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Induction of Erythrocytic Nuclear Abnormalities by Permitted Concentration of Cadmium in Common Carp (Cyprinus carpio L.)

Vesela I. Mitkovska[1*], Hristo A. Dimitrov[1], Tsenka G. Chassovnikarova[1,2]

1 - University of Plovdiv Paisii Hilendarski, Faculty of Biology, Department of Zoology, 24 Tzar Asen Street, 4000 Plovdiv, BULGARIA
2 - Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel Blvd., 1000 Sofia, BULGARIA
*Corresponding author: mitkovska.v@gmail.com

Abstract. The induction of micronuclei and other nuclear abnormalities (NAs) - nuclear buds, nucleoplasmic bridges, binucleated cells, as well as notched, blebbed, lobed and eight-shaped nuclei - was analyzed in peripheral blood erythrocytes of common carp (*Cyprinus carpio* L.) treated with cadmium (Cd) at legally allowed concentration. Young specimens of the common carp were exposed to the lowest of the four permitted concentrations of Cd (0.45 µg/L) in surface waters, according to Directive 2013/39/EU, for 72 h and 144 h in laboratory conditions. Blood smears were stained with acridine orange and tested for the presence of micronuclei and other NAs. We report that Cd exposure for 72 h resulted in a significantly increased number of blebbed and lobed nuclei and eight-shaped nuclei, as well as an increase in the frequency of Total NAs. Cd exposure for 144 h resulted in an increase in the number of cells with notched nuclei and nucleoplasmic bridges, as well as an increased occurrence of binucleated cells. Our results highlight the cytotoxic and genotoxic effects of Cd, even at low permissible levels, and confirm the use of NAs as an effective biomarker. The elevated levels of NAs observed after just 144 h of exposure to Cd highlight the need for further research. Once confirmed, changes in national and EU legislation of permitted concentrations of Cd in surface waters might be required.

Key words: cadmium, cytotoxicity, genotoxicity, nuclear abnormalities, *Cyprinus carpio.*

Introduction

Cadmium is a highly toxic ubiquitous trace metal that is released to the aquatic environment from anthropogenic sources, such as industrial, agricultural and urban effluents, and natural sources, such as rocks and soils. Persistent pollution continues to cause Cd accumulation in the aquatic food web and thereby impacts the health of organisms in aquatic ecosystems (BERVOETS *et al.*, 2009). The International Agency for Research on Cancer (IARC, 1993) has classified Cd and its compounds as carcinogenic to humans and experimental animals. Moreover, Cd has been considered as a priority toxic substance in surface waters according to Directive 2013/39/EU (EC, 2013) of the European Parliament and of the Council.

Fish are very susceptible and vulnerable to heavy metal contamination due to their sensitivity to low concentrations of genotoxic substances in the aquatic environment (ÇAVAŞ & ERGENE-GÖZÜKARA,

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2005). It has been reported that fish can accumulate Cd at levels up to 10 – 1000 times higher than those found in ambient water (WHO, 1989). The increase in temperature of the water basins due to global warming is presumably connected to the increase in toxicity levels of Cd for the hydrobionts. Cd exposure (0.5 mg/L) had a deteriorative effect on the growth and health of Nile tilapia (*Oreochromis niloticus*) and the cadmium's hazardous effect increased as water temperature increased (ABDEL-TAWWAB & WAFEEK, 2014). Cd is a non-essential heavy metal that does not play any known physiological role, but very often induces anemia in fish due to its adverse effect on iron uptake and metabolism and direct damage to erythrocytes (CAMBIER *et al.*, 2010; WITESKA *et al.*, 2011; KONDERA & WITESKA, 2013). Irrespective of the exposure or accumulation levels, Cd is found in all cellular compartments, in both the gill and liver of fish organism (KAMUNDE, 2009). A high positive correlation between Cd levels and metallothioneins (responsible for binding heavy metals in aquatic organisms and reducing their toxicity) was detected in fish organs. The Cd distribution levels were in the following order: liver > kidney > gills > muscles (ABDEL-TAWWAB & WAFEEK, 2014). The toxic effects of Cd are known to be due to its interaction with a myriad of molecules at the cellular level (LUPARELLO *et al.*, 2011).

