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A Chloroflexi bacterium dechlorinates polychlorinated biphenyls in marine sediments under *in situ*-like biogeochemical conditions

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ABSTRACT

We investigated the reductive dechlorination of Aroclor 1254 PCBs by a coplanar PCB-dechlorinating microbial community enriched from an actual site contaminated marine sediment of the Venice lagoon in sterile slurry microcosms of the same sediment suspended in its site water, *i.e.*, under biogeochemical conditions that closely mime those occurring *in situ*. The culture dechlorinated more than 75% of the penta- through hepta-chlorinated biphenyls to tri- and tetra-chlorinated congeners in 30 weeks. The dechlorination rate was reduced by the addition of H_2 and short chain fatty acids, which stimulated sulfate-reduction and methane production, and markedly increased by the presence of vancomycin or ampicillin. DGGE analysis of 16S rRNA genes on PCB-spiked and PCB-free cultures ruled out sulfate-reducing and methanogenic bacteria and revealed the presence of a single Chloroflexi phylotype closely related to the uncultured bacteria m-1 and SF1 associated to PCB dechlorination. These findings suggest that a single dechlorinator is responsible for the observed extensive dechlorination of Aroclor 1254 and that a Chloroflexi species similar to those already detected in freshwater and estuarine contaminated sediments mediates PCB dechlorination in the marine sediment adopted in this study under biogeochemical conditions resembling those occurring *in situ* in the Brentella Canal of Venice Lagoon.

1. Introduction

Polychlorinated biphenyls (PCBs) were widely used in several industrial contexts during the past century. Despite their production was banned since the 1980s, PCBs are still occurring in several anaerobic freshwater and marine sediments, where they are persisting because of their very high hydrophobicity, chemical stability and recalcitrance to biodegradation [1]. In addition, due to their lipophilic nature, they are bioaccumulating and biomagnifying through the food chain up to humans, where they can exert multiple adverse health effects [2,3]. For these reasons, PCBs are still considered contaminants of great environmental concern and are included among the 21 worldwide priority persistent organic pollutants (POPs) [4].

Microbial PCB reductive dechlorination processes replace chlorine atoms with hydrogen atoms, thus converting highly chlorinated congeners into low chlorinated PCBs, that have lower bioaccumulation potential and toxicity and are readily degraded by native aerobic bacteria [5,6]. Microbial PCB reductive dechlorination, if properly stimulated, might therefore mediate the natural decontamination of the sediment *in situ* by reducing the need or the extension of quite expensive and poorly environmental sustainable sediment dredging operations [7].

Anaerobic dechlorination of PCBs has been documented in laboratory slurry microcosms and in a few sediment-free cultures derived from contaminated rivers, lakes and estuaries [5,6,8,9]. During the last decade, a number of studies have investigated the microorganisms involved in PCB dechlorination combining selective enrichment procedures with the use of molecular techniques targeting the 16S rRNA genes. Bacterium *o*-17, a Chloroflexi bacterium from estuarine sediments of the Baltimore Harbour having 89% 16S rDNA sequence similarity with *Dehalococcoides ethenogenes*, was the first reported to dechlorinate a PCB congener (2,3,5,6-pentachlorobiphenyl) in the *ortho* positions [10]. In the same period [11], a phylogenetically similar bacterium, *i.e.*, DF-1, was identified in cultures enriched from estuarine sediments of the Charleston Harbour as the dechlorinator of 2,3,4,5-CB, 3,4,5-CB and 2,3,4-CB congeners in the doubly flanked *meta* and *para* positions. Other members of the Chloroflexi phylum closely related to the *o*-17/DF-1 group and to *Dehalococcoides* spp. have been identified in a number of cultures from freshwater and estuarine habitats [12–19]. Since this group of microorganisms links the growth to the

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respiration of chlorinated compounds, their detection in contaminated sediments is a reliable indication of the sediment potential to undergo intrinsic bioremediation and detoxification. However, PCB dechlorinators identified so far are able to dechlorinate a limited range of individual PCB congeners and, with the single exception of a pure culture of *Dehalococcoides* sp. strain CBDB1 [20], the dechlorination of complex commercial mixtures of PCBs has been attributed to the occurrence of multiple dechlorinating Chloroflexi strains. In addition, all studies were performed on sediments from contaminated freshwater or estuarine habitats. Finally, since PCB dechlorinating microorganisms have been so far studied in sediment slurries or sediment-free cultures established in defined culture media [10–20], their dechlorination activity under actual site biogeochemical conditions is unknown.

Less is known on microbial PCB reductive dechlorination and PCB dechlorinating microorganisms in marine environments [5,6,8,9], which typically display biogeochemical and microbiological features quite different from those of freshwater and estuarine habitats [21]. We previously demonstrated the occurrence of microbial reductive dechlorination of weathered and spiked PCBs in slurry microcosms of contaminated marine sediments of the Venice lagoon suspended in their own site water, *i.e.*, under laboratory biogeochemical conditions very close to those occurring *in situ* [22–24]. PCB dechlorination rates and extents differed from those detected when the sediment was suspended in synthetic culture medium [22], indicating that the PCB dechlorination processes observed in the laboratory might be strongly affected by the biogeochemical conditions created in the microcosms. From the same sediment and under *in situ*-like biogeochemical conditions, we recently enriched the indigenous microbial community able to extensively dechlorinate five of the most toxic coplanar PCBs [25].

The objectives of this work were (i) to further enrich, under the same *in situ*-like biogeochemical conditions, the PCB dechlorinating microbial population of the same Venice lagoon sediments, (ii) to characterize the dechlorinating capability of the obtained enriched cultures towards Aroclor 1254 PCBs and (iii) to identify the dehalogenating members of the community. The results obtained indicate that a single, non-*Dehalococcoides* Chloroflexi carries out the *meta* and *para* reductive dechlorination of Aroclor 1254 PCBs according to the dechlorination process H′ under marine biogeochemical conditions closely miming those occurring *in situ*. To very best of our knowledge, this is the first paper in which a PCB dechlorinator obtained from a marine habitat through enrichments performed under biogeochemical conditions mimicking those occurring *in situ* is reported in the literature.

