

Detection of Dioxin-like Contaminants in Soil from the Area of Oil Refineries in Vojvodina Region of Serbia

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Abstract In this study level of soil contamination by polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) in two oil refineries in Vojvodina region of Serbia was assessed using combined bio/chemical approach. Toxicity of the samples, determined by micro-EROD analysis, could not be exclusively attributed to the content of measured PCBs and PAHs, but also to the presence of unknown dioxin-like compounds (DLC), and/or positive interactions among similarly acting chemicals. The results proved that biotests, when applied in ecotoxicological assessments, should be used either as a screening tool or initial step in effect-directed analyses.

Keywords Dioxin-like compounds · EROD · Oil refinery · Soil

Various industrial activities contribute to widespread contamination of the environment by different classes of persistent organic pollutants (POPs) such as polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs). Among these, oil industry has been shown to have considerable environmental impact on different ecosystems (Cetin et al. 2003; Wake 2005; Nadal et al. 2007). During Kosovo conflict in 1999, two oil refineries, Novi Sad and Pancevo, both located in Northern Serbian Province of Vojvodina, were severely damaged, which resulted in

releases of considerable amounts of crude oil, oil derivatives and consequently POPs in the environment. It has been reported (UNEP 1999) that in Oil refinery Novi Sad approximately 73,000 tonnes of crude oil and oil products had been burnt (almost 90%) or leaked, whereas in Oil refinery Pancevo about 62,000 tonnes of crude oil and petroleum were combusted. As a result of fire and explosions, various combustion products were formed. Apart from historical pollution, due to the nature of industrial activities, some spots in the areas of the Refineries are continually subject to constant input of polycyclic organic pollutants.

The aim of this study was to get insight into POPs contamination of soil at industrial complex of two oil refineries 5 years after the accident. Soil samples were analyzed on the presence of PCBs by gas chromatography–electron capture detection (GC/ECD) and PAHs by gas chromatography–mass selective detection (GC/MSD). Biological screening tool, micro-ethoxyresorufin-*O*-deethylase (microEROD) analysis on primary rat hepatocytes was used to detect dioxin-like compounds (DLC) in complex mixtures extracted from soil samples.

Materials and Methods

In total, 15 soil samples were collected from the two oil refineries, Novi Sad (10 samples, enumerated as NS1–NS10) and Pancevo (5 samples, enumerated as PA1–PA5). Samples labelled as NS8, NS9, PA2 and PA5 were collected from the sites directly contaminated by heavy leakages and/or combustion caused by facility destruction in spring 1999. Sample labelled NS2 was taken from the site of severe accidental leakage, while the rest of the samples (NS1, NS3, NS4, NS5, NS6, NS7, PA1, PA3 and

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PA4) were collected from the locations subject to constant exposure to various by-products due to the continual industrial activity. Control samples (NS10 and PA0) were taken either at the outskirts of Oil refinery Novi Sad, not subject to any direct impact of the industrial process, or, in case of Oil Refinery Pancevo, from the residential area downtown. Soil samples were taken by “T” type sampling tube from 0 to 30 cm depth, placed into glass jars, and transferred to the laboratory where they were kept on -20°C wrapped in aluminium foil until the extraction.

Dry soil (25 g) was extracted by 50 mL of acetone:hexane mixture (1:1), on a shaker for 24 h. After that, acetone and hexane layers were separated and remaining hexane extract was cleaned up by adding the 2–3 mL portions of concentrated H_2SO_4 (in total: 22–175 mL of acid, depending on cleanness of sample) until discoloration of the extract. Residues of sulphuric acid were then washed with distilled water. Samples were then evaporated to dryness, dissolved in 1 mL of hexane, and concentration of PCBs was determined by GC/ECD analysis. The rest of the extract was evaporated to dryness, dissolved in DMSO, labelled as refined hexane extracts and tested for toxicity in microEROD analysis.

For PAHs determination, 7 g of soil sample was extracted in methylene-chloride:hexane mixture (1:1) and cleaned up on silica gel column in accordance with EPA 3630C. Fraction containing PAHs was eluted by pentane:methylene-chloride mixture (1:1.5). After chemical analysis on the presence of PAHs using GC/MSD (GC HP5890 Series II with HP5971A MSD) on a HP5-ms column (J&W Scientific), extract was evaporated to dryness, dissolved in DMSO and used for bioanalysis.

