ETHOXYRESORUFIN-O-DEETHYLASE INDUCTION POTENCY IN SEDIMENT SAMPLES FROM RIVERS LEPENICA AND MORAVA – SURROUNDING AREA OF KRAGUJEVAC "HOT SPOT"

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Introduction

Activation of aryl hydrocarbon receptor (AhR) and induction of CYP1A1 izozyme, whose activity can be determined by measuring activity of 7-ethoxyresorufin-o-deethylase (EROD) in various species and test systems, are well known bioanalyses for assessment of toxicity of persistent toxic compounds (PTCs). Whereas analytical methods determine the concentrations of known substances, bioanalyses with EROD as an end point may also detect the joint activities of non-analyzed EROD inducing compounds in environmental samples[1].

The main environmental effects of the bombardments (in 1999) of factory Zastava-Kragujevac were damaged transformers which contained Pyralene oil, leaking of several tons of PCBs into the rivers Zdraljica, Lepenica and Morava, and contamination of groundwater by PCBs and heavy metals. Up to 2,500 kg of oil containing PCBs was released into the environment. Extremely high levels of PCBs were detected in water ecosystems in the area of accident – in the river Lepenica PCB levels were 400-1000mg/l of water [2]. As a consequence of explosions, fires, and PCB combustions, very high levels of PCDDs/Fs were found in soil samples from that area (up to 100,000ng I-TEQ/kg) few months after bombardments[3]. Very high levels of PCBs and PCDDs/Fs were also found in samples taken around the transformers of the power plant (70-74 g/kg of PCBs and 10,200 ng I-TEQ/kg of PCDDs/Fs)[4]. Right after spillage there was a flood wave from the river Lepenica that caused transport and sedimentation of the pollutants along nearby agricultural area.

In 2000, UNEP/UNOPS Clean-up Project YUG 00-R71 was performed for clean up action of environmental damage of hot spots in SCG[4]. However, further investigations which covered hot spot Kragujevac as a zone possibly contaminated with PCBs revealed that there are still ppm concentrations of PCBs as determined by GC/ECD[2]. In this study we explored if these

contaminants still remained in sediments from rivers Lepenica and Morava. Sediment samples were analyzed by microEROD and GC/ECD analyses.

Methods and Materials

Sampling locations. Sediment samples of river Lepenica were taken from four spots (1-4) at the local depressions in the river vicinity, where the flooding surface water (bearing various pollutants including PCBs) retained for the longest period in 1999. Samples of river Morava were taken from one spot, but from three different depths (0.3m, 0.6m and 1m) within the alluvial sediments in the riverbed, after Lepenica mouth.

Samples preparation. Samples were dried at room temperature, and 25g of each sample were extracted by 50ml of acetone:hexane mixture (1:1) on the magnetic stirrer for 24h. After that, acetone and hexane layer were separated, evaporated to dryness, dissolved in DMSO, and exposed to microEROD bioanalysis. Such specimens were marked as crude acetone or hexane extracts. Whole crude extracts, containing both acetone and hexane fraction, were also used for bioanalysis.

Parallel extraction of 5g of each sample was done with dichloromethane using Buchi automatic extractor. Sulfur was removed by treatment with powder cooper. The whole extracts were refined by sulfuric acid activated silica gel column. Hexane was used as solvent, and concentration of PCBs was determined by gas chromatography (GC). The rest of the fraction of each sample was evaporated, dissolved in DMSO, and exposed to microEROD bioanalysis. Such specimens were marked as cleaned up extracts.

GC/ECD analysis. Samples were analyzed on GC/ECD HP 5890 for PCB28, PCB52, PCB101, PCB118, PCB153, PCB138 and PCB180 (EPA 7 congeners).

MicroEROD analysis. The bioassay was done on primary rat hepatocyte culture. Isolation of rat hepatocytes was done according to a method originally described by Seglen[5], with minor modifications. After isolation, cells were counted and plated in sterile 96-well collagen-coated culture plates at the density 25,000 cells/well in 0.1ml of the culture medium supplemented with 10% fetal calf serum. After cell attachment, the medium was removed and fresh medium containing different concentrations of extracts samples was added. Hepatocytes were incubated for 48h or 72h, and measurement of EROD activity was done according to Donato et al.[6]. EROD activity in wells was analyzed fluorometrically using 544 nm excitation and 590 emission filter in a Fluoroscan Ascent FL plate reader (ThermoLabsystems). Amount of formed resorufin was calculated relative to standard curve (range 1.2nM to 78nM).

