Frequency of micronucleated erythrocytes in wild fish from natural freshwater bodies

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The frequency of micronucleated erythrocytes (MNE) was examined in 60 individual fish belonging to five species, captured at five different places with different levels of water pollution. For a total, 20 perches (*Perca fluviatilis* L., Percidae), 4 pike-perches (*Stiziostedion lucioperca* L., Percidae), 19 roaches (*Rutilus rutilus* L., Cyprinidae), 15 common breams (*Abramis brama* L., Cyprinidae), and 2 silver breams (*Blicca bjoerkna* L., Cyprinidae) were analysed. A total of 10,000 mononucleated erythrocytes were examined for each fish. The frequency of MNE was dependent on both fish species and capture site. Higher frequencies of MNE were found at more polluted sites. A comparison of the same species in different waterbodies and different species in the same waterbody showed that the most sensitive species in our study was perch, followed by roach and common bream. Thus, analysis of MNE in perch can be recommended as a suitable method for *in situ* detection of environmental genotoxins.

Key words: micronuclei, genotoxicity, freshwater fish, perch, roach, bream

INTRODUCTION

Pollution of the environment through the release of various chemical substances is one of the major concerns of modern society. A significant part of these substances get into surface waters, raising potential risks to water organisms as well as humans.

Genotoxicity of such contaminated water is well documented using standard genotoxicity assays *in vitro* (White et al., 1998; Vahl et al., 1997). In addition, aquatic species present in a contaminated environment were examined for the effects of genotoxins *in situ* (Reichert et al., 1998; Harvey et al., 1999).

Fish have often been considered as the "sentinel" organisms for the health of the water environment (De L. G. Solbé, 1993), because they are capable of inhabiting practically all zones of the aquatic habitat and have a great commercial and recreational value. No wonder that fish are used for many purposes of toxicological and ecotoxicological research (American…, 1991). It may be convenient to extend ecotoxicological studies with fish by adding some genotoxicological tests. One of the most popular and promising tests is analysis of micronuclei in erythrocytes of fish (for review, see (Al-Sabti et al., 1995)). This method has frequently been used for *in situ* assessing genotoxicity in water. Although micronuclei may be studied in both marine and freshwater fish, studies with freshwater fish are more abundant (Al-Sabti et al., 1995).

Surface waters of Lithuania have been shown to be contaminated by potential genotoxic compounds – aromatic hydrocarbons and organochlorine pesticides (Ellington et al., 1996; Sabaliūnas et al., 2000). Genotoxicity *in vitro* of aquatic pollutants sampled from several water sources in Lithuania has been shown as well (Sabaliūnas et al., 2000). Therefore, it was interesting whether surface waters of Lithuania may be of genotoxic risk to water organisms. To elucidate this, an *in situ* study was carried out. During this study, the frequency of micronucleated erythrocytes was examined in fish captured at five different places probably representing different levels of water contamination.

MATERIALS AND METHODS

Study locations

Six locations in Lithuania were selected for the study: (1) at the upper part of the Nemunas River

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near to the tributary Juodoji Ančia, (2) at the middle part of the Nemunas River near the tributary Verknė, (3) at the Curonian Bay near the Nemunas River outfall, (4) at Lake Ginkūnai near the Šiauliai city, (5) at the Švedė pond located in Šiauliai district, and (6) the Bubliai pond near the Kėdainiai town.

Although the Nemunas River is the biggest one in Lithuania accumulating pollutants from the entire Southern Lithuania, the first study site is located in a relatively clean part with no industry and non-intensive agriculture.

The second study site is located in a rural region of Lithuania with some health-resort settlements upstream.

The third study site is located near the outfall of the Nemunas River with a lot of industrial towns upstream.

Near the fourth study site – Lake Ginkūnai – a big tannery is operating. The lake is also flowed by the Kulpė River – one of the most polluted rivers in Lithuania (Lithuanian…, 1999). Most of the Kulpė water flow is formed of the municipal and industrial wastewater discharge of the Šiauliai city receiving a very limited treatment.

The fifth study site – the pond Švedė – is located near the rubbish dump of the Šiauliai city and neighboring settlements.

The Bubliai pond is the sixth study location situated near the Kėdainiai town. This pond is abounding in fish and supposed to be unpolluted.

