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Assessment of the Sediment Toxicity in Bulgarian and Turkish Rivers Using the Biomarkers in *Chironomus riparius* Mg. (Diptera: Chironomidae)

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Abstrcat: As a model organism we used *Chironomus riparius* Mg. – a widely distributed species that can be reared in the laboratory conditions and has excellent salivary gland chromosomes.

The study showed that the genome at cytogenetical and biochemical levels is a sensitive biomarker and can serve as early – warning indicators of environmental impact of chemicals. Analysis of trace metals in sediment of Chaya River (Asenovgrad, Bulgaria, 2010) and Derincay River (Turkey, 2010), indicated higher concentrations of trace metals (Pb, Cr, Cu, Cd) in comparison with reference data. The response at cytogenetical level is determined by changes of gene expression of key structures (BRs and NOR) and increased in structural chromosome somatic aberrations. Changes of gene expression are indicated by decreasing the transcriptional activity of BRs and NOR: very often they occurred in the intermediate state of activity or BRs are in collapse. The cells with somatic rearrangements of *C. riparius* from polluted Derincay and Chaya River were in 16.94% and 36.36% respectively, which were significantly higher than those of the control (Derincay River: $G = 125.53$, $P<0.001$; Chaya River: $G = 73.81$, $P<0.001$). Somatic index was the highest in the sample from Chaya River – 3.25, following by Derincay River -1.58, while it was 0.13 in the control sample.

The response of the *C. riparus* at biochemical level is characterized by increaseing amount of metallothionein (MT) higher in Chaya River in comparison of Derincay River. Similarly, the other studied enzyme activities, GST and EROD, showed on the average 20% and 24% higher activities respectively, in Derincay than in Chaya. In addition, in accordance with above enzyme activities, the level of AChE inhibition is about 10% higher in Derincay River than in Chaya River. As a result, alterations in these biochemical parameters could be regarded as valid reflections of the increased trace metals in Derincay and Chaya Rivers.

It could be concluded that the environmental diagnose quality by multilevel approach (cytogenetical and biochemical) will enable better understanding of the impact of pollutants on organisms and should be successful implemented in environmental monitoring procedures.

Key words: Balbiani rings, Chironomidae, enzymes activities, Nucleolar Organizer, polytene chromosomes, somatic alterations

Introduction

Environmental damage is one of the most critical questions in the environmental field in general. Selection of the most informative method and indicative group of organisms is therefore very important. From the perspective of assessing the pollution by biomarker studies, benthic larval stages of Chironomids (Diptera) have been considered a typical bio-monitoring model for eco-toxicological tests (Warwick 1988) and are included in both Extended Biotic Index (De Pauw *et al.* 1992) and Annex V.1.2.6 of EC Water Framework Directive.

Chironomus riparius Mg. is recognized as very suitable species for studying the effect of contaminants in aquatic ecosystems (Ankley *et al.* 1994) and fulfils the criteria for using the environmental monitoring and laboratory toxicity (Choi 2004, Michailova 2011). It is a widely distributed species with short life cycle and can easily rear in laboratory conditions. Its larva stage is sensitive to many pollutants, responsive to different stress agents in the environment. The larvae possess excellent salivary gland chromosomes with great resolution which makes them a prospective model for cytogenetic monitoring. It was shown that the somatic rearrangements in Chironomids polytene chromosomes appeared as promising tools for detection the stress agents in the environment (Michailova *et al.* 2012).

Also, biomarkers that employ enzyme activity measurements detecting low levels of pollution are heavily used in ecological risk assessments of aquatic ecosystems. Acetylcholinesterase (AChE) inhibition or increased metallothionein (MT) contents have been widely used in terrestrial and freshwater aquatic systems as an indicator of organophosphorous (OPs), carbamate (CBs) exposure and heavy metal contamination (Kägi, Schäffer 1988). Measurement of etoxyresorufin O-deethylase (EROD) and glutathione S-transferase activities (GST) activities are using as the exposure index, thus enabling the identification of areas contaminated by industrial or domestic pollutants (Burgeot *et al.* 2001).

