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Traceability of polychlorinated dibenzo-dioxins/furans pollutants in soil and their ecotoxicological effects on genetics, functions and composition of bacterial community

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HIGHLIGHTS

- Dioxin-pollution is estimated near potential industrial dioxins sources.
- The highest dioxin-pollution is detected in the vicinity of petroleum refineries.
- Soil microbial diversity and functionality is affected by dioxin-pollution.
- Transcript of *AD α-subunit* is abundant in the heavily dioxin-polluted soil.
- No relationship is observed between *CYPBM3* expression and dioxin level in soil.

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ABSTRACT

Dioxins (PCDD/Fs) are persistent organic pollutants. Their accumulation in soil is a crucial step in their transmission through the ecosystem. Traceability of dioxin in soil was evaluated in four sites A, B, C and D considered as potential industrial PCDD/Fs sources in Syria. Our results showed that the highest pollution with dioxin ($\geqslant$50 ppt) was found in site C (vicinity of Homs refinery). In parallel, analysis of physico-chemical proprieties and bacterial density of soil samples were carried out. Bacterial density differed significantly among samples between 68×10^4 and 64×10^6 CFU g^{-1} DW. Analysis of 16S rRNA encoding sequences showed that the genus *Bacillus* was the most abundant (74.7%) in all samples, followed by the genera *Arthrobacter* and *Klebsiella* with 5.2% and 4.7%, respectively. The genera *Microbacterium*, *Pantoea*, *Pseudomonas*, *Enterobacter* and *Exiguobacterium* formed between 2.1% and 2.6%. *Cellulomonas*, *Kocuria*, *Lysinibacillus*, *Staphylococcus* and *Streptomyces* were in a minority (0.5–1%). The bacterial richness and biodiversity, estimated by D_{Mg} and H' index, were highest in the heavily polluted site. Molecular screening for angular dioxygenase (*AD α-subunit*) and the cytochrome P450 (*CYPBM3*) genes, led to identification of 41 strains as AD-positive and 31 strains as CYPBM3-positive. RT-real-time PCR analysis showed a significant abundance of *AD α-subunit* transcript in the heavily dioxin-polluted soils, while the expression of *CYPBM3* was highest in the moderately polluted soils. Our results illustrate the microbial diversity and functionality in soil exposed to dioxin pollution. Identification of dioxin-degrading bacteria from polluted sites should allow bioremediation to be carried out.

1. Introduction

Polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzodioxins (PCDDs), collectively termed "dioxins" are persistent organic pollutants (POPs) covered by the Stockholm Convention. Depending on chlorination level ($P = 1–8$), dioxins form a generic group of 75 PCDD congeners and 135 PCDF congeners with varying degrees of toxicity. Congeners with chlorine atoms substituted in the lateral 2, 3, 7 and 8 positions of the aromatic rings are, however, considered as the most toxic. Toxicity is expressed in Toxicological Equivalence or TEQ units. Of these, 2,3,7,8-TCDD is considered the most toxic and the remaining congeners have their toxicity compared to 2,3,7,8-TCDD using toxic

Abbreviations: TCDD, 2,3,7,8-polychlorinated dibenzo-p-dioxins; D_{Mg}, Margalef's index; H', Shannon–Wiener index; *AD*, angular dioxygenase; *CYPBM3*, *B. megaterium* cytochrome P450; RT-QPCR, real-time quantitative PCR.

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equivalency factors (TEFs) (WHO, 1998). Dioxins are majorly produced by industrial, municipal and domestic incineration and combustion processes (Tuppurainen et al., 2003) and during industrial processes involving chlorinated aromatic and aliphatic compounds, such as pesticides and herbicides synthesis (Hutzinger et al., 1985; Meharg and Osborn, 1995). Currently, the incineration of solid waste contributes the most to the release of these compounds into the environment (WHO, 1999).

Due to their stability, persistence in the environment and high fat solubility, PCDD/Fs are subject to lipophilic bio-concentration and accumulation in the food chain. Their adverse health effects are now well known for example; wasting syndrome (Huuskonen et al., 1994); immunotoxicity (McConkey et al., 1988), teratogenicity (Baker et al., 1995); dysfunctional immune and reproductive systems (Carney et al., 2006); and carcinogenesis (Toth et al., 1979). Due to their ability to associate with soil organic matter, PCDD/Fs have a high organic carbon–water partition (KOC) coefficient. This low mobility together with low water solubility results in accumulation in soil, with very little water leaching. Therefore, along with sediment, soil contains the greatest reservoirs of these contaminants. The accumulation of xenobiotic affects "soil quality" which can be monitored by many bioindicators notably the diversity of the microbial community (Giller et al., 1998). The soil microbiota play significant roles in maintenance of soil structure and detoxification of noxious chemicals, including PCDD/Fs (Cerniglia et al., 1979). Because soil microorganisms can respond rapidly, their composition may reflect a hazardous environment and is, therefore, useful for monitoring soil status. A number of soil microbiological parameters, notably microbial population and enzyme activity, have been employed as indicators of soil environmental quality in national and international monitoring programs (Yao et al., 2000). Due to the fact that the accumulation of PCDD/Fs in soil has serious implications, it is extremely important to gain knowledge about the presence of PCDD/Fs and the responses of soil microbial communities to PCDD/Fs exposure. Therefore, two issues could be addressed in the present study; the first is to determine levels of PCDD/Fs in soils collected from different regions of Syria with significant industrial activities; the second is to study the effects of PCDD/Fs on the structure of the microbial community, on the genetic and functional diversity of microbiota in soils and their possible ecological implications. The observations and related findings will establish a useful scientific basis for soil ecological risk assessment and potential for microbial of remediation PCDD/Fs.

