Arch. Biol. Sci., Belgrade, 63 (1), 37-42, 2011 DOI:10.2298/ABS110137V

EXPRESSION OF CYP1A PROTEIN IN THE FRESHWATER CLAM *CORBICULA FLUMINEA* (MÜLLER)

JELENA VRANKOVIĆ[1], SVETLANA LABUS-BLAGOJEVIĆ[2] and M. PAUNOVIĆ[1]

[1]*Department of Hydrobiology, Institute for Biological Research "Siniša Stanković", University of Belgrade,* 11060 Belgrade, Serbia
[2]*Institute for Public Health of Serbia "Dr Milan Jovanović-Batut",* 11000 Belgrade, Serbia

Abstract – We investigated the expression of CYP1A in the foot, gill and visceral mass of the freshwater clam *Corbicula fluminea* in relation to polychlorinated biphenyls (PCBs) exposure. Different PCBs congeners were found in the foot and visceral mass, while the expression of CYP1A was observed only in the visceral mass. However the level of CYP1A expression in the visceral mass was not related to the level of PCBs present in the tissue. Our results indicate a higher rate of biotransformation and lower threshold of CYP1A induction in the visceral mass compared with other tissues.

Key words: CYP1A, PCBs, *Corbicula fluminea*

UDC 594.141.17:577.218

INTRODUCTION

In recent years, the rapid increase of anthropogenic activities has led to a continual influx of both organic and inorganic xenobiotics into the aquatic environment. The uptake and accumulation of xenobiotics in the tissues of aquatic organisms occur from the sediment, contaminated water column and food chain that cause deleterious effects. Aquatic organisms possess some intrinsic detoxifying mechanisms to eliminate accumulated xenobiotics. The xenobiotic metabolizing catalyst CYP1A (EC 1.14.14.1) is a phase I biotransformation enzyme widely used as a biomarker of exposure to organic compounds (Whyte et al., 2000). The CYP-dependent monooxygenase system has a wide tissue distribution, but is generally found in the highest levels in those tissues concerned with the processing of food, e.g. the liver of fish, hepatopancreas of crustaceans and digestive gland of molluscs. Filtering organisms such as bivalves and especially mussels are able to bioconcentrate organic compounds in their tissues and so are used widely as sentinel organisms to assess the concentration of pollutants in aquatic ecosystems (Vidal et al., 2001).

Persistent organic pollutants (POPs) are characterized by their persistence or longevity before degradation, thereby retaining high chronic toxicity (Hansen, 1998). Among POPs, polychlorinated biphenyls (PCBs) are considered as the most serious threat to biota. PCBs are liposoluble: they can build up in animal fat and along the food chain lead to various pathological conditions. PCBs are a bioaccumulative class of organic compounds that are highly resistant to metabolic biotransformation in invertebrates and fish (Safe, 1994).

Chemical analysis (i.e. gas chromatography/mass spectrometry) on the levels of POPs in environmental samples (water, sediment, biota) provide invaluable information on the severity of contamination (Cheung et al., 1997; Wong et al., 1999). Together with chemical analysis, biomarker responses

could be considered as a supplementary approach to determine the biological impact of environmental contaminant (van der Oost et al., 1997). Since PCBs induce CYP1A protein (Kleinow et al., 1987), the level of CYP1A expression was suggested as useful biomarker of PCBs exposure (van der Oost et al., 2003).

In this work, we examined the expression of CYP1A protein and concentrations of PCBs in *Corbicula fluminea* (Müller, 1774) tissues. The aim of this study was to find out whether the expression of this protein correlates with potential exposure to PCBs in this species. The examination of CYP1A expression described in this work was the first of its kind to be carried out in Serbia.

MATERIAL AND METHODS

Study area and sampling

Animals from wild populations were collected (during spring 2008) by benthic hand nets (Kick & Sweep multihabitat semi-quantitative technique, David et al., 1998) at the locality Ratno Ostrvo (44°50'18.1"N and 20°25'21.8"E), in the Danube River, upstream from Belgrade and the confluence of the Sava River.

