Benzoylecgonine exposure induced oxidative stress and altered swimming behavior and reproduction in *Daphnia magna*

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ABSTRACT

Several monitoring studies have shown that benzoylecgonine (BE) is the main illicit drug residue commonly measured in the aquatic system worldwide. Few studies have investigated the potential toxicity of this molecule towards invertebrate and vertebrate aquatic non-target organisms focusing on effects at low levels of the biological organization, but no one has assessed the consequences at higher ones. Thus, the present study was aimed at investigating the toxicity of a 48-hour exposure to two concentrations of BE, similar to those found in aquatic ecosystems (0.5 µg/L and 1.0 µg/L), on the cladoceran *Daphnia magna* at different levels of the ecological hierarchy. We relied on a multi-level approach focusing on the effects at biochemical/biomolecular (biomarkers), individual (swimming activity) and population (reproduction) levels. We measured the amount of reactive oxygen species and of the activity of antioxidant (SOD, CAT, and GPx) and detoxifying (GST) enzymes to assess if BE exposure can alter the oxidative status of *D. magna* specimens, while the lipid peroxidation (TBARS) was measured as a marker of oxidative damage. Moreover, we also measured the acetylcholinesterase (AChE) activity because it is strictly related to behavioral changes in aquatic organisms. Changes in swimming behavior were investigated by a video tracking analysis, while the consequences on reproduction were assessed by a chronic toxicity test. Our results showed that BE concentrations similar to those found in aquatic ecosystems induced oxidative stress and inhibited AChE activity, affecting swimming behavior and the reproduction of *Daphnia magna* individuals.

Capsule: benzoylecgonine exposure induced adverse effects at different levels of the biological organization in *Daphnia magna*

Keywords: Benzoylecgonine, biomarkers, behavioral effects, chronic toxicity, *Daphnia magna*

1. INTRODUCTION

Cocaine (COC) is a psychostimulant that affects behavior and brain physiology by altering dopamine release from dopaminergic neurons (Jeon et al., 2008). Differently from other illicit drugs, COC use declined worldwide as a result of the consumption trends in North America and Europe, but it has been estimated that globally 18.3 million people aged 15-64 is still a cocaine user (UNODC, 2016). However, COC remains the most used illicit stimulant in Europe, and its market accounts for about one half of the global COC market (UNODC, 2015). As consequence of its use, COC and its metabolites are the most abundant illicit drugs found in surface waters (Pal et al., 2013 and references therein). After a dose consumption, COC is metabolized by the liver and excreted through the urine as two main metabolites, the benzoylecgonine (BE, 45% of the swallowed dose) and the ecgonine methyl ester (EME, 40%), while only a limited amount (1–9%) is eliminated unchanged (Baselt, 2004). Considering human metabolism, BE is the main COC-related molecule measured in freshwater, reaching concentrations up to 7,500 ng/L and 3,425 ng/L in inlet and outlet of wastewater treatment plants (WWTPs; Pal et al., 2013 and references therein; Mendoza et al., 2014). Since WWTP efficiency in removing BE is incomplete (Zuccato et al., 2008), this molecule enters the surface water, where it was measured at concentrations up to 316 ng/L (Pal et al., 2013 and references therein).

Even though the current BE levels in freshwaters are quite low, the risks for the aquatic community cannot be neglected. Because of its pseudo-persistency and molecular activity, BE may exert different adverse effects towards aquatic non-target organisms. For instance, a 14-day exposure to 1 µg/L of BE imbalanced the antioxidant activity and caused oxidative and genetic damage in the zebra mussel (Parolini et al., 2013). Results from a companion study of functional proteomics showed that a 14-day exposure to BE altered the protein profile of gills from the zebra mussel, modulating the expression of proteins involved in diverse functions, including energy and amino acidic metabolism, stress response, and protein biosynthesis (Binelli et al., 2014). Moreover, a redox-proteomics approach showed that BE caused oxidative modifications in different classes of gill proteins involved in cytoskeleton, energetic metabolism and stress response (Pedriali et al., 2014). A recent study showed that the exposure of zebrafish embryos to increasing BE concentrations (0.01 µg/L-10 µg/L range) caused the overproduction of reactive oxygen species (ROS) and altered the gene expression and the activity of antioxidant enzymes, leading to cytogenetic damage in 96 h post fertilization larvae (Parolini et al., 2017). Lastly, Spasiano and coauthors (2016) investigated the potential adverse effects induced by BE and its transformation by-

