Sub-lethal effects induced by morphine to the freshwater biological model *Dreissena polymorpha*

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ABSTRACT

Opioids are considered as emerging contaminants in aquatic ecosystems, mainly due to their large illicit consume worldwide. Morphine (MOR) is the main opiate and it was commonly found at measurable concentrations in freshwaters. Even though its occurrence is well documented, just limited information is available regarding its hazard to non-target organisms. The aim of this study was of the evaluation of sub-lethal effects induced by MOR to the freshwater bivalve *Dreissena polymorpha*. We exposed mussels to two MOR concentrations (0.05 µg/L and 0.5 µg/L) for 14 days and we investigated the sub-lethal effects by a suite of biomarkers. The Neutral Red Retention Assay (NRRA) was used as a test of cytotoxicity, while the oxidative stress was evaluated by the activity of antioxidant and detoxifying enzymes, namely catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), and by measuring the levels of lipid peroxidation (LPO) and protein carbonylation (PCC). The genetic damage was assessed by the Single Cell Gel Electrophoresis (SCGE) assay, the DNA diffusion assay and the micronucleus test (MN test). Finally, the filtration rate of *D. polymorpha* was evaluated in order to investigate possible physiological effects. Both tested concentrations reduced the lysosome membrane stability of bivalves, but only the highest MOR concentration induced significant changes in the activity of antioxidant enzymes (SOD, CAT and GPx) and increase in lipid peroxidation levels. Slight increase in primary DNA fragmentation was noticed, while no fixed genetic damage and alterations of the filtering rate were found.

Keywords: Morphine, Biomarkers, *Dreissena polymorpha*

1. INTRODUCTION

The scientific community has recently shown a growing interest for problems related to the presence of new contaminants such as pharmaceuticals and personal care products (PPCPs) and illicit drugs (Pal *et al*., 2013) in the aquatic environment. Once used, these chemicals and their metabolites enter the sewage waters through urines and feces (Ternes, 1998; Zuccato *et al*., 2000;

Heberer, 2002; Castiglioni *et al*., 2006). The wastewater treatment plants (WWTPs) are built to remove the organic matter and nutrients and are not suitable for the removal of most PPCPs and drugs of abuse (Reungoat *et al*., 2011; Pal *et al*., 2013). Many monitoring studies showed measurable concentrations (in the ng/L-µg/L range) of several PPCPs and illicit drugs in both European and US WWTP effluents and surface waters (Fent *et al*., 2006; Santos *et al*., 2010). Among pharmaceuticals, analgesics are topical pain relievers and can be divided into two groups: non-opioids (non-narcotic analgesics) and opioids (narcotic analgesic). The first family reduces pain and inflammation interfering with the synthesis of prostaglandin hormones (Julien 1997), while the latter group causes a muscular relaxation interacting with specific opioid receptor (MOP), a class of G-protein-coupled receptors (Suzuki and Misawa, 1997). Considering their pharmacological features, opioids are used as pharmaceuticals in human medicine, but also as drugs of abuse. The latest World Drug Report (UNODC, 2013) has estimated that about 16.5 million people worldwide, accounting for the 0.4% of the population aged 15-64, have used opiates as drugs of abuse at least once in 2012. These chemicals are not the most common illicit drugs used worldwide, since cannabis (3.9 % of the global population) and amphetamines (0.7 % of the global population) showed a higher use prevalence (UNODC, 2013). However, their use trend remains stable over the last years, with high prevalence in South-Western and Central Asia, Eastern and South-Eastern Europe and North America (UNODC, 2013). Opiates are opium derivatives, a substance extracted from *Papaver somniferum* and *Papaver setigerum*, historically prescribed for the care of cough, anemia and diarrhea (Nicholson, 2003). Opium contains many active alkaloid compounds, mainly morphine (MOR). In humans, MOR acts on the nervous system by binding to opioid receptors, reducing pain and smoothing muscle contraction (Zhu *et al*., 2005). MOR is metabolized for 87% from hepatic carboxylase, and the main metabolite is represented by morphine-3β-D-glucuronide (75%, Baselt *et al*., 2004). In addition, MOR is a metabolite of heroin, which has low affinity for opioid receptors and only when it is converted into MOR (4%), 6-acetylmorphine (1,3%) and morphine-3β-D-glucuronide (38%) performs a pharmacological action (Baselt *et al*., 2004; Maurer *et al*., 2006). For many decades, studies on vertebrates were focused on the pharmacological effects of exogenous MOR and exogenous morphine-like compounds, but after the discovery of the binding of MOR with opioid receptors, endogenous opioids have been identified (Lord *et al*., 1977). The presence of endogenous MOR is not a prerogative of vertebrates, as shown by studies performed on different species of invertebrates (Stefano *et al*., 2000). For instance, it is known that mussels have opioid receptors in their nervous system (Stefano and Scharrer, 1996). The MOR-opioid receptors interaction in these bivalves involves a release of dopamine (Zhu *et al*., 2005), a crucial neurotrasmitter involved in oogenesis. Despite the abovementioned evidences, very few

studies have been carried out on aquatic organisms to evaluate the effects of MOR towards non-target organisms. Mantione and co-workers (2002) showed that nitric oxide (NO) is released by the pedal ganglia in *Mytilus edulis*, after the stimulation by the interaction between opioid receptors and MOR metabolites. Other studies performed on microglia and immunocytes of *Mytilus edulis* suggested an immunosuppressive activity of MOR (Stefano 1989; Stefano *et al*., 1993), similar to that described in humans (Stefano *et al*., 1994; Makman *et al*., 1995). A recent investigation by Gagné and co-workers (2010) showed the neurochemical consequence of MOR exposure to the freshwater bivalve *Elliptio complanata*. After injections of increasing MOR doses in the adductor muscle (0.07; 0.15 and 0.75 mg/g wet weight), reductions in levels of serotonin and acetylcholinesterase (AChE), as well as increases in dopamine and γ-aminobutyric acid (GABA) levels, were noticed. Similar effects were obtained in the same mussel species exposed to a WWTP effluent extract, in which MOR was detected at 0.1 μg/L concentration (Gagné *et al*., 2004). However, to date no one investigation was performed to study neither the MOR cyto-genotoxicity nor the involvement of oxidative stress in the mechanism of action of this drug towards non-target organisms. Considering that MOR is frequently detected in European surface waters with an average concentration of 50-55 ng/L (Karolak *et al*., 2010; Terzic *et al*., 2010; Jurado *et al*., 2012; Martinez Bueno *et al*., 2011; Rosa Boleda *et al*., 2011), and that the continual input of this drug can lead to the exposure for the entire life-cycle of aquatic organisms, the investigation of its potential sub-lethal toxicity is new and pivotal in freshwater ecotoxicology. The aim of this study was to investigate the effects of MOR on the zebra mussels *Dreissena polymorpha,* using an *in vivo* multi-biomarkers approach. Thanks to its physio-ecological features, this bivalve species is commonly used in ecotoxicology, showing a good sensitivity to different emerging aquatic pollutants (Binelli *et al*., 2009a,b; Parolini *et al*., 2010; Parolini and Binelli 2011; 2012), including illicit drugs (Parolini *et al*., 2013; Parolini and Binelli, 2013; Parolini and Binelli, 2014). Moreover, this filter-feeding species has a great filtration rate (mean=200 mL/h/mussels) and it is more prone than other biological models to introduce the aquatic pollutants into the organism, pointing out rapidly their potential toxic effects. We exposed *D. polymorpha* specimens for 14 days to two low MOR concentrations: 0.05 μg/L and 0.5 μg/L. The end-points of twelve different biomarkers were measured to assess MOR sub-lethal effects. Cytotoxicity was evaluated on hemocytes by the Neutral Red Retention Assay (NRRA), while the activity of antioxidant and detoxifying enzymes, namely catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST), as well as the lipid peroxidation (LPO) and the protein carbonyl content (PCC) were applied as indices of oxidative stress in mussel homogenates. Primary (DNA strand breaks) and fixed (apoptotic and micronucleated cell frequency) genetic damage was investigated on *D.*

106 *polymorpha* hemocytes by the Single Cell Gel Electrophoresis (SCGE) assay, the DNA diffusion
107 assay and the micronucleus test (MN test), respectively. Finally, the filtration rate was evaluated as
108 physiological biomarker.
109
110 2. MATERIALS AND METHODS
111 The MOR standard (CAS number 57-27-2) was purchased from Alltech-Applied Science (State
112 College, PA, USA), while all the reagents used for biomarker analyses were purchased from Sigma-
113 Aldrich (Steinheim, Germany). We diluted the methanol stock solution (1 g/L) to 10 mg/L in
114 ultrapure water (working solution), which was then used to obtain the MOR concentration in
115 experimental aquaria.
116
117 2.1 *Experimental design*
118 *D. polymorpha* specimens were collected in September 2012 by a scuba diver at a depth of 4-6 m in
119 Lake Lugano (Northern Italy), which is considered a reference site due to its low drug pollution
120 (Zuccato *et al*., 2008). The mussels were gently cut off from the rocks, quickly transferred to the
121 laboratory in bags filled with lake water and placed in 15 L glass-holding aquaria filled with tap and
122 lake water (50:50 v/v) to avoid a drastic change in the chemical composition of the water and to
123 guarantee a food supply for the mussels during the first 24 h of acclimation. Mussel (n=60), having
124 the same shell length (15±4 mm), were placed within 5 L beakers filled with 4 L of tap and
125 deionized water (50:50 v/v), previously de-chlorinated by aeration, under a natural photoperiod with
126 constant temperature (20±1 °C), pH (7.5) and oxygenation (>90% of saturation). In order to avoid
127 the so-called tank effect, we prepare three beakers *per* treatment, including control. The bivalves
128 were fed daily with lyophilized algae *Spirulina* spp., and the water was regularly renewed every two
129 days for 2 weeks to gradually purify the mollusks by any possible pollutants that had previously
130 accumulated in their soft tissues. Only specimens that were able to re-form their byssus were used
131 in the experiments. Mussel viability was checked daily by the Trypan blue exclusion method and
132 was 93±2%, whereas biomarker baseline levels were checked weekly. Mussels were exposed to
133 MOR concentrations only when target biomarker levels were comparable with baseline ones
134 obtained in our previous laboratory studies (Parolini *et al*. 2010; 2011a,b; 2013; Parolini and Binelli
135 2013). Exposure assays were performed under semi-static conditions for 14 days. Control and
136 exposure beakers were processed at the same time and the whole water volume (4 L) was renewed
137 on a daily basis. Mussels were exposed to 0.05 µg/L (0.17 nM, Low) and 0.5 µg/L (1.7 nM, High)
138 of MOR. The first concentration was similar to the levels found in European surface waters (Pal *et*
139 *al*., 2013), while the second one was the same tested in previous studies investigating the toxicity of

cocaine metabolites, benzoylecgonine (BE; Parolini *et al*., 2013), ecgonine methyl ester (EME; Parolini and Binelli, 2013), and Δ-9-tetrahydrocannabinol (Δ-9-THC; Parolini and Binelli, 2014) in order to allow a comparison among drug toxicity administered at the same dose. Exact volumes of working solution (10±0.6 mg/L) were added daily to the exposure aquaria until reaching the selected concentrations. Specimens were fed 2 h before the daily change of water and chemicals to avoid the adherence of the drugs to food particles and to prevent the reduction of their bioavailability. Every 3 days, 8 specimens were randomly collected from each tank (24 specimens *per* treatment) to evaluate MOR-induced sub-lethal effects. Hemolymph was withdrawn by 10 bivalves and cyto-genotoxicity was evaluated on hemocytes. After the withdrawal, the soft tissue of mussels was immediately frozen in liquid nitrogen and stored at -80 °C until LPO and PCC analyses. Lastly, the soft tissue of the other 14 specimens was frozen in liquid nitrogen and stored at –80 °C until the enzymatic activity was measured. Simultaneously, 10 zebra mussels were placed in other control and exposure 500 mL beakers (three replicates *per* treatment), maintained at the same condition described above and exposed at the same concentrations to assess the variation in filtration rate due to MOR treatments.