The common carp (*Cyprinus carpio* L.) is a common fish species which is often reared and used for fish culture. Multiple studies have highlighted its role as an excellent bioindicator for heavy metal contamination, as well as for Cd bioaccumulation. ARKCHIPCHUK & GARANKO (2005) observed that cadmium (0.005 and 1.0 mg/L) ions increased the number of nucleoli in common carp fin cells. JIA *et al.* (2011) demonstrated that the exposure of low concentrations of Cd (0.41, 0.52, 0.69, 1.03 and 2.06 mg/L, respectively) for 7 days induced oxidative stress and DNA damage in the livers of *Cyprinus carpio* var. *color*. The data presented by KONDERA & WITESKA (2013) suggest that Cd exposure in concentrations equal to 100% of 96hLC50 (6.50 mg/dm^3) for 3 h or for 4 weeks to 10% of 96hLC50 (0.65 mg/dm^3) may affect the hematological and immune status of *C. carpio* by disturbing the process of hematopoiesis.

The peripheral blood of fish is commonly used in genotoxic studies due to its high mitotic index. It also provides thousands of scorable erythrocytes with large nuclei (UDROIU, 2006), which are in direct contact with the toxicant during its transportation (RUAS *et al.*, 2008). In addition, the use of peripheral blood erythrocytes avoids the complex procedures involved in cell preparation and animal sacrifice (BOLOGNESI *et al.*, 2006). The effects of short-term and long-term *in vivo* exposure to Cd (3 h exposure at 6.5 mg/l; 4 weeks – at 0.65 mg/l) on the morphology of juvenile carp erythrocytes were evaluated by WITESKA *et al.* (2011). It was observed that all exposures resulted in a significant increase in the frequency of abnormal erythrocytes. Their results confirmed the genotoxic and cytotoxic properties of Cd and demonstrated that fish erythrocytes are a good model for cytotoxicity studies. The comparison between the effects of different *in vitro* toxicity levels of various cadmium salts on these cells in the same experiment showed that cadmium nitrate induced more anomalies in carp erythrocytes than chloride and sulfate (WITESKA *et al.*, 2011).

Since CARRASKO *et al.* (1990) first described morphological alterations in nuclei and suggested their use as indicators of damage in genotoxicity surveys, NAs (lobed, notched, blebbed and eight-shaped nuclei, buds, binucleated cells and binucleated cells with nucleoplasmic bridges), along with micronuclei (MN), have been recommended as reliable biomarkers in fish toxicology (FERRARO *et al.*, 2004; ÇAVAŞ & ERGENE-GÖZÜKARA, 2005; BOLOGNESI *et al.*, 2006; ANBUMANI & MOHANKUMAR, 2011; BOLOGNESI & HAYASHI, 2011). Although the mechanism of their occurrence is still not

fully determined, they are associated with errors in cell division, DNA replication and chromatin condensation, chromosome fragments lacking telomeres and centromeres, apoptosis, and genotoxicity and/or mutagenicity (LINDBERG *et al.*, 2007; FENECH, 2007; FENECH *et al.*, 2011). In recent years, NAs have complemented MN scoring in routine genotoxic procedures and environmental studies, and have therefore been shown to be a sensitive biomarker in the testing of *in vivo* genotoxic effects of heavy metals in fish *in situ* (BARŠIENĖ *et al.*, 2014; FURNUS *et al.*, 2014; REBOK *et al.*, 2017) and *ex situ* (ROCHA *et al.*, 2011; STANKEVIČIŪTĖ *et al.*, 2016; MITKOVSKA *et al.*, 2017). NAs have also been used as effective biomarkers for Cd-induced cytotoxicity and genotoxicity in fish erythrocytes (GÜNER *et al.*, 2011; JINDAL & VERMA, 2015) and in *C. carpio* erythrocytes (ÇAVAŞ *et al.*, 2005; WITESKA *et al.*, 2011). By induction of micronuclei and NAs in blood of Cd-injected juvenile individuals of gilthead seabream (*Spratus aurata*) COSTA & COSTA (2007) suggest the fluorescence microscopy based on acridine orange (AO) staining as a more reliable genotoxicity assessment method for fish erythrocytes compared to traditional bright-field techniques. Due to the specificity of AO staining to DNA and RNA, discrimination of NAs with this approach proved to be less time-consuming and more accurate, since non-nucleic acid artefacts are not stained and there is better contrast to discriminate small structures, like micronuclei and nuclear buds.

Some scientific experiments have examined the genotoxic effect of different concentrations of soluble salts of Cd on fish erythrocytes – 0.001, 0.01 and 0.1 mgL^{-1} $CdCl_2$ (ZHU *et al.*, 2004); 0.005-0.1 mg/L of $CdCl_2$ (ÇAVAŞ *et al.*, 2005); 0.1 ppm and 1 ppm of $CdSO_4.8H_2O$ (GÜNER *et al.*, 2011); 0.65 and 6.5 mg/L of $CdCl_2$ .2 ½ H_2O (WITESKA *et al.*, 2011); 0.37 and 0.67 mg l^{-1} of $CdCl_2$, (JINDAL & VERMA, 2015). However, the possibility of inducing NAs (including MN) from very low, permitted Cd concentrations has not yet been studied. Here, we aimed to assess the genotoxic and cytotoxic potential of the Maximum Allowable Concentration (MAC) of Cd (at the lowest permit level – 0.45 µg/L), considered safe by regulatory agencies, trough the *ex situ* induction of NAs (including MN) in the circulating erythrocytes of exposed specimens of the common carp (*Cyprinus carpio* L.).

Material and Methods

Experimental design

For the purpose of this study, juvenile forms of the same size group (total length 10.6 cm ± 1.5; total weight 20 g ± 0.5) of the common carp (*Cyprinus carpio* L.) were exposed to permitted Cd concentration for 72 h and 144 h in laboratory conditions. According to the Bulgarian legislation (Regulation on environmental quality standards for priority substances and certain other pollutants, 2015) based on Directive 2013/39/EU (EC, 2013) of the European Parliament and of the Council, the MAC of Cd and its compounds in surface waters is based on the water hardness (5 classes). It ranges from 0.45 µg/L (1st and 2nd class) to 1.5 µg/L (5th class). The lowest permitted MAC (0.45 µg/L) was selected in order to test the effect of Cd in the lower permitted levels. The Cd concentration (soluble nitrate salt, $Cd(NO_3)_2.xH_2O$) in our experiment was prepared for 100 L of water (45 µg/100 L) for each tank. Fish were obtained from the Institute of Fisheries and Aquaculture (Plovdiv, Bulgaria). They presented with no external pathological abnormalities. After transportation, the fish were acclimatized for four days in glass aquaria containing well aerated, dechlorinated (by evaporation) water at constant temperature and a photoperiod prior to heavy metal treatment. Fish were not fed prior or during the experiment. The physico-chemical characteristics of the aquarium water, including pH, temperature, oxygen levels and conductivity, were measured once a day according to a standard procedure using a

combined field-meter (WTW, 162 Germany). The experiment was 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 (EC, 2010). After acclimatization, the individuals were divided into two groups in 100 l tanks: exposed group to the MAC of Cd for 72 h (n=10) and 144 h (n=8), and a control group of untreated fish (n=10) was used as a negative control for result comparisons. No fish mortality was recorded during the exposure period. Following the total acute time of exposure (72 h and 144 h), blood probes from individuals were collected using an intracardiac puncture and whole blood smears were prepared.

Slide preparation and scoring criteria for different types of NAs

Whole blood smears were prepared 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 an 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 a uniformly spread monolayer of non-overlapping cells were targeted. As a result of AO metachromasia, the cytoplasm of immatures polychromatic erythrocytes (PCEs) emits red fluorescence, the cytoplasm of mature normochromatic erythrocytes (NCEs) emits green fluorescence, and the nuclei emit yellow-green or yellow fluorescence. Analyses consisted of detecting and counting erythrocytes with NAs (including MN), as distinguished by notched, lobed, or blebbed nuclei according to the criteria of CARASCO *et al.* (1990). Furthermore, an additional two types of NAs were observed: these were denominated as "eight-shaped" and "bud". Binucleated cells and nucleoplasmic bridges were also reported. MN were defined as clearly separated from the main nucleus, no larger than 1/3th of the size of the main nucleus, of round or oval shape, and displaying the same staining (yellow-green) and focusing pattern as the main nucleus. Notched nuclei (NotchN) exhibited a substantial notch into the nucleus; lobed nuclei (LobeN) displayed large evaginations (lobes) of the nuclear membrane; blebbed nuclei (BlebN) exhibited relatively small evaginations of the nuclear membrane which contained euchromatin; nuclear buds (NBud) were defined as nuclei containing euchromatin and having a relatively small evagination (bud) of the nuclear membrane that was partially separated from the nucleus; eight-shaped nuclei (EN) (according to FURNUS *et al.*, 2014) represented a constriction resembling the shape of the number eight; binucleates (BN) were defined as cells with two nuclei of approximately equal sizes; nucleoplasmic bridges (NPB) represented two daughter cells connected by a chromatin bridge.

The average frequency of nuclear abnormalities (NAsF) represented the number of cells with NAs per a minimum of 2000 counted erythrocytes (both PCEs and NCEs), expressed as per mille. This was calculated for each animal tested and for every type of NAs separately. Total NAsF (‰) was expressed as a sum of the frequencies of all abnormality types:

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

For the statistical analysis, the different types of NAs are counted separately for each exposure period and expressed as a frequency per a minimum of 2000 observed erythrocytes, per mille. The overall frequency of NAs (Total NAsF, ‰) for each period of exposure is presented as the sum of all types of abnormalities per 2000 erythrocytes.

Using these indicators, statistically significant differences from the control were reported for each period of exposure ($P < 0.05$).

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 4.0. Data were tested for normal distribution using the D'Agostino & Pearson omnibus normality test. As the data were not normally distributed, significant differences between the control and treated groups were assessed using a nonparametric Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. The results were expressed as a mean ± standard error. The differences were considered significant at $P<0.05$.

Results

The physico-chemical properties of the water showed relatively constant values in the control and experimental tanks. The conditions in the control tank were as follows: pH – 8.1 ± 0.5, conductivity – 435 µS/cm ± 1.5, temperature – 20.5˚C ± 1.5 and oxygen level – 6.8 mg/l ± 0.5; and the conditions in the experimental tank: pH – 7.9 ± 0.3; conductivity – 461 µS/cm ± 3.5, temperature – 20.5 ˚C ± 1.5 and oxygen level – 6.5 mg/l ± 1.5, respectively. Therefore, it is likely that the changes, observed in the fish were not due to physico-chemical properties, but due to the Cd exposure.

Our results show that the exposure of the lowest MAC of Cd (0.45 µg/L) can induce multiple types of NAs in the erythrocytes of the common carp: BlebN, LobeN, EN, NotchN, NPB and BN (Fig. 1). In contrast, the control group exhibited very few types of abnormalities and at a much lower frequency. Control group erythrocytes were packed with hemoglobin and presented with elliptically-shaped nuclei. A statistically significant increase in the frequency of MN ($P = 0.557$) and in NBud ($P = 0.339$) was not registered either at the 72 h or the 144 h exposure time point.

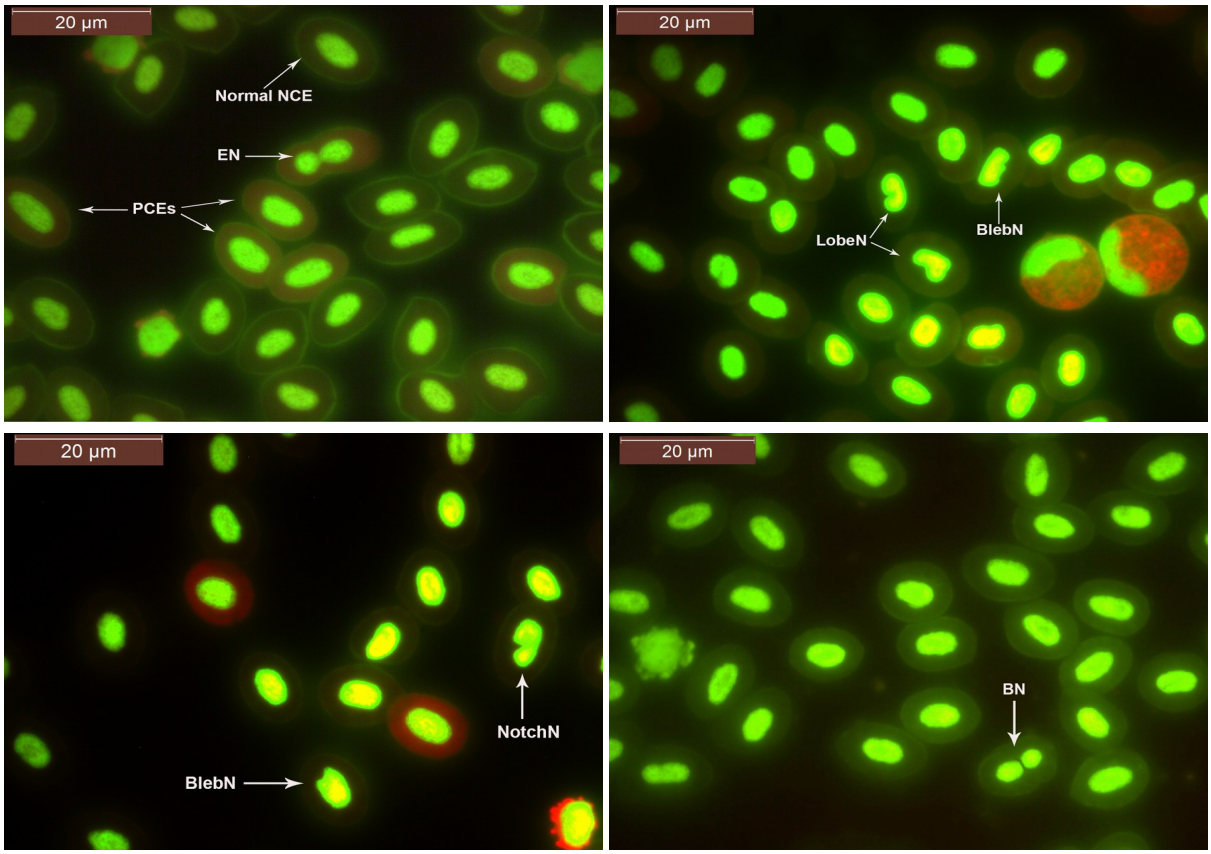

Fig. 1. Microphotographs of blood smears representing common observed NAs in acridine orange stained erythrocytes of *C. carpio* exposed to the lowest Maximum Allowable Cd Concentration of 0.45 µg/L (1000×, *in immersion*).

Statistical analysis of the data (Fig. 2) showed a statistically significant increase in the frequency of certain types of NAs – BlebN, LobeN ($P < 0.0001$) and EN ($P = 0.0003$) – when compared with the control group, as early as 72 h post-exposure to the MAC of Cd. These three types of NAs, as well as NotchN, were most frequently observed at both the 72 h exposure ($BlebN_{72h} = 110.5 \pm 44.34$; $NotchN_{72h} = 23.0 \pm 26.2$; $LobeN_{72h} = 8.80 \pm 7.33$; $EN_{72h} = 4.81 \pm 4.82$), and 144 h exposure ($BlebN_{144h} = 198.6 \pm 41.78$; $NotchN_{144h} = 49.7 \pm 21.8$; $LobeN_{144h} = 10.0 \pm 5.27$; $EN_{144h} = 9.40 \pm 6.16$). The number of NAs observed nearly doubled with the increased exposure, indicating a time-dependent induction of these abnormalities. Multiple comparison with Dune's post-test showed that BlebN, LobeN and EN had statistically significant differences with controls after 72 and 144 hours of exposure, while NotchN differred from the controls only after 144 hours of exposure.

In addition to statistically significant increases in the frequencies of BlebN, LobeN, EN and Total NAs at the 144 h exposure, we also detected a statistically significant increase in the frequency of BN ($P < 0.0001$) and NPB ($P = 0.0039$). The frequencies of these anomalies at 144 hours ($BN_{144h} = 2.07 \pm 1.07$; $NPB_{144h} = 1.68 \pm 1.66$) differed statistically with both the control group (with zero values) and 72 h exposed group ($BN_{72h} = 0.396 \pm 0.856$; $NPB_{72h} = 0.272 \pm 0.860$).

At the 72 h exposure, we detected a statistically significant increase in the frequency of Total NAs ($P < 0.0001$). The average frequency of Total NAs (Total $NAsF_{72h} = 148 \pm 66.2$) nearly doubled at the 144 h exposure (Total $NAsF_{144h} = 272 \pm 41.8$) when compared to the control group ($P < 0.0001$).

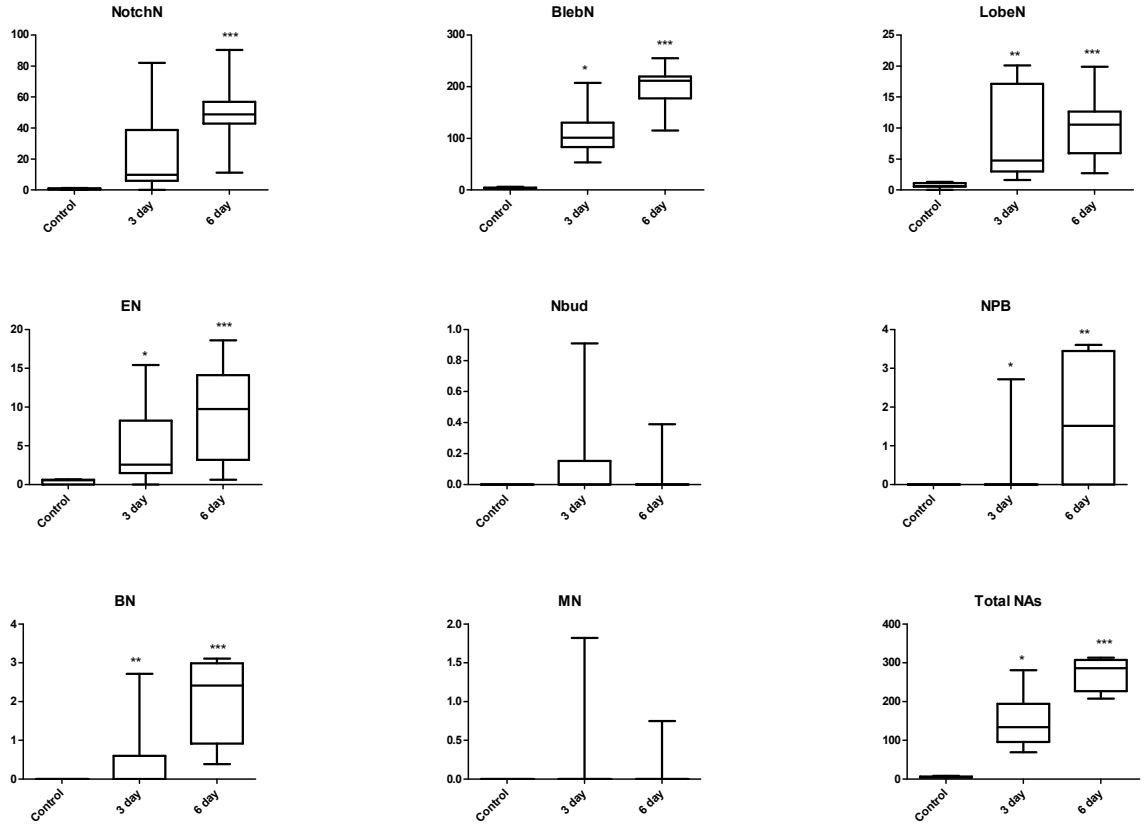

Fig. 2. Frequencies (‰) of different NAs in circulating erythrocytes of *C. carpio* induced *in vivo* by the lowest Maximum Allowable Concentration of Cd (0.45 µg/L) at the beginning of the experiment (0 hour) and after 72 h (3 days) and 144 h (6 days) exposure.
* $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$, compared to negative control.

Discussion

The genotoxic and cytotoxic potential of Cd and its compounds are well-documented. The International Agency for Research on Cancer has classified Cd as a Group I carcinogen (IARC, 2012). Due to the accumulation of Cd in food chains, the risk of environmental exposure has increased, making it essential to develop and improve biomonitoring approaches and tools. By applying biomarkers at different levels of the biological organization, the toxic effects of different concentrations of Cd and its compounds have been tested in aquatic organisms, especially fish (Zhu *et al.*, 2004; Kamunde, 2009; Kondera & Witeska, 2013; Abdel-Tawwab & Wafeek, 2014; Dubey & Tripathi, 2014; Onukwufor *et al.*, 2017). Very few of the conducted experiments have tested the effect of lower permitted levels of Cd. It was observed earlier that the 72 h exposure in the allowable Cd concentration of 0.45 µg/L is accompanied with an increase in the respiration rate index, compared with the control, as well as significant histological changes on gills of juvenile carps, which were grouped as proliferative and degenerative ones (Yancheva *et al.*, 2016). This means that the tested fish are sensitive even at exposure to low concentrations of Cd. However, it is still unclear what levels of Cd are actually safe to living organisms and whether the permitted allowable Cd concentrations in water exhibit no cytotoxic and genotoxic effect on fish erythrocytes. The main aim of this study, therefore, was to assess the cytotoxic and genotoxic potential of the lowest current MAC of Cd considered safe by regulatory agencies.

In recent years, considerable attention has been directed towards the use of NAs and MN in the piscine micronucleus test (Baršienė *et al.*, 2014; Furnus *et al.*, 2014; Stankevičiūtė *et al.*, 2016; Mitkovska *et al.*, 2017; Rebok *et al.*, 2017). Among current cytogenetic techniques, the presence of NAs and MN is considered an indicator of cytotoxicity and genotoxicity, respectively (Çavaş *et al.*, 2005; Grisolia *et al.*, 2009). MN are products of chromosome fragments or whole chromosomes that lag at cell division due to a lack of centromere, a damaged centromere or a defect in cytokinesis (Heddle *et al.*, 1991). The formation of NBuds may reflect the clastogenic action of the agent, as well as the differential capacity of organisms to deal with failed DNA replication, improperly condensed chromatin, or chromosome fragments lacking telomeres and centromeres (Lindberg *et al.*, 2007). It seems likely that MN, NBud and BlebN share a common origin and can therefore all be applied as genotoxicity analogues. It is assumed that the initiation of the breakage-fusion-bridge cycle to separate entangled or attached chromosomes can lead to LobeN, 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). The induction of bi-nucleated cells containing nucleoplasmic bridges is an indicator of dicentric chromosomes (Fenech, 2007). Bi-nucleated erythrocytes are formed during abnormal cell division due to a block in cytokinesis (Çavaş *et al.*, 2005). The formation of 8-shaped erythrocytes, considered to be remnants of the mitotic spindle, is also used as a cytotoxicity marker because it reflects a failure in erythropoiesis (Baršienė *et al.*, 2014).

Here we report a statistically significant increase in the frequency of BlebN, LobeN and EN in the erythrocytes of the common carp after just 72 h of exposure to Cd. Furthermore, we found that of the different types of NAs, the frequency of BlebN is most markedly increased at both 72 h and 144 h of exposure to Cd. Our results are in agreement with those reported by Witeska *et al.* (2011), who show a highest frequency of erythrocytes with nonuniform distribution of chromatin at the nuclear border in carp exposed to various Cd salts. Certain authors suggest that both BlebN and LobeN can give rise to MN (Shimizu *et al.*, 1998; Anbumani

& MOHANKUMAR, 2012). It has been established that the increase in the frequency of occurrence is dose-dependent and can be explained by the cellular mechanism of dealing with excess chromatin, during which the excess genetic material is incorporated into micronuclei such that the cell can dispose of it as a "double minute" (SHIMIZU *et al.*, 1998).

The eight-shape nuclei were described as a separate type of nuclear abnormality in a relatively small amount of studies. This is most likely due to some authors including them in broader terms like "lobed nuclei". They were described by FURNUS *et al.* (2014) examining the baseline micronuclei and nuclear abnormalities frequencies in native fish. In our study, this abnormality was described separately and it stood out with a statistically-significant higher values in the first 72h. Increased frequencies of eight-shaped erythrocytic nuclei were observed also by BARŠIENĖ *et al.* (2014) during their assessment *in situ* of the genotoxic risk to flounder (*Platichthys flesus*), herring (*Clupea harengus*) and cod (*Gadus morhua*) at chemical munitions dumping zones in the southern Baltic Sea. The induction of EN, as well as binucleated cells, was used as a cytotoxicity endpoint by the authors, while the frequency of MN, NBuds and NPB in erythrocytes was used as a genotoxicity endpoint.

We observed a statistically significant increase in the frequency of NPB after 144 h of exposure to the lowest MAC of Cd, thereby highlighting the genotoxic effect of Cd even at such low, allowable concentrations. Nucleoplasmic bridges are formed during anaphase, when the centromeres of dicenteric chromosomes are pulled in opposite directions during mitosis. When the anaphase bridge fails to separate, the nuclear membrane encloses the daughter nucleus as well as the anaphase bridge, thereby leaving the two cells connected via their nuclei (FENECH, 2007; FENECH *et al.*, 2011). Nucleoplasmic bridges are therefore a commonly used biomarker of DNA damage resulting from radiation-induced clastogenic events (CHEONG *et al.*, 2013; MEENAKSHI *et al.*, 2017). By using the fluorescent dye acridine orange in our experiments, we could accurately detect this type of anomaly.

On the other hand, we did not observe a statistically significant increase in the frequencies of either MN or NBud at either exposure time point. MN arise during cell division and can appear at different times following the DNA damage (BOLOGNESI & HAYASHI, 2011). GRISOLIA & CORDEIRO (2000) evaluated the time-dependent response of MN formation during hematopoiesis in the kidney and the micronucleus peak in circulating blood erythrocytes after exposing fish to cytochalasin B. The experiment showed that micronuclei formed in young kidney erythrocyte cells were detected in the peripheral blood 2–4 days later, and that this time lag is species- and clastogen-dependent. In this regard, both 72 h and 144 h of exposure to Cd in our experiment were sufficient to induce MN in carp erythrocytes.

Induction of MN in polychromatic erythrocytes of *C. carpio* by Cd treatment was demonstrated by ZHU *et al.* (2004). It was found that micronuclei frequencies increased with the rise of $CdCl_2$ concentrations. In carp exposed to 0.1 mgL^{-1} Cd^{2+} and 0.01 mgL^{-1} Cd^{2+}, micronuclei erythrocyte frequencies were higher than those in the control group in this study. The MN assay has also been applied *in vivo* by ÇAVAŞ *et al.* (2005) in three different fish species, *Corydoras paleatus*, *Carassius gibelio* and *Cyprinus carpio*, to show the potential of Cd (0.005 – 0.1 mg/L) to cause genotoxicity. It has been observed that fish species and their tissues showed differential sensitivity to the heavy metal treatment; gill and liver cells showed higher frequencies of micronuclei and binuclei than erythrocytes. On the other hand, *C. carpio* was the most resistant of the three fish species exposed to different doses of cadmium. The time-dependent response was observed in all three studied fish species with all clastogens tested, and must therefore be determined before extensive testing of a given species.

The treatment with four sub-lethal concentrations of cadmium chloride i.e., 1.0, 2.0, 4.0 and 8.0 mg/l for 24, 48, 72 and 96 hrs of exposure induced higher MN frequencies in gills, red blood cells and kidney cells of fresh water fish, *Channa punctatus* (DUBEY & TRIPATHI, 2014). However, it has also been observed that MN frequencies increase in tissues in a concentration- and time-dependent manner in *Labeo rohita* specimens acutely exposed to relatively low and environmentally realistic concentrations (0.37 and 0.67 mg l^{-1}) of $CdCl_2$ (JINDAL & VERMA, 2015). The concentrations used in the afore mentioned studies more often use Cd concentrations of tens and hundreds times higher than the MAC applied in our study. The low levels of Cd exposure (0.45 µg/L) could explain the lack of MN and NBud in our experiment and also indicates that the lowest MAC of Cd does not induce these types of anomalies, but induces other abnormalities (BlebN, LobeN, EN, NPB, BN). In support of our findings, GÜNER *et al.* (2011) also established that at Cd exposure (0.1 ppm and 1 ppm) of *Gambusia affinis* for 1 and 2 weeks, NAs were significantly induced compared to control groups, even though Cd did not significantly increase micronuclei frequency.

In mammals, the observed correlation between MN, NPB and NBud suggest that these biomarkers of genomic instability may share a common mechanism leading to the formation of dicentric chromosomes (FENECH *et al.*, 2011; MEENAKSHI *et al.*, 2017). Such a correlation could explain the lack of a statistically significant increase in NBud and MN in our experiment. BOLOGNESI *et al.* (2006) also reported the existence of a strong correlation between the induction of MN and the induction of NBud in fish erythrocytes, which is in accordance with our results. NBuds are characterized by the same morphology as MN, with the exception that they are connected to the nucleus by a stalk of nucleoplasmic material depending on the stage of the budding process. While it is thought that NBud are precursors to MN, we previously reported that the statistically significant increase in the frequency of NBud formation in carp erythrocytes following 72 h of exposure to allowable concentrations of Pb and Ni was not accompanied by a similar increase in the formation of MN (MITKOVSKA *et al.*, 2017). On the other hand, such a correlation between MN, NPB and NBud in mammals could not explain the increased frequencies of NPB that we observe after 144 h of exposure to Cd; these, however, could be associated with the observed increase in the frequency of BN. Cd-mediated increases in the frequency of BN in carp erythrocytes, gill and liver cells have also been reported by ÇAVAŞ *et al.* (2005). Cadmium ions also demonstrated a genotoxic capacity with a tendency of formation of erythrocytes with double nuclei in crucian carp (*Carassius auratus gibelio*) (ARKCHIPCHUK & GARANKO, 2005). Binucleation is an indicator of abnormal cell division because of a block in cytokinesis. This abnormal cell division could result in a genetic imbalance in the cells, which may also be involved in carcinogenesis (RODILLA, 1993).

The statistically significant increases in the total values of NAsF that we observed at both the 72 h and 144 h time point indicate that even the lowest MAC of Cd (0.45 µg/L), as specified by current law, can induce cytotoxic and genotoxic effects in the erythrocytes of the common carp. YANCHEVA *et al.* (2016) also observed that the 72 h exposure in the same permitted Cd concentration affects the gill structure and respiration rate of *C. carpio*. Therefore, it turns out that the juvenile carps prove to be sensitive even at such a low Cd concentration as is the lowest MAC of Cd.

Conclusion

Here we show that while the MAC of Cd does not induce the formation of MN or NBud, a number of structural abnormalities were observed at the nuclear level in *C. carpio* erythrocytes when exposed to the lowest of the four MAC of Cd (0.45 µg/L). The induction of BlebN, LobeN, EN and

Total NAs that we observed was time-dependent. Even short-term exposure at the low MAC of Cd affects the nuclei of erythrocytes. Our results demonstrate the cytotoxic and genotoxic effects of Cd, even at low permissible levels, and confirm the use of NAs as an effective biomarker. The significant increases in NAs, such as nucleoplasmic bridges and binucleated cells, following 144 h of acute exposure to Cd indicate the need for further research into the MAC of Cd. Changes in national and EU legislation regarding the MAC of Cd in surface waters might be required.

Acknowledgments

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