www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 18: 313-324 (2018) DOI: 10.4194/1303-2712-v18_2_10

PROOF RESEARCH PAPER

Bioindication of Cardboard-Paper Mill Effluents Using Molecular Responses of Fish *Carassius auratus* and Bivalve Mollusk *Unio tumidus*

Halina I. Falfushynska[1,2], Lesya L. Gnatyshyna[1,2], Inna Goch[3], Oksana B. Stoliar[1,*]

[1] Ternopil National Pedagogical University, 2, M. Kryvonosa Str., Ternopil, 46027, Ukraine..
[2] I.Ya. Horbachevsky Ternopil State Medical University, 1, Maidan Voli Ternopil, 46001, Ukraine.
[3] WWF Danube-Carpathians Programme in Ukraine, 18, Simona Petlyury Str, apt.3, Kyiv, 02035, Ukraine.

* Corresponding Author: Tel.: : +380.352 522448; Fax: +380.352 436055;
E-mail: Oksana.Stolyar@gmail.com

Received 25 March2017
Accepted 28 June 2017

Abstract

The effluents of cardboard-paper mill (CPM) industry belong to most abundant pollutants of surface water. The aim of this study was to elucidate the toxicity of effluents downstream the CPM by the bioindication. The specimens of fish *Carassius auratus* (Cyprinidae) and mollusk *Unio tumidus* (Unionidae) from the suspected polluted area (SP) and pristine area as control (C) were compared. The fish and mollusk from SP demonstrated plural signs of toxicity and stress: 2.5−3.7 times higher levels of DNA fragmentation, nuclear abnormalities, lipid peroxidation products and lipofuscin (determined only in fish) in comparison with C-groups. The exposure to the certain xenobiotics was confirmed by the elevated by 2−3 times levels of metallothionein (response to toxic metals), CYP450-related activity and vitellogenin-like proteins (responses to endocrine disrupters, in particular, chlorinated organic substances). Cholinesterase activity in the brain of fish was the same in the C- and SP-groups proving the low evidence of typical agricultural pollution. Chemical analysis of water confirmed the highest level of pollution by phenols, chlorine, sulphate, nitrogen and organic compounds only directly downstream the wastewater treatment facilities of the CPM. These results prove the molecular bioindication to be the most valid approach to assess the toxicity of CPM effluents.

Keywords: Paper mill effluents; *Carassius auratus; Unio tumidus;* oxidative damage; metallothionein; Vitellogenesis; hepatic cytochrome P450.

Introduction

Due to the complex nature of the aquatic pollution and the ability of some substances to act even at low ppt–ppb concentrations or to undergo rapid degradation, the chemical analysis could give only the preliminary evidence of the toxicity of aquatic environment. The validity of molecular biomarkers of stress and exposure to different types of pollution in aquatic animals is proved in a broad number of experimental exposures (van der Oost, Beyer, & Vermeulen, 2003; Viarengo, Lowe, Bolognesi, Fabbri, & Koehler, 2007; Hook, Gallagher, & Batley, 2014). However, their applying in the in the environmentally realistic situations needs further development (**Dondero,** Banni, Negri, Boatti, Dagnino, & Viarengo**,** 2011).

Paper industry is considered as one of the most polluters among different kinds of industry in the world (Owens, 1991; Hoffman et al., 2015). The wastewaters generated from its production contain a mixture of potentially toxic products: reduced sulfur (sulfides, bisulfites), alkaline, high-chlorine compounds, chlorinated and plural other organic substances derived from polyglucans and terpenes. Additionally, pulping technology is water intensive (see for review Ince, Ince, & Cetecioglu, 2011). Despite the paper mill effluents represent the mix of toxic substances, their toxicity or effectiveness of purification (remediation) is mostly evaluated from the physico-chemical analysis (Devi, Yadav, Shihua, Singh, & Belagali, 2011; Mishra et al., 2013). The expertise of the toxicity of these effluents is realized by the experimental exposures *in vivo* or *in vitro* to their extracts during short period (Gagné and Blaise, 1993). The main manifestations of injury detected in these trials are genotoxicity and endocrine disruption (see for the review Hewitt, 2011). The bioindication of the consequences to environment is mainly devoted to the parameters of populations (Meriläinen & Oikari, 2008; Manríquez, Llanos-Rivera, Galaz, & Camaño, 2013) or bioaccumulation of toxic compounds (Hayer, Wagner, & Pihan, 1996) whereas the analysis of molecular responses of the inhabitants of polluted areas is rather scant (Cajaraville, Cancio, Ibabe, & Orbea, 2003; Oakes & Van Der Kraak, 2003).

The repeated accidents with mass fish death in

the small Ukrainian river (Pryp'yat basin, West Ukraine) were registered during two seasons of 2016 y. The situation was widely discussed in the local periodicals and social network (published only in Ukrainian), and the effluents of cardboard-paper mill (CPM) that is located upstream the river were proposed to be causal factors for these incidents. However, the traditional set of physico-chemical indices of the water was not able to prove this origin. Additionally, the signs of genotoxicity and cytotoxicity and high mortality were detected in the laboratory exposures of different aquatic organisms to the effluents (http://moemisto.info/index.php?option=com_news&Itemid=54&newsId=127985; http://cityukraine.info/?citynews=110024). However, the responses of feral animals were not studied, and the reasons for the toxicity of effluents were not investigated.

A key goal of this study was to develop a sensitive biotesting approach for the evaluation of particular impact of the paper mill effluents with the utilizing of the animal models of fish Cyprinidae and bivalve mollusk Unionidae inhabiting this area. Among aquatic animals, the biomarkers of effect are most verified in fish (van der Oost et al., 2003). Gibel carp *Carassius auratus* is widely distributed in freshwaters, and its feeding habits expose it to many different types of environmental contaminants. Despite the well-known tolerance of this species to environmental effects related to the morphological and biochemical peculiarities in their gills and hepatocytes (Eyckmans, Blust, & De Boeck, 2012), several incidents resulting in high mortality of this fish have occurred, including in April and October of 2016 y in the river Sluch in the rural area (https://baranivka.blogspot.com/2016/04/blog-post_17.html). Bivalve mollusks Unionidae are sessile filters that accumulate different pollutants including toxic metals and persistent organic substances and demonstrate the sensitive molecular responses in the exposures to xenobiotics (Hayer et al., 1996; Parolini & Binelli, 2012; Falfushynska, Gnatyshyna, Gyori, & Stoliar, 2014a; Faria et al., 2014; Blaise, Gagné, & Burgeot, 2016; El-Shenawy, Loutfy, Soliman, Tadros, & Abd El-Azeez, 2016). On the other hand, they can withstand rather high level of pollution by the effective mechanisms of detoxification and avoiding the excessive accumulation of pollution, so their survival in the toxic environment is suspected (Guénard, von der Ohe, de Zwart, Legendre, & Lek, 2011; Ahmad et al., 2012; Manfrin et al., 2012). To the best of our knowledge, the mussels are rare utilised for the evaluation of paper mill effluents toxicity. For example, at the time of writing, a search in Scopus database returned only 12 documents for the keywords "TITLE-ABS-KEY ('paper AND mill' AND mollusk" including Burgeot, His, and Galgani (1995), Cajaraville et al. (2003).