Fish

Sixty individual fish belonging to five species were captured using nets during the years 1999–2000. For a total, 20 perches (*Perca fluviatilis* L., Percidae), 4 pike-perches (*Stiziostedion lucioperca* L., Percidae), 19 roaches (*Rutilus rutilus* L., Cyprinidae), 15 common breams (*Abramis brama* L., Cyprinidae), and 2 silver breams (*Blicca bjoerkna* L., Cyprinidae) were captured and analyzed for the frequency of micronucleated erythrocytes.

Micronucleus tests

A drop of blood from the caudal vessels was directly smeared on slides and air-dried. After fixation in methanol for 10 min, the slides were stained with 10% Giemsa solution for 8 min, rinsed with distilled water and dried.

The frequency of micronucleated erythrocytes was evaluated by scoring at a 1000× magnification. A total of 10,000 mononucleated erythrocytes were examined for each fish. Only cells with intact cellular and nuclear membrane were scored. Round or ovoid-shaped non-refractory particles with the colour and structure similar to chromatin, with a diameter 1/3–1/50 of the main nucleus and clearly detached from it were interpreted as micronuclei (MN). The mean frequency of micronucleated erythrocytes (MNE) expressed as the number of MNE per 1000 erythrocytes (‰) was calculated for each species and study location.

Statistical analysis

Statistical analysis was performed by means of analysis of variance (ANOVA). Before the analysis, MNE frequency was a mean square root transformed ($Y = 0.5[\sqrt{X} + \sqrt{X+1}]$, where X is the frequency of MNE per 10,000 erythrocytes and Y is the transformed quantity). Such transformation was effective in normalizing the distribution and stabilizing the dispersion (data not shown).

RESULTS AND DISCUSSION

Pooled frequencies of micronucleated erythrocytes (MNE) in fish captured at different locations were: in silver bream – 0.05 ± 0.05‰ (range 0–0.1‰), in common bream – 0.09 ± 0.03‰ (range 0–0.3‰), in pike-perch – 0.18 ± 0.06‰ (range 0–0.3‰), in roach – 0.19 ± 0.05‰ (range 0–0.7‰), and in perch – 0.36 ± 0.07‰ (range 0–1.2‰). The differences in MNE frequency among different species are statistically significant at a significance level $P < < 0.01$. These differences, however, cannot be solely explained by inter-species variation, because the mean frequency of MNE is also different ($P < 0.01$) among the study locations (Table 1).

The lowest frequency of MNE was found in fish captured at the Upper Nemunas and the highest frequency in fish captured in the pond Švedė. Increased frequencies of MNE were found in the Middle Nemunas and in the Curonian Bay near the Nemunas outfall as compared to the Upper Nemunas. The frequency of MNE in the Curonian Bay (0.12 ± 0.04‰) was slightly lower than in the Middle Nemunas (0.17 ± 0.05‰). Such a result, however, may be due to a low MNE frequency in common bream (0.06 ± 0.03‰) captured in the Curonian Bay. If only results of MNE analysis in roach (which was present in all free locations related to the Nemunas River) are taken into account, a clear increase in the frequency of MNE is seen along the river's length (Fig. 1). In general, there was quite an obvious relation among the frequencies of MNE in different species captured in different locations: in Lake Ginkūnai and pond Švedė all species tended to have a higher frequency of MNE as compared to the Upper or Middle Nemunas.

Table 1. **Frequency of micronucleated erythrocytes (MNE) in fish captured at different sites of natural water bodies**

Place and date of fish capture	Species	Number of individuals	Frequency of MNE, ‰ ± SEM	Mean frequency of MNE, ‰ ± SEM
The Upper	*Perca fluviatilis* L.	1	0	0.05 ± 0.03
Nemunas,	*Rutilus rutilus* L.	3	0.06 ± 0.06	
07/20/99	*Blicca bjoerkna* L.	2	0.05 ± 0.05	
The Middle	*Perca fluviatilis* L.	2	0.30 ± 0.00	0.17 ± 0.05
Nemunas,	*Rutilus rutilus* L.	4	0.10 ± 0.04	
07/22/99				
Kuršių Bay,	*Rutilus rutilus* L.	2	0.25 ± 0.25	0.12 ± 0.04
11/08/99	*Abramis brama* L.	8	0.06 ± 0.03	
	Stizostedion lucioperca L.	4	0.18 ± 0.06	
Lake	*Perca fluviatilis* L.	5	0.38 ± 0.09	0.24 ± 0.06
Ginkūnai,	*Rutilus rutilus* L.	3	0.27 ± 0.21	
01/10/99	*Abramis brama* L.	7	0.13 ± 0.04	
Pond Švedė,	*Perca fluviatilis* L.	6	0.63 ± 0.14	0.51 ± 0.1
14/10/99	*Rutilus rutilus* L.	4	0.33 ± 0.13	
Pond Bubliai,	*Perca fluviatilis* L.	6	0.17 ± 0.04	0.11 ± 0.04
18/08/00	*Rutilus rutilus* L.	3	0	

