only if *G.metallireducens* could provide electrons at that energy state, requiring the implication of biological disproportion mechanisms or other. It could also be good to allow for the methanogen to grow at the same time on both electron transfer from the *G.metallireducens* and some other electron source: this could require again a knock-out strain and molar calculation of the yields could be harder.

7 Dissemination activities

7.1 Publications

7.1.1 Full papers on international scientific journals

1) **Hosseinkhani B, Nuzzo A, Zanaroli G, Fava F, Boon N.** 2015**.** Assessment of catalytic dechlorination activity of suspended and immobilized bio-Pd NPs in different marine conditions. *Appl. Catal. B Environ*. 168-169:62–67. http://www.sciencedirect.com/science/article/pii/S0926337314007887.
2) **Valentino F, Brusca AA, Beccari M, Nuzzo A, Zanaroli G, Majone M.** 2013. Start-up of biological sequencing batch reactor (SBR) and short-term biomass acclimation for polyhydroxyalkanoates production. *J. Chem. Technol. Biotechnol*. 88:261–270. http://doi.wiley.com/10.1002/jctb.3824.
3) **Zanaroli G, Negroni A, Vignola M, Nuzzo A, Shu H-Y, Fava F.** 2012. Enhancement of microbial reductive dechlorination of polychlorinated biphenyls (PCBs) in a marine sediment by nanoscale zerovalent iron (NZVI) particles. *J. Chem. Technol. Biotechnol.* 87:1246–1253. http://doi.wiley.com/10.1002/jctb.3835.

7.1.2 Papers submitted or in preparations

1) **Nuzzo. A., Zanaroli G., Negroni A., Fava F.** SUBMITTED. PCB dechlorination activities associated to different Chloroflexi bacteria in marine sediments
2) **Nuzzo A., Hosseinkhani B., Boon N., Zanaroli G., Fava F.** IN PREPARATION Impact of biogenic Pd nanoparticles on the microbial reductive dechlorination in a marine sediment of the Venice lagoon
3) **Nuzzo A., Zanaroli G., Raddadi N., Fava F.** IN PREPARATION Bioremediation lab-scale tests for PCB-impacted anaerobic marine sediments.

7.2 Conferences

7.2.1 Extended abstracts in proceedings of national and international conferences

1) **Nuzzo A., Andria Y., Zanaroli G., Fava F.,** 2013 Biostimulation of microbial reductive dechlorination of polychlorinated biphenyls in marine sediments of the Venice Lagoon, *Ecomondo 2013, Reclaim Expo*, ORAL PRESENTATION
2) **Nuzzo A., Negroni A., Zanaroli G., Fava F.,** 2012 Assessment of the polychlorinated biphenyls (PCBs)-dechlorinating potential of the indigenous microbial communities of

contaminated marine sediments of the Venice Lagoon, *Ecomondo 2012, Reclaim Expo,* POSTER PRESENTATION

7.2.2 Abstracts in proceedings of national and international conferences

1) **Nuzzo A., Zanaroli G., Raddadi N., Fava F.,** 2015, Bioaugmentation and combined biostimulation enhanced PCB reductive dechlorination in a marine sediment, *EBC-VI Conference* (June 29th - July 2nd, Chania, Crete, Greece). ORAL PRESENTATION
2) **Nuzzo A., Hosseinkhani B., Boon N., Zanaroli G., Fava F.,** 2015, Impact of bio-Pd NPs on the microbial dechlorinating community enriched from a marine sediment of the Venice lagoon, *EBC-VI Conference* (June 29th - July 2nd, Chania, Crete, Greece). POSTER PRESENTATION
3) **Nuzzo A., Zanaroli G., Raddadi N., Fava F.,** 2014 Enhancement of PCB microbial reductive dechlorination in marine sediments via bioaugmentation and biostimulation approaches. *MedRem-2014 Conference* (16-18 January, Hammamet, Tunis). ORAL PRESENTATION
4) **Nuzzo A., Zanaroli G., Negroni A., Fava F**. 2014 PCB dechlorination activities associated to different Chloroflexi bacteria in marine sediments. *MedRem-2014 Conference* (16-18 January, Hammamet, Tunis). ORAL PRESENTATION
5) **Hosseinkhani B., Nuzzo A., Zanaroli G., Hernandez Sanabria E., Fava F., Boon N.** 2014 Biogenic palladium nano-catalysts for dechlorination of TCE and PCBs in marine sediments. *MedRem-2014 Conference* (16-18 January, Hammamet, Tunis). ORAL PRESENTATION
6) **Zanaroli G., Nuzzo A., Negroni A., Fava F.** 2013 Anaerobic biodegradation of polychlorinated biphenyls in marine sediments. *SIMGBM 2013* (18-21 September, Ischia, Italy). ORAL PRESENTATION
7) **Nuzzo A., Hosseinkhani B., Boon N., Zanaroli G., Fava F.,** 2013 Impact of biogenic Pd nanoparticles on the microbial reductive dechlorination in a marine sediment of the Venice lagoon, *Ecomondo 2013, Reclaim Expo.* POSTER PRESENTATION
8) **Nuzzo A., Andria Y., Zanaroli G., Fava F.,** 2013 Biostimulation of microbial reductive dechlorination of polychlorinated biphenyls in marine sediments of the Venice Lagoon, *GENT BIOBASED ECONOMY Summer School 2013.* POSTER PRESENTATION
9) **Nuzzo A., Negroni A., Zanaroli G., Fava F.,** 2012 Polychlorinated biphenyl (PCB) microbial reductive dechlorination potential in contaminated marine sediments of the Venice Lagoon, *EMB 2012, Section 4.2.* ORAL PRESENTATION

8 References

Abhilash PC, Jamil S, Singh N. 2009. Transgenic plants for enhanced biodegradation and phytoremediation of organic xenobiotics. *Biotechnol. Adv.* **27**:474–88.

Abraham W-R, Nogales B, Golyshin PN, Pieper DH, Timmis KN. 2002. Polychlorinated biphenyl-degrading microbial communities in soils and sediments. *Curr. Opin. Microbiol.* **5**:246–253.

Abramowicz D. 1995. Aerobic and anaerobic PCB biodegradation in the environment. *Environ. Health Perspect.* **10**:241–251.

Adrian L, Szewzyk U, Wecke J, Görisch H. 2000. Bacterial dehalorespiration with chlorinated benzenes. *Nature* **408**.

Adrian L, Dudková V, Demnerová K, Bedard DL. 2009. "Dehalococcoides" sp. strain CBDB1 extensively dechlorinates the commercial polychlorinated biphenyl mixture aroclor 1260. *Appl. Environ. Microbiol.* **75**:4516–24.

Agarwal S, Al-Abed SR, Dionysiou DD. 2009. A feasibility study on Pd/Mg application in historically contaminated sediments and PCB spiked substrates. *J. Hazard. Mater.* **172**:1156–62.

Agarwal S, Al-Abed SR, Dionysiou DD. 2007. In Situ Technologies for Reclamation of PCB-Contaminated Sediments: Current Challenges and Research Thrust Areas. *J. Environ. Eng.* **133**:1075–1078.

Agency for Toxic Substances and Disease Registry (US Department of Health and Human Services). 2000. TOXICOLOGICAL PROFILE FOR POLYCHLORINATED BIPHENYLS (PCBs). *US Dep. Heal. Hum. Resour. Agency Toxic Subst. Dis. Regist.* Atalanta, GA.

Ahlborg UG, Hanberg A, Kenne K. 1992. Risk assessment of polychlorinated biphenyls (PCBs).

Ahmad M, Simon MA, Sherrin A, Tuccillo ME, Ullman JL, Teel AL, Watts RJ. 2011. Treatment of polychlorinated biphenyls in two surface soils using catalyzed H_2O_2 propagations. *Chemosphere* **84**:855–62.

Van Aken B, Correa P a, Schnoor JL. 2010. Phytoremediation of polychlorinated biphenyls: new trends and promises. *Environ. Sci. Technol.* **44**:2767–76.

Alder AC, Haggblom MM, Oppenheimer SR, Young LY. 1993. Reductive dechlorination of polychlorinated biphenyls in anaerobic sediments. *Environ. Sci. Technol.* **27**:530–538.

Amann R, Ludwig W, Schleifer K. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:143–169.

Aresta M, Caramuscio P, De Stefano L, Pastore T. 2003. Solid state dehalogenation of PCBs in contaminated soil using NaBH4. *Waste Manag.* **23**:315–9.

Aulenta F, Fuoco M, Canosa A, Petrangeli Papini M, Majone M. 2008. Use of poly-beta-hydroxy-butyrate as a slow-release electron donor for the microbial reductive dechlorination of TCE. *Water Sci. Technol.* **57**:921–5.

Aulenta F, Canosa A, Reale P, Rossetti S, Panero S, Majone M. 2009. Microbial reductive dechlorination of trichloroethene to ethene with electrodes serving as electron donors without the external addition of redox mediators. *Biotechnol. Bioeng.* **103**:85–91.

Aulenta F, Majone M, Tandoi V. 2006. Enhanced anaerobic bioremediation of chlorinated solvents: environmental factors influencing microbial activity and their relevance under field conditions. *J. Chem. Technol. Biotechnol.* **81**:1463–1474.

Azizian MF, Marshall IPG, Behrens S, Spormann AM, Semprini L. 2010. Comparison of lactate, formate, and propionate as hydrogen donors for the reductive dehalogenation of trichloroethene in a continuous-flow column. *J. Contam. Hydrol.* **113**:77–92.

Bach H-J, Tomanova J, Schloter M, Munch JC. 2002. Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. *J. Microbiol. Methods* **49**:235–45.

Baker EL, Landrigan PJ, Glueck CJ, Zack MM, Liddle JA, Burse VW, Housworth WJ, Needham LL. 1980. Metabolic consequences of exposure to polychlorinated biphenyls (PCB) in sewage sludge. *Am. J. Epidemiol.* **112**:553–63.

Ballschmiter K, Zell M. 1980. Analysis of polychlorinated biphenyls (PCB) by glass capillary gas chromatography. *Fresenius' Zeitschrift für Anal. Chemie* **302**:20–31.

Banat IM, Makkar RS, Cameotra SS. 2000. Potential commercial applications of microbial surfactants. *Appl. Microbiol. Biotechnol.* **53**:495–508.

Barry T, Colleran G, Glennon F. 1991. The 16s / 23s ribosomal spacer region as a target for DNA probes to identify eubacteria service:1–2.

Bedard D. 2003. Polychlorinated Biphenyls in Aquatic Sediments: Environmental Fate and Outlook for Biological Treatment. In: Häggblom, MM, Bossert, ID, editors. *Dehalogenation. Microb. Process. Environ. Appl.* Boston: Kluwer Academic Publishers.

Bedard D, Bunnell S, Smullen L. 1996. Stimulation of microbial para-dechlorination of polychlorinated biphenyls that have persisted in Housatonic River sediment for decades. *Environ. Sci. …* **30**:687–694.

Bedard D, Van Dort H, Deweerd K. 1998. Brominated Biphenyls Prime Extensive Microbial Reductive Dehalogenation of Aroclor 1260 in Housatonic River Sediment. *Appl. Environ. Microbiol.* **64**:1786–95.

Bedard DL. 2008. A case study for microbial biodegradation: anaerobic bacterial reductive dechlorination of polychlorinated biphenyls-from sediment to defined medium. *Annu. Rev. Microbiol.* **62**:253–70.

Bedard DL, Bailey JJ, Reiss BL, Jerzak GVS. 2006. Development and Characterization of Stable Sediment-Free Anaerobic Bacterial Enrichment Cultures That Dechlorinate Aroclor 1260 **72**:2460–2470.

Bedard DL, Pohl E a, Bailey JJ, Murphy A. 2005. Characterization of the PCB substrate range of microbial dechlorination process LP. *Environ. Sci. Technol.* **39**:6831–8.

Bedard DL, Quensen JF. 1995. Microbial Reductive Dechlorination of Polychlorinated Biphenyls. In: Young, LY, Cerniglia, CE, editors. *Microb. Transform. Degrad. Toxic Org. Compd.* New York: Wiley-Liss, pp. 137–216.

Bedard DL, Ritalahti KM, Löffler FE. 2007. The Dehalococcoides population in sediment-free mixed cultures metabolically dechlorinates the commercial polychlorinated biphenyl mixture aroclor 1260. *Appl. Environ. Microbiol.* **73**:2513–21.

Bell G. 2005. The co-distribution of species in relation to the neutral theory of community ecology. *Ecology*.

Bellucci L., Frignani M, Raccanelli S, Carraro C. 2000. Polychlorinated Dibenzo-p-dioxins and Dibenzofurans in Surficial Sediments of the Venice Lagoon (Italy). *Mar. Pollut. Bull.* **40**:65–76.

Bellucci LG, Frignani M, Paolucci D, Ravanelli M. 2002. Distribution of heavy metals in sediments of the Venice Lagoon: the role of the industrial area. *Sci. Total Environ.* **295**:35–49.

Berkaw M, Sowers KR, May HD. 1996. Anaerobic ortho Dechlorination of Polychlorinated Biphenyls by Estuarine Sediments from Baltimore Harbor. *Appl. Environ. Microbiol.* **62**:2534–9.

Beurskens JEM, Mol GAJ, Barreveld HL, van Munster B, Winkels HJ. 1993. Geochronology of priority pollutants in a sedimentation area of the rhine river. *Environ. Toxicol. Chem.* **12**:1549–1566.

Bierman VJ. 1990. Equilibrium partitioning and biomagnification of organic chemicals in benthic animals. *Environ. Sci. Technol.* **24**:1407–1412.

Blaise C. 1991. Microbiotests in aquatic ecotoxicology: Characteristics, utility, and prospects. *Environ. Toxicol. Water Qual.* **6**:145–155.

Blazak WF, Marcum JB. 1975. Attempts to Induce Chromosomal Breakage in Chicken Embryos with Aroclor 1242. *Poult. Sci.* **54**:310–312.

Bodour AA, Miller-Maier RM. 1998. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. *J. Microbiol. Methods* **32**:273–280.

Bond DR, Lovley DR. 2003. Electricity Production by Geobacter sulfurreducens Attached to Electrodes. *Appl. Environ. Microbiol.* **69**:1548–1555.

Bond DR. 2010. Electrodes as electron acceptors, and the bacteria who love them. In: . *Geomicrobiol. Mol. Environ. Perspect.*, pp. 385–399.

Borgå K, Gabrielsen G, Skaare J. 2001. Biomagnification of organochlorines along a Barents Sea food chain. *Environ. Pollut.* **113**:187–198.

Borja J, Taleon DM, Auresenia J, Gallardo S. 2005. Polychlorinated biphenyls and their biodegradation. *Process Biochem.* **40**:1999–2013.

Bossert I, Häggblom M, Young L. 2003a. Microbial ecology of dehalogenation. In: Häggblom, MM, Bossert, ID, editors. *Dehalogenation*. Boston: Kluwer Academic Publishers.

Bossert ID, Häggblom MAXM, Young LY. 2003b. Microbial ecology of dehalogenation. In: Bossert, ID, Häggblom, MM, Young, LY, editors. *Dehalogenation Microb. Process. Environ. Appl.* Springer US, pp. 33–52.

Bowman JP, McCammon SA, Nichols DS, Skerratt JH, Rea SM, Nichols PD, McMeekin TA. 1997. Shewanella gelidimarina sp. nov. and Shewanella frigidimarina sp. nov., novel Antarctic species with the ability to produce eicosapentaenoic acid (20:5 omega 3) and grow anaerobically by dissimilatory Fe(III) reduction. *Int. J. Syst. Bacteriol.* **47**:1040–1047.

Boyle AW, Blake CK, Price WA, May HD. 1993. Effects of polychlorinated biphenyl congener concentration and sediment supplementation on rates of methanogenesis and 2,3,6-trichlorobiphenyl dechlorination in an anaerobic enrichment. *Appl. Environ. Microbiol.* **59**:3027–31.

Boyle AW, Silvin CJ, Hassett JP, Nakas JP, Tanenbaum SW. 1992. Bacterial PCB biodegradation. *Biodegradation* **3**:285–298.

Braathen M, Mortensen AS, Sandvik M, Skåre JU, Arukwe A. 2009. Estrogenic effects of selected hydroxy polychlorinated biphenyl congeners in primary culture of Atlantic Salmon (Salmo salar) hepatocytes. *Arch. Environ. Contam. Toxicol.* **56**:111–22.

Breivik K, Sweetman A, Pacyna JM, Jones KC. 2002a. Towards a global historical emission inventory for selected PCB congeners — a mass balance approach: 1. Global production and consumption. *Sci. Total …* **290**:181–198.

Breivik K, Sweetman A, Pacyna J, Jones K. 2002b. Towards a global historical emission inventory for selected PCB congeners — a mass balance approach: 2. Emissions. *Sci. Total Environ.* **290**:199–224.

Breivik K, Sweetman A, Pacyna JM, Jones KC. 2007. Towards a global historical emission inventory for selected PCB congeners--a mass balance approach: 3. An update. *Sci. Total Environ.* **377**:296–307.

Brown JF, Bedard DL, Brennan MJ, Carnahan JC, Feng H, Wagner RE. 1987. Polychlorinated biphenyl dechlorination in aquatic sediments. *Science* **236**:709–12.

Bryant MP, Wolin EA, Wolin MJ, Wolfe RS. 1967. Methanobacillus omelianskii, a symbiotic association of two species of bacteria. *Arch. Mikrobiol.* **59**:20–31.

Buchner D, Loganathan M, Behrens S, Laskov C, Schoendorf T, Haderlein S. 2011. Enhanced natural attenuation - determining the potential for reductive dechlorination in an oxic aquifer. *IAHS-AISH Publ.*:283–286.