The expertise included a set of biomarkers of general stress and specific effects (neurotoxicity, metal-related toxicity, endocrine disruption) (Viarengo et al., 2007). Some utilised biomarkers were attested only in fish whereas their applying in mollusc is considered as doubtful (Van der Oost et al., 2003; Viarengo et al., 2007). The genotoxicity was analysed as DNA stability and nuclear abnormalities. The oxidative injury was assessed from lipid peroxidation level and accumulation of lipofuscin as the end-product of lipid and protein peroxidation in the lysosomes. We determined cholinesterase (ChE) activity in fish as a marker of neurotoxicity, which is oppressed mainly by thiocarbamate and phosphate pesticides, metallothionein concentration (marker of the pollution by toxic metals and stress-related protein), the level of vitellogenin-like protein (Vtg-LP) as a marker of endocrine disruption, which is elevated by environmental oestrogens in male specimens. The suspected endocrine disrupters in CPM effluents are chlorinated organic substances and phenols. The activity of ethoxyresorufin-*O*-deethylase (EROD) as a marker of the microsomal biotransformation of polycyclic hydrocarbons and halogenated aromatic hydrocarbons was measured in the liver of fish. Commonly used for the attesting of water quality chemical parameters were also measured in some points upstream and downstream the location of CPM discharges.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, USA) or Merck (Synbias, Kyiv, Ukraine), and were of the analytical grade or higher.

Experimental Groups

The experiments were carried out in October of 2016. We utilised two aquatic species, crucian carp *Carassius auratus* and bivalve mollusc *Unio tumidus.* We collected about two-year-old male specimens of *Carassius sp.* of 23±2 cm length with weight 276±24 g. Fish was collected by net and bivalve manually from 0.5-1 m depth. The both species' specimens were collected from two sites. One was the rural pond near the springs of the Seret River (tributary of Dniester River) in the forestry area with several fishing ponds in surrounding (village Hayi-Roztotski, 49°49′ N, 25°24′ E) where no industrial contamination should be detected. Specimens from this site represented the control groups (C). The other site (SP site) is situated at the place of Khomora River falling in the Sluch River (basin of Horyn/Pripyat River, close to the Pripyat Marshes) in the vicinity of the Baranivka city (50° 14′ N, 27° 38′ E), where the mass death of fish was fixed (last mentioning at October 10, http://www.zhitomir.info/news_160376.html). This site

received effluent from the paper mill industry dispersed in the sites located at the upper portions of the rivers Khomora and Sluch (in the village Poninka, 50°11′ N, 27°33′ E) (Figure 1). The sampling of fish, mollusks and water for analysis was carried out within the same week in both areas. About 20 individuals of fish and mollusks from each site were transported to the laboratory in 40 L cages with aerated native water (dissolved oxygen levels were 8.67 ± 0.51 mg/L) and analysed within a day after the sampling procedure. The mortality was not observed until dissection. The temperature of water was indicated in the time of sampling. Water samples were collected at a reference site and four sites along the SP area, sealed and transferred to the laboratory in iced packs to analyse chemical composition.

Experiments were performed in accordance with the national and institutional guidelines for the protection of animal welfare with approval of the Committee on the Bio-Ethics at Ternopil National Pedagogical University (No 1/15.10.2016). Fish put to sleep by clove oil (Goulet, HÚlie, & Vachon, 2010), and heparinized blood was collected from the heart. Liver was immediately removed. For serum preparation, whole blood was allowed to clot and centrifuged for 10 min at 1500 ×g for 10 min.

Nuclear abnormalities were assessed in the erythrocytes of fish, Vtg-LP were determined in blood plasma of fish or gonads of mollusks, ChE activity − in the brain of fish, while all other biomarkers – in the liver of fish or digestive gland of mollusk. Samples of tissues, blood serum (from fish) in each group were prepared individually and kept at –40°C until analyses except microsomal fraction separation, Vtg-LP concentration and micronucleus test which were carried out immediately. Hepatic/digestive gland tissue was homogenized (1:10 w:v) in 0.1 M pH 7.4 phosphate buffer containing 100 mM KCl, 1 mM EDTA and 0.1 mM PMSF to inhibit proteolysis. Microsomal pellet obtained by calcium (80 mM $CaCl_2$) precipitation of the postmitochondrial supernatant of liver homogenates was centrifuged for 20 min at 12,000 *x g* in 10 mM Tris-HCl buffer, pH 7.4 (Cinti, Moldeus, & Schenkman, 1972). Homogenization was carried out at 4 °C using 12–15 strokes of a motor driven Teflon Potter-Elvehjem homogenizer and centrifuged at 6,000 g for 10 min at 4° C. PMSF (0.1 mM) was also added to serum to inhibit proteolysis. Protein concentration in the supernatant, microsomal pellet and blood serum was measured by the method of Lowry et al. (1951) with using bovine serum albumin as a standard. The absorbance values were measured on the UV/Vis spectrophotometer "LOMO-56" (LOMO, Russian Federation), and the fluorescence was measured on the f-max fluorescence microplate reader (Molecular Device, USA). Each procedure of tissue sampling was carried out at a temperature of 4 °C.

Chemical Analysis of Water

Water parameters were measured by routine analytical tests: pH; ammonia, nitrite, nitrate, phosphate and phenol content by colorimetric methods; oxidizability (permanganate-related), chloride, sulphate, hardness, biochemical oxygen demands and dissolved oxygen by standard titration methods has been described elsewhere (www.epa.ie/rivermap/docs/Parameters.pdf).

Biomarkers of General Toxicity

To assay the genotoxicity, levels of protein-free DNA strand breaks in fish hepatocytes and mollusks

Figure 1. Location of the sampling sites in West Ukraine. Sites: C, control site. SP, suspected polluted area; I – Khomora river, Poninka village, upper from waste site of fish and mollusk sampling); IV – Khomora river, Pershotravensk urban village. The map from https://www.google.com.ua/url?sa=i&rct=j&q=&esrc=s&source=imgres&cd=&ved=0ahUKEwjRgLXjiNzSAhVIBSwKHWm4CV4QjxwIAw&url=http%3A%2F was utilised.

digestive gland cells were determined by the alkaline DNA precipitation assay (Olive, 1988) based on using Hoescht 33342 dye in the presence of 0.4 M NaCl, 4 mM sodium cholate, and 0.1 M Tris (pH 9) (Bester, Potgieter, & Vermaak, 1994). The micronucleated erythrocytes (MN) in peripheral blood were determined as miniature nuclei in the cytoplasm of postmitotic cells (Barsiene, Andreikenaite, & Rybakovas, 2006). For scoring of micronuclei, a criterion devised by Fenech et al. (2003) was adopted. The frequencies of MN were expressed per 1,000 cells studied. Also nuclear lesions were scored into other categories: lobed nuclei (L), dumbbell-shaped or segmented nuclei (S), and kidney-shaped nuclei (K). Cells with these abnormalities (NA) were counted and result was expressed as a mean value of the sums (L+S+K) for all the individual lesions observed per 1,000 cells (Pacheco & Santos, 1998).

Lipofuscin concentration in the liver of fish was determined using chloroform : methanol (2:1, v/v) extraction of the homogenate. The lower clear chloroform phase obtained after the centrifugation was dried and redissolved in chloroform. The fluorescent signal of lipofuscin was detected by using an *f*-max fluorescence plate-reader [excitation = 350 nm, emission = 450 nm]. A freshly prepared solution of quinine sulphate (1 μg·mL^{-1} of 0.1N H_2SO_4) was used as a standard (Manibabu & Patnaik, 1997).

Lipid peroxidation (LPO) was analyzed in the protein-free supernatant of the fish liver and mollusks digestive gland homogenate by the production of thiobarbituric acid-reactive substances (TBARS) (Ohkawa, Ohishi, & Tagi, 1979). A molar extinction coefficient of $1.56 \cdot 10^5$ M^{-1}·cm^{-1} was used.

Metallothioneins (MTs) as MT-related thiols (MT-SH) were determined in fish liver and mollusks digestive gland after ethanol/chloroform extraction with DTNB as described by Viarengo, Ponzano, Dondero, and Fabbri (1997) and calculated by assuming the relationship: 1 mol MT-SH = 20 mol GSH and expressed as μg of MTs per gram of fresh weighted (FW) tissues.

