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RESEARCH ARTICLE

Early genotoxic response and accumulation induced by waterborne copper, lead, and arsenic in European seabass, *Dicentrarchus labrax*

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Abstract Cu, Pb, and As, which are among the most abundant metals in the aquatic environment, are also among the most health-threatened by causing diverse cellular injuries. The aim of this study was to assess and compare the potential early induction of genotoxic effects after waterborne Cu, Pb, and As exposure in European seabass, *Dicentrarchus labrax*, a commercial widely cultured fish, using the micronucleus (MN) assay in peripheral blood erythrocytes. Fish were exposed under laboratory conditions to nominal solutions ranging 0–10 mg/L for 24 and 96 h. Furthermore, actual metal ion concentrations were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or differential pulse anodic stripping voltammetry (DPASV) in water and four fish tissues differentially related to environmental exposition and metal accumulation, i.e. the gills, liver, muscle, and brain. Dose-dependent increases of micronuclei (MNi) frequency were observed after these very short exposures; based on measured metal concentrations in water, the genotoxic effect ordered as Cu>As>Pb. Significant genotoxic effect at 0.009 mg/L Cu, 0.57 mg/L Pb, and 0.01 mg/L As was seen. For Cu and Pb these are only slightly higher, but for As it is notably lower than the USEPA criteria of maximum concentration to prevent acute toxicity in aquatic organisms. Furthermore, genotoxicity was differentially related to metal accumulation. MNi frequency correlated positively with the content of Pb in all the organs, with the content of As in liver and gills and only with the content of Cu in the brain. In conclusion, our findings raised environmental concerns because these depicted a genotoxic potential of Cu, Pb, and As after a very short exposure to low but environmentally relevant concentrations, too close to regulatory thresholds. In addition, the MN test in *D. labrax* could be considered an early biomarker of genotoxicity induced by these metals in fish.

Keywords Arsenic · Copper · Fish · Genotoxicity · Micronuclei · Lead

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Introduction

Among the wide panoply of detrimental effects caused by contaminants of aquatic environment, genetic alterations are the most dangerous as their effects may exert a damage beyond that of individual and may be active through several generations (Russo et al. 2004; Bolognesi and Hayashi 2011). Genetic damage can result in subsequent biological changes such as enzyme dysfunction, cytotoxicity, immunotoxicity, metabolic and reproductive disorders, growth inhibition, or carcinogenesis (Ohe et al. 2004; Bolognesi and Hayashi 2011; Baršienė et al. 2013). Their evaluation presents new challenges for the regulator (Pratt and Barron 2003). Thus, the use of histological, biochemical, cytogenetic, and other biomarkers is increasingly required and applied in environmental monitoring and protection. Vertebrate are suitable models to estimate possible risks in the aquatic environment due to their ability to efficiently metabolize, concentrate, and store waterborne pollutants (Yadav

and Trivedi 2009; Cavas 2011). Above all, fish are excellent specimens for the study of the mutagenic or carcinogenic potential because they are particularly sensitive to pollutants that interact with DNA (Schnurstein and Braunbeck 2001). Hence, a broad range of both in vitro and in vivo DNA damage biomarkers in fish are being widely used to implement environmental directives from regulatory agencies (USEPA 2009; EC 2008/56/EC).

The micronucleus (MN) test and the comet assay have been proved to be greatly useful in genotoxicity measurement of toxicants (Russo et al. 2004; Ahmed et al. 2011a; Barbosa et al. 2010; Monteiro et al. 2011; Cavas 2011). Particularly, the MN test is considered as the most suitable technique in assessing the genotoxic effects of a wide range of compounds in fish (Al-Sabti and Metcalfe 1995; Sanchéz-Galán et al. 1999; Rodriguez-Cea et al. 2003; Bolognesi et al. 2006; Udroiu 2006; Koca et al. 2008; Ahmed et al. 2011a, b; Bolognesi and Hayashi 2011; Carrola et al. 2014). Micronuclei (MNi) are small masses of cytoplasmic chromatin separated from the main cell nucleus, thus are an index of chromosomal breaking or mitotic spindle dysfunctions (Schmid 1975). Thus, the evaluation of MNi allows addressing both clastogenic and aneugenic potentials of environmental agents. The MN test can be carried out in any proliferating active tissue. The blood, gill, liver, kidney, and fin cells have been mainly used (Al-Sabti and Metcalfe 1995; Cavas et al. 2005; Udroiu 2006; Cavaş 2008; Ahmed et al. 2011b). However, peripheral erythrocytes are the most commonly employed cells in the piscine MN test because it avoids the complex cellular dissociation required in other tissues, which may lead to mechanical stress and cell damage, as well as the killing of the animals (Bolognesi et al. 2006; Cavaş 2008).

Metals are naturally occurring elements in the biosphere, where they are ubiquitously distributed as part of the natural background of chemicals. However, in the aquatic environment they are contaminants of great concern as their concentrations may largely increase as a result of mobilization derived from multiple anthropogenic activities. Due to their properties of non-biodegradability, bioaccumulation, and biomagnification, they have attracted significant ecological and public health attention. Importantly, heavy metals are known to cause DNA damage and cancer (Barbosa et al. 2010).

