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Does biodiversity of macroinvertebrates and genome response of Chironomidae larvae (Diptera) reflect heavy metal pollution in a small pond?

Paraskeva Michailova · Elżbieta Warchałowska-Śliwa · Ewa Szarek-Gwiazda · Andrzej Kownacki

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Abstract The investigation was carried out on a small pond situated on a recent mine spoil at Bolesław in the Olkusz region with Zn–Pb ore deposits. Water of the pond had pH 7.2–8.5 and low concentrations of heavy metals. Concentrations of Pb (487 μg g^{-1}) and Zn (1,991 μg g^{-1}) in the sediment were very high and potentially could lead to toxicological effects. In the pond, 48 taxa of macroinvertebrates belonging to Oligochaeta and water stages of Ephemeroptera, Odonata, Megaloptera, Trichoptera, Heteroptera, Coleoptera and Diptera (mainly Chironomidae family) were found. The influence of heavy metals on macroinvertebrates diversity was not found. Effect of heavy metal pollution was observed on the appearance of chromosome aberrations in the polytene chromosomes of Chironomidae larvae. It was manifested by two ways: (1) in *Kiefferulus tendipediformis* and *Chironomus* sp. chromosome rearrangements in fixed state (tandem fusion and homozygous inversions), indicated intensive process of speciation; (2) in *Chironomus* sp., *K. tendipediformis, Glyptotendipes gripekoveni* (Chironomidae) somatic chromosome rearrangements (inversions, deficiencies, specific puffs, polyploidy) affected few cells of every individual. The somatic functional and structural alterations in Chironomidae species are particular suitable as biomarkers—they can be easily identified and used for detecting toxic agents in the environment.

Keywords Water body · Pb · Zn · Macroinvertebrate · Diversity · Genotoxicity

P. Michailova
Institute of Zoology, Bulgarian Academy of Sciences, 1 Tzar Osvoboditel boulv, Sofia, Bulgaria
e-mail: michailova@zoology.bas.bg

E. Warchałowska-Śliwa
Institute of Systematic and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Krakow, Poland

E. Szarek-Gwiazda (✉) · A. Kownacki
Institute of Nature Conservation, Polish Academy of Sciences, al. Adama Mickiewicza 33, 31-120 Krakow, Poland
e-mail: szarek@iop.krakow.pl

Introduction

Contamination of the environment by chemical wastes poses one of the most serious threats to the quality of freshwater ecosystem. Pollution by trace metals as a result of mine drainage is a particularly serious problem in many parts of the world. It is known that heavy metals are genotoxic and neurotoxic and affect many physiological and cellular processes in different invertebrates which have been studied so far (Ross et al. 2002; Bonacker et al. 2005; Florea and Büsselberg 2006). It is necessary to find sensitive and inexpensive ways of assessing the degree of environmental damage arising from such contaminants. Very important in this aspect is the identification of a good biological system which can be used as an indicator of pollution in the environment. In rivers and lakes, composition and abundance of benthic invertebrate fauna are important elements for classification of ecological status (Directive 2000/60/EC). Among the benthic invertebrate Chironomidae larvae are considered as a suitable biomonitoring model for ecotoxicological assessment (Warwick 1990). The larvae are widely distributed and inhabit every type and condition of aquatic habitat (Warwick 1990). They are included in biotic indices (De Pauw et al. 1992). They possess excellent salivary gland chromosomes which make them very suitable organisms for genotoxicological studies (Michailova et al. 2003, 2009a, b).

The aim of the study was to use biodiversity of macroinvertebrates and the appearance of chromosome aberrations of Chironomidae (Diptera) larvae in monitoring of small pond, which sediments are heavily contaminated by heavy metals. The genome response of the Chironomid species was studied for the first time. Investigation was carried out in a small pond within Olkusz Industrial Region with Zn–Pb deposits near Kraków (Poland).

Materials and methods

Study area

The study was conducted in a small pond situated on a recent mine spoil in Bolesław, in the area of zinc–lead ore deposits in Olkusz Industrial Region (Southern Poland, geographical coordinates: 50°17′26.61″ E, 19°26′36.93″ N (Fig. 1). It is 52 m in length and 17 m in wide. Its maximum depth is 1.2 m. The bottom sediment contains both sand and mud. A large part of the pond (ca. 75%) is overgrown by emergent macrophytes, mainly *Glyceria maxima* (Hartm. Holmb.), *Phragmites australis* (Cav.) and *Typha latifolia* (L.). Open water occurred only as a narrow belt in the middle part of the pond. Now on the spoil recultivation processes are being conducted.

The mining of the Olkusz Industrial Region started in the twelfth century and has lasted up to the present day. Many Zn–Pb mines, smelters and a sulphur acid plant have operated in this area (Cabała 2001; Cabała and Sutkowska 2006).

Investigations were conducted during the spring and summer of 2001, 2002 and 2008. Samples of water and sediment (upper layer 0–5 cm) for physicochemical analysis, macroinvertebrates for biodiversity analysis and larvae of (Chironomidae, Diptera) for cytogenetical analysis were collected.

Physicochemical and biological analysis of samples

Water temperature, conductivity and pH (also sediment) were measured in situ. Dissolved oxygen was determined according to the Winkler method. Chloride, sulphate, hydrocarbonate and nitrate anions were analyzed using ion chromatography (DIONEX, IC25 Ion Chromatograph), while ion chromatography (DIONEX, ICS-1000) was used for cations (Mg, Ca, K and P). Ammonia was analyzed with the nesslerization method, while P-tot (after mineralization) with the molybdenum blue method (APHA 1992).

Sediment samples were passed through a 0.063-mm sieve. For the study of total metal concentrations, sediment samples (three subsamples from each station) were digested with 65% HNO_3 using DigiPREP HT (Tusnovics Instruments). Fractionations of the heavy metals were analyzed using the operationally defined BCR procedure (Larner et al. 2006). The BCR procedure aims to fractionate metals into four operationally defined phases of (F1) acid extractable, (F2) reducible, (F3) oxidisable and (F4) residual, with steps targeting exchangeable and carbonate bound metals, iron and manganese oxide/hydroxide associated metals, metals bound to sulphide and organic phases and mineral phases, respectively.