Vitellogenin-like proteins (Vtg-LP) were evaluated in fish blood serum and in mollusks' gonads as alkali labile phosphate level (Nagler, Ruby, & Idler, 1987; Blaise, Gagne, Pellerin, & Hansen, 1999). The method is based on principle that the trichloroacetic acid or t-butyl methyl ether-extracted lipophosphoproteins are subjected to an alkali treatment in order to release labile phosphates. The content of free phosphates was determined by the phosphomolybdenum assay.

Microsomal ethoxyresorufin-*O*-deethylase (EROD) activity as an indicator of the activity of the cytochrome P450 family I (CYP450 I) enzymes was analyzed in the postmitochondrial supernatant of liver homogenates centrifuged for 20 min at 12,000 x *g*. EROD activity was detected by measuring the absorbance of resorufin at 572 nm (Klotz, Stegeman, & Walsh, 1984) in the microsomal pellet obtained by calcium (80 mM $CaCl_2$) precipitation of the postmitochondrial supernatant in 10 mM Tris-HCl buffer, pH 7.4 (Cinti et al., 1972). The reaction at 30°C was initiated by the addition of 0.5 mM NADPH. EROD activity was calculated using a molar extinction coefficient of 73.2 mM^{-1} cm^{-1} and standardized to the microsomal protein content.

Cholinesterase (ChE, EC 3.1.1.7) activity was determined in the fish brain as the acetylthiocholine-cleaving ChE activity at 25°C according to the colorimetric method of Ellman, Courtney, Andres, and Featherstone (1961). Enzyme activity was calculated using a molar extinction coefficient of $13.6 \cdot 10^3$ M^{-1}·cm^{-1} and standardized to the soluble protein content.

Statistical Analysis

For all biological traits and experimental treatment groups, sample size was 5-6. The data are presented as means ± standard deviation (SD) unless indicated otherwise. Data were tested for normality and homogeneity of variance by using Kolmogorov-Smirnoff and Levene's tests, respectively. Whenever possible, data were normalized by Box-Cox common transforming method. One-way ANOVA was used to test the effect of experimental exposures, followed by post hoc procedures. For the data that were not normally distributed, non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney U-test) were performed. All statistical calculations were performed with Statistica v. 12.0 and Excel for Windows-2013. Differences were considered significant if the probability of Type I error was less than 0.05.

Results

General Signs of Toxicity and Stress

The evaluation of the genotoxicity has shown the elevated level of DNA fragmentation in the liver /digestive gland of specimens from SP-site (by 2.7 − 3.1 times) (Figure 2 A, F). The rate of the erythrocytes with the micronuclei and nuclear abnormalities in SP-group of fish was also higher by 2.5−3.7 times than in the C-group. The specimens from SP groups were characterized by high levels of TBARS (by 42.6% in fish and by 2.23 times in mollusks) and lipofuscin (by 34 %) in the liver of fish in comparison with the specimens from C-groups (Figure 2 D, E, G).

Markers of Exposure to Specific Xenobiotics

The concentration of metallothioneins in the liver of fish and in the digestive gland of mollusk was different in the specimens from SP and C groups (Figure 3A, E). In both cases, its level was by 2.5 − 3.6 times highly in the SP groups. The level of Vtg-

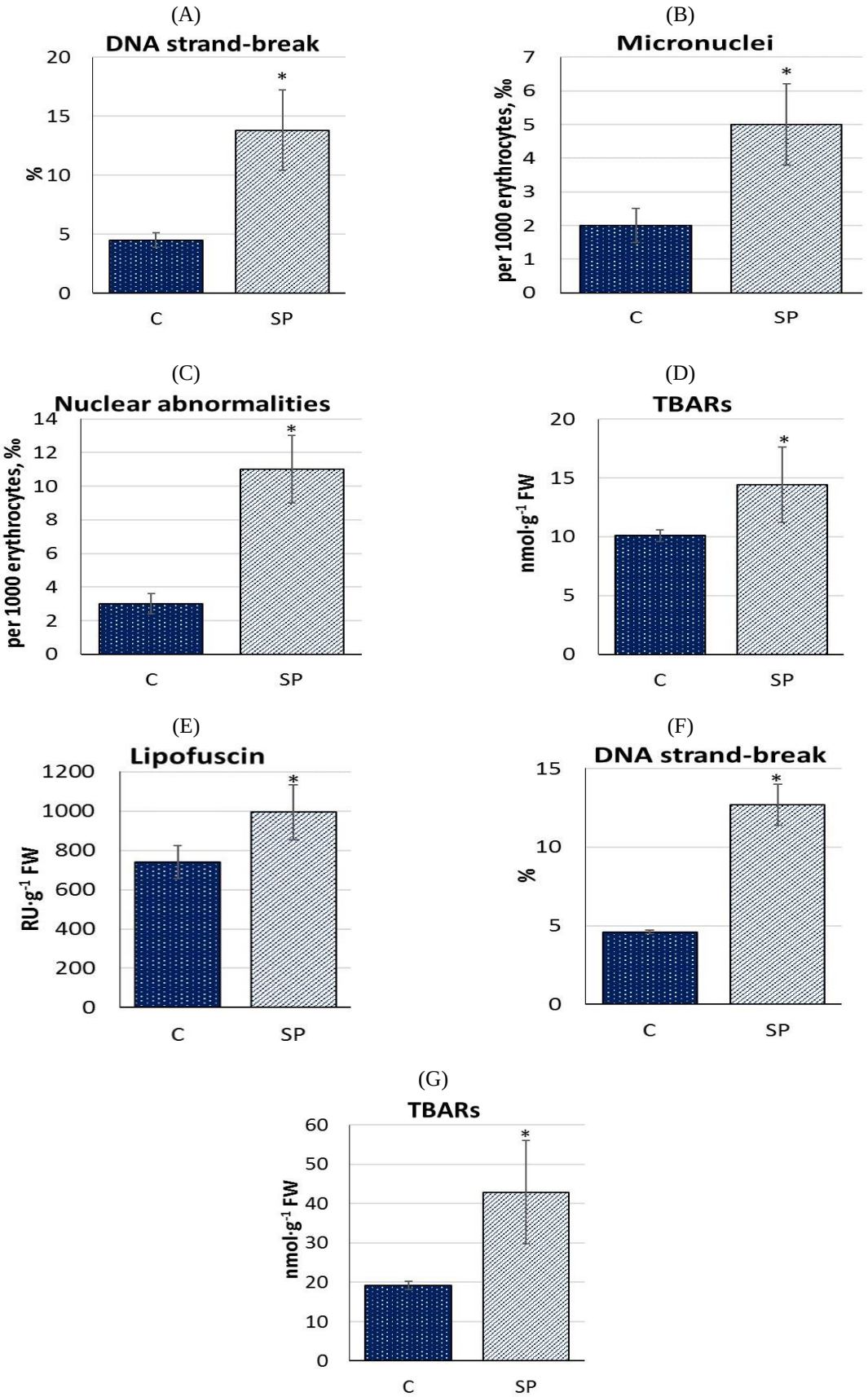

Figure 2. The indices of toxicity and stress in the fish *Carassius auratus* (A, C, D, E) and mollusk *Unio tumidus* (F, G) from control (C) and suspected polluted (SP) sites: DNA strand breaks in the fish liver (A) and mollusks digestive gland (F); micronuclei (B) and nuclear abnormalities (C) frequency in the fish erythrocytes; TBARS concentration in the fish liver (D) and mollusks digestive gland (G); lipofuscin concentration in the fish liver (E). Data are presented as means ± SD (N=6 for fish and 5 for mollusk). In figures 2-3: * indicates the values that are not significantly different (P > 0.05).