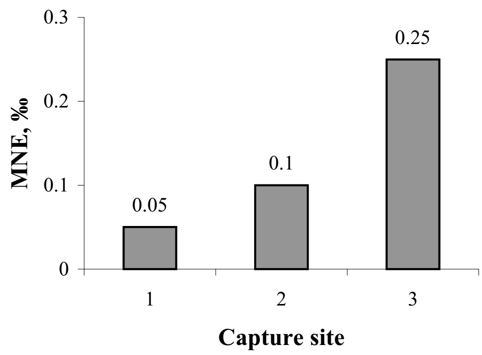

Fig. 1. Frequency of micronucleated erythrocytes (MNE) in blood smears of roach (*Rutilus rutilus* L.) captured at different sites of the Nemunas River: *1* = the Upper Nemunas, *2* – the Middle Nemunas, *3* – the Curonian Bay near the Nemunas outfall

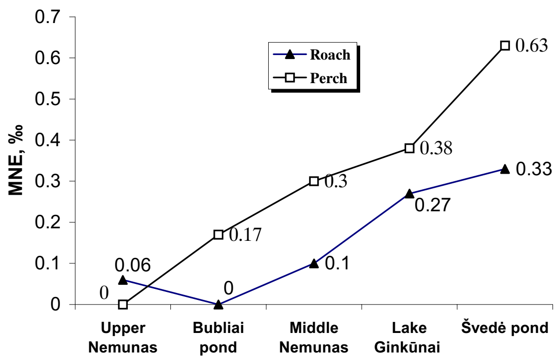

Fig. 2. Comparison of the frequency of micronucleated erythrocytes (MNE) in perch (*Perca fluviatialis* L.) and roach (*Rutilus rutilus* L.) captured at different water bodies. Capture sites are arranged in the order of increasing MNE frequency

Two fish species – perch and roach – were captured in five out of six study locations. These two species (perch and roach) and five locations (the Upper Nemunas, the Middle Nemunas, Lake Ginkūnai, pond Švedė and pond Bubliai) were selected for the analysis of variance in order to find out what variables were significant for the frequency of MNE. Results of the analysis are shown in Table 2. It may be concluded that both the species ($P = 0.0025$) and the site of fish capture ($P = 0.007$) significantly contributed to the variation of MNE frequency observed in the present study. The frequency of MNE in perch was almost systematically higher than in roach (Fig. 2). The highest difference between these two species was seen in the Švedė pond where the highest frequencies of MNE were found.

Table 2. **Results of analysis of variance of mean square root transformed frequency of micronucleated erythrocytes in perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) captured at four different locations**

Source of variability	df	Sum of squares	Mean square	F ratio	P
Main efects:					
Site of capture	4	8.896	2.1256	6.41	0.007
Species	1	3.5896	3.5896	10.82	0.0025
Residual	31	10.2869	0.3318		
Total (*corrected*)	36	23.6288			

Our results indicate that the micronucleus test in fish can be used for detection of genotoxic pollution in natural water bodies. This is in general agreement with earlier and more recent findings of other researchers (Hose et al., 1987; Minissi et al., 1996; Ayllón et al., 2000). Increased frequencies of MNE in fish captured at certain locations (the Curonian Bay, Lake Ginkūnai and the Švedė pond) also indicate that some Lithuanian waterbodies or their certain parts can be contaminated with genotoxic substances. Previous reports indicating the presence of dangerous substances in Lithuanian surface waters and their genotoxicity *in vitro* (Ellington et al., 1987; Sabaliūnas et al., 2000) also substantiate this conclusion.