As a reference compound in bioassay PCB126 was used. PCB126 dose response curve was calculated using logyt-log model, while sample dose response curves were calculated using logyt-ln model. EC25 value of PCB126 was used for calculating toxic equivalent quotient (bio-TEQ) for each sediment sample extract. Bio-TEQ was expressed as

$$\text{bio-TEQ (pg/g)} = \frac{\text{PCB126 } EC_{25}\text{ (pg/ml)}}{\text{Extract } EC_{25PCB126}\text{ (g/ml)}}$$

As test toxicants were used PCB153 as well as following polycyclic aromatic hydrocarbons (PAHs): dibenzo(a,h)anthracene (DBA), benzo(a)pyrene, benzo(a)anthracene and chrysene.

Results and Discussion

For determination of EROD inducing potency of investigated samples, we used PCB126 as a reference compound. This congener is present in environmental samples and it is the most potent of all PCB congeners in comparison to TCDD[7]. On the other hand, DBA was used as a model of polycyclic aromatic hydrocarbons (PAHs) because it revealed high EROD-inducing potency. The order of relative potencies among the PAHs tested was: DBA > benzo(a)pyrene > benzo(a)anthracene ≈ chrysene.

EROD activities of primary rat hepatocytes in the presence of PCB126 and DBA after two incubation periods (48h and 72h) are shown in Fig.1. The shape of concentration-response curve and EC25 value for PCB126 are similar regardless of incubation time. On the other hand, PCB153 as di-ortho congener induced no EROD activity, which confirmed that it is not an AhR agonist.

In the case of DBA, decreased EROD activity was detected at lower concentrations after 72h of exposure in comparison to 48h, and it could be result of its degradation. In parallel, higher concentrations exhibit reverse effect – EROD activity was higher after 72h of incubation. Since PAHs are readily metabolized, the levels of DBA after 72h of incubation were probably decreased, and therefore an increase in EROD activity was detected. It is the consequence of the reduction of substrate inhibition which possibly occurred during shorter incubation period. Namely, lower EROD activity in the presence of high DA concentrations 48 h after exposure could be result of substrate competition of unmetabolized DA and 7-ethoxyresorufin at the catalytic site of the CYP1A1 enzyme. The same mode of action of PAHs was reported by Smeets et al.[8] In the case of PCB126 such response was not observed, because PCBs are very persistent and have much lower rate of metabolism than PAHs.

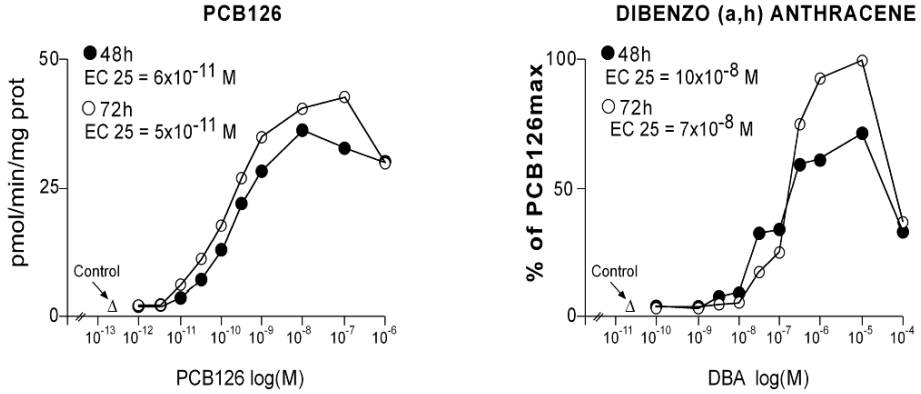

Figure 1. EROD activity in primary rat hepatocytes after 48h (•) and 72h (o) of incubation with different concentrations of PCB126 and dibenzo(a,h)anthracene (DBA). Activity of DBA is expressed as % of maximal production in the presence of PCB126.

Similar results as in the case of DBA, were observed with various extracts of sediment sample of river Morava 0.3m - separated hexane and acetone fractions, and cleaned-up fraction at two time points (Fig. 2). Certain inhibition of EROD activity was observed at higher concentrations after 48h

of incubation, while after 72h EROD signal was much higher. These results suggested that in all extracts, apart from PCBs, certain EROD inducers with high metabolic rate are present.