2.2 *Evaluation of MOR concentrations*

In order to guarantee the reliability of the experimental design, the MOR concentration in both working solution and exposure beakers were measured. At each time of biomarker analysis, water was sampled 1 h after the contamination from both the three control and exposure beakers and integrated in a unique sample (100 mL) per treatment. Water samples were spiked with 0.2 µg/L of MOR-D_3 as internal recovery standard. The concentration of the MOR was checked in LC-MS/MS by using a HCT Ultra (Bruker, Germany) using a Phenomenex Luna PFP (2 x 50 mm-5 µm) column after purification and concentration by SPE (HLB 1 cm^3, Waters). After cartridge activation (2 mL methanol and 3 mL of water), 5 mL of each sample with internal standard were load on SPE and then resuspended with 50 µL of water. 20 µL of each sample were then injected and analyzed in LC-MS/MS. MOR quantification in water was performed by a calibration curve (0.025-1 µg/L; R^2=0.99) and internal standard recoveries were >90%. The analysis of MOR concentration in working solution, control and exposure beakers was performed in triplicate.

2.3 *Biomarkers of cytotoxicity*

The NRRA was performed to assess cytotoxicity following the method proposed by Lowe and Pipe (1994) and applied on mussel hemocytes. Slides were examined systematically thereafter at 15 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to

174 the cytosol. Tests finished when dye loss was evident in at least 50% of the hemocytes. The mean
175 retention time was then calculated from five replicates.
176
177 *2.4. Oxidative stress biomarkers*
178 The activity of SOD, CAT, GPx, and GST was measured in triplicate (n=3) in the cytosolic fraction
179 extracted from a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 100 mM
180 phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) with dithiothreitol (DTT, 100 mM) using a
181 Potter homogenizer. Specific protease inhibitors (1:10) were also added to the buffer:
182 phenanthroline (Phe, 10 mM) and trypsin inhibitor (Try, 10 mg/mL). The homogenate was
183 centrifuged at 15.000 g for 1 hour at 4 °C. The sample was held in ice and immediately processed
184 for the determination of protein and enzymatic activities. The total protein content of each sample
185 was determined according to the Bradford method (1976) using bovine serum albumin as a
186 standard. Enzymatic activities were determined spectrophotometrically as described by Orbea *et al*.
187 (2002). Briefly, the CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm
188 using 50 mM of H_2O_2 substrate in 67 mM potassium phosphate buffer (pH 7). The SOD activity
189 was determined by measuring the degree of inhibition of cytochrome c (10 µM) reduction at 550
190 nm by the superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50
191 µM) reaction. The activity is given in SOD units (1 SOD unit=50% inhibition of the xanthine
192 oxidase reaction). The GPx activity was measured by monitoring the consumption of NADPH at
193 340 nm using 0.2 mM H_2O_2 substrate in 50 mM potassium phosphate buffer (pH 7) containing
194 additional glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL), and
195 NADPH (120 µM). Lastly, the GST activity was measured by adding reduced glutathione (1 mM)
196 and 1-chloro-2,4 dinitrobenzene in phosphate buffer (pH 7.4) to the cytosolic fraction; the resulting
197 reaction was monitored for 1 min at 340 nm. LPO and PCC were measured in triplicate (n=3) from
198 a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 50 mM phosphate buffer (pH
199 7.4; KCl 100 mM, EDTA 1 mM) containing 1 mM DTT and 1 mM PMSF using a Potter
200 homogenizer. LPO level was assayed by the determination of thiobarbituric acid-reactive substances
201 (TBARS) according to Ohkawa (1979). The absorbance was read at 532 nm after removal of any
202 fluctuated material by centrifugation. The amount of thiobarbituric acid reactive substances
203 (TBARS) formed was calculated by using an extinction coefficient of 1.56*105 M/cm and
204 expressed as nmol TBARS formed/g fresh weight. For carbonyl quantification the reaction with
205 2,4-dinitrophenylhydrazine (DNPH) was employed according to Mecocci *et al*. (1999). The
206 carbonyl content was calculated from the absorbance measurement at 370 nm with the use of molar
207 absorption coefficient of 22 000 mol/cm and expressed as nmol/(mg protein).

208 2.5 *Genotoxicity biomarkers*

209 Since methods and procedures of genotoxicity biomarkers applied in this study were described in
210 detail by Parolini *et al*. (2010), only a brief description of the followed techniques was reported
211 here. The alkaline (pH>13) SCGE assay was performed on hemocytes according to the method
212 adapted for the zebra mussel by Buschini *et al*. (2003). Fifty cells *per* slide were analyzed using an
213 image analysis system (Comet Score®), for a total of 500 analyzed cells per specimen (n=10). Two
214 SCGE assay end-points were evaluated: the ratio between migration length and comet head
215 diameter (LDR) and the percentage of DNA in tail. The apoptotic cell frequency was evaluated
216 through the protocol described by Singh (2000). Two hundred cells per slide were analyzed for a
217 total of 1000 cells per sample (n=5). The MN test was performed according to the method of
218 Pavlica *et al*. (2000). Four hundred cells were counted per each slide (n=10) for a total of 4000
219 cells/treatment. Micronuclei were identified by the criteria proposed by Kirsch-Volders *et al*.
220 (2000), and the MN frequency was calculated (MN‰).

221

222 2.6 *Filtration rate*

223 Mussel filtration rate was measured according to a procedure adapted from Faria *et al*. (2009) and
224 Palais *et al*. (2012) and based on the loss of neutral red dye particles from the water column as a
225 result of mussel filtration activity (Coughlan, 1969). 10 zebra mussels per beaker (three replicates
226 per treatment) were placed in 500 mL and were exposed to 0.05 µg/L and 0.5 µg/L MOR
227 concentrations. Every 3 days for 14 days a 250 µg/L neutral red solution was added in the beakers.
228 After 15 min of acclimation, bivalve were allowed to filter for 3 h in the dark at 20 °C. Dye particle
229 concentration in the test beakers was then measured spectrophotometrically (340 nm) at the
230 beginning and at the end of the experiment, using a standard curve for neutral red solution. The
231 filtration rate (f), expressed in mL of water *per* individual and *per* hour (mL/ind/h), was calculated
232 using the following formula:

233 $$f=[V/(n*t)]*\log (C_0/C_t)$$

234

235 where V is the volume (mL) of the dye solution in the beaker, n the number of mussels, t the
236 duration of the filtration period (h), C_0 and C_t the initial and final dye particle concentrations in the
237 beaker (Coughlan, 1969).

