Diversity of dehalorespiring bacteria and selective enrichment of aryl halides-dechlorinating consortium from sedimentary environment near an oil refinery

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Abstract

This study demonstrated the potential for reductive dechlorination of aryl halides in a sedimentary environment by naturally occurring bacteria. A laboratory sediment microcosm and highly enriched, stable dechlorinating cultures were established using hydrogen as electron donor and 2,3-dichlorophenol (2,3DCP), monochlorophenol (MCP) and dichlorobenzene (DCB) as electron acceptors. 16S rRNA gene sequencing of dominant DGGE bands assigned detected phylotypes in chlorophenols (CPs) amended sediment microcosms to the genera Anaerospora, Pseudomonas, Desulfotobacterium, Clostridium, Mycobacterium, and Peptoclostridium, beside uncultured marine Bacterioplankton, Halochromatium and Sedimentibacter. Chlorobenzene (CB) amended sediments showed the same community in addition to Bacillus sp. Major operational taxonomic units (OTUs) in CPs enrichment cultures were assigned to Anaerospora hongkongensis, Pseudomonas stutzeri, Pseudomonas pseudocaligenes, Clostridium sp., Desulfotobacterium dichloroeliminans, beside unidentified marine bacterioplankton. Enrichment on DCB resulted in predominance of the same populations in addition to Peptoclostridium sp. and Dehalococccoides mccartyi. Dechlorination in enrichment cultures was mainly assigned to Desulfotobacterium and Dehalococcoides, which are both known for their ability to couple dechlorination to growth in a halorespiration way. Enrichment cultures containing Desulfotobacterium showed ortho dechlorination activity of 2,3DCP to 2 chlorophenol (2CP) and 3 chlorophenol (3CP). These results provided compelling evidence that sedimentary environments in the vicinity of oil refineries do harbor dehalorespiring bacteria capable of reductive dechlorination of various aryl halides pollutants.

Introduction

Aryl halides like chlorophenols and chlorobenzenes are greatly harmful to all organisms owing to their mutagenic, genotoxic, cytotoxic, immunogenicity and carcinogenic properties [1]. The transformation of CPs in particular may result in an elevated toxicity of intermediary or end products compounds attributable to the development of electrophilic metabolites that may damage DNA or its products [2]. Due to these properties, CPs and CBs are considered as significant pollutants and potential human carcinogens by the World Health Organization and the United States Environmental Protection Agency [3,4]. The main sources for environmental pollution with CPs and CBs include effluents from the pulp and paper production, petrochemical, chemical, oil refineries, coking plants, pharmaceuticals, plastic, pesticides industries and textiles manufacturing [5]. The common methods for removal of aryl halides from wastewater include ion exchange, adsorption, chemical oxidation, advanced oxidation processes and liquid–liquid extraction [5,6]. The previous methods are costly, not green and technically challenging. Some methods and technologies for microbial bioremediation have been investigated and valued in the field, greenhouse and using microbial consortia, applying biostimulation and bioaugmentation strategies [7,8]. These strategies are valuable, green and economically feasible. CPs could efficiently be biodegraded under different environmental conditions [4]. CPs and CBs, like many chlorinated aromatic compounds, are amenable to reductive dehalogenation and are bio-transformed in anaerobic soils, sediments and sewage sludge. In microbial degradation of CPs and/or CBs, certain microbial communities expressed their degrading capability under limited anaerobic conditions; initiated by reductive dehalogenation prior to ring cleavage and downstream catabolic activities [9,10]. Anaerobic microorganisms have the ability to use aryl halides like CPs or CBs as electron acceptors in their respiration to support their growth with oxidation of simple electron acceptors. 16S rRNA gene sequencing of dominant DGGE bands assigned detected phylotypes in chlorophenols (CPs) amended sediment microcosms to the genera Anaerospora, Pseudomonas, Desulfotobacterium, Clostridium, Mycobacterium, and Peptoclostridium, beside uncultured marine Bacterioplankton, Halochromatium and Sedimentibacter. Chlorobenzene (CB) amended sediments showed the same community in addition to Bacillus sp. Major operational taxonomic units (OTUs) in CPs enrichment cultures were assigned to Anaerospora hongkongensis, Pseudomonas stutzeri, Pseudomonas pseudocaligenes, Clostridium sp., Desulfotobacterium dichloroeliminans, beside unidentified marine bacterioplankton. Enrichment on DCB resulted in predominance of the same populations in addition to Peptoclostridium sp. and Dehalococcoides mccartyi. Dechlorination in enrichment cultures was mainly assigned to Desulfotobacterium and Dehalococcoides, which are both known for their ability to couple dechlorination to growth in a halorespiration way. Enrichment cultures containing Desulfotobacterium showed ortho dechlorination activity of 2,3DCP to 2 chlorophenol (2CP) and 3 chlorophenol (3CP). These results provided compelling evidence that sedimentary environments in the vicinity of oil refineries do harbor dehalorespiring bacteria capable of reductive dechlorination of various aryl halides pollutants.
donating substrates leading to the formation of less toxic and more biodegradable compounds [11]. In this sense, reductive dechlorination, halorespiration or chloridogenesis have been well documented [12,13]. Reductive dechlorination or dehalorespiration represent one of the most promising approaches for detoxification of the environment from a variety of chlorinated pollutants [14].

Dehalorespiring bacteria are quite diverse and include Dehalobacter, Desulfitobacterium, Anaeromyxobacter, Geobacter, Desulfomonile, Desulfuromonas, Desulfovibrio, Sulfurospirillum, Dehalogenimonas, Dehalobium and Dehalococcoides [15]. Among all dehalorespiring bacteria, Desulfitobacteria were mainly reported for its ability to reductively dechlorinate various aryl halides [16]. Many microorganisms either individually or in the consortium have been characterized for their selective reductive dechlorination and ultimately the degrada-tion of a various array of aryl halides including CPs and CBs [17–19]. Activated sludge and sedimentary environments in particular were reported to contain complex communities of microorganisms which are able to reductively dechlorinate various chlorinated compounds [20,21]. Microbial community capable of degrading 4CP, 2,4DCP and 2,4,6-trichlorophenol were studied and found to contain collaborative members of Proteobacteria, as Rhodococcus, Novosphingobium and Xanthomonas, in a sludge granule as a stable consortium for efficient dechlorination activity [22]. Chen et al. [23] reported that variations in the microbial population during repetitive anaerobic dechlorination of pentachlorophenol showed a predominance of Methanosarcina, Syntrophobatus, Anaeromusa, Zoogloea and Treponema as PCP dechlorination consortium, and demonstrated that the iron-cycling bacteria Syntrophobatus, Anaeromusa and Zoogloea were likely to play an important role in PCP dechlorination.

Different environments were found to harbour novel microbial communities capable of dechlorination of various aryl halides pollutants [24,25]. Among all studied environments, sedimentary environments represent one of the most promising sites for isolation of microbial communities that are able to chlorinate and detoxify various aryl halides pollutants. Diversity of CPs and/or CBs dehalorespiring communities in different sedimentary environments is not greatly exploited [26–28]. Studying microbial diversity in contaminated sites is crucially important in understanding the role and interactions among aryl halides-utilizing microorganisms. This study was designed to determine the diversity and dynamics of bacterial community in sedimentary environments near an oil refinery due to the selective pressure of persistent aryl halides pollutants like CPs and CBs attempting to establish highly enriched microbial consortia containing reductive dechlorinating bacterial strains.

Materials and methods

Sediment samples collection

The contaminated sediment samples with petroleum oil derivatives were collected from red sea surrounding areas of oil processing refineries at Yanbu industrial city, KSA. Collected samples had a different texture and colours which refers to the level of contamination. The contaminated sediment samples were taken from two sites; S1, at 5 m depth (24°04′21.72″N, 38°03′05.54″E) and S2, at 10 m depth (23°53′36.36″N, 38°11′42.54″E) under the sea surface. Collected samples then placed into sterile bottles, closed tightly under the water and transferred to the laboratory for further microbiological processing.

Establishment of anaerobic sediment and enrichment microcosms

Bacterial sediment microcosms were established in a leak proof screw caped 250 ml sterile Duran bottles containing 20% (w/v) sediment in Basal Mineral Medium (BMM) [29]. Microcosms were amended with 2,3DCP, MCP and DCB (Sigma-Aldrich, Deisenhofen, Germany) to a final concentration of 100 µM. Bottles were flushed with hydrogen gas (99.9% purity) to ensure a head space of H2 and then stored in such an anaerobic condition in dark at 30°C for one month prior to use. BMM used in this study was composed of (per litre): K2HPO4, 4.36 g; NaH2PO4, 3.45 g; (NH4)2SO4, 1.26 g; MgSO4.6H2O, 0.91 g; trace salt solution, 1 ml. Trace salt solution contained (per 100 ml): CaCl2.2H2O, 4.77 g; FeSO4.7H2O, 0.37 g; CoCl2.6H2O, 0.37 g; MnCl2, 0.1 g; Na2MoO4.2H2O, 0.02 g. All microcosms were incubated at 30°C for monitoring purposes. Unamended microcosms were prepared as a control. Enrichment microcosms were prepared by successive transfer of 10% (v/v) from aryl halides-amended microcosms into 250 ml Duran Bottles containing fresh 100 ml of BMM. Microcosms were supplemented with chlorinated hydrocarbons to a final concentration of 100 µM as electron acceptors and 0.5 g/L yeast extract as a carbon source. Enrichment microcosms were injected with hydrogen gas as an electron donor and incubated at 30°C for a period of one month prior to the examination.

Establishment of enrichment anaerobic cultures

Enrichment anaerobic dechlorinating cultures were established in fresh BMM in 50 ml serum bottles capped with a teflon-coated butyl rubber stoppers and sealed with aluminium caps under hydrogen headspace. Chlorinated hydrocarbons were added to cultures as a main terminal electron acceptor in a final concentration of 100 µM. Yeast extract (0.5 g/L) was used as a carbon source. Enrichment sediment-free cultures were
obtained after at least two successive transfers of cultures (10% (v/v)) into a new medium containing chlorinated phenols as electron acceptors. All cultures were incubated at 30°C under a headspace of H2. Selected cultures were monitored for dechlorination activities by gas chromatography analysis.

**Extraction of genomic DNA**

For denaturing gradient gel electrophoresis (DGGE) analysis, genomic DNA was extracted from cultures by using UltraClean™ Soil DNA Purification Kit (MO BIO™ Laboratories, USA) according to the manufactured instruction manual.