Copper (Cu) is a transition metal that serves as an essential cofactor for many key components of metabolism, including several oxidative-stress-related enzymes (Tchounwou et al. 2012). However, at high concentrations it becomes highly toxic, particularly to aquatic organisms. In fact, Cu is a priority pollutant for environmental agencies (USEPA 2009). High levels of Cu have been reported in water and sediments, especially from harbors and marinas due to its use as therapeutant and antifoulants. Besides, mining activities near aquatic ecosystems usually increase the pollution by Cu (Vicente-Martorell et al. 2009). Toxicity mechanisms associated with waterborne copper exposition have been linked to the occurrence of oxidative stress and genotoxic effects (Machado et al. 2013).

Lead (Pb) is perhaps the most used and best recognized toxic environmental chemical (Johnson 1998). Pb largely enters the environment through anthropogenic activities (Monteiro et al. 2011). Its persistence in ecosystems and its adverse effects on living organisms are still a major concern (Cavaş 2008) so that it is a priority pollutant for most regulatory agencies (USEPA 2009). Lead has been shown to exert a wide range of biological effects, from enzyme dysfunction to histopathological alterations (Tchounwou et al. 2012; Oliva et al. 2013). Furthermore, lead compounds are known genotoxicants by both aneugenic and clastogenic mechanisms (Bonacker et al. 2005) involving direct interaction with DNA (Woźniak and Blasiak 2003) or the inhibition of DNA repair (Hartwig 1994).

Arsenic (As) is also a priority pollutant, which is released in the aquatic environment from natural and anthropogenic sources (USEPA 2009). The contamination of water resources by As, mainly in the eastern hemisphere, is one of the most relevant environmental concern at the global scale (Frisbie et al. 2002; Ahmed et al. 2011a). Mining and industrial waste largely facilitate the entering of arsenic compounds into the environmental compartments and the human exposition (Yadav and Trivedi 2009). Both organic and inorganic compounds are present in the environment, the last being more toxic (Frisbie et al. 2002; Ahmed et al. 2011a). Arsenic is a well-documented genotoxic compound and is stated as a human carcinogen (Koedrith et al. 2013).

Since these three metals accumulate in different tissues of aquatic organisms and can enter the food chain, the assessment of early biological effects of Cu, Pb, and As excess is an urgent need from the environmental, nutritional, and toxicological points of view. While the evaluation of non-genetic targeted toxicity of these metals has previously received much attention, there is a paucity of information on the genotoxic effects. This study is part of a research project that focused on the evaluation of diverse cytotoxic effects induced by these priority heavy metals on fish species that may be of special interest for their economic and ecotoxicological importance. Hence, our main aims are (1) to assess and compare the potential early induction of genotoxic effects after waterborne Cu, Pb, and As exposure using the micronucleus assay in peripheral blood erythrocytes and (2) to elucidate the use of this procedure in the European seabass (*Dicentrarchus labrax* (Linnaeus, 1758)), one of the most important commercial fish widely cultured in the Mediterranean, as an easy and reliable early biomarker of genotoxicity.

Materials and methods

Fish and experimental design

Specimens of *D. labrax* (Linnaeus, 1758) (weight range 33.50–65.82 g and length range 15.5–18.0 cm) were obtained from the aquaculture facilities of the Faculty of Marine and Environmental Sciences (University of Cadiz, Spain). The fish were disease-free and did not have any history of previous chemical exposure. Before the experiments, fish were acclimated in large aquaria containing marine water supplied by seawater well under laboratory conditions with oxygen-saturated water at 25 °C and 12/12 dark/light cycle for 1 week. No mortality was observed during the acclimatization period.

Then, they were randomly grouped into five duplicated groups (n=12/group) in 30 L tanks and exposed under laboratory conditions to 0 (control), 0.01, 0.1, 1, and 10 mg/L nominal concentrations of heavy metal solutions prepared by dissolving $Pb(CH_3COOH)_2$, As_2O_3, and Cu powder (as $CuSO_4$) (purchased from Sigma-Aldrich, USA) for 24 and 96 h. Test concentrations were selected based on previous literature data on the potential genotoxic effect of these metals, on reported metal toxicity bioassays, and on environmental concentrations at polluted sites. Fish were not fed during the experimental period.

All experimental procedures were performed in compliance with the Directive 2010/63/EU on the protection of animals used for scientific purposes.

Physicochemical parameters of water

Temperature, pH, dissolved oxygen (DO), conductivity, total dissolved solids (TDS), and salinity of water from tanks were measured every day in situ with an electrochemical portable device (HI 9828, Hanna Instruments, Spain). An amount of 50 mL of water sample was taken from each tank to evaluate the dissolved organic carbon (DOC) using TOC analyzer (Analytic Jena 3100, Germany) and 100 mL to test the spiked metal concentration. The mean values of these physicochemical parameters in water were as follows: temperature 19.5± 0.9 °C, pH 8.39±0.27, dissolved oxygen 7.34±0.41 mg/L, conductivity 52.1±1.3 mS/cm, total dissolved solids 28.9± 0.9 g/L, salinity 38.8±0.4 g/L, and DOC 6.09±1.65 mg/L. The physicochemical properties of the test water did not vary significantly among treatments throughout the experimental procedure.