238

239 2.7 *Statistical analysis*

240 Data normality and homoscedasticity were verified using the Shapiro-Wilk and Levene's tests,
241 respectively. To identify dose/effect and time/effect relationships a two-way analysis of variance

(ANOVA) was performed using time and MOR concentrations as variables, while biomarker end-points served as cases. The ANOVA was followed by a Fisher LSD post-hoc test to evaluate significant differences (*$p<0.05$; **$p<0.01$) between treated samples and related controls (time to time), as well as among exposures. All statistical analyses were performed using the STATISTICA 7.0 software package.

3. RESULTS

3.1 MOR concentration in exposure tanks

The MOR concentration in the control tanks was <0.025 µg/L, which is the limit of detection of the used equipment. In the exposure tanks, MOR level was close to the nominal values (0.05 µg/L and 0.5 µg/L). We found an average value of 0.045±0.005 µg/L for the lower concentration and a value of 0.35±0.01 µg/L for the higher tested concentration, accounting for the 90% and 70% of the nominal values, respectively. Considering that the coefficient of variation of the method was ±20%, our analyses confirmed the reliability of the whole experimental design.

3.2 *Baseline levels of applied biomarkers*

During the 14-day experiment, very low mortality was observed in the control (0.6%) and exposure (< 3%) tanks. Baseline levels of cyto-genotoxic and oxidative stress biomarkers were similar to those obtained in our previous laboratory studies (Binelli *et al*., 2009a,b; Parolini *et al*. 2010; 2011a,b; 2013; Parolini and Binelli 2011; 2013). The baseline filtration rate of zebra mussel specimens ranged between 0.74±0.29 and 1.92±0.17 mL/individual/h, according to values measured in the same species with a similar method by Palais *et al*. (2012).

3.3 *Biomarkers of cytotoxicity*

The NRRA showed a significant destabilization of hemocytes lysosome membranes (Figure 1) according to time- ($F=27.87$, $p<0.01$) and concentration-dependent ($F=48.56$, $p<0.01$) relationships. Both the MOR concentrations were able to significantly increase generic cellular stress in mollusks: 0.05 µg/L treatment caused a significant decrease ($p<0.01$) of NRRT starting to 11 days of exposure, while at 0.5 µg/L a significant destabilization ($p<0.01$) of the lysosomal membranes was notice as early as 7 days of exposure.

3.4 *Biomarkers of oxidative stress*

The activity of antioxidant enzymes (SOD, CAT and GPx) and detoxification enzymes (GST) showed some significant changes compared to controls during the exposure tests (Figure 2A, B, C, D). Although the activity of GST was not significantly altered ($p>0.05$) after exposure to the two

MOR tested concentrations (Figure 2A), the activity of SOD showed a significant time-dependent ($F=3.07$, $p<0.05$) and dose-dependent ($F=11.54$, $p<0.01$) inhibition already after 4 days of exposure to 0.5 µg/L (Figure 2B), reaching at the end of the test values lower than 40% compared to controls. Regarding the GPx, a significant time- ($F=4.64$, $p<0.01$) and concentration-dependent ($F=10.46$, $p<0.01$) increase was noticed after 7 days of exposure at the highest MOR concentration (Figure 2C). Accordingly, CAT showed significant activity increase ($p<0.05$) compared to baseline values (Figure 2D) at the end of the exposure to both the treatments. Finally, significant time-dependent ($F=5.37$; $p<0.01$) and concentration-dependent ($F=4,36$; $p<0,05$) differences were found in the lipid peroxidation levels (Figure 3A) with an increase of 15% compared to controls at the end of exposure. In contrast, no significant differences ($p>0.05$) of protein carbonylation compared to baseline levels were found, except for a single value obtained after 11 days at the lowest treatment (Figure 3B).

3.5 Biomarkers of genotoxicity and filtration rate

Even if no significant increase in LDR parameter was found (data non shown), significant increase in DNA fragmentation was noticed at the end of the 0.5 µg/L exposure, as pointed out by the raise of percentage of DNA in the hemocyte comet tail with respect to control ($F= 2.57$, $p<0.05$; Figure 4A). No significant increase ($p>0.05$) in frequencies of apoptotic (Figure 4B) and micronucleated cells (Figure 4C), whose levels were similar to the baseline ones throughout the test, even if a significant time-dependent relationship ($F=2.91$, $p<0.05$) was noticed for the latter end-point. Lastly, the filtration rate (ranged between 1.43±0.25 and 3.11±0.28 mL/individual/h for 0.05 µg/L MOR concentration and ranged between 1.19±0.17 and 3.05±0.66 for 0.5 µg/L) followed a bell-shaped curves for both MOR tested concentrations, with no significant changes ($p>0.05$) compared to controls (data not shown).

4. DISCUSSION

4.1 Sub-lethal effects of MOR

Although the lowest MOR concentration did not induced any significant variation ($p>0.05$) for all the investigated end-points, the exposure to 0.5 µg/L MOR caused a notable cytotoxicity to *D. polymorpha* specimens, as pointed out by the significant ($p<0.01$) time- and concentration dependent decrease of NRRT (Figure 1). This showed a progressive aggravation of the bivalve health status, suggesting that bivalves suffer a situation of general cellular stress, which could be linked to the induction of oxidative stress (Lowe *et al*. 1995). The destabilization of lysosome membranes in aquatic organisms could be caused by the production of reactive oxygen species

(ROS) following exposure to pollutants (Regoli *et al*., 1998). The ROS are mainly produced as side-products of oxygen metabolism and among these the biotransformation of xenobiotics is an oxidative process in which the production of ROS and the formation of more polar (reactive) intermediates occur (Gagné *et al*., 2010). In vertebrates, MOR is biotransformed by cytochrome P450 3A4 and 2C19, which involves oxidative N-dealkylation, hydroxylation and conjugation to glucuronide (Charney *et al*., 2001). Even if no information regarding the biotransformation of MOR in the zebra mussel is currently available, the observed trends of antioxidant enzymes suggested that this drug could induce the production of ROS. Variation in antioxidant levels, in fact, indirectly supply information on the changes of pollutant-induced reactive oxygen species (ROS) levels in different aquatic organisms (Viarengo *et al*. 2007), including *D. polymorpha* (Parolini *et al*., 2010; 2013). The inhibition of SOD activity (Figure 2B) suggested an increase of superoxide anion in bivalves (O^{2-}; Verlecar *et al*., 2008), as found in our previous study exposing zebra mussel to cocaine metabolites (Parolini *et al*., 2013; Parolini and Binelli, 2013). Since dismutation of O^{2-} leads to the production of hydrogen peroxide, this particular trend could be due to a phenomenon of product inhibition, according to a negative feedback mechanism (Vlahogianni and Valavanidis, 2007). The inhibition of SOD should therefore indicate both an accumulation of O^{2-} and an overproduction of H_2O_2, which could be also produced through the spontaneous conversion of superoxide anion mediated by non-enzymatic pathways (Gwoździński *et al*., 2010). The significant time-dependent trend in the levels of GPx and CAT (Figure 2C and D) confirmed that MOR was able to increase the levels of H_2O_2, whose toxicity seems to be counterbalanced by the antioxidant shield of bivalves. However, it is important to note that the accumulation of superoxide radical caused by SOD inhibition, combined with the increase of H_2O_2 caused by the activation of CAT and GPx, could lead to the formation of hydroxyl radicals through the Haber-Weiss reaction, with the consequent increase in the levels of lipid peroxidation and protein carbonylation (Verlecar *et al*., 2008). The analysis of lipid peroxidation levels and protein carbonyl content just partially confirmed this hypothesis, since we observed a significant ($p<0.01$) increase in the levels of lipid peroxidation (Figure 3A), despite no variations in protein carbonylation were noticed (Figure 3B). Accordingly, MOR treatments caused negligible genotoxic effects to zebra mussels, since just slight significant ($p<0.01$) increase of DNA fragmentation was found at the end of exposure to 0.5 µg/L (Figure 4A). Although several studies showed that the increase in DNA fragmentation is one of the main factor leading to the onset of fixed genetic damage in *D. polymorpha* specimens (Binelli *et al*., 2009a,b; Parolini and Binelli, 2012), no significant ($p>0.05$) increases in frequency of apoptotic cells and MN were found (Figure 4B and 4C). Lastly, even if our data showed that low MOR concentrations could alter antioxidant status of the zebra mussel leading to low oxidative damage,

no physiological effect was noticed, as pointed out by the lack of significant ($p>0.05$) alteration of bivalve filtration rate (data not shown). Despite of the moderate MOR-induced adverse effects found in the zebra mussel, the potential toxicity of this illicit drug cannot be neglected since it could cause other deleterious effects that further studies should have to investigate. For instance, being a psychotropic substance, MOR could act as neurotoxic compound, as pointed out by a recent *in vivo* study on the freshwater bivalve *Elliptio complanata* in which the exposure to three MOR concentrations (0.07; 0.15 and 0.75 mg/g wet weight) induced decreases in serotonin and AChE, and increases in dopamine and GABA levels, suggesting the induction of a relaxation state in mussels (Gagnè *et al*., 2010).

4.2 Comparison of MOR toxicity with the effects of other illicit drugs

Considering the moderate sub-lethal effects of MOR to treated zebra mussel, the comparison between its toxicity and that of previously analyzed illicit drugs, namely benzoylecgonine (BE; Parolini *et al*., 2013), ecgonine methyl ester (EME; Parolini and Binelli, 2013) and Δ-9-tetrahydrocannabinol (Δ-9-THC; Parolini and Binelli, 2014), should allow the drawing of a toxicity scale to individuate the most dangerous compound towards our biological model, laying the bases for further in-depth investigations. Although it is well-known that the assessment of several biomarkers is the best approach to the understanding of adverse effects and mechanism of action of pollutants on organism (Viarengo *et al*., 2007; Sforzini *et al*., 2011), the simple examination of the simultaneous changes of dissimilar biological parameters is considered insufficient to rank the hazard of different pollutants because of the wide variability in biomarker responses. For example, by comparing present data and those from our experiments on the sub-lethal effects induce by other illicit drugs (Parolini *et al*., 2013; Parolini and Binelli, 2013; 2014) we can note a remarkable variability in most of investigated end-points, depending on tested compound and probably due to dissimilarities in their mechanism of action, which prevents from ranking their toxicity. In fact, even though all psychotropic substances were able to induce significant destabilization of lysosome membranes, substantial differences in the activity of antioxidant/detoxification enzymes, as well as in oxidative and genetic damage were found. For this reason, the application of procedures able to integrate the biomarker responses within a simple synthetic index could help to minimize the variation of responses, allowing to draw an accurate scale of toxicity. To compare the sub-lethal toxicity of tested illicit drugs we integrated the whole biomarker dataset obtained at 0.5 μg/L for MOR into a synthetic index called Biomarker Response Index (BRI), previously described by Parolini *et al*. (2013). We excluded from integration analysis the results from filtration rate since it is not assessed in previous studies. Briefly, since changes in each specific biomarkers follow

different trends (increasing, decreasing or bell-shaped curves, Hagger *et al*., 2010), we calculated the percentage of alteration level (AL) of each biomarker *per* exposure time compared to the correspondent control. To calculate the BRI, we attributed a specific score to each obtained AL value according to Parolini *et al*. (2013) and each biomarker was then weighted in relation to its level of biological organization (Hagger *et al*., 2010). Finally, we compared the BRI value obtained for MOR with those calculated for BE and EME (Parolini and Binelli, 2013), and Δ-9-THC (Parolini and Binelli, 2014), deriving the following toxicity scale:

$$\Delta\text{-9-THC} > \text{BE} \approx \text{EME} >> \text{MOR}$$

The toxicity of Δ-9-THC (BRI=8.78; Parolini and Binelli, 2014) seems to be slightly higher than that of BE (BRI=8.22) and EME (BRI=8.06; Parolini and Binelli 2013), while MOR (BRI=6.17) showed the lowest value among the tested illicit drugs, suggesting its possible lowest hazard towards the zebra mussel for the measured end-points at least.