After sampling the animals were identified and immediately transferred to the laboratory for processing. Individuals of the same size class were selected to ensure a uniform sample. The collected clams were then frozen and preserved at –80°C until further treatment.

PCBs content in the soft tissue

After the shells were removed, the clams were dissected to obtain feet, gills and visceral masses (taken from as many individuals as necessary to get a critical mass of tissue needed for further analysis). The tissue samples were minced and homogenized in 1 volume of 0.9% NaCl solution using Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogenizer at 4°C (Lionetto et al., 2003). The homogenates were saponified, extracted with *n*-hexane, and further cleaned by column chromatography (adsorbent: florisil, 60-100 mesh, ALDRICH; effluent: *n*-hexane, MERCK).

The concentrations of PCBs were measured by methods described by AOAC (Association of Official Agricultural Chemists), 983.21 (AOAC, 1995). The PCBs were evaluated by gas chromatography (GC) using an electron capture detector (ECD) and flame ionization detector (FID). The concentrations of PCBs in the samples were calculated using Aroclor 1248 standard (Sigma-Aldrich). The absence of an individual peak was not reported as zero but as less than the detection limit.

Isolation of the cytosolic fraction

The soft tissue was minced and homogenized in 5 volumes of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5 using Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogenizer (Rossi et al., 1983) and sonicated for 15 s at 10 kHz on ice (Takada et al., 1982). Sonicates were centrifuged at 100 000 x g for 90 min at 4°C and the resulting supernatant was used as the cytosolic fraction for further analysis.

SDS-polyacrylamide gel electrophoresis and Western blot analysis

Protein concentrations were determined according to Lowry et al. (1951). For SDS-polyacrylamide gel electrophoresis (SDSPAGE), the proteins (20 μg) were loaded onto 4% stacking/12% separating slab gels as described by Laemmli (1970). The gels were stained using Coomassie Brilliant Blue R-250. Proteins separated by SDSPAGE were electroblotted onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech). Immunoblot analysis was performed according to Towbin et al. (1979) using a polyclonal antibody to fish CYP1A (CP226, Biosense Laboratories, Norway). Immunoreactive bands were identified with an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology). Antigen-antibody complexes were analyzed and

quantified with Total Lab (Phoretix) electrophoresis software (version 1.10).

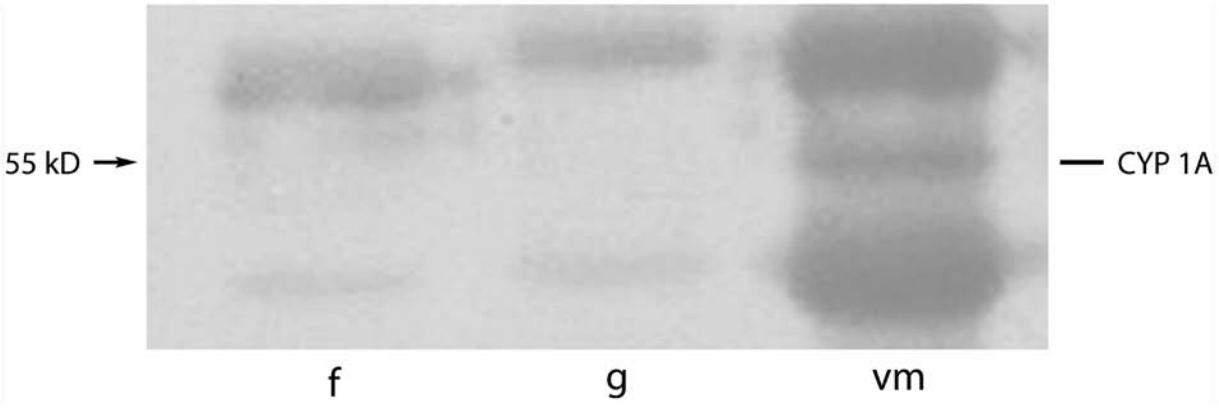
RESULTS AND DISCUSSION

Chemical analysis of the environment and animal tissues provides information concerning the presence of specific xenobiotic compounds. However, this data on its own is not particularly indicative of the concentrations to which the animals have been exposed and cannot serve as bioaccumulation markers for exposure assessment (van der Oost et al., 1997). The induction of CYP1A is an early-warning signal that reflects adverse biological responses to environmental toxins (Bucheli and Fent, 1995; Mihailović et al., 2010).