LP was also higher in the male specimens from SP-groups, particularly in fish (by 3.7 times) (Figure 3B). The EROD activity in the liver of fish from SP-area was 3.7 times higher than in C-specimens (Figure 3C). However, ChE activity in the brain of fish did not show differences between the groups from two sites (Figure 3D).

Chemical Characteristics of Water

From the comparison of the chemical indices of water samples (Table 1) it is shown that the site III (located directly downstream the CPM plant) was characterized by the elevation of permitted levels for most indices. In this site the elevated levels of reduced nitrogen compounds, phenols, sulphate,

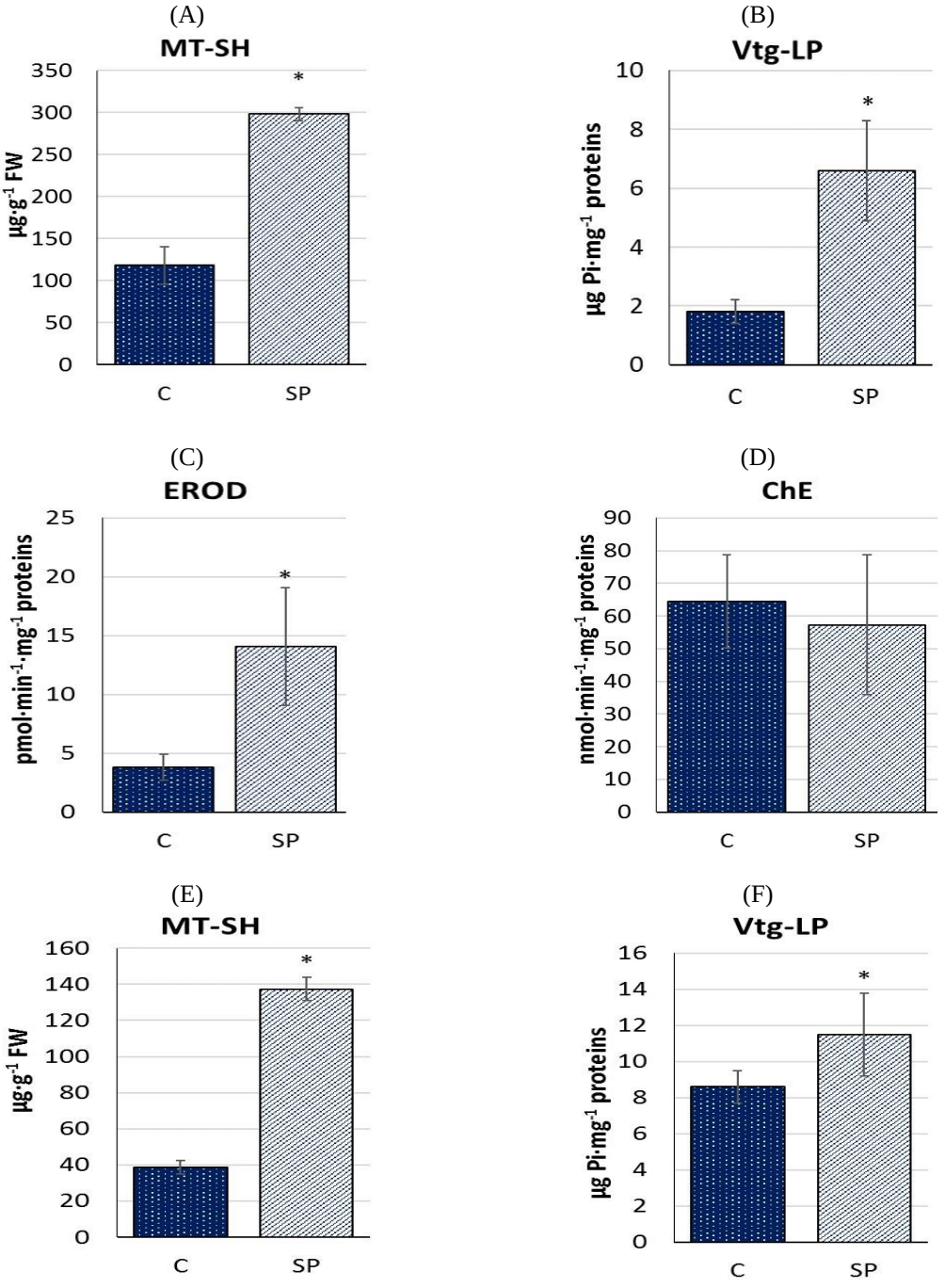

Figure 3. Markers of exposure in the tissues of fish *Carassius auratus* (A, B, C, D) and mollusk *Unio tumidus* (E, F) from control (C) and suspected polluted (SP) sites: metallothionein total concentration (MT-SH) in the fish liver (A) and mollusk digestive gland (E); vitellogenin-like protein concentration (Vtg-LP) in the fish liver (B) and mollusk gonads (F); ethoxyresorufin-*O*-deethylase activity (EROD) in fish liver (C); cholinesterase activity (ChE) in fish brain (D). Data are presented as means ± SD (N=6 for fish and 5 for mollusk).

Table 1. Physico-chemical parameters of water samples, M ± SD, *N = 3*

Parameters	Groups				
	C	III (SP)	I	II	IV
pH	7.72±0.09	6.83±0.05	7.35±0.05	6.59±0.05	6.65±0.05
Ammonia, mg N-NH_4^+/L	0.55±0.06	2.55±0.05*	0.95±0.04	1.35±0.05	1.90±0.04
Nitrites, mg N–NO_2^- /L	0.05±0.01	3.21±0.12**	0.48±0.27*	0.06±0.05	0.37±0.08*
Nitrates, mg N–NO_3^- /L	1.0±0.1	11.5±1.2	13.5±1.3	1.8±0.2	1.8±0.2
Phosphates, μmol/L	6.5±0.3	23.5±0.2	12.2±0.1	13.4±0.8	15.1±0.2
Phenols, μg/L	0.32±0.07	2.82±0.69*	0.67±0.09	0.71±0.06	0.60±0.06
Chlorides, mg/L	12.3±1.3	70.9±5.5	15.6±1.4	35.5±2.5	35.5±2.5
Iron (total content), μg/L	36.4±2.54	120.1±8.4	80.5±0.5	25.0±1.6	75.2±6.6
Sulphates, mg/L	42.3±5.2	516±36*	276±12*	240±48	312±24*
Total hardness, mmol/L	3.5±0.2	5.67±0.61	4.03±0.15	3.10±0.10	3.83±0.06
Oxidizability, mg O_2/L	16.3±2.1	62.2±2.4**	15.4±0.3*	15.8±0.4*	25.9±0.4*
Dissolved oxygen, mg O_2/L	10.3±1.5	6.7±0.7	10.4±0.6	10.8±0.1	8.0±0.3
Dry residue, mg/L	257±24	620±17	232±30	386±25	450±45
BOD, mg O_2/L	2.10±0.30	6.25±0.35*	5.75±0.32*	5.18±0.25*	5.86±0.18*

*, mean value exceeded maximum permitted concentration allowed for the protection of freshwater aquatic life, P<0.05

oxidizability and also highest levels of chloride, iron, dry residue were detected. In the other sites the lesser values of all indices were detected with the most similarity to the standard values in the point II (Markivka), most distanced from the plant.