Buckman AH, Brown SB, Small J, Muir DCG, Parrott J, Solomon KR, Fisk AT. 2007. Role of Temperature and Enzyme Induction in the Biotransformation of Polychlorinated Biphenyls and Bioformation of Hydroxylated Polychlorinated Biphenyls by Rainbow Trout (Oncorhynchus mykiss). *Environ. Sci. Technol.* **41**:3856–3863.

Bunge M, Adrian L, Kraus A, Opel M, Lorenz WG, Andreesen JR, Görisch H, Lechner U. 2003. Reductive dehalogenation of chlorinated dioxins by an anaerobic bacterium. *Nature* **421**:357–60.

Busset G, Sangely M, Montrejaud-Vignoles M, Thannberger L, Sablayrolles C. 2012. Life cycle assessment of polychlorinated biphenyl contaminated soil remediation processes. *Int. J. Life Cycle Assess.* **17**:325–336.

Capone D, Kiene R. 1988. Comparison of microbial dynamics in marine and freshwater sediments: Contrasts in anaerobic carbon catabolism. *Limnol. Oceanogr.* **33**:725–749.

Chang B V, Liu WG, Yuan SY. 2001. Microbial dechlorination of three PCB congeners in river sediment. *Chemosphere* **45**:849–56.

Chen IM, Chang FC, Hsu MF, Wang YS. 2001. Comparisons of PCBs dechlorination occurrences in various contaminated sediments. *Chemosphere* **43**:649–54.

Cho Y-M, Ghosh U, Kennedy AJ, Grossman A, Ray G, Tomaszewski JE, Smithenry DW, Bridges TS, Luthy RG. 2009. Field Application of Activated Carbon Amendment for In-Situ Stabilization of Polychlorinated Biphenyls in Marine Sediment. *Environ. Sci. Technol.* **43**:3815–3823.

Choi H, Al-Abed SR, Agarwal S. 2009. Catalytic Role of Palladium and Relative Reactivity of Substituted Chlorines during Adsorption and Treatment of PCBs on Reactive Activated Carbon. *Environ. Sci. Technol.* **43**:7510–7515.

Chun CL, Payne RB, Sowers KR, May HD. 2013. Electrical stimulation of microbial PCB degradation in sediment. *Water Res.* **47**:141–52.

Colores GM, Macur RE, Ward DM, Inskeep WP. 2000. Molecular analysis of surfactant-driven microbial population shifts in hydrocarbon contaminated soils. *Appl. Environ. Microbiol.* **66**:2959.

Commission of the European Communities. 2006a. Directive 2006/13/EC. Vol. 2000.

Commission of the European Communities. 2006b. Regulation 1881/2006. Vol. 2006.

Compeau GC, Bartha R. 1985. Sulfate-Reducing Bacteria: Principal Methylators of Mercury in Anoxic Estuarine Sediment. *Appl. Envir. Microbiol.* **50**:498–502.

Cooper DG, Goldenberg BG. 1987. Surface-active agents from two bacillus species. *Appl. Environ. Microbiol.* **53**:224–9.

De Corte S, Hennebel T, De Gusseme B, Verstraete W, Boon N. 2012. Bio-palladium: From metal recovery to catalytic applications. *Microb. Biotechnol.* **5**:5–17.

Council of the European Union. 1996. Directive 96/59/EC. Official Journal L 243, 24/09/1996.

Cutter L a, Watts JE, Sowers KR, May HD. 2001. Identification of a microorganism that links its growth to the reductive dechlorination of 2,3,5,6-chlorobiphenyl. *Environ. Microbiol.* **3**:699–709.

Daffonchio D, Ferrer M, Mapelli F, Cherif A, Lafraya A, Malkawi HI, Yakimov MM, Abdel-Fattah YR, Blaghen M, Golyshin PN, Kalogerakis N, Boon N, Magagnini M, Fava F. 2013. Bioremediation of Southern Mediterranean oil polluted sites comes of age. *N. Biotechnol.* **30**:743–8.

Daffonchio D, Mapelli F, Cherif A, Malkawi HI, Yakimov MM, Abdel-Fattah YR, Blaghen M, Golyshin PN, Ferrer M, Kalogerakis N, Boon N, Magagnini M, Fava F. 2012. ULIXES, unravelling and exploiting Mediterranean Sea microbial diversity and ecology for xenobiotics' and pollutants' clean up. *Rev. Environ. Sci. Bio/Technology* **11**:207–211.

Desai JD, Banat IM. 1997. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* **61**:47–64.

DiStefano TD, Baral R, Duran M, Speece RE. 2001. A Comparison of Complex Electron Donors for Anaerobic Dechlorination of PCE. *Bioremediat. J.* **5**:131–143.

Dobson S, van Esch GJ. 1993. 140: Polychlorinated biphenyls and terphenyls. In: . *Environ. Heal. Criteria* 2nd ed. Geneva, Switzerland: Geneva: World Health Organization.

Dolfing J. 2000. Energetics of anaerobic degradation pathways of chlorinated aliphatic compounds. *Microb. Ecol.* **40**:2–7.

Dolfing J, Harrison B. 1992. Gibbs free energy of formation of halogenated aromatic compounds and their potential role as electron acceptors in anaerobic environments. *Environ. Sci. Technol.* **26**:2213–2218.

Donato F, Magoni M, Bergonzi R, Scarcella C, Indelicato A, Carasi S, Apostoli P. 2006. Exposure to polychlorinated biphenyls in residents near a chemical factory in Italy: the food chain as main source of contamination. *Chemosphere* **64**:1562–72.

Duarte-Davidson R, Jones KC. 1994. Polychlorinated biphenyls (PCBs) in the UK population: estimated intake, exposure and body burden. *Sci. Total Environ.* **151**:131–152.

Dudkova V, Demnerova K, Bedard DL. 2009. Microbial reductive dechlorination of polychlorinated biphenyls. *Nov. Biotechnol.* **9**:119–123.

Dunaway-Mariano D, Babbitt P. 1994. On the origins and functions of the enzymes of the 4-chlorobenzoate to 4-hydroxybenzoate converting pathway. *Biodegradation*:259–276.

Dyksterhouse S, Gray J, Russel P, Cano Lara J, Staley JT. 1995. Cycloclasticus pugetii gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. *Int. …* **45**:116–123.

Dzantor EK, Chekol T, Vough LR. 2000. Feasibility of using forage grasses and legumes for phytoremediation of organic pollutants. *J. Environ. Sci. Heal. Part A* **35**:1645–1661.

Elder D., Villeneuve J. 1977. Polychlorinated biphenyls in the Mediterranean Sea. *Mar. Pollut. Bull.* **8**:19–22.

EPA. 1986. Broad scan analysis of the FY82 national human adipose tissue survey specimens. *Vol. III - Semi-volatile Org. Compd.* Washington DC.

Esser a, Gaum PM, Schettgen T, Kraus T, Gube M, Lang J. 2015. Effect of Occupational Polychlorinated Biphenyls Exposure on Quality-Adjusted Life Years Over Time at the HELPcB Surveillance Program. *J. Toxicol. Environ. Health. A* **78**:132–50.

European Parliament, Council of the European Union. 2000. Directive 2000/60/EC.

European Parliament, Council of the Union. 2008. Directive 2008/105/EC.

Exon JH, Talcott PA, Koller LD. 1985. Effect of lead, polychlorinated biphenyls, and cyclophosphamide on rat natural killer cells, interleukin 2, and antibody synthesis. *Fundam. Appl. Toxicol.* **5**:158–164.

Fagervold SK, May HD, Sowers KR. 2007. Microbial reductive dechlorination of aroclor 1260 in Baltimore harbor sediment microcosms is catalyzed by three phylotypes within the phylum Chloroflexi. *Appl. Environ. Microbiol.* **73**:3009–18.

Fagervold SK, Watts JEM, May HD, Sowers KR. 2005. Sequential reductive dechlorination of meta-chlorinated polychlorinated biphenyl congeners in sediment microcosms by two different Chloroflexi phylotypes. *Appl. Environ. Microbiol.* **71**:8085–90.

Fagervold SK, Watts JEM, May HD, Sowers KR. 2011. Effects of bioaugmentation on indigenous PCB dechlorinating activity in sediment microcosms. *Water Res.* **45**:3899–907.

Fava F, Agathos S. 2006. Uncertainty and research needs in the area of the biological restoration of contaminated sediments. In: Reible, D, Lanczos, T, editors. *Assess. Remediat. Contam. …*. Dordrecht: Springer Netherlands. Nato Science Series: IV: Earth and Environmental Sciences, Vol. 73,.

Fava F, Bertin L. 1999. Use of exogenous specialised bacteria in the biological detoxification of a dump site-polychlorobiphenyl-contaminated soil in slurry phase conditions. *Biotechnol. Bioeng.* **64**:240–9.

Fava F, Gioia D Di. 1998. Effects of Triton X-100 and Quillaya Saponin on the ex situ bioremediation of a chronically polychlorobiphenyl-contaminated soil. *Appl. Microbiol. Biotechnol.* **50**:623–630.

Fava F, Bertin L, Fedi S, Zannoni D. 2003a. Methyl-beta-cyclodextrin-enhanced solubilization and aerobic biodegradation of polychlorinated biphenyls in two aged-contaminated soils. *Biotechnol. Bioeng.* **81**:381–90.

Fava F, Gentilucci S, Zanaroli G. 2003b. Anaerobic biodegradation of weathered polychlorinated biphenyls (PCBs) in contaminated sediments of Porto Marghera (Venice Lagoon, Italy). *Chemosphere* **53**:101–9.

Fava F, Zanaroli G, Young LY. 2003c. Microbial reductive dechlorination of pre-existing PCBs and spiked 2,3,4,5,6-pentachlorobiphenyl in anaerobic slurries of a contaminated sediment of Venice Lagoon (Italy). *FEMS Microbiol. Ecol.* **44**:309–18.

Fennell DE, Nijenhuis I, Wilson SF, Zinder SH, Häggblom MM. 2004. Dehalococcoides ethenogenes Strain 195 Reductively Dechlorinates Diverse Chlorinated Aromatic Pollutants. *Environ. Sci. Technol.* **38**:2075–2081.

Fernández P, Grimalt JO. 2003. On the Global Distribution of Persistent Organic Pollutants. *Chim. Int. J. Chem.* **57**:514–521.

Ferry JG, Lessner DJ. 2008. Methanogenesis in marine sediments. *Ann. N. Y. Acad. Sci.* **1125**:147–157.

Fetzner S. 1998. Bacterial dehalogenation. *Appl. Microbiol. Biotechnol.* **50**:633–657.

Fetzner S, Lingens F. 1994. Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications. *Microbiol. Rev.* **58**:641–685.

Fiedler H. 1997. Polychlorinated Biphenyls (PCBs): Uses and Environmental Releases. In: . *Polychlorinated Biphenyls Uses Environ. Releases*. United Nations Environment Programme.

Fiedler H. 2001. Global and local disposition of PCBs. *PCBs Recent Adv. Environ. Toxicol. Heal. Eff.*:11–15.

Field J a, Sierra-Alvarez R. 2008. Microbial transformation and degradation of polychlorinated biphenyls. *Environ. Pollut.* **155**:1–12.

Frame GM. 1997a. A collaborative study of 209 PCB congeners and 6 Aroclors on 20 different HRGC columns 2. Semi-quantitative Aroclor congener distributions. *Fresenius. J. Anal. Chem.*

Frame GM. 1997b. A collaborative study of 209 PCB congeners and 6 Aroclors on 20 different HRGC columns 1. Retention and coelution database. *Fresenius. J. Anal. Chem.* **357**:701–713.

Franks AE, Nevin KP. 2010. Microbial fuel cells, a current review. *Energies* **3**:899–919.

Frederiksen M, Meyer HW, Ebbehøj NE, Gunnarsen L. 2012. Polychlorinated biphenyls (PCBs) in indoor air originating from sealants in contaminated and uncontaminated apartments within the same housing estate. *Chemosphere* **89**:473–9.

Freeman MD, Kohles SS. 2012. Plasma levels of polychlorinated biphenyls, non-Hodgkin lymphoma, and causation. *J. Environ. Public Health* **2012**:258981.

Friedman ES, Rosenbaum MA, Lee AW, Lipson DA, Land BR, Angenent LT. 2012. A cost-effective and field-ready potentiostat that poises subsurface electrodes to monitor bacterial respiration. *Biosens. Bioelectron.* **32**:309–13.

Frignani M, Bellucci LG, Carraro C, Favotto M. 2004. Accumulation of polychlorinated biphenyls in sediments of the Venice Lagoon and the industrial area of Porto Marghera. *Chemosphere* **54**:1563–72.

Frignani M, Bellucci L., Carraro C, Raccanelli S. 2001a. Polychlorinated biphenyls in sediments of the Venice Lagoon. *Chemosphere* **43**:567–575.

Frignani M, Bellucci LG, Carraro C, Favotto M. 2001b. Accumulation of Polychlorinated Dibenzo-p-Dioxins and Dibenzofurans in Sediments of the Venice Lagoon and the Industrial Area of Porto Marghera. *Mar. Pollut. Bull.* **42**:544–553.

Frignani M, Bellucci LG, Favotto M, Albertazzi S. 2005. Pollution historical trends as recorded by sediments at selected sites of the Venice Lagoon. *Environ. Int.* **31**:1011–22.

Furukawa K, Fujihara H. 2008. Microbial degradation of polychlorinated biphenyls: biochemical and molecular features. *J. Biosci. Bioeng.* **105**:433–49.

Gantzer CJ, Wackett LP. 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ. Sci. Technol.* **25**:715–722.

Gertler C, Gerdts G, Timmis KN, Golyshin PN. 2009. Microbial consortia in mesocosm bioremediation trial using oil sorbents, slow-release fertilizer and bioaugmentation. *FEMS Microbiol. Ecol.* **69**:288–300.

Gobas F, Morrison H. 2000. Bioconcentration and Biomagnification in the Aquatic Environment. In: Boethling, RS, editor. *Handb. Prop. Estim. …* 1st ed. Boca Raton: CRC Press LLC, pp. 189–202.

Golyshin PM, Fredrickson HL, Giuliano L, Rothmel R, Timmis KN, Yakimov MM. 1999. Effect of novel biosurfactants on biodegradation of polychlorinated biphenyls by pure and mixed bacterial cultures. *New Microbiol.* **22**:257–67.

Gomes HI, Dias-Ferreira C, Ribeiro AB. 2013. Overview of in situ and ex situ remediation technologies for PCB-contaminated soils and sediments and obstacles for full-scale application. *Sci. Total Environ.* **445-446**:237–60.

Grandjean P, Landrigan PJ. 2006. Developmental neurotoxicity of industrial chemicals. *Lancet* **368**:2167–78.

Green S, Sauro FM, Friedman L. 1975. Lack of dominant lethality in rats treated with polychlorinated biphenyls (Aroclors 1242 and 1254). *Food Cosmet. Toxicol.* **13**:507–510.

Gregory KB, Bond DR, Lovley DR. 2004. Graphite electrodes as electron donors for anaerobic respiration. *Environ. Microbiol.* **6**:596–604.

Gribble GW. 1994. The natural production of chlorinated compounds. *Environ. Sci. Technol.* **28**:310A–319A.

Grieger KD, Fjordbøge A, Hartmann NB, Eriksson E, Bjerg PL, Baun A. 2010. Environmental benefits and risks of zero-valent iron nanoparticles (nZVI) for in situ remediation: risk mitigation or trade-off? *J. Contam. Hydrol.* **118**:165–83.

Haba E, Espuny MJ, Busquets M, Manresa a. 2000. Screening and production of rhamnolipids by Pseudomonas aeruginosa 47T2 NCIB 40044 from waste frying oils. *J. Appl. Microbiol.* **88**:379–387.

Hagenmaier H, She J, Lindig C. 1992. Persistence of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans in contaminated soil at Maulach and Rastatt in Southwest Germany. *Chemosphere* **25**:1449–1456.

Harker M, Hirschberg J, Oren a. 1998. Paracoccus marcusii sp. nov., an orange gram-negative coccus. *Int. J. Syst. Bacteriol.* **48 Pt 2**:543–548.

Harms H, Schlosser D, Wick LY. 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. *Nat. Rev. Microbiol.* **9**:177–92.

Hattula ML. 1985. Mutagenicity of PCBs and Their Pyrosynthetic Derivatives in Cell-Mediated Assay. *Environ. Health Perspect.* **60**:255.

Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time quantitative PCR. *Genome Res.* **6**:986–994.

Hendrickson ER, Payne JA, Young RM, Starr MG, Perry MP, Fahnestock S, Ellis DE, Ebersole RC. 2002. Molecular Analysis of Dehalococcoides 16S Ribosomal DNA from Chloroethene-Contaminated Sites throughout North America and Europe. *Appl. Environ. Microbiol.* **68**:485–495.

Hennebel T, Simoen H, Windt W De. 2009. Biocatalytic dechlorination of trichloroethylene with bio-palladium in a pilot-scale membrane reactor. *Biotechnol. Bioeng.* **102**:995–1002.

Hermanson MH, Johnson GW. 2007. Polychlorinated biphenyls in tree bark near a former manufacturing plant in Anniston, Alabama. *Chemosphere* **68**:191–8.

Hinck JE, Norstrom RJ, Orazio CE, Schmitt CJ, Tillitt DE. 2009. Persistence of organochlorine chemical residues in fish from the Tombigbee River (Alabama, USA): Continuing risk to wildlife from a former DDT manufacturing facility. *Environ. Pollut.* **157**:582–91.

Hiraishi A. 2008. Biodiversity of Dehalorespiring Bacteria with Special Emphasis on Polychlorinated Biphenyl/Dioxin Dechlorinators. *Microbes Environ.* **23**:1–12.