MicroEROD analysis was done on primary rat hepatocytes, isolated by liver perfusion method originally described by Seglen (1973), with minor modifications. Cells were plated in 96-well collagen-coated culture plates (5 μg collagen/well) at the density 25,000 cells/well in 0.1 mL of the Williams' Medium E (WME) culture medium supplemented with 10% foetal calf serum (FCS). After 24 h of recovery period, the medium was removed and fresh serum-free medium containing different amounts of extracted sample equivalents, or different concentrations of PCB126 as a referent compound was added. After 48 h of exposure, measurement of EROD activity was done according to Donato et al. (1993), with minor modifications. Briefly, medium was removed, cells were washed with PBS and 0.1 mL of WME containing 8 μM 7-ethoxyresorufin and 10 μM dicumarol was added. After incubating for 1 h at 37°C , medium with formed resorufin from each well was transferred to black 96-well plates and double amount of methanol was added. The amount of resorufin was measured fluorometrically using excitation/emission wavelengths of 544/590 nm on a Fluoroscanner ascent FL plate

reader (ThermoLabsystems), calculated relative to resorufin standard curve (range 1.2–78 nM), and normalized to protein content determined for each well. In cell monolayer the protein content was determined by fluorescamine protein assay. Cells were washed with PBS, and 0.15 mL of PBS and 0.05 mL of 1.08 mM fluorescamine in acetonitrile was added. The amount of formed fluorescamine–protein adduct was measured fluorometrically using excitation/emission wavelengths of 400/460 nm on a Fluoroscanner ascent FL plate reader (ThermoLabsystems) and the protein concentration was calculated relative to bovine serum albumin standard curve. Toxic equivalent quotient (bio-TEQ) for each soil sample extract was calculated using EC_{25} value of PCB126 and corresponding value for each sample.

The residual levels of PCBs in refined hexane extracts of soil samples were determined by GC/ECD using HP 6890 gas chromatography with capillary column HP 19091J-416 HP-5 5% phenyl-methyl-siloxane (initial working temperature – 130°C , maximum temperature – 300°C), and electron capture detector Varian 3400. Concentration of PCBs was determined according to Riedel-de-Haen standard mixture of seven PCB congeners: PCB28, PCB52, PCB101, PCB138, PCB153, PCB180 and PCB209. Method detection limit (MDL) for all compounds was 0.05 ng/g.

PAH concentration in soil samples was calculated according to internal standard calibration for 16 compounds: naphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene. Phenanthrene-d10 was used as an internal standard. Practical quantisation limit (PQL) and method detection limit (MDL) for all compounds was 5 and 2 ng/g, respectively. Recovery for applied method for PAH analyses was determined for phenanthrene and chrysene using spiked soil samples at the level of 20 $\mu\text{g}/\text{kg}$. Recovery values from three measurements of phenanthrene and chrysene were $64\pm 5\%$ and $78\pm 7\%$, respectively.

Results and Discussion

Levels of PCBs in the soil samples from Oil refinery Novi Sad ranged from as low as 0.05–581.44 ng/g, while in samples taken from Oil refinery Pancevo PCB levels were in the range of 1.91–122.65 ng/g (Table 1). PCB congener profile varied among the samples, especially in Oil refinery Novi Sad, what suggests probably diverse sources of contamination in that area. Higher-chlorinated PCB congeners have longer half-life in soil and comprise an increasingly bigger proportion of ΣPCB with time (Alcock et al. 1996; Sinkkonen and Paasivirta 2000). Accordingly, it can be

Table 1 PCB levels (ng/g d.w.) in soil samples from Oil refineries in Novi Sad and Pancevo

Samples	PCB congener ^a							Σ PCB ^a
	28	52	101	138	153	180	209	
NS1	0.15	0.88	<0.05	0.12	0.12	0.14	0.06	1.47
NS2	0.26	0.06	<0.05	0.13	0.11	0.09	0.29	0.94
NS3	0.55	3.56	<0.05	0.23	0.27	0.34	<0.05	4.95
NS4	484.72	65.44	12.40	10.24	8.64	<0.05	<0.05	581.44
NS5	0.28	<0.05	<0.05	0.09	<0.05	0.05	0.13	0.55
NS6	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
NS7	0.42	0.06	0.12	0.41	0.40	0.43	<0.05	1.84
NS8	0.21	<0.05	<0.05	0.45	0.13	0.69	<0.05	1.48
NS9	0.80	0.29	1.97	5.23	5.88	3.29	0.68	18.14
NS10	<0.05	<0.05	<0.05	0.14	1.09	0.20	0.50	1.93
PA1	0.93	9.16	31.15	44.31	26.88	10.22	<0.05	122.65
PA2	<0.05	0.73	2.99	4.91	2.69	0.96	<0.05	12.28
PA3	<0.05	0.39	1.75	2.97	1.64	0.78	<0.05	7.53
PA4	<0.05	<0.05	0.35	0.84	0.72	<0.05	<0.05	1.91
PA5	<0.05	0.80	2.68	3.18	3.03	0.60	<0.05	10.29
PA0	<0.05	<0.05	0.60	11.01	1.09	0.48	<0.05	13.18