**PCR-DGGE**

For DGGE analysis, primers (EUB341F-GC) (5′-GCCTACGCGGCGCCTGG-3′) and (EUB517R) (5′-ATTACCGCGGCTGCTGG-3′) were used to amplify the 16SrRAN gene, which corresponds to positions 341 and 517 in *Escherichia coli* [30]. Amplification reaction mixture (25 µl) consisted of 0.5 U Taq DNA polymerase (Invitrogen, USA), 1 µl of total DNA, 2 µl of 10× PCR buffer, each primer at a concentration of 0.25 µM, and a mixture containing each deoxynucleoside triphosphate at a concentration of 100 µM. PCR was performed on 2720 thermal cycler (Applied Biosystems, USA). For specific amplification, A step-down PCR program was used as follows: initial denaturation at 95°C for 5 min, followed by five cycles of 94°C for 40 s, annealing at 65°C for 40 s, and extension at 72°C for 40 s; 5 cycles of 94°C for 40 s, annealing at 60°C for 40 s and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 50°C for 40 s. PCR products were verified by electrophoresis on 1% agarose gels with the size markers (1 kb Plus DNA ladder, Invitrogen, USA).

DGGE was performed using Dcode Mutation Detection System (Bio Rad Laboratories Ltd., Hertfordshire, UK). PCR products were electrophoresed with 0.5× TAE buffer (1× TAE buffer is 0.04 M Tris base, 0.02 M sodium acetate and 10 mM EDTA [pH 7.4]) on 8% polyacrylamide gel containing 25–50% denaturing gradient of formamide and urea. DGGE was conducted at 60°C for 5 h at a voltage of 200 V. The gel was stained with SYBR Green I Nucleic acid gel stain (Cambrex Bio Science Rockland, USA), photographed and analysed with Quantity One 1D software (BioRad, UK). Intensity of DNA bands relative to the total amount of DNA was densitometrically measured and used to express the relative richness of each phylotype [32,33].

**Sequence analysis and phylogenetics**

The obtained 16S rDNA sequences were edited by Genetyx-Win MFC application software version 4.0 and then used as a query in a BLASTn search against the non-redundant DNA database (http://www.ncbi.nlm.nih.gov/BLASTP/) to identify sequence similarities with their closest matches and nearest phylogenetic neighbours in GenBank. The reference 16S rRNA gene sequences were downloaded from the GenBank database (National Center for Biotechnology Information, National Library of Medicine, USA). Multi sequence alignments were performed by Clustal W1.83 XP [34] and phylogenetic trees were constructed with MEGA4 software [35] using the neighbour-joining method [36].

**Gas chromatography/FID analysis**

Chlorinated hydrocarbons were analysed after extraction from enrichment cultures with methylene chloride. Resolved organic phase was centrifuged at 15,000 rpm for 10 min, and supernatant was analysed using high-resolution GC/FID on a Thermo Scientific Trace 1300 series instrument equipped with a split/splitless injector, a flame ionization detector and a TG-5MS (Restek, USA) fused silica capillary column (30 m, 0.25 mm i.d., film thickness 0.25 µm). The following program was used to achieve a maximum separation between peaks: column oven temperature, 40°C for 3 min, 40°C to 80°C at 8°C/min, 80°C to 190°C at 44°C/min, 190°C for 5 min. Helium was used as a carrier gas.

**Results**

**Bacterial community structure in sediment microcosms**

Bacterial community structure in a laboratory established anaerobic microcosms amended with different aryl halides was monitored by DGGE fingerprinting over one month of incubation (Figure 1A). The DGGE fingerprints obtained in this study with metagenomic DNA from microcosms amended with 2,3-DCP, MCP or DCB showed a change in the bacterial community structure.
Figure 1. DGGE fingerprints of 16S rRNA gene fragments from established sediment microcosms (A) and dechlorinating enrichment cultures (B). Lanes S1 and S2 represent bacterial fingerprint of original unamended sediments from 5 and 10 km depths, respectively. Sediment microcosms amended with 2,3DCP, MCP or DCB are indicated above each lane. Each sediment microcosm and/or enrichment culture was analysed in duplicates.

from original sediment. 16S rRNA gene sequencing of dominant DGGE bands assigned detected operational taxonomic units (OTUs) in original unamended sediments to Anaerospora hongkongensis, Pseudomonas stutzeri, Clostridium sp., Desulfotobacterium dichloroeliminans, Pseudomonas pseudoalcaligenes, Mycobacterium sp., Peptoclostridium sp., Bacillus sp., and uncultured members of Sedimentibacter sp. and marine bacterioplankton (Table 1).

OTUs in amended sediment microcosms were diverse and rich compared to original sediments. Figure 2 shows relative abundances and species richness in established sediment microcosms amended with 2,3DCP, MCP or DCB. 2,3DCP amended sediment microcosms showed a predominance of about 11 major OTUs identified as Anaerospora hongkongensis, Pseudomonas stutzeri, Pseudomonas pseudoalcaligenes, Clostridium sulfidigenes, Clostridium sp., Desulfotobacterium dichloroeliminans, Mycobacterium sp., uncultured marine bacterioplankton, uncultured bacterium, uncultured Halochromatium sp. and uncultured Sedimentibacter sp. MCP amended sediment microcosms were dominated by Anaerospora hongkongensis, Pseudomonas stutzeri, Pseudomonas pseudoalcaligenes, Clostridium sulfidigenes, Clostridium sp., Desulfotobacterium dichloroeliminans, Mycobacterium sp. and uncultured members of Sedimentibacter sp. and marine bacterioplankton. OTUs detected in DCB-amended sediments were affiliated to Anaerospora hongkongensis, Pseudomonas stutzeri, Pseudomonas pseudoalcaligenes, Clostridium sulfidigenes, Clostridium sp., Desulfotobacterium dichloroeliminans, Mycobacterium sp., Bacillus sp. and uncultured Sedimentibacter sp.

