*Research Article*

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# *In Vivo* Genotoxicity and Cytotoxicity Assessment of Allowable Concentrations of Nickel and Lead: Comet Assay and Nuclear Abnormalities in Acridine Orange Stained Erythrocytes of Common Carp (*Cyprinus carpio* L.)

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**Abstract:** The aim of the present study was to assess the possibility of *in vivo* genotoxicity by nickel and lead concentrations considered safe by regulatory agencies. In order to evaluate their genotoxic and cytotoxic potential, young specimens of the common carp (*Cyprinus carpio* L.) were exposed for 72 h in laboratory conditions to different concentrations of Ni and Pb, considered as annual average according to Directive 2008/105/EC. The alkaline comet assay in circulating erythrocytes was applied and blood smears stained with acridine orange were tested for the presence of micronuclei and other nuclear abnormalities. The comet parameters % tail DNA and tail moment indicated statistically significant genotoxic damage for both metals at 100%, 75% and 50% of the permissible concentrations, as compared to controls. Increased levels of micronuclei were not observed in carp erythrocytes, but we found a significant increase in other nuclear abnormalities – notched, blebbed, lobed, eight-shaped nuclei and nuclear buds. This demonstrated evident cytotoxic effects and confirmed the use of nuclear abnormalities as an effective biomarker. The obtained results confirmed the genotoxic and cytotoxic effects of Ni and Pb, even at low permissible levels, and illustrated the need for additional investigations in order to reduce the allowable concentrations of heavy metals in water basins.

**Key words:** genotoxicity, cytotoxicity, nickel, lead, *Cyprinus carpio*, comet assay, nuclear abnormalities

## Introduction

Heavy metal contamination is one of the most important environmental issues to be tackled by modern society. Due to their high toxicity and the property to become concentrated in living organisms they pose a direct hazard to public health (Çavaş 2008). The presence of heavy metals in aquatic ecosystems has long been considered a serious issue and aimed by both the national and the European policy. One of the steps taken for prevention and control of chemical pollution of surface waters is the list of priority substances established by the European Parliament and the Council, for which allowable concentrations have been introduced, considered safe for living organisms and the environment (Directive 2008/105/EC). Ni and Pb are referred to as priority substances in surface waters according to Directive 2008/105/EC, Directive on Environmental Quality Standards (EQSs).

Aquatic organisms, such as fish, accumulate hazardous agents directly from contaminated water or indirectly through ingestion of contaminated water (Matsumoto *et al.* 2006), and along the food chain, through contaminated food (Ribeiro *et al.* 2000,

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Cestari *et al.* 2004). Therefore, such substances can lead to contamination not only of aquatic organisms but also of the whole ecosystem. Since the response of fish to toxic substances is often similar to that of other higher vertebrates, they prove to be extremely useful bioindicators in the screening of chemicals potentially capable of inducing a teratogenic and carcinogenic effect in humans (Al-Sabti & Metcalfe 1995). Teleosts are sensitive bioconcentrators even at low levels of contaminants, including heavy metals, to which nowadays they are often chronically exposed in natural basins (Russo *et al.* 2004, Ivanova *et al.* 2016).

The significance of fish as bioindicators of water pollution is also related to the fact that they are rich sources of protein and lipids and their health is very important to humans. The dominant species in a given site are the most informative bioindicators as they reflect the full impact on the habitat for a longer period. The carp was selected to be the subject of the study because it is a common species that is often reared and consumed by people, and because of its ecological features, and its obvious importance as an indicator species in a number of laboratory (Gustavino *et al.* 2001, Zhu *et al.* 2004, Arkhipchuk & Garanko 2005, Çavaş *et al.* 2005, Ali & Ganaie 2014, Al-Tamimi *et al.* 2015) and field studies (Pellacani *et al.* 2006, Klobučar *et al.* 2010, Çok *et al.* 2011).

The methods of monitoring contaminants causing geno- and cytotoxic effects in living organisms are constantly being improved and modified within the context of achieving an accurate and rapid assessment of anthropogenic pollution. Comet assay is a sensitive technique for the detection of DNA damage, widely used in biomonitoring, practically applicable in all nuclear eukaryotic cells and biological species and particularly relevant in confirming DNA damage in aquatic organisms (Mitchelmore & Chipman 1998, Kim & Hyun 2006, Frenzilli *et al.* 2009) and in particular, in fish (Russo *et al.* 2004, Wirzinger *et al.* 2007, Çok *et al.* 2011, Ferreira *et al.* 2015). The method involves single-strand and double-strand DNA breaks, as well as alkali-labile sites and reparation, even when exposed to low concentrations of toxicants.

Another widely used method for genotoxicity testing is the micronucleus test (MN test) which in recent years has been significantly optimized by using fluorescent dye acridine orange (AO), increasing many times the sensitivity of the method as compared to routine dyes (Costa & Costa 2007, Çavaş 2008, Polard *et al.* 2011). The analysis of micronucleus frequency in fish, however, shows significant differences between the species (Bolognesi & Hayashi 2011) and many factors such as metabolic capacity, DNA repair efficiency and defence mechanisms may be responsible for such different sensitivity (Rodriguez *et al*., 2003). Since Carrasko *et al.* (1990) first described the morphological alterations in nuclei, along with MN, different nuclear abnormalities (NAs) (buds, lobed, notched, blebbed, eight-shaped and donut-shaped nuclei, broken eggs), have been used as reliable biomarkers in several studies concerning fish exposed to mutagenic substances (Anbumani & Mohankumar 2011, Ali & Ganaie 2014, Hoshina *et al.* 2015, Srivastava & Singh 2015, Ivanova *et al.* 2016). Although the mechanism of their occurrence is still not fully determined, some authors define NAs as cytotoxic biomarkers (Rocha *et al.* 2011), others relate the presence of certain NAs (lobed and notched nuclei) with the cytotoxic or with genotoxic effect (nuclear buds) (Bolognesi & Hayashi 2011). At all events, however, their occurrence is associated with errors in cell division, the process of cell death and genotoxicity and / or mutagenicity; therefore, in many studies testing the effects of different genotoxicants NAs are recommended as biomarker complementing MN reporting in routine genotoxic procedures (Ferraro *et al.* 2004, Çavaş 2008, Hoshina *et al.* 2008).

The aim of the present study was the detection of possible *in vivo* genocytotoxicity of nickel and lead concentrations, considered safe by regulatory agencies. In order to evaluate their possible genotoxic and cytotoxic potential even at permissible levels *ex situ* comet assay and observation for NAs (including MN) were applied in circulating erythrocytes from exposed specimens of common carp (*Cyprinus carpio* L.).

# Materials and Methods

### Experimental design

Young specimens (mean body weight 21.02 ± 13.15 g; mean body length 11.73 ± 2.16 cm) of common carp (*Cyprinus carpio* L.) were exposed to 100%, 75%, 50% and 25% of the allowable Ni and Pb concentrations for 72 h in laboratory conditions. According to the Bulgarian legislation based on Directive 2008/105/EC the allowable concentrations which is set as annual average (AA) of Ni and Pb in surface waters is 20 μg/L and 7.2 μg/L respectively. The heavy metal concentrations were prepared for 50 L water, as follows: Ni 1000 μg (100% AA); Ni 750 μg (75% AA); Ni 500 μg (50% AA); Ni 250 μg (25% AA); Pb 360 μg (100% AA); Pb 270 μg (75% AA); Pb 180 μg (50% AA); Pb 90 μg (25% AA). Fish were obtained from the Institute of Fisheries and Aquaculture (Plovdiv, Bulgaria). There were no external pathological abnormalities. After transportation, the fish were acclimatized for four days in glass aquaria containing well-aerated, dechlorinated (by evaporation) water at con-

stant temperature and photoperiod before the heavy metals treating. After acclimatization the individuals were divided into nine groups (eight groups exposed to the four concentrations of Ni and Pb and a control group of untreated fish)(n=10 in each experimental tank and for control). After the total acute time of exposure (72 h) blood probes from all individuals were collected through intracardiac puncture in tubes with lithium heparine for further analyses. Blood from a control group of untreated fish was used as negative control for comparison of the results. Fish were not fed prior or during the experiment. The physico-chemical characteristics of the aquarium water such as: pH, temperature, oxygen level and conductivity were measured once on the 0, 24$^{th}$ and 72$^{nd}$ hour according to a standard procedure with a combined field-meter (APHA 2005). All experiments were conducted in accordance with national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes according to DIRECTIVE 2010/63/EU.

**Comet assay**

The comet assay was carried out according to the alkaline version of Sighn *et al.* (1988), with some modifications. A mixture of 5 μL of blood sample collection with 95 μL low melting point agarose (LMA) (0.7% in PBS) was added onto degreased microscope slides previously covered with 1% normal melting agarose (NMA) for the first layer and immediately covered with a coverslip. Two slides for each individual were prepared and kept for 5 min in refrigerator to solidify. After the solidification of the gel, coverslips were gently removed and a third layer with LMA (100 μL) were added. The procedure with cover slides was repeated and after the cover slides removing the slides were immersed in cold, freshly made lysis solution (2.5M NaCl, 100mM $Na_2EDTA$, 10mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) in a Coplin jar and refrigerated at 4°C overnight in the dark. After the lysis, the excess salt was removed by rinsing the slides in cold distilled water. Then the slides were placed side by side in a specifically designed horizontal electrophoresis tank (Model CSL-COM20, Cleaver Scientific Ltd., UK, Max. volts 250 V, Max. current 500 mA) and alkaline DNA-unwinding was carried out in a freshly prepared buffer (1mM $Na_2EDTA$, 300mM NaOH, pH>13) for 20 min at 4°C. The electrophoresis was performed in the same buffer for 20 min at 25 V and 300 mA. The steps above were carried out both at 4°C provided by a cooling apparatus and under the yellow light to minimise additional temperature- and UV-induced DNA damage. After the electrophoresis the slides were gently removed from the tank, and neutralizing buffer (0.4M Tris-HCl, pH 7.5) was added to the slides dropwise three times, letting it sit for 5 minutes each time. To allow scoring in appropriate time, the gels were fixed in absolute ethanol for 20 min and dried at room temperature. The slides were rehydrated with SYBR Green I (1 : 10 000 dilution) immediately before fluorescence examination.