5. CONCLUSION

Our findings showed that MOR exposure could induce moderate adverse effects to this freshwater bivalve species, highlighting its possible hazard to freshwater communities. Even if current environmental MOR levels seem not cause any deleterious effect to bivalves, 14-day treatment to 0.5 μg/L concentration affected the oxidative status of bivalves and induced slight oxidative damage to cellular macromolecules. Although our findings suggest that oxidative stress seems to be involved in the mechanism of action of MOR in zebra mussel, further studies using powerful techniques, as well as the analysis of different end-points (i.e. neurotoxicity parameters), should be necessary to confirm it. Although the integrated MOR toxicity obtained at 0.5 μg/L treatment resulted lower compared to that of other common illicit drugs previously tested at the same experimental conditions, its environmental hazard cannot be underestimated. In fact, considering that in the real environment organisms are exposed to MOR concentrations for their whole life span, and its levels could increase due to the stable use of opiates worldwide, MOR effects could be more deleterious with respect to those highlighted in the present study. For this reason, further investigations should be necessary to enhance knowledge on MOR sub-lethal effects and its mechanism of action in non-target aquatic organisms.

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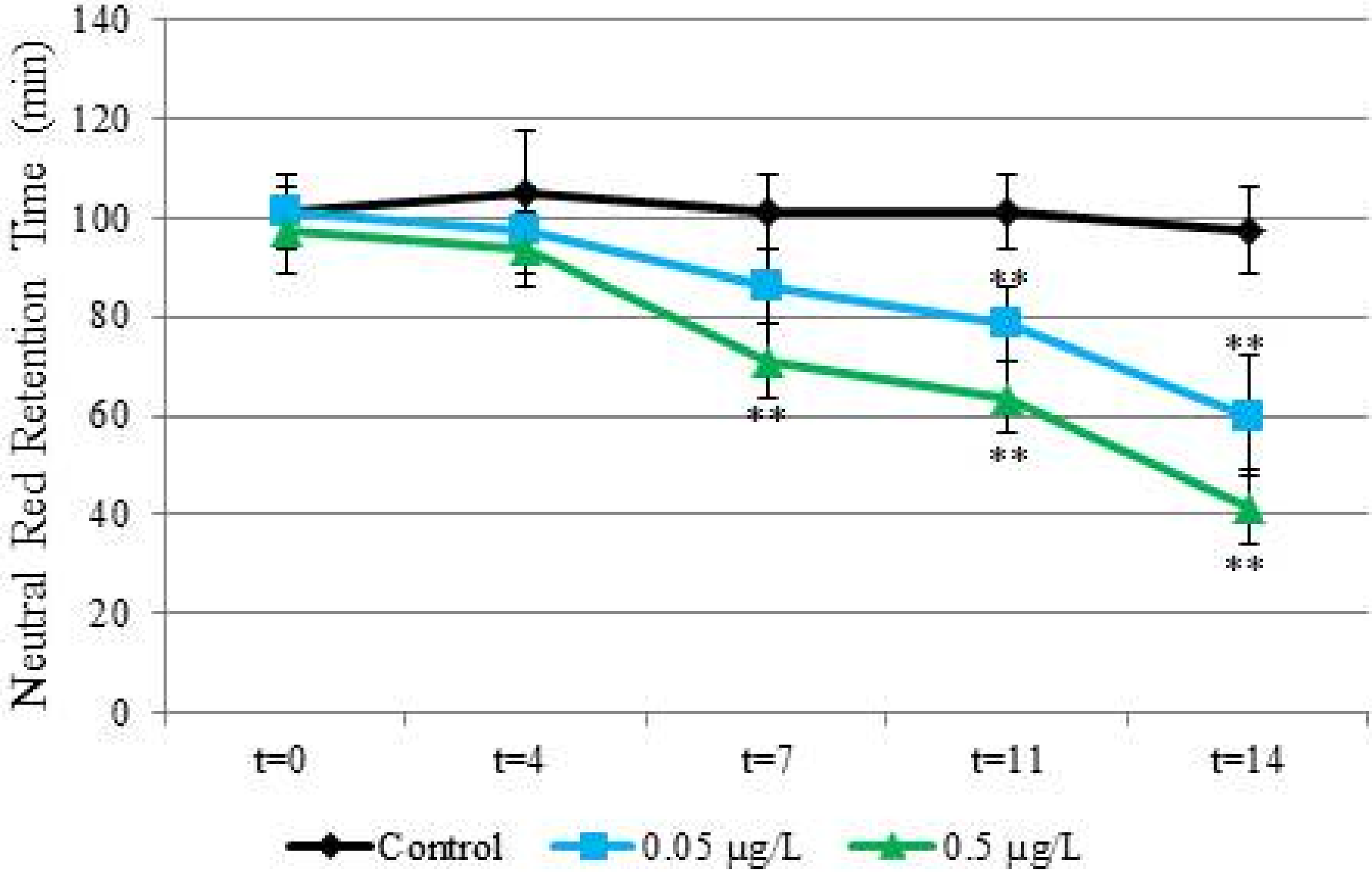
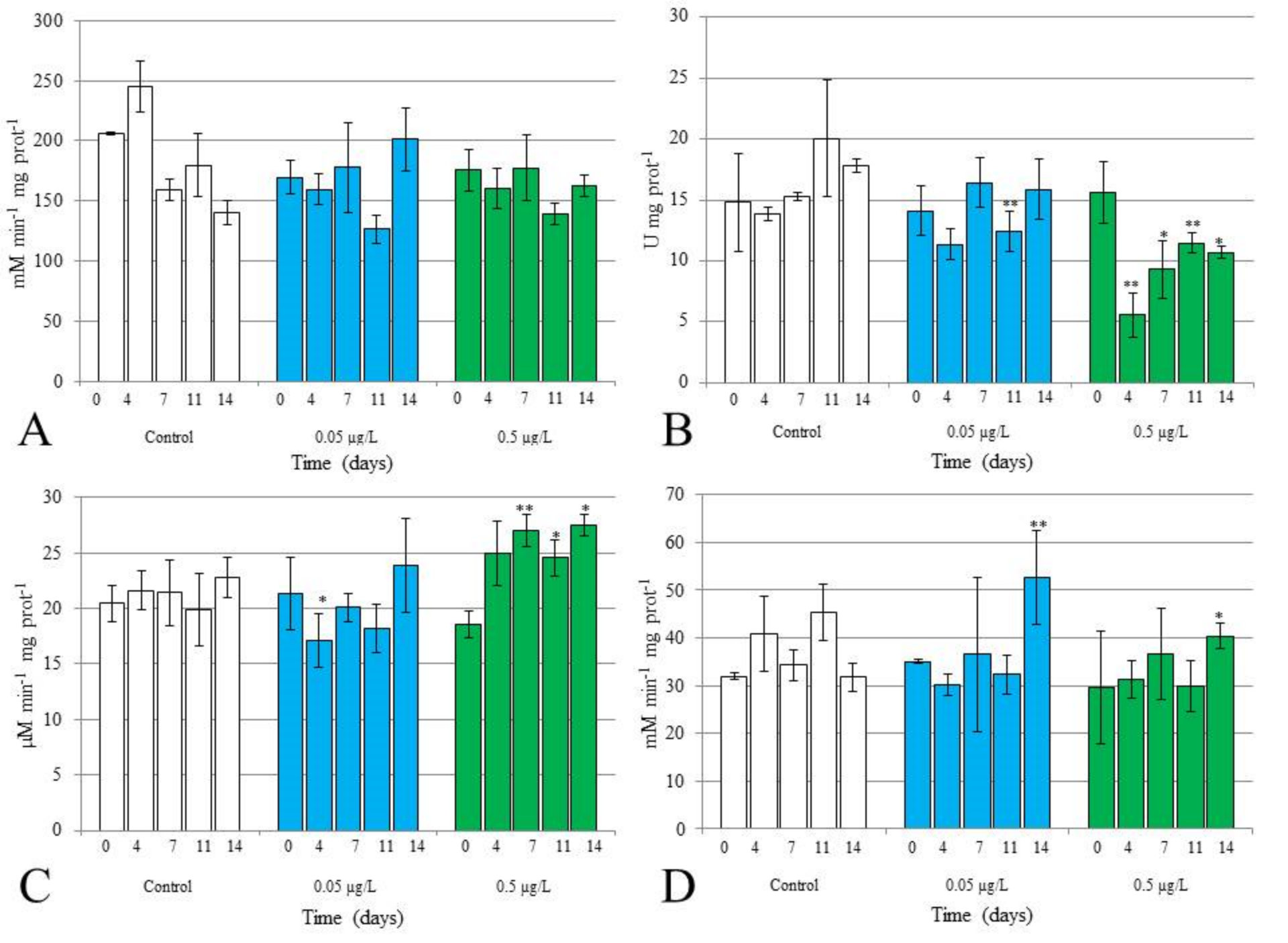
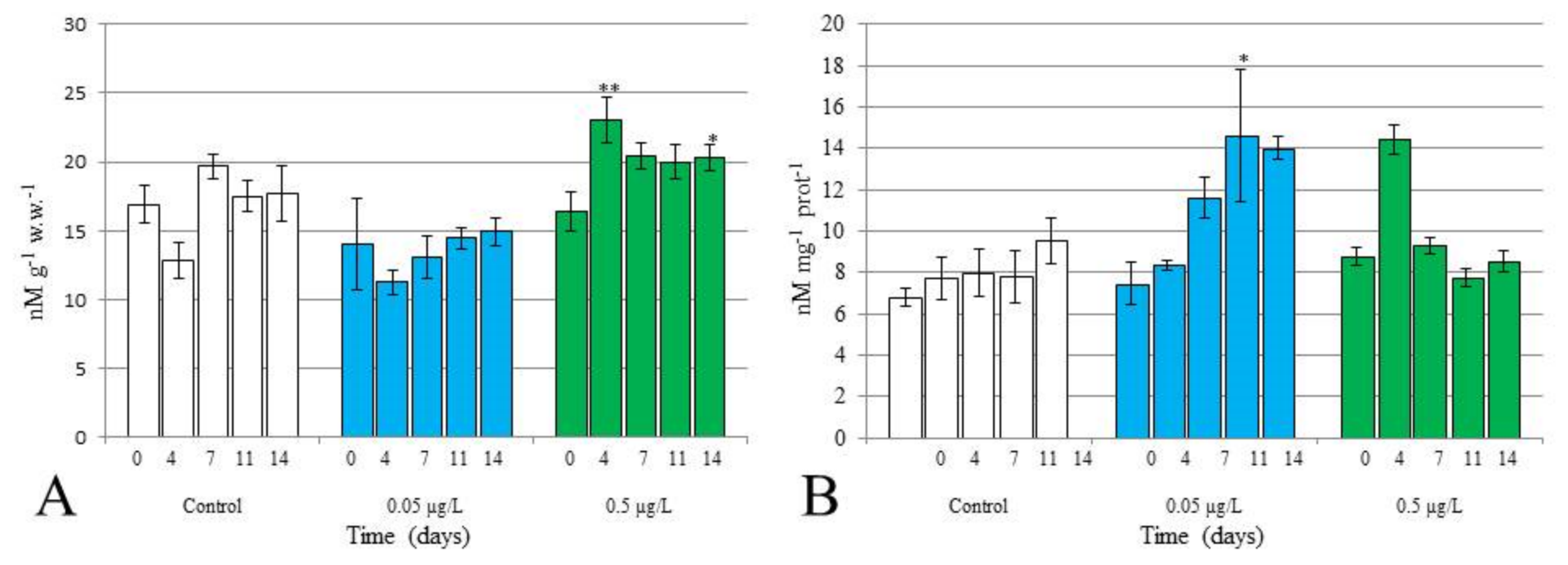
566
567 FIGURES:
568 Fig. 1: Assessment of lysosomal membrane stability (Neutral Red Retention Time-mean±SEM)
569 found in the hemocytes of treated bivalves (n=5). Asterisks indicate significant differences between
570 the treated and the corresponding controls (two-way ANOVA, Fisher LSD post-hoc test, *$p<0.05$,
571 **$p<0.01$).
572
573 Fig. 2: Mean values (±SEM) of the activity of glutathione-S-transferase (GST, A), superoxide
574 dismutase (SOD, B), glutathione peroxidase (GPx, C) and catalase (CAT, D), measured in the
575 bivalves (n=3, pool of 3 individuals) exposed to both MOR concentrations. The significant
576 differences (two-way ANOVA, Fisher LSD post-hoc test, *$p<0.05$; **$p<0.01$) refer to the
577 comparison of exposed with the corresponding baseline value.
578