It has previously been recommended that for *in situ* studies all fish be sampled from all locations on approximately the same date (Al-Sabti et al., 1995). This term was violated in our study, because fish from the Curonian Bay, Lake Ginkūnai and the Švedė pond were captured 3–4 months later than from the Upper and Middle Nemunas. However, it is hardly probable that these differences in fish capture time could exert a significant influence on the results of our study. First, there was almost a threefold increase in the frequency of MNE in the Middle Nemunas as compared to the Upper Nemunas, although fish in both locations were captured practically at the same time. Second, in an extensive study with barbel (*Barbus plebejus*) from two Italian rivers no significant seasonal differences were observed (Minissi et al., 1996).

According to the literature data (Al-Sabti et al., 1995), the spontaneous frequency of MN in control (untreated or captured in clean water body) fish varied from 0 to 15.8‰. The frequency of MNE in fish from the cleanest site in our study – the Upper Nemunas – was 0.05 ± 0.03‰. This frequency corresponds to the lowest spontaneous frequency so far reported in the field studies (Minissi et al., 1996). Interestingly, in one study performed in Estonia, which is geographically quite close to Lithuania, about tenfold higher frequencies of MN were reported for the same species as in our study (perch, roach and bream) captured in the cleanest "reference" water bodies (Palm et al., 1995). However, a direct comparison of MN frequency in fish from Estonia and Lithuania is impossible because of the lack of precise data on the real levels of pollution at study locations.

A comparison of the same species in different waterbodies and different species in the same waterbody showed that the most sensitive species in our study was perch, followed by roach. It may be deduced that common bream is less sensitive to pollution than roach, but the data are too flimsy. The high sensivity of perch to genotoxic pollutants has also been reported in some other studies (Palm et al., 1995; Al-Sabti et al., 1990). Thus, analysis of MNE in perch can be recommended as a suitable method for *in situ* detection of environmental genotoxins. The difference in the sensivity of fish species to MN induction could be explained by their feeding peculiarities: perch feeds on smaller fish, common bream mainly on chironomid and other insect larvae, while roach feeds on *Dreissena* mussels. A similar conclusion was made by Hose et al. (Hose et al., 1987) when comparing the sensivity of two fish species (*Paralabrax clathratus* and *Genyonemus lineatus*) from Southern California. Alternatively, other researchers claimed that variability in sensivity to the induction of MN among fish species is not always in accordance with the stage on the trophic food web (Al-Sabti et al., 1990). This is not surprising, since not only feeding but also metabolism differences can exist among different species. It is obvious that this point should be clarified by further more extensive studies.

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LIETUVOS VANDENS TELKINIŲ GENOTOKSIKOLOGINIAI TYRIMAI, TAIKANT ŽUVŲ ERITROCITŲ MIKROBRANDUOLIŲ METODĄ

S a n t r a u k a

1999–2000 m. buvo atliekami Lietuvos vidaus vandens telkinių genotoksikologiniai tyrimai žuvų eritrocitų mikrobranduolių metodu. Tyrimams buvo pasirinktos 6 vietos. Iš viso ištirta 60 žuvų (5 rūšys).

Tirtų vandens telkinių genotoksiškumo lygis buvo įvertintas apskaičiavus bendrą eritrocitų su mikrobranduoliais (EMB) dažnį atskirai kiekvienam telkiniui. Transformuotos eritrocitų su mikrobranduoliais (TEMB) reikšmės panaudotos dispersinei analizei. Šiai analizei buvo pasirinktos penkios vietos (Nemunas ties J. Ančia, Nemunas ties Verkne, Ginkūnų ež., Švedės tv., Bublių tv.) bei dvi žuvų rūšys, t. y. ešerys (*Perca fluviatilis* L.) ir kuoja (*Rutilus rutilus* L.), kurios sugautos kiekvienoje iš minėtų vietų. Gauti duomenys rodo patikimą skirtumą tiek tarp vietų (P = 0,007), tiek tarp abiejų tirtų rūšių (P = 0,0025). Iš gautų duomenų matyti, jog žemiausias EMB dažnis yra Nemune ties J. Ančia (0,05‰), o aukščiausias – Švedės tvenkinyje (0,42‰). Taip pat pastebėta, kad aukščiausi EMB dažniai yra ešerio (jautriausia rūšis), antroje vietoje – kuoja. Palyginus ešerių ir kuojų EMB vidutines reikšmes (tų telkinių, kuriuose sugautos abiejų rūšių žuvys), galima pamatyti tokią tendenciją – ešerių EMB reikšmės yra sistemingai didesnės nei kuojų.

Raktažodžiai: mikrobranduoliai, genotoksiškumas, gėlavandenės žuvys, ešerys, kuoja, karšis