In the present study, following Western analysis with a polyclonal antibody to CYP1A (Fig. 1A) and quantification of antigen-antibody complexes (Fig. 1B), CYP1A was detected in the cytosolic fraction of the visceral mass, but no expression in the cytosolic fractions of the foot and gill of *C. fluminea* was observed. These could indicate that the visceral mass is exposed to higher concentrations of pollutants compared to the other tissues.

Chemical analysis of the foot and visceral mass showed the presence of PCBs, but the concentration and composition of PCB congeners were different. PCBs observed in the foot were PCB47, PCB154 and PCB171 at concentrations of 51.05, 26.65 and 52.75 ng/g of wet tissue, respectively. The results also revealed the presence of PCB98 (33.5 ng/g of wet tissue) and PCB171 (27.25 ng/g of wet tissue) in the visceral mass (Table 1). Gills were not analyzed for PCBs concentrations due to a lack of available material. As the limit of detection of PCBs using a

A



B

Corbicula fluminea		
foot	gill	visceral mass
0	0	46.8

Fig. 1.
A. Immunoblot analysis with anti-CYP1A antibody. Lane f – foot; g – gill; vm – visceral mass. Proteins (20 μg) were subjected to 12% SDS-PAGE and electroblotted to membranes. Immunoblotting was performed with a polyclonal antibody for CYP1A.
B. Quantification of antigen-antibody complexes. Antigen-antibody complexes (changes of the relative concentrations of CYP1A), were analyzed by densitometry using Total Lab (Phoretix) electrophoresis software (version 1.10).

GC column is 1 ng/g of wet tissue, the presence of PCBs at lower concentrations cannot be ruled out. The PCBs body burden is not in positive correlation with the expression level of CYP1A comparing the foot and visceral mass. The cytosolic fractions of the foot exhibited a remarkable lack of CYP1A sensitivity to halogenated hydrocarbons, showing no response to higher accumulated concentrations of PCBs. The apparently elevated concentrations of PCBs in the foot, without detected CYP1A, probably resulted from long term exposure to concentrations far below the threshold for CYP1A in the muscle.

The observed induction of CYP1A and lower concentrations of PCBs in the visceral mass (including digestive gland) primarily resulted from a high biotransformation rate in the digestive gland of *C. fluminea* and the efficient excretion of xenobiotic. This biochemical response is only the first signal of temporal exposure to contaminants and is usually reversible, contrary to the changes manifested at higher levels of organization of an organism, the population, community and ecosystem (van der Oost et al., 2003).

PCBs (and polycyclic aromatic hydrocarbons-PAHs) in the aquatic environment lead to a dose-dependent induction of the transcription of CYP1A genes and resulting increased concentrations of CYP1A proteins. Besides the concentrations of pollutants in the environment, the period of exposure to contaminants is also important. The continuously dosed regimes of organic contaminants produced a stronger response in aquatic organisms than pulsed regimes, and the examined parameters may not be useful indicators of contaminant exposure under conditions where "no dose" periods are a component of the experimental design or are an environmental fact (Richardson et al., 2008).

