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CYP1A expression in the hepatopancreas of *Mullus barbatus*, *Merluccius merluccius* and *Trigla lucerna* at the mouth of the river Bojana

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Abstract

Background and Purpose: *CYP1A is one of the most sensitive biomarkers in fish of exposure to polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). These compounds induce a dose-dependent transcriptional induction of the CYP1A gene that results in increased protein concentrations. The aim of this study was the detection of changes in CYP1A protein levels as a result of the exposure of fish to PAHs and PCBs at the mouth of the river Bojana in the spring.*

Materials and Methods: *The PAH and PCB contents in seawater and sediment were determined by gas chromatography. CYP1A induction was examined in the hepatopancreas of Red mullet (*Mullus barbatus*), European hake (*Merluccius merluccius*) and Tub gurnard (*Trigla lucerna*) by immunoblot analysis.*

Results and Conclusions: *Chemical analyses of seawater and sediment revealed the presence of several PAHs and PCBs. CYP1A was detected in the hepatic microsomal fraction in all examined fish species as an adaptive response to the presence of pollutants. The reported changes at the molecular level represent an early-warning signal that reflected the biological response of fish to elevated toxin concentrations in the environment.*

INTRODUCTION

In order to assess exposure to or effects of environmental pollutants on aquatic ecosystems, different biomarkers can be examined: biotransformation enzymes (phase I and II), oxidative stress parameters, biotransformation products, stress proteins, metallothioneins, immunological parameters, reproductive and endocrine parameters, genotoxic parameters, physiological, histological and morphological parameters *(1)*. The phase I biotransformation enzymes, notably CYP1A, belong to the most sensitive fish biomarkers known at present.

Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants in the aquatic environment. They induce a dose-dependent transcriptional induction of CYP1A gene and resulting increased concentration of protein *(2, 3)*. The induction of CYP1A occurs through ligand binding of PAHs and PCBs to a cytoplasmic receptor. Upon heterodimerisation with the aryl hydrocarbon nuclear translocator, the nuclear translocation of the complex proceeds. This complex, together with further co-activators, is thought to specifically bind to dioxin- or xenobiotic-responsive ele-

ments at the DNA upstream of promoters of CYP1A genes. Transcription factors now have ready access to the promoter region of the CYP1A gene. This results in an upregulation of gene transcription and subsequent rise in CYP1A mRNA, CYP1A protein level and CYP1A catalytic activity *(4)*. Generally, a good correlation is observed between CYP1A mRNA, protein levels and the activity of the enzyme.

In this study we performed Western-blot analysis in order to characterize the ecological impact of chemical contaminants in view of their toxicological potential. For that reason the relative changes of CYP1A concentrations in the hepatopancreas of the Red mullet-*Mullus barbatus*, European hake-*Merluccius merluccius* and Tub gurnard-*Trigla lucerna* were examined in the spring, at the mouth of the river Bojana, the Adriatic Sea, Montenegro. The selected species, with characteristic habitats and feeding behaviour , have been used previously in bioaccumulation studies of organochlorinated compounds in the western Mediterranean *(5)*. These fish are also of considerable commercial importance. In the hepatic microsomal fractions that were prepared from these species, increased CYP1A was observed, signifying activation of adaptive response to the presence of pollutants.

MATERIAL AND METHODS

Animals

Specimens of *Mullus barbatus*, *Merluccius merluccius* and *Trigla lucerna* were collected by trawling at the mouth of the river Bojana, as shown in Figure 1. The investigations took place in the spring (25th May). At least seven (and up to nine) individual fish of one species were pooled. The fish were killed immediately by spinosectomy according to standard animal care regulations. The hepatopancreas was quickly removed, washed in ice-cold 0.15 M NaCl and frozen in liquid nitrogen. Individuals of the same size were selected to ensure uniformity of samples.

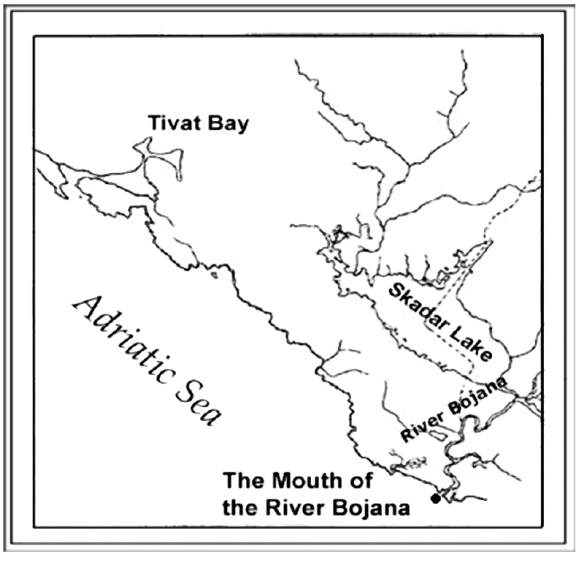

Figure 1. *A map of the Adriatic coast of Montenegro. The location in the region of the mouth of the river Bojana where the sea water samples were collected and the fish caught is indicated.*

