16S rRNA gene-based Bacterial Community in Polychlorinated Biphenyls (PCBs) contaminated site using PCR- Single-Strand Conformation Polymorphism (SSCP)

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Abstract: The oxidation pond site in Sadat city, Egypt had been polluted with PCBs compounds more than a decade ago because of the wastes collected from different manufactures. Culture-independent approach generate a more accurate view for the bacterial community of the PCBs contaminated site. The diversity of bacterial populations in site contaminated with polychlorinated biphenyls (PCBs) was investigated using Cultivation-independent technique PCR-based single-strand conformation polymorphism (SSCP) for genetic profiling of PCR-amplified 16S rRNA genes. SSCP was performed using Com primer set targeting the 16S rRNA genes. The SSCP analysis showed increasing of the microbial communities from phyla *Proteobacteria* and certainly in the family *Sphingomonadaceae* correlated with increasing of PCBs in the contaminant site. This finding may benefit as an effective tool for bioremediation by facilitating the detection and monitoring of the PCBs degrader with SSCP technique.
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1. Introduction

Sadat City is one of the largest industrial city in Egypt, located in the west of Al-Menufiya Province, at 93 km from Cairo. Area: 500 km^2, Population: 900,200. It is a desert area and includes more than 109 factories with several industrial activity such as iron-steel industry, paints, ceramic, chemicals, foods, fertilizers, biocides, organic products, textile, paper, batteries, dyes, printing materials and recycled plastics. The final outputs of industrial as well as urban effluents are expelling out of the populated area of the city. The treatment of such emissions is carried out depending on the oxidation pond system (Elaxada-ponds). However, the sweeping overflow of this discharge move downward to the lower land forming large pools (2.6-4 hectares, 2-4 m depth). The Oxidation Pond site in Sadat city, Egypt had been polluted with PCBs compounds.

PCBs are man-made chemicals and synthesized by substituting variable number of chlorine atoms (from 1 to 10 chlorine atoms) onto the biphenyl aromatic molecular structure to produce 209 congeners. These congeners were used widely as coolant in power transformers, insulators in capacitors, heat transfer fluids, fire resistance, and plasticizers as well as in consumer products such as ink, paper and paints. PCBs are a main category of persistent organic pollutants (POPs) present as contaminants in the environment (IPCS, 1993; 1997). Exposure to elevated levels of PCBs has caused birth defects and cancer in laboratory animals, and they are a suspected cause of cancer and adverse skin and liver effects in humans (USEPA, 2008).

Cultivation of microorganisms isolated from PCBs contaminated sites or any variety of ecosystem samples by any chosen cultivation approach will inevitably favor the growth of some community members while others are inhibited or not cultured and allows only around 0.1 to 1% of the total viable bacterial cells present in a variety of ecosystems can be cultured (Amann *et al*., 1995). It is unlikely that any cultivation method will not allow a full description of the microbial diversity. Therefore, adapting or developing dedicated molecular methods for the culture-independent survey of microorganisms take advantage producing complete information for PCBs contaminated sites.

A developed protocol was used, which allows the application of single-strand-conformation polymorphism (SSCP) (Orita *et al*.,1989; Hayashi, 1991) for the culture-independent assessment of microbial-community diversity (Schwieger and Tebbe, 1998; Jean and Georges, 2008; Keskes *et al*., 2012). The SSCP method has the potential to be more easily applied (Lee *et al*., 1996) and the SSCP produced a number of sharp bands and differentiated the bacterial community structures (Tomoyuki Hori *et al*., 2006). SSCP was optimized to analyze only one of the complementary single strands (Schwieger and Tebbe, 1998; Meng-zhi *et al*., 2008), by preferentially degrading with lambda exonuclease the one strand generated with a phosphorylated primer. This development aims to avoid heteroduplex formations, or overlapping of forward-reverse strands from different amplicons during separation, allowing the separation of mixtures of fragments of identical size

but different in sequence. The application of this modified technique was focused on studies of taxonomic shifts in microbial communities by targeting 16S rRNA genes (Peters *et al.*, 2000; Schwieger and Tebbe, 2000; Schmalenberger *et al.*, 2001; Schmalenberger and Tebbe, 2003). However, a potential application to assess diversity of functional genes was foreseen (Stach and Burns, 2002).
This study tries to identify the bacterial community in the Oxidation pond (Elaxada-ponds) in Sadat City as one of PCBs contaminated site by culture independent method via SSCP

2. Material and Methods

DNA Extraction from the four soil samples.
Total DNA from the Four PCBs-contaminated soil samples collected from the different treatments stages of the oxidation pond which contain all the final outputs of 109 factories The soil samples were stored at 4°C until use within 7 days. DNA were then extracted according to the protocol for DNA extraction with the Fast prep DNA kit for soil (Bio 101). DNA was visualized on 1% agarose gels. Yield of genomic DNA was determined spectrophotometrically by measuring the absorbance at 260 nm. Purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm (a pure DNA has an A_{260}/A_{280} ~ 2.0) The DNA extracts from PCBs-contaminated samples containing approximately 200 ng ml^{-1} DNA were 50- or 100-fold diluted in Tris-HCl buffer (10 mM, pH 8.0) and used as template DNA in PCR.

SSCP for the four soil samples.
Com1 (5`CAGCAGCCGCGGTAATAC3`) targeting the position (519-536) and Com2-Ph (5`CCGTCA ATTCCTTTGAGTTT3`) targeting the position (907-926) were chosen for the amplification of bacterial 16S rRNA genes (Schwieger and Tebbe 1998). Single-stranded DNA (ssDNA) from PCR products was obtained as previously described (Schwieger and Tebbe, 1998). Briefly, PCR has performed with one of the primers being 5` phosphorylated, PCR products were eluted from agarose gels (Figure 1), and the phosphorylated strands were digested by lambda exonuclease (NEB). The remaining single-strands were purified with Qiaquick PCR Cleaning Kit (Qiagen), dried by vacuum centrifugation, resuspended in 6µl of loading buffer (95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol), and denatured for 5 minutes at 94 °C, followed by instant cooling on water ice bath for 3 minutes. The separation conditions were standardized in a DCode System for PCR-SSCP, optimized running parameters were 120V (10 mA) for 18 h at a constant temperature of 26°C on 20 cm x 20 cm x 0,75 mm 0.6X MDE gels in 0.7X TBE (Sambrook *et al.*, 1989) as a running buffer. Optimal results were obtained when ssDNA obtained from 100 - 400 ng dsDNA was loaded onto the gels and a slightly enhanced resolution was achieved when the amplified single-strands of the reverse primer were subjected to PCR-SSCP analysis. For nucleic acid detection, gels were silver stained as reported previously (Bassam *et al.*, 1991). Single-strand electrophoretic mobilities corresponding to different conformations were excised from dried gels (Figure 2), and DNA extracted by the "Crush and Soak" method (Sambrook *et al.*, 1989) PCR reamplification of the excised and eluted single-strands was made with the same primers used to generate the original dsDNA fragment (Figure 3).

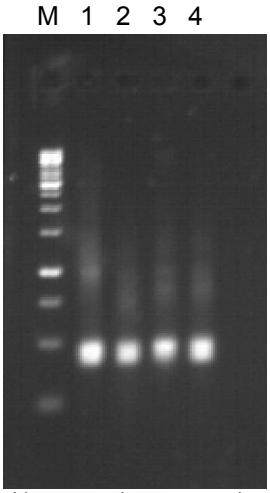

Figure 1 PCR with com primers targeting the 16SrRNA genes for the four DNA extracted samples from the four soil samples collected from the Oxidation-ponds (lanes 1, 2, 3, 4) and M is 1Kb DNA Ladder GeneRuler™, Fermentas

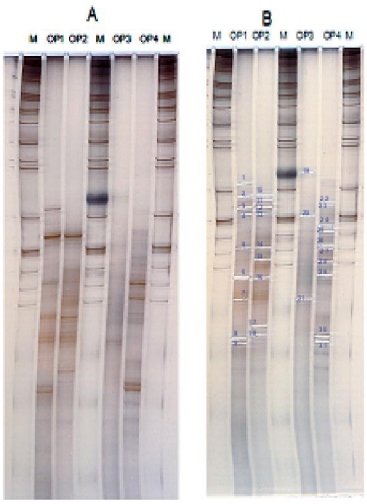

Figure 2 (A) show SSCP on a polyacrylamide gel for the four different soil samples before cutting the bands, (B) after cutting the bands.

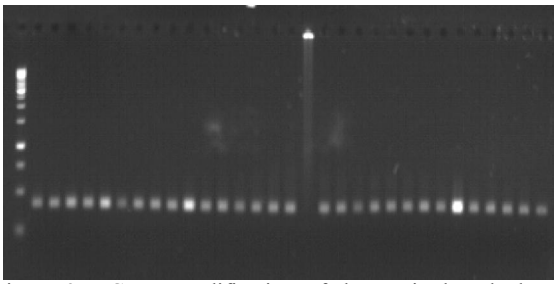

Figure 3. PCR reamplification of the excised and eluted single-strands with the same com primers to generate the original dsDNA fragment lans 1-31 are the product of the reamplification , lan 17 with the missed product, M is 1Kb DNA Ladder GeneRuler™, Fermentas

Data deposition

The sequence reported in this study has been deposited in the GenBank database (29 sequences for the independent culture bacteria accession numbers HQ829977- HQ830005.

3. Results

Com Primer set were used to amplify the eubacterial 16S rRNA gene sequences including the variable regions yielded complex SSCP patterns, SSCP community profiling showed highly diverse and distinct microbial communities for the soil samples, the OP-4 soil sample displayed the largest numbers of bands, while sample 3 (OP-3) displayed less of them (Figure 2). The SSCP profiles for the four PCBs contaminated soil shown in Figure.2. By PCR, the opposite strands were regenerated and the products were reamplified. SSCP gel electrophoresis was used to evaluate the purities and identities of the reamplified 31 products, as shown for products obtained from PCR targeting the hypervariable samples 16S rRNA genes only 30 were appeared (Figure. 3). In most cases, reamplification products corresponded to the expected positions in the community patterns and no additional products were observed. These products were then directly used for cloning and DNA sequencing. To identify the predominant products by DNA sequencing, a total of 30 different DNA single strands ("bands") were excised, only 29 sequences were in good appearance. By comparing the 29 sequences Acc. Nr. from HQ829977 to HQ830005 with the related taxa, revealed 18 different operational taxonomic units OTUs of bacteria, and that the compositions of the communities of the four soil samples were all common. These OTUs were most closely related to *Sphingomonas* sp. and *Pseudomonas* sp (Table 1).

Table (1) Phylogenetic assignment of sequences of prominent bands in SSCP gel profiles of the four soil samples communities.

OTU# & Acc. No.	Most closest related to	Similarity (%)	Bp	Acc. No.
1 (HQ829977)	*Sphingomonas* sp. Ens32	76	358	DQ339627
2 (HQ829978)	*Sphingomonas faeni (T); MA-olki*	93	396	AJ429239
3 (HQ829979)	uncultured alpha proteobacterium, *ATB-LH-6119*	91	399	FJ535117
4 (HQ829980)	uncultured bacterium; 4_G07	97	386	FN421770
5 (HQ829981)	uncultured bacterium; 4_H07	91	391	FN421778
6 (HQ829982)	uncultured bacterium; 10_D09	95	396	FN421920
7 (HQ829983)	uncultured alpha proteobacterium; ATB-LH-6119	93	393	FJ535117
8 (HQ829984)	uncultured bacterium; 1_C07	96	385	FN421584
9 (HQ829985)	*Pseudomonas sp. TSBY-92*	93	392	DQ173037
10 (HQ829986)	*Sphingomonas* sp. J05	97	390	AJ864842
11 (HQ829987)	uncultured bacterium; 5_H07	97	395	FN421995
12 (HQ829988)	uncultured Sphingomonas sp.; 437D	96	391	AY571827
13 (HQ829989)	uncultured bacterium; BF0002B019	97	387	AM697069
14 (HQ829990)	uncultured bacterium; 3_H05	96	391	FN421710
15 (HQ829991)	uncultured bacterium; 4_H07	97	382	FN421778
16 (HQ829992)	uncultured bacterium; 4_H03	96	384	FN421776
17 (HQ829993)	uncultured bacterium; BF0001B024	97	387	AM696998
18 (HQ829994)	uncultured bacterium; BF0002B019	98	381	AM697069
19 (HQ829995)	uncultured bacterium; 4_H07	55	351	FN421778
20 (HQ829996)	uncultured bacterium; 2_E05	96	353	FN421872
21 (HQ829997)	uncultured Sphingomonas sp.; O11	96	386	AM691108
22 (HQ829998)	uncultured bacterium; BF0002B019	96	387	AM697069
23 (HQ829999)	uncultured bacterium; BF0002B019	97	388	AM697069
24 (HQ830000)	*Sphingomonas* sp. clone jing-G-41	97	383	HM123760
25 (HQ830001)	*Sphingomonas faeni* (T); MA-olki	95	390	AJ429239
26 (HQ830002)	uncultured alpha proteobacterium; ATB-LH-6119	98	385	FJ535117
27 (HQ830003)	uncultured alpha proteobacterium; ATB-LH-6119	98	384	FJ535117
28 (HQ830004)	uncultured alpha proteobacterium; ATB-LH-6119;	97	388	FJ535117
29 (HQ830005)	uncultured alpha proteobacterium; ATB-LH-6119;	96	388	FJ535117