Discussion

Biomarkers Reflect the Toxicity and Stress Responses

Despite the genotoxicity represents a long-time formed damage, accumulated during the lifespan of the cells (Bolognesi & Hayashi, 2011), the micronucleus test is well approved in the short-term laboratory exposures for fish (for example, Barsienė et al., 2006; Bücker, Carvalho, Conceição, & Alves-Gomes, 2012). From the studied biological parameters only genotoxicity is officially valid for the proving of aquatic pollution in Ukraine determined in the short-term laboratory exposures (SSTU 7387:2013.6). According to the results of micronucleus test in the official expertise of water from the studied site, the level of the cells with micronuclei was ~ 8 ‰ for erythrocytes, gills and fin of fish *Danio rerio* exposed during 96 h to the analyzed water (http://cityukraine.info/?citynews=110024). However, the information concerning the frequency ratio in comparison with the control samples was not represented. The genotoxicity of CPM effluents was confirmed by different experimental groups and using different test systems in model experiments (Hewitt, 2011; Roa, Yeber, & Venegas, 2012). In general, the data concerning the results of micronucleus test in different fish species and different exposures are highly distinct. For example, in the experiments with perch (*Perca fluviatilis L.*) exposed during ten days to 0.25, 0.5 or 1.0 ppm of crude oil the frequency of micronuclei varied from 0.23 to 0.35 ‰, whereas the control group of perch showed the lowest value (0.07‰ of MN, the frequency ratio 3.3 to 5.0) (Barsienė et al., 2006). Bücker, Carvalho, Conceição, & Alves-Gomes (2012) detected the time and concentration dependent elevation of the erythrocytes with the micronuclei (from 0.14‰ in control up to 4.00‰ after exposure during 96 h of the electric fish *Apteronotus bonapartii* to highest (25 ppm) concentration of benzen. Reported level of micronuclei frequency in the *Carassius sp*. is varying from 0.0−0,18 to 13 (Bolognesi & Hayashi, 2011). From our previous experience, the exposure of *Carassius sp.* to thiocarbamate or organochlorine pesticides, copper and manganese ions during fourteen days caused the elevation of the micronuclei frequency in the erythrocytes from 2‰ to 6.5−16‰ in the specimens depending on the origin of fish from the pristine or agricultural sites correspondingly (Falfushynska, Gnatyshyna, Stoliar, & Nam, 2011; Falfushynska, Gnatyshyna, & Stoliar, 2012).

In the present study, the frequency of the cells with nuclear abnormalities in fish from SP site was higher than it was detected previously (1‰ - 4‰) in two field sites with different levels of pollution during three seasons (Falfushynska, Gnatyshyna, Priydun, Stoliar, & Nam, 2010b). This manifestation witnesses the stable genotoxic press in the area. Moreover, in the present study the genotoxicity was undoubtedly confirmed from the high level of DNA instability both in fish and mollusks from the SP-group.

The comparison of the signs of oxidative lesions in the C- and SP-groups confirms the remarkable adverse effect in the SP site. From our experience of the field studies of *Carassius* sp. and Unionidae mussels in the corresponding area in Ukraine (Falfushynska et al., 2010b, Falfushynska et al., 2014a), the level of TBARS in the digestive tissues did not reflect so severe damage that in the present study. The oxidative lesions are confirming by the difference in the level of lipofuscin that is a stable

final product of lipid and protein oxidation accumulated in the lysosomes (Viarengo et al., 2007). The oxidative damage in fish caused by the CPM effluents was shown also by Oakes and Van Der Kraak (2003).

Consequently, the comparison of the cytotoxicity and general stress response in the studied groups has shown the strong and chronic adverse conditions in the SP-area. Furthermore, the comparison with the data available for the representatives of the same fila and in the common area confirms that this adverse effect prominently higher than the typical pressure in the agricultural and municipal sites and is corresponding to the emergency situation.

Specific Effects of Pollution Detected by the Biomarkers of Exposure

The attempt to detect the effect of specific kinds of pollution related to CPM effluents was undergoing in the present study by the evaluation of the markers of effect.

The elevated level of metallothionein in the fish liver and particularly in the bivalve tissues is the well-recognized marker of the pollution by toxic metals (Viarengo et al., 2007). This kind of pollution is not typical for the agricultural activity. On the other hand, the CPM industry is a valuable source of the pollution by toxic metals (Ince et al., 2011; Hoffman et al., 2015). Therefore, our finding confirms this specific source of pollution in the river. The elevated level of metallothioneins was highly sensitive biomarker to the pulp and paper mill effluents even in the acute exposure of fingerling trout (*Oncorhynchus mykiss*) (Gagné & Blaise, 1993).

Egg-yolk precursor Vtg is one of the most widely used biomarkers of exposure to estrogens in freshwater fish (Segner, 2009; Hinfray et al., 2010). The endocrine disruptive effect of the CPM effluents is well known about three decades in the exposures of fish in laboratory or in *in vitro* tests. Even the improved wastewater treatment did not totally remove it (Orrego et al., 2009; Waye et al., 2014). The precise structure of the endocrine disrupters in the CPM effluents is of the main contemporary research interest but it is not clarified exactly yet (for the review see van den Heuvel, 2010; Hewitt, 2011; Rutherford, 2011). Most expected compounds are the chlorinated organic substances, phytoestrogens and resin acids (Denslow, Kochera, Sepulveda, Gross, & Holm, 2004). The phytosterol represented in these extracts as well as two extracts increased VTG levels in rainbow trout (*Oncorhynchus mykiss* after 4, 7, 14, 21 and 28 days of exposure, both in males and females (Orrego, Guchardi, Krause, & Holdway, 2010). Decrease of the estrogens and Vtg in the blood plasma of female specimens and/or androgenic activation in the male under the exposure to the paper mill effluents was shown (Denslow et al., 2004). However, the opposite shift of endocrine disruption, the up-regulation of Vtg in the male specimens was detected in the present study for both species. In the mussels, the Vtg-LP in the hemolymph is not totally approved as a marker of endocrine disruption (Riffesern & Hock, 2002; Puinean et al., 2006; Oehlmann et al., 2007). However, the determination of Vtg-LP in gonads has been successfully used for the detection of oestrogenic effects in male mussels in the field sites with the municipal effluents discharge and pollution with the chlorinated aromatic compounds (Gagné, Blaise, & Hellou, 2004; Rittschof & McClellan-Green, 2005; Porte et al., 2006;). The signs of activated vitellogenesis in fish and bivalve were reflected earlier in field studies on fish and mollusks in the polluted sites in West Ukraine (Falfushynska et al., 2010a,b; Falfushynska et al., 2014b). However, they had not so regular and prominent manifestations as in the present study for aquatic animals in the SP-groups.

The CYP450-related microsomal activity represents the Phase I of the transformation of steroids and persistent organic xenobiotics, namely polyaromatic hydrocarbons and their chlorinated derivatives, such as dioxin (2,3,7,8-tetracholordibenzo-p-dioxin). Its elevated activity in fish is recommended as the biomarker of the pollution by these substances (Whyte, Jung, Schmitt, & Tillitt, 2000). In the field studies the elevated EROD activity is typical for the fish sampled in the sites with bleached kraft mill and industrial effluents, contaminated sediments, and chemical spills. Up-regulation of CYP-dependent enzymes activity and their expression was reported in the exposures of fish to the effluents of paper mill (Gagné & Blaise, 1993; Denslow et al., 2004). In rainbow trout (*Oncorhynchus mykiss*) EROD activity was up-regulated by the exposure to pulp mill effluents during 2-3 weeks, and the limits of this elevation reached threefold or more (Hodson et al., 1996). In the Channel catfish *Ictalarus punctatus* exposed to 0, 10, 20, and 40% bleached kraft effluent EROD activity was increased in the dose-dependent manner (Mather-Mihaich & Di Giulio, 1991). In the field study white sucker (*Catostomus commersoni*) collected in the vicinities of three pulp and paper mills with different technologies and in different seasons were compared. Fish collected downstream the paper mill exhibited elevated hepatic cytochrome P4501A activity. However, this response was not regular (Oakes & Van Der Kraak, 2003). The evaluation of EROD activities in the fish chub (*Leuciscus cephalus*) from five French rivers has shown the up-regulation of EROD in the four polluted sites, combined with the elevation of Vtg in males in most polluted site, however, the nature of the inducers for these responses was not analyzed (Hinfray et al., 2010). On the other hand, the successful recovery from the pollution by paper mill industry was confirmed by the stable level of EROD activity in wild and exposed to

sediments fish (Ratia, Vehniäinen, Rusanen, & Oikari, 2014).