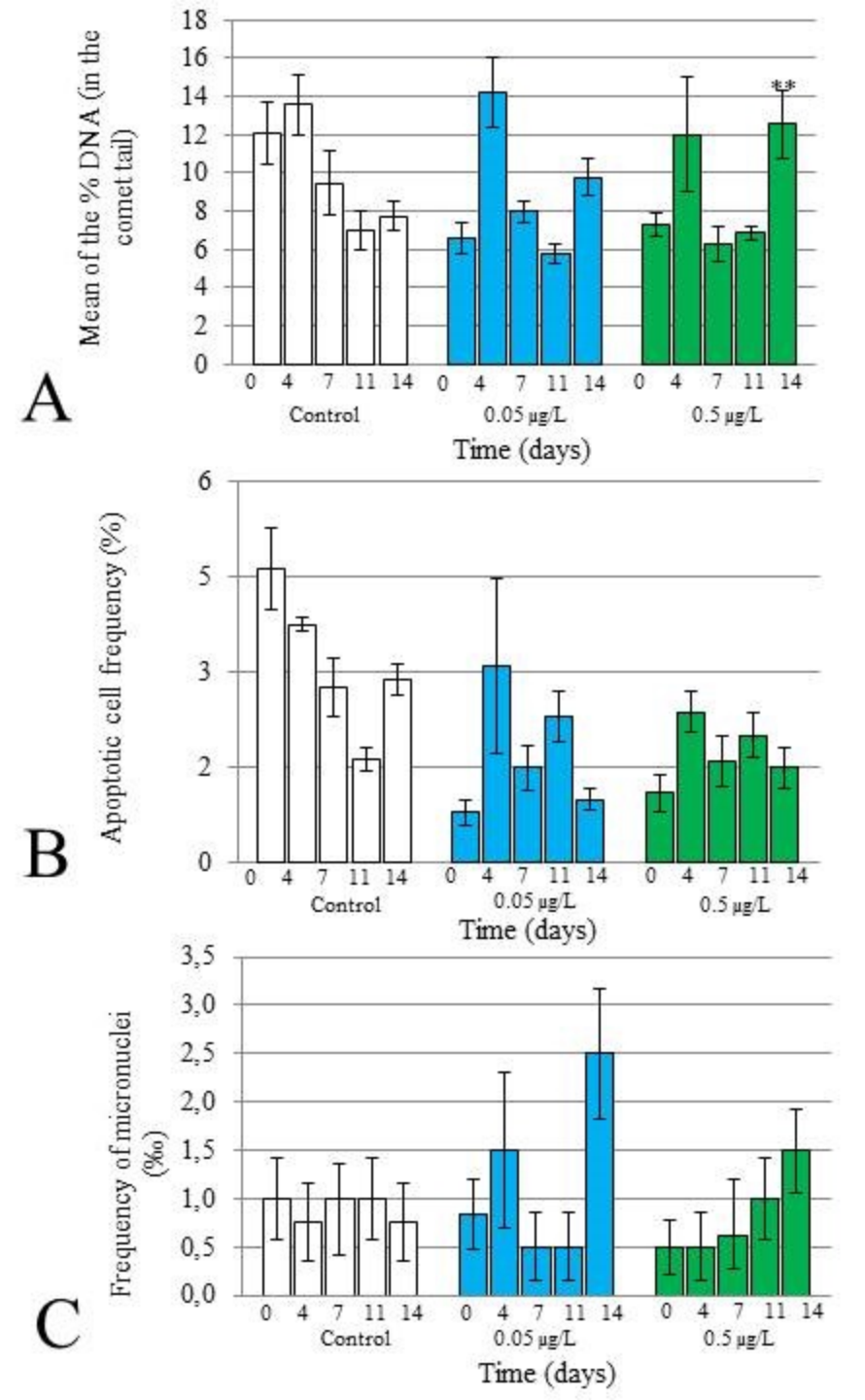
Fig. 3: Mean values (±SEM) of lipid peroxidation levels (LPO, A) and protein carbonylation (PCC, B) found in the bivalves (n=3, pool of 3 individuals) exposed to both MOR concentrations. The significant differences (two-way ANOVA, Fisher LSD post-hoc test, *$p<0.05$, **$p<0.01$) relate to the comparison between the exposed and corresponding baseline value.

Fig. 4: DNA percentage in the comet tails (A; mean±SEM) of the bivalve hemocytes (n=8) exposed to both MOR concentrations. The significance (two-way ANOVA, Fisher LSD post-hoc test, **$p<0.01$) refers to the comparison between the exposed and controls. Mean (±SEM) apoptotic cells frequency (%; B) and micronucleated frequency (‰MN; C) showed no significant differences compared to the corresponding controls (two-way ANOVA, $p>0.05$).


Neutral Red Retention Time (min)
140
120
100
80
60
40
20
0
t=0
t=4
t=7
t=11
t=14
**
**
**
**
**
Control
0.05 µg/L
0.5 µg/L


A
mM min-1 mg prot-1
300
250
200
150
100
50
0
0 4 7 11 14 0 4 7 11 14 0 4 7 11 14
Control
0.05 µg/L
0.5 µg/L
Time (days)
B
U mg prot-1
30
25
20
15
10
5
0
**
*
**
*
**
*
C
µM min-1 mg prot-1
30
25
20
15
10
5
0
*
**
*
*
D
mM min-1 mg prot-1
70
60
50
40
30
20
10
0
**
*


30
25
20
15
10
5
0
nM g-1 w.w.-1
**
*
0 4 7 11 14 0 4 7 11 14 0 4 7 11 14
A
Control
0.05 µg/L
0.5 µg/L
Time (days)
20
18
16
14
12
10
8
6
4
2
0
nM mg-1 prot-1
*
0 4 7 11 14 0 4 7 11 14 0 4 7 11 14
B
Control
0.05 µg/L
0.5 µg/L
Time (days)


A
Mean of the % DNA (in the comet tail)
18
16
14
12
10
8
6
4
2
0
**
0 4 7 11 14 0 4 7 11 14 0 4 7 11 14
Control
0.05 µg/L
0.5 µg/L
Time (days)
B
Apoptotic cell frequency (%)
6
5
3
2
0
0 4 7 11 14 0 4 7 11 14 0 4 7 11 14
Control
0.05 µg/L
0.5 µg/L
Time (days)
C
Frequency of micronuclei (‰)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0 4 7 11 14 0 4 7 11 14 0 4 7 11 14
Control
0.05 µg/L
0.5 µg/L
Time (days)