The phylogenetic tree (Figure. 4) constructed from the partial sequences of the 16S rRNA amplicons from the 4 soil samples showed a predominance of *Alphaproteobacteria*, and the SSCP bands (OP1-1, OP1-7, OP1-9, OP2-10, OP3-20, OP4-24, OP4-26, OP4-27, OP4-28, OP4-29) were far from any closet published 16S rRNA in the public database (Figure 4), OP1-4 band is very close to Uncultured bacterium partial 16S rRNA gene, clone 4_H03(Acc. No. FN421776) from phyllosphere of soybean in Switzerland. P4-14 band is very close to the partial sequence of the 16S rRNA of Pseudomonas sp. TSBY-92 (Acc. No. DQ173037) from frozen soil in China. Bands no OP2-12 and OP2-16 are very close to Uncultured bacterium partial 16S rRNA gene clone 10- D09 (Acc. No. FN421920) and Uncultured bacterium partial 16S rRNA gene clone 10- H07 (Acc. No. FN421995) from the phyllosphere of clover in Switzerland, and also Bands no. OP1-2 and OP4-22 are very close to many 16S rRNA from the phylosphere of soybean and clover plants in Switzerland. Band no. OP4-23 is very close to Uncultured *Sphingomonas* sp. 16S rRNA gene, isolate O11 (Acc. No. AM691108). Band no. OP4-18 is very close to *Sphingomonas faenia* partial 16S rRNA gene, strain MA-olki (Acc. No. AJ429239) (Figure. 2C).

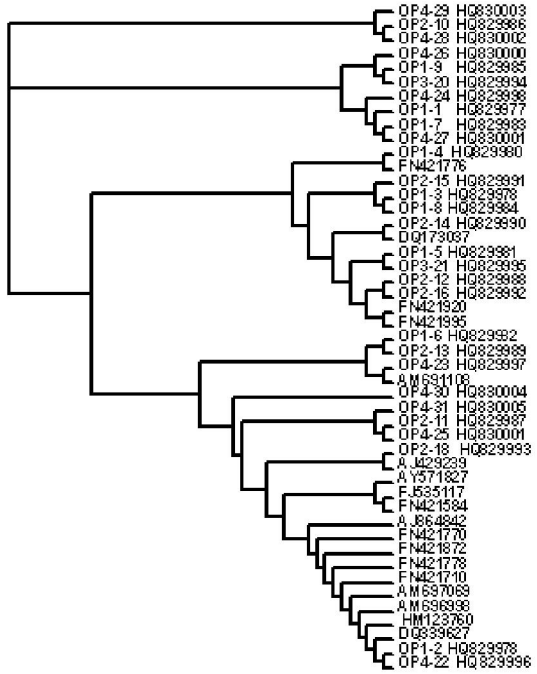

Figure 4 Phylogenetic tree of partial 16S rRNA gene sequences for the SSCP bands and its high similarity

4. Discussions

Soil microorganisms play important roles in maintaining soil quality and ecosystem health. Development of effective methods to analysis bacterial community structures and encourages the growth of selected microorganisms that are capable of transforming PCBs are challenges for successful bioremediation of PCBs- Contaminated soils.

The cultivation independent methods, based on amplification of environmental DNA followed by acrylamide gel electrophoresis, separate sequence specific DNA fragments of the same length, have the potential for accurate comparison of environmental samples in a short period of time. In this study, we have shown that SSCP analysis of 16S rRNA genes amplified from directly extracted DNA from soil samples can be used to visualize such community structures of highly PCBs contaminated sites, and also this indicate the high potential of this technique to monitor microbial communities and their variation qualitatively and quantitatively (Peters *et al*., 2000).

Soil sample OP.4 displayed the largest bands than the other bands maybe possibly due to the slightly higher organic carbon content in this sample and this emphasis that the higher chlorinated PCBs provide the organisms neither with energy nor with carbon to support the degradation process and the microbial degradation of PCBs in the environment is influenced by various biological, chemical and physical factors as well as the survival of microorganisms in cases where bioaugmentation is the application of choice (Blumenroth and Wagner-Döbler, 1998; Barriault *et al*., 1999; Ahn *et al*., 2001).

As more gene sequences become available, PCR-SSCP-mediated monitoring of different subgroups or microorganisms, due to optimized primer design, will become even more attractive, almost all of these OTUs from the indirect culture (Table 1) in this study are belonging to the phyla *Proteobacteria. a*nd certainly in the family *Sphingomonadaceae* which includes a large number of strains, which have been reported to be capable of degrading and assimilating PCBs and dioxin like compounds via dioxygenation (Akira, 2003).

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