Ho CH, Liu SM. 2011. Effect of coplanar PCB concentration on dechlorinating microbial communities and dechlorination in estuarine sediments. *Chemosphere* **82**:48–55.

Holliger C, Regeard C, Diekert G. 2003. Dehalogenation by Anaerobic Bacteria. In: Häggblom, M, Bossert, I, editors. *Dehalogenation SE - 5*. Springer US, pp. 115–157.

Holliger C, Schraa G. 1994. Physiological meaning and potential for application of reductive dechlorination by anaerobic bacteria. *FEMS Microbiol. Rev.* **15**:297–305.

Hölscher T, Krajmalnik-Brown R, Ritalahti KM, Von Wintzingerode F, Görisch H, Löffler FE, Adrian L. 2004. Multiple nonidentical reductive-dehalogenase-homologous genes are common in Dehalococcoides. *Appl. Environ. Microbiol.* **70**:5290–7.

Hong PKA, Nakra S, Jimmy Kao CM, Hayes DF. 2008. Pressure-assisted ozonation of PCB and PAH contaminated sediments. *Chemosphere* **72**:1757–64.

Hosseinkhani B, Hennebel T, Van Nevel S, Verschuere S, Yakimov MM, Cappello S, Blaghen M, Boon N. 2014. Biogenic nanopalladium based remediation of chlorinated hydrocarbons in marine environments. *Environ. Sci. Technol.* **48**:550–557.

Hosseinkhani B, Nuzzo A, Zanaroli G, Fava F, Boon N. 2015. Assessment of catalytic dechlorination activity of suspended and immobilized bio-Pd NPs in different marine conditions. *Appl. Catal. B Environ.* **168-169**:62–67.

Høyer AP, Grandjean P, Jørgensen T, Brock JW, Hartvig HB. 1998. Organochlorine exposure and risk of breast cancer. *Lancet* **352**:1816–20.

Huang S, Gibson GG. 1991. Differential induction of cytochromes P450 and cytochrome P450-dependent arachidonic acid metabolism by 3,4,5,3′,4′-pentachlorobiphenyl in the rat and the guinea pig. *Toxicol. Appl. Pharmacol.* **108**:86–95.

Hungate RE. 1969. Chapter IV A Roll Tube Method for Cultivation of Strict Anaerobes. *Methods Microbiol.* **3**:117–132.

Ibbinia J, Santharamb S, Davis L, Erickson L. 2010. Laboratory and field scale bioremediation of tetrachloroethene (PCE) contaminated groundwater. *JJMIE* **4**:35–44.

Jablonski P. 1992. Reductive dechlorination of trichloroethylene by the CO-reduced CO dehydrogenase enzyme complex from Methanosarcina thermophila. *FEMS Microbiol. Lett.* **96**:55–60.

James MO, Kleinow KM. 2014. Seasonal influences on PCB retention and biotransformation in fish. *Environ. Sci. Pollut. Res. Int.* **21**:6324–33.

James RC, Busch H, Tamburro CH, Roberts SM, Schell JD, Harbison RD. 1993. Polychlorinated Biphenyl Exposure and Human Disease. *J. Occup. Environ. Med.* **35**.

Janssen DB, Pries F, van der Ploeg JR. 1994. Genetics and biochemistry of dehalogenating enzymes. *Annu. Rev. Microbiol.* **48**:163–91.

Jensen. 1966. Report of a new chemical hazard. *New Sci.* **32:612**:445.

Jensen S, Johnels AG, Olsson M, Otterlind G. 1969. DDT and PCB in Marine Animals from Swedish Waters. *Nature* **224**:247–250.

Jones WJ, Paynter MJB, Gupta R. 1983. Characterization of Methanococcus maripaludis sp. nov., a new methanogen isolated from salt marsh sediment. *Arch. Microbiol.* **135**:91–97.

Jørgensen BB. 1977. Bacterial sulfate reduction within reduced microniches of oxidized marine sediments. *Mar. Biol.* **41**:7–17.

Kalinovskaya NI, Ivanova EP, Alexeeva Y V, Gorshkova NM, Kuznetsova T a, Dmitrenok AS, Nicolau D V. 2004. Low-molecular-weight, biologically active compounds from marine Pseudoalteromonas species. *Curr. Microbiol.* **48**:441–446.

Kassenga GR, Pardue JH. 2006. Effect of competitive terminal electron acceptor processes on dechlorination of cis-1,2-dichloroethene and 1,2-dichloroethane in constructed wetland soils. *FEMS Microbiol. Ecol.* **57**:311–23.

Kelly BC, Gobas FAPC, McLachlan MS. 2004. Intestinal absorption and biomagnification of organic contaminants in fish, wildlife, and humans. *Environ. Toxicol. Chem.* **23**:2324.

Kerkvliet NI, Baecher-Steppan L, Smith BB, Youngberg JA, Henderson MC, Buhler DR. 1990. Role of the Ah locus in suppression of cytotoxic T lymphocyte activity by halogenated aromatic hydrocarbons (PCBs and TCDD): structure-activity relationships and effects in C57Bl/6 mice congenic at the Ah locus. *Fundam. Appl. Toxicol.* **14**:532–41.

Khan FI, Husain T, Hejazi R. 2004. An overview and analysis of site remediation technologies. *J. Environ. Manage.* **71**:95–122.

Kim J, Rhee GY. 2001. Reductive dechlorination of polychlorinated biphenyls as affected by sediment characteristics. *Chemosphere* **44**:1413–20.

Kim J, Rhee G-Y. 1999. Reductive dechlorination of polychlorinated biphenyls: Interactions of dechlorinating microorganisms with methanogens and sulfate reducers. *Environ. Toxicol. Chem.* **18**:2696–2702.

Kittelmann S, Friedrich MW. 2008. Novel uncultured Chloroflexi dechlorinate perchloroethene to trans-dichloroethene in tidal flat sediments. *Environ. Microbiol.* **10**:1557–70.

Kjellerup B V, Sun X, Ghosh U, May HD, Sowers KR. 2008. Site-specific microbial communities in three PCB-impacted sediments are associated with different in situ dechlorinating activities. *Environ. Microbiol.* **10**:1296–309.

Klasson KT, Barton JW, Evans BS, Reeves ME. 1996. Reductive microbial dechlorination of indigenous polychlorinated biphenyls in soil using a sediment-free inoculum. *Biotechnol. Prog.* **12**:310–315.

Koenigsberg SS, Lapus KA, Sandefur CA. 2000. Time-release electron donor technology for accelerated biological reductive dechlorination. In: . *Contam. Soil 2000 Proc. Seventh Int. FZK/TNO Conf. Contam. Soil, 18-22 Sept. 2000, Leipzig, Ger.* Thomas Telford, Vol. 2, p. 968.

Komancová M, Jurčová I, Kochánková L, Burkhard J. 2003. Metabolic pathways of polychlorinated biphenyls degradation by Pseudomonas sp. 2. *Chemosphere* **50**:537–543.

Krumins V, Park J-W, Son E-K, Rodenburg L a, Kerkhof LJ, Häggblom MM, Fennell DE. 2009. PCB dechlorination enhancement in Anacostia River sediment microcosms. *Water Res.* **43**:4549–58.

Kuratsune M, Yoshimura T, Matsuzaka J, Yamaguchi A. 1972. Epidemiologic study on Yusho, a Poisoning Caused by Ingestion of Rice Oil Contaminated with a Commercial Brand of Polychlorinated Biphenyls. *Environ. Health Perspect.* **1**:119–128.

Laanbroek HJ, Veldkamp H, Postgate JR, Lynch JM, Roux NL. 1982. Microbial Interactions in Sediment Communities [and Discussion]. *Philos. Trans. R. Soc. B Biol. Sci.* **297**:533–550.

Lake JL, Pruell RJ, Osterman FA. 1992. An examination of dechlorination processes and pathways in New Bedford Harbor sediments. *Mar. Environ. Res.* **33**:31–47.

Lamon L, von Waldow H, MacLeod M, Scheringer M, Marcomini A, Hungerbühler K. 2009. Modeling the Global Levels and Distribution of Polychlorinated Biphenyls in Air under a Climate Change Scenario. *Environ. Sci. Technol.* **43**:5818–5824.

Lamoureux E, Brownawell B. 1999. Chemical and biological availability of sediment-sorbed hydrophobic organic contaminants. *Environ. Toxicol. …* **18**:1733–1741.

Lane DJ. 1991. 16S/23S rRNA sequencing. In: Goodfellow, M, Stackebrandt, E, editors. *Dev. Appl. Nucleic Acid Probes*. Chichester: John Wiley & Sons, pp. 115–175.

Lanthier M, Villemur R, Lépine F, Bisaillon J-G, Beaudet R. 2001. Geographic distribution of Desulfitobacterium frappieri PCP-1 ad Desulfitobacterium spp. in soils from the province of Quebec, Canada. *FEMS Microbiol. …* **36**.

LaRoe SL, Fricker AD, Bedard DL. 2014. Dehalococcoides mccartyi strain JNA in pure culture extensively dechlorinates Aroclor 1260 according to polychlorinated biphenyl (PCB) dechlorination Process N. *Environ. Sci. Technol.* **48**:9187–96.

Lauby-Secretan B, Loomis D, Grosse Y, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, Guha N, Baan R, Mattock H, Straif K. 2013. Carcinogenicity of polychlorinated biphenyls and polybrominated biphenyls. *Lancet. Oncol.* **14**:287–8.

Leonards PEG, Broekhuizen S, De Voogt P, Van Straalen NM, Brinkman UAT, Cofino WP, Van Hattum B. 1998. Studies of bioaccumulation and biotransformation of PCBs in mustelids based on concentration and congener patterns in predators and preys. *Arch. Environ. Contam. Toxicol.* **35**:654–665.

Leonards PEG, van Hattum B, Leslie H. 2008. Assessing the risks of persistent organic pollutants to top predators: a review of approaches. *Integr. Environ. Assess. Manag.* **4**:386–98.

Liu F, Rotaru A-E, Shrestha PM, Malvankar NS, Nevin KP, Lovley DR. 2014. Magnetite compensates for the lack of a pilin-associated c-type cytochrome in extracellular electron exchange. *Environ. Microbiol.*

Liu L, Tindall J a., Friedel MJ. 2007. Biodegradation of PAHs and PCBs in Soils and Sludges. *Water. Air. Soil Pollut.* **181**:281–296.

Liu W, Marsh T, Cheng H, Forney L. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Envir. Microbiol.* **63**:4516–4522.

Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards E a, Konstantinidis KT, Müller J a, Fullerton H, Zinder SH, Spormann AM. 2013. Dehalococcoides mccartyi gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation,

belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and famil. *Int. J. Syst. Evol. Microbiol.* **63**:625–35.

Logan BE. 2009. Exoelectrogenic bacteria that power microbial fuel cells. *Nat. Rev. Microbiol.* **7**:375–381.

Logan MSP, Newman LM, Schanke CA, Wacket LP. 1993. Cosubstrate effects in reductive dehalogenation byPseudomonas putida G786 expressing cytochrome P-450CAM. *Biodegradation* **4**:39–50.

Lohner ST, Deutzmann JS, Logan BE, Leigh J, Spormann AM. 2014. Hydrogenase-independent uptake and metabolism of electrons by the archaeon Methanococcus maripaludis. *ISME J.* **8**:1673–81.

Long Y-Y, Zhang C, Du Y, Tao X-Q, Shen D-S. 2014. Enhanced reductive dechlorination of polychlorinated biphenyl-contaminated soil by in-vessel anaerobic composting with zero-valent iron. *Environ. Sci. Pollut. Res. Int.* **21**:4783–92.

Loomis D, Browning SR, Schenck a P, Gregory E, Savitz D a. 1997. Cancer mortality among electric utility workers exposed to polychlorinated biphenyls. *Occup. Environ. Med.* **54**:720–728.

López-López A, Pujalte MJ, Benlloch S, Mata-Roig M, Rosselló-Mora R, Garay E, Rodríguez-Valera F. 2002. Thalassospira lucentensis gen. nov., sp. nov., a new marine member of the alpha-Proteobacteria. *Int. J. Syst. Evol. Microbiol.* **52**:1277–1283.

Lovley DR, Giovannoni SJ, White DC, Champine JE, Phillips EJP, Gorby YA, Goodwin S. 1993. Geobacter metallireducens gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch. Microbiol.* **159**:336–344.

Lovley DR. 2006. Bug juice: harvesting electricity with microorganisms. *Nat. Rev. Microbiol.* **4**:497–508.

Lovley DR. 2012. Electromicrobiology. *Annu. Rev. Microbiol.* **66**:391–409.

Lovley DR, Dwyer DF, Klug MJ. 1982. Kinetic analysis of competition between sulfate reducters and methanogens for hydrogen in sediments. *Appl. Environ. Microbiol.* **43**:1373–1379.

Lovley DR. 2011a. Live wires: direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. *Energy Environ. Sci.*

Lovley DR. 2011b. Powering microbes with electricity: Direct electron transfer from electrodes to microbes. *Environ. Microbiol. Rep.* **3**:27–35.

Ludewig G, Robertson LW. 2013. Polychlorinated biphenyls (PCBs) as initiating agents in hepatocellular carcinoma. *Cancer Lett.* **334**:46–55.

Mackiewicz M, Wiegel J. 1998. Comparison of Energy and Growth Yields forDesulfitobacterium dehalogenans during Utilization of Chlorophenol and Various Traditional Electron Acceptors. *Appl. Environ. Microbiol.* **64**:352–355.

Mackova M, Prouzova P, Stursa P, Ryslava E, Uhlik O, Beranova K, Rezek J, Kurzawova V, Demnerova K, Macek T. 2009. Phyto/rhizoremediation studies using long-term PCB-contaminated soil. *Environ. Sci. Pollut. Res. Int.* **16**:817–29.

Magar VS. 2001. Natural recovery of contaminated sediments. *J. Environ. Eng.*:473–474.

Magar VS, Brenner RC, Johnson GW, Quensen JF. 2005. Long-term recovery of PCB-contaminated sediments at the Lake Hartwell superfund site: PCB dechlorination. 2. Rates and extent. *Environ. Sci. Technol.* **39**:3548–54.

Magar V, Chadwick D, Bridges T, Fuchsman P, Conder J, Dekker T, Steevens J, Gustavson K, Mills M. 2009. Monitored Natural Recovery at Contaminated Sediment Sites. *... Nat. Recover. …*.

Malisch R, Kotz A. 2014. Dioxins and PCBs in feed and food--review from European perspective. *Sci. Total Environ.* **491-492**:2–10.

Malvankar NS, Lovley DR. 2012. Microbial nanowires: A new paradigm for biological electron transfer and bioelectronics. *ChemSusChem* **5**:1039–1046.

Manciocco A, Calamandrei G, Alleva E. 2014. Global warming and environmental contaminants in aquatic organisms: the need of the etho-toxicology approach. *Chemosphere* **100**:1–7.

Marsili E, Baron DB, Shikhare ID, Coursolle D, Gralnick J a, Bond DR. 2008. Shewanella secretes flavins that mediate extracellular electron transfer. *Proc. Natl. Acad. Sci. U. S. A.* **105**:3968–73.

Marzorati M, Negroni A, Fava F, Verstraete W, Boon N. 2013. Application of a molecular based approach for the early detection of short term 3-chloroaniline shock loads on activated sludge bacterial community and functionality. *N. Biotechnol.* **30**:763–71.

Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W. 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environ. Microbiol.* **10**:1571–81.

Master ER, Lai VWM, Kuipers B, Cullen WR, Mohn WW. 2002. Sequential anaerobic-aerobic treatment of soil contaminated with weathered Aroclor 1260. *Environ. Sci. Technol.* **36**:100–3.

May HD, Miller GS, Kjellerup B V, Sowers KR. 2008. Dehalorespiration with polychlorinated biphenyls by an anaerobic ultramicrobacterium. *Appl. Environ. Microbiol.* **74**:2089–94.

Maymo-Gatell X, Chien Y, Gossett J, Zinder S. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science (80-.).* **276**:1568–1571.

McInerney MJ, Bryant MP. 1981. Anaerobic degradation of lactate by syntrophic associations of Methanosarcina barkeri and Desulfovibrio species and effect of H2 on acetate degradation. *Appl. Environ. Microbiol.* **41**:346–354.

McKinney JD, Waller CL. 1994. Polychlorinated biphenyls as hormonally active structural analogues. *Environ. Health Perspect.* **102**:290–7.

Mishra S, Singh SN. 2012. Microbial degradation of n-hexadecane in mineral salt medium as mediated by degradative enzymes. *Bioresour. Technol.* **111**:148–154.

Mohn W, Tiedje J. 1991. Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in Desulfomonile tiedjei. *Arch. Microbiol.* **157**:1–6.

Moret I, Gambaro a, Piazza R, Ferrari S, Manodori L. 2005. Determination of polychlorobiphenyl congeners (PCBs) in the surface water of the Venice lagoon. *Mar. Pollut. Bull.* **50**:167–74.

Moret I, Piazza R, Benedetti M, Gambaro A, Barbante C, Cescon P. 2001. Determination of polychlorobiphenyls in Venice Lagoon sediments. *Chemosphere* **43**:559–565.

Morris PJ, Mohn WW, Quensen JF, Tiedje JM, Boyd S a. 1992. Establishment of polychlorinated biphenyl-degrading enrichment culture with predominantly meta dechlorination. *Appl. Environ. Microbiol.* **58**:3088–94.

Mouhamadou B, Faure M, Sage L, Marçais J, Souard F, Geremia RA. 2013. Potential of autochthonous fungal strains isolated from contaminated soils for degradation of polychlorinated biphenyls. *Fungal Biol.* **117**:268–74.

Mueller JG, Lantz SE, Blattmann BO, Chapman PJ. 1991. Bench-scale evaluation of alternative biological treatment processes for the remediation of pentachlorophenol- and creosote-contaminated materials. Solid-phase bioremediation. *Environ. Sci. Technol.* **25**:1045–1055.

Müller G, Nkusi G, Schöler H. 1996. Natural organohalogens in sediments. *J. für Prakt. Chemie/ …* **338**:23–29.

Mulligan C., Yong R., Gibbs B. 2001. Surfactant-enhanced remediation of contaminated soil: a review. *Eng. Geol.* **60**:371–380.

Mulr DCG, Norstrom RJ, Simon M. 1988. Organochlorine Contaminants in Arctic Marine Food Chains : Accumulation of Specific Polychlorinated Biphenyls and Chlordane-Related Compounds **22**:1071–1079.

Murena F, Gioia F. 2009. Solvent extraction of chlorinated compounds from soils and hydrodechlorination of the extract phase. *J. Hazard. Mater.* **162**:661–7.

Muyzer G, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Envir. Microbiol.* **59**:695–700.

Nadeau RJ, Davis RA. 1976. Polychlorinated biphenyls in the Hudson river (Hudson Falls-Fort Edward, New York state). *Bull. Environ. Contam. Toxicol.* **16**:436–444.

Nagarajan H, Embree M, Rotaru A-E, Shrestha PM, Feist AM, Palsson BØ, Lovley DR, Zengler K. 2013. Characterization and modelling of interspecies electron transfer mechanisms and microbial community dynamics of a syntrophic association. *Nat. Commun.* **4**:2809.

Natarajan MR, Nye J, Wu W-M, Wang H, Jain MK. 1997. Reductive Dechlorination of PCB Contaminated Raisin River Sediments by Anaerobic Microbial Granules. *Biotechnol. Bioeng.* **55**:182–190.

Natarajan M, Wu W, Nye J. 1996. Dechlorination of polychlorinated biphenyl congeners by an anaerobic microbial consortium. *Appl. Microbiol. …*:673–677.

Natarajan M, Wu W, Wang H, Bhatnagar L, Jain M. 1998. Dechlorination of spiked PCBs in lake sediment by anaerobic microbial granules. *Water Res.* **32**:3013–3020.

Nies L, Vogel T. 1990. Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediments. *Appl. Environ. Microbiol.* **56**:2612–2617.

Nilsson B, Ramel C. 1974. Genetic tests on Drosophila melanogaster with polychlorinated biphenyls (PCB). *Hereditas* **77**:319–322.

Nollet H, Verstraete W. 2003. Development of microbial granules for PCB dechlorination. *Commun. Agric. Appl. Biol. Sci.* **68**:185–189.

Oberg G. 2002. The natural chlorine cycle--fitting the scattered pieces. *Appl. Microbiol. Biotechnol.* **58**:565–81.

Occulti F, Roda GC, Berselli S, Fava F. 2008. Sustainable decontamination of an actual-site aged PCB-polluted soil through a biosurfactant-based washing followed by a photocatalytic treatment. *Biotechnol. Bioeng.* **99**:1525–34.

Ockenden W a., Prest HF, Thomas GO, Sweetman A, Jones KC. 1998. Passive Air Sampling of PCBs: Field Calculation of Atmospheric Sampling Rates by Triolein-Containing Semipermeable Membrane Devices. *Environ. Sci. Technol.* **32**:1538–1543.

Offenberg JH, Baker JE. 1999. Influence of Baltimore's Urban Atmosphere on Organic Contaminants over the Northern Chesapeake Bay. *J. Air Waste Manage. Assoc.* **49**:959–965.

Øfjord GD, Puhakka JA, Ferguson JF. 1994. Reductive dechlorination of aroclor 1254 by marine sediment cultures. *Environ. Sci. Technol.* **28**:2286–94.

Oh K-H, Ostrofsky EB, Cho Y-C. 2008. Molecular characterization of polychlorinated biphenyl-dechlorinating populations in contaminated sediments. *J. Microbiol.* **46**:165–73.

Ohtsubo Y, Kudo T, Tsuda M, Nagata Y. 2004. Strategies for bioremediation of polychlorinated biphenyls. *Appl. Microbiol. Biotechnol.* **65**:250–8.

Okey AB. 1983. The Ah Receptor: A Specific Site for Action of Chlorinated Dioxins? In: Tucker, RE, Young, AL, Gray, AP, editors. *Hum. Environ. Risks Chlorinated Dioxins Relat. Compd.* Boston, MA: Springer US, pp. 423–440.

Oliver BG, Niimi AJ. 1988. Trophodynamic analysis of polychlorinated biphenyl congeners and other chlorinated hydrocarbons in the Lake Ontario ecosystem. *Environ. Sci. Technol.* **22**:388–397.

Onozuka D, Yoshimura T, Kaneko S, Furue M. 2009. Mortality after exposure to polychlorinated biphenyls and polychlorinated dibenzofurans: a 40-year follow-up study of Yusho patients. *Am. J. Epidemiol.* **169**:86–95.

Pakdeesusuk U, Lee CM, Coates JT, Freedman DL. 2005. Assessment of natural attenuation via in situ reductive dechlorination of polychlorinated biphenyls in sediments of the Twelve Mile Creek arm of Lake Hartwell, SC. *Environ. Sci. Technol.* **39**:945–52.

Park J-W, Krumins V, Kjellerup B V, Fennell DE, Rodenburg L a, Sowers KR, Kerkhof LJ, Häggblom MM. 2011. The effect of co-substrate activation on indigenous and bioaugmented PCB dechlorinating bacterial communities in sediment microcosms. *Appl. Microbiol. Biotechnol.* **89**:2005–17.

Park SS, Jaffé PR. 1996. Development of a sediment redox potential model for the assessment of postdepositional metal mobility. *Ecol. Modell.* **91**:169–181.

Van de Pas B a., Smidt H, Hagen WR, van der Oost J, Schraa G, Stams a. JM, de Vos WM. 1999. Purification and Molecular Characterization ofortho-Chlorophenol Reductive Dehalogenase, a Key Enzyme of Halorespiration in Desulfitobacterium dehalogenans. *J. Biol. Chem.* **274**:20287–20292.

Payne RB, May HD, Sowers KR. 2011. Enhanced reductive dechlorination of polychlorinated biphenyl impacted sediment by bioaugmentation with a dehalorespiring bacterium. *Environ. Sci. Technol.* **45**:8772–9.

Payne RB, Fagervold SK, May HD, Sowers KR. 2013. Remediation of polychlorinated biphenyl impacted sediment by concurrent bioaugmentation with anaerobic halorespiring and aerobic degrading bacteria. *Environ. Sci. Technol.* **47**:3807–3815.

Peters RW. 2001. Hazardous wastes: Sources, pathways, receptors by Richard J. Watts John Wiley & Sons New York, NY (1998) 784 pages hardcover ISBN 0-471-00238-0 U.S. list price: $98.95. *Environ. Prog.* **20**:D11–D11.

Picardal FW, Arnold RG, Couch H, Little AM, Smith ME. 1993. Involvement of cytochromes in the anaerobic biotransformation of tetrachloromethane by Shewanella putrefaciens 200. *Appl. …* **59**:3763–3770.

Pieper DH. 2005. Aerobic degradation of polychlorinated biphenyls. *Appl. Microbiol. Biotechnol.* **67**:170–91.

Poster DL, Chaychian M, Neta P, Huie RE, Silverman J, Al-Sheikhly M. 2003. Degradation of PCBs in a Marine Sediment Treated with Ionizing and UV Radiation. *Environ. Sci. Technol.* **37**:3808–3815.

Prince MM, Ruder AM, Hein MJ, Waters M a., Whelan E a., Nilsen N, Ward EM, Schnorr TM, Laber P a., Davis-King KE. 2006. Mortality and Exposure Response among 14,458 Electrical Capacitor Manufacturing Workers Exposed to Polychlorinated Biphenyls (PCBs). *Environ. Health Perspect.* **114**:1508–1514.

Pulliam Holoman TR, Elberson MA, Cutter LA, May HD, Sowers KR, Holoman T. 1998. Characterization of a Defined 2,3,5,6-Tetrachlorobiphenyl-ortho-Dechlorinating Microbial Community by Comparative Sequence Analysis of Genes Coding for 16S rRNA. *Appl. Envir. Microbiol.* **64**:3359–3367.

Purkey HE, Palaninathan SK, Kent KC, Smith C, Safe SH, Sacchettini JC, Kelly JW. 2004. Hydroxylated polychlorinated biphenyls selectively bind transthyretin in blood and inhibit amyloidogenesis: rationalizing rodent PCB toxicity. *Chem. Biol.* **11**:1719–28.

Qaker JE, Eisenreich SJ, Johnson TC, Halfman BM. 1985. Chlorinated hydrocarbon cycling in the benthic nepheloid layer of Lake Superior. *Environ. Sci. Technol.* **19**:854–61.

Quensen JF, Tiedje JM, Boyd SA. 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. *Science* **242**:752–4.

Quensen JF, Boyd S a, Tiedje JM. 1990. Dechlorination of Four Commercial Polychlorinated Biphenyl Mixtures (Aroclors) by Anaerobic Microorganisms from Sediments. *Appl. Environ. Microbiol.* **56**:2360–2369.

Quensen JF, Mousa MA, Boyd SA, Sanderson JT, Froese KL, Giesy JP. 1998. Reduction of aryl hydrocarbon receptor-mediated activity of polychlorinated biphenyl mixtures due to anaerobic microbial dechlorination. *Environ. Toxicol. Chem.* **17**:806–813.

Rabaey K, Rodríguez J, Blackall LL, Keller J, Gross P, Batstone D, Verstraete W, Nealson KH. 2007. Microbial ecology meets electrochemistry: electricity-driven and driving communities. *ISME J.* **1**:9–18.

Rabaey K, Rozendal R a. 2010. Microbial electrosynthesis - revisiting the electrical route for microbial production. *Nat. Rev. Microbiol.* **8**:706–16.

Rakowska MI, Kupryianchyk D, Harmsen J, Grotenhuis T, Koelmans a a. 2012. In situ remediation of contaminated sediments using carbonaceous materials. *Environ. Toxicol. Chem.* **31**:693–704.

Reddy KR, Adams JA, Richardson C. 1999. Potential Technologies for Remediation of Brownfields. *Pract. Period. Hazardous, Toxic, Radioact. Waste Manag.* **3**:61–68.

Risebrough RW, Walker W, Schmidt TT, De Lappe BW, Connors CW. 1976. Transfer of chlorinated biphenyls to Antarctica. *Nature* **264**:738–739.

Ritalahti KM, Amos BK, Sung Y, Wu Q, Koenigsberg SS, Löffler FE. 2006. Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple Dehalococcoides strains. *Appl. Environ. Microbiol.* **72**:2765–74.

Robinson J a., Tiedje JM. 1984. Competition between sulfate-reducing and methanogenic bacteria for H2 under resting and growing conditions. *Arch. Microbiol.* **137**:26–32.

Robles-González I V, Fava F, Poggi-Varaldo HM. 2008. A review on slurry bioreactors for bioremediation of soils and sediments. *Microb. Cell Fact.* **7**:5.

Rodrigues J, Kachel C. 2006. Degradation of Aroclor 1242 dechlorination products in sediments by Burkholderia xenovorans LB400 (ohb) and Rhodococcus sp. strain RHA1 (fcb). *Appl. ...* **72**:2476–2482.

Romanenko L a., Zhukova N V., Rohde M, Lysenko AM, Mikhailov V V., Stackebrandt E. 2003. Pseudoalteromonas agarivorans sp. nov., a novel marine agarolytic bacterium. *Int. J. Syst. Evol. Microbiol.* **53**:125–131.

Rosenbaum M a, Franks AE. 2014. Microbial catalysis in bioelectrochemical technologies: status quo, challenges and perspectives. *Appl. Microbiol. Biotechnol.* **98**:509–18.

Rosenbaum M, Aulenta F, Villano M, Angenent LT. 2011. Cathodes as electron donors for microbial metabolism: which extracellular electron transfer mechanisms are involved? *Bioresour. Technol.* **102**:324–33.

Rosenberg E, Ron EZ. 1999. High- and low-molecular-mass microbial surfactants. *Appl. Microbiol. Biotechnol.* **52**:154–162.

Rosenberg E, Zuckerberg a, Rubinovitz C, Gutnick DL. 1979. Emulsifier of Arthrobacter RAG-1: isolation and emulsifying properties. *Appl. Environ. Microbiol.* **37**:402–8.

Rotaru AE, Shrestha PM, Liu F, Ueki T, Nevin K, Summers ZM, Lovley DR. 2012. Interspecies electron transfer via hydrogen and formate rather than direct electrical connections in cocultures of Pelobacter carbinolicus and Geobacter sulfurreducens. *Appl. Environ. Microbiol.* **78**:7645–7651.

Rotaru A-E, Shrestha PM, Liu F, Shrestha M, Shrestha D, Embree M, Zengler K, Wardman C, Nevin KP, Lovley DR. 2014. A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to Methanosaeta for the reduction of carbon dioxide to methane. *Energy Environ. Sci.* **7**:408.

De Roy K, Marzorati M, Van den Abbeele P, Van de Wiele T, Boon N. 2013. Synthetic microbial ecosystems: An exciting tool to understand and apply microbial communities. *Environ. Microbiol.* **16**:1472–1481.

Royal CL, Preston DR, Sekelsky AM, Shreve GS. 2003. Reductive dechlorination of polychlorinated biphenyls in landfill leachate. *Int. Biodeterior. Biodegradation* **51**:61–66.

Ruiz M, Pasadakis N, Kalogerakis N. 2006. Bioremediation and toxicity determination of natural seawater polluted with weathered crude oil by salt-tolerant consortia in a SBR. *Mar. Pollut. Bull.* **52**:1490–3.

Rysavy JP, Yan T, Novak PJ. 2005. Enrichment of anaerobic polychlorinated biphenyl dechlorinators from sediment with iron as a hydrogen source. *Water Res.* **39**:569–78.

Safe S, Bandiera S, Sawyer T, Robertson L, Safe L, Parkinson A, Thomas PE, Ryan DE, Reik LM, Levin W. 1985. PCBs: structure–function relationships and mechanism of action. *Environ. Health Perspect.* **60**:47.

Safe SH. 1994. Polychlorinated Biphenyls (PCBs): Environmental Impact, Biochemical and Toxic Responses, and Implications for Risk Assessment. *Crit. Rev. Toxicol.* **24**:87–149.

Safe S, Hutzinger O. 1984. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology, and mechanism of action. *CRC Crit. Rev. Toxicol.* **13**:319–395.

Sánchez A, Recillas S, Font X, Casals E, González E, Puntes V. 2011. Ecotoxicity of, and remediation with, engineered inorganic nanoparticles in the environment. *TrAC Trends Anal. Chem.* **30**:507–516.

Sandefur CA, Koenigsberg SS. 1999. The Use of Hydrogen Release Compound for the Accelerated Bioremediation of Anaerobically Degradable Contaminants: The Advent of Time-Release Electron Donors. *Remediat. J.* **10**:31–53.

Sargent L, Roloff B, Meisner L. 1989. In vitro chromosome damage due to PCB interactions. *Mutat. Res. Toxicol.* **224**:79–88.

Sass AM, Sass H, Coolen MJ, Cypionka H, Overmann J. 2001. Microbial communities in the chemocline of a hypersaline deep-sea basin (Urania basin, Mediterranean Sea). *Appl. Environ. Microbiol.* **67**:5392–402.

Sato T, Todoroki T, Shimoda K, Terada A, Hosomi M. 2010. Behavior of PCDDs/PCDFs in remediation of PCBs-contaminated sediments by thermal desorption. *Chemosphere* **80**:184–9.

Schantz SL, Widholm JJ, Rice DC. 2003. Effects of PCB Exposure on Neuropsychological Function in Children. *Environ. Health Perspect.* **111**:357–376.

Schink B. 1997. Energetics of Syntrophic Cooperation in Methanogenic Degradation **61**:262–280.

Schink B, Stams AJM. 2006. Syntrophism among Prokaryotes. *Prokaryotes Vol. 2 Ecophysiol. Biochem.* **2**:309–335.

Schneiker S, Martins dos Santos V a P, Bartels D, Bekel T, Brecht M, Buhrmester J, Chernikova TN, Denaro R, Ferrer M, Gertler C, Goesmann A, Golyshina O V, Kaminski F, Khachane AN, Lang S, Linke B, McHardy AC, Meyer F, Nechitaylo T, Pühler A, Regenhardt D, Rupp O, Sabirova JS, Selbitschka W, Yakimov MM, Timmis KN, Vorhölter F-J, Weidner S, Kaiser O, Golyshin PN. 2006. Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium Alcanivorax borkumensis. *Nat. Biotechnol.* **24**:997–1004.

Schoeny RS, Smith CC, Loper JC. 1979. Non-mutagenicity for salmonella of the chlorinated hydrocarbons aroclor 1254, 1,2,4-trichlorobenzene, mirex and kepone. *Mutat. Res. Toxicol.* **68**:125–132.

Schrand AM, Rahman MF, Hussain SM, Schlager JJ, Smith DA, Syed AF. 2010. Metal-based nanoparticles and their toxicity assessment. *Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology*.

Schulz DE, Petrick G, Duinker JC. 1989. Complete characterization of polychlorinated biphenyl congeners in commercial Aroclor and Clophen mixtures by multidimensional gas chromatography-electron capture detection. *Environ. Sci. Technol.* **23**:852–859.

Schwarzenbach RP, Gschwend PM, Imboden DM. 2005. Environmental organic chemistry. John Wiley & Sons.

Semkiw ES, Dybas MJ, Barcelona MJ. 2009. Evaluation of Three Electron-Donor Permeable Reactive Barrier Materials for Enhanced Reductive Dechlorination of Trichloroethene. *Bioremediat. J.* **13**:7–20.