^a Riedel-de-Haen standard of seven PCB congeners No. 28, 52, 101, 138, 153, 180, 209

noticed that at the spots where contamination could be a result of leakages that occurred in 1999 (NS8 and NS9) the maximum concentration of higher-chlorinated PCB congeners was recorded, while at other spots, constantly exposed to the pollutants (NS1, NS3–NS7), lower-chlorinated biphenyls dominate. Sample NS4 is characterized by high content of PCB28 what suggests recent contamination. On the other hand, congener-specific PCB analysis of soil samples from Oil refinery Pancevo showed that all samples demonstrated very similar congener profile, with PCB138 as a dominant congener, following by PCB101 and PCB 153 (Table 1). Total PCB level in those samples was lower than 15 ng/g, except in sample PA1 where 8 times higher content was recorded. Uniform PCB pattern detected along the studied area, with domination of higher PCB congeners, indicates the similar pattern of historical pollution at all examined spots.

Refined hexane extracts were checked by microEROD analysis on the presence of dioxin-like compounds. Obtained results indicated that soil samples from Pancevo were characterized by relatively uniform and low bio-TEQ values which did not exceed 122 pg PCB126/g d.w. (Table 2). Although interpretation of bio-TEQ values as indicators of ecotoxicological status in the literature cautiously goes further than its rough estimation, some threshold bio-TEQ levels could be assumed. Engwall et al. (1996) classify samples with bio-TEQ value of 3.2 pg TCDD-eq./g d.w. as fairly unpolluted by EROD-inducing compounds. Our previous studies revealed that potential threshold bio-TEQ value in our experimental conditions on primary rat hepatocyte culture could be 30 pg PCB126-eq./g d.w. (Zoric et al. 2004).

Table 2 Bio-TEQ values (pg PCB126/g d.w.) and PCB levels (ng/g d.w.) in soil samples from Oil refinery Pancevo

Sample	Bio-TEQ ^a	Σ PCB ^b
PA1	70.18	122.65
PA2	68.52	12.28
PA3	121.57	7.53
PA4	108.21	1.91
PA5	72.60	10.29
PA0	62.34	13.18

^a Bio-TEQ = PCB126EC₂₅/SampleEC₂₅PCB126

^b As described in Table 1

Taking into account that PCB126 has ten times lower potency than TCDD in primary rat hepatocytes (Zeiger et al. 2001), these data are quite comparable. In light of these data, bio-TEQ values of soil samples from Oil refinery Pancevo indicate their low dioxin-like toxic potency. On the contrary, bio-TEQ values of refined hexane extracts of soil samples from Oil refinery Novi Sad are in the range from as low as 14 pg PCB126/g d.w. to 911 pg PCB126/g d.w. (Table 3). Six samples had toxic potency higher than 100 pg PCB126/g d.w. suggesting potential contamination of this area.

It is known that complex environmental mixtures contain different POP compounds among which are AhR agonists, i.e., CYP1A1-inducers (Hilscherova et al. 2001). EROD-inducing potency indicates to the contamination with prototypic AhR ligands, including PCDD/Fs, dioxin-like PCBs and polybrominated biphenyls, as well as polybrominated diphenyl ethers. The presence of PAHs

Table 3 Bio-TEQ values (pg PCB126/g d.w.), PCB and PAH levels (ng/g d.w.) in soil samples from Oil refinery Novi Sad

Sample	Refined hexane extract		PAH fraction	
	Bio-TEQ ^a	ΣPCBs ^b	Bio-TEQ ^a	ΣPAHs ^c
NS1	13.72	1.47	NA	NA
NS2	241.22	0.94	742.30	175.9
NS3	76.99	4.95	NA	NA
NS4	444.97	581.44	924.72	3,928.6
NS5	224.74	0.55	NA	NA
NS6	911.22	<0.05	18,701.49	507.1
NS7	127.18	1.84	NA	NA
NS8	15.22	1.48	425.47	<5
NS9	235.74	18.14	244.54	<5
NS10	25.7	1.93	47.31	<5

NA sample was not analysed on the presence of PAHs and corresponding bio-TEQ value

^a Bio-TEQ = PCB126EC₂₅/SampleEC₂₅PCB126

^b As described in Table 1

^c Standard mixture of 16 components: acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3,-cd)pyrene, naphthalene, phenanthrene, pyrene

which are also well known EROD-inducers (Hilscherova et al. 2001; Brack and Schirmer 2003) in refined hexane extracts is questionable due to clean-up procedure with concentrated sulphuric acid, which is used to eliminate non-persistent organic compounds from the extract (Yoo et al. 2006). However, since the amount of sulphuric acid used for cleanup was at most 175 ml/25 g of sample (1:7 = sample:acid in volume), it is quite possible that some parent and/or oxidized derivatives of PAHs were still present in the refined hexane extracts. Besides, contribution of other persistent organic compounds and potentially harmful unknowns with AhR agonist-activity could not be neglected.

In order to assess contribution of PAHs in total dioxin-like contamination, corresponding fractions of several soil samples from Oil refinery Novi Sad were analysed by GC/MS and microEROD analysis (Tables 3, 4). Instrumental analysis revealed presence of PAHs in only three of examined samples (NS2, NS4 and NS6). In these samples, 4 out of 16 standard PAHs used in GC/MS analysis, pyrene, chrysene, benzo(a)pyrene and phenanthrene were generally recorded at highest concentrations (Table 4). At the same time, calculated bio-TEQ values were in the range from 47 pg PCB126/g d.w. in control sample NS10 to as high as 18,701 pg PCB126/g d.w. in sample NS6. Such high bio-TEQ value is partly the result of the presence of benzo(a)pyrene (230 ng/g) and chrysene (180 ng/g) as known EROD inducers (Bosveld et al. 2002; Till et al.

Table 4 PAH levels (ng/g d.w.) in soil samples from Oil Refinery Novi Sad

Compound ^a	RAF NS2	RAF NS4	RAF NS6	RAF NS8	RAF NS9	RAF NS10
Acenaphthene	<2.0	<5.0	<2.0	<2.0	<2.0	<2.0
Acenaphthylene	<5.0	<5.0	<5.0	<2.0	<2.0	<2.0
Anthracene	<5.0	23.0	<5.0	<5.0	<5.0	<5.0
Benzo(a)anthracene	<5.0	83.0	<5.0	<5.0	<5.0	<5.0
Benzo(a)pyrene	48.0	380.0	230.0	<5.0	<5.0	<5.0
Benzo(b)fluoranthene	15.0	32.0	24.0	<5.0	<5.0	<5.0
Benzo(k)fluoranthene						
Benzo(g,h,i)perylene	5.0	7.6	13.0	<2.0	<5.0	<5.0
Chrysene	83.0	940.0	180.0	<5.0	<5.0	<5.0
Dibenzo(a,h)anthracene	5.3	<5.0	7.1	<2.0	<5.0	<5.0
Fluoranthene	<5.0	97.0	<5.0	<5.0	<5.0	<5.0
Fluorene	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
Indeno(1,2,3,-cd)pyrene	14.0	16.0	32.0	<2.0	6.8	<5.0
Naphthalene	<5.0	<5.0	<5.0	<2.0	<2.0	<2.0
Phenanthrene	<5.0	750.0	5.0	<5.0	<5.0	<5.0
Pyrene	5.6	1,600.0	11.0	<5.0	<5.0	<5.0
ΣPAH (ng/g d.w.)	175.9	3,928.6	507.1	<5.0	<5.0	<5.0

^a As described in Table 3

1999), but strong biological response induced by this sample also indicate high concentration of EROD-inducing unknowns. Sample with the highest PAH concentration (NS4) also exhibited relatively high toxic potency (942.72 pg PCB126/g d.w.). This could be again assigned to the presence of chrysene (940 ng/g) and benzo(a)pyrene (380 ng/g), and also to other EROD-inducing PAHs present in lower concentrations, since pyrene (1,600 ng/g) and phenanthrene (750 ng/g), although present at high concentrations, do not induce EROD activity in primary rat hepatocytes (Till et al. 1999). Nevertheless, considering generally high toxic potency in all examined samples, it can be assumed that they still contain some other EROD-inducing compounds that were not removed in the process of sample extraction. This especially refers to the samples NS8 and NS9, with PAH content below the detection limit, but bio-TEQ values far above background level.

Sources of contamination in the areas of two oil refineries, Novi Sad and Pancevo, are probably diverse. In spite of rather low PCB and PAH levels in examined samples, one of the factors that could contribute to relatively high bio-TEQ values might be the characteristic of the study area. Namely, in the area of Oil refineries, releases of various by-products in the environment might occur. Aromatic structure of these products, and their interactions with different compounds, make them potential AhR ligands, while their presence in the examined samples can not be identified by standard analytical procedure.

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