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First evidence on the occurrence and dynamics of *Dehalococcoides mccartyi* PCB-dechlorinase genes in marine sediment during Aroclor1254 reductive dechlorination

B. Matturro [a], M. Di Lenola [a], C. Ubaldi [b], S. Rossetti [a,*]

[a] Water Research Institute, IRSA-CNR, Via Salaria km 29,300, Monterotondo (RM), Italy
[b] ENEA, Technical Unit for Environmental Characterization, Prevention and Remediation, UTPRA, C. R. Casaccia, Italy

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ABSTRACT

The present study evaluates the PCB-dehalorespiring capabilities and dynamics of indigenous *Dehalococcoides mccartyi* population in a PCB contaminated marine sediment. Specialized PCB-dechlorinase genes *pcbA1*, *pcbA4* and *pcbA5* previously characterized in pure cultures of *D. mccartyi*, were here found for the first time in environmental samples. Reductive dechlorination was stimulated by spiking Aroclor1254 to the sediment and by imposing strictly anaerobic conditions both with and without bioaugmentation with a *Dehalococcoides mccartyi* enrichment culture. In line with the contaminant dechlorination kinetics, *Dehalococcoides* population increased during the entire incubation period showing growth yields of 4.94E + 07 *Dehalococcoides* per μmol Cl^{-1} and 7.30E + 05 *Dehalococcoides* per μmol Cl^{-1} in the marine sediment with and without bioaugmentation respectively. The *pcbA4* and *pcbA5* dechlorinase genes, and to a lesser extent *pcbA1* gene, were enriched during the anaerobic incubation suggesting their role in Aroclor1254 dechlorination under salinity conditions.

1. Introduction

Polychlorinated biphenyls (PCBs) are a family of 209 congeners largely used in several industrial processes and applications (*e.g.* lubricants, dielectric fluids, plasticizers, pesticides) because they are stable, inert and versatile organic molecules. Although their production was forbidden in the 1970s, they are continuously being released into the environment, consequently air, water, soil, sediments and living organisms have been exposed to PCBs for several decades. Nowadays, PCBs are considered among the most toxic, ubiquitous and persistent contaminants and their presence into the environment consequently poses a threat to the health of human being and ecosystems, including marine life (Gomes et al., 2014, Wang and He, 2013a, 2013b, Field and Sierra-Alvarez, 2008, Quero et al., 2015).

Although physical and chemical treatments are available, biological processes are the most attractive options for PCB removal. Bioremediation is a sustainable and cost-effective approach mediated by an anaerobic respiration, well known as reductive dechlorination process, in which specialized microorganisms use chlorinated compounds as electron acceptors in presence of H_2 or organic fermentable substrate as electron donors. *In situ* microbial reductive dechlorination of PCBs was firstly reported in 1987 and organisms responsible for the reductive dechlorination were found widespread in many environmental matrices including aquifers, freshwater sediments, soils and sludges (Brown et al., 1987; Bedard et al., 2007; Bedard, 2014; Borja et al., 2005). However, only a little is known about the identity of PCB dechlorinating microbes from marine sediments (Fava et al., 2003a, 2003b; Häggblom et al., 2003; Zanaroli et al., 2006, 2010, Zanaroli et al., 2012a, 2012b).

Within phylum *Chloroflexi*, *Dehalococcoides mccartyi* (*Dhc*) and other related species *(i.e. Dehalobium chlorocoercia DF-1, strain o-17, phylotypes SF-1*) were shown to be involved in PCB dechlorination (Cutter et al., 2001; Fagervold et al., 2007; Wu et al., 2002). Key reductive dehalogenase (RDase) genes of *Dhc* with a specific affinity to different chlorinate substrates, were described and, among these, *pceA, tceA, bvcA* and *vcrA* have been characterized (He et al., 2003, 2005; Bedard, 2014; Richardson, 2013). However, less is known about RDases strictly involved in PCB dechlorination. Only recently, the *Dhc* strains CG1, CG4, CG5 and JNA were demonstrated to be involved in PCB reductive dechlorination (Wang et al., 2014, 2015). They harbor RDase genes able to degrade PCB (*pcbA1*, *pcbA4*, *pcbA5* and orthologues) whose role is strictly related to the dechlorination of highly chlorinated PCB congeners. In detail, *pcbA1, pcbA4* and *pcbA5* address the reduction of esa-CBs and epta-CBs (congeners 128, 138, 149, 153, 170, 174, 180 and 187) to tetra-CBs (congeners 47, 49, 53) *via* penta-CBs (congeners 85, 90, 91, 95, 99, 101). In addition, it has been recently demonstrated that they have different bifunctional RDase genes to catalyze chlorine removal from both PCBs and PCE. Indeed, it has been reported that