2. Materials and methods

2.1. Sites and soil sampling

Sampling sites (A, B and C) are situated in the vicinity of potential industrial PCDD/Fs sources and located in the middle and coastal regions of Syria. Site D was chosen as an unpolluted site. Table S1 (Supplementary materials) presents basic information about the sampling sites. Site A is located around Baniyas's refinery which is a part of Tartous Governorate, on the northwestern Mediterranean coast of Syria. Site B is a part of Frunlluq coastal forest, which is located north of Lattakia city. Site C is located in the middle to western region of Syria, Homs Governorate. Site D is located near Tartous city. Sixteen surface soil samples (S1–S16) (0–5 cm depth) were collected, placed in sterile zip lock bags and transported to the laboratory, where they were immediately stored at −20 °C freezer until use.

2.2. Nitrogen content analysis in soil samples

The air-dried samples were 1 mm sieved to eliminate mollusk shell debris and other coarse materials. Content of total organic nitrogen, NO_3 and NH_4 were estimated according to Kjeldahl method (Kjeldahl, 1883).

2.3. Bacteria isolation and culture conditions

Each gram of sample was suspended in 10 mL of 1 X PBS (Phosphate buffer Saline, pH 7.2) and shaken vigorously for 2 min. Then the soil suspensions were serially diluted in 1 X PBS, and the 100 μL of dilutions from 10^{-1} to 10^{-6} were plated on Luria–Bertani (LB) agar plates. The LB agar plates were incubated at 30 °C until some colonies had formed (Tabata et al., 2013). Each single colony was subcultured onto new LB plate and kept at 4 °C for further identification. Bacterial population density was determined by the number of total bacteria (CFU) per gram dry weight soil. All the selected isolates were stored at −80 °C in LB containing 20% glycerol.

2.4. Extraction and cleanup of PCDD/Fs

Soil samples were air-dried and manually ground and sifted before extraction. PCDD/Fs extraction was carried out as described by Casanovas et al. (1994). In brief, 5 g of soil were mixed with 6 g of anhydrous sodium sulfate. 20 mL of hexane containing 20% acetone was added and the mix was shaken for 1 h. The extraction phase was separated by centrifugation at 3000 rpm for 5 min. The organic phase was carefully taken and immediately mixed with 8 mL of concentrated sulfuric acid. After manually shaking for 2 min, the upper phase was carefully transferred to a clean 40-mL vial. The extract was cleaned up with a small column (0.5 g anhydrous Na_2SO_4 on top, 1.0 g of florisil at the bottom). This column was activated with 3 mL of dichloromethane/hexane/methanol (50:45:5). PCDD/Fs were eluted with 5 mL of the same solvents mix. The extract volume was reduced to 1 mL under nitrogen. One hundred microlitres of DMSO (Dimethyl sulfoxide) was added to the extract and the mix was dried to remove all trace of solvents. Finally, 400 μL of DMSO was added and the final volume of the extract was adjusted to 1 mL by deionized water. Dilution of 1:10 with 50% DMSO in deionized water was used in an ELIZA screening. To validate our extraction procedure, a control soil sample was spiked with known concentration of TCDD (100 ppt) and subjected to the same extraction protocol.

2.5. Determination of TCDD by enzyme-linked immunosorbent assay

PCDD/Fs content was quantified in the cleaned extracts by using Abraxis TCDD-ELISA kit as described by the manufacturer's instructions (Abraxis LLC, USA). The absorbance was measured at 450 nm using a microplate reader (Multiskan EX, Thermo/Labsystems, USA). All steps of the ELISA procedure were carried out at room temperature.

2.6. DNA isolation, primers and PCR conditions for 16S rDNA amplification

Genomic DNA was extracted from bacteria isolates using a Genomic DNA extraction kit (BIOTOOLS – B&M Labs, Spain) as described in the manufacturer's manual. The pelleted DNA was dissolved in 50 μL of distilled water. Then, its concentration was adjusted to 200 ng μL^{-1} and stored until needed at −20 °C. Fragments of 1450 bp of the 16S rRNA genes were amplified using the universal bacterial primers 27F and 1492R (Table S2, supplementary materials) following the protocol established by Marcial Gomes et al., 2008. The PCR amplification was performed in 25 μL reaction final volume containing 3 mM $MgSO_4$, 200 μM each of the four dNTPs, 10 μM of each primers and 2.5 U *Taq* DNA polymerase. PCR conditions involved one first step denaturation at

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