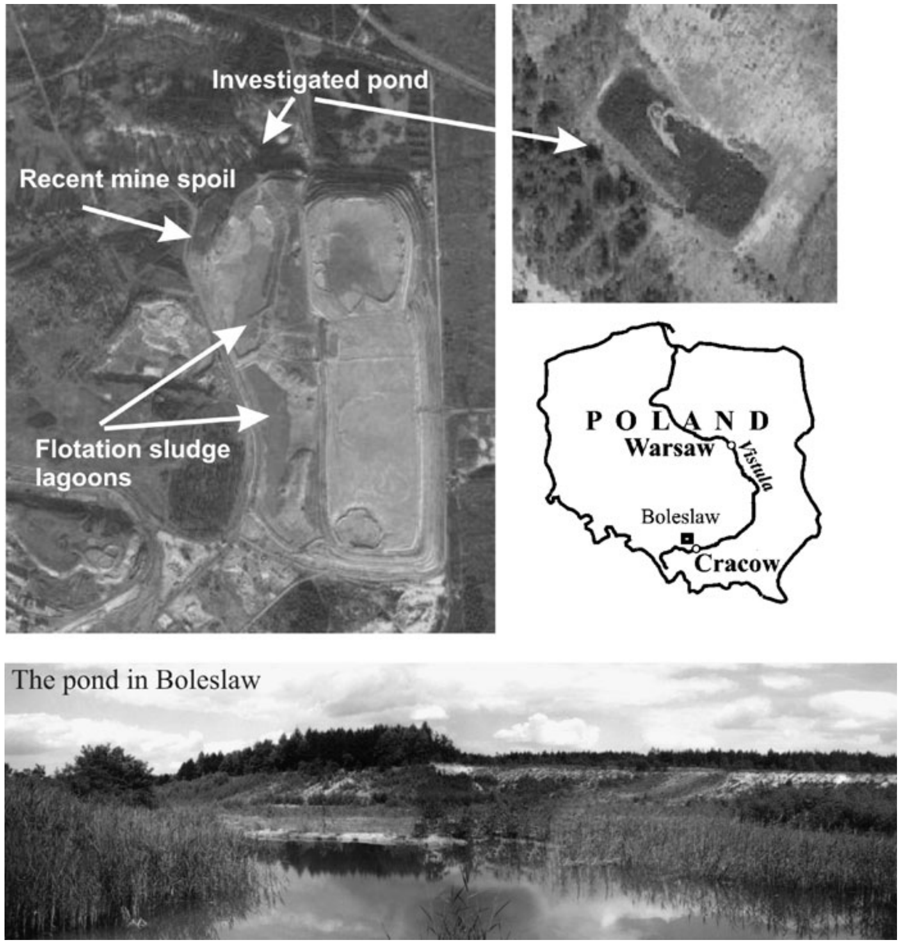

Fig. 1 Location and general view of investigated pond in Bolesław (satellite map according to http://wikimapia.org/)

Heavy metals in the water and sediment were analyzed with the AAS method using a Varian (Spektr AA-20) atomic absorption spectrophotometer. The sum of the concentrations obtained with the BCR procedure was compared to the total metal concentration. A good agreement between them was found. SPS-SW1 Quality Control Material was used to determine analytical accuracy for water samples, while Standards Reference Material (NCS DC 73308) was used for sediment samples. Comparisons of measured and certified values of analytical standard concentrations are given in Table 1.

To estimate the risk of contamination of the water in the pond in Bolesław by the elements deposited in the sediment individual contamination

Table 1 Comparison of measured and certified values of analytical standards for water SPS-SW1 and sediment NCS DC 73308

	Cd	Pb	Cu	Cr	Ni	Mn
Water (μg dm^{-3})						
Measured values	0.48 ± 0.01	4.9 ± 0.1	19.5 ± 0.05	2.08 ± 0.07	9.8 ± 0.15	10.4 ± 0.3
Certified values	0.5 ± 0.01	5.0 ± 0.1	20 ± 1	2.0 ± 0.02	10.0 ± 0.1	10.0 ± 0.1
Sediment (μg g^{-1})						
Measured values	1.21 ± 0.05	28.2 ± 0.20	21.2 ± 1.2	132.4 ± 4.8	28.9 ± 0.9	990 ± 8.2
Certified values	1.12 ± 0.08	27 ± 2	22.6 ± 1.3	136 ± 10	30 ± 2	1010 ± 29

Table 2 Number of individuals and cells of the cytogenetically studied species from families Chironomidae

Species	Time of collections	Number of studied larvae of Chironomids	Number of studied cells in the Chironomids salivary gland chromosomes
Chironomis sp.	06.2001	15	370
Kiefferulus tendipediformis Cytotype 2	06.2002	20	382
Glyptotendipes gripekoveni	07.2008	8	256

factors (ICF) according to the modified formula of Ikem et al. (2003) were calculated:

$$ICF = (C_{F1} + C_{F2} + C_{F3})/C_{F4}$$

where: C_{F1}, C_{F2} and C_{F3} are the heavy metal concentrations in the potentially "mobile" fractions (F1, F2 and F3) of the sediment, while C_{F4} is heavy metal concentration in the "immobile" residual fraction.

The samples for diversity of invertebrates had a qualitative character. Aquatic macroinvertebrates were collected from sediment and between macrophytes by a hand net covered with a 0.3-mm mesh gauze. The taxonomy of macroinvertebrates corresponded to Fauna Europea (2009).

Cytogenetic methods

Salivary gland chromosomes were prepared by the method of Michailova (1989). Together with chromosome preparations, from each larva, a preparation of the larval head capsule was performed. The larvae used for genotoxicological analysis were identified cytotaxonomically as *Glyptotendipes gripekoveni* Kieffer, 1913 [valid name *Glyptotendipes cauliginellus* (Kieffer 1913)], *Kiefferulus tendipediformis* (Goetghebuer, 1921)—cytotype 2 and *Chironomus* sp. The identification to the species level was done on the basis of species-specific cytogenetical markers (Michailova 1989; Michailova et al. 2005). The number of individuals and cells of Chironomidae species for cytogenetical analysis is presented in Table 2.