* Corresponding author.
E-mail address: rossetti@irsa.cnr.it (S. Rossetti).

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pceA gene, commonly present in *Dhc* strains able to grow on chlorinated ethenes, is also present in *Dhc* strains specialized in PCB dechlorination (Wang et al., 2014, 2015).

To date, little is still known about the diversity of *Dhc* and related functional genes involved in PCB dechlorination in the marine environment. Indeed, a recent study conducted on a PCB contaminated marine sediment reported only 0.15% of the entire indigenous *Dhc* population was carrying known RDase genes typically associated to the RD of chlorinated ethenes pointing out the need of further research efforts (Matturro et al., 2016b). Additionally, shotgun metagenome libraries revealed an intriguing higher diversity in reductive dehalogenase homologue genes than previously known, suggesting that marine sediments might harbor new functional genes involved in reductive dechlorination process in saline environments (Zanaroli et al., 2015; Kawai et al., 2007).

Moreover, it has been demonstrated that a critical mass of dehalorespiring microbes is necessary to stimulate effective PCB dechlorination and low numbers of indigenous dehalorespiring bacteria might explain why substantial natural attenuation of PCBs is slow and it is rarely observed in the environment (Bedard et al., 2007). Nevertheless, the effectiveness and rate of PCB biodegradation at field scale may be unpredictable because it does not only depends on the presence of dehalorespiring microbes, but also on many environmental factors including proper reaction environment, electron donor availability and PCB concentration. Despite microcosm study have been previously performed on marine sediment and different positive effects have been reported upon biostimulation, only a few bioaugmentation experiments have been performed so far (Matturro et al., 2016a,b; Zanaroli et al., 2015).

In the present study, an harbor marine sediment, affected by a pre-existing low PCBs contamination, was spiked with Aroclor1254 under strictly anaerobic conditions with and without bioaugmentation with a *Dhc*-enriched culture in order to *i)* evaluate the intrinsic PCB dehalorespiring capabilities of indigenous *Dhc* population and *ii)* assess the impact of bioaugmentation on PCB reductive dechlorination in the marine environment.

In detail, microcosms were setup and monitored over the course of 200 days during which the RD process performances were examined. Moreover, *Dhc* cells and RDase genes including *pceA, tceA, bvcA, vcrA* and novel PCB-dechlorinase genes recently characterized only in a laboratory culture (*pcbA1, pcbA4, pcbA5*), were monitored and quantified over time.

2. Materials and methods

2.1. Microcosm preparation

Two sets of anaerobic microcosms were prepared in duplicate in sterile 250 mL serum bottles containing 60 g of dry marine sediment (original marine sediment presented 53.07% of humidity), collected from the La Spezia harbor (lat.: 44.086; long.: 9.873), and 80 mL of synthetic marine water (composition: NaCl 22 g L^{-1}, $MgCl_2 \cdot 6H_2O$ 9.7 g L^{-1}, Na_2SO_4 3.7 g L^{-1}, $CaCl_2$ 1 g L^{-1}, KCl 0.65 g L^{-1}, $NaHCO_3$ 0.2 g L^{-1}, H_3BO_3 0.023 g L^{-1}). The serum bottles were sealed with Teflon-faced butyl rubber stoppers and fluxed for 10 min with a mixture of N2/CO2. One set of microcosms was setup without addition of external dehalorespiring bacteria (hereafter cited as LSA) and one set (hereafter cited as LSB) was bioaugmented with the *Dhc*-mixed culture (Matturro et al., 2013a) at the final abundance of 2.57E + 06 *Dhc* cells g^{-1} dry sediment.

Both LSA and LSB were spiked with a total of 6 mg of Aroclor1254® (final concentration 100 μg g^{-1} dry sediment), the latter mainly composed by highly chlorinated PCBs. No electron donors were added to the microcosms. A biotic control was constructed by incubating the sediment under anaerobic conditions without the addition of Aroclor1254. All microcosms were incubated at 20 °C under rotation.

2.2. PCB extraction and quantification

PCBs were analyzed in the original marine sediment, immediately after microcosm setup ($t = 0$) and 80 and 200 days after anaerobic incubation. At each sampling, 4 g of slurry from each microcosm were collected in 30-mL glass tubes and stored at −20 °C until further processing. The PCBs were extracted from both the original marine sediment and the slurry microcosms with an Accelerated Solvent Extractor and then quantified by gas chromatography (GC) with a mass spectrometric detector as described in detail in (Matturro et al., 2016a,b). Chemical analyses were conducted on 18 PCB congeners quantified during the anaerobic incubation of the microcosms with and without bioaugmentation.

2.3. Sampling for biomolecular analysis

Biomolecular analyses were conducted on each replicate microcosm and performed on the original marine sediment and on samples taken anaerobically with sterile spatulas under N_2 flux.

For DNA extraction and subsequent qPCR applications 1 g of slurry was collected and immediately stored at −20 °C.

2.4. DNA extraction and qPCR

DNA was extracted from 0.25 g of marine sediment (dry weight) in the original sample and from each microcosm at different sampling times. The extraction was performed with PowerSoil DNA Isolation kit (MoBio, Italy) according to the manufacturer's instructions. DNA was eluted in 100 μL of sterile water and 1:100 dilutions were used for qPCR absolute quantification assays targeting *Dhc* 16S rRNA and functional genes *tceA, bvcA, vcrA, pcbA1, pcbA4, pcbA5.*

qPCR reactions targeting *Dhc 16S rRNA, tceA, bvcA, vcrA* genes were performed with TaqMan® chemistry in 20 μL total volume of *SsoAdvanced™ Universal Probes Supermix* (Biorad, Italy), including 3 μL of DNA as template, 300 nM of each primer and 300 nM of TaqMan® probe composed by 6-carboxyfluoresceine (FAM) as the 5′ end reporter fluorophore and N,N,N,N,-tetramethyl-6-carboxyrhodamine (TAMRA) as the 3′ end quencher. Additionally, qPCR reactions targeting *pceA, pcbA1, pcbA2, pcbA3* genes were performed with SybrGreen chemistry in 20 μL total volume of *SsoAdvanced™ Universal SYBR® Green Supermix* (Biorad, Italy) including 3 μL of DNA as template and 300 nM of each primer. Primers and probes used for each reaction are listed in table S1. Standard curves for the absolute quantification were constructed by using the long amplicons method previously reported in (Matturro et al., 2013b). Each reaction was performed in triplicate with CFX96 TouchTM Real-Time PCR Detection System (Biorad, Italy). Quantitative data were expressed as gene copy numbers g^{-1} sediment, and error bars were calculated with Microsoft Excel® on triplicate reactions for each sample.

3. Results

3.1. PCB reductive dechlorination

A total PBC concentration (≈20 ng g^{-1}) was found in the marine sediment collected from La Spezia harbor (Italy). In detail, esa-CB and epta-CB congeners were found at concentration ≤ 5 ng g^{-1} dry sediment while other low chlorinated congeners were at concentrations ≤2 ng g^{-1} dry sediment.

After the spike of Aroclor1254 (final concentration of 100 μg g^{-1} dry sediment), microcosms were monitored over the incubation period. PCB concentrations in the not bioaugmented microcosms (LSA) remained quite unvaried after 80 days of incubation and among highly chlorinated CBs only a weak decrement (−8%) was observed for the esa-CBs 156 (Fig. 1a). Among the lower chlorinated congeners, tetra-CB 52 decreased by 15%, tri-CB mixture 28 + 31 and 18 decreased by

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