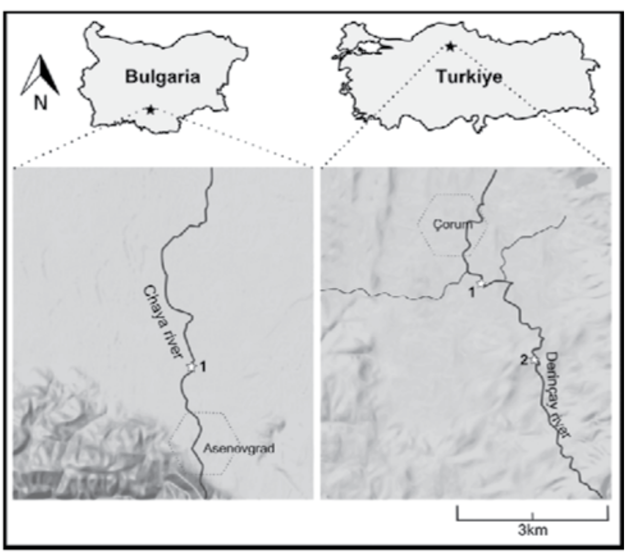

Fig. 1. The studied stations – Chaya River in Bulgaria and Derincay River in Turkey.

In this study the integrated system of genotoxic damage is proposed to be used. It will evaluate the somatic structure and functional alterations of the salivary gland chromosomes of *Chironomus ripsrius* Mg. as well as the changes of enzymes activities of GST, EROD, AChE and MT in this species, collected from anthropogenically polluted water basins in Bulgaria and Turkey. These basins are closed to factories which waste are pour in the studied stations and their contaminants are mainly by trace metals.

Material and Methods

Study area and materials

The samples of sediments and *C. riparius* larvae were collected in 2010 year from two field polluted stations: one from Bulgaria (Chaya River, near Asenovgrad) and one in Turkey (Derincay River, near Corum) (Fig. 1). Chaya is one of the most contaminants rivers that is originated from the factory producing different metals and pour its waste in river Derincay is polluted by some factories (sugar refinery, leader, poultry and metals), domestic sewage and agricultural runoff.

C. riparius larvae were not found in unpolluted basins of both studied countries as a control site for cytogenetic data we used data from the literature – Corio, Italy (Sella *et al.* 2004) where concentration of trace metals were less than those of the reference (Forstner, Salomons, 1980).

Samling the sediment and its analysis

Sediment samples were air dried overnight and then dried in an oven 60 °C until constant weight. Digestion was carried out utilizing Anton Paar Multiwave 3000 microwave system.

Water samples acidified with nitric acid, hydrochloric acid and hydrofluoric acid, following by complexation with acid and nitric acid, hydrogen peroxide digestion was also performed. Cr, Mn, Cu determined was performed with ICP-OES (Perkin Elmer Optoma 4300 DV) and for Cd and Pl – ICP-MS (Perkin Elmer ELAN DRCII) was used. All measurements were performed for two sets of samples and in 3 replicates. Rh was used as internal standard in IPC-MS measurements. NIST- 1643 Trace Elements in water was used as reference material for water samples.

Sampling the Chironomid larvae

Chironomid larvae were collected from sediments of the studied stations using special nets and sieves. After cleaning from the mud the largest larvae were separated and fixed for cytogenetical analysis.

Cytogenetic method

Chironomid larvae were fixed in alcohol: acetic acid (3:1). Fourth instars larvae, phase 6-7 (Wülker, Götz 1968) were used for preparing salivary gland chromosomes and external morphology preparations of the larvae. Chromosome preparations were done according to Michailova (1989). Both external morphology and polytene chromosomes done from each larva were maintained in the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences. *C. riparius*, an abound species, was identified by the specific chromosome markers (Michailova 1989, Kiknadze *et al.*1991). Polytene chromosome map of *C. riparius* of Hägele (1970) and Kiknadze *et al.* (1991) were used as standard. Number of studied specimens and cells was shown on Table 1.

Biochemical methods

Before starting the enzyme analysis collected *C. riparius* larvae were preserved in dry ice. Homogenization and preparation of cytosolic and microsomal fractions performed on ice according to Schenkman, Cinti (1978). Aliquots of fractions were stored at -80 °C for biomarker analysis. Protein concentrations of cytosolic and microsomal fractions were determined by the method of Lowry *et al*. (1951). Cytosolic AChE (Acethylcholinesterase) activities were assayed according to the Ellman *et al*. (1961) in a medium containing 0.6 mM 5,5'-dithiobis 2-nitrobenzoic acid (3,3'-6) (DTNB) and 7.5 mM ATC in 100 mM Tris-HCl buffer pH 8.5.