S1 samples were taken from about 5 m deep-sediments of shallow water opposite to an oil refinery plant. Unamended sediments from (S1) showed diverse relative abundance, 23% uncultured bacteria, 19% Pseudomonas stutzeri, 17% Mycobacterium sp., 16% Clostridium sp., 8% Bacillus sp. 11% Pseudomonas pseudoalcaligenes and 4% for one OTUs affiliated to Desulfotobacterium dichloroeliminans. The amended S1 samples showed various changes in the development of microcosm. The uncultured bacterium remained unchanged in the presence of 2,3DCP, but was significantly reduced in the presence of DCB and not detected in MCP. Pseudomonas stutzeri was reduced 60% in samples amended with 2,3DCP and MCP and completely eliminated in DCB microcosm. OTU resemble Clostridium sp. was reduced 50% and 40% in sample amended with 2,3DCP and MCP, respectively; however, in the presence of DCB, it was flourished 1.7 folds. Amendment of sediment samples with chlorinated hydrocarbons allowed other selective strains to grow. Anaerospora hongkongensis was detected at a relative abundance of 9% in the presence of 2,3DCP and MCP, nevertheless, reached 18% in sample amended with
Table 1. OTUs identified in established anaerobic sediment microcosms.

| DGGE Band | Identity | Similarity (%) | Accession No. | Phylogenetic affiliation |
|-----------|----------|----------------|--------------|-------------------------|
| A1        | Anaerospora hongkongensis HKU15 | 96 | NR_115223 | Firmicutes/Sporomusaceae |
| A2        | Unidentified marine bacterioplankton ES05B_E078 | 97 | KC002542 | Bacteria/environmental sample |
| A3        | Pseudomonas stutzeri NIOSSD012248 | 97 | KY606664 | Gammaproteobacteria/Pseudomonadaceae |
| A4        | Clostridium sp. JM-1 | 99 | KF924760 | Firmicutes/Clostridiaceae |
| A5        | Desulfotobacterium dichloroeliminans LMG P-21439 | 97 | AJ665938 | Firmicutes/Psuedomonadaceae |
| A6        | Pseudomonas pseudoalcaligenes strain E1205 | 99 | KY667526 | Gammaproteobacteria/Pseudomonadaceae |
| A7        | Mycobacterium sp. SM-2017 | 98 | LC218398 | Mycobacterium/Sporomusaceae |
| A8        | Uncultured bacterium NT61a9_15084 | 98 | JQ383993 | Bacteria/environmental sample |
| A9        | Clostridium sulfidigenes Marseille-P1446 | 99 | LT223655 | Firmicutes/Clostridiaceae |
| A10       | Uncultured Halochromatium sp. G0B2-IINAL | 97 | GQ242422 | Gammaproteobacteria/environmental sample |
| A11       | Uncultured Sedimentibacter sp. OTU 545 | 100 | LT024545 | Firmicutes/Clostridiaceae |
| A12       | Uncultured bacterium NT61a9_15084 | 98 | JQ383993 | Bacteria/environmental sample |
| A13       | Desulfotobacterium dichloroeliminans LMG P-21439 | 97 | AJ665938 | Firmicutes/Psuedomonadaceae |
| A14       | Pseudomonas pseudoalcaligenes strain E1205 | 99 | KY667526 | Gammaproteobacteria/Pseudomonadaceae |
| A15       | Peptoclostridium sp. 37 | 100 | KX170738 | Firmicutes/Psuedomonadaceae |
| A16       | Clostridium sp. H2 | 100 | LC194786 | Firmicutes/Clostridiaceae |
| A17       | Bacillus sp. 3T3 | 100 | HM804387 | Firmicutes/Clostridiaceae |
| A18       | Clostridium sp. AN-B51C | 98 | FR872936 | Firmicutes/Clostridiaceae |

Figure 2. Numerical analysis of the DGGE fingerprints revealing bacterial diversity, relative abundances (%) and species richness in sediment microcosms amended with 2,3DCP, MCP or DCB. S1 and S2 represent unamended control samples.

DCB. Desulfotobacterium dichloroeliminans was detected in microcosm amended with 2,3DCP at a relative abundance of 8.5%. Furthermore, another OTU of Desulfotobacterium dichloroeliminans was enriched and flourished two folds in the presence of MCP and DCB. Detection of other strains possibly linked to dehalorespiration was also observed as Clostridium sulfidigenes in all amended samples. Uncultured Sedimentibacter sp. and unidentified marine bacterioplankton were detected in samples amended with 2,3DCP and MCP, and the uncultured Halochromatium sp. was detected in the presence of 2,3DCP.

Sample (S2), on the other hand, was obtained from approximately 10 m deep-sediments, 5 km away from the seashore. The relative abundance of microbial strains in the unamended samples was predominated by two OTUs of Clostridium sp. comprised 61% of total detected strains reflecting the anaerobic nature of this site. Unamended samples were dominated by uncultured bacteria, unidentified marine bacterioplankton and uncultured Sedimentibacter sp. with the relative abundance of 21%, 14% and 4%, respectively. Anaerospora hongkongensis was detected in the presence of 2,3DCP, MCP or DCB and their relative abundance reached 15%, 17% and 8%, respectively. Pseudomonas stutzeri, Mycobacterium sp., Clostridium sulfidigenes and Desulfotobacterium dichloroeliminans were detected only in samples amended with MCP and DCB, at relative abundance levels ranging from 4 up to 27%. Likewise, Pseudomonas pseudoalcaligenes and Desulfotobacterium dichloroeliminans were detected only in the presence of 2,3DCP at relative abundance of 46% and 17%, respectively. Conversely, the OTUs resembling Clostridia were reduced from 45% in unamended sample to 5% and 16% in the presence of 2,3DCP and DCB, respectively. Another OTU of Clostridium sp. was not significantly changed but detected only in the presence of DCB.
This study also attempts to monitor the microorganisms responsible for the dechlorination in sediment microcosms or enrichment cultures. The DGGE sediment microcosm OTU A5 and A13 have been assigned to the genus *Desulfitobacterium*, which is known for its ability to couple dechlorination to growth in a halorespiration way. Presence of *Desulfitobacterium* in such sediment microcosms represents an evidence for the corresponding dechlorination activity.

### Bacterial community structure in anaerobic enrichment cultures

Enrichment on chlorinated hydrocarbons resulted in the elimination of most bacterial species and enrichment of those survived the provided conditions. After successive transfers to obtain sediment-free cultures, enrichment dechlorinating cultures showed a remarkable change in bacterial community structure. Bacterial populations were reduced in all amended cultures. Basically, enrichment leads to increasing populations only involved in dechlorination process which can be revealed by decreasing OTUs in enrichment cultures.

The DGGE fingerprints of enrichment cultures with different chlorinated hydrocarbons (Figure 1(B)) showed a change in the bacterial community structure from sediment microcosms as well as original sediment. Most of the bacterial populations were deteriorated in both S1 and S2 original unamended sediments which were dominated by only fewer phytypes. Table 2 shows the OTUs identified in established anaerobic enrichment cultures. Generally, enrichment cultures from both unamended sediments (S1 and S2) showed the survival of few members identified as *Pseudomonas pseudoalcaligenes*, *Pseudomonas sp.*, *Desulfitobacterium dichloroeliminans*, *Dehalococcoides mccartyi* and *Clostridium* sp. Figure 3 shows the relative abundances and species richness of bacterial strains dominating the anaerobic enrichment cultures. 16S rRNA gene sequencing of dominant DGGE bands from 2,3DCP enrichment cultures assigned detected major OTUs to members of *Anaerospora hongkongensis*, *Pseudomonas stutzeri*, *Pseudomonas pseudoalcaligenes*, *Clostridium* sp., *Desulfitobacterium dichloroeliminans*, beside unidentified marine bacterioplankton.

**Table 2. OTUs identified in established anaerobic highly enrichment cultures.**

| DGGE band | Identity | Similarity (%) | Accession No. | Phylogenetic affiliation |
|-----------|----------|----------------|---------------|-------------------------|
| B1        | *Anaerospora hongkongensis* HKU15 | 96 | NR_115223 | Firmicutes/Sporomusaecae |
| B2        | Unidentified marine bacterioplankton E305B_278 | 97 | KO002542 | Bacteria/environmental sample |
| B3        | *Desulfitobacterium dichloroeliminans* LMG P-21439 | 97 | AJ565938 | Firmicutes/Peptococcaceae |
| B4        | *Pseudomonas pseudoalcaligenes* strain E1205 | 99 | KY867526 | Gammaproteobacteria/Pseudomonadaceae |
| B5        | Unidentified marine bacterioplankton E305B_278 | 97 | KO002542 | Bacteria/environmental sample |
| B6        | *Clostridium* sp. IM-1 | 99 | KE934760 | Firmicutes/Clostridiaceae |
| B7        | *Pseudomonas stutzeri* NIOSSD012#48 | 97 | KY606664 | Gammaproteobacteria/Pseudomonadaceae |
| B8        | Uncultured bacterium NT61a9_15084 | 97 | JQ383993 | Firmicutes/Clostridiaceae |
| B9        | Uncultured bacterium clone: O502TCLN009 | 97 | AB695741 | Firmicutes/Clostridiaceae |
| B10       | *Bacillus* sp. 22-B | 99 | KX816421 | Firmicutes/Bacillaceae |
| B11       | *Peptoclostridium* sp. 37 | 100 | KX170738 | Firmicutes/Peptostreptococcaceae |
| B12       | *Dehalococcoides mccartyi* BAV1 | 97 | CP000688 | Chloroflexi/Dehalococcoidaceae |
| B13       | *Pseudomonas stutzeri* NIOSSD012#48 | 97 | KY606664 | Gammaproteobacteria/Pseudomonadaceae |
| B14       | *Peptoclostridium* sp. 37 | 100 | KX170738 | Firmicutes/Peptostreptococcaceae |

* Pseudomonas pseudoalcaligenes and *Pseudomonas* sp. comprised all community in S1 non-enriched samples. *Pseudomonas* sp. was then detected only in the sample enriched with DCB and its relative abundance reached 53% by an increase of 4.4 folds. However, the relative abundance of *Pseudomonas pseudoalcaligenes*, that was reduced 35%, 26% and 70%, yet, comprised 57%, 65% and 26% of samples enriched with 2,3DCP, MCP and DCB, respectively. *Desulfitobacterium dichloroeliminans* was enriched and detected in all enrichment cultures. The relative abundance of *Desulfitobacterium dichloroeliminans* reached 17%, 13% and 7% in samples enriched with 2,3DCP, MCP and DCB, respectively. *Clostridium* sp. and *Pseudomonas stutzeri* were detected in the enrichment cultures with MCP and 2,3DCP, respectively. *Anaerospora hongkongensis* was enriched in the presence of 2,3DCP and DCB, while, *Pseudomonas* sp. and uncultured bacterium were detected in the presence of DCB.

The relative abundance of *Pseudomonas pseudoalcaligenes* and *Pseudomonas* sp. comprised 72% of S2-non-enriched samples. *Pseudomonas* sp. was then detected only in sample enriched with DCB with a relative abundance of 18% by an increase of 3.4 folds. The relative abundance of *Pseudomonas pseudoalcaligenes* reached 46%, 29% and 37% in samples enriched with 2,3DCP, MCP and DCB, respectively. The relative abundance of *Desulfitobacterium dichloroeliminans* increased from 18% in the non-enriched sample to 22% in the presence of 2,3DCP and reduced to 2% in the presence of DCB. *Peptoclostridium* sp. and *Anaerospora hongkongensis* were detected in enrichments with DCB. *Dehalococcoides mccartyi* was detected in cultures enriched on DCB and its relative abundance was increased after enrichment by 3.2 folds.

S1 sediments enriched on MCP were dominated by *Pseudomonas pseudoalcaligenes* (65%), *Desulfitobacterium dichloroeliminans* (13%), *Clostridium* sp. (11%), unidentified marine bacterioplankton (6%),
**Pseudomonas stutzeri** (5%). However, S2 sediments showed privilege of **Pseudomonas pseudoalcaligenes** (29%), **Desulfitobacterium dichloroeliminans** (22%), uncultured marine bacterium (25%), 2 OUTs of uncultured bacteria (29%), **Pseudomonas stutzeri** (10%) and **Bacillus** sp. (7%). Enrichment of S1 sediments on DCB resulted in the predominance of **Pseudomonas** sp. (53%), **Pseudomonas pseudoalcaligenes** (26%), and 7% of both **Anaerospora hongkongensis** and **Desulfitobacterium dichloroeliminans**. However, S2-DCB enriched cultures were dominated by **Pseudomonas pseudoalcaligenes** (37%), **Pseudomonas** sp. (18%), **Dehalococcoides mccartyi** (14%), **Peptoclostridium** sp. (11%), **Clostridium** sp. (8%), uncultured bacterium (6%), **Anaerospora hongkongensis** (4%) and **Desulfitobacterium dichloroeliminans** (2%).

The enrichment of S1 with 2,3DCP resulted in a community structure comprising **Pseudomonas pseudoalcaligenes** (57%), **Desulfitobacterium dichloroeliminans** (17%), **Pseudomonas stutzeri** (10%), **Clostridium** sp. (8%) and 4% of both **Anaerospora hongkongensis** and unidentified marine bacterioplankton. Furthermore, 2,3DCP-enriched S2 cultures favoured the growth of **Pseudomonas pseudoalcaligenes** (45%), **Desulfitobacterium dichloroeliminans** (22%), **Pseudomonas stutzeri** (14%), uncultured marine bacterium (11%), **Clostridium** sp. (5%) and unidentified marine bacterioplankton (2%).

### Dechlorinating anaerobic enrichment cultures

In this study, highly enriched dechlorinating cultures could be obtained after selective enrichments for aryl halides respiring bacteria using hydrogen as an electron donor and chlorinated hydrocarbons as electron acceptors. Highly enriched dechlorinating cultures showed unique bacterial fingerprints. Enrichment had led to the reduction in most bacterial populations in original sediment and increasing populations only involved in dechlorination process. DGGE analysis of these cultures showed a strong selection of specific bacterial populations represented by four OTUs affiliated to the genera **Anaerospora**, **Pseudomonas**, **Clostridium** and **Desulfitobacterium**. The DGGE OUT-B3 from 2,3DCP-amended dechlorinating enrichment culture has been assigned to genus **Desulfitobacterium** (Table 2), which is known for its ability to grow via reductive dechlorination pathway. Existence of **Desulfitobacterium** in enrichment cultures was regarded as a preliminary evidence for reductive dechlorination on chlorinated hydrocarbons due to its ability to couple their growth to dechlorination via the halorespiratory mechanism. Confirmation for dechlorination activity was possible using GC analysis of provided 2,3DCP. Enrichment cultures maintained under optimum conditions of temperature and pH showed a dechlorination activity of provided 2,3DCP. Figure 4 shows the GC analysis and detection of 2CP and 3CP as dechlorination products from enrichment cultures amended with 2,3DCP after one month of incubation at 30°C. All these results provided compelling evidence that detected **Desulfitobacterium** sp. was responsible for the dechlorination activity.

Detection of the well-known dehalorespiring **Dehalococcoides** as well (OUT-B12) represents another indication for reductive dechlorination activity in established enrichment cultures. Presence of **Dehalococcoides** in DCB-enriched cultures indicating a selective behaviour of such bacterium toward dechlorination of chlorinated benzenes rather than chlorinated phenols.

### Phylogenetic analysis of dechlorinating bacteria

Enriched cultures with different chlorinated hydrocarbons were dominated by seven major genera including **Anaerospora**, **Pseudomonas**, **Clostridium**, **Peptoclostridium**, **Desulfitobacterium**, **Bacillus** and **Dehalococcoides**.

**Figure 3.** Numerical analysis of the DGGE fingerprints revealing bacterial diversity, relative abundances (%) and species richness in dechlorinating enrichment cultures amended 2,3DCP, MCP or DCB. S1 and S2 represent unamended control samples.
Figure 4. GC-FID chromatograms showing the dechlorination of 2,3DCP to 2CP and 3CP in all enrichment cultures on 2,3DCP after prolonged period of incubation at 30°C. (A–D) represent sediments S1–1, S1–2, S2–1 and S2–2 enrichment cultures, respectively, on 2,3DCP.

*Desulfitobacterium* as dechlorinating bacterium was distributed mainly in 2,3DCP enrichment cultures and being limited to fewer cultures enriched on MCP.

*Dehalococcoides*, on the other hand, was not detected in CPs cultures but was existed in DCB enrichment cultures only. Figure 5 shows the Neighbour-joining phylogenetic tree demonstrating the relationship between detected dechlorinating *Desulfitobacterium* and *Dehalococcoides* strains and closest matches from GenBank database. Phylogenetic analysis of the 16S rDNA fragments corresponding to OTUs A5, A13 and B3 showed their close relation to *Desulfitobacterium dichloroeliminans* strain LMG P-21439 and *Desulfitobacterium chlororespirans* strain Co23. However, it was clustered in a separate phylogenetic branch indicating a possibility of a novel strain which might be different from already characterized Desulfitobacteria. This is supported by relative low sequence similarity (97%) and different clustering from all known Desulfitobacteria including *Desulfitobacterium hafniense*, *Desulfitobacterium* sp. PCE, *Desulfitobacterium metallireducens* and *Desulfitobacterium dehalogenans*. On the other hand, OTU-B12 from enrichment cultures showed it close relation to *Dehalococcoides* group with a sequence similarity of 97% to *Dehalococcoides mccartyi* BAV1. Although OUT-B12 was assigned to *Dehalococcoides* group, it was clustered in a separate phylogenetic branch away from known *Dehalococcoides* strains which might also indicate a possibility of a novel strain.

**Discussion**

Chlorinated hydrocarbons from an oil refinery in general are known as hazardous pollutants when being released to the environment [5]. Specifically, aryl halides like CPs and CBs are massively released to the environment as byproducts for oil refinery and as wastes from many industrial activities [37]. CPs and CBs have been proved to be carcinogenic and/or mutagenic [38,39] and therefore being listed in the priority pollutants to be monitored [1].

Although chlorinated hydrocarbons can be biodegraded under aerobic conditions, its accumulation in anaerobic habitats like sedimentary environments requires different biodegradation mechanisms. In anaerobic environments, the biodegradation of chlorinated aromatic compounds may occur through reductive dehalogenation leading to the formation of less toxic and more biodegradable compounds [10]. Dehalorespiring bacteria and corresponding reductive dechlorination activities are greatly affected by provided environmental conditions [40]. Bacterial strains reductively dechlorinating chlorinated hydrocarbons under different environmental conditions are quite diverse [4,12,28]. In this investigation, chlorinated hydrocarbons-amended microcosms from sediments near an oil refinery were investigated and found to be dominated by members of *Anaerospora hongkongensis*, *Pseudomonas stutzeri*, *Clostridium* sp., *Desulfitobacterium dichloroeliminans*, *Pseudomonas pseudoalcaligenes*,...
Figure 5. Neighbour-joining phylogenetic tree showing the relationship between detected dechlorinating Desulfitobacterium and Dehalococcoides strains and their closest matches from GenBank data base. The bar represents 0.05 substitutions per site. The phylogenetic tree was generated using MEGA 4 software, bootstrap values (n = 1000) are displayed.

Mycobacterium sp., Peptoclostridium, Bacillus sp., beside uncultured bacteria including uncultured Halochromatium, uncultured Sedimentibacter and unidentified marine bacterioplankton. Detection of the well-known dehalorespiring Desulfitobacterium in sediments represents an evidence for corresponding reductive dechlorination of CPs and CBs [41]. Several species of Desulfitobacterium with dehalogenating capabilities toward various CPs and CBs have been isolated and characterized [16,42]. In this study, enrichment on chlorinated hydrocarbons resulted in the establishment of dechlorinating cultures with a remarkable change in its endogenous bacterial community structure. Bacterial populations were reduced in all amended cultures to those which are most probably involved in the dechlorination process. Enrichment cultures on 2,3DCP were dominated by members of Anaerospora hongkongensis, Pseudomonas stutzeri, Clostridium sp., Desulfitobacterium dichloroeliminans, beside unidentified marine bacterioplankton. Enrichment of Desulfitobacterium in dechlorinating cultures provides another clear evidence for corresponding dechlorination of CPs. Dechlorinating cultures developed from River Nile sediment on 2,3-dichlorophenol (2,3DCP) and 2,4,6-trichlorophenol (2,4,6TCP) showed enrichment of bacterial populations enclosing Desulfitobacterium to which dechlorination was assigned [43]. Vandermeeren et al. [28] demonstrated the presence of many organohalide-respiring strains in sediment samples originating from river sludge containing Dehalobacter, Dehalococcoides mccartyi, Desulfitobacterium and Sedimentibacter sp.
Confirmation for dechlorination activity was possible using GC analysis of provided 2,3DCP. Enrichment cultures maintained under optimum conditions of temperature and pH showed a dechlorination activity of 2,3DCP to 2CP and 3CP. This pattern of dechlorination is most similar to that reported for Desulfitobacterium chlororespirans which was able to dechlorinate ortho chlorines of polysubstituted phenols to monohalophenols as end product [44]. It has been reported that recalcitrant chlorophenols can be reductively dehalogenated into phenols with less chlorine atoms, which may be further mineralized more easily [4]. However, in this study, dechlorination beyond monochlorophenols was not detected. These results are in consensus also with El-Sayed [43] who has characterized an anaerobic reductive dechlorinating consortium form River Nile sediment showing restricted dechlorination to ortho chlorine of 2,3-dichlorophenol (2,3DCP) and 2,4,6-trichlorophenol (2,4,6TCP). It has been reported that most of Desulfitobacteria strains exclusively cause the reductive ortho dehalogenation of chlorophenols. Only Desulfitobacterium strains belonging to the species D. hafniense had the ability to perform various reductive dehalogenation patterns catalyzing ortho, meta and para dechlorinations [16,45].

In this investigation, dehalorespiring Dehalococcoides was also detected. However, it was only existed in DCB enriched cultures indicating a selective behaviour of such bacterium toward dechlorination of chlorinated benzenes rather than chlorinated phenols. Dehalococcoides strains are known to dechlorinate a range of chlorinated pollutants including various aryl halides, most specifically chlorinated benzenes, and couple dechlorination to its growth [46–48]. Presence of both Desulfitobacterium and Dehalococcoides in enrichment cultures represents an evidence for the existence of the reductive dechlorination potential for organohalides in the sedimentary environment. It’s well established that, highly enriched consortia may exhibit more potential dechlorinating activities than pure cultures. Synthrophic relationship among different microorganisms would accelerate the intercellular communication of metabolites of recalcitrant substance like chlorinated hydrocarbons for efficient biodegradation [49]. Here, we report the establishment of highly enriched dechlorinating cultures containing both Desulfitobacterium and Dehalococcoides as well-known reductive dechlorinating bacteria that could be exploited in various bioremediation protocols.

It has been reported that organohalide-respiring bacteria are phylogenetically clustered with the genera Dehalobacter, Anaeroamyxobacter, Geobacter, Desulfovomonile, Desulfuromonas, Desulfovibrio, Sulfurospirillum, Desulfitobacterium and Dehalococcoides [15]. In this study, phylogenetic analysis of the 16S rDNA fragment corresponding to DGGE fingerprints from enrichment cultures shows its close relation to Desulfitobacterium dichloroeliminans strain LMG P-21439 and Desulfotobacterium chlororespirans strain Co23. However, it was clustered in a separate phylogenetic branch indicating a novel strain which might be different from already characterized Desulfotobacteria. Detected Desulfotobacterium strain clustered differently from all known Desulfotobacteria including Desulfitobacterium hafniense, Desulfotobacterium sp. PCE, Desulfotobacterium metalireducens and Desulfotobacterium dehalogenans. DGGE analysis also revealed the existence of one phylotype in enrichment cultures which had a sequence similarity of 97% to Dehalococcoides mccartyi BAV1. However, it was also clustered in a separate phylogenetic branch away from known Dehalococcoides strains indicating a possibility of a novel strain. Speciation and strain characterization in Dehalococcoides are highly linked to its substrate specificity. Dehalococcoides sp. (OTU-B12) has been enriched in CB enrichment cultures indicating a selective dechlorination activity for CBs. However, its closest match, Dehalococcoides mccartyi BAV1 which was previously known as Dehalococcoides sp. strain BAV1 had been characterized as a member of the Chloroflexi which reductively dechlorinate chlorinated ethenes but not aryl halides [50]. Difference in such respiratory substrates represents another supporting evidence for suggesting Dehalococcoides sp. (OTU-B12) as a novel strain.

Conclusion

This study was designed to determine the diversity and dynamics of bacterial communities in sedimentary environments near an oil refinery and revealing their potential for reductive dechlorination of aryl halides pollutants. Bacterial community structures in sediments collected from the vicinity of an oil refinery were analysed by DGGE fingerprinting. Established CPs or CBs amended sediment microcosms were dominated by members of Anaerospora, Pseudomonas, Desulfitobacterium, Clostridium, Mycobacterium, Peptoclostridium and Bacillus sp., beside several uncultured members belonging to marine Bacterioplankton, Halochlor matium and Sedimentibacter. Enrichment on CPs resulted in the predominance of Anaerospora hongkongensis, Pseudomonas stutzeri, Pseudomonas pseudoalcaligenes, Clostridium sp., Desulfitobacterium dichloroeliminans, beside unidentified marine bacterioplankton. Enrichment on DCB resulted in the same bacterial profile beside Peptoclostridium sp. and Dehalococcoides mccartyi. Flourishment of key dechlorinating (dehalorespiring) bacteria like Desulfitobacterium and Dehalococcoides in enrichment cultures represents a clear and explicit evidence for dechlorination activity. Dechlorination activity in enrichment cultures was exclusive to reductive ortho dechlorination. Dechlorination beyond monochlorophenols was not detected. These results provided potential evidence
that sedimentary environments in the vicinity of oil refineries do harbour dehalorespiring bacterial populations capable of reductive dechlorination of various chlorinated pollutants and therefore considered useful for bioremediation applications especially in anaerobic environments.

Disclosure statement
No potential conflict of interest was reported by the authors.

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