Isolation of the microsomal fraction

The microsomal fraction of the hepatopancreas was prepared following the procedure of Krauss *et al. (6)*. The tissues were excised and homogenized (1g liver/ 1mL) in STM buffer: 0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 1 mM PMSF) and pelleted at 10,000× g, 4°C for 25 min. The obtained post mitochondrial supernatant was then centrifuged at 150,000 × g, 4°C for 60 min. The obtained microsomal pellets were resuspended in STM buffer.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis

For SDS-polyacrylamide gel electrophoresis (SDS--PAGE) 20 mg of microsomal proteins were loaded onto 4% stacking/12% separating slab gels as described by Laemmli *(7)*. The gels were stained using Coomassie Brilliant Blue R-250. Proteins separated by SDS-PAGE were electro blotted onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech). Immunoblot analysis was performed according to Towbin *et al. (8)* using a polyclonal antibody to fish CYP1A (CP226, Biosense laboratories, Norway). Immunoreactive bands were identified by an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions. Protein concentrations were determined according to Lowry *et al. (9)*.

Determination of PCBs and PAHs in water and sediment

PAHs from the filtrated seawater were extracted using a BAKERBONDspe™ PAH AQUA, 6 mL column (JT Baker products). PAH concentrations were determined by gas chromatography (GC) with a FID detector and a linear programmable temperature vaporizer (PTV) injector. PCBs from filtrated seawater were extracted using a BAKERBOND spe™ C_{18}, 6 mL, 500 mg column (JT Baker products). PCBs from sediment were extracted using Soxhlet apparatus in combination with silica gel column. PCB concentrations were determined by gas chromatography (GC) with an ECD detector and linear programmable temperature vaporizer (PTV) injector. The absence of individual peaks was not reported as zero but as less than the detection limit.

RESULTS

The chemical analyses of the PAH contents in seawater at the mouth of the river Bojana in the spring are shown in Table 1A. The analyses revealed the presence of anthracene and benzo(A)pyrene (2089 ng/L and 200 ng/L, respectively). Chemical analysis of the PCB contents in seawater and sediment at the examined locality are shown in Table 1B and C. PCBs in seawater were not detected (Table 1B), while pcb28 and pcb101 were de-

tected in sediment in concentrations of 147 ng/100g and 457 ng/ 100g, respectively (Table 1C).

The observed presence of PAHs and PCBs in seawater and sediment at the indicated locality led us to examine the induction of CYP1A, a well-established biomarker of exposure of fish to aromatic hydrocarbons. Proteins prepared from the hepatopancreatic microsomal fraction of *Mullus barbatus*, *Merluccius merluccius* and *Trigla lucerna* were separated by SDS-PAGE and stained with Coomassie Blue. Interspecies differences in protein profiles were established (Figure 2A, lanes 1, 2, 3). Following Western analysis with a polyclonal antibody to CYP1A species-specific levels of CYP1A expression were observed (Figure 2B). Comparison of the relative concentration of CYP1A in the hepatopancreas microsomal fraction from three analyzed fish species revealed that in *Mullus barbatus* and *Merluccius merluccius* CYP1A presence was similar (Figure 2B, lane 1 and 2) while in the sample from *Trigla lucerna* the detected CYP1A relative concentration was the highest (Figure 2B, lane 3).

DISCUSSION

The Adriatic Sea is relatively shallow and virtually land-locked, differing from other seas in its physico-chemical and biological characteristics *(10)*. Also, the Adriatic Sea is the final recipient for terrestrial wastewaters that contain chemical pollutants and its pollution state depends largely on the anthropogenic input. The DDT and PCB content and distribution along the South Adriatic and Montenegrin coast was studied previously *(11)*.

The maximal recommended values of xenobiotics in sea water and the minimal concentrations of xenobiotics that exert a negative impact on marine organisms are different in different countries. Compared to the values reported in the Environment Quality Standards for the Mediterranean Sea in Israel *(12)* the measured concentrations of PAHs and PCBa (Table 1) at the mouth of the river Bojana are under or at an acceptable level. Since the data about the effects of anthracene are insufficient, its maximal recommended concentration in seawater has not been established (Nagpal *(13)* and the Water Quality Criteria for PAHs in Canada), while the maximal recommended value of benzo(A)pyrene is 10 ng/L. Considering the maximal recommended values in Canada, the measured concentration of anthracene and benzo(A)pyrene in this study (Table 1) were above the recommended values. In the light of the recommended maximal total PCB concentration of 0.1 ng/L *(14)* and considering that the limit of detection of PCBs using the GC column is 10 ng/L, the presence of other PCBs cannot be ruled out. In a similar study, carried out in another part of the Montenegrin coast (at Platamuni, Valdanos and near the Bar harbour), correlation between CYP1A expression and the PAH and PCB content was observed *(15)*. Regardless of the differences between the minimal toxic concentrations, any change in xenobiotic concentrations should be considered as an indicator for potential contamination

TABLE 1

Concentrations of PAHs (A) and PCBs (B) in seawater and PCBs (C) in sediment collected from the locality at the mouth of the river Bojana.

A	
PAHs	ng/L
Acenaphthylene	<10
Fluorene	<10
Phenantrene	<10
Anthracene	2089
Pyrene	<10
Benz(A)anthracene	<10
Chrysene	<10
Benzo(B)fluoranthene	<10
Benzo(K)fluoranthene	<10
Benzo(A)pyrene	200
Benzoperylene	<10
Indeno(1.2.3.cd)pyrene	<10
Dibenzo(A)anthracene	<10
B	
PCBs	ng/L
2,4,4'-trichlorobiphenyl (pcb28)	<10
2,2',5,5'-tetrachlorobiphenyl (pcb52)	<10
2,2',4,5,5'-pentachlorobiphenyl (pcb101)	<10
2,2',3,4,4',5'-heksachlorobiphenyl (pcb138)	<10
2,2',4,4',5,5'-heksachlorobiphenyl (pcb153)	<10
2,2',3,4,4',5,5'-heptachlorobiphenyl (pcb180)	<10
C	
PCBs	ng/100g
2,4,4'-trichlorobiphenyl (pcb28)	147
2,2',5,5'-tetrachlorobiphenyl (pcb52)	<10
2,2',4,5,5'-pentachlorobiphenyl (pcb101)	457
2,2',3,4,4',5'-heksachlorobiphenyl (pcb138)	<10
2,2',4,4',5,5'-heksachlorobiphenyl (pcb153)	<10
2,2',3,4,4',5,5'-heptachlorobiphenyl (pcb180)	<10

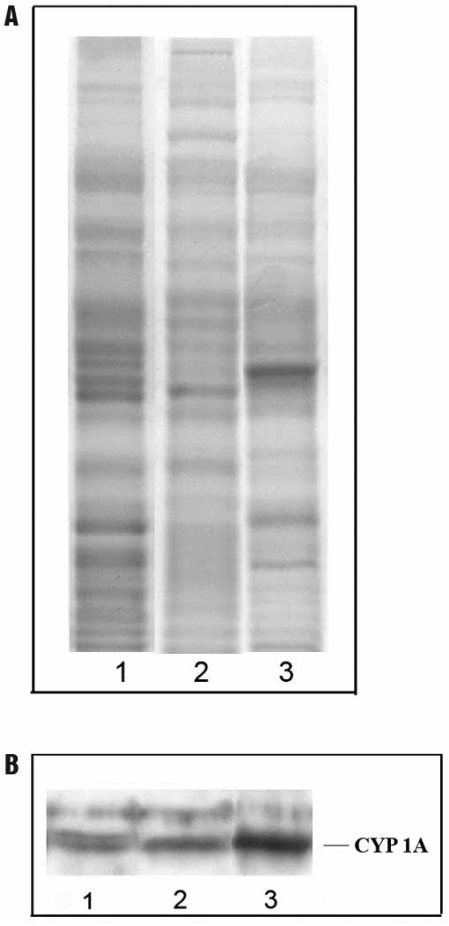

Figure 2. *SDS-PAGE profiles (A) and immunoblot analysis (B) of the microsomal fraction proteins prepared from the hepatopancreas of* Mullus barbatus *(1),* Merluccius merluccius *(2) and* Trigla lucerna *(3) with anti-CYP1A antibody. Twenty mg of proteins were subjected to 12% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R-250 (A). Immunoblotting was performed with a polyclonal antibody for CYP1A.*

that might lead to long-term and irreversible changes in ecosystems.

Together with chemical analysis, biomarker responses represent an additional approach in studies of the biological impact of environmental contaminants. The sudden death of large numbers of fish often indicates severe pollution. The effects of exposure to sublethal levels of pollutants can be measured in terms of biochemical, physiological or histological responses of the fish organism *(16)*. The activation of a set of intracellular antioxidant enzymes that eliminate reactive oxygen species and restore free radical homeostasis represents one such response *(17)*. However, no correlation between PAH concentrations and activity of the antioxidant defense enzyme system was observed in marine organisms *(18)*. In different fish species, hepatic CYP1A protein level increases are a very sensitive biomarker of exposure to PAHs and PCBs, particularly as the response-inducing xenobiotic concentrations are too low to be detected by other methods *(19)*.

Immuno blot analysis with a polyclonal antibody to CYP1A (Figure 2B) revealed the presence of CYP1A in hepatopancreas from all the examined fish species. In addition, species-specific differences in CYP1A expression were observed. Namely, the highest CYP1A concentration was observed in hepatopancreas of *Trigla lucerna* compared to that in *Mullus barbatus* and *Merluccius merluccius*.

The physiological values of many parameters in fish are susceptible to seasonal variations and are related to the species of fish, age and sex *(16, 19, 20)*. Therefore, it is important to use indicators independent of such physiological fluctuations. CYP1A induction represents a sensitive indicator of the entry of pollutants into an organism and their distribution in tissues. This biochemical response is the first signal of exposure to contaminants; it is usually reversible, contrary to the changes manifested at higher levels of organization of an organism, the population, community and ecosystem *(21)*.

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