GST (*Glutathione S-transferase)* activities using CDNB (1-Chloro 2,4 dinitrobenzene) as a substrate were determined at room temperature spectrophotometrically at 340 nm. Enzyme activities towards CDNB were calculated using extinction co-

Table 1. Number of studied individuals and cells of *C. riparius* from studied regions

Locality	Corio, Italy (control)	Chaya River, Bulgaria	Derincay River, Turkey
Number of studied individuals	46	4	12
Number of studied cells	1349	44	248

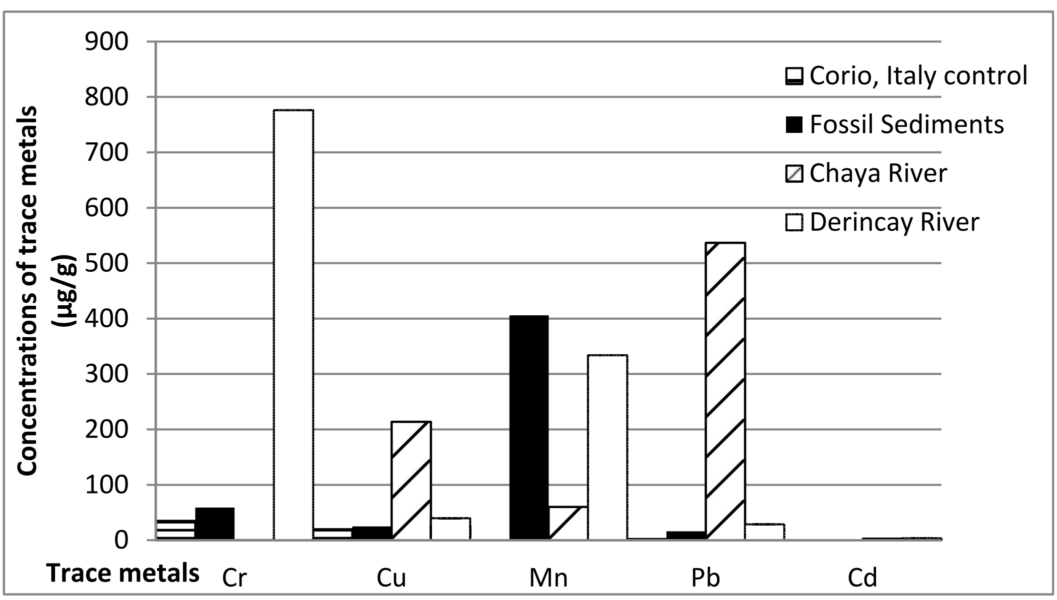

Fig. 2. Concentrations of the trace metals (μg/g) in the sediments of the studied rivers.

efficient of 9.6 $mM^{-1} \cdot cm^{-1}$ (Habig *et al*. 1974).

Microsomal EROD (7-Ethoxyresorufin-O-deethylase) activities were assayed using the methods of Burke, Mayer (1974), as optimised by Arinc, Sen (1994), using 7-ethoxyresorufin as a substrate. The fluorescence increase (λ_{ex} = 535 nm; λ_{em} = 585 nm) was recorded using a Cary Eclipse fluorometer for 120 seconds.

MT (Metallothionein) contents of *C. riparius* were determined as described by Viarengo *et al.* (1997), as optimised by Ozdemir *et al.* (2011) by evaluating the concentration of reduced sulphydryl by reading the absorbance at 412 nm, utilizing GSH as a reference standard.

Statistical analysis

Somatic structural alterations in the polytene chromosomes were calculated in percentages. On the basis of these chromosome aberrations, the somatic index of *C. riparius* from the studied polluted sites was calculated (Sella *et al.* 2004). The level of functional activity as some indicator of the degree of transcription of the BRs and NOR on chromosome G was analyzed. Three levels of puffing (Beermann 1971) were used including high activity (++/++), intermediate activity (+) and little or no activity (-). Somatic functional activities of BRc/BRb were calculated by Student's t-test. Levels of (BRc/BRb) and NOR activities were compared with standard activities of the 4th instars larva previously reported (Kiknadze 1978).

The variations in the cytosolic AChE, GST and microsomal EROD activities and MT contents between sampling sites were analyzed by one way analyses of multivariation test (one way ANOVA) and the two samples – test in Minitab 10.3.

Results and Discussion

Fig. 2 shows the concentrations of trace metals (Cr, Cu, Mn, Pb and Cd) in studied Bulgarian and Turkish stations. As a reference data we used fossil sediments of rivers in Germany (Forstner, Salomons 1980). In the sediment of Bulgarian station (Asenovgrad – Chaya River) the concentrations of Pb was 33.54, Cd was 14.3, and Cu was 10.69 times higher than that of fossil sediment (Forstner, Salomons 1980). In Turkey, in Derincay River Cd concentration was more than 18, Cr was 13.15, Pb was 1.79 and Cu 1.1 times higher than that of the reference.

The effect of trace metals on *C. riparius* was analyzed at three level of organization:

At morphological level – detecting the mouth deformities of the larvae.

The appearance of malformations in Chironomids larvae is well known phenomenon (Warwick 1988). The assessment of mouthpart deformities offers a powerful and successful tool to monitor the overall toxicity of contaminated sediments, especially exposed to trace metals (Vermeulen *et al*. 2000). No malformations were detected in individuals of *C. riparius* from both studied rivers.

At cytogenetical level – studying the somatic structural and functional alterations of the polytene chromosomes of *C. riparius* from the trace metal polluted Bulgarian and Turkish rivers.

The species has chromosome set 2n = 8, with chromosome arm combinations AB CD EF and G

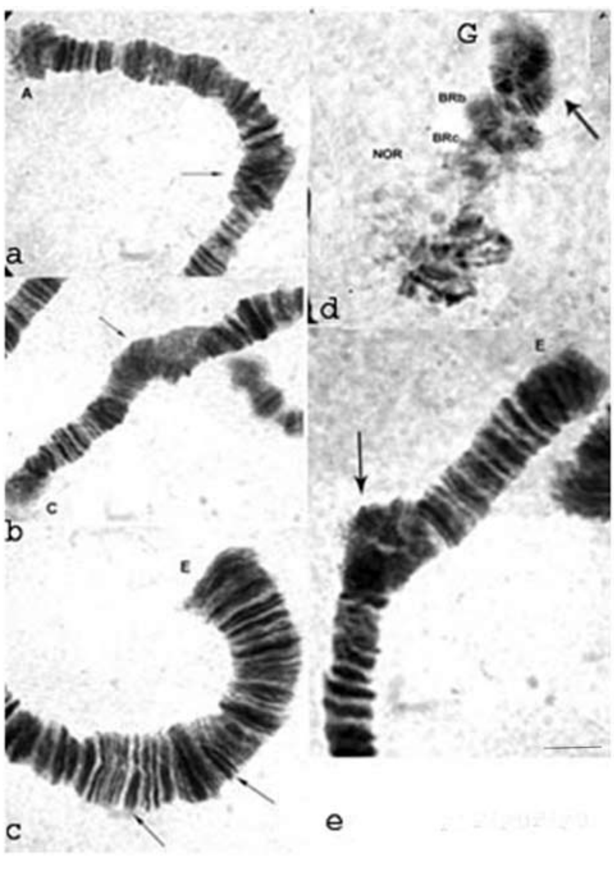

Fig. 3. Somatic chromosome aberrations in *C. riparius*: Bar 10 μm:
a) pericentric heterozygous inversion in AB chromosome; b) heterozygous inversion in arm C; c) amplification in EF chromosome (from paper by Michailova *et al.* 2010); d) heterozygous inversion in G chromosome; e) pericentric heterozygous inversion in EF chromosome (from paper by Michailova *et al.* 2009).

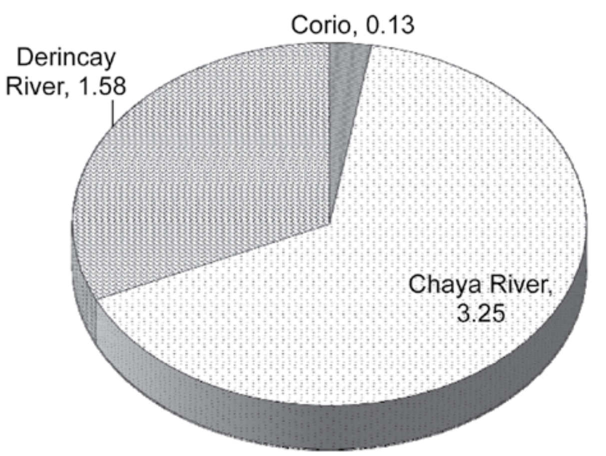

Fig. 4. Somatic index.

(cytocomplex ‘thummi’).Chromosome G has three Balbiani rins (BRa, BRb and BRc and a Nucleolar Organizer (NOR)). BRa is active in few cells only.

It is important to underline that the genome of *C. riparius* was very sensitive – every individual has either structural or functional alterations in their salivary gland chromosomes. The somatic structural chromosome rearrangements observed in the polytene chromosomes of *C. riparius* (heterozygous inversions, deficiencies, deletions and amplifications) (Fig. 3a, b, c, d, e) occurred in a significant higher frequency than that in a control station (Corio, Italy). The cells with somatic rearrangements of *C. riparius* from polluted Derincay and Chaya Rivers were in 16.94% and 36.36% respectively, which were significantly higher than those of the control (Derincay River: G=125.53, P< 0.001; Chaya River: G = 73.81, P<0.001). Somatic index of *C. riparius* was the highest in Chaya River – 3.25, following by Derincay River – 1.58, while it was 0.13 in the control site – Corio, Italy (Fig. 4).

Also, the transcription activity of key structures as Balbiani rings- BRc/BRb and Nucleolar Organizer decreased which was manifested by increasing of their intermediate state of activity instead the normal high activity of these structures (Fig. 5 a, b).

At biochemical level- analyzing the enzyme activities.

We have measured cytosolic GST, AChE activities and microsomal EROD activity together with total MT levels in *C. riparius* samples collected from Bulgarian and Turkish polluted rivers. GST activities of the specimens from Derincay River were significantly higher then from Chaya (Fig. 6a). In addition to GST activities, an activity of EROD indicated PAHs (Polycyclic Aromatic Hydrocarbons) contamination was higher in Turkish than Bulgarian samples. Particularly, Derincay River samples showed 1.3 fold higher microsomal EROD activities (Fig. 6c). As shown in Fig. 6b Derincay River cytosolic AChE inhibitions demonstrate approximately 25% than Chaya River. Moreover, we determined total MT contents of *C. riparius* collected from Bulgaria and Turkey to detect effect of trace metal contamination. According to total MT content measurements, approximately 10% more induction determine in Chaya than in Derincay River (Fig. 6d).

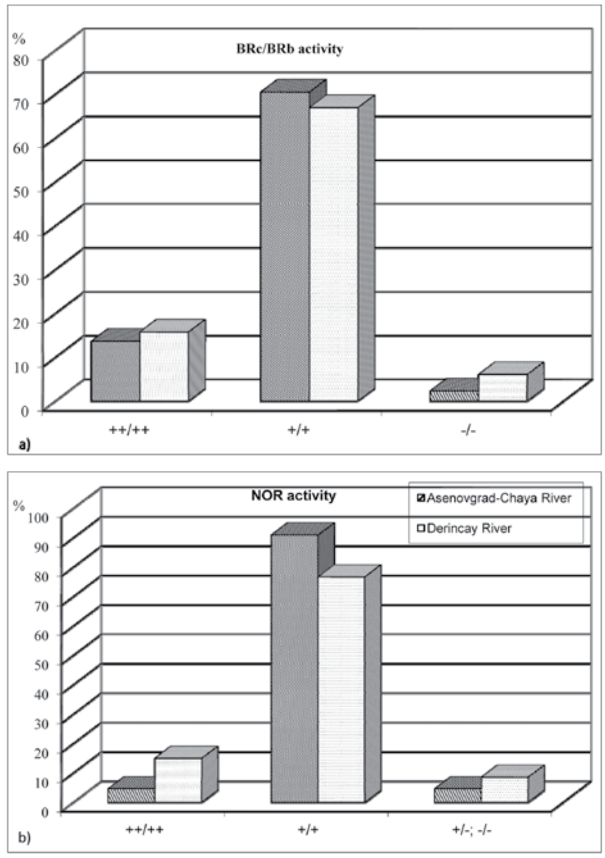

Fig. 5. Activity of BRs and NOR of *C. riparius*:
a) BRc/BRb activity; b) NOR activity; ++ high level of activity; + intermediate level of activity; – low or no activity.