In Chironomidae larvae, the percentage of cells with chromosome rearrangements was assessed. In order to evaluate the genotoxic effect of trace metals in Chironomidae larvae, we considered somatic chromosome rearrangements, affecting only a few cells of a single individual (Sella et al. 2004). The level of functional activity of the salivary gland chromosomes of chironomid species was evaluated from the amount of puff activity of the Balbiani rings (BRs) and Nucleolar Organizer Region (NOR; as an indicator of the degree of transcription) in the chromosomes following Beermann (1971): high (++), intermediate (+), little or no activity (−). Somatic (S) index of every species was calculated by dividing the number of somatic chromosome aberrations to the number of studied individuals (Sella et al. 2004).

Table 3 Physicochemical parameters of the water from pond in Bolesław

Parameters		Range
pH		7.2–7.2
Conductivity	$\mu S\ cm^{-1}$	1401–1554
Dissolved oxygen	$mg\ dm^{-3}$	2.4–6.7
Chloride	$mg\ dm^{-3}$	3.4–4.1
Sulphate	$mg\ dm^{-3}$	641–915
Hydrocarbonates (HCO_3^-)	$mg\ dm^{-3}$	241–418
Nitrate	$mg\ dm^{-3}$	nd–0.143
$N\text{-}NH_4$	$mg\ dm^{-3}$	0.09–0.38
BOD_5	$mg\ dm^{-3}$	0.5–1.4
Ca	$mg\ dm^{-3}$	165–250
Mg	$mg\ dm^{-3}$	74.4–100
K	$mg\ dm^{-3}$	16–17
Na	$mg\ dm^{-3}$	17.2–24.4

nd not determined

Table 4 Contents of the heavy metals in the water and sediment of the pond in Bolesław

	Water	Sediment (μg g $^{-1}$)	
	(μg dm $^{-3}$)	with macrophytes	without macrophytes
Cd	0.05–0.14	3.7–9.4	9.8–11.0
Pb	1.1–2.4	290–682	3,670–3,860
Cu	1.4–3.3	70–128	650–696
Zn	29.5–80.0	1,673–2,168	7,840–7,901
Cr	1.0–3.1	14.0–48.7	15.8–46.1
Ni	1.0–1.1	23.1–31.0	28.9–34.5
Mn	49.1–82.2	175–336	1,490–1,578
Fe	147–275	9,340–22,244	37,940–38,710

Results

Physicochemical parameters of water and sediment of the pond

Water of the pond had pH from about neutral to slightly alkaline and high amounts of salt (expressed as conductivity, Table 3). SO_4^{2-}, HCO_3^-, Ca^{2+} and Mg^{2+} were dominant anions and cations, respectively (Table 3). The amounts of nitrate, phosphate, organic matter (expressed as BOD_5) and heavy metals in the water were low (Tables 3 and 4). There were small differences in the physicochemical parameters of the water between studied stations, i.e. with and without macrophytes.

Sediment at both stations was characterized by neutral pH (6.9–7.2) and a low amount of LOI (ca. 6%). Concentrations of Cr and Ni in the sediments were low, Cd and Cu elevated, while Pb and Zn were very high (Table 4). Despite the small size of the pond, a great variability was found in the content of most heavy metals in the sediment between studies sites in the pond. Higher metal contents were found in the sediment without macrophytes compare to the sediment overgrow by macrophytes.