DNA damage was visualized in circulating erythrocytes with a Leica DM1000 epifluorescence microscope equipped with a suitable for SYBR Green filter and a camera, at a magnification of 400×. The images were processed using a Comet Assay Score Programme (CASPlab, Wroclaw, Poland). For each animal, 100 nucleoids were measured. For all specimens 2 parameters were traced: % tail DNA (%TDNA) and tail moment (TM). %TDNA is the total DNA that migrates from the nucleus into the comet tail. TM is the product of the tail length (TL) and %TDNA, which gives a more integrated measurement of overall DNA damage in the cell. In order to visually determine the predominant type of comets, these are categorized according to the degree of damage in 5 different classes, from 0 (no tail) to 4 (almost all DNA in tail)(Collins 2004).

**Observation for NAs**

Whole blood smears were done on clean glass slides immediately after sampling, dried at room temperature, fixed with absolute ethanol for 20 min, and stained with acridine orange (AO) (0.003% in PBS) at the time of analysis (Ueada *et al.* 1992). Two smears were prepared from each individual. The frequency of different NAs was manually scored at a magnification of 1000× using epifluorescence microscope (Leica DM 1000) equipped with an appropriate filter and photo camera. Only nucleated erythrocytes with intact cellular and nuclear membrane were scored. On each slide, only areas with uniform spread in monolayer without overlapping cells were targeted. As a result of AO metachromasia the cytoplasm of the immatures polychromatic erythrocytes (PCEs) emits red fluorescence, unlike greenish cytoplasm of mature normochromatic erythrocytes (NCEs), while the cells nuclei fluorescence yellow-green or yellow. Analyses consisted of count and detection of erythrocytes with NAs (including MN) distinguished in notched, lobed, and blebbed nuclei according to the criteria of Carasco *et al.* (1990). Furthermore, two types of NAs observed in the studied individuals, which were denominated "eight-shaped" and "bud", were added. Erythrocytes having a main nucleus with a clearly separated smaller nucleus with rounded or oval shape, that is not larger than 1/5 of

the main nucleus and displaying the same staining (yellow-green) and focusing pattern like main nucleus were accepted as micronuclei (MN). Nuclei with a substantial notch into the nucleus were noted as notched nuclei (NotchN); lobed nuclei (LobeN) had larger evaginations (lobe), including those with several lobes. Blebbed nuclei (BlebN) had a relatively small evaginations of the nuclear membrane and contained euchromatin; nuclei containing euchromatin and having a relatively small evagination (bud) on the nuclear membrane that was partially separated from the nucleus are accepted as nuclear bud (NBud); Eight-shaped nuclei (EN) (according to Furnus 2014) presenting a constriction acquiring an eight shape. The average nuclear abnormalities frequency (NAsF), which represents the number of cells with NAs per minimum 2000 accounted erythrocytes (both PCEs and NCEs) expressed as per mille were calculated for each animal tested.

$$\text{NAsF ‰} = \frac{\text{Number of cells containing NAs}}{\text{Total number of cells scored}} \times 1000$$

**Statistical analysis**

Statistical analysis of the data was performed by GraphPad Prism 4.0 statistical software. Data was tested for normal distribution using Kolmogorov-Smirnov test. Since data was normally distributed, significance of differences between the control and treated groups were tested using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The results were expressed as mean±standard error. The differences were considered significant at $p \leq 0.05$.

# Results

**Alkaline comet assay**

The comet parameters %TDNA and TM showed statistically significant genotoxic damage for both metals at 100%, 75% and 50% of AA compared to controls (Fig. 1). The most intensive DNA migration for Ni (%TDNA = 26.72 ± 4.84; TM = 2.43 ± 1.09) and Pb (%TDNA = 26.37 ± 8.12; TM = 3.35 ± 1.60) was observed at 100% of AA, with no statistical difference between the both metals. The lowest values of TM were recorded at 25%, with no statistical difference from the controls. The results of the visual classification of comets showed that comets class 1, 2 and 3 predominated in the treated specimens; less frequently, however, comets of class 4 were also observed, especially at 100% and 75% of AA of the both metals. This indicated a certain degree of genetic damage (Fig. 2a) confirmed by the increased value of the parameter %TDNA in all treated specimens. Comets class 0, with intact head and symmetrical halo dominated mainly in specimens of the control group, followed by comets of class 1 (Fig. 2b).