It is worth underlying that the detected by us the highest Somatic index in Chaya River corroborate with high concentrations of Cu and Pb and highest enzymes activities of AChE and MT (Fig. 6 b, d). It is well known that MTs have a high affinity to some heavy metals (Cu, Cd, etc.) (Amiard *et al*. 2006) and serves as a protective mechanism. Similarly to our results, several studies showed that MT contents are increased when the metal gradient (Cd, Cu, Zn)

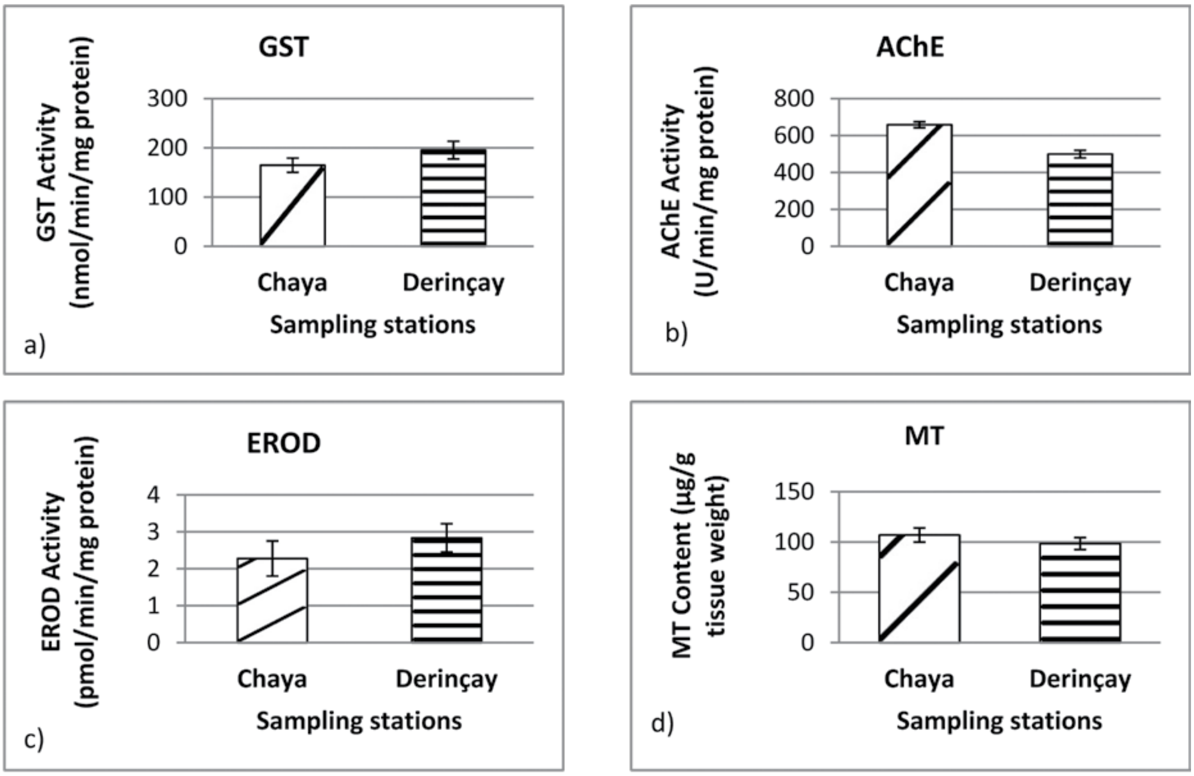

Fig. 6. Biomarker enzyme activities of *C. riparius* collected from Bulgaria and Turkey.
a) Cytosolic GST (Glutathione S-transferase), b) Cytosolic AChE (Acethylcholinesterase); c) Microsomal EROD (7-Ethoxyresorufin-O-deethylase) and d) Total MT (Metallothionein).

of pollution is excessive (Mourgaud *et al*. 2002; Amiard *et al*. 2006). MTs play a major role in the homeostasis of essential metals (such as Zn and Cu) and as well as they are involved in the process of detoxification of nonessential metals such as Ag, Hg and Cd (Amiard *et al*. 2006). High metal concentrations in cell induces an increase in MT concentration and the use of MTs as biomarkers of Ag, Cd, Cu, Hg contamination has been evaluated by several authors for different animal species (Amiard *et al*. 2006).

The results obtained in this study showed the high sensitivity to contaminants of the genome system of *C. riparius* at cytogenetical and biochemical levels. It can be used as integrated biomarker of environmental diagnoses quality and could be successful implemented in environmental monitoring program.

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