2. Experimental

2.1. PCB dechlorinating cultures

A microbial community able to dechlorinate coplanar PCBs, which was previously enriched from a marine sediment of the Brentella canal (Venice lagoon) contaminated by approximately 1.6 mg of a mixture of PCBs partially ascribed to Aroclor 1242 and Aroclor 1254 per kg of dry sediment [25], was sub-cultured in the presence of the commercial mixture of PCBs Aroclor 1254 in anaerobic sterile slurries consisting of autoclave-sterilized sediment of the Brentella canal suspended at 2% (v/v) in filter-sterilized water collected from the same site. Anaerobic sterile slurries (29.4 ml) were prepared in 50 ml serum bottles flushed with an O_2-free, 0.22 μm filter-sterilized N_2:CO_2 (70:30) mixture with a Hungate-similar apparatus as described previously [25]. Sterile slurries were then inoculated with 0.6 ml (2%, v/v) of the slurry culture grown in the previous sub-culturing step and spiked with a 20,000 mg L^{-1} solution of Aroclor 1254 PCBs in acetone to obtain a final PCB concentration of 1000 mg kg^{-1} dry sediment. Killed controls were prepared at each sub-culturing step by autoclaving the culture for 1 h at 121 °C on three consecutive days. Killed controls were spiked with Aroclor 1254 after the autoclave treatment. At the first sub-culturing step (sub-culture M1), non-amended microcosms were prepared. At the second sub-culturing step (sets of sub-cultures M2), additional microcosms were monthly amended with electron donors, *i.e.*, H_2 (5 ml) plus a mixture of short chain organic acids (formate, acetate, propionate and butyrate, 20 mM each), whereas some others were monthly supplemented with electron donors plus antibiotics (vancomycin or ampicillin, 100 μg ml^{-1} each). At the third sub-culturing step (sets of sub-cultures M3), electron donors and antibiotics were monthly supplied separately to additional parallel microcosms. In addition, a parallel set of PCB-free microcosms was also set up for each culture condition. PCB-free cultures were amended with the same amount of acetone used to supplement Aroclor 1254 in the PCB-spiked cultures. All cultures were set-up in triplicate. The overall sub-culturing procedure is described in Fig. 1. Cultures were incubated statically in the dark at 28 °C and periodically sampled in order to analyze the volume and the composition of the head-space gas, as well as the concentration of each PCB congener and of SO_4^{2-}. At the end of incubation of the cultures of the third sub-culturing step (set of sub-cultures M3), an additional 10 ml slurry aliquot was sampled to extract metagenomic DNA for the analysis of microbial populations. The sampling of the microcosms was performed according to Fava et al. [23].

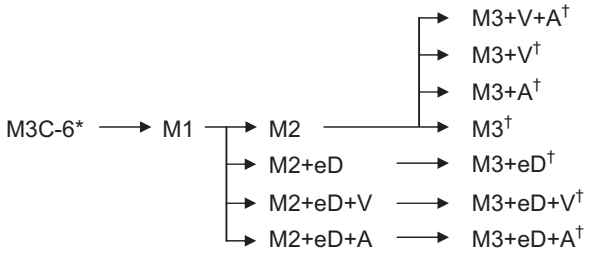

Fig. 1. Cultures developed in the study. eD: H_2 (5 ml) + formate, acetate, propionate and butyrate (20 mM each); V: vancomycin (100 μg ml^{-1}); A: Ampicillin (100 μg ml^{-1}). (*) PCB dechlorinating culture enriched on coplanar PCBs [24] used as inoculum in this study. (†) PCB-free cultures were also established under these conditions.

2.2. PCBs extraction and analytical procedures

PCB batch extraction was performed in duplicate from each replicate culture as described by Fava et al. [23]. The qualitative and quantitative analysis of the extracted PCBs was performed with a gas chromatograph (6890N) 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, Palo Alto, CA, USA) under the analytical conditions described elsewhere [23]. The concentrations were expressed as μmol of PCB kg^{-1} of dry sediment. The chlorination degree was reported as average number of Cl per biphenyl, whereas the initial and average PCB dechlorination rates were expressed as μmol of Cl released kg^{-1} of dry sediment $week^{-1}$, as described in Zanaroli et al. [25].

Gas production in the microcosms was measured with an airtight syringe while its composition was analyzed with an Agilent Technologies 6890 gas chromatograph equipped with a Carboxen 1010 PLOT fused silica column (30 m by 530 μm internal diameter, 25 μm film thickness) (Supelco Inc., Bellefonte, PA, USA) and a thermal conductivity detector (Agilent Technologies Italia S.p.A., Italy) as described by Fava et al. [23].

The concentration of SO_4^{2-} in the water phase was determined with a Dionex DX-120 ion chromatograph equipped with an IonPac

Table 1
PCB dechlorination, sulfate consumption and methane production in the cultures throughout the sub-culturing program. Values are the average from three independent replicate cultures (±SD).

Cultures	Lag phase	Initial PCB Dechlorination rate[a]	Average PCB Dechlorination rate[a]	Sulfate consumption rate[b]	Methane produced (ml)
M1	7 weeks	143 ± 8	147 ± 23	307 ± 15	2.0 ± 0.2
M2	–	133 ± 18	147 ± 29	385 ± 36	0
M2 + Ed	–	144 ± 21	157 ± 19	500 ± 39	7.1 ± 1.9
M2 + eD + V	–	192 ± 19	163 ± 27	517 ± 2	10.6 ± 5.4
M2 + eD + A	–	217 ± 17	159 ± 44	175 ± 3	14.1 ± 4.1
M3[c]	6 weeks	131 ± 13	111 ± 14	355 ± 35	0
M3 + eD[c]	6 weeks	59 ± 7	66 ± 10	390 ± 20	39.1 ± 1.5
M3 + eD + V[c]	–	118 ± 17	87 ± 14	224 ± 16	12.9 ± 0.9
M3 + eD + A[c]	–	201 ± 26	95 ± 31	44 ± 16	7,8 ± 0.3
M3 + V[c]	–	386 ± 65	189 ± 40	131 ± 21	0
M3 + A[c]	–	252 ± 44	161 ± 22	14 ± 5	0
M3 + V + A[c]	–	185 ± 23	145 ± 14	26 ± 5	0

eD: H_2 (5 ml) + sodium formate, acetate, propionate and butyrate (20 mM each). V: vancomycin (100 μg ml^{-1}), A: ampicillin (100 μg ml^{-1}).
[a] μmol of Cl removed kg^{-1} (dry sediment) $week^{-1}$.
[b] mg of SO_4^{2-} consumed L^{-1} $week^{-1}$.
[c] PCB-free cultures were also established under these conditions.