**NAs observation**

The fluorescent observation of blood smears indicated no increase in the frequency of MN in circulating erythrocytes but there was a significant increase in the frequency of other NAs (Table 1). The following were observed: BlebN, LobeN, NotchN, EN, NBuds (Fig. 3), as the first two types of abnormalities are the most common (Table 1). Nucleoplasmic bridges and binucleated cells were not found in any of the tested concentrations. Different types of NAs are reported separately as a certain number per minimum 2000 observed erythrocytes (Table 1) expressed as permille but in the statistical processing of the data the overall average frequency of NAs (total NAsF) for each concentration was used. Using this indicator, statistically significant differences from the control were reported for both metals at 100%, 75% and 50% of AA ($p<0.05$), as there are no statistical differences between the both metals.

# Discussion

The common carp (*Cyprinus carpio* L.) is a species widely used in the evaluation of the genotoxic potential of agents and, over the last decade, also in comet assay, both in laboratory and in field conditions (Kim & Hyum 2006, Pellacani *et al.* 2006, Klobučar *et al.* 2010, Çok *et al.* 2011). In tests of erythrocytes of young carps exposed *in situ* to disinfectants for potabilization, Bushini *et al.* (2004) registered an immediate response (after 3 hours) with comet assay to genotoxic agents, unlike MN, whose peak was registered on the 20$^{th}$ day. For assessment of genotoxic contamination of surface water, Pellacani *et al.* (2006) found *in vivo* genotoxicity by comet assay of carps, which also confirmed the method as a reliable biomarker and the species as a suitable bioindicator for water quality. Klobučar *et al.* (2010) used caged carps for genotoxicity assessment of freshwater, where specimens from the place with strong anthropogenic influence indicated a high degree of DNA damage proved by comet assay and micronucleus test. The authors defined the comet assay as more sensitive. The comparative analysis between *in vivo* comet assay and *in vivo* MN test of three fish species, including carp, showed dose- and time-dependent increase in the examined parameters after treatment with mutagens. A higher correlation of concentrations was reported in the comet assay than in the MN test.

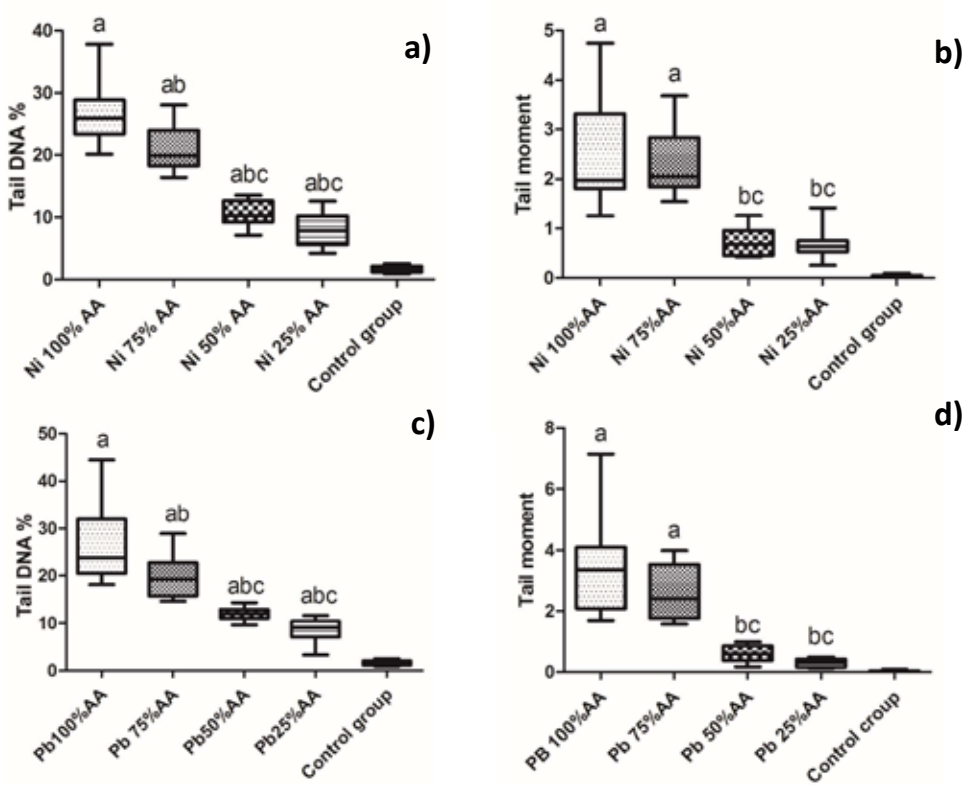


**Fig. 1.** Investigated comet assay parameters in circulating erythrocytes of *C. carpio* exposed *in vivo* to different allowable (annual average – AA) Ni and Pb concentrations: a) %TDNA – Ni; b) TM – Ni; c) %TDNA – Pb; d) TM – Pb
Values are expressed as mean ± standard error and statistical significance was determined by one way ANOVA followed by Tukey's post hoc test. "a" indicates statistical significance of differences with respect to corresponding control group($p<0.05$); "b" indicates statistical significance of differences between 100% AA and other exposed groups; "c" indicates statistical significance of differences between 75% AA and other exposed groups. No statistical significance of differences was observed between 50% AA and 25% AA.