These findings support the selection of indicator organisms that inhabit bottom sediments (benthos, benthic taxa), rather than those that predominantly live in water medium (neuston), since pollutants could be attached to sediment fractions for longer periods of time, and consequently bottom living organisms are constantly exposed to the influence of sediment quality (continuously dosed regimes), even if the pollutants are not constantly present in the water column. On the other hand, if the particular pollution is not constantly present in the water column, organisms that are predominantly associated with the water column are only occasionally exposed to the pollutants (pulsed regime). Consequently, the reaction that could be used as biomarker is weaker. Moreover, due to the capacity of fine sediment to bind and accumulate certain pollutants (Slobodník et al., 2005), bottom living organisms are better indicator taxa than those primarily connected with the water column. Additionally, benthic species are usually less mobile in comparison to neuston.

Since the biotransformation mechanisms are localized principally in the digestive gland, the induction of CYP1A into the visceral mass of *C. fluminea* when compared to the other tissues may be due to the presence of other xenobiotics or a mixture of several contaminants. Our findings do not necessarily imply deleterious effects, as links between the levels of exposure, the degree of tissue contamination, and early adverse effects on the Asiatic clam need to be established further. Taken together, the results in this work indicated a tissue-specific response of CYP1A in relation to the PCBs presence.

Since the presence of CYP1A is still controversial in invertebrates, and numerous studies have demonstrated enzymatic and genetic differences with respect to vertebrates (Livingstone et al., 1995), the aim of our research was to identify one or more protein bands that could be attributed to CYP1A in *C. fluminea*. The CYP1A detected in the visceral mass clearly shows the existence of a cytochrome P450-mediated mixed function-oxidase system in *C. fluminea*. Therefore, it is important to measure CYP1A induction in tissues proximate to the ambient water, such as the gills, as well. (Levine and Oris, 1999).

Acknowledgments - This study was supported by Ministry of Science and Technological Development of Republic of Serbia (Grant No. 173025).

REFERENCES

AOAC (1995). Organochlorine Pesticide and Polychlorinated Biphenyl Residues in Fish: Gas Chromatographic Method. Sec. 10.2.01, Method 983.21., In: Official Methods of Analysis of AOAC International, 16th ed., P.A. Cunniff (Ed.), 11-12. AOAC International, Gaithersburg, MD.

Bucheli, T. D., and *K. Fent* (1995). Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Crit. Rev. Environ. Sci. Technol*. **25**, 201-268.

Cheung, Y. H., *Neller, A., Chu, K. H.*, *Tam, N. F. Y.*, *Wong, C. K.*, *Wong, Y. S.*, and *M. H. Wong* (1997). Assessment of sediment toxicity using different trophic organisms. *Arch. Environ. Contam. Toxicol.* **32,** 260–267.

David, S. M., Somers, K. M., Reid, R. A., Hall, J., and *R. E. Girard* (1998). Sampling protocols for the rapid bioassessment of streams and lakes using benthic macroinvertebrates, (Eds), Queen's Printer, Ontario.

Hansen, J. C. (1998). The human health programme under AMAP. AMAP human health group. Arctic monitoring and assessment program. *Int. J. Circumpolar Hlth.* **57**, 280–291.

Kleinow, K., *Melancon, M.*, and *J. J. Lech* (1987). Biotransformation and induction: implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. *Environ. Hlth. Persp.* **61**, 105–119.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* **227**, 680-685.

Levine, S. L., and *J. T. Oris* (1999). CYP1A expression in liver and gill of rainbow trout following waterborne exposure: implications for biomarker determination. *Aquat. Toxicol.* **46**, 279–287.

Lionetto, M. G., *Caricato, R.*, *Giordano, M. E.*, *Pascariello, M. F.*, *Marinosci, L.*, and *T. Schettino* (2003). Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymes activities) in *Mytilus galloprovincialis* and *Mullus barbatus* in an Italian coastal marine area. *Mar. Poll. Bull.* **46**, 324-330.

Livingstone, D. R., Lemaire, P., Matthews, A., Peters, L. D., Porte, C., Fitzpatrick, P. J., Forlin, L., Nasci, C., Fossato, V., Wootton, N., and *P. Goldfarb* (1995). Assessment of the impact of organic pollutants on goby (*Zosterisessor ophiocephalus*) and mussel (*Mytilus galloprovincialis*) from the Venice lagoon, Italy: biochemical studies. *Mar. Environ. Res.* **39**, 235–240.