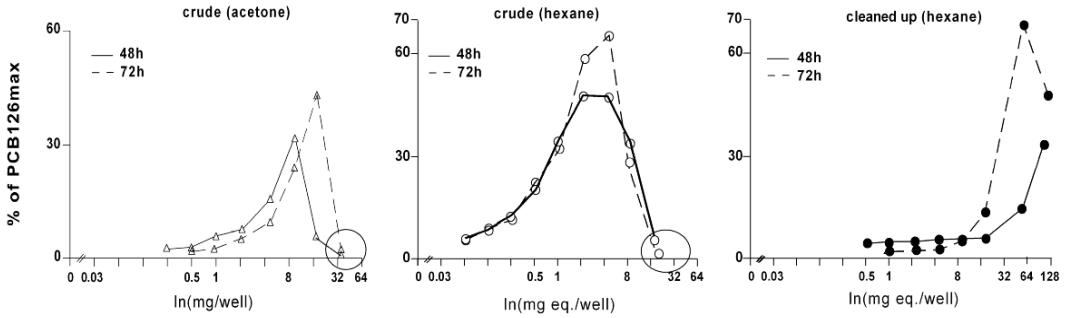

Figure 2. EROD activity in primary rat hepatocytes after 48h (•) and 72h (o) of incubation with differently prepared extracts of sediment samples of river Morava 0.3m. Activity is expressed as % of maximal production in the presence of PCB126. Circled points represent cytotoxic concentrations determined by measuring protein concentrations in wells.

Analyses of EROD activity of crude and cleaned up extracts of three sediment samples from river Morava are shown in Fig. 3. The results demonstrated that dose-response curves for crude extracts were bell-shaped plots. The EROD induction was enhanced with increasing concentrations up to the maximum level and followed by inhibition at higher concentrations. These results are in accordance with Hollert et al.[1] who showed high EROD-induction potency in acetone-extracted sediment samples from the River Neckar. On the other hand, cleaned up extracts demonstrated smaller EROD-inducing capacity in comparison to crude ones. These results suggest that some of the AhR active residues were removed during preparation of cleaned up samples. It was supposed that these active compounds may be different heterocyclic aromatyc hydrocarbons (HAHs), and other "non-priority" pollutants that can induce EROD activity.

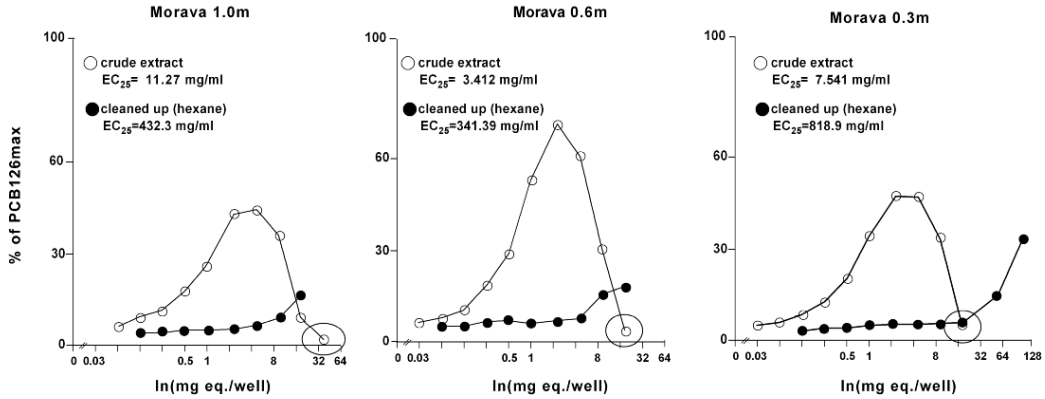

Figure 3. EROD activity in primary rat hepatocytes after 48h of incubation with crude (o) and cleaned up (•) extracts of sediment samples of river Morava. Activity is expressed as % of maximal production in the presence of PCB126. Circled points represent cytotoxic concentrations determined by measuring protein concentrations in wells.

GC/ECD analyses revealed relatively low levels of PCBs in sediment samples from rivers Lepenica and Morava, in range of ppb or less. In addition, bio-TEQ values of cleaned up extracts are relatively low and in the case when PCB levels were less than 1 μg/kg no EROD activity was detected (Tab. 1).

Table 1. Calculated bio-TEQ values and PCB levels determined by GC/ECD analyses in cleaned up extracts of sediment samples

Sample	bio-TEQ (pg/g)	GC/ECD (μg/kg)
Lepenica 1	56.05	6.32
Lepenica 2	31.68	6.92
Lepenica 3	no response	0.96
Lepenica 4	no response	0.99
Morava 1 (0.3m)	28.49	43.64
Morava 1 (0.6m)	68.34	58.1
Morava 1 (1m)	54.00	51.57

Our results elucidated that crude extracts expressed high EROD activity, what suggests the presence of complex mixtures of toxicants. They may include various compounds that can induce EROD activity, but that could not be identified by standard chemical analysis. But, significance of such determination is the measurement of combined effects of "priority chemicals" and chemically non-analyzed compounds in environmental samples, especially if such compounds occur in toxicologically relevant concentrations in contaminated environments[9]. Therefore, microEROD analysis is a useful screening tool for identification of different types of EROD-inducers in investigated samples and for prioritizing samples which require further chemical investigation.

Acknowledgements
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