The values that we received for %TDNA and TM in the treatment with 100% of AA for both metals are close to the values of these parameters obtained after the comet assay of erythrocytes of carps from contaminated basins (Çok *et al.* 2011). This indicates that the genetic damage resulting from exposure to permissible levels of heavy metals registered by us in laboratory conditions is comparable to an actually existing genotoxic effect in the environment resulting from anthropogenic influence.

On the one hand the absence of MN in carp erythrocytes upon treatment with heavy metals in our study is surprising but, on the other hand, it could be caused by various reasons. Staining of blood smears with the fluorescent dye AO removes any doubts about the deficiencies in their scoring (Costa & Costa 2007, Çavaş 2008, Polard *et al.* 2011). AO is a fluorochrome binding specifically with nucleic acids and showing metachromasia, by staining DNA in yellow-green, and RNA in orange-red against a background of green cytoplasm of the cells. Such specificity eliminates the reporting of all kinds of non-nucleic artifacts, such as, for example, protein granules, which are often confused with MN in the bright-field microscopy.

In order to explain the absence of MN in the present study it should be considered that the increase in MN-frequencies in fish, caused by different contaminants is dose- and time-dependent (Ali & Ganaie 2014), species- and tissue-dependent (Arkhipchuk & Garanko 2005, Çavaş *et al.* 2005, Çavaş 2008), reaching different peak-time. In general, spontaneous micronucleus frequency in fish is not high. It is assumed that this is related to the fact that the intensity of the cell cycle in fish as poikilothermic animals depends on temperature (Al-Sabti & Metcalfe 1995). Bolognesi & Hayashi (2011) synthesize the result of the analysis of fish baseline micronucleus frequency reported by various researchers and state that it varies from 0 to 13 per 1000 cells, and most frequently articles report data from 0 to 1. This variability may be associated with the interspecies differences in metabolitic competency and DNA repair mechanisms, as well as cell proliferation in the target organ affecting

**Table 1.** Frequency of carp erythrocytes NAs, expressed as per mille from different Ni and Pb concentrations

| Concentrations | N | Frequency of different types of NAs ‰ | | | | | | Total NAsF | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NotchN | BlebN | LobeN | EN | NBud | MN | Mean | Min | Max | X± SD |
| Ni 100% 20 µg/L | 10 | 15.5 | 32.3 | 30.7 | 6.4 | 4.7 | 0 | 92.83* | 70.53 | 135.0 | 21.03 |
| Ni 75% 15 µg/L | 10 | 9.2 | 30.9 | 28.3 | 2.7 | 1.2 | 0 | 75.58* | 51.76 | 86.00 | 11.16 |
| Ni 50% 10 µg/L | 10 | 4.2 | 14.5 | 13.2 | 1.1 | 0.25 | 0.1 | 33.25* | 24.00 | 44.00 | 5.47 |
| Ni 25% 5 µg/L | 10 | 1.8 | 7.6 | 8.6 | 0.6 | 0.1 | 0 | 22.95 | 12.00 | 27.63 | 4.78 |
| Pb 100% 7.2 µg/L | 10 | 12.3 | 44.4 | 40.3 | 6.3 | 3.4 | 0.3 | 100.6* | 30.63 | 150.0 | 34.35 |
| Pb 75% 5.4 µg/L | 10 | 8.7 | 18.1 | 15.2 | 4.1 | 0.9 | 0.1 | 47.94* | 29.41 | 67.00 | 12.57 |
| Pb 50% 3.6 µg/L | 10 | 9.4 | 13.9 | 10,5 | 2.5 | 0.7 | 0 | 37.54* | 27.00 | 48.00 | 6.63 |
| Pb 25% 1.8 µg/L | 10 | 1.9 | 8.0 | 6.3 | 1.1 | 0.1 | 0 | 17.74 | 13.40 | 24.00 | 3.41 |
| Control group | 10 | 0 | 3.2 | 2.9 | 0 | 0 | 0 | 5.36 | 4.00 | 8.00 | 1.35 |

* $p < 0.05$ compared to negative control.

the MN expression (Rodriguez *et al.* 2003). Other authors have also reported significant differences in the frequency of MN and their peak in *Cyprinus carpio* (Nepomuceno & Spanó 1995, Nepumoceno *et al.* 1997, Gustavino *et al.* 2001, Bushini *et al.* 2004, Çavaş *et al.* 2005). Apart from the mechanism of action of the toxin, the latter may be due to different biotic factors such as age, gender, genetic make-up, treatment conditions or different reporting criteria.