Lowry, O. H., Rosebrough, N.J., Farr, A. L., and *R. J. Randall* (1951). Protein measurement with the Folin phenol reagent. *J. Biol.Chem*. **193**, 265-275.

Mihailović, M., Petrović, M., Grdović, N., Dinić, S., Uskoković, A., Vidaković, M., Grigorov, I., Bogojević, D., Ivanović-Matić, S., Martinović, V., Arambašić, J., Joksimović, D., Labus-Blagojević, S., and *G. Poznanović* (2010). CYP1A and metallothionein expression in the hepatopancreas of *Merluccius merluccius* and *Mullus barbatus* from the Adriatic Sea. *J. Serbian Chem. Soc.* **75,** 1149-1159.

Richardson, B. J., Mak, E., De Luca-Abbott, S. B., Martin, M., McClellan, K., and *P. K. S. Lam* (2008). Antioxidant responses to polycyclic aromatic hydrocarbons and organochlorine pesticides in green-lipped mussels (*Perna viridis*): Do mussels "integrate" biomarker responses?. *Mar. Poll. Bull.* **57**, 503–514.

Rossi, M. A., Cecchini, G., and *M. U. Dianzani* (1983). Glutathione peroxidase, glutathione reductase and glutathione transferase in two different hepatomas and in normal liver. *IRCS Med. Sci. Biochem.* **11**, 805.

Safe, S. H. (1994). Polychlorinated biphenyls (PCBs): Environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit. Rev. Toxicol.* **24**, 87-149.

Slobodník, J., Hamchevichi, C., Liška, I., Shearman, A., Csányi, B., Makovinská, J., Paunović, M., Tóthová, L., Stahlschmidt-Allner, P., and *B. Allner* (2005). Final report on sampling, chemical analysis and ecotoxicological studies. Deliverable BASIN R5.11, October 2005. EC Framework 6 Project AquaTerra, Project no. 505428 (GOCE). www.eu-aquaterra.de

Stegeman, J. J., and *M. E. Hahn* (1994). Biochemistry and molecular biology of monooxygenase: current perspective on forms, functions, and regulation of cytochrome P450 in aquatic species, In: *Aquatic Toxicology; Molecular, Biochemical*, *and Cellular Perspectives,* 87-206. (Eds. D. C. Malins and G. K. Ostrander). Lewis Publishers, CRC Press, Boca Raton.

Takada, Y., Noguchi, T., Okabe, T., and *M. Kajiyama* (1982). Superoxide dismutase in various tissues from rabbits bearing the Vx-2 carcinoma in the maxillary sinus. *Can. Res.* **42**, 4233-4235.

Towbin, H., Staehelin, T., and *J. Gordon,* (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA*. **76**, 4350-4354.

Van der Oost, R., *Vindimian, E.*, *Brink, P. J. V. D.*, *Satumalay, K.*, *Heida, M.*, and *N. P. E. Vermeulen* (1997). Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*). III. Statistical analyses of relationships between contaminant exposure and biomarkers. *Aquat Toxicol*. ***39***, 45–75.

Van der Oost, R., Beyer, J., and *N. P. E. Vermeulen* (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol*. **13**, 57-149.

Vidal, M. L., *Bassères, A.*, and *J. F. Narbonne* (2001). Potential biomarkers of trichloroethylene and toluene exposure in *Corbicula fluminea*. *Environ. Toxicol. Pharmacol.* **9**, 87–97.

Whyte, J. J., Jung, R. E., Schnitt, C. J., and *D. E. Tillit* (2000). Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Crit. Rev. Toxicol.* **30**, 374–570.

Wong, C. K. C., *Cheung, R. Y. H.*, and *M. H. Wong* (1999). Toxicological assessment of coastal sediments in Hong Kong using a flagellate, *Dunaliella tertiolecta*. *Environ. Poll.* **105**, 175–183.