Depletion of ChE activity is utilized as a biomarker of exposure to carbamate and organophophorus pesticides and is typical for fish from agricultural polluted field sites (Dorval, Leblond, Deblois, & Hontela, 2005; Linde-Arias, Inácio, de Alburquerque, Freire, & Moreira, 2008; Valbonesi, Brunelli, Mattioli, Rossi, & Fabbri, 2011; Hook et al., 2014). Therefore the absence of the ChE depletion in the studied fish from SP-site attests the mild level of agricultural pollution in the area in this season.

The results of chemical analysis of water confirm that they could not make the self-reliant evidence of the toxicity of CPM effluents for the aquatic animals. No doubt, the biomonitoring of water quality with the utilizing of early molecular responses of fish and mollusks must be included in this expertise. The Carassius sp. seems to be most relevant model due to the possibility to detect specific responses to the certain compounds. Similarly, in the acute toxicity tests for CPM effluents, the fish *Salmo gairdneri* was the more sensitive indicative species than *Daphnia pulex* or bacterial tests (Leal, Rocha, & Lema, 1997). However, in the case of the inability to sampling fish, the bivalve could represent the valuable test-organism according to our results.

Conclusions

The main question for the environmental expertise is to distinguish between CPM effluents and non-pointed sources of pollution typical for the rural area and represented by the mix of agricultural wastes, personal care and pharmaceutical products. The presented results attest the severity of lesions due to genototoxicity and indicate the complex pollution by toxic metals and organic substances that affects the cellular system of biotransformation, causes endocrine disruption and disturbance of metal homeostasis. These compounds are typical for CPM effluents, whereas impact of agricultural pollution in this area was not evident due to the absence of the neurotoxicity in fish. The further studies of the CPM effluents toxicity must be accented on the environmental monitoring with the elucidation of the specificity of the responses in comparison with the responses to agricultural wastes.

References

Ahmad, I., Mohmood, I., Coelho, J.P., Pacheco, M., Santos, M.A., Duarte, A.C., & Pereira, E. (2012). Role of non-enzymatic antioxidants on the bivalves' adaptation to environmental mercury: Organ-specificities and age effect in *Scrobicularia plana* inhabiting a contaminated lagoon. *Environmental Pollution*, 163, 218-25. doi: 10.1016/j.envpol.2011.12.023

Barsiene, J., Andreikenaite, L., & Rybakovas, A., (2006). Cytogenetic damage in perch (*Perca fluviatilis L.*) and duck mussel (*Anodonta anatina L.)* exposed to crude oil. *Ekologija*, 1, 25-31. Retrieved from http://www.elibrary.lt/resursai/LMA/Ekologija/Eko_025_03 1.pdf

Bester, M.J., Potgieter, H.C., & Vermaak, W.J.H. (1994). Cholate and pH reduce interference by sodium dodecyl sulfate in the determination of DNA with Hoechst. *Analytical Biochemistry*, 223(2), 299-305. doi: 10.1006/abio.1994.1588

Blaise, C., Gagné, F., & Burgeot, T. (2016). Three simple biomarkers useful in conducting water quality assessments with bivalve mollusks. *Environmental Science and Pollution Research, in press* doi: 10.1007/s11356-016-6908-6

Blaise, C., Gagne, F., Pellerin, J., & Hansen, P.D. (1999). Determination of vitellogenin-like properties in *Mya arenaria* hemolymph (Saguenay Fjord, Canada): A potential biomarker for endocrine disruption. *Environmental Toxicology and Chemistry*, 14(5), 455-465. doi: 10.1002/(SICI)1522-7278(199912)14:5<455::AID-TOX2>3.0.CO;2-8

Bolognesi, C., & Hayashi, M. (2011). Micronucleus assay in aquatic animals. *Mutagenesis*, 26(1), 205-213. doi: 10.1093/mutage/geq073

Bücker, A., Carvalho, M.S., Conceição, M.B., & Alves-Gomes, J.A. (2012). Micronucleus test and comet assay in erythrocytes of the Amazonian electric fish *Apteronotus bonapartii* exposed to benzene. *Journal of the Brazilian Society of Ecotoxicology*, 7(1), 65-73. doi: 10.5132/jbse.2012.01.010

Burgeot, T., His, E., & Galgani, F. (1995). The micronucleus assay in *Crassostrea gigas* for the detection of seawater genotoxicity**.** *Mutation Research/Genetic Toxicology***,** 342(3-4), 125-140. http://dx.doi.org/10.1016/0165-1218(95)90022-5

Cajaraville, M.P., Cancio, I., Ibabe, A., & Orbea, A. (2003). Peroxisome proliferation as a biomarker in environmental pollution assessment. *Microscopy Research and Technique*, 61(2), 191-202. doi: 10.1002/jemt.10329

Cinti, D.L., Moldeus, P., & Schenkman, J.B. (1972). Kinetic parameters of drug metabolizing enzymes in Ca^{2+}-sedimented microsomes from rat liver. *Biochemical Pharmacology*, 21(24), 3249–3256. http://dx.doi.org/10.1016/0006-2952(72)90089-5

Denslow, N.D., Kochera, J., Sepulveda, M.S., Gross, T., & Holm, S.E. (2004). Gene expression fingerprints of largemouth bass (*Micropterus salmoides*) exposed to pulp and paper mill effluents. *Mutation Research*, 552(1-2), 19-34. doi: 10.1016/j.mrfmmm.2004.06.001

Devi, N.L., Yadav, I.C., Shihua, Q.I., Singh, S., & Belagali, S.L. (2011). Physicochemical characteristics of paper industry effluents - a case study of South India Paper Mill (SIPM). *Environmental Monitoring and Assessment*, 177(1), 23-33. doi:10.1007/s10661-010-1614-1

Dondero, F., Banni, M., Negri, A., Boatti, L., Dagnino, A., & Viarengo, A. (2011). Interactions of a pesticide/heavy metal mixture in marine bivalves: a transcriptomic assessment. *BMC Genomics*, 12, 195. doi: 10.1186/1471-2164-12-195

Dorval, J., Leblond, V., Deblois, C., & Hontela, A. (2005). Oxidative stress and endocrine endpoints in white sucker (*Catostomus commersoni*) from a river impacted by agricultural chemicals. *Environmental Toxicology and Chemistry*, 24(5), 1273-1280.

doi: 10.1897/03-523.1

Ellman, G.L., Courtney, K.D., Andres, V.J., & Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, 7(2), 88-95. http://dx.doi.org/10.1016/0006-2952(61)90145-9

El-Shenawy, N.S., Loutfy, N., Soliman, M.F., Tadros, M.M., & Abd El-Azeez, A.A. (2016). Metals bioaccumulation in two edible bivalves and health risk assessment. *Environmental Monitoring and Assessment*, 188(3), 139. doi: 10.1007/s10661-016-5145-2

Eyckmans, M., Blust, R., & De Boeck, G. (2012). Subcellular differences in handling Cu excess in three freshwater fish species contributes greatly to their differences in sensitivity to Cu. *Aquatic Toxicology*, 118-119, 97-107.