BlebN and LobeN are considered to precede MN (Shimizu *et al.* 1998; Anbumani & Mohankumar 2012). In our study, we find statistically significant increase in NAsF at 100%, 75% and 50% of AA for Ni and Pb, and Table 1 clearly shows that this is due to the BlebN and LobeN. These two types of abnormalities are average 75% of the NAs in all of the experimental groups. Their increased frequency is dose-dependent and can be explained by a cellular mechanism for coping with excess chromatin, which will separate the genetic material of micronucleus and then it will throw it out of the cell as *double minute* (Shimizu *et al.* 1998). It is assumed that exactly the initiation of the Breakage-Fusion-Bridge Cycle for separation of the tangled and attached chromosomes associated with gene amplification can lead to LobeN or BlebN, NBuds, nucleoplasmatic bridges and MN during the isolation of the amplified DNA from the nucleus (Shimizu *et al.* 1998, 2000; Fenech *et al.* 2011). According to Fenech *et al.* (2011) NBuds are associated with the processes of amplified DNA extraction, DNA repair complexes and probably the unnecessary chromosomes of aneuploid cells. Shimizu *et al.* (1998, 2000) used *in vitro* experiments with mammalian cells to demonstrate that the amplified DNA is selectively localized in specific places along the periphery of the nucleus and is eliminated by nuclear budding in the S phase of the cell cycle. The amplified DNA can be eliminated by the chromosomes by recombination of homologous regions from amplified sequences forming mini-circles of acentric and double minutes. NBuds are characterized by the same morphology as MN, with the exception that they are connected to the nucleus by a narrow or a wide stalk of nucleoplasmic material depending on the stage of the budding process. In support thereof Bolognesi *et al.* (2006) report the existence of a strong correlation between induction of MN and NBuds in fish erythrocytes. On the other hand, the results of experiments with telomeric and centromeric samples in human lymphocytes conducted by Lindbergh *et al.* (2007) demonstrate putative partial differences in the mechanisms of occurrence of NBuds and MN. It appears that the interstitial DNA without centromeric or telomeric regions occurs to a greater extent in NBuds (43%) than in the MN (13%), while only telomeric DNA or simultaneously centromeric and telomeric DNA – more frequently in MN (62 and 22%, respectively) compared to NBuds (44 and 10%, respectively). Although some issues pertaining to the origin of the NAs are still open to debate, all literature data clearly show that they are a biomarker of genotoxic events and chromosomal instability.

Acute exposure is associated with causing significant damage to the cells, including apoptosis,

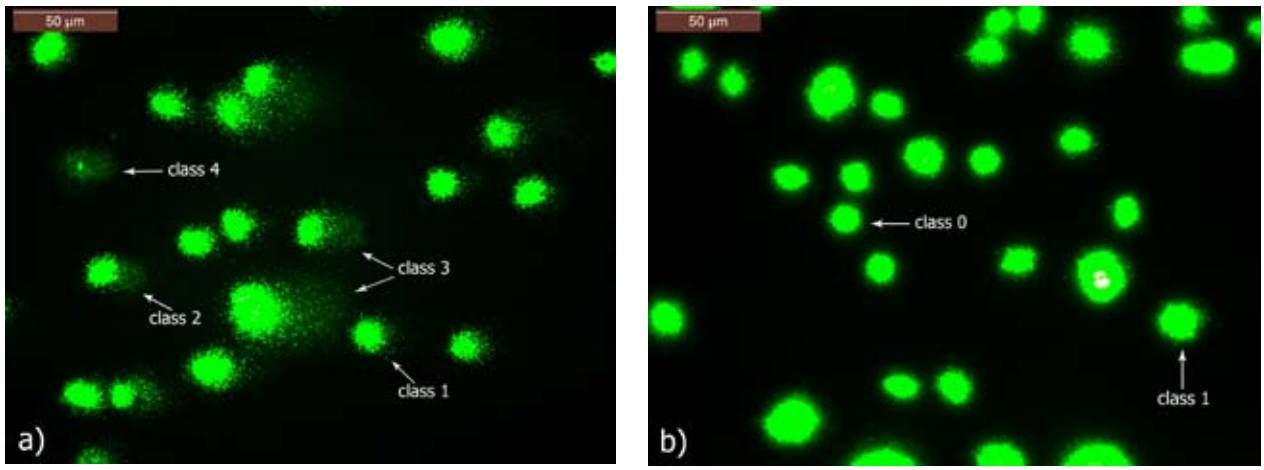


**Fig. 2.** Comet cells from *C. carpio*: a) DNA damage in erythrocytes exposed to 100% AA of Pb demonstrated by different comet classes; b) Class 0 and class 1 comets in *C. carpio* from control group (400×)

in contrast to chronic exposure, which allows time for adjustment and repair of damage (Anbumani & Mohankumar 2012). It is assumed that extremely high frequency of BlebN in the erythrocytes of barbell continuously exposed to chronic contamination *in situ*, activate mechanisms such as nuclear budding, for example, for excess chromatin removal (Ivanova *et al.* 2016). The frequency of NBuds registered in our study is considerably lower compared to BlebN and LobeN. This gives us grounds to assume that the allowable concentration of both metals in 72 hours of acute exposure induce BlebN and LobeN, which at a later stage involving intensification of the mechanisms of amplified DNA discharge could transform into NBuds and, accordingly, MN.The treatment with these concentrations of heavy metals that are not high, such as the annual average approved by European legislation, will probably most often induce BlebN and LobeN and less often NBuds, which is supported by the values dose-dependent decrease.

Vinodhini & Narayanan (2008) show accumulation of heavy metals in the kidney of *Cyprinus carpio* in the following order: Pb>Cd>Cr>Ni. Given that the kidney is a hematopoietic organ in fish, the damage in the kidney stem cells as a result of bioaccumulation of heavy metals at a later stage may be registered through potential damage to the circulating erythrocytes.

The mutagenic and clastogenic activity of the lead results from the disruption of enzyme regulation associated with the replication, translation and reparation of the genetic material (Goyer & Moor 1974). Therefore, even in the cases of low concentrations, such as the AA which we can accept for surface water, the circulating erythrocytes of the carp in our study show a high degree of geno- and cytotoxic damage. In accord with our results from chronic exposure to $Pb^{2+}$ in the diet of neotropic fish *Hoplias malabaricus,* a lower micronucleus frequency has also been registered, which the authors (Cestari *et al.* 2004) associated rather with the inhibitory effect of the lead on the cell cycle than with the induction of reparation mechanisms in cells. To examine the effects of long-term exposure to lead (64 days), the same authors reported through comet assay an increased frequency of comets by which they evidence a lead-induced DNA damage as well. By comet assay mutagenic potential of inorganic lead (PbII) in the same species *(Hoplias malabaricus)* was also evidenced and even though again there were no significant differences with the controls in regard to MN, statistically significant differences in erythrocytes with different NAs types were recorded (Ferraro *et al.* 2004). Treatment with lead acetate (10 µg/L, 50 µg/L and 100 µg/L) in *Carassius auratus auratus* for 2, 4 and 6 days induced an increased frequency of MN in blood erythrocytes, gill and fin epithelial cells of different tissue sensitivity, but it did not cause any significant increase in NBuds, except the highest dose and longest exposure (Çavaş 2008). Russo *et al.* (2004) suggest that MN and the increased migration of DNA in *Gambusia holbrooki* registered by them is due to the evidenced presence of heavy metals in the tested waters, as the analyzed sediments contain Pb as well.

It is well known that the compounds of Ni induce specific structural chromosome damage and MN (Arrouijal *et al.* 1990), and DNA-protein crosslinks and oxidative DNA base damage were observed in cells treated with Ni (II) (Kasprzak 1991). Furthermore, Ni genotoxicity may be potentiated through the generation of DNA-damaging reactive oxygen species and the inhibition of DNA