products due to UV_{254}/H_2O_2 process in four different model species. BE and its by-products did not affect the growth of *Raphidocelis subcapitata* and the viability of *D. magna* individuals, even if an increase of lipid droplets within the body of cladocerans were noted. Differently, the viability of *Caenorabditis elegans* was seriously influenced by the exposure to both BE and its by-products, while a marked genotoxicity was found in *Vicia faba* individuals, showing an increase of cytogenetic damage during the cell mitosis of primary roots.

All these studies highlighted the potential sub-lethal toxicity of BE towards aquatic non-target organisms and suggested a central role of oxidative stress in the mechanism of action of this molecule. However, they were only focused at biochemical and/or cellular levels of the bio-ecological organization, while no investigations concerning the potential consequences at higher hierarchical levels have been performed. The first effect induced by the exposure to a toxicant appears at the sub-organism level and then it tends to propagate to the higher hierarchical levels of the bio-ecological organization through a bottom-up mechanism. The propagation of that signal can lead to a plethora of adverse effects that can influence the eco-ethological performances of exposed individuals and, consequently, populations. In addition, this effect can propagate at community level, impairing ecological relationships (e.g. the prey-predator relationship). The investigation on the linkage between responses at different levels of the ecological hierarchy remains a challenge in ecotoxicology (Amiard-Triquet 2009). Some recent studies of aquatic organisms have related biomarkers endpoints involved in crucial physiological responses with behavioral responses (e.g., Castro et al. 2004; Wallace and Estephan 2004; Sandahl et al. 2005; Kennedy and Farrell 2006; Ballesteros et al. 2009; Gravato and Guilhermino 2009). In fact, behavior is linked to diverse contaminant-induced stress responses, and alterations in some behavioral endpoints have been associated with biochemical and/or physiological changes (e.g., Weis et al. 2001; Peakall et al. 2002; Moreira et al. 2006; Gravato and Guilhermino 2009). For instance, the impairment in locomotion has been related to changes of neural, metabolic and endocrine processes in aquatic animals (Baatrup, 2009). Locomotor alterations can induce detrimental consequences also at higher levels of the biological organization causing direct or indirect effects on the population growth rate and changes in the intra- and inter-specific relationships. In spite of these findings, the effect of an illicit drug at different levels of the ecological hierarchy in an aquatic non-target species has never been investigated so far.

The present study was aimed at evaluating the adverse effects induced by the main cocaine metabolite, the benzoylecgonine (BE) at two environmentally relevant concentrations (0.5 µg/L and 1.0 µg/L) in the cladoceran *Daphnia magna*. We decided to test the toxicity of these concentrations because they both fall in the range of concentrations found in aquatic system worldwide. In detail,