Shrestha PM, Rotaru A-E. 2014. Plugging in or going wireless: strategies for interspecies electron transfer. *Front. Microbiol.* **5**:237.

Shrestha PM, Rotaru A-E, Aklujkar M, Liu F, Shrestha M, Summers ZM, Malvankar N, Flores DC, Lovley DR. 2013. Syntrophic growth with direct interspecies electron transfer as the primary mechanism for energy exchange. *Environ. Microbiol. Rep.* **5**:904–10.

Singer a. C, Gilbert ES, Luepromchai E, Crowley DE. 2000. Bioremediation of polychlorinated biphenyl-contaminated soil using carvone and surfactant-grown bacteria. *Appl. Microbiol. Biotechnol.* **54**:838–843.

Sivey JD, Lee CM. 2007. Polychlorinated biphenyl contamination trends in Lake Hartwell, South Carolina (USA): sediment recovery profiles spanning two decades. *Chemosphere* **66**:1821–8.

Smidt H, de Vos WM. 2004. Anaerobic microbial dehalogenation. *Annu. Rev. Microbiol.* **58**:43–73.

Sokol RC, Bethoney CM, Rhee G-Y. 1994. Effect of hydrogen on the pathway and products of PCB dechlorination. *Chemosphere* **29**:1735–1742.

Song B, Springer J. 1996a. Determination of Interfacial Tension from the Profile of a Pendant Drop Using Computer-Aided Image Processing1. Experimental. *J. Colloid Interface Sci.*

Song B, Springer J. 1996b. Determination of Interfacial Tension from the Profile of a Pendant Drop Using Computer-Aided Image Processing2. Experimental. *J. Colloid Interface Sci.* **184**:77–91.

Song B, Palleroni NJ, Häggblom MM. 2000. Isolation and Characterization of Diverse Halobenzoate-Degrading Denitrifying Bacteria from Soils and Sediments. *Appl. Environ. Microbiol.* **66**:3446–3453.

Sowers KR, May HD. 2013. In situ treatment of PCBs by anaerobic microbial dechlorination in aquatic sediment: are we there yet? *Curr. Opin. Biotechnol.* **24**:482–8.

Springer U, Klee J. 1954. Prüfung der Leistungsfähigkeit von einigen wichtigeren Verfahren zur Bestimmung des Kohlenstoffs mittels Chromschwefelsäure sowie Vorschlag einer neuen Schnellmethode. *Zeitschrift für Pflanzenernährung, Düngung, Bodenkd.* **64**:1–26.

Srinivasa Varadhan A, Khodadoust AP, Brenner RC. 2011. Effect of biostimulation on the microbial community in PCB-contaminated sediments through periodic amendment of sediment with iron. *J. Ind. Microbiol. Biotechnol.* **38**:1691–707.

Stackerbrandt E, Goebel BM. 1994. Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *Int. J. Syst. Bacteriol.*

Stams a. JM. 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.*

Stauffer CE. 1965. The Measurement of Surface Tension by the Pendant Drop Technique. *J. Phys. Chem* **69**:1933–1938.

Stegeman JJ, Schlezinger JJ, Craddock JE, Tillitt DE. 2001. Cytochrome P450 1A expression in midwater fishes: potential effects of chemical contaminants in remote oceanic zones. *Environ. Sci. Technol.* **35**:54–62.

Stelmack PL, Gray MR, Pickard M a. 1999. Bacterial adhesion to soil contaminants in the presence of surfactants. *Appl. Environ. Microbiol.* **65**:163–168.

Stockholm Convention. 2004. Listing of POPs in the Stockholm Convention (updated May 2013). http://chm.pops.int/TheConvention/ThePOPs/ListingofPOPs/tabid/2509/Default.aspx.

Sugiura K. 1992. Congener-specific PCB analyses of a sediment core of lake Shinji, Japan. *Chemosphere* **24**:427–432.

Summers ZM, Fogarty HE, Leang C, Franks AE, Malvankar NS, Lovley DR. 2010. Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science* **330**:1413–1415.

Suthersan SS, Payne FC. 2004. In situ remediation engineering. CRC Press.

Sweetman AJ, Valle MD, Prevedouros K, Jones KC. 2005. The role of soil organic carbon in the global cycling of persistent organic pollutants (POPs): interpreting and modelling field data. *Chemosphere* **60**:959–72.

Sylvestre M, Macek T, Mackova M. 2009. Transgenic plants to improve rhizoremediation of polychlorinated biphenyls (PCBs). *Curr. Opin. Biotechnol.* **20**:242–7.

Tanabe S, Kannan N, Subramanian A, Watanabe S, Tatsukawa R. 1987. Highly toxic coplanar PCBs: Occurrence, source, persistency and toxic implications to wildlife and humans. *Environ. Pollut.* **47**:147–163.

Tang NH, Myers TE. 2002. PCB removal from contaminated dredged material. *Chemosphere* **46**:477–484.

Taniguchi S, Miyamura A, Ebihara A, Hosomi M, Murakami A. 1998. Treatment of pcb-contaminated soil in a pilot- scale continuous decomposition system. *Chemosphere* **37**:2315–2326.

Tartakovsky B, Michotte A, Cadieux J-C. 2001. Degradation of Aroclor 1242 in a single-stage coupled anaerobic/aerobic bioreactor. *Water Res.* **35**:4323–4330.

Teng Y, Xu Z, Luo Y, Reverchon F. 2012. How do persistent organic pollutants be coupled with biogeochemical cycles of carbon and nutrients in terrestrial ecosystems under global climate change? *J. Soils Sediments* **12**:411–419.

Thompson IP, van der Gast CJ, Ciric L, Singer AC. 2005. Bioaugmentation for bioremediation: the challenge of strain selection. *Environ. Microbiol.* **7**:909–15.

Tiedje JM, Quensen JF, Chee-Sanford J, Schimel JP, Boyd S a. 1994. Microbial reductive dechlorination of PCBs. *Biodegradation* **4**:231–40.

Tilston EL, Collins CD, Mitchell GR, Princivalle J, Shaw LJ. 2013. Nanoscale zerovalent iron alters soil bacterial community structure and inhibits chloroaromatic biodegradation potential in Aroclor 1242-contaminated soil. *Environ. Pollut.* **173**:38–46.

Traunecker J, Preuß A, Diekert G. 1991. Isolation and characterization of a methyl chloride utilizing, strictly anaerobic bacterium. *Arch. Microbiol.* **156**:416–421.

Tsai P-C, Ko Y-C, Huang W, Liu H-S, Guo YL. 2007. Increased liver and lupus mortalities in 24-year follow-up of the Taiwanese people highly exposed to polychlorinated biphenyls and dibenzofurans. *Sci. Total Environ.* **374**:216–22.

Undeman E, Brown TN, Wania F, McLachlan MS. 2010. Susceptibility of human populations to environmental exposure to organic contaminants. *Environ. Sci. Technol.* **44**:6249–55.

UNEP. 1999. Guidelines for the identification of PCBs and materials containing PCBs. UNEP Chemicals.

UNEP UNEP. 2003. Standardized Toolkit for Identification and Quantification of Dioxin and Furan Releases.

Unterman R. 1996. Bioremediation Principles and Applications. Ed. Ronald L. Crawford, Don L. Crawford. Cambridge: Cambridge University Press.

US GPO GPO. 2007. Protection of Environment. Code of Federal Regulations (annual edition).

Utkin I, Woese C, Wiegel J. 1994. and characterization of Desulfitobacterium dehalogenans gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. *Int. J. …* **44**:612–619.

Vaneechoutte M, De Beenhouwer H, Claeys G, Verschraegen G, De Rouck A, Paepe N, Elaichouni A, Portaels F. 1993. Identification of Mycobacterium species by using amplified ribosomal DNA restriction analysis. *J. Clin. Microbiol.* **31**:2061–2065.

Varanasi P, Fullana A, Sidhu S. 2007. Remediation of PCB contaminated soils using iron nano-particles. *Chemosphere* **66**:1031–8.

Vasilyeva GK, Strijakova ER. 2007. Bioremediation of soils and sediments contaminated by polychlorinated biphenyls. *Microbiology* **76**:639–653.

Venkataraman A, Rosenbaum MA, Perkins SD, Werner JJ, Angenent LT. 2011. Metabolite-based mutualism between Pseudomonas aeruginosa PA14 and Enterobacter aerogenes enhances current generation in bioelectrochemical systems. *Energy Environ. Sci.*

Vila J, Nieto JM, Mertens J, Springael D, Grifoll M. 2010. Microbial community structure of a heavy fuel oil-degrading marine consortium: Linking microbial dynamics with polycyclic aromatic hydrocarbon utilization. *FEMS Microbiol. Ecol.* **73**:349–362.

Volkering F, Breure A, Rulkens W. 1997. Microbiological aspects of surfactant use for biological soil remediation. *Biodegradation* **8**:401–17.

Voogt PDE, Brinkman UAT. 1989. Production, properties and usage of polychlorinated biphenyls. *Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins Relat. Prod.*:3–45.

Vos JG, de Roij T. 1972. Immunosuppressive activity of a polychlorinated biphenyl preparation on the humoral immune response in guinea pigs. *Toxicol. Appl. Pharmacol.* **21**:549–555.

Wang S, Chng KR, Wilm A, Zhao S, Yang K-L, Nagarajan N, He J. 2014. Genomic characterization of three unique Dehalococcoides that respire on persistent polychlorinated biphenyls. *Proc. Natl. Acad. Sci. U. S. A.* **111**:12103–8.

Wang S, He J. 2013a. Phylogenetically distinct bacteria involve extensive dechlorination of aroclor 1260 in sediment-free cultures. *PLoS One* **8**:e59178.

Wang S, He J. 2013b. Dechlorination of commercial PCBs and other multiple halogenated compounds by a sediment-free culture containing Dehalococcoides and Dehalobacter. *Environ. Sci. Technol.* **47**:10526–34.

Wang Y, Sheng H-F, He Y, Wu J-Y, Jiang Y-X, Tam NF-Y, Zhou H-W. 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Appl. Environ. Microbiol.* **78**:8264–71.

Wania F, Mackay D. 1993. Global fractionation and cold condensation of low volatility organochlorine compounds in polar regions. *Ambio*. Vol. 22.

Wania F, Daly GL. 2002. Estimating the contribution of degradation in air and deposition to the deep sea to the global loss of PCBs. *Atmos. Environ.* **36**:5581–5593.

Watts JE, Wu Q, Schreier SB, May HD, Sowers KR. 2001. Comparative analysis of polychlorinated biphenyl-dechlorinating communities in enrichment cultures using three different molecular screening techniques. *Environ. Microbiol.* **3**:710–9.

Watts JEM, Fagervold SK, May HD, Sowers KR. 2005. A PCR-based specific assay reveals a population of bacteria within the Chloroflexi associated with the reductive dehalogenation of polychlorinated biphenyls. *Microbiology* **151**:2039–46.

Weber R. 2007. Relevance of PCDD/PCDF formation for the evaluation of POPs destruction technologies--review on current status and assessment gaps. *Chemosphere* **67**:S109–17.

Weisglas-Kuperus N, Vreugdenhil HJI, Mulder PGH. 2004. Immunological effects of environmental exposure to polychlorinated biphenyls and dioxins in Dutch school children. *Toxicol. Lett.* **149**:281–5.

West CC, Harwell JH. 1992. Surfactants and subsurface remediation. *Environ. Sci. Technol.* **26**:2324–2330.

Wiegel J, Wu Q. 2000. Microbial reductive dehalogenation of polychlorinated biphenyls. *FEMS Microbiol. Ecol.* **32**:1–15.

Winchell LJ, Novak PJ. 2008. Enhancing polychlorinated biphenyl dechlorination in fresh water sediment with biostimulation and bioaugmentation. *Chemosphere* **71**:176–82.

Windt W De, Aelterman P, Verstraete W, De Windt W. 2005. Bioreductive deposition of palladium (0) nanoparticles on Shewanella oneidensis with catalytic activity towards reductive dechlorination of polychlorinated biphenyls. *Environ. Microbiol.* **7**:314–325.

Winneke G, Ranft U, Wittsiepe J, Kasper-Sonnenberg M, Fürst P, Krämer U, Seitner G, Wilhelm M. 2014. Behavioral sexual dimorphism in school-age children and early developmental exposure to dioxins and PCBs: a follow-up study of the Duisburg Cohort. *Environ. Health Perspect.* **122**:292–8.

Wittebolle L, Marzorati M, Clement L, Balloi A, Daffonchio D, Heylen K, De Vos P, Verstraete W, Boon N. 2009. Initial community evenness favours functionality under selective stress. *Nature* **458**:623–6.

Wong MH, Wu SC, Deng WJ, Yu XZ, Luo Q, Leung a OW, Wong CSC, Luksemburg WJ, Wong a S. 2007. Export of toxic chemicals - a review of the case of uncontrolled electronic-waste recycling. *Environ. Pollut.* **149**:131–40.

Wu Q, Sowers KR, May HD. 1998a. Microbial reductive dechlorination of aroclor 1260 in anaerobic slurries of estuarine sediments. *Appl. Environ. Microbiol.* **64**:1052–8.

Wu Q, Sowers KR, May HD. 2000. Establishment of a polychlorinated biphenyl-dechlorinating microbial consortium, specific for doubly flanked chlorines, in a defined, sediment-free medium. *Appl. Environ. Microbiol.* **66**:49–53.

Wu Q, Wiegel J. 1997. Two anaerobic polychlorinated biphenyl-dehalogenating enrichments that exhibit different para-dechlorination specificities. *Appl. Environ. Microbiol.* **63**:4826–32.

Wu Q, Bedard D, Wiegel J. 1996. Influence of Incubation Temperature on the Microbial Reductive Dechlorination of 2,3,4,6-Tetrachlorobiphenyl in Two Freshwater Sediments. *Appl. Environ. …* **62**:4174–4179.

Wu Q, Bedard D, Wiegel J. 1997a. Effect of Incubation Temperature on the Route of Microbial Reductive Dechlorination of 2,3,4,6-Tetrachlorobiphenyl in Polychlorinated Biphenyl (PCB)-Contaminated and PCB-Free Freshwater Sediments. *Appl. Environ. …* **63**:2836–2843.

Wu Q, Bedard D, Wiegel J. 1997b. Temperature Determines the Pattern of Anaerobic Microbial Dechlorination of Aroclor 1260 Primed by 2,3,4,6-Tetrachlorobiphenyl in Woods Pond Sediment. *Appl. Environ. …* **63**:4818–4825.

Wu Q, Bedard DL, Wiegel J. 1999. 2,6-Dibromobiphenyl Primes Extensive Dechlorination of Aroclor 1260 in Contaminated Sediment at 8−30 °C by Stimulating Growth of PCB-Dehalogenating Microorganisms. *Environ. Sci. Technol.* **33**:595–602.

Wu Q, Milliken CE, Meier GP, Watts JEM, Sowers KR, May HD. 2002a. Dechlorination of chlorobenzenes by a culture containing bacterium DF-1, a PCB dechlorinating microorganism. *Environ. Sci. Technol.* **36**:3290–4.

Wu Q, Watts JEM, Sowers KR, May HD. 2002b. Identification of a bacterium that specifically catalyzes the reductive dechlorination of polychlorinated biphenyls with doubly flanked chlorines. *Appl. Environ. Microbiol.* **68**:807–812.

Wu T. 2008. Environmental Perspectives of Microwave Applications as Remedial Alternatives: Review. *Pract. Period. Hazardous, Toxic, Radioact. Waste Manag.* **12**:102–115.

Wu W-M, Nye J, Jain MK, Hickey RF. 1998b. Anaerobic dechlorination of trichloroethylene (TCE) to ethylene using complex organic materials. *Water Res.* **32**:1445–1454.

Yakimov MM, Golyshin PN, Lang S, Moore ER, Abraham WR, Lünsdorf H, Timmis KN. 1998. Alcanivorax borkumensis gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *Int. J. Syst. Bacteriol.* **48 Pt 2**:339–348.

Yakimov MM, Denaro R, Genovese M, Cappello S, D'Auria G, Chernikova TN, Timmis KN, Golyshin PN, Giluliano L. 2005. Natural microbial diversity in superficial sediments of Milazzo Harbor (Sicily) and community successions during microcosm enrichment with various hydrocarbons. *Environ. Microbiol.* **7**:1426–1441.

Yakimov MM, Gentile G, Bruni V, Cappello S, D'Auria G, Golyshin PN, Giuliano L. 2004. Crude oil-induced structural shift of coastal bacterial communities of rod bay (Terra Nova Bay, Ross Sea, Antarctica) and characterization of cultured cold-adapted hydrocarbonoclastic bacteria. *FEMS Microbiol. Ecol.* **49**:419–432.

Yamada Y, Katsura K, Kawasaki H, Widyastuti Y, Saono S, Seki T, Uchimura T, Komagata K. 2000. Asaia bogorensis gen. nov., sp. nov., an unusual acetic acid bacterium in the alpha-Proteobacteria. *Int. J. Syst. Evol. Microbiol.* **50 Pt 2**:823–9.

Yan T, Lapara TM, Novak PJ. 2006. The reductive dechlorination of 2,3,4,5-tetrachlorobiphenyl in three different sediment cultures: evidence for the involvement of phylogenetically similar Dehalococcoides-like bacterial populations. *FEMS Microbiol. Ecol.* **55**:248–61.

Ye D, Quensen JI, Tiedje JM, Boyd S a. 1995. Evidence for para dechlorination of polychlorobiphenyls by methanogenic bacteria. *Appl. Environ. Microbiol.* **61**:2166–71.

Ye D, Quensen J. 1992. Anaerobic Dechlorination of Polychlorobiphenyls (Aroclor 1242) by Pasteurized and Ethanol-Treated Microorganisms from Sediments I11. *Appl. …* **58**:1110–1114.

Ye D, Quensen J. 1999. 2-Bromoethanesulfonate, Sulfate, Molybdate, and Ethanesulfonate Inhibit Anaerobic Dechlorination of Polychlorobiphenyls by Pasteurized Microorganisms. *Appl. …* **65**:327–329.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**:134.

Yoshida N, Ye L, Baba D, Katayama A. 2009. Reductive Dechlorination of Polychlorinated Biphenyls and Dibenzo-p-Dioxins in an Enrichment Culture Containing Dehalobacter Species. *Microbes Environ.* **24**:343–346.

Zanaroli G, Balloi A, Negroni A, Borruso L, Daffonchio D, Fava F. 2012a. A Chloroflexi bacterium dechlorinates polychlorinated biphenyls in marine sediments under in situ-like biogeochemical conditions. *J. Hazard. Mater.* **209-210**:449–57.

Zanaroli G, Balloi A, Negroni A, Daffonchio D, Young LY, Fava F. 2010. Characterization of the microbial community from the marine sediment of the Venice lagoon capable of reductive dechlorination of coplanar polychlorinated biphenyls (PCBs). *J. Hazard. Mater.* **178**:417–26.

Zanaroli G, Negroni A, Vignola M, Nuzzo A, Shu H-Y, Fava F. 2012b. Enhancement of microbial reductive dechlorination of polychlorinated biphenyls (PCBs) in a marine sediment by nanoscale zerovalent iron (NZVI) particles. *J. Chem. Technol. Biotechnol.* **87**:1246–1253.

Zanaroli G, Pérez-Jiménez JR, Young LY, Marchetti L, Fava F. 2006. Microbial reductive dechlorination of weathered and exogenous co-planar polychlorinated biphenyls (PCBs) in an anaerobic sediment of Venice Lagoon. *Biodegradation* **17**:121–9.

Zeliger HI. 2013. Lipophilic chemical exposure as a cause of type 2 diabetes (T2D). *Rev. Environ. Health* **28**:9–20.

Zhang T, Gannon SM, Nevin KP, Franks AE, Lovley DR. 2010. Stimulating the anaerobic degradation of aromatic hydrocarbons in contaminated sediments by providing an electrode as the electron acceptor. *Environ. Microbiol.* **12**:1011–1020.

Zhang WX. 2003. Nanoscale iron particles for environmental remediation: An overview. *J. Nanoparticle Res.* **5**:323–332.

Zhang X, Wiegel J. 1990. Sequential anaerobic degradation of 2,4-dichlorophenol in freshwater sediments. *Appl. Envir. Microbiol.* **56**:1119–1127.

ZoBell C. 1941. Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J Mar Res* **4**:42–75.

Zonta R, Botter M, Cassin D, Pini R, Scattolin M, Zaggia L. 2007. Sediment chemical contamination of a shallow water area close to the industrial zone of Porto Marghera (Venice Lagoon, Italy). *Mar. Pollut. Bull.* **55**:529–42.

Zrafi-Nouira I, Khedir-Ghenim Z, Bahri R, Cheraeif I, Rouabhia M, Saidane-Mosbahi D. 2009. Hydrocarbons in seawater and water extract of Jarzouna-Bizerte coastal of Tunisia (Mediterranean Sea): Petroleum origin investigation around refinery rejection place. *Water. Air. Soil Pollut.* **202**:19–31.

Zuckerberg A, Diver A, Peeri Z, Gutnick DL, Rosenberg E. 1979. Emulsifier of Arthrobacter RAG-1: chemical and physical properties. *Appl. Environ. Microbiol.* **37**:414–20.

Zwiernik MJ, Quensen JF, Boyd SA. 1998. FeSO 4 Amendments Stimulate Extensive Anaerobic PCB Dechlorination. *Environ. Sci. Technol.* **32**:3360–3365.

9 Appendix

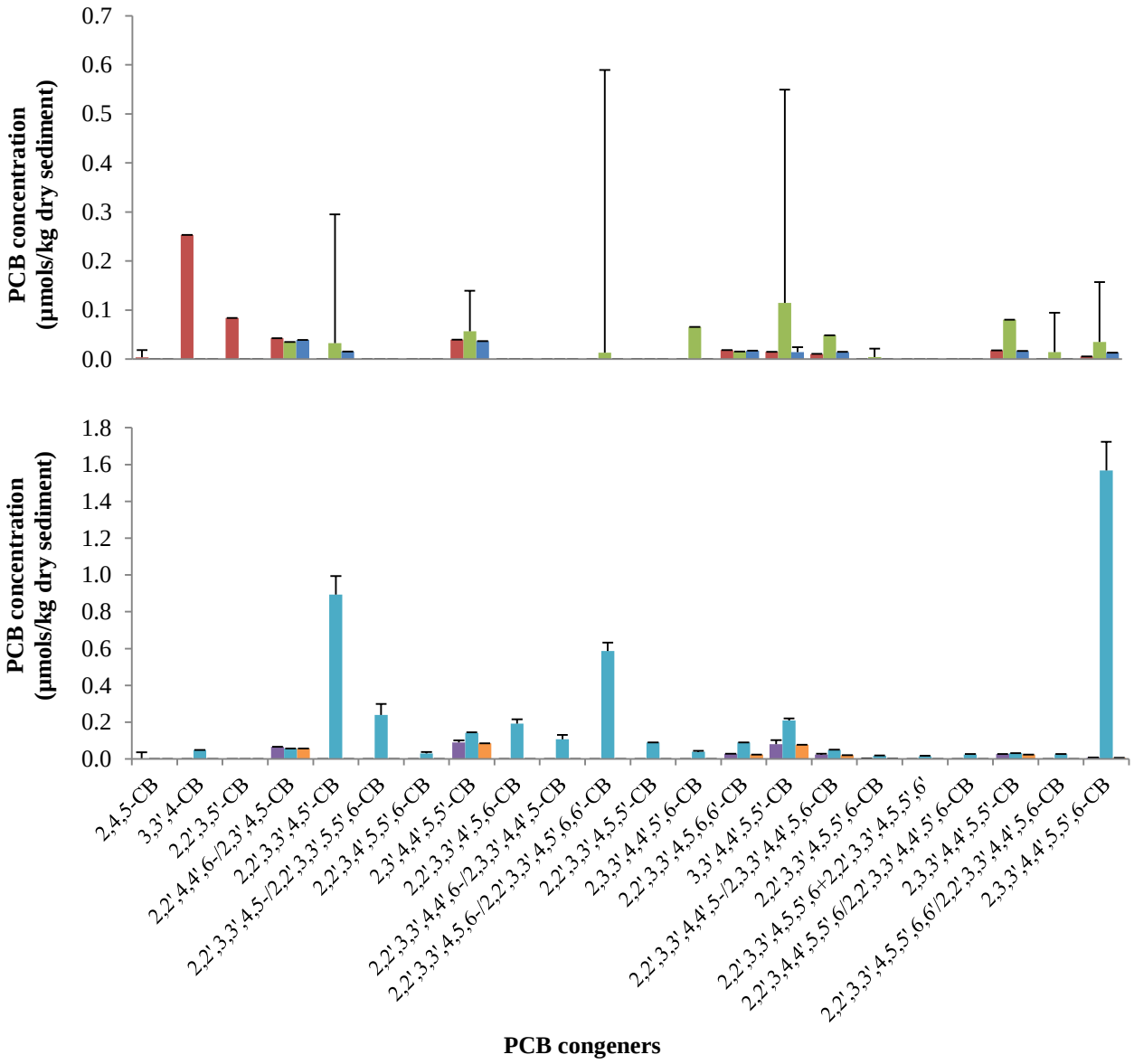

Figure 9.1:PCB congeners constituting more than 1% w/w of the original contamination in sediment A, B1, B2. C, D and E (red, green, blue, violet, cyan and orange bars, respectively). Values are average of duplicate extractions and error bars represent standard deviations.

IUPAC Name	Congener Number	Descriptor*	CASRN
2-Chlorobiphenyl	1	CP1	2051-60-7
3-Chlorobiphenyl	2	CP0	2051-61-8
4-Chlorobiphenyl	3	CP0	2051-62-9
2,2'-Dichlorobiphenyl	4		13029-08-8
2,3-Dichlorobiphenyl	5	CP1	16605-91-7
2,3'-Dichlorobiphenyl	6	CP1	25569-80-6

IUPAC Name	Congener Number	Descriptor*	CASRN
2,4-Dichlorobiphenyl	7	CP1	33284-50-3
2,4'-Dichlorobiphenyl	8	CP1	34883-43-7
2,5-Dichlorobiphenyl	9	CP1	34883-39-1
2,6-Dichlorobiphenyl	10		33146-45-1
3,3'-Dichlorobiphenyl	11	CP0, 2M	2050-67-1
3,4-Dichlorobiphenyl	12	CP0	2974-92-7
3,4'-Dichlorobiphenyl	13	CP0	2974-90-5
3,5-Dichlorobiphenyl	14	CP0, 2M	34883-41-5
4,4'-Dichlorobiphenyl	15	CP0, PP	2050-68-2
2,2',3-Trichlorobiphenyl	16		38444-78-9
2,2',4-Trichlorobiphenyl	17		37680-66-3
2,2',5-Trichlorobiphenyl	18		37680-65-2
2,2',6-Trichlorobiphenyl	19		38444-73-4
2,3,3'-Trichlorobiphenyl	20	CP1, 2M	38444-84-7
2,3,4-Trichlorobiphenyl	21	CP1	55702-46-0
2,3,4'-Trichlorobiphenyl	22	CP1	38444-85-8
2,3,5-Trichlorobiphenyl	23	CP1, 2M	55720-44-0
2,3,6-Trichlorobiphenyl	24		55702-45-9
2,3',4-Trichlorobiphenyl	25	CP1	55712-37-3
2,3',5-Trichlorobiphenyl	26	CP1, 2M	38444-81-4
2,3',6-Trichlorobiphenyl	27		38444-76-7
2,4,4'-Trichlorobiphenyl	28	CP1, PP	7012-37-5
2,4,5-Trichlorobiphenyl	29	CP1	15862-07-4
2,4,6-Trichlorobiphenyl	30		35693-92-6
2,4',5-Trichlorobiphenyl	31	CP1	16606-02-3
2,4',6-Trichlorobiphenyl	32		38444-77-8
2,3',4'-Trichlorobiphenyl	33	CP1	38444-86-9
2,3',5'-Trichlorobiphenyl	34	CP1, 2M	37680-68-5
3,3',4-Trichlorobiphenyl	35	CP0, 2M	37680-69-6
3,3',5-Trichlorobiphenyl	36	CP0, 2M	38444-87-0
3,4,4'-Trichlorobiphenyl	37	CP0, PP	38444-90-5
3,4,5-Trichlorobiphenyl	38	CP0, 2M	53555-66-1
3,4',5-Trichlorobiphenyl	39	CP0, 2M	38444-88-1
2,2',3,3'-Tetrachlorobiphenyl	40	4CL, 2M	38444-93-8
2,2',3,4-Tetrachlorobiphenyl	41	4CL	52663-59-9
2,2',3,4'-Tetrachlorobiphenyl	42	4CL	36559-22-5
2,2',3,5-Tetrachlorobiphenyl	43	4CL, 2M	70362-46-8
2,2',3,5'-Tetrachlorobiphenyl	44	4CL, 2M	41464-39-5
2,2',3,6-Tetrachlorobiphenyl	45	4CL	70362-45-7

IUPAC Name	Congener Number	Descriptor*	CASRN
2,2',3,6'-Tetrachlorobiphenyl	46	4CL	41464-47-5
2,2',4,4'-Tetrachlorobiphenyl	47	4CL, PP	2437-79-8
2,2',4,5-Tetrachlorobiphenyl	48	4CL	70362-47-9
2,2',4,5'-Tetrachlorobiphenyl	49	4CL	41464-40-8
2,2',4,6-Tetrachlorobiphenyl	50	4CL	62796-65-0
2,2',4,6'-Tetrachlorobiphenyl	51	4CL	68194-04-7
2,2',5,5'-Tetrachlorobiphenyl	52	4CL, 2M	35693-99-3
2,2',5,6'-Tetrachlorobiphenyl	53	4CL	41464-41-9
2,2',6,6'-Tetrachlorobiphenyl	54	4CL	15968-05-5
2,3,3',4-Tetrachlorobiphenyl	55	CP1, 4CL, 2M	74338-24-2
2,3,3',4'-Tetrachlorobiphenyl	56	CP1, 4CL, 2M	41464-43-1
2,3,3',5-Tetrachlorobiphenyl	57	CP1, 4CL, 2M	70424-67-8
2,3,3',5'-Tetrachlorobiphenyl	58	CP1, 4CL, 2M	41464-49-7
2,3,3',6-Tetrachlorobiphenyl	59	4CL, 2M	74472-33-6
2,3,4,4'-Tetrachlorobiphenyl	60	CP1, 4CL, PP	33025-41-1
2,3,4,5-Tetrachlorobiphenyl	61	CP1, 4CL, 2M	33284-53-6
2,3,4,6-Tetrachlorobiphenyl	62	4CL	54230-22-7
2,3,4',5-Tetrachlorobiphenyl	63	CP1, 4CL, 2M	74472-34-7
2,3,4',6-Tetrachlorobiphenyl	64	4CL	52663-58-8
2,3,5,6-Tetrachlorobiphenyl	65	4CL, 2M	33284-54-7
2,3',4,4'-Tetrachlorobiphenyl	66	CP1, 4CL, PP	32598-10-0
2,3',4,5-Tetrachlorobiphenyl	67	CP1, 4CL, 2M	73575-53-8
2,3',4,5'-Tetrachlorobiphenyl	68	CP1, 4CL, 2M	73575-52-7
2,3',4,6-Tetrachlorobiphenyl	69	4CL	60233-24-1
2,3',4',5-Tetrachlorobiphenyl	70	CP1, 4CL, 2M	32598-11-1
2,3',4',6-Tetrachlorobiphenyl	71	4CL	41464-46-4
2,3',5,5'-Tetrachlorobiphenyl	72	CP1, 4CL, 2M	41464-42-0
2,3',5',6-Tetrachlorobiphenyl	73	4CL, 2M	74338-23-1
2,4,4',5-Tetrachlorobiphenyl	74	CP1, 4CL, PP	32690-93-0
2,4,4',6-Tetrachlorobiphenyl	75	4CL, PP	32598-12-2
2,3',4',5'-Tetrachlorobiphenyl	76	CP1, 4CL, 2M	70362-48-0
3,3',4,4'-Tetrachlorobiphenyl	77	CP0, 4CL, PP, 2M	32598-13-3
3,3',4,5-Tetrachlorobiphenyl	78	CP0, 4CL, 2M	70362-49-1
3,3',4,5'-Tetrachlorobiphenyl	79	CP0, 4CL, 2M	41464-48-6
3,3',5,5'-Tetrachlorobiphenyl	80	CP0, 4CL, 2M	33284-52-5
3,4,4',5-Tetrachlorobiphenyl	81	CP0, 4CL, PP, 2M	70362-50-4
2,2',3,3',4-Pentachlorobiphenyl	82	4CL, 2M	52663-62-4
2,2',3,3',5-Pentachlorobiphenyl	83	4CL, 2M	60145-20-2
2,2',3,3',6-Pentachlorobiphenyl	84	4CL, 2M	52663-60-2

IUPAC Name	Congener Number	Descriptor*	CASRN
2,2',3,4,4'-Pentachlorobiphenyl	85	4CL, PP	65510-45-4
2,2',3,4,5-Pentachlorobiphenyl	86	4CL, 2M	55312-69-1
2,2',3,4,5'-Pentachlorobiphenyl	87	4CL, 2M	38380-02-8
2,2',3,4,6-Pentachlorobiphenyl	88	4CL	55215-17-3
2,2',3,4,6'-Pentachlorobiphenyl	89	4CL	73575-57-2
2,2',3,4',5-Pentachlorobiphenyl	90	4CL, 2M	68194-07-0
2,2',3,4',6-Pentachlorobiphenyl	91	4CL	68194-05-8
2,2',3,5,5'-Pentachlorobiphenyl	92	4CL, 2M	52663-61-3
2,2',3,5,6-Pentachlorobiphenyl	93	4CL, 2M	73575-56-1
2,2',3,5,6'-Pentachlorobiphenyl	94	4CL, 2M	73575-55-0
2,2',3,5',6-Pentachlorobiphenyl	95	4CL, 2M	38379-99-6
2,2',3,6,6'-Pentachlorobiphenyl	96	4CL	73575-54-9
2,2',3,4',5'-Pentachlorobiphenyl	97	4CL, 2M	41464-51-1
2,2',3,4',6'-Pentachlorobiphenyl	98	4CL	60233-25-2
2,2',4,4',5-Pentachlorobiphenyl	99	4CL, PP	38380-01-7
2,2',4,4',6-Pentachlorobiphenyl	100	4CL, PP	39485-83-1
2,2',4,5,5'-Pentachlorobiphenyl	101	4CL, 2M	37680-73-2
2,2',4,5,6'-Pentachlorobiphenyl	102	4CL	68194-06-9
2,2',4,5',6-Pentachlorobiphenyl	103	4CL	60145-21-3
2,2',4,6,6'-Pentachlorobiphenyl	104	4CL	56558-16-8
2,3,3',4,4'-Pentachlorobiphenyl	105	CP1, 4CL, PP, 2M	32598-14-4
2,3,3',4,5-Pentachlorobiphenyl	106	CP1, 4CL, 2M	70424-69-0
2,3,3',4',5-Pentachlorobiphenyl	107	CP1, 4CL, 2M	70424-68-9
2,3,3',4,5'-Pentachlorobiphenyl	108	CP1, 4CL, 2M	70362-41-3
2,3,3',4,6-Pentachlorobiphenyl	109	4CL, 2M	74472-35-8
2,3,3',4',6-Pentachlorobiphenyl	110	4CL, 2M	38380-03-9
2,3,3',5,5'-Pentachlorobiphenyl	111	CP1, 4CL, 2M	39635-32-0
2,3,3',5,6-Pentachlorobiphenyl	112	4CL, 2M	74472-36-9
2,3,3',5',6-Pentachlorobiphenyl	113	4CL, 2M	68194-10-5
2,3,4,4',5-Pentachlorobiphenyl	114	CP1, 4CL, PP, 2M	74472-37-0
2,3,4,4',6-Pentachlorobiphenyl	115	4CL, PP	74472-38-1
2,3,4,5,6-Pentachlorobiphenyl	116	4CL, 2M	18259-05-7
2,3,4',5,6-Pentachlorobiphenyl	117	4CL, 2M	68194-11-6
2,3',4,4',5-Pentachlorobiphenyl	118	CP1, 4CL, PP, 2M	31508-00-6
2,3',4,4',6-Pentachlorobiphenyl	119	4CL, PP	56558-17-9
2,3',4,5,5'-Pentachlorobiphenyl	120	CP1, 4CL, 2M	68194-12-7
2,3',4,5',6-Pentachlorobiphenyl	121	4CL, 2M	56558-18-0
2,3,3',4',5'-Pentachlorobiphenyl	122	CP1, 4CL, 2M	76842-07-4
2,3',4,4',5'-Pentachlorobiphenyl	123	CP1, 4CL, PP, 2M	65510-44-3

IUPAC Name	Congener Number	Descriptor*	CASRN
2,3',4',5,5'-Pentachlorobiphenyl	124	CP1, 4CL, 2M	70424-70-3
2,3',4',5',6-Pentachlorobiphenyl	125	4CL, 2M	74472-39-2
3,3',4,4',5-Pentachlorobiphenyl	126	CP0, 4CL, PP, 2M	57465-28-8
3,3',4,5,5'-Pentachlorobiphenyl	127	CP0, 4CL, 2M	39635-33-1
2,2',3,3',4,4'-Hexachlorobiphenyl	128	4CL, PP, 2M	38380-07-3
2,2',3,3',4,5-Hexachlorobiphenyl	129	4CL, 2M	55215-18-4
2,2',3,3',4,5'-Hexachlorobiphenyl	130	4CL, 2M	52663-66-8
2,2',3,3',4,6-Hexachlorobiphenyl	131	4CL, 2M	61798-70-7
2,2',3,3',4,6'-Hexachlorobiphenyl	132	4CL, 2M	38380-05-1
2,2',3,3',5,5'-Hexachlorobiphenyl	133	4CL, 2M	35694-04-3
2,2',3,3',5,6-Hexachlorobiphenyl	134	4CL, 2M	52704-70-8
2,2',3,3',5,6'-Hexachlorobiphenyl	135	4CL, 2M	52744-13-5
2,2',3,3',6,6'-Hexachlorobiphenyl	136	4CL, 2M	38411-22-2
2,2',3,4,4',5-Hexachlorobiphenyl	137	4CL, PP, 2M	35694-06-5
2,2',3,4,4',5'-Hexachlorobiphenyl	138	4CL, PP, 2M	35065-28-2
2,2',3,4,4',6-Hexachlorobiphenyl	139	4CL, PP	56030-56-9
2,2',3,4,4',6'-Hexachlorobiphenyl	140	4CL, PP	59291-64-4
2,2',3,4,5,5'-Hexachlorobiphenyl	141	4CL, 2M	52712-04-6
2,2',3,4,5,6-Hexachlorobiphenyl	142	4CL, 2M	41411-61-4
2,2',3,4,5,6'-Hexachlorobiphenyl	143	4CL, 2M	68194-15-0
2,2',3,4,5',6-Hexachlorobiphenyl	144	4CL, 2M	68194-14-9
2,2',3,4,6,6'-Hexachlorobiphenyl	145	4CL	74472-40-5
2,2',3,4',5,5'-Hexachlorobiphenyl	146	4CL, 2M	51908-16-8
2,2',3,4',5,6-Hexachlorobiphenyl	147	4CL, 2M	68194-13-8
2,2',3,4',5,6'-Hexachlorobiphenyl	148	4CL, 2M	74472-41-6
2,2',3,4',5',6-Hexachlorobiphenyl	149	4CL, 2M	38380-04-0
2,2',3,4',6,6'-Hexachlorobiphenyl	150	4CL	68194-08-1
2,2',3,5,5',6-Hexachlorobiphenyl	151	4CL, 2M	52663-63-5
2,2',3,5,6,6'-Hexachlorobiphenyl	152	4CL, 2M	68194-09-2
2,2',4,4',5,5'-Hexachlorobiphenyl	153	4CL, PP, 2M	35065-27-1
2,2',4,4',5,6'-Hexachlorobiphenyl	154	4CL, PP	60145-22-4
2,2',4,4',6,6'-Hexachlorobiphenyl	155	4CL, PP	33979-03-2
2,3,3',4,4',5-Hexachlorobiphenyl	156	CP1, 4CL, PP, 2M	38380-08-4
2,3,3',4,4',5'-Hexachlorobiphenyl	157	CP1, 4CL, PP, 2M	69782-90-7
2,3,3',4,4',6-Hexachlorobiphenyl	158	4CL, PP, 2M	74472-42-7
2,3,3',4,5,5'-Hexachlorobiphenyl	159	CP1, 4CL, 2M	39635-35-3
2,3,3',4,5,6-Hexachlorobiphenyl	160	4CL, 2M	41411-62-5
2,3,3',4,5',6-Hexachlorobiphenyl	161	4CL, 2M	74472-43-8
2,3,3',4',5,5'-Hexachlorobiphenyl	162	CP1, 4CL, 2M	39635-34-2

IUPAC Name	Congener Number	Descriptor*	CASRN
2,3,3',4',5,6-Hexachlorobiphenyl	163	4CL, 2M	74472-44-9
2,3,3',4',5',6-Hexachlorobiphenyl	164	4CL, 2M	74472-45-0
2,3,3',5,5',6-Hexachlorobiphenyl	165	4CL, 2M	74472-46-1
2,3,4,4',5,6-Hexachlorobiphenyl	166	4CL, PP, 2M	41411-63-6
2,3',4,4',5,5'-Hexachlorobiphenyl	167	CP1, 4CL, PP, 2M	52663-72-6
2,3',4,4',5',6-Hexachlorobiphenyl	168	4CL, PP, 2M	59291-65-5
3,3',4,4',5,5'-Hexachlorobiphenyl	169	CP0, 4CL, PP, 2M	32774-16-6
2,2',3,3',4,4',5-Heptachlorobiphenyl	170	4CL, PP, 2M	35065-30-6
2,2',3,3',4,4',6-Heptachlorobiphenyl	171	4CL, PP, 2M	52663-71-5
2,2',3,3',4,5,5'-Heptachlorobiphenyl	172	4CL, 2M	52663-74-8
2,2',3,3',4,5,6-Heptachlorobiphenyl	173	4CL, 2M	68194-16-1
2,2',3,3',4,5,6'-Heptachlorobiphenyl	174	4CL, 2M	38411-25-5
2,2',3,3',4,5',6-Heptachlorobiphenyl	175	4CL, 2M	40186-70-7
2,2',3,3',4,6,6'-Heptachlorobiphenyl	176	4CL, 2M	52663-65-7
2,2',3,3',4,5',6'-Heptachlorobiphenyl	177	4CL, 2M	52663-70-4
2,2',3,3',5,5',6-Heptachlorobiphenyl	178	4CL, 2M	52663-67-9
2,2',3,3',5,6,6'-Heptachlorobiphenyl	179	4CL, 2M	52663-64-6
2,2',3,4,4',5,5'-Heptachlorobiphenyl	180	4CL, PP, 2M	35065-29-3
2,2',3,4,4',5,6-Heptachlorobiphenyl	181	4CL, PP, 2M	74472-47-2
2,2',3,4,4',5,6'-Heptachlorobiphenyl	182	4CL, PP, 2M	60145-23-5
2,2',3,4,4',5',6-Heptachlorobiphenyl	183	4CL, PP, 2M	52663-69-1
2,2',3,4,4',6,6'-Heptachlorobiphenyl	184	4CL, PP	74472-48-3
2,2',3,4,5,5',6-Heptachlorobiphenyl	185	4CL, 2M	52712-05-7
2,2',3,4,5,6,6'-Heptachlorobiphenyl	186	4CL, 2M	74472-49-4
2,2',3,4',5,5',6-Heptachlorobiphenyl	187	4CL, 2M	52663-68-0
2,2',3,4',5,6,6'-Heptachlorobiphenyl	188	4CL, 2M	74487-85-7
2,3,3',4,4',5,5'-Heptachlorobiphenyl	189	CP1, 4CL, PP, 2M	39635-31-9
2,3,3',4,4',5,6-Heptachlorobiphenyl	190	4CL, PP, 2M	41411-64-7
2,3,3',4,4',5',6-Heptachlorobiphenyl	191	4CL, PP, 2M	74472-50-7
2,3,3',4,5,5',6-Heptachlorobiphenyl	192	4CL, 2M	74472-51-8
2,3,3',4',5,5',6-Heptachlorobiphenyl	193	4CL, 2M	69782-91-8
2,2',3,3',4,4',5,5'-Octachlorobiphenyl	194	4CL, PP, 2M	35694-08-7
2,2',3,3',4,4',5,6-Octachlorobiphenyl	195	4CL, PP, 2M	52663-78-2
2,2',3,3',4,4',5,6'-Octachlorobiphenyl	196	4CL, PP, 2M	42740-50-1
2,2',3,3',4,4',6,6'-Octachlorobiphenyl	197	4CL, PP, 2M	33091-17-7
2,2',3,3',4,5,5',6-Octachlorobiphenyl	198	4CL, 2M	68194-17-2
2,2',3,3',4,5,5',6'-Octachlorobiphenyl	199	4CL, 2M	52663-75-9
2,2',3,3',4,5,6,6'-Octachlorobiphenyl	200	4CL, 2M	52663-73-7
2,2',3,3',4,5',6,6'-Octachlorobiphenyl	201	4CL, 2M	40186-71-8

IUPAC Name	Congener Number	Descriptor*	CASRN
2,2',3,3',5,5',6,6'-Octachlorobiphenyl	202	4CL, 2M	2136-99-4
2,2',3,4,4',5,5',6-Octachlorobiphenyl	203	4CL, PP, 2M	52663-76-0
2,2',3,4,4',5,6,6'-Octachlorobiphenyl	204	4CL, PP, 2M	74472-52-9
2,3,3',4,4',5,5',6-Octachlorobiphenyl	205	4CL, PP, 2M	74472-53-0
2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl	206	4CL, PP, 2M	40186-72-9
2,2',3,3',4,4',5,6,6'-Nonachlorobiphenyl	207	4CL, PP, 2M	52663-79-3
2,2',3,3',4,5,5',6,6'-Nonachlorobiphenyl	208	4CL, 2M	52663-77-1
Decachlorobiphenyl	209	4CL, PP, 2M	2051-24-3

Table 9.1: Table of PCB species by Congener Number, revised as of November 2003 (Source: US EPA website http://www.epa.gov/epawaste/hazard/tsd/pcbs/pubs/congeners.htm). CP0/CP1 indicate 68 co-planar congeners which include 20 with chlorine substitution at none (CP0, non-ortho) and 48 with chlorine substitution at only one (CP1, mono-ortho) of the 2, 2', 6, or 6' positions; 4CL indicates169 congeners which have a total of four or more chlorine substituents (regardless of position); PP indicates 54 congeners which have both para positions (4 and 4') chlorinated; 2M indicates 140 congeners which have two or more of the meta positions (3, 3', 5 and 5') chlorinated; when all four of the above mentioned descriptors are displayed, the congener is also called "dioxin-like". The Congener Number is presented in the table as from the data published by Ballschmiter and Zell (1980). CASRN is Chemical Abstracts Service (CAS) Registry Number.

Isolate	Media	Assay	Days of incubation						
			0	**3**	**8**	**10**	**11**	**14**	**16**
N1 E2 3	HLB/10	OD	0.00	0.00	0.00	-	0.00	-	0.00
		EI_{24}	0.00	0.00	0.00	-	0.00	-	-
N1 E9 3N	HLB/10+CA	OD	0.00	0.15	0.11	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	0.00	-	-
N2 E2 1A	HLB/10	OD	0.00	0.00	0.00	-	-	0.00	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E2 1A	ONR7+CA+YE	OD	0.00	0.00	0.03	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E2 1A	ONR7+CA+YE	OD	0.00	0.28	0.02	-	-	0.19	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E2 2B	Zobell	OD	0.00	1.76	1.88	-	-	-	-
		EI_{24}	0.00	0.00	0.44	-	-	-	0.12
N2 E2 2C	Zobell	OD	0.00	1.46	1.68	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.46
N2 E2 2F	ONR7+CA+YE	OD	0.00	1.03	1.33	-	-	-	-
		EI_{24}	0.00	0.41	0.44	-	0.40	-	-
N2 E2 3A	HLB	OD	0.00	0.09	1.01	-	-	0.42	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E2 3A	HLB/10	OD	0.00	0.00	0.00	-	-	0.00	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E2 3A	HLB	OD	0.00	0.00	0.00	-	-	-	-
		EI_{24}	0.00	0.00	0.56	-	-	0.00	-
N2 E2 3B	Zobell	OD	0.00	1.66	1.68	-	-	-	-
		EI_{24}	0.00	0.00	0.46	-	-	-	0.44
N2 E2 3N	HLB/10	OD	0.00	0.00	0.00	-	-	0.00	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-

Isolate	Media	Assay	Days of incubation						
			0	3	8	10	11	14	16
N2 E2 3N	HLB/10+CA	OD	0.00	0.00	0.03	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E2 3N	HLB+CA	OD	0.00	0.68	0.75	-	-	0.26	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E2 4C	HLB	OD	0.00	0.22	0.56	-	-	0.32	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E2 4C	HLB/10	OD	0.00	0.00	0.00	-	-	0.00	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E2 4C	HLB/10	OD	0.00	0.00	0.00	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E2 5C	HLB/10	OD	0.00	0.00	0.00	-	-	0.00	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E2 5C	ONR7+CA+YE	OD	0.00	0.29	0.26	-	-	0.20	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E2 5C	ONR7+CA+YE	OD	0.00	0.00	0.03	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E2 5D	ONR7+CA+YE	OD	0.00	1.65	1.73	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	0.06	-	-
N2 E5 1G	ONR7+CA+YE	OD	0.00	1.00	-	1.49	-	1.68	-
		EI_{24}	0.00	0.47	-	0.53	-	0.48	-
N2 E5 1H	ONR7+CA+YE	OD	0.00	1.12	-	1.66	-	1.64	-
		EI_{24}	0.00	0.00	-	0.43	-	0.51	-
N2 E5 3A	Zobell	OD	0.00	1.69	2.24	-	-	-	-
		EI_{24}	0.00	0.00	0.36	-	-	-	0.09
N2 E5 3D	HLB	OD	0.00	0.00	2.04	-	-	1.00	-
		EI_{24}	0.00	0.00	0.57	-	-	0.73	-
N2 E5 3D	HLB	OD	0.00	0.32	0.00	-	-	0.00	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E5 3D	Zobell	OD	0.00	0.22	0.00	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E5 3F	HLB	OD	0.00	0.05	0.04	-	-	0.90	-
		EI_{24}	0.00	0.00	0.00	-	-	0.72	-
N2 E5 3F	HLB/10	OD	0.00	0.00	0.01	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E5 3F	HLB/10	OD	0.00	0.00	0.00	-	-	0.00	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E5 4D	HLB	OD	0.00	0.34	0.32	-	-	0.25	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E5 4D	HLB/10	OD	0.00	0.00	0.00	-	-	0.00	-
		EI_{24}	0.00	0.00	0.00	-	-	0.00	-
N2 E5 4D	ONR7+CA+YE	OD	0.00	0.00	0.04	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E5 4L	HLB/10+CA	OD	0.00	0.00	0.05	-	-	-	-
		EI_{24}	0.00	0.00	0.00	-	-	-	0.00
N2 E5 4L	ONR7+CA+YE	OD	0.00	2.48	1.64	-	-	0.99	-

Isolate	Media	Assay	Days of incubation						
			0	3	8	10	11	14	16
N2 E5 5D	HLB/10	EI_{24}	0.00	0.00	0.68	-	-	0.75	-
		OD	0.00	0.00	0.45	-	-	-	-
N2 E5 TQ N	HLB/10+CA	EI_{24}	0.00	0.00	0.00	-	-	0.00	-
		OD	0.00	0.92	-	0.70	-	0.70	-
N2 E9 2S	ONR7+CA+YE	EI_{24}	0.00	0.11	-	0.00	-	0.22	-
		OD	0.00	1.36	-	1.77	-	1.78	-
N2 E9 3F	HLB/10+CA	EI_{24}	0.00	0.41	-	0.00	-	0.00	-
		OD	0.00	0.03	-	1.67	-	1.77	-
N2 E9 3M	HLB/10+CA	EI_{24}	0.00	0.00	-	0.54	-	0.21	-
		OD	0.00	0.86	1.08	-	-	-	-
N2 E9 4F	HLB/10	EI_{24}	0.00	0.00	0.00	-	0.00	-	-
		OD	0.00	1.78	-	1.81	-	1.81	-
N2 E9 4G	ONR7+CA+YE	EI_{24}	0.00	0.44	-	0.32	-	0.21	-
		OD	0.00	0.60	1.13	-	-	-	-
N2 E9 4L	HLB/10	EI_{24}	0.00	0.00	0.00	-	0.49	-	-
		OD	0.00	0.00	0.00	-	-	0.00	-
N2 E9 4L	HLB+CA	EI_{24}	0.00	0.00	0.00	-	-	0.00	-
		OD	0.00	0.32	0.28	-	-	0.24	-
N2 E9 4L	ONR7+CA+YE	EI_{24}	0.00	0.00	0.00	-	-	0.00	-
		OD	0.00	0.00	0.05	-	-	-	-
N2 E9 4L	ONR7+CA+YE	EI_{24}	0.00	0.00	0.00	-	-	-	0.00
		OD	0.00	1.74	-	1.81	-	1.81	-
N2 E9 5n	Zobell	EI_{24}	0.00	0.00	-	0.00	-	0.00	-
		OD	0.00	1.68	2.92	-	-	-	-
N2 E9 5P	Zobell	EI_{24}	0.00	0.00	0.00	-	-	-	0.38
		OD	0.00	0.00	2.72	-	-	-	-
N2 E9 5S	Zobell	EI_{24}	0.00	0.00	0.44	-	-	-	0.57
		OD	0.00	0.42	1.64	-	-	-	-
N2 E9 6f	HLB/10	EI_{24}	0.00	0.00	0.00	-	-	-	0.27
		OD	0.00	0.00	0.04	-	-	-	-
N2 E9 6F	ONR7+CA+YE	EI_{24}	0.00	0.00	0.00	-	-	-	0.00
		OD	0.00	0.32	0.28	-	-	0.22	-
N2 E9 6G	ONR7+CA+YE	EI_{24}	0.00	0.00	0.00	-	-	0.00	-
		OD	0.00	1.33	-	1.79	-	1.80	-
		EI_{24}	0.00	0.09	-	0.29	-	0.16	-

Table 9.2: Isolates screening from mixed culture E2, E5 and E9. Media indicates the solid media on which the isolate was obtained, where CA and YE represent the presence of crude oil (0.5% w/v) and yeast extract (0.1 g/l), respectively.