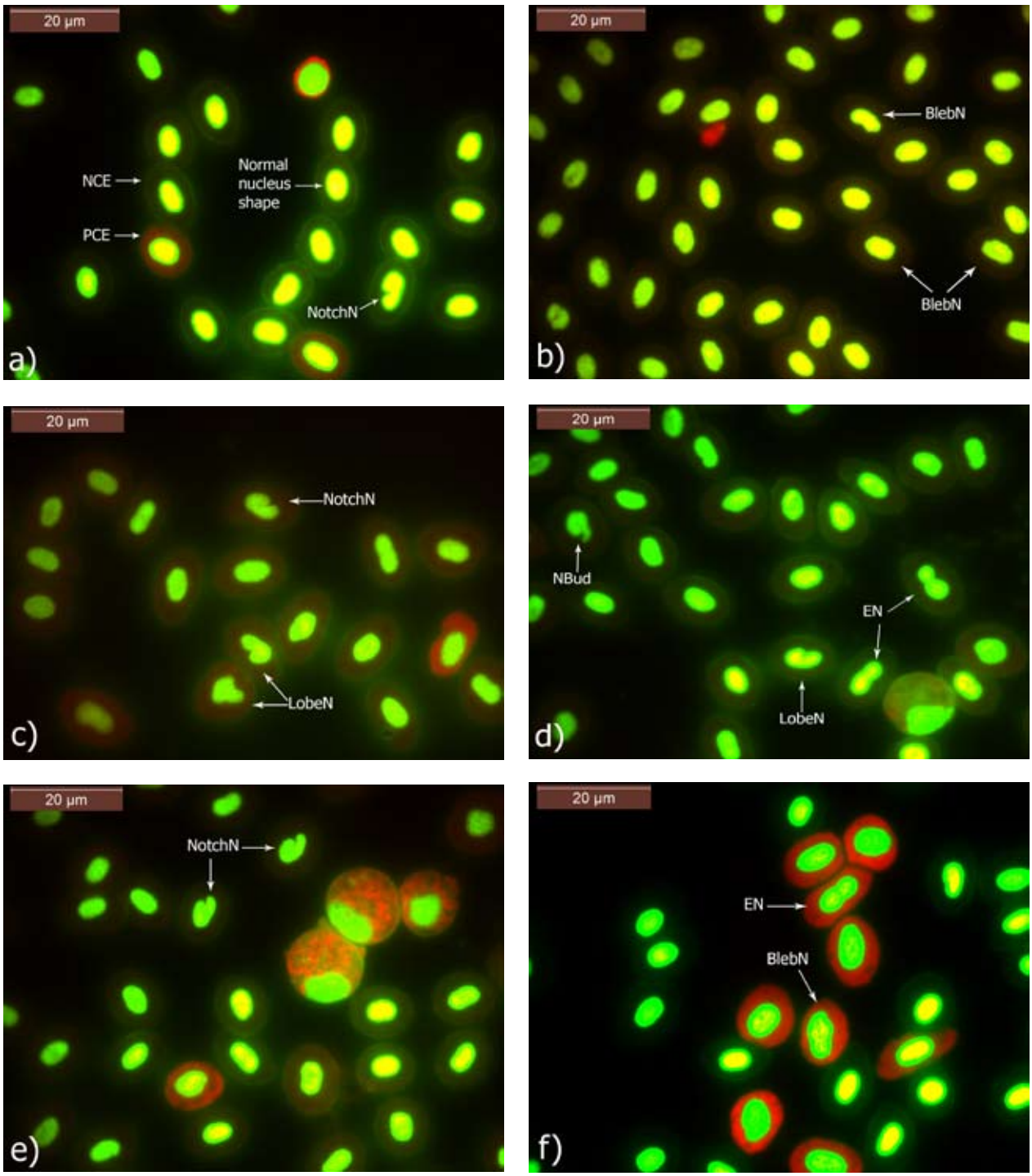


**Fig. 3.** Microphotographs of blood smears representing different NAs observed in acridine orange stained erythrocytes of *C. carpio* exposed to allowable Ni and Pb concentrations: a) 50% AA of Ni; b)75% AA of Ni; c) 100% AA of Ni; d) 100% AA of Pb; e) 75% AA of Pb; f) 50% AA of Pb (1000×, *in imersion*)

repair by this metal (Kasprzak *et al.* 2003). Ergene *et al.* (2005) report that in waters contaminated by heavy metals (including Ni and Pb), they observe an increased frequency of MN and NAs in the erythrocytes of 3 fish species, and find a clear correlation between MN and NBuds. Data on the effects of this metal on carp erythrocytes *ex situ*, as evaluated by comet assay or NAs, are not found in the available literature. In this sense, values obtained in our study may serve for comparison in future studies.

One of the main issues in the genotoxicology concerns the concentration of toxicants that does not have effect on the organism. The results of our study show that the allowable concentrations of Ni and Pb also have *ex situ* geno- and cytotoxic effect in erythrocytes of carp. Comet assay shows an im-

mediate response; the different types of NAs whose frequency is dose dependent demonstrate this toxic effect, and possibly MN will reach their peak at a later stage, after 72 hours of exposure when the genotoxic damage generated in the kidney stem cells will be expressed in the circulating erythrocytes. The toxic effect of the same concentrations may even be stronger in environmental conditions in the presence of other additional toxicants because it has long been known that the combined effect of heavy metals on living organisms has a synergistic effect.

# Conclusions

The metal exposed groups showed significant variation in the frequency of NAs as well as an extent of a DNA damage compared to the control group. These frequencies increased significantly ($p<0.05$) in a concentration dependent manner. The significantly higher comet assay parameters (%TDNA, TM) of the exposed fishes compared with control group ($p<0.05$) prove the existence of genotoxic effect at allowable heavy metal levels; The 72 h exposure time of the investigated concentrations was not effective for the formation of MN but demonstrated evident cytotoxic effect and confirmed formation of NAs as effective biomarker; The lowest values (for TM and NAs) were registered at 25% and the lack of statistical differences with controls showed the harmlessness of this concentration; The obtained results proved the geno- and cytotoxic effects of Ni and Pb, even at low permissible levels and illustrated the need of additional investigations in order to reduce allowable concentrations of heavy metals in water basins.

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