AS14 4 × 250 mm column, a conductivity detector combined to an ASRS-II Ultra conductivity suppressor system (Dionex, Sunnyvale, CA, USA) as described elsewhere [23].

2.3. 16S rRNA gene PCR-DGGE analysis

Metagenomic DNA was extracted from approximately 250 mg of wet sediment with the UltraClean Soil DNA kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. For Bacterial DGGE analysis, PCR amplification was performed with primers GC-357f and 907r [26] as described elsewhere [25]. PCR amplification of the 16S rRNA gene of the dechlorinating members of Chloroflexi was performed with the primers pair GC-Chl348f and Dehal884r using PCR conditions described previously [12]. Archeal 16S rRNA gene DGGE analysis was performed with primers GC-344f and 915r [27] and PCR conditions described previously [25].

PCR products were resolved with a D-Code apparatus (Bio-Rad, Milan, Italy) on a 7% (w/v) polyacrylamide gel (acrylamide-*N*,*N*′-methylenebisacrylamide, 37:1) in 1× TAE with a denaturing gradient from 40% to 60% denaturant for Bacteria, from 45% to 50% for Chloroflexi, and from 40% to 70% for Archea, where 100% denaturant is 7 M urea and 40% (v/v) formamide. The electrophoresis was run at 90 V for 15 h at 60 °C [25]. The gel was stained in a solution of 1× SYBR Green I (Sigma–Aldrich, Milwaukee, WI) in 1× TAE for 30 min and its image captured in UV transillumination with a digital camera supported by a Gel Doc apparatus (Bio-Rad, Milan, Italy). Bands were cut from the gel with a sterile scalpel and DNA was eluted by incubating the gel fragments for 16 h in 50 μL of sterile deionized water at 4 °C. Eight μL of the solution were then used as template to re-amplify the band fragment using the same primers without the GC-clamp and the same PCR conditions described above.

2.4. Sequencing and phylogenetic analysis

The obtained bacterial, Chloroflexi and archaeal amplicons were then sequenced with primer 907r, Dehal884r and 915r, respectively. Sequencing reactions and runs were performed by PRIMM (Milano, Italy). Nucleotide sequences obtained from DGGE analysis were deposited in the GenBank database under the accession numbers FR827870 to FR827894.

The closest relative match to each sequence was obtained with BLASTN facilities [28] and Classifier v.6.0 program at the Ribosomal Database Project (RDP) 10 (http://rdp.cme.msu.edu/classifier/classifier.jsp). Sequences belonging to the Chloroflexi phylum were phylogenetically aligned using ClustalW software (http://clustalw.genome.ad.jp/). Alignments were checked manually and corrected for any misalignments that had been generated. Tree construction was done with Treecon v.1.3b using neighbor-joining methods and a bootstrap analysis with 1000 replicates.

2.5. PCR detection of Dehalococcoides spp.

Metagenomic DNA of all the samples was screened for the presence of *Dehalococcoides* spp. with forward primer DHC 1 and reverse primer DHC 1377 as described by Hendrickson and colleagues [29]. DNA from *Dehalococcoides* spp. strain BAV1 was used as positive control.

3. Results

3.1. Enrichment and activity of the PCB dechlorinating community

No PCB dechlorination was detected throughout the whole incubation period in the sterile controls developed at each sub-culturing step (data not shown). On the contrary, the first biologically active sub-cultures developed (M1) exhibited a remarkable dechlorination activity after 7-weeks of incubation. PCB dechlorination occurred with an initial rate of 143 ± 8 μmol of Cl removed kg^{-1} (dry sediment) $week^{-1}$ and proceeded up to the 30th week of incubation with an average dechlorination rate of 147 ± 23 μmoles of Cl removed kg^{-1} (dry sediment) $week^{-1}$ (Table 1), leading to a final depletion of 73.5% of the overall initial amount of *penta*-, *hexa* and *hepta*-chlorinated PCBs of Aroclor 1254 and to the accumulation of stoichiometric amounts of *tri*- and *tetra*-chlorinated congeners (Fig. 2). The initial average chlorination degree of Aroclor 1254 PCBs was reduced by about 20% (*i.e.*, from 5.1 to 4.1 chlorines per biphenyl) during the same incubation period. PCB congeners 2,2′,4-CB, 2,2′,5-CB, 2,3′,4-CB, 2,3′,5-CB, 2,3′,6-CB, 2,2′,4,4′-CB, 2,2′,4,5′-CB and 2,2′,5,5′-CB (CB = chlorobiphenyl) mainly accumulated in the same microcosms (Fig. 2), indicating that dechlorination was selective towards the *meta* chlorines of 2,3- and 2,3,4-chlorophenyl groups and the *para* chlorines of 3,4- and 2,4,5-chlorophenyl groups according to the dechlorination pattern H′ [5].

The second sub-culturing step was performed in the absence of amendments (sub-cultures M2) as well as in the presence of electron donors (sub-cultures M2 + eD) and of electron donors plus vancomycin or ampicillin (M2 + eD + V and M2 + eD + A sub-cultures). Under all these culture conditions, no lag phases preceding PCB dechlorination were observed (Table 1). PCB dechlorination rates and extents, as well as dechlorination patterns

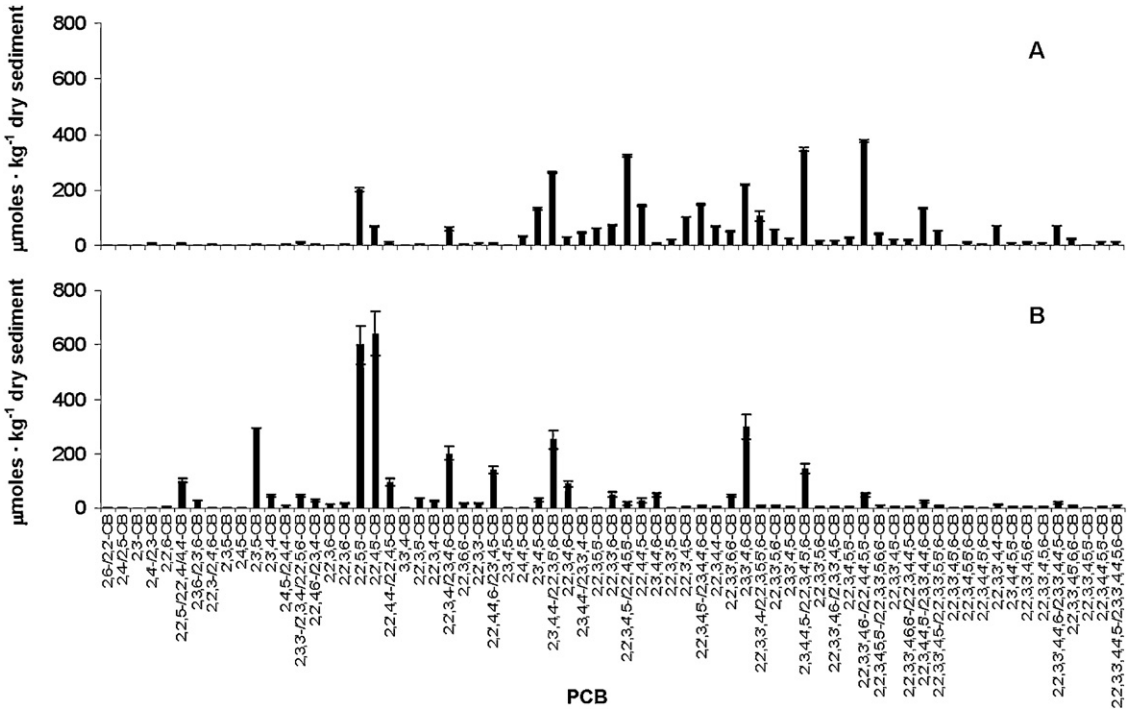

Fig. 2. PCB congener profiles and concentration in M1 microcosms at the beginning (A) and after 30 weeks (B) of incubation. Values are the average of duplicate batch extractions from three independent replicate cultures. Error bars represent standard deviations.

comparable to those detected in M1 sub-cultures were observed in the non-amended sub-cultures M2 and in the parallel ones with electron donors (M2 + eD) (Table 1). On the contrary, the addition of vancomycin or ampicillin together with H_2 and short chain fatty acids (M2 + eD + V and M2 + eD + A sub-cultures) resulted in an increase of the initial PCB dechlorination rate by about 30% and 50% (as compared to M2 + eD), respectively, without any significant effect on the average PCB dechlorination rate (Table 1) and the final PCB dechlorination extent and pattern (data not shown).

During the third sub-culturing step, each sub-culture was transferred under the same culturing conditions used at the second step. In addition, the non-amended sub-cultures M2 were also cultured in the presence of vancomycin, ampicillin or a mixture of the two antibiotics in the absence of electron donors (M3 + V, M3 + A and M3 + V + A, respectively). In the non amended sub-cultures M3, the observed PCB dechlorination rates, extents and patterns were comparable to those detected in M1 and M2 (Table 1). On the contrary, M3 + eD, M3 + eD + V, M3 + eD + A displayed initial and average PCB dechlorination rates lower by 59%, 57% and 38% and by 44%, 7% and 40%, respectively, than those observed in the corresponding cultures of the previous sub-culturing step. In addition, the initial and/or average PCB dechlorination rates displayed by the same sub-cultures were in general lower than those detected in the corresponding non-amended sub-cultures M3 (Table 1).

Finally, initial PCB dechlorination rates higher by 190%, 89% and 39% were observed in M3 + V, M3 + A and M3 + V + A sub-cultures, respectively, than in the non-amended ones (M3) (Table 1). A significant increase of the average PCB dechlorination rate was also detected in the same sub-cultures (Table 1), even though dechlorination extent and patterns very similar to those observed in the non-amended ones were recorded at the end of the incubation (data not shown).

3.2. Sulfate-reducing and methanogenic activities

No sulfate reduction or methanogenic activity was detected throughout the incubation in the sterile controls developed during each sub-culturing step (data not shown).

The first sub-cultures (M1) exhibited a strong sulfate-reducing activity, which resulted in the complete depletion of the initial sulfate (2148 ± 105 mg L^{-1}) during the first 7 weeks of incubation, and a very limited methanogenic activity. Comparable sulfate-reducing activities were observed in the non-amended sub-cultures M2 and M3, where however no methane production was detected (Table 1).

The addition of H_2 and short chain organic acids at the second sub-culturing step (M2 + eD) resulted in sulfate-reducing activity and methane production significantly higher than those observed in the parallel non-supplemented cultures (Table 1). The addition of vancomycin (M2 + eD + V) did not change the time course of sulfate-reduction and methanogenesis observed in its absence (M2 + eD), whereas the addition of ampicillin (M2 + eD + A) remarkably decreased sulfate-reduction, which was 65% of that detected in M2 + eD (Table 1).

Enhanced sulfate reduction and methanogenesis activities were observed in the cultures generated with the third sub-culturing step in the presence of electron donors (M3 + eD) (Table 1). The co-occurrence of vancomycin or ampicillin (*i.e.*, M3 + eD + V and M3 + eD + A) resulted in a reduction of 42% and 89%, respectively, of sulfate depletion rate, and in methane productions lower by 67% and 80%, respectively. Similarly, a remarkable reduction of the sulfate-reducing rate (from 63% to 96%) and no methanogenesis were detected in the presence of antibiotics (M3 + V, M3 + A, M3 + V + A) (Table 1). Finally, in the PCB-free sub-cultures established at the third sub-culturing step, sulfate reduction and methanogenic activities comparable to those detected in the corresponding PCB-spiked sub-cultures were observed (data not shown).

3.3. Characterization of the microbial communities

The structure of the bacterial and archaeal populations selectively enriched in each of the three replicate cultures during the third sub-culturing step under each condition was investigated *via* DGGE analysis of the 16S rRNA genes. The analysis was limited to the sub-cultures that were not amended with H_2 and short chain fatty acids (*i.e.*, M3, M3 + V, M3 + A and M3 + V + A), where higher PCB dechlorination rates were observed (Table 1). In addition, the analysis was performed on the corresponding PCB-free controls to reveal phylotypes whose enrichment was associated with PCB dechlorination.

Similar DGGE profiles were obtained from the replicates of sub-cultures M3 and M3 + V. Conversely, some differences were detected between profiles from replicates of M3 + A and M3 + A + V sub-cultures, both in the presence and in the absence of PCBs (Fig. 3). This indicates that ampicillin had less reproducible inhibitory effects than vancomycin, which may be due to a more rapid inactivation/degradation of this antibiotic and/or to a more frequent acquisition of resistance to ampicillin in the marine microbial community enriched.

The bacterial population detected in the absence of amendments (M3) was composed by Deltaproteobacteria belonging to the *Desulfosarcina*, *Desulfococcus* and *Pelobacter* genera (DGGE bands 5, 6 and 8), a Gammaproteobacterium (DGGE band 1), a Bacteroidetes (DGGE band 4) and an unidentified bacterium (DGGE band 7) (Fig. 3, Table 2). The supplementation of vancomycin resulted in the disappearance of the Bacteroidetes, the Gammaproteobacterium and the unidentified bacterium, and in the detection of a *Desulfovibrio* sp. (DGGE band 9). On the contrary, the addition of ampicillin selected for a different Bacteroidetes bacterium (band 4 disappeared and band 3 was detected), induced the loss of sulfate-reducing Deltaproteobacteria belonging to the genera *Desulfosarcina* and *Desulfococcus*, and resulted in the detection of other Deltaproteobacteria having low sequence identity to the genera *Desulfocapsa* (DGGE bands 11 and 12), *Desulfuromonas* (DGGE band 14), and to an unidentified bacterium (band 10, Fig. 3 and Table 2). In addition, a Chloroflexi phylotype (DGGE band 13) with 100% sequence identity with the uncultured bacterium m^{-1} was detected in the ampicillin-supplemented sub-cultures (Fig. 3, Table 2). Finally, the concomitant supplementation of vancomycin and ampicillin (M3 + V + A) resulted in a combination of the effects produced by each single antibiotic, *i.e.*, in the disappearance of the Bacteroidetes (band 4), the Gammaproteobacterium (band 1) and the Deltaproteobacteria of the genera *Desulfosarcina* and *Desulfococcus*, along with the detection of the Chloroflexi phylotype and of other Deltaproteobacteria (Fig. 3, Table 2). It is noteworthy that the addition of ampicillin, alone or in combination with vancomycin, resulted in the loss of most of the sulfate-reducing bacteria of the culture, as it was also evidenced by the strong decrease of the sulfate reducing activity observed in the presence of this antibiotic (Table 1).

The sub-culturing of M3 microbial community in the absence of PCBs resulted in the loss the *Desulfosarcina* phylotype (band 5) and the appearance of a different Bacteroidetes phylotype (band 3, Fig. 3). However, a *Desulfosarcina* phylotype was detected both in the presence and in the absence of PCBs in the vancomycin-amended sub-cultures (M3 + V) (band 2, Fig. 3); in addition, no *Desulfosarcina* spp. were detected in the PCB-spiked sub-cultures amended with ampicillin and vancomycin plus ampicillin (M3 + A and M3 + V + A), where a remarkable dechlorination activity was observed. This observation indicates that *Desulfosarcina* spp. were not involved in the dechlorination of PCBs. No significant differences were detected between the DGGE banding pattern of the PCB-spiked and PCB-free sub-cultures with vancomycin (M3 + V). Conversely, the Chloroflexi phylotype (band 13) was not detected in the DGGE profile of the PCB-free controls amended with ampicillin (M3 + A) and with vancomycin plus ampicillin (M3 + V + A) (Fig. 3).

When the DGGE analysis was performed with primers specific for the dechlorinating members of the Chloroflexi phylum, including both *Dehaloccoides* spp. and the o-17/DF1 and m^{-1}/SF1 groups, a Chloroflexi phylotype (VL-CHL1) identical to that identified with universal primers in M3 + A and M3 + V + A was detected in all the sub-cultures supplemented with Aroclor 1254 PCBs and not in their corresponding PCB-free controls (Fig. 4). Three additional Chloroflexi phylotypes, namely VL-CHL2, VL-CHL3 and VL-CHL4, were detected regardless of the presence of PCBs (Fig. 4). VL-CHL2, VL-CHL3 and VL-CHL4 had sequence identities higher than 96% to the PCB-dechlorinating bacterium OTU-5 [AY559068], the uncultured eubacterium t0.6.f [AF005745] and the uncultured bacterium clone SPG12_273_283 [FJ746266], respectively, and were previously detected in marine sediments regardless of the occurrence of PCB contamination (Fig. 5). Finally, no amplification products were obtained from all cultures with a 16S rDNA PCR assay specific for *Dehalococcoides* spp.

DGGE analysis of the archeal community showed at each sub-culturing step the dominance of Euryarchaeota and the presence of a single Crenaechaeota with a low sequence identity with a *Caldisphaerales*. Within the Euryarchaeota, methanogens belonging to the orders Methanobacteriales, Methanomicrobiales, Methanosarcinales and one Thermoplasmatales were detected (Table 3). The addition of antibiotics slightly reduced the archeal community richness with the disappearance of the Uncultured Thermoplasmatales phylotype. Identical DGGE profiles were obtained in the presence and in the absence of PCBs (data not shown), indicating that Archea have not any active role in the degradation of PCBs.

4. Discussion

A very few cultures able to extensively dechlorinate commercial PCB mixtures have been characterized so far; all of them were enriched from freshwater or estuarine sediments in low-sulfate mineral media [18,30], or in sediment-free and sulfate-free synthetic mineral media [16,17]. Although the use of well defined cultural media might facilitate the obtainment and the physiological characterization of PCB dehalorespiring bacteria [31], the information and microbial isolates obtained under such conditions might be of limited relevance for interpreting the dechlorination processes occurring *in situ* and designing site specific strategies for intensifying PCB dechlorination activities in progress at the actual site [7]. In this work, the indigenous microbial community of a PCB-contaminated marine sediment of the Brentella Canal of the Venice lagoon previously enriched on five coplanar PCBs [25] was sub-cultured in the presence of high concentrations of Aroclor 1254 under biogeochemical conditions miming those occurring *in situ*. 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 (Table 1, Fig. 2) by exhibiting the same selectivity (pattern H′) reported previously for the same culture during its enrichment on coplanar PCBs [25]. 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 chlorination degree and substitution can also be expressed by the culture *in situ* in the reductive dechorination 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.

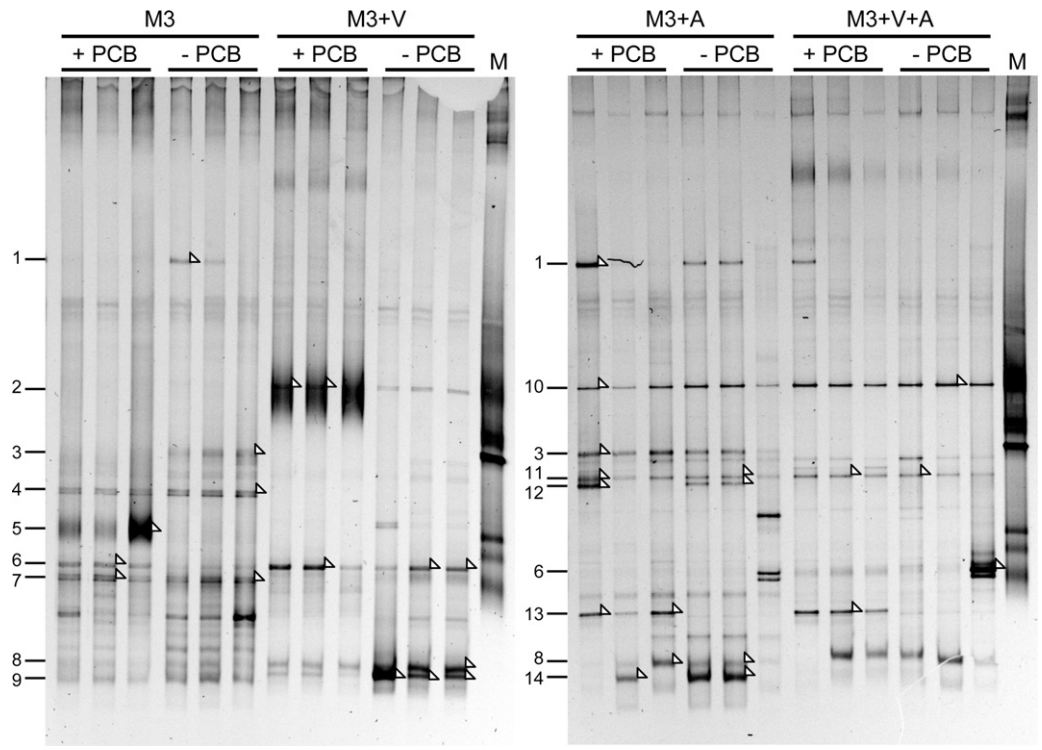

Fig. 3. DGGE analysis of the bacterial population enriched after the third sub-culturing step in the PCB-spiked and PCB-free cultures not amended with electron donors. M: marker. Arrows indicate the excized and sequenced bands. Band numbers are indicated on the left side of each gel. The analysis was performed independently on the three replicate cultures set up under each condition.

The adoption of different electron donors and microbial inhibitors in combination with an integrated chemical, microbiological and molecular biology monitoring methodology allowed us to collect key insights into the major PCB dechlorinators of the culture. Hydrogen and/or short chain fatty acids were employed as they have been frequently found to sustain PCB dechlorination in sediment slurries and sediment-free cultures enriched from freshwater or estuarine sediments in low-sulfate or sulfate-free synthetic media [6,8,11,12,18,20]. However, in this case their repeated supplementation resulted in detrimental effects on PCB dechlorination rate and in the remarkable stimulation of both sulfate-reducing and methanogenic activities (Table 1), probably as a consequence of the different origin of the PCB dechlorinating culture, *i.e.*, marine, and the different biogeochemical conditions adopted, *i.e.*, the use of actual marine site water. This suggests (i) that neither sulfate-reducing bacteria nor methanogens were directly involved in PCB dechlorination and (ii) that the addition of H_2 and the mixture of short chain fatty acids under the

Table 2
Phylogenetic affiliation of bands excized from the bacterial community DGGE gel obtained from M3, M3 + V, M3 + A and M3 + V + A sub-cultures.

DGGE band	Closest relative [accession #]	bp identity (%)	Closest described relative [accession #]	Identity %	Phylogenesis
3	*Prolixibacter bellariavorans* [AY918928]	504/512 (99%)			Bacteroidetes
4	Uncultured Bacteroidetes clone VHS-B3-78 [DQ394963]	520/529 (99%)	*Prolixibacter bellariavorans* [AY918928]	90%	
1	Uncultured Gamma proteobacterium [AM229493]	525/531 (99%)	*Thiomicrospira chilensis* strain Ch-1 [AF013975]	99%	Gamma proteobacteria
2	*Desulfosarcina variabilis* [M34407]	548/549 (99%)			
5	Uncultured Delta proteobacterium 56 EDB1 [AM882603]	526/528 (99%)	*Desulfosarcina variabilis* [M34407]	99%	
6	*Desulfococcus multivorans* [AF418173]	540/540 (100%)			
8	*Pelobacter carbinolicus* DSM 2380 [CP000142]	531/538 (99%)			Delta proteobacteria
9	Desulfovibrionaceae bacterium enrichment culture clone MS_H2_U7 [HQ400841]	510/536 (96%)	*Desulfovibrio zosterae* [Y18049]	93%	
11	Delta proteobacterium LacK10 [AY771932]	526/544 (97%)	*Desulfocapsa sulfexigens* [Y13672]	97%	
12	Uncultured bacterium clone s78 [AY171314]	521/538 (97%)	*Desulfocapsa sulfexigens* [Y13672]	98%	
14	Desulfuromonadales bacterium Tc37 [AB260047]	544/547 (99%)			
13	Uncultured bacterium m1 [DQ113418]	496/496 (100%)	*Dehalococcoides* sp. BAV1 [CP000688]	92%	Chloroflexi
7	Uncultured bacterium clone TopBa59 [FJ748805]	522/548 (96%)	*Thermolithobacter ferrireducens* [AF282253]	84%	Firmicutes
10	Uncultured Delta Proteobacterium [AJ889125]	451/473 (96%)	*Eubacterium tortuosum* [L34683]	83%	