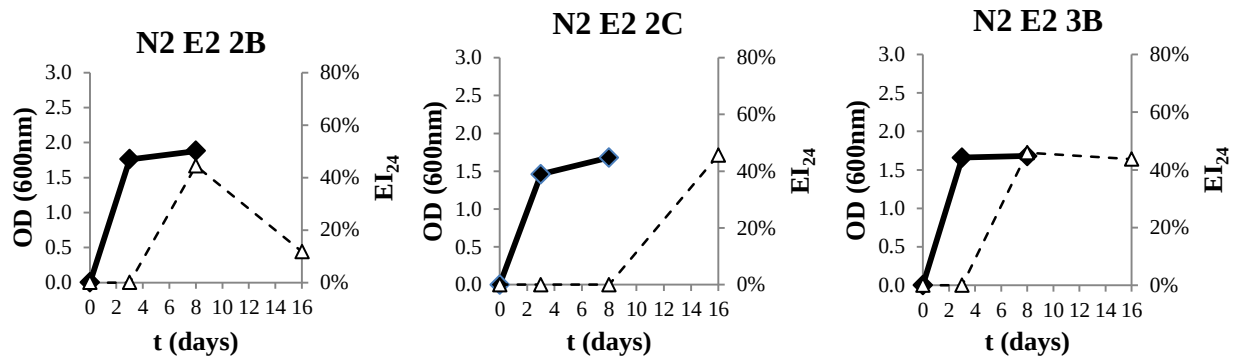

Figure 9.2: Characterization in terms of OD_{600} (solid line) and EI_{24} (dashed line) of isolates related to *Thalassospira sp.* M65-3N obtained from culture E2

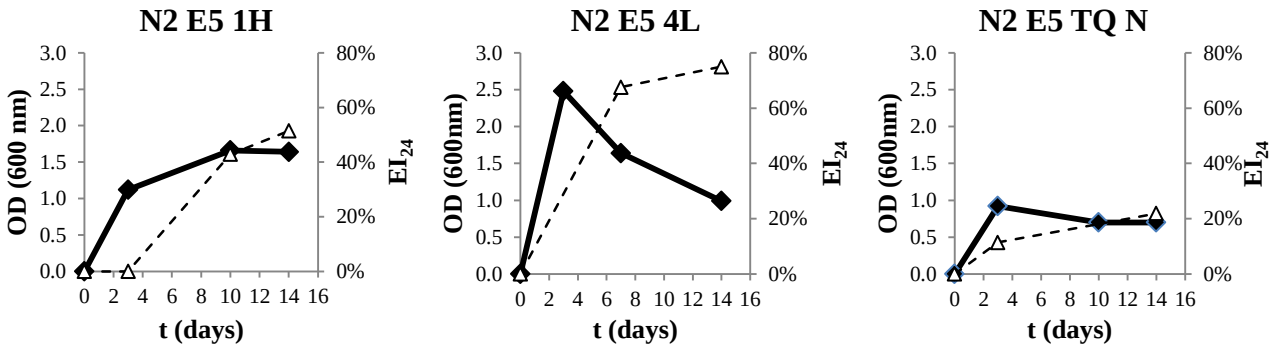

Figure 9.3: Characterization in terms of OD_{600} (solid line) and EI_{24} (dashed line) of isolates related to *Thalassospira sp.* M65-3N obtained from culture E5

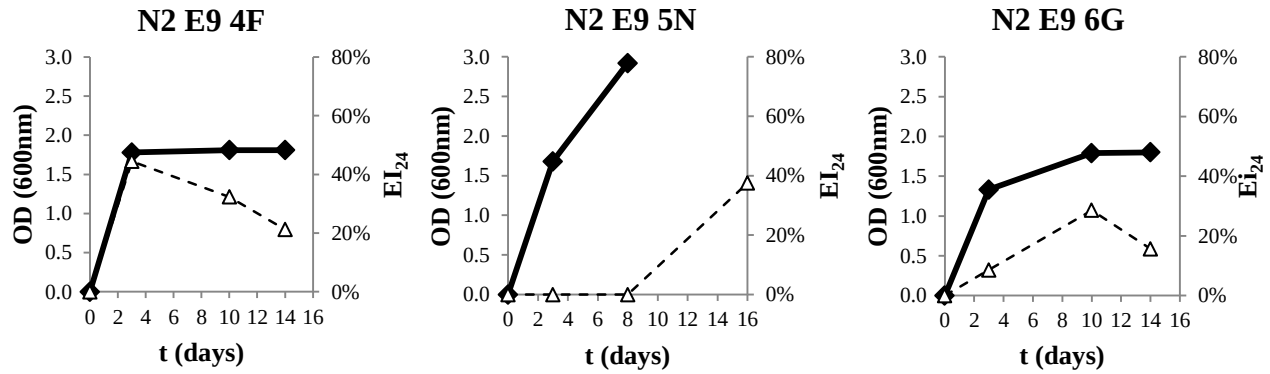

Figure 9.4: Characterization in terms OD_{600} (solid line) and EI_{24} (dashed line) of isolates related to *Thalassospira sp.* M65-3N obtained from culture E9

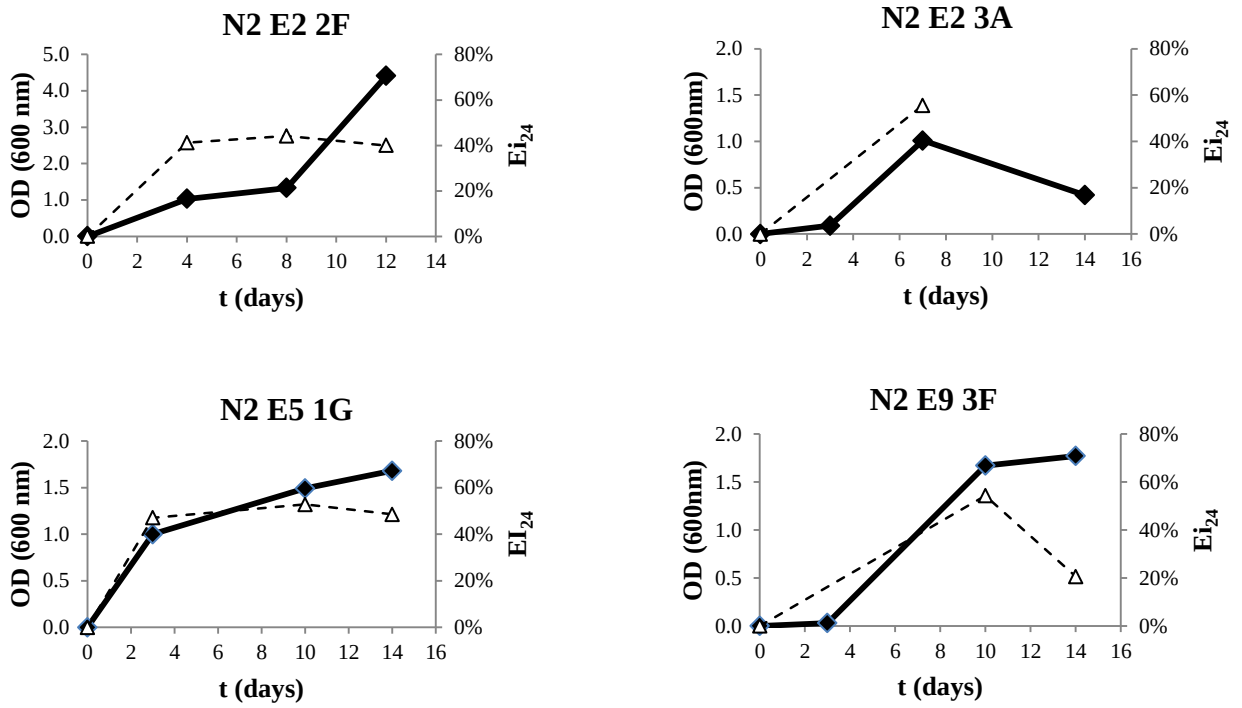

Figure 9.5: Characterization in terms of OD_{600} (solid line) and EI_{24} (dashed line) of isolates related to *Thalassospira lucentensis* obtained from mixed culture E2, E5 and E9

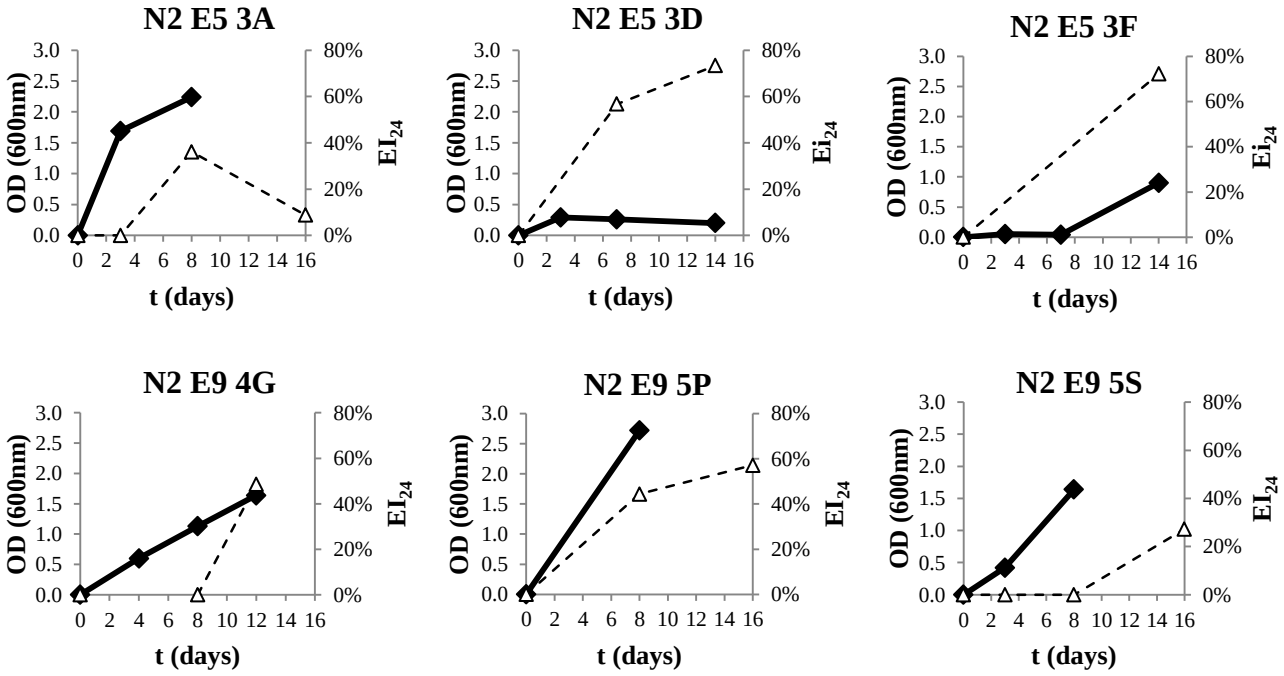

Figure 9.6: Characterization in terms of OD_{600} (solid line) and EI_{24} (dashed line) of unclassified isolates obtained from mixed culture E2, E5 and E9.

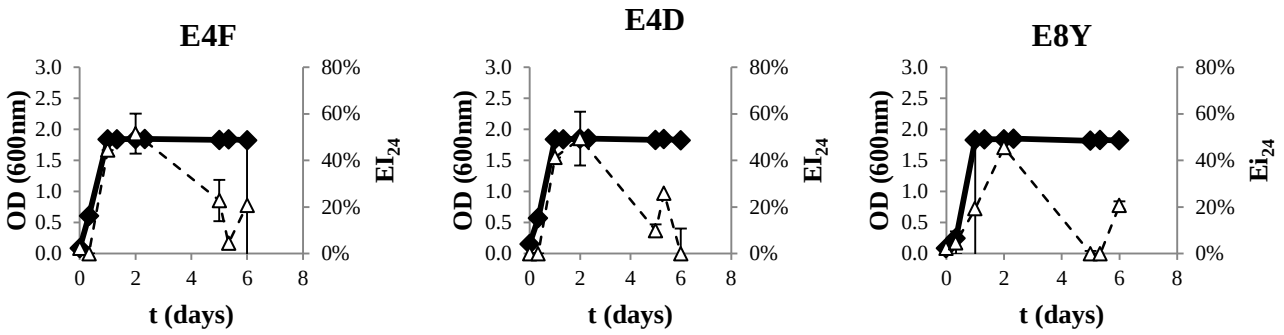

Figure 9.7: Characterization in terms of OD_{600} (solid line) and EI_{24} (dashed line) of isolates provided by the partner of the project related to *Alkanivorax borkumensis* SK2. Values are average of duplicate cultures.

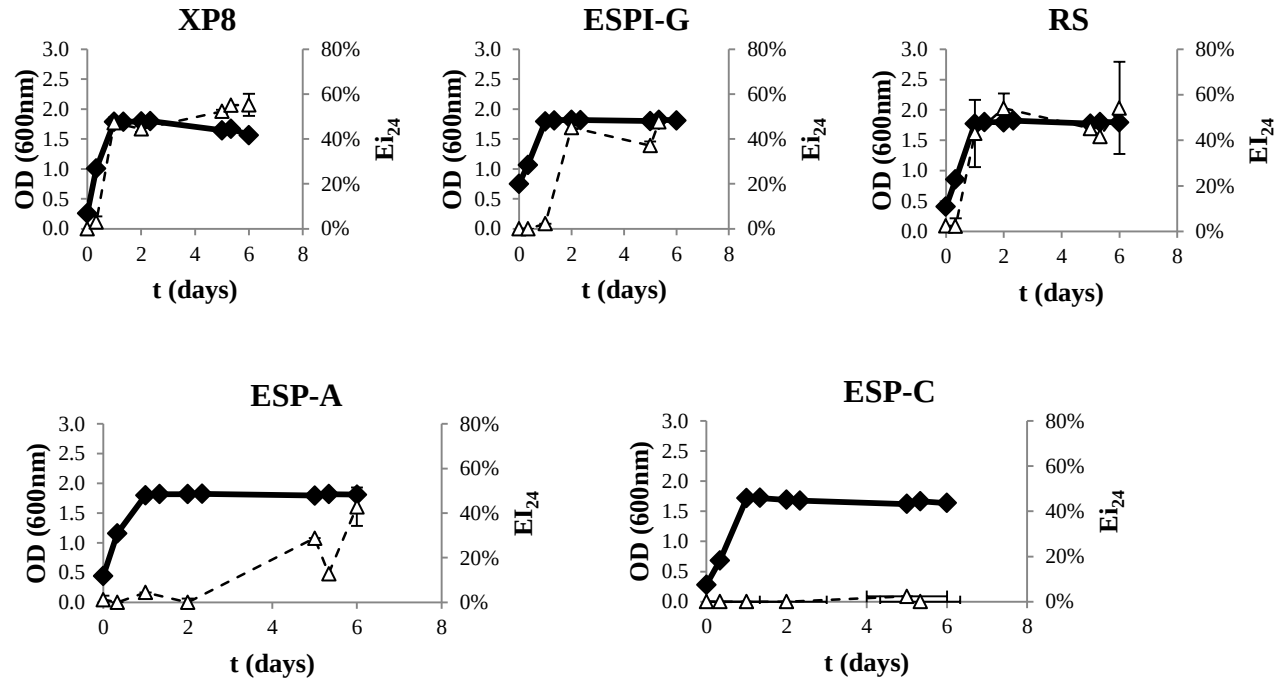

Figure 9.8: Characterization in terms of OD_{600} (solid line) and EI_{24} (dashed line) of isolates provided by the partner of the project grown on rich marine medium. Values are average of duplicate cultures.

10 Glossary

11 Acknowledgements

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