doi: 10.1016/j.aquatox.2012.03.019

Falfushynska, H.I., Gnatyshyna, L.L., & Stoliar, O.B. (2012). Population-related molecular responses on the effect of pesticides in *Carassius auratus gibelio*. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 155(2), 396-406. doi: 10.1016/j.cbpc.2011.11.001

Falfushynska, H.I., Gnatyshyna, L.L., Farkas, A., Vehovszky, A., Gyori, J., & Stoliar, O.B. (2010a). Vulnerability of biomarkers in the indigenous mollusk *Anodonta cygnea* to spontaneous pollution in a transition country. *Chemosphere*, 81(10), 1342-1351. doi: 10.1016/j.chemosphere.2010.08.016

Falfushynska, H.I., Gnatyshyna, L.L., Gyori, J., & Stoliar, O.B. (2014a). Metallothioneins, caspase-3 and oxidative stress responses in the multi-marker study of freshwater mussel inhabiting sites of various human impact. *Turkish Journal of Fisheries and Aquatic Sciences (TrJFAS)*, 14(3), 591-603. doi: 10.4194/1303-2712-v14_3_01

Falfushynska, H.I., Gnatyshyna, L.L., Osadchuk, O.Y., Farkas, A., Vehovszky, A., Carpenter, D.O., Gyori, J., & Stoliar, O.B. (2014b). Diversity of the molecular responses to separate wastewater effluents in freshwater mussels. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*,164, 51-58. doi: 10.1016/j.cbpc.2014.04.007

Falfushynska, H.I., Gnatyshyna, L.L., Priydun, C.V., Stoliar, O.B., & Nam, Y.K. (2010b). Variability of responses in the crucian carp *Carassius carassius* from two Ukrainian ponds determined by multi-marker approach. *Ecotoxicology and Environmental Safety*, 73(8), 1896-1906. doi: 10.1016/j.ecoenv.2010.08.029

Falfushynska, H.I., Gnatyshyna, L.L., Stoliar, O.B., & Nam, Y.K. (2011). Various responses to copper and manganese exposure of *Carassius auratus gibelio* from two populations. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 154(3), 242–253. http://dx.doi.org/10.1016/j.cbpc.2011.06.001

Faria, M., Ochoa, V., Blázquez, M., Juan, M.F.S., Lazzara, R., Lacorte, S., … Barata, C. (2014). Separating natural from anthropogenic causes of impairment in zebra mussel (*Dreissena polymorpha*) populations living across a pollution gradient. *Aquatic Toxicology, 152*, 82-95. http://dx.doi.org/10.1016/j.aquatox.2014.03.020

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., & Zeiger, E. (2003). HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research*, 534(1-2), 65-75. doi: 10.1016/S1383-5718(02)00249-8

Gagné, F., & Blaise, C. (1993). Hepatic metallothionen level and mixed function oxidase activity in fingerling trout (*Oncorhynchus mykiss*) after acute exposure to pulp and paper mill effluents. *Water Research*, 27(11), 1669-1682.

http://dx.doi.org/10.1016/0043-1354(93)90131-Z

Gagné, F., Blaise, C., & Hellou, J. (2004). Endocrine disruption and health effects of caged mussels, *Elliptio complanata*, placed downstream from a primary-treated municipal effluent plume for one year. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 138(1), 33-44. doi: 10.1016/j.cca.2004.04.006

Goulet, F., HÚlie, P., & Vachon, P. (2010). Eugenol anesthesia in African clawed frogs (*Xenopus laevis*) of different body weights. *Journal of the American Association for Laboratory Animal Science*, 49, 460-463. doi: 10.1111/j.1467-2995.2006.00316

Guénard, G., von der Ohe, P.C., de Zwart, D., Legendre, P., & Lek, S. (2011). Using phylogenetic information to predict species tolerances to toxic chemicals. *Ecological Applications*, 21(8), 3178-3190. doi: 10.1890/10-2242.1

Hayer, F., Wagner, P., & Pihan, J.C. (1996). Monitoring of extractable organic halogens (EOX) in chlorine bleached pulp and paper mill effluents using four species of transplanted aquatic mollusks. *Chemosphere*, 33 (11), 2321-2334. http://dx.doi.org/10.1016/0045-6535(96)00323-2

Hewitt, M. (2011). Effect-directed analysis of complex environmental contamination. In W. Brack (Ed.), The Handbook of Environmental Chemistry (pp. 267-283). Berlin, Springer-Verlag Berlin Heidelberg, 365 pp.

Hinfray, N., Palluel, O., Piccini, B., Sanchez, W., Aït-Aïssa, S., Noury, P., Porcher, J.M. (2010). Endocrine disruption in wild populations of chub (*Leuciscus cephalus*) in contaminated French streams. *Science of the Total Environment*, 408(9), 2146-2154. doi: 10.1016/j.scitotenv.2010.01.003

Hodson, P.V., Efler, S., Wilson, J.Y., el-Shaarawi, A., Maj, M., & Williams, T.G. (1996). Measuring the potency of pulp mill effluents for induction of hepatic mixed-function oxygenase activity in fish. *Journal of Toxicology and Environmental Health*, 49(1), 83-110.

Hoffman, E., Bernier, M., Blotnicky, B., Golden, P.G., Janes, J., Kader, A., Walker, T.R. (2015). Assessment of public perception and environmental compliance at a pulp and paper facility: a Canadian case study. *Environmental Monitoring and Assessment*, 187(12), 766. doi: 10.1007/s10661-015-4985-5

Hook, S.E., Gallagher, E.P., & Batley, G.E. (2014). The role of biomarkers in the assessment of aquatic ecosystem health. *Integrated Environmental Assessment and Management*, 10(3), 327-341. doi: 10.1002/ieam.1530

Ince, B.K., Ince, O., & Cetecioglu, Z. (2011). Pollution prevention in the pulp and paper industries. In E. Broniewicz (Ed.), Environmental Management in Practice (pp. 117-174). Croatia, InTech, Open Access Publisher, 448 pp.

Klotz, A.V., Stegeman, J.J., & Walsh, C. (1984). An

alternative 7-ethoxyresorufin *O*-deethylase activity assay: a continuous visible spectrophotometric method for measurement of cytochrome P-450 monooxygenase activity. *Analytical Biochemistry*, 140(1), 138-145. http://dx.doi.org/10.1016/0003-2697(84)90144-1

Leal, H.E., Rocha, H.A., & Lema, J.M. (1997). Acute toxicity of hardboard mill effluents to different bioindicators. *Environmental Toxicology*, 12(1), 39-42. doi:10.1002/(SICI)1098-2256(1997)12:1<39::AID-TOX6>3.0.CO;2-9

Linde-Arias, A.R., Inácio, A.F., de Alburquerque, C., Freire, M.M., & Moreira, J.C. (2008). Biomarkers in an invasive fish species, *Oreochromis niloticus*, to assess the effects of pollution in a highly degraded Brazilian River. *Science of the Total Environment*, 399(1-3), 186-192. doi: 10.1016/j.scitotenv.2008.03.028

Manfrin, C., de Moro, G., Torboli, V., Venier, P., Pallavicini A., & Gerdol, M. (2012). Physiological and molecular responses of bivalves to toxic dinoflagellates. *Invertebrate Survival Journal*, 9, 184-199. Retrieved from http://www.isj.unimo.it/articoli/ISJ282.pdf

Manibabu, P.V., & Patnaik, B.K. (1997). Lipofuscin concentration of the brain shows a reduction with age in male garden lizard. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 117(3), 229-232. http://dx.doi.org/10.1016/S0742-8413(97)00054-6