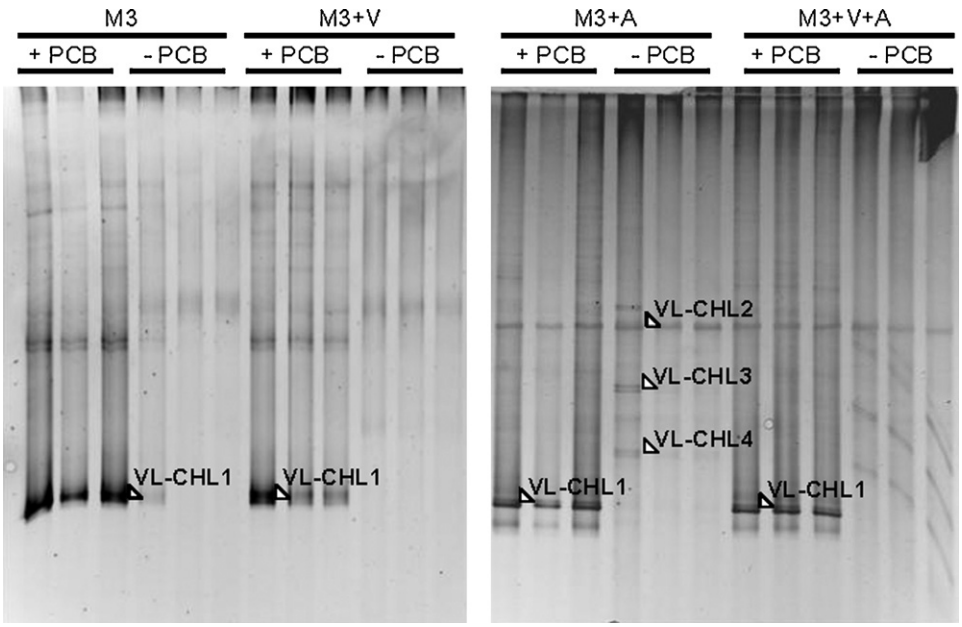

Fig. 4. DGGE analysis of the Chloroflexi population enriched after the third sub-culturing step in the PCB-spiked and PCB-free cultures not amended with electron donors. Arrows indicate the excized and sequenced bands. The analysis was performed independently on the three replicate cultures set up under each condition.

biogeochemical conditions adopted in this study adversely affected PCB dechlorination by stimulating the non specialized members of the culture, such as sulfidogenic and methanogenic bacteria, which compete with PCB dehalorespirers for H_2 and other electron donors [32]. The evidence that PCB dechlorination rate was increased in microcosms where sulfate-reducing and methanogenic activities were remarkably inhibited by the presence of vancomycin and ampicillin (Table 1) supported such hypothesis and indicated that PCB dechlorinators of the investigated cultures were resistant to vancomycin and ampicillin.

DGGE analysis with Archaeal and Bacterial primers confirmed that neither methanogens, nor bacteria of the Proteobacteria,

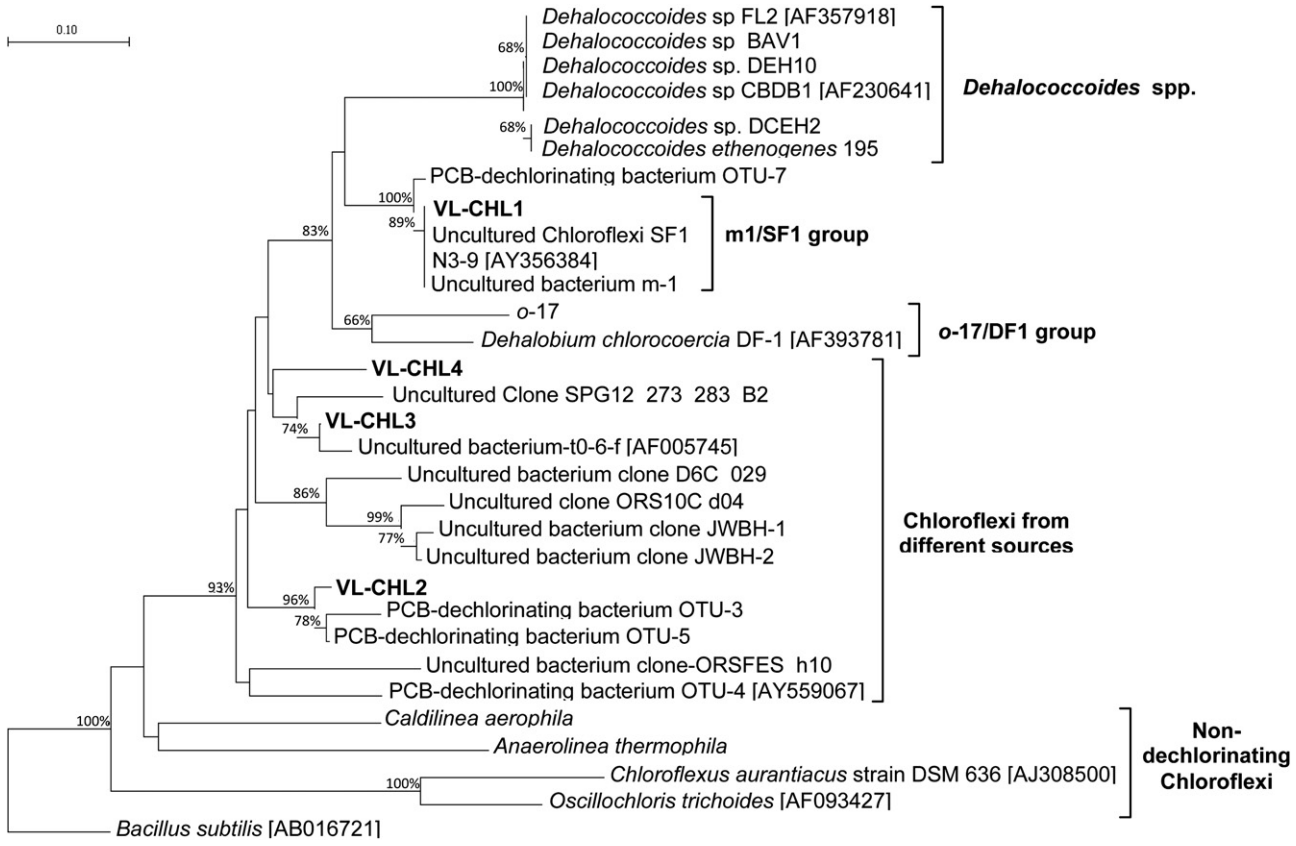

Fig. 5. Phylogenetic placement of the Chloroflexi members of the enriched microbial communities.

Table 3
Phylogenetic affiliation of the Archaeal members of the microbial community of M3, M3 + V, M3 + A and M3 + V + A sub-cultures.

DGGE band	Closest relative [accession #]	Identity %	Closet described relative [accession #]	Identity %	Phylogenetic affiliation [confidence]
1A	Uncultured archaeon clone NKtzac17 [EU983172]	95%	Methanobacteriaceae archaeon RMAS [AB523785]	76%	Euryarchaeota[93%] Methanobacteria[21%] Methanobacteriales[21%]
2A	Uncultured archaeon clone MOB7-5 [DQ841240]	100%	*Methanosaeta harundinacea* [AY970347]	99%	Euryarchaeota[100%] Methanomicrobia[100%] Methanosarcinales[100%]
3A	Uncultured euryarchaeote clone 56S_3A_18 [DQ837286]	98%	*Methanoculleus receptaculi* [DQ787475]	93%	Euryarchaeota[100%] Methanomicrobia[100%] Methanomicrobiales[100%]
4A	Uncultured Thermoplasmatales [FJ477308]	100%	Methanogenic archaeon WGK1 [GQ339877]	79%	Euryarchaeota[87%] Thermoplasmata[23%] Thermoplasmatales[23%]
5A	Uncultured *Methanosarcina* sp. clone LW4 [DQ155311]	99%	*Methanosarcina semesiae* [AJ012742]	97%	Euryarchaeota[100%] Methanomicrobia[100%] Methanosarcinales[100%]
6A	Uncultured crenarchaeote clone EI_H09 [AY454579]	99%	*Ignisphaera aggregans* [DQ060321]	80%	Crenarchaeota[83%] Thermoprotei[83%] Caldisphaerales[42%]
7A	Uncultured *Methanogenium* sp. isolate PSW29 [EF043530]	99%	*Methanogenium cariaci* [M59130]	98%	Euryarchaeota[100%] Methanomicrobia[100%] Methanomicrobiales[100%]

Bacteroidetes and Fimicutes phyla were involved in PCB dechlorination, since their detection was not related to the presence of PCBs and of PCB dechlorinating activity under all the culture conditions tested (Fig. 3, Table 3). With primers specific for the dechlorinating members of the phylum Chloroflexi, conversely, 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 (Fig. 4). This clearly indicates that the presence of VL-CHL1 was strictly related to the presence of the dechlorination activity, thus confirming that VL-CHL1 was the PCB dechlorinator enriched in the culture. The fact that VL-CHL1 was detectable with nonspecific primers only in M3 + A and M3 + V + A subcultures and not in M3 and M3 + V (Fig. 3), despite similar final extents of PCB dechlorination were detected under all culture conditions, might be attributed to remarkable differences of the growth of sulfate-reducing bacteria among subcultures amended (M3 + A and M3 + V + A) and non amended (M3 and M3 + V) with ampicillin. In particular, the remarkably lower growth of sulfate reducers throughout incubation in the presence of ampicillin might have contributed to the increase of the final relative abundance of VL-CHL1 with respect to the overall community above the DGGE detection limit, that has been estimated around 1% relative abundance [33,34]. Conversely, the higher growth of sulfate reducers in the absence of ampicillin (*i.e.*, M3 and M3 + V) might have contributed to maintain the final relative abundance of VL-CHL1 with respect to the overall bacterial community under the DGGE detection limit. By using group specific primers targeting Chloroflexi, differences in the abundance of the non Chloroflexi members of the microbial community, such as of sulfate reducers, did not affect the detection of VL-CHL1, which was detected in all cultures. 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 [17,35–37]. It is also consistent with the detection of lower initial PCB dechlorination rates in the presence of ampicillin, where a remarkable inhibition of the sulfate reduction rate was detected, as compared to vancomycin, since the growth of the sole *Dehalococcoides*-like PCB dehalorespiring bacterium isolated so far, to which VL-CHL1 is closely related, is favored by the growth of sulfate-reducing bacteria [31]. This is the first time that a Chloroflexi strain is reported as the PCB dechlorinator in a culture enriched from a contaminated marine sediment, which typically display biogeochemical and microbiological features quite different from those occurring in freshwater and estuarine habitats [21]. In addition, VL-CHL1 is also the *Dehalococcoides*-related strain with the broadest dechlorination activity towards PCBs among those described so far. Indeed, bacterium m-1 and a very closely related phylotype previously identified in estuarine and river sediment slurry cultures [13,38] displayed a very narrow selectivity, from one specific congener to singly flanked and unflanked *meta* dechlorination of a restricted group of coplanar PCB congeners. Several of the other PCB dechlorinators of the Chloroflexi phylum obtained from freshwater or estuarine sediments exhibited a dechlorination selectivity limited to one or a few chlorine configurations [10–12,38]. There is only a case of *Dehalococcoides sp.* (strain CBDB1) obtained from freshwater sediment able to dechlorinate Aroclor 1260 PCBs in pure culture in a defined medium [20], whereas the majority of the few studies available reports on the reductive dechlorination of commercial mixtures of PCBs by populations of multiple *Dehalococcoides* or *Dehalococcoides*-related strains [16–18].

In conclusion, bacterium VL-CHL1 was found to be the PCB dechlorinator occurring in the PCB contaminated marine sediments of Venice Lagoon. It is the first dechlorinator identified so far in marine sediments and it displays a dechlorination activity and a specificity much wider than those of the other *Dehalococcoides*-like bacteria described so far in the literature. Further, it is the first PCB dechlorinator able to display such a broad and extensive dechlorination activity under biogeochemical conditions close to those occurring *in situ* in a marine habitat. This feature is of special relevance in the perspective to develop a site tailored strategy for the stimulation of *in situ* dechlorination of wheatered PCBs occurring in the Brentella Canal.

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