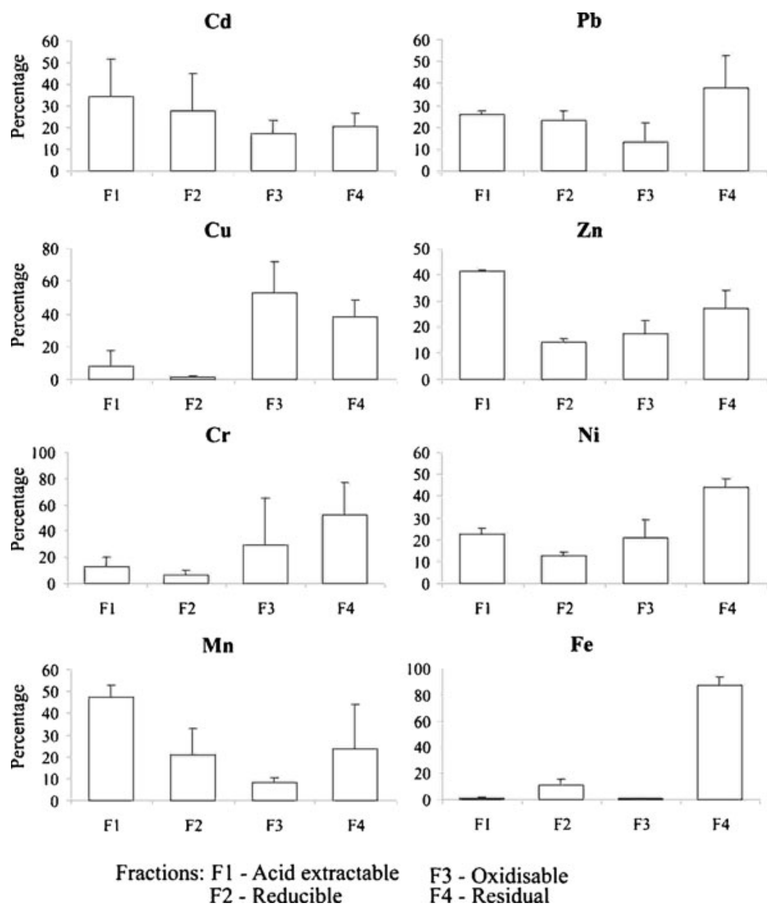

Fig. 2 Binding form of the heavy metals in the sediment of the pond in Bolesław

Table 5 List of macroinvertebrates from the pond in Bolesław

OLIGOCHAETA	
Nais communis Piguet. 1906	
Pristina longiseta Ehrenberg. 1828	
Pristina aequiseta Bourne. 1981	
Tubifex ignatus (Stolc. 1886)	
EPHEMEROPTERA	
Caenis horaria (Linnaeus. 1758) (l)	
Cloeon dipterum (Linnaeus. 1761) (l)	
ODONATA	
Anax imperator Leach. 1815 (l)	
Coenagrion pulchellum (Vander Linden. 1825) (l)	
Enallagma cyathigerum (Charpentier. 1840) (l)	
Sympetrum sanguineum (Műller. 1764) (l)	
MEGALOPTERA	
Sialis lutaria (Linnaeus. 1758) (l)	
TRICHOPTERA	
Cyrnus flavidus McLachlan. 1864 (l)	
Cyrus insolutus McLachlan. 1878 (l)	
Phryganeidae (p)	
Oecetis sp. (l)	
HETEROPTERA	
Notonecta glauca Linnaeus. 1758	
Ilyocoris cimicoides (Linnaeus. 1758)	
Sigara sp.	
COLEOPTERA	
Helophorus sp.	
Haliplus sp.	
DIPTERA	
LIMONIIDAE	
Limonia sp. (l)	
CERATOPOGONIDAE (l) (non det.)	
CHIRONOMIDAE	
Procladius (*Holotanypus*) sp. (l)	
Ablabesmyia longistyla Fittkau. 1962 (p)	
Zavrelimyia sp. (l)	
Clinotanypus nervosus (Meigen. 1818) (p)	
Corynoneura sp. (l)	
Cricotopus (*Isocladius*) *sylvestris* (Fabricius. 1794) (l)	
Psectrocladius psilopterus- gr. (p)	
Paracladius sp. (l)	
Dicrotendipes sp. (p)	
Chironomus luridus Strenzke. 1959 (♂. p. l)	
Chironomus sp. I (p)	
Kiefferulus tendipediformis (Goetghebuer. 1921) (♂. p. l)	
Synendotendipes impar (Walker. 1856) (l) (syn. *Endochironomus impar* Walker. 1856)	
Glyptotendipes cauliginellus (Kieffer. 1913) (p) (syn. *Glyptotendipes gripekoveni* Kieffer. 1913)	
Microtendipes sp. (l)	
Polypedilum (*P.*) *nubeculosum* (Meigen. 1804) (p)	
Cladotanytarsus sp. (l)	
Paratanytarsus laccophilus (Edwards. 1929) (p)	
Paratanytarsus bituberculatus (Edwards. 1929) (p)	
Paratanytarsus grimmii Schneider. 1885 (p)	
Micropsectra sp. (l)	
Tanytarsus usmaensis- gr. (p. l.)	
Tanytarsus Pe 4 (sensu Langton. 1991) (p)	
Tanytarsus Pe 14 (sensu Langton. 1991) (p)	
DIXIDAE	
Dixella sp. (l)	
STRATIOMYIDAE (l.) (non det.)	

l larvae, *p* pupae, ♂ imago

Results of the sequential extraction of the sediment indicated that trace elements displayed different degrees of association with the targeted fractions (Fig. 2). Most of the Mn and Zn (ca. 40–50%), Cd (ca. 35%), Pb and Ni (20–30%) was found in F1 (acid extractable phase). A large part of Cd, Pb and Mn (20–30%) was associated with F2 (reducible phase). The majority of Cu (ca. 50%) but also Cr (ca. 30%) and a smaller part of Cd, Pb, Zn, and Ni (ca. 10–20%) was associated with F3 (oxidisable phase). To the immobile F4 (residual phase) in the highest amount was bound Fe (ca. 85%), Cr (ca. 51%) and Ni (ca. 44%). The obtained results indicate that the elements (except for Fe) are characterized by a potentially high mobility.

The values of ICF, estimating the risk of contamination of water reservoirs by the elements deposited in the sediment, were the following: Cd 3.8, Pb and Cu 1.6, Zn 2.7, Cr 0.9, Ni 1.3, Mn 3.2, and Fe 0.1. According to ICF values, Cd and Mn pose the highest risks to water contamination. ICF values higher than 1 indicate that more than 50% of the total amounts of the elements were bound to potentially "mobile" phases (F1, F2 and F3). This means that Pb, Cu, Ni and Cr also have great potential to be remobilized from the sediment.

Biodiversity of aquatic macroinverebrates

A total of 48 macroinvertebrate taxa were identified in the pond (Table 5). Chironomidae was most diverse (24 taxa), found both in sediment with and without macrophytes. The Ephemeroptera *Caenis horaria* and *Cloeon dipterum* were important among the vegetation. Other groups were Oligochaeta, Odonata, Trichoptera, Megaloptera, Heteroptera and Coleoptera, which are typical for small ponds. They were less diverse and less abundant. Molusca and Malacostraca, also typical for this ecosystem, were absent.

Genome response of Chironomidae

Cytogenetic characteristics of the salivary gland chromosomes of Chironomidae

Chironomus sp. belongs to cytocomplex "*pseudothummi*" (Keyl 1962) with $2n = 8$ and chromosome arm combinations: AE, BF, CD and G. Chromosomes I (AE), II (BF) and III (CD) are metacentric whereas the IV (G) is acrocentric. Chromosome G has two BRs and a NOR (Fig. 3a). However, the band sequences differ from those of the "pseudothummi" cytocomplex. For instance: chromosome AE has a species specific fixed homozygous inversion.

G. gripekoveni This species has the chromosome set $2n = 8$, with chromosome arm combinations AB CD EF and G. Chromosome G has two BRs and a NOR at the telomere (Fig. 5a). The band sequences do not differ from the standard described by Michailova (1989).

K. tendipediformis In the polluted pond in Bolesław was found cytotype 2 (Michailova et al. 2005) which has ($2n = 6$) with chromosome arm combinations AB CD EFG. The EFG chromosome was produced by fixed specific chromosome rearrangements, i.e. the tandem fusion of two acrocentric chromosomes EF and G of cytotype 1 (Michailova et al. 2005). It has two BRs and one NOR; one NOR is at the telomere of arm A.

Chromosome alterations

Chironomus sp. We observed both somatic and functional alterations in this species. Somatic alterations (inversions, deficiencies, deletions) affected different chromosome arms at a low frequency (Table 6). Deletion in chromosome G (Fig. 3c) affected higher number of the studied

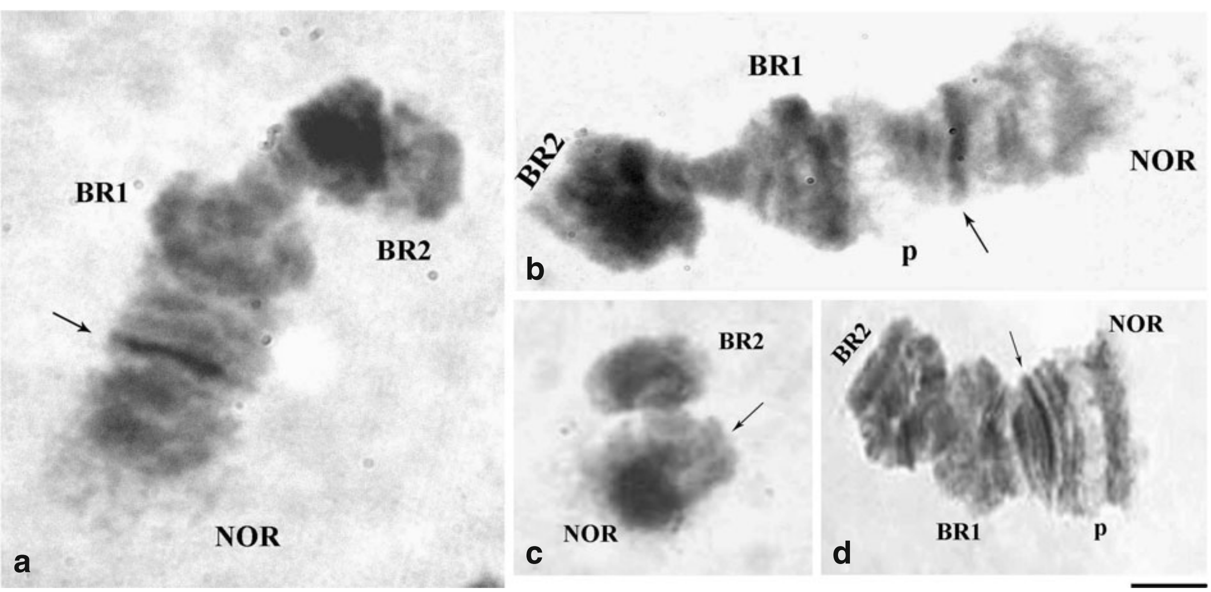

Fig. 3 **a**–**d** Chromosome G of *Chironomus* sp. **a** Chromosome G—standard; **b** Chromosome G—BR1 in an intermediate activity, BR2 in collapse, NOR in a high activity; puff before BR1. **c** Chromosome G—a deletion and BR2 in intermediate activity; **d** Chromosome G—BR1 in intermediate activity, BR2 in collapse, a puff after NOR. *BR* Balbiani ring, *NOR* nucleolar organizer, *arrow* centromere region, *p* puff. *Bar* 10 μm

Table 6 Somatic chromosome rearrangements in *Chironomus* sp., *Kiefferulus tendipediformis*, *Glyptotendipes gripekoveni*

Species and chromosomes with alterations	% of individuals with alterations	% of cells with alterations
Chironomus sp.		
Heterozygous inversion in arm B	6.67	0.27
Heterozygous inversion in arm E	6.67	0.27
Heterozygous inversion in arm F	6.67	0.27
Heterozygous inversion in arm G	6.67	0.27
Perizentric inversion in BF	6.67	0.27
Heterozygous deficiency in arm F	6.67	0.27
Deletion in arm G	20.00	1.62
Kiefferulus tendipediformis (cytotype 2)		
Heterozygous inversion in arm A	5	0.26
Heterozygous inversion in arm B	5	0.26
Heterozygous inversion in arm C (nest to the centromere region)	5	0.26
Heterozygous inversion in arm C (near to the telomere)	5	0.52
Heterozygous inversion in arm D	15	1.047
Heterozygous inversion in arm E	5	0.26
Heterozygous inversion in arm G	15	0.79
Heterozygous deficiency in arm B	5	0.26
Heterozygous deficiency in arm C	10	0.79
Heterozygous deficiency in arm D	10	0.52
Glyptotendipes gripekoveni		
Chromosome G completely with unpaired homologues	75	76.56
Chromosome G with partly unpaired homologues	25	23.43
Chromatid break	12.5	0.39
Heterozygous deficiency in arm F	12.5	0.39
Heterozygous deficiency in arm D	12.5	0.39
Heterozygous deficiency in arm B	12.5	0.39
Heterozygous inversion in arm B	12.5	0.39
Heterozygous inversion in arm C	25	0.78
Specific puff in arm C	50	10.54

individuals (Table 6). NOR occurred in two states: high activity (++/++ 55.14%) and intermediate activity (+/+ 44.86%). Three activity states were detected in BR1: high activity (++/++ 58.38%), intermediate activity (+/+ 23.51%; Fig. 3a) and low or no activity (−/−18.11%). BR2 occurred in two states of activity: intermediate and low (2.70%) or no activity (97.30%, Fig. 3b–d). Specific puffs occurred on chromosome G (after NOR–6.22% (Fig. 3d) and before BR1–4.05% (Fig. 3b)), in single cells it was in a heterozygous state. Interesting activity was observed for chromosome G: in most cases when puffs occurred on chromosome G (before BR1 and after NOR), no activity was detected in BR2. The somatic index (S) of the species was 0.47.

K. tendipediformis-cytotype 2. Inherited and somatic chromosome rearrangements were detected in this species. For the first time, we found three inherited heterozygous inversions: a pericentric heterozygous inversion in chromosomes AB (30%), a heterozygous inversion in chromosome arm G (10%) and a heterozygous inversion in chromosome arm D (5%). Somatic aberrations affected chromosome arms A, B, C, D, E and G, occurring in low frequencies (Table 6, Fig. 4c). Changes in functional activities were also observed. The NOR (arm G) and BR2

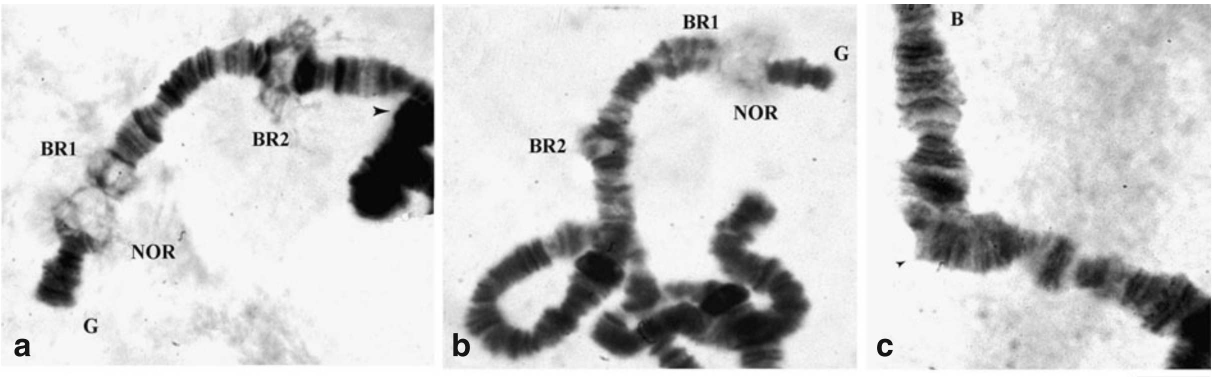

Fig. 4 **a**–**c** Chromosome GEF of *K. tendipediformis* (in the picture is seen a part of the chromosome—the chromosome G only) (**a**, **b**) and chromosome arm B (**c**). **a** Chromosome G—NOR and BR2 in a high activity; BR1 in an intermediate activity. **b** Chromosome G—NOR and BR2 in a high activity, BR1 in a collapse. **c** Somatic heterozygous inversion in chromosome arm B. *BR* Balbiani ring, *NOR* nucleolar organizer, *long arrow* centromere region, *small arrow* heterozygous inversion. *Bar* 10 μm

occurred almost in a normal state of high activity, 99.74% and 99.21%, respectively (Fig. 4a). The NOR in arm A was always very active. However, BR1 often appeared in a state of collapse or had low activity (97.12%, Fig. 4b). In single cells, a specific puff was observed in chromosome arms

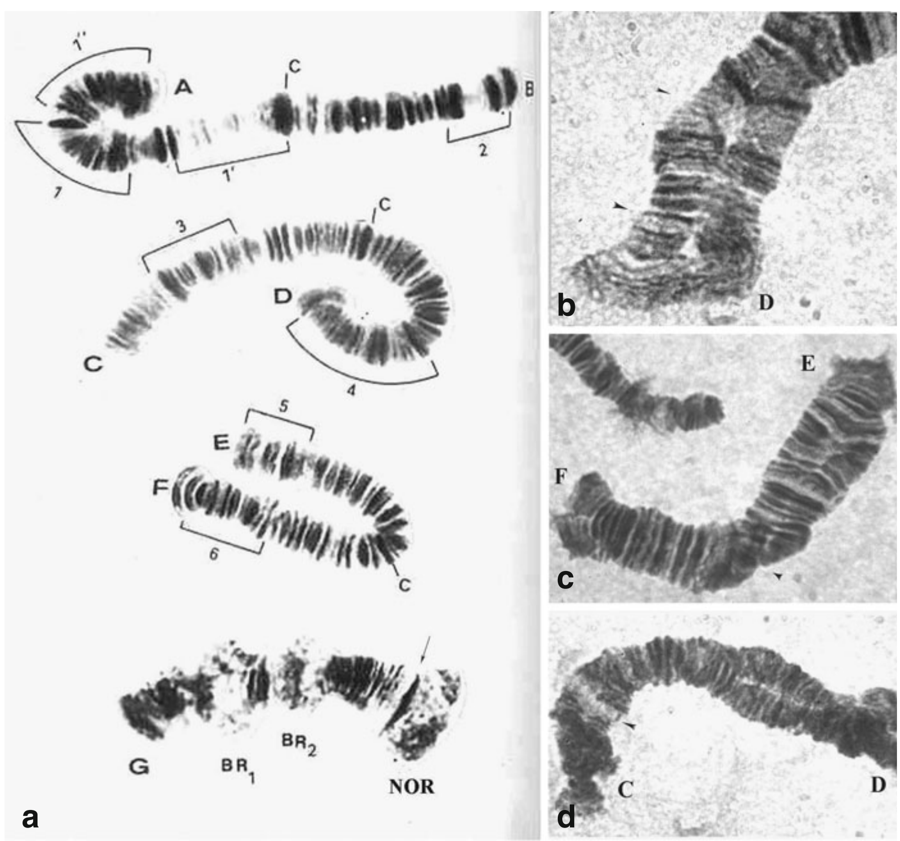

Fig. 5 **a**–**d** *G. gripekoveni*. **a** Chromosome AB, CD, EF and G.(indications *1*, *1′*, *1″*, *2*, *3*, *4*, *5*, *6*—markers of the chromosomes). **b** Chromosome CD unpaired regions in arm D. **c** Somatic heterozygous inversion in chromosome EF. **d** Specific puff in chromosome arm C. *BR* Balbiani ring, *NOR* nucleolar organizer, *long arrow* centromere region, *small arrow* chromosome alterations. *Bar* 10 μm

B, C, D. The somatic index (S) of the species was 0.50.

G. gripekoveni Inherited and somatic chromosome rearrangements were detected in the genome of this species. Inherited heterozygous inversions were observed in chromosomes AB (37.5%), CD (25%) and EF (25%). All somatic alterations (inversions and deficiencies) are unique and were observed for the first time in the studied species (Table 6). They appeared both structural and functional aspects. Six different somatic alterations were detected, including heterozygous inversions (Fig. 5c), deficiencies and chromatid breaks. In the case of functional alterations, we observed a specific puff in chromosome arm C, appearing in 50% of the studied individuals and in more that 10% of the cells (Fig. 5d). In comparison with the standard, chromosome G occurred with completely unpaired homologues or partly unpaired state. Also, unpaired regions along the chromosomes were often observed in the polytene chromosomes of all studied individuals (Fig. 5b). The somatic index (S) of this species was 0.75.

Discussion

Physicochemical parameters of water and sediment of the pond

The high concentrations of sulphates and hydrocarbonates in the water of pond are associated with the geochemical background of the Olkusz Industrial Region. Sulphides, oxidized and carbonate minerals dominated in Zn–Pb ore deposits (Cabała 2001). Low concentrations of heavy metals in the water indicated that they had precipitated to the bottom sediment. In the water of Biała Przemsza River, flowing through this region, considerably higher concentrations of heavy metals (up to 13 μg dm^{-3} Cd, 520 μg dm^{-3} Cu, 738 μg dm^{-3} Pb and 1,295 μg dm^{-3} Zn) were found (Suschka et al. 1994). Similar quality of the water of the pond in Bolesław was found earlier by Michailova et al. (2005).

Elevated concentrations of Cd and Cu and very high concentrations of Pb and Zn in the sediment of the pond in Bolesław were associated both with the geochemical background and also the neighbouring Zn–Pb mine spoil. Mine spoils created during Zn–Pb mining in this region are highly polluted by Zn, Pb, Fe and Cd (Szarek-Łukaszewska and Niklińska 2002). The sediments of various water bodies usually contained high concentrations of Cd, Pb and Zn because of the regional Zn and Pb ore deposits, their mining and the processing of mineral ores (Schintu et al. 1991; Cappuyns et al. 2006). Mean concentrations of Cd in the sediment of pond were about 2.2 times, Pb 15 times and Zn 10 times higher than the probable effect level (PEL; CCME 1997). Therefore, the sediment can be considered as contaminated and potentially toxic for organisms.

Part of the heavy metals deposited in the sediment may be remobilized and made available for biota under changing environmental conditions including pH, redox conditions and salinity (Bervoets and Blust 2000; Bidwel and Gorrie 2006). Both the fractionation study and the values of ICF showed that the studied heavy metals (except Fe) are characterized by a potentially high mobility. A large part of the Mn, Zn, Cd, Pb and Ni was found in F1, including exchangeable (the most mobile and hazardous) and carbonate (moderately mobile and available for organisms; Förstner 1986) bound metals. These metals may be released from the sediment via a decrease in pH. Most Cd, Pb and Mn were associated with F2, the moderately mobile phase, from which release can occur under reductive conditions (Van der Berg et al. 1998). The majority of Cu, but also a smaller portion of Cr, Cd, Pb, Zn and Ni were associated with F3, including metals bound to sulphide and organic phases. The amount of organic matter in the sediment was small; therefore metals were probably associated mainly with sulphides and may be released in oxide conditions. In summary, both the high total concentrations of Cd, Pb and Zn as well as their high potential mobility indicated that they are potentially hazardous to the environment.

Diversity

The diversity of the aquatic macroinvertebrates (48 taxa) in the pond in Bolesław was typical for a small pond. Williams et al. (2003) in individual pond in the agricultural landscape of southern England recorded from five to 67 invertebrate species. Martens et al. (2008) consider that older ponds may have higher biodiversity levels than new ponds on the basis of a study of 162 natural, extensive and intensive ponds in Belgium, polluted to a variable extent by heavy metals. They also recorded that the Chironomidae was an important group, as in our study. Our finding that Mollusca were absent from the pond in Bolesław confirmed Martens et al. (2008) results that snails occurred in only 12–24% of pools.

In spite of the high level of pollution in the studied area, we did not detect the impact of trace metals on the diversity of the different studied animal groups, identified by conventional external morphology.

Genome response of Chironomidae (Diptera)

The Chironomidae species have been exposed to trace metals for many generations. As mentioned above, this site is an old mining region where Zn–Pb has been deposited since the twelfth century to the present day. Our results show that their genome is very sensitive to contaminants existing in the environment. Chromosome alterations in a mosaic appearance were detected in insects from the polluted environment, but not from unpolluted regions (Michailova 1989; Michailova et al. 2005). Although most of the genome of all studied individuals of the studied species were affected by the stress agent, phenotypically only one malformation was observed in the chironomids.

The genomic changes in the studied species were manifested in two ways:

1. Fixed chromosome rearrangements, having an important role in speciation (King 1993).

A good example of this process is the monophyletic genus *Kiefferulus*. In Bulgaria, the species identified by external morphology as *K. tendipediformis* collected from unpolluted regions has $2n = 8$, with chromosome arm combination AB CD EF G (cytotype 1). The same species collected from a highly polluted area of Bolesław has $2n = 6$ with chromosome arm combination AB, CD, GEF. Thus the same species occurs in two forms, termed cytotype 1 ($2n = 8$) and cytotype 2 ($2n = 6$) (Michailova et al. 2005). Due to fixed tandem fusion, the chromosome set is reduced to $2n = 6$ and a new submetacentric chromosome (EFG) is produced. Almost the same process has been observed in *Chironomus* sp., collected from the pond in Bolesław. This species can be distinguished from other species of the cytocomplex "psedothummi" by fixed homozygous inversions, detected in arms A and specific band patterns in arms B, C, D and G.

Due to permanent chromosome rearrangements (tandem fusions and fixed homozygous inversions), new gene linkage groups were created, indicating an intensive microevolutionary process in this highly polluted old mine site. It is important to underline that these two species lived in the sediment of the pond which has been chronically exposed to high heavy metal concentrations. Morgan et al. (2007) also reported some evidence for the existence of genetically differentiated invertebrate populations in a metal-polluted environment.

2. Somatic chromosome rearrangements

Lagadic and Caquet (1998) proposed these aberrations to be used for detecting the genotoxic effect of contaminants in the environment. These alterations were detected in chironomidae larvae. The chironomid genome reacts to contaminants by somatic and functional alterations. The somatic alterations (inversions, deficiencies) observed in *Chironomus* sp., *K. tendipediformis* (cytotype 2), and *G. gripekoveni* affected very small regions of the polytene chromosomes and only a few cells (Table 6). Michailova et al. (1996, 2000, 2009a, b) presented a high spectrum of unique somatic rearrangements in different Chironomidae species living in trace metal contaminated freshwaters. In *G. gripekoveni*, the larvae of which inhabit the stems and leaves of *T. latifolia* and *P. australis*, 6.25% of individuals have somatic alterations although their cells have few (2.73%) chromosome alterations. Both *T. latifolia* and *P. australis* are known to take up metals from the sediment, accumulate them mostly in the roots and rhizome tissues, and to a very small degree transfer the metals to stem and leaf tissues (Keller et al. 1998; Ye et al. 1997a, b). Lower heavy metal contents in stems and leaves of *T. latifolia* and *P. australis* create better conditions and might lower the toxic effect on larvae of *G. gripekoveni* compared to conditions in the sediment of the pond, where larvae of *K. tendipediformis* ($2n = 6$) and *Chironomus* sp. (undergoing the process of microevolutionary differentiation and somatic rearrangements) were collected. The observed functional alterations in the studied chironomids showed a species-specific occurrence. Very interesting is the presence of both BRs, as they constitute the sites of intensive transcription of genes encoding silk proteins (Wieslander 1994). Silk proteins are very important for chironomid larvae for the construction of their tubes in which they live and develop. However, in *K. tendipediformis* BR1 had low activity or was not active. In *Chironomus* sp. in almost all cells, BR2 was not active or showed intermediate activity. Therefore, these data indicate that the development of larvae may be disturbed. However, in these species we observed additional transcriptional activity, expressed by specific puffs in chromosome G of *Chironomus* sp., chromosome arms B, C, and D of *K. tendipediformis* and chromosome arm C of *G. gripekoveni.* Especially revealing is the appearance of new puffs (before BR1 and after NOR) when BR2 is not expressed. In *Chironomus acidophilus*, so-called compensatory mechanism was detected (Michailova et al. 2009b), characterized by the appearance of a specific puff at the telomere of chromosome G, however, without the expression of standard BR2. Thus, it cannot be excluded that the same mechanism may operate in chironomids, studied by us.

Another key structure is the Nucleolar Organizer, the function of which is essential for cellular maintenance and the ribosomal production machinery and very often is the direct target for toxicity (Planello et al. 2007). The observed intermediate state of activity of NOR in *Chironomus* sp. manifested the transcription inactivation of this structure. Planello et al. (2007), using a 28S ribosomal DNA probe, established that Cd induced nucleolar inactivation and underlined that NOR plays a key role in monitoring and responding to cellular stress. Changes in the functional activity of key structures (BRs and NOR) have been observed in a model *Chironomus* species, *Chironomus riparius*, after laboratory treatment with lead, copper and aluminum (Michailova et al. 2003, 2006).

However, it is important to emphasize that the smallest chromosome in the genome of the studied chironomid species, chromosome G, is very sensitive. For instance, chromosome G of *Chironomus* sp. very often, due to deletion, it loses a great amount of genetic material and changes its appearance. The same tendency has been described by Michailova et al. (1998) in the model species *C. riparius*, in which chromosome G has converted into the so called “pompon” chromosome. It also carries key structures (BRs and NOR) very sensitive to stress agents. Because the chromosome G is the smallest in the genome, it can be recognized easily and used to test the presence of genotoxic concentrations of heavy metals in aquatic ecosystems.

The genome alterations of the studied insects not affected the biodiversity and phenotype morphology of Chironomids (in fact one larva has the malformation only). There are many regulatory mechanisms which might be involved in the species living in the polluted regions and preserve them in this stress conditions. One of them is the synthesis of HSP. For instance, Warchalowska-Sliwa et al. (2005) established a high amount of HSP 70 in some orthopteran species from polluted Bolesław region. Appearing the specific fractions of HSP 70 has been detected also in some Chironomid species treated with different concentrations of Cr ions (Todorova et al. 2000). Also, synthesis of specific proteins can help the species to survive in the polluted areas. In this study, many new puffs in Chironomid species were expressed and show that high transcription process connected with synthesis of proteins is going. However, further molecular analysis is necessary to perform in order to know which proteins are responsible for this process.

Genome alterations of Chironomidae larvae in aquatic ecosystems may be a response to complex stress agents in the environment. Both a high total metal concentration and its binding form in the sediment showed a great potential hazard for biota. However, it must be mentioned that also interactions among metals (synergistic effects) or other stressors such as organic pollution may influence genome alterations. In the future, laboratory experiments may help in recognizing the effect of specific environmental agents on genome alterations.

Conclusions

1. In a study pond concentrations of heavy metals Zn and Pb in the sediment were high and could have a toxic effect on organisms.
2. The diversity of aquatic macroinvertebrates was rich and typical for small ponds. The influence of heavy metals on macroinvertebrates diversity was not found; therefore, biodiversity is not suitable indicators of those contaminants.
3. Genome instability of Chironomidae larvae was manifested by two ways: (a) chromosome rearrangements in a fixed state indicated intensive speciation processes, (b) structural and functional chromosome rearrangements (inversions, deficiencies, deletions and changes the activity of key structures: Balbiani rings and Nucleolar Organizer and new puffs) in a somatic state.
4. The somatic functional and structural alterations in the polytene chromosomes of Chironomidae species are particular suitable as biomarkers—they can be easily identified and use for detecting toxic agents in the environment.

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