Manríquez, P.H., Llanos-Rivera, A., Galaz, S., & Camaño, A. (2013). Ecotoxicological studies with newly hatched larvae of *Concholepas concholepas* (Mollusca, Gastropoda): bioassay with secondary-treated kraft pulp mill effluents. *Ecotoxicology and Environmental Safety*, 98, 110-118. doi: 10.1016/j.ecoenv.2013.09.015

Mather-Mihaich, E., & Di Giulio, R.T. (1991). Oxidant, mixed-function oxidase, and peroxisomal responses in channel catfish exposed to bleached kraft mill effluent. *Archives of Environmental Contamination and Toxicology*, 20(3), 391-397. doi:10.1007/BF01064409

Meriläinen, P., & Oikari, A. (2008). Exposure assessment of fishes to a modern pulp and paper mill effluents after a black liquor spill. *Environmental Monitoring and Assessment*, 144(1), 419-435. doi:10.1007/s10661-007-0004-9

Mishra, S., Mohanty, M., Pradhan, C. Patra, H.K., Das, R., & Sahoo, S. (2013). Physico-chemical assessment of paper mill effluent and its heavy metal remediation using aquatic macrophytes – a case study at JK Paper mill, Rayagada, India. *Environmental Monitoring and Assessment*, 185(5), 4347-4359. doi:10.1007/s10661-012-2873-9

Nagler, J.J., Ruby, S.M., & Idler, D.R. (1987). Serum phosphoprotein phosphorus and calcium levels as reproductive indicators of vitellogenin in highly vitellogenic mature female and estradiol-injected immature rainbow trout (*Salmo gairdneri*). *Canadian Journal of Zoology*, 65(10), 2421-2425. doi: 10.1139/z87-365

Oakes, K.D., & Van Der Kraak, G.J. (2003). Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. *Aquatic Toxicology*, 63(4), 447-463. doi:10.1016/S0166-445X(02)00204-7

Oehlmann, J., Di Benedetto, P., Tillmann, M., Duft, M., Oetken, M., & Schulte-Oehlmann, U. (2007). Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. *Ecotoxicology*, 16(1), 29-43. doi: 10.1007/s10646-006-0109-x

Ohkawa, H., Ohishi, N., & Tagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, 95(2), 351-358. http://dx.doi.org/10.1016/0003-2697(79)90738-3

Olive, P.L. (1988). DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. *Environmental and Molecular Mutagenesis*, 11(4), 487-495. doi: 10.1002/em.2850110409

Orrego, R., Guchardi J., Krause, R., & Holdway, D. (2010). Estrogenic and anti-estrogenic effects of wood extractives present in pulp and paper mill effluents on rainbow trout. *Aquatic Toxicology*, 99(2), 160-167. doi: 10.1016/j.aquatox.2010.04.016.

Orrego, R., Guchardi, J., Hernandez, V., Krause, R., Roti, L., Armour, J., … Holdway, D. (2009). Pulp and paper mill effluent treatments have differential endocrine-disrupting effects on rainbow trout. *Environmental Toxicology and* Chemistry, 28(1), 181-188. doi: 10.1897/08-191.1

Owens, J.W. (1991). The hazard assessment of pulp and paper effluents in the aquatic environment: a review. *Environmental Toxicology and Chemistry*, *10*(12), 1511–1540. doi: 10.1002/etc.5620101202

Pacheco, M., & Santos, M.A. (1998). Induction of liver EROD and erythrocytic nuclear abnormalities by cyclophosphamide and PAHs in *Anguilla anguilla L*. *Ecotoxicology and Environmental Safety*, 40(1-2), 71-76. doi: 10.1006/eesa.1998.1644

Parolini, M., & Binelli, A. (2012). Cyto-genotoxic effects induced by three brominated diphenyl ether congeners on the freshwater mussel *Dreissena polymorpha*. *Ecotoxicology and Environmental Safety*, 79, 247–255. doi: 10.1016/j.ecoenv.2012.01.008

Porte, C., Janer, G., Lorusso, L.C., Ortiz-Zarragoitia, M., Cajaraville, M.P., Fossi, M.C., & Canesi, L. (2006). Endocrine disruptors in marine organisms: approaches and perspectives. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*. 143(3), 303-315. doi: 10.1016/j.cbpc.2006.03.004

Puinean, A.M., Labadie, P., Hill, E.M., Osada, M., Kishida, M., Nakao, R., … Rotchell, J.M. (2006). Laboratory exposure to 17beta-estradiol fails to induce vitellogenin and estrogen receptor gene expression in the marine invertebrate *Mytilus edulis*. *Aquatic Toxicology*, 79(4), 376-383. doi: 10.1016/j.aquatox.2006.07.006

Ratia, H., Vehniäinen, E.-R., Rusanen, A., & Oikari, A. (2014). Recovery of historically contaminated watercourse polluted by chemical wood industry - EROD activity in fish as biomarker. *Soil and Sediment Contamination: An International Journal*, 23(2), 211-225. doi: 10.1080/15320383.2014.812613

Riffeser, M., & Hock, B. (2002). Vitellogenin levels in mussel hemolymph – a suitable biomarker for the exposure to estrogens? *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 132(1), 75-84. http://dx.doi.org/10.1016/S1532-0456(02)00048-0

Rittschof, D., & McClellan-Green, P. (2005). Molluscs as multidisciplinary models in environment toxicology.

Marine Pollution Bulletin, 50(4), 369-373. doi: 10.1016/j.marpolbul.2005.02.008

Roa, O., Yeber, M.C., & Venegas, W. (2012). Genotoxicity and toxicity evaluations of ECF cellulose bleaching effluents using the *Allium cepa* L. test. *Brazilian Journal of Biology*, 72(3), 471-477. http://dx.doi.org/10.1590/S1519-69842012000300009

Rutherford, R.J.G. (2011). Improving understanding of endocrine-active compounds in pulp and paper mill condensates using a mummichog (*Fundulus heteroclitus*) bioassay (M.Sc thesis). Wilfrid Laurier University, Canada. Retrieved from http://scholars.wlu.ca/cgi/viewcontent.cgi?article=2019&context=etd

Segner, H. (2009). Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 149(2), 187-195. http://dx.doi.org/10.1016/j.cbpc.2008.10.099

Valbonesi, P., Brunelli, F., Mattioli, M., Rossi, T., & Fabbri, E. (2011) Cholinesterase activities and sensitivity to pesticides in different tissues of silver European eel, *Anguilla anguilla*. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 154(4), 353-359. doi: 10.1016/j.cbpc.2011.07.003

van den Heuvel, M.R. (2010). Recent progress in understanding the causes of endocrine disruption related to pulp and paper mill effluents. *Water Quality Research Journal of Canada*, 45(2), 137-144

Van der Oost, R., Beyer, J., & Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology***,** 13(2), 57-149. http://dx.doi.org/10.1016/S1382-6689(02)00126-6

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., & Koehler, A. (2007). The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 146(3), 281-300. http://dx.doi.org/10.1016/j.cbpc.2007.04.011

Viarengo, A., Ponzano, E., Dondero, F., & Fabbri, R. (1997). A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic Molluscs. *Marine Environmental Research*, 44(1), 69-84. http://dx.doi.org/10.1016/S0141-1136(96)00103-1

Waye, A., Annal, M., Tang, A., Picard, G., Harnois, F., Guerrero-Analco, J.A., … Arnason, J.T. (2014). Canadian boreal pulp and paper feedstocks contain neuroactive substances that interact in vitro with GABA and dopaminergic systems in the brain. *Science of the Total Environment*, 15 (468-469), 315-325. doi: 10.1016/j.scitotenv.2013.08.040

Whyte, J.J., Jung, R.E., Schmitt, C.J., & Tillitt, D.E. (2000). Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology*, 30(4), 347-570. doi: 10.1080/10408440091159239