Heavy metal analysis in water

Metal concentrations were analyzed in water samples by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Iris Intrepid spectrometer, Thermo Elemental, UK) or differential pulse anodic stripping voltammetry (DPASV) using a Metrohm 757 VA Computrace Stand controlled by PC software (VA Computrace 2, Metrohm, Switzerland) depending on the spiked metal concentration. Analytical methods were checked using LGC 6016 (estuarine water) and BCR 610 (groundwater) certified reference water samples and by analyzing three replicates for each sample, obtained good recoveries (≥90 %); blanks and detection limits were also evaluated for all metals (Table 1: a and b).

Heavy metal analysis in fish tissues

Fish tissue subsamples were taken from the liver, gills, muscle, and brain, making two pools of three specimens from each tank. Freeze-dried tissue samples (0.1–0.3 g) were acid-digested by microwave heating using 7 mL of 65 % HNO_3 (Suprapur grade) for the muscle and gills, and 4 mL of 65 % HNO_3 (Suprapur grade) and 2 mL of 30 % H_2O_2 (Suprapur grade) for the liver and brain when As and Cu were analyzed. For Pb analysis, all tissues were digested with 4 mL of 65 % HNO_3 (Suprapur grade) and 2 mL 30 % H_2O_2 (Suprapur grade). After digestion, the samples were diluted with Milli-Q deionized water and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (X7 Series plasma scan sequential ICP-MS, Thermo Elemental, UK). Analytical parameters were evaluated, and the accuracy of the methodology applied was satisfactorily evaluated using two certified reference biological materials of the National Research Council Canada (NRCC) DOLT-3 (dogfish liver) and DORM-2 (dogfish muscle) (Table 1 (c) and (d)).

Micronucleus assay

The frequencies of micronuclei (MNi) were evaluated in peripheral blood erythrocytes. Slide preparation and scoring criteria for MNi were performed as previously described (Cavas et al. 2005). Briefly, peripheral blood samples were withdrawn by caudal puncture and smeared on clean microscope slides. After fixation in pure ethanol for 20 min, the slides were air-dried and then stained using 5 % Giemsa solution for 30 min. Only the cells clearly isolated from the surrounding cells were scored. The criteria for the identification of micronuclei were as follows: (a) MNi must be smaller than one third of the main nuclei, (b) MNi must be clearly separated from the main nuclei, and (c) MNi must be on the same plane of focus and have the same color. To prevent bias, the slides were coded and scored blind independently by two researchers.

The frequency of micronuclei was evaluated by scoring the slides under oil immersion at ×1000 magnification using Nikon Eclipse 400 microscope (Tokyo, Japan) and expressed as number of MNi per 1000 erythrocytes. The blind scoring of micronuclei was performed on randomized and coded slides to avoid any technical variation.

Table 1 Analytical quality control data for metal analysis in water and fish tissues (n=10 for LD; n=3 for analysis of reference material)

Reference material	Metal	LD (μg/L)	Blank concentration (μg/L)	Found concentration (μg/L)	Certified concentration (μg/L)
(a) Metal analysis in water by DPASV					
LGC 6016	Cu	0.069	1.944	189.3±3.2	190.0±2.0
	Pb	0.153	0.843	196.2±4.0	196.0±1.5
BCR 610	As	0.098	<LD	9.6±0.9	10.8±0.4
(b) Metal analysis in water by ICP-AES					
LGC 6016	Cu	6.3	<LD	189.3±3.8	190.0±2.0
	Pb	69.3	<LD	199.8±31.1	196.0±1.5
(c) Metal analysis in muscle by ICP-MS					
DORM-2 (dogfish muscle)	Cu[a]	0.049	0.142	2.23±0.10	2.34±0.16
	Pb[b]	0.075	0.202	0.062±0.016	0.065±0.007
	As[a]	0.645	<LD	16.2±0.4	18.0±1.1
(d) Metal analysis in liver by ICP-MS					
DOLT-3 (dogfish liver)	Cu[b]	0.004	0.023	31.7±0.6	31.2±1.0
	Pb[b]	0.025	0.266	0.40±0.09	0.32±0.05
	As[b]	0.293	<LD	10.6±0.1	10.2±0.5

LD limit of detection

[a] Sample digested with 7 mL 65 % HNO_3

[b] Sample digested with 4 mL 65 % HNO_3 and 2 mL 30 % H_2O_2

Statistical analysis

The experiment was performed with 12 fish per treatment tank and in duplicate (n=24 for each metal concentration). Data from micronuclei were assessed by one-way ANOVA followed by the Duncan's post hoc test. To analyze the correlation between metal concentrations and MNi frequencies, Pearson correlation test was employed. The data are expressed as means±SD (standard deviation). All statistical analyses were conducted at an alpha level of 0.05.

Results

Heavy metal concentrations in water and fish tissues

To monitor the nominal metal concentrations in the water of tanks during the experiments, the actual metal concentrations were measured by ICP-AES or DPASV at initial time, 24 and 96 h (Table 2). Furthermore, in order to correlate MNi frequency with metal accumulation in fish, the level of metals was measured in four different toxicologically relevant fish tissues: liver, gills, muscle, and brain (Table 2).

As compared to the nominal concentration for each dose, the actual levels of Cu in water showed a trend toward a decrease in the upper Cu doses (Table 2). Average concentrations of Cu in the fish tissues of *D. labrax* for each test are also shown in Table 2. The liver exhibited the highest metal concentration and they were arranged as follows: liver>gills>brain>muscle. Nonetheless, these results could be altered for the gills and brain by the mortality observed at the higher Cu doses.

The concentrations of Pb in water and fish tissues for each experimental dose are depicted in Table 2. As compared to the nominal concentration for each treatment, the actual levels of Pb in water showed a trend toward a decrease, mainly at higher doses. A marked dose and time-dependent accumulation of Pb was observed in all studied tissues, arranged as gills>brain>liver>muscle.

As shown in Table 2, the actual As concentrations in water were rather similar to the nominal concentrations, being slightly lower at the higher exposure concentration. In regard to the fish tissues, the absolute concentrations of As ranged as liver=gills>muscle>brain.

Induction of MNi

As shown in Fig. 1, a trend toward a metal concentration-dependent increase in MNi frequencies was recorded. MNi frequencies induced by the treatment with copper are depicted in Fig. 1a. In Cu-treated fish a full mortality was recorded for the nominal dose of 10 mg/L after 24 and 96 h and for that of 1 mg/L after 96 h. After the 24 h period, only the dose of 1 mg/L induced a significant increase in the MNi frequency; a trend to higher MNi frequencies was also observed with lower copper concentrations, but they did not reach statistical significance. After the 96 h copper exposure, the two non-lethal doses enhanced significantly the MNi frequencies. The maximal 24-h non-lethal dose of 1 mg/L caused a 3.6-fold increase

Table 2 Metal concentrations in water (mg/L)[a] and fish tissues (mg/kg)[b] from the exposure tests

Test (mg/L)	Cu at 0 h	Cu at 24 h					Cu at 96 h				
	Water	Water	Liver	Gills	Muscle	Brain	Water	Liver	Gills	Muscle	Brain
Control	0.0018±0.0002	0.0071±0.0001	83.83±24.12	3.27±0.71	0.99±0.43	3.98±0.2	0.0061±0.0003	100.30±51.28	3.69±0.39	0.76±0.13	4.13±1.47
0.01	0.009±0.001	0.0102±0.0004	59.44±11.18	3.85±1.26	1.01±0.64	3.80±0.10	0.0088±0.0003	85.31±16.37	3.76±0.34	0.74±0.01	4.14±0.42
0.1	0.098±0.006	0.089±0.005	81.99±35.09	4.35±0.64	0.76±0.24	4.15±0.17	0.090±0.002	83.58±25.70	5.71±2.96	0.84±0.23	4.21±0.42
1	0.957±0.015	0.999±0.007	95.23±39.57	11.39±1.28	0.92±0.19	4.13±0.15	0.557±0.008	†	†	†	†
10	10.46±0.18	3.55±0.023	†	†	†	†	3.972±0.067	†	†	†	†
Test (mg/L)	Pb at 0 h	Pb at 24 h					Pb at 96 h				
	Water	Water	Liver	Gills	Muscle	Brain	Water	Liver	Gills	Muscle	Brain
Control	0.0028±0.0003	0.0102±0.0008	0.013±0.006	0.925±0.171	0.052±0.016	0.068±0.030	0.0168±0.0005	0.048±0.046	1.163±1.565	0.056±0.050	0.120±0.000
0.01	0.0088±0.0005	0.0111±0.0003	0.010±0.003	2.909±1.795	0.030±0.010	0.014±0.000	0.0110±0.0005	0.158±0.073	5.918±0.363	0.047±0.032	0.079±0.024
0.1	0.054±0.001	0.047±0.001	0.085±0.040	2.803±1.712	0.043±0.025	0.031±0.006	0.051±0.001	0.395±0.093	14.589±8.555	0.060±0.038	0.616±0.075
1	0.588±0.061	0.576±0.046	0.540±0.117	9.611±5.554	0.080±0.036	0.519±0.050	0.565±0.036	1.426±0.397	24.514±12.202	0.200±0.077	2.409±0.506
10	4.82±0.13	3.547±0.053	1.588±0.957	12.706±3.811	0.290±0.123	2.070±0.010	2.089±0.065	2.981±0.532	21.663±4.023	0.312±0.157	5.218±0.031
Test (mg/L)	As at 0 h	As at 24 h					As at 96 h				
	Water	Water	Liver	Gills	Muscle	Brain	Water	Liver	Gills	Muscle	Brain
Control	0.0047±0.0003	0.0038±0.0003	2.19±0.05	2.00±1.34	2.20±0.04	1.33±0.01	<LD[c]	2.25±0.47	1.60±0.23	1.85±0.12	1.50±0.05
0.01	0.012±0.001	0.0106±0.0005	2.09±0.84	1.58±0.04	2.14±0.26	1.23±0.07	0.0097±0.0002	1.92±0.26	1.96±0.52	1.76±0.07	1.29±0.09
0.1	0.079±0.007	0.079±0.001	2.14±0.20	2.07±1.82	2.00±0.00	1.21±0.31	0.074±0.001	1.90±0.10	1.67±0.12	1.83±0.22	1.04±0.15
1	0.822±0.037	0.807±0.017	3.13±0.44	2.55±0.85	2.19±1.07	1.17±0.02	0.742±0.007	2.16±0.33	2.03±0.44	1.77±0.14	1.10±0.26
10	7.065±0.091	6.628±0.082	7.87±2.77	7.08±2.64	4.12±0.26	1.44±0.08	5.765±0.068	3.92±1.10	3.05±0.54	2.46±0.68	1.47±0.17