the lowest tested concentration was close to the highest BE concentration found in surface waters, while the highest one was similar to the mean concentration of BE measured in the influents of wastewater treatment plants worldwide (see Pal et al., 2013 and references therein). In addition, in our previous studies we assessed the toxicity of the same BE concentrations on the zebra mussel *Dreissena polymorpha* (Parolini et al., 2013) and on zebrafish (*Danio rerio*) embryos (Parolini et al., 2017). BE-induced adverse effects were studied by a multi-level approach at biochemical/biomolecular (biomarkers), individual (swimming activity) and population (reproduction) levels. Regarding biomarkers, we mainly focused on oxidative stress-related endpoints because previous studies, conducted on aquatic organisms treated with BE, showed an overproduction of ROS, the impairment of antioxidant defenses and the occurrence of oxidative damage (Parolini et al., 2013; Parolini et al., 2017). Thus, we measured the amount of ROS, the activity of antioxidant (SOD, CAT, and GPx) and detoxifying (GST) enzymes, as well as the lipid peroxidation (TBARS). In addition, we also measured the acetylcholinesterase (AChE) inhibition because it is directly/indirectly involved in crucial functions for the survival, growth and reproduction, in both invertebrate and vertebrate species (Rosenberry, 2006). For instance, contaminant-induced changes in AChE activity may affects behavioral endpoints related to locomotion and feeding activity in aquatic species, including *D. magna*, which may results in reduced growth and reproduction, as well as in changes of predator avoidance behavior (e.g., Lovern et al., 2007). At individual level, the swimming activity of *D. magna* was investigated by a video tracking approach, while a chronic toxicity test was performed to assess the potential effects of BE on reproduction. Effects of BE on biomarker and swimming behavior were investigated in *D. magna* individuals (8-day old at the beginning of the exposure) after a 48 hours of exposure, while effects on reproduction were evaluated following the reproductive cycle of single daphnids (younger than 24 hours old at the beginning of the exposure) for 21 days. We investigated sub-individual and individual effects *D. magna* specimens after 48-h of exposure because we would like to evaluate the capability of BE to induce oxidative stress, to modulate AChE activity and to alter the swimming behavior by excluding any potential confounding effects of reproduction, which were then investigated by a standard 21-d reproduction test (OECD, 2012). Our choice was also due to experimental constraints because for video-tracking analyses we had to use 8-day old *D. magna* individuals, which were sufficiently large to be recorded and their movements tracked. So, to avoid effects of reproduction we could not expose *D. magna* specimens more than 48 hours because after the tenth day of life the most of individuals begins parthenogenic reproduction. Our multi-level approach allowed to investigate and to follow the propagation of BE-induced effects at different

levels of the ecological hierarchy, as well as to interpret these effects on individuals in a broader ecological context.

2. MATERIAL AND METHODS

The analytical standards of benzoylecgonine (BE) and benzoylecgonine-d3 (BE-d3) were purchased from Cerilliant Corporation (Round Rock, Texas, USA) as liquid solutions in methanol. Methanol for pesticide analysis, and hydrochloric acid (37%) were from Carlo Erba (Italy); ammonium hydroxide solution (25%) and acetic acid for LC-MS (> 99%) were obtained from Fluka (Buchs, Switzerland). Acetonitrile for LC-MS was purchased from Riedel de Haen (Seelze, Germany). A MILLI-RO PLUS 90 apparatus (Millipore, Molsheim, France) was used to obtain the HPLC grade Milli-Q water used throughout the study. The cartridges employed for solid phase extraction were 3-mL disposable Oasis MCX (60 mg) from Waters Corp. (Milford, MA, USA). The chromatographic separation was performed using an Atlantis T3 column (2.1 x 150 mm, 3 µm) from Waters Corp. (Milford, MA, USA). All the reagents used for biomarker analyses were purchased from Sigma-Aldrich (Steinheim, Germany).