LD limit of detection

† Full mortality was recorded

[a] Metal mean concentration in water from two replicate tanks

[b] Metal mean concentration in fish tissues from two replicate tanks and obtaining two pools of each tank, being pools of $n=3$ for liver, gills, and muscle and $n=5$ for brain

[c] Measured by DPASV

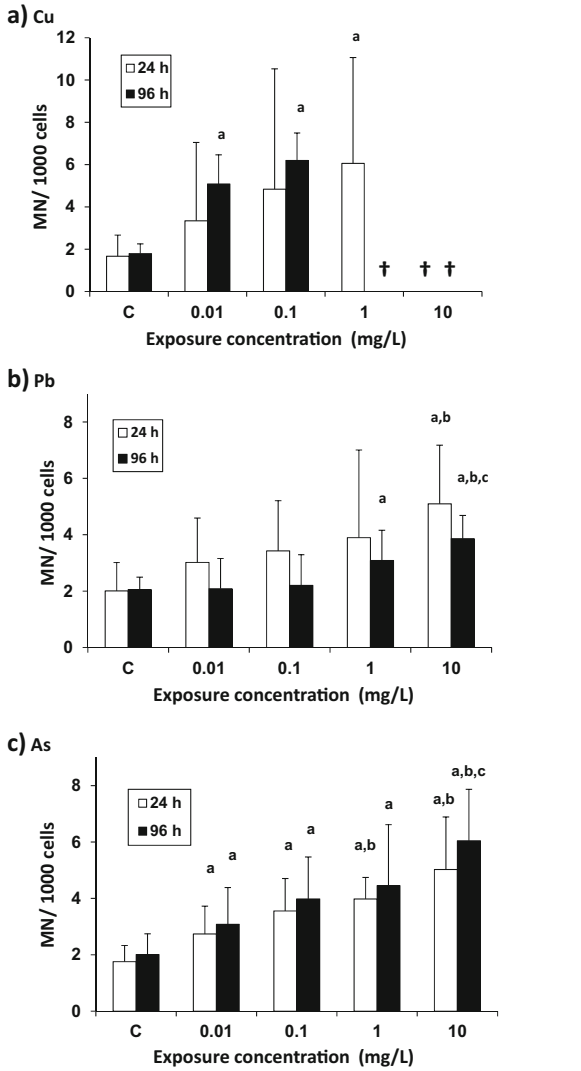

Fig. 1 Frequency of micronuclei induced by Cu, Pb, and As in peripheral erythrocytes of fish *D. labrax* after 24 and 96 h exposure. **a** Copper (full mortality was recorded for 10 mg/L Cu after 24 and 96 h and for 1 mg/L Cu 1 after 96 h (denoted as *dagger*); **b** lead; **c** arsenic. Data are shown as mean±SD. a $p<0.05$ vs. control; b $p<0.05$ vs. 0.01 mg/L; c $p<0.05$ vs. 0.1 mg/L

of MNi frequency as compared to controls, whereas the maximal 96-h non-lethal dose of 0.1 mg/L caused a 3.5-fold increase. No difference was observed between the MNi frequencies at 24 and 96 h exposure for each dose. Finally, correlation between measured Cu concentrations in water and MNi frequency was not significant for the 24-h exposure but it was significant for the 96-h exposure (Table 3). In addition, no correlation was observed between metal content in the tissues and MNi frequencies measured for the non-lethal doses, with the exception of a positive correlation with the brain Cu level at 96 h (Table 3).

In regard to the experiments on exposure to Pb, no death was induced; and a clear trend toward increasing frequencies of MNi in relation to the lead dose was observed for both exposure periods (Fig. 1b). After 24 h, the enhanced MNi frequency was only significant for the 10 mg/L dose, whereas after 96 h MNi frequencies were significantly higher in fish exposed to 1 and 10 mg/L. Maximal dose of Pb produced 2.5- and 1.9-fold increases in MNi frequency after 24 and 96 h exposition, respectively. By comparing the same dose, no significant difference was observed in the MNi frequencies between the 24 and 96 h. A significant correlation was established between the actual Pb concentrations in water and MNi frequencies for the 24- and 96-h exposure periods (Table 3). Furthermore, MNi frequency correlated positively with Pb content in the liver, gills, muscle, and brain at 24 and 96 h (Table 3).

Exposure to increasing concentrations of arsenic resulted in a dose-dependent increase in MNi frequencies after both 24 and 96 h (Fig. 1c), which was evidenced by the lower As dose (0.01 mg/L). The maximal dose of 10 mg/L induced 2.9- and 3.2-fold increases in MNi frequency after 24 and 96 h, respectively. However, for each dose no differences were detected between these two experimental periods. In As-exposed fish, MNi frequencies correlated positively with the measured As concentrations in water for the 24- and 96-h exposure periods (Table 3), as well as with the As levels in the liver and gills at both 24 and 96 h (Table 3).

To compare the potential of the three heavy metals to induce the occurrence of MNi, the mean MNi frequencies were plotted against the actual metal concentrations in water

Table 3 Significant correlation coefficients ($p<0.05$) and p value between frequency of MN and the measured metal concentration in water and tissues

Metal	Test (h)	Correlation coefficient (p value) [a]				
		Water	Liver	Gills	Muscle	Brain
Cu	24	ns	ns	ns	ns	ns
	96	0.5186 (0.0329)	ns	ns	ns	0.6680 (0.0033)
Pb	24	0.5029 (0.0046)	0.5396 (0.0021)	0.5179 (0.0034)	0.5722 (0.0010)	0.5896 (0.0024)
	96	0.5896 (0.0024)	0.6491 (0.0001)	0.4269 (0.0186)	0.6993 (0.0000)	0.6923 (0.0002)
As	24	0.5737 (0.0009)	0.6114 (0.0003)	0.3629 (0.0487)	ns	ns
	96	0.5143 (0.0101)	0.5724 (0.0010)	0.5539 (0.0015)	ns	ns

ns not significant

(Fig. 2). Though at lower concentrations all metals showed similar capacity to induce MNi after the 24-h exposure, at higher concentrations Cu appeared to induce higher levels of MNi than Pb and As, which showed similar potentials (Fig. 2a). After the 96-h exposure, Cu continued as being a more potent inducer of MNi than the other two metals (at least in the range of sublethal concentrations), but As was over the capacity of Pb throughout all the range of metal concentrations evaluated (Fig. 2b).

Discussion

In the present study the assessment of early genotoxic effects of three priority metals of public health as Cu, Pb, and As was carried out by determining the capacity to induce the formation of MNi in European seabass (*D. labrax L.*, 1758). The results showed that all these three waterborne heavy metals had the potential to accumulate in different fish tissues and to enhance dramatically the MNi frequency in blood erythrocytes after 24 and 96 h exposure.

D. labrax, an autochthon euryhaline fish inhabiting in the European Atlantic and Mediterranean area, was selected for the present study because it has been previously used as a sentinel species in monitoring studies and ecotoxicological assays (Giari et al. 2006). Additionally, it is one of the most important commercial fish cultured in the Atlantic to Mediterranean region, with the subsequent high human consumption (Fernandes et al. 2009). In our study, exposure to metals was carried out through the tank water because it better mimics the natural exposure of metals usually dissolved or suspended in the water (Rodriguez-Cea et al. 2003). Since we were interested at elucidating an early response biomarker of genotoxicity and long-term exposures may result in adaptive responses in fish (Cavas et al. 2005; Ahmed et al. 2011a), an experimental period of 24 and 96 h was selected. Taking into account these short-term periods and to avoid any alteration in the experimental conditions that would result in distress of fish, a static model was preferred. In addition, a wide range of metal concentrations was evaluated. A possible inconvenience of this model is that the metal concentration may vary along the time. Thus, the actual metal concentrations at the study points were checked. Certainly, while the measured As levels remained rather similar to the nominal ones, the measured levels of Cu and Pb in water showed a trend toward a small decrease, mainly at the higher doses and the longer exposures (though very far of an order of magnitude).

Exposure of fish to Cu caused a full mortality in the groups treated with a nominal dose of 10 mg/L after 24 and 96 h and of 1 mg/L after 96 h. This demonstrates the high toxicity potential of Cu for *D. labrax* even at the short term; as previously observed in other fish species. *Saba senegalensis* exposed to 1 mg/L copper showed a corrected mortality of 26.6 % at 24 h and 100 % at 96 h exposure (Oliva et al. 2009; Torres et al. 1987), while in *Scyliorhinus canicula* 4 mg/L of copper produced 50 % mortality at 48 h exposure. On the other hand, we observed that exposure to the sublethal concentrations of Cu resulted in a dramatic elevation of the MNi frequency. The potential of Cu to induce MNi in fish erythrocytes had been previously addressed but the results have not been actually consistent. Zhu et al. (2004) could not find any significant effect of Cu ranging from 0.01 to 1 mg/L after 48 or 96 h exposure in carp (*Cyprinus carpio*); by contrast, the same concentrations of Cr and Cd dose-dependently enhanced MNi frequency. Similarly, Sanchéz-Galán et al. (1999) found that in brown trout (*Salmo trutta*) intraperitoneal injections (1.7 mg/kg body weight) of cadmium and mercury induced MNi in renal erythrocytes after 24 h treatment, but copper failed to do it; furthermore, European minnow (*Phoxinus phoxinus*) was not sensitive to copper and

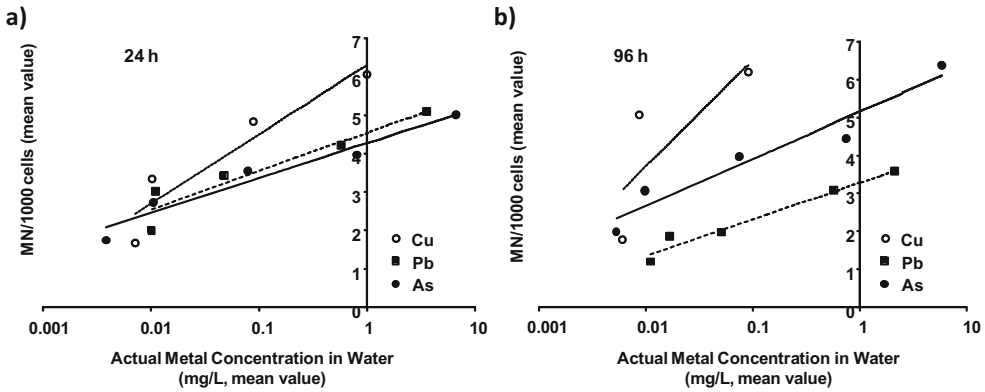

Fig. 2 Regression analysis of mean data for actual metal concentration in water and frequency of micronuclei in fish exposed to Cu, Pb, and As. The best fitting was achieved by logarithmic regression. **a** After 24 h exposure [the best-fit logarithmic equations for these data were $y=6.306+0.781 \ln x$ ($r^2=0.887$) for Cu, $y=4.540+0.433 \ln x$ ($r^2=0.903$) for Pb, and $y=4.272+0.384 \ln x$ ($r^2=0.951$) for As]. **b** After 96 h exposure [the best-fit logarithmic equations for these data were $y=9.331+1.219 \ln x$ ($r^2=0.606$) for Cu, $y=3.298+0.414 \ln x$ ($r^2=0.965$) for Pb, and $y=5.165+0.538 \ln x$ ($r^2=0.933$) for As]

mercury, only to cadmium. Analogous results were also observed in the eel *Anguilla anguilla* (Sanchéz-Galán et al. 2001; Gravato et al 2006). In this same species exposed during 7 days to 0.2 μM Cu, Oliveira et al. (2008) did not observed significant changes in the DNA integrity (measured by DNA alkaline unwinding assay) in the blood, gill, liver, and kidney, but a significant increase in erythrocyte nuclear abnormalities (ENA) frequency was observed. Cavas et al. (2005) evaluated the induction of blood MNi by 0.01 and 0.21 mg/L Cu after a 21-day exposure and found a non-significant effect in Prussian carp (*Carassius gibelio*), an induction of MNi in common carp (*C. carpio*), and a full mortality in peppered cory (*Corydoras paleatus*). On the other hand, exposure of gilthead sea bream (*Sparus aurata*) to 0.1 ppm of Cu during 20 days resulted in significant DNA damage in erythrocytes as measured by the comet assay (Gabbianelli et al 2003), and the frequency of blood MNi increased after exposure of rainbow trout (*Oncorhynchus mykiss*) to $CuSO_4$ (0.04, 0.08, and 0.16 mg/L) for 96 h (Bolognesi et al. 2006). Enhanced erythrocyte MNi frequencies were also observed in *Channa punctata* exposed to 0.4 mg/L copper for 24 to 168 h (Yadav and Trivedi 2009) and in the estuarine guppy *Poecilia vivipara* after a 96-h exposure to 9 mg/L Cu (Machado et al. 2013). Finally, in vitro studies showed that incubation of rainbow trout (*O. mykiss*) gill homogenates with cooper resulted in altered DNA integrity by inducing single- and double-stranded DNA breaks (Arabi and Alaeddini 2005), though DNA damage was not observed in erythrocyte of this same species in response to copper exposure (Fedeli et al 2010). Taken altogether, these findings indicate that as related to lethality and the induction of MN, there is a differential sensitivity to Cu by different fish species and also a differential response to different metals in each fish species, with *D. labrax* being highly sensitive.

In our study, no mortality was observed in fish subjected to Pb exposure, but the MNi frequency was increasingly enhanced with higher lead doses both at 24 and 96 h. Although laboratory studies performed on the genotoxic effect of lead in fish through the MN assay are rather scarce, previous data also showed a genotoxic effect of Pb as evaluated by this or other DNA-damage assays. In short-term cell kidney cultures of the tropical fish *Hoplias malabaricus*, chromosomal aberrations were observed after a long-term treatment (18 and 46 days) with Pb (Cestari et al. 2004). This same group reported a significant increase in blood MNi frequency in the same species after 2 months (Ferraro et al. 2004). Cavaş (2008) found that lead acetate treatments at doses from 0.01 to 0.1 mg/L for 2 to 6 days significantly induced the formation of MNi in erythrocytes, gills, and fin cells of *Carassius auratus*. Interestingly, the blood response demonstrated to be the most sensitive as MNi frequencies were significantly increased from the lowest exposure concentration. Similarly to our data, maximal increase reached approximately a 2.7-fold increase as compared to controls and no differences were observed between the exposure time periods. More recently, in the freshwater fish *Prochilodus lineatus* treated with 5 mg/L lead nitrate for 6 to 96 h, Monteiro et al. (2011) reported that while the comet assay showed a genotoxic effect in blood, gill, and liver cells only after 96 h, MNi frequency did not increase at any exposures; nonetheless, the frequency of other erythrocyte nuclear abnormalities showed a significant increase after 24 and 96 h. Likewise, by means of the comet assay, concentration-dependent DNA damage was shown in the gills, kidney, and liver of freshwater climbing perch *Anabas testudineus* exposed to 0.1–2 mg/L lead chloride for 96 h (Ahmed et al. 2011b).

In regard to As, in our study MNi frequencies increased gradually with increasing As concentrations since the lowest evaluated doses for both 24 and 96 h exposure. These results are in agreement with the limited previous studies in other species. Yadav and Trivedi (2009) observed a gradual time-dependent increase in blood MNi frequency induced by As_2O_3 (6.94 mg/L) for 24 to 168 h of exposure in *C. punctata*. In *Oreochromis mossambicus* exposed to $NaAsO_2$ (3, 28, and 56 mg/L) for 48 to 192 h, Ahmed et al. (2011a) found that blood MNi increased in a concentration-dependent manner, and the highest value was recorded after 96 h. Of note, a 56-fold increase in MNi frequency was also showed earlier by Ramírez and García (2005) in gill cells of zebra fish (*Danio rerio*) maintained in waters from wells with As contents ranging from 0.395 to 0.630 mg/L.

Recently, research is focusing on genotoxic effects in field-collected aquatic animals and on assays with sediments using fish (Costa et al 2008). A number of this kind of studies regarding Cu, Pb, or As in marine environments have been reported (Edwards et al 2001; Ergene et al 2007; Costa et al 2008; Della Torre et al 2010; Martínez-Gómez et al. 2012). Though these studies are highly useful for monitoring the adverse biological effects occurring at specific areas suffering from complex mixtures of contaminants, it is often very difficult to ascertain the specific effect of a particular xenobiotic. By contrast, an important issue of laboratory studies is that the effect of a unique metal can be isolated and accurately related to definite exposure concentrations. In our study, MNi frequency correlated positively with the actual Pb and As concentrations in water at 24 and 96 h. However, for Cu this correlation was shown only for the 96 h; which may be likely related to lethality observed at the higher Cu concentrations. By comparing the MNi frequency in relation to the actual water concentrations of the three metals, the genotoxic potential was ordered as Cu>As>Pb.

A key aim of the present investigation was to assess whether the aforesaid genotoxicity ranking was related to the burden of metal accumulation in the fish bodies. Hence, we evaluated the metal content in four representative organs with key physiological functions and differentially related to environmental

exposition and accumulation of xenobiotics, i.e., the gills (related to xenobiotic uptake), the liver (related to xenobiotic metabolism), the muscle (which accounts for the higher mass of fish body), and the brain (related to possible neurotoxic and behavioral effects). Each metal showed a differential pattern of bioaccumulation in these potentially important target organs, and this was also the case for the relationships between the induction of MNi and the metal levels. In the case of Pb, these were significant for all of the organs at 24 and 96 h. MNi frequency correlated positively with As content in liver and gills at 24 and 96 h. For Cu, a correlation was only established with the brain at 96 h. This suggests that for Pb and As, the levels of metal accumulation, mainly in liver and gills, may explain to some extent genotoxicity measured by the blood MN assay. However, the Cu content in these organs mostly involved in pollutant contact and metabolism was not related to the genotoxic damage in erythrocytes; though again this must be carefully assumed due to the lethal response observed, which reduced the statistical power.

In order to assess the actual importance of the results in relation to the potential hazardous impact of these metals in the environmental setting, we compared the observed genotoxic effects at the measured metal concentrations with quality guidelines for protection of aquatic life (USEPA 2009). In regard to Cu, we observed a significant genotoxic effect at the actual concentration of 0.009 mg/L after 96 h, which is only slightly higher than the 0.005 and 0.003 mg/L proposed by EPA criteria of maximum concentration (CMC) and continuous concentration (CCC) to prevent from acute and chronic toxicity to aquatic organisms, respectively. Therefore, since this minimal dose evaluated in our study was demonstrated as genotoxic, the evaluation of lower doses and longer exposures is deserved in future studies to confirm whether the threshold for the genetic damage is in accordance with the USEPA recommendations. In fact, local concentrations of copper in seawater can be very high, i.e., in a natural habitat of *D. Labrax* in the south of Spain, between the Atlantic Ocean and the Mediterranean Sea, Cu reached 0.021–0.072 mg/L in different shore sample sites (Oliva et al. 2013). Thus, in this place the aquatic organisms, at least this fish species, are at risk of genotoxicity. In regard to Pb, the minimal measured metal concentrations at which MNi frequency was significantly increased in our study (0.57 mg/L at 96 h) were higher, though not so much, than the USEPA CMC value of 0.210 mg/L. Finally, in the case of As, a significant increase in MNi frequency was observed by the lowest measured metal concentration of 0.01 mg/L at both 24 and 96 h. This is notably lower than the USEPA CMC and CCC of 0.069 and 0.036 mg/L, respectively. These results, which show a very early genotoxic effect at very low As concentrations in *D. labrax*, raised important environmental concerns and may question water quality criteria for the protection of aquatic life recommended by regulatory agencies about this metal.

Conclusions

This study revealed significant dose-dependent increases of MNi frequency in peripheral erythrocytes of *D. labrax* after Cu, Pb, and As exposure for 24 and 96 h. These findings are of particular environmental concern because depicted a genotoxic potential of Cu, Pb, and As after a very short exposure to low but environmentally relevant concentrations, too close to the thresholds established by regulatory agencies. In addition, the blood MN test in this species, one of the most important commercial fish widely cultured in the Mediterranean, could be considered an appropriate early biomarker to monitor genotoxicity induced by heavy metals in the natural or farmed aquatic environments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals All experimental procedures were performed in compliance with the Directive 2010/63/EU on the protection of animals used for scientific purposes.

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