2.1 Experimental plan

Adult *Daphnia magna* specimens came from a single clone obtained from the Istituto Superiore di Sanità (Roma, Italy) and they were cultured (30 individuals/L) in a commercial mineral water (San Benedetto® - conductivity 415 µS cm^{-1} at 20 °C; pH 7.42; 301 mg/L HCO_3^-, 48.6 mg/L Ca^{2+}; 28.2 mg/L Mg^{2+}). Daphnids were cultured in 400 mL beakers (40 individuals/L) and fed *ad libitum* with a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (8×10^6 cells ind^{-1} day^{-1} until they were 8-day old, then 16×10^6 cells ind^{-1} day^{-1}) and the yeast *Saccharomyces cerevisiae* (15×10^6 cells mL^{-1}) three times a week. The culture medium was renewed every second day. Culture medium and exposure solutions were maintained at 20.0 ± 0.5 °C under a 16 h light: 8 h dark photoperiod, which are conditions ensuring continuous amictic parthenogenetic reproduction (Frey, 1982). Algae were cultured in ISO 8692:1989 medium in 2 L flask at 20.0 ± 2.0 °C under continuous light and shaken through aeration. Algae were harvested during their exponential growth and let for sedimentation in the dark at 4 °C for a week, supernatant was discharged and cell density was measured using a Burker chamber under a brightfield light microscope. For BE exposures we planned two experiments: a short-term BE exposure (48h) and a 21 day-exposure (*D. magna* chronic test). Short-term exposure was performed using 8-day old individuals from the fourth

reproduction cycle because they reached the minimum dimension allowing the video tracking of their swimming activity (personal observation). Exposures were performed in 200 mL beaker under semi-static conditions renewing BE solution (1 mg/L in ultrapure water) after 24 hours from the beginning of the experiment. To confirm the effectiveness of the exposures, we collected a water sample from both control and exposure beakers one hour after the spiking of BE and after 24-h of exposure. Twenty 8-day old individuals were transferred into each beaker and exposed for 48-h to two concentrations of BE (0.5 µg/L and 1.0 µg/L). Negative control beaker containing only culture water without chemical was included in all experimental replicate. Individuals were not fed during the experiments. We performed three independent experimental replicates per treatment to assess oxidative stress-related endpoints, and AChE activity. At the end of 48-h exposures individuals were transferred to a 1.5 mL Eppendorf tube, frozen in liquid nitrogen and stored at – 80 °C until the biochemical analyses.

2.2 Chemical analysis of BE in water

The chemical analysis of water samples to check BE concentrations was carried out by solid phase extraction (SPE) followed by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) adapting a methodology previously published (Castiglioni et al., 2011). Aliquots of 5 mL for control samples, samples spiked at 0.5 µg/L and samples spiked at 1.0 µg/L were extracted using mixed reverse-phase cation exchange cartridges (Oasis-MCX). Before extraction, the pH of each aliquot was adjusted to 2.0 with 37% HCl, and 2 ng of the labeled deuterated analog (benzoylecgonine-d3) was added to be used as internal standard. MCX cartridges were conditioned before use by washing with 5 mL methanol, 3 mL Milli-Q water, and 3 mL water acidified to pH 2. Samples were then passed through the cartridges at a flow rate of 5 mL/min. Cartridges were vacuum-dried for 10 min and eluted with 2 mL of methanol and 2 mL of a 2% ammonia solution in methanol. The eluates were pooled and dried under a gentle nitrogen stream. Dried samples were redissolved in 100 µL of Milli-Q water, centrifuged for 2 min at 2,500 rpm, and transferred into glass vials for HPLC injection. HPLC-MS/MS determination was performed using a 1200 Series Binary Pump and Autosampler (Agilent Technologies, Santa Clara, CA, USA) coupled to a mass spectrometer with a triple quadrupole detector and a turbo ion spray source (API 5500, Applied Biosystems–Sciex, Thornhill, Ontario, Canada). The chromatographic separation was performed by gradient elution using 0.1% acetic acid in Milli-Q water as solvent A and acetonitrile as solvent B at a flow rate of 200 µL/min. The analysis started with 99% of eluent A for 3 min, followed by a 20-min linear gradient to 60% of eluent B and a 1-min linear gradient to 100% of eluent B, which was maintained for 3 min. The initial conditions (99% of eluent A) were then achieved in 0.5 min and

were maintained for 8 min to equilibrate the column. The injection volume was 4 µL and the column was kept at room temperature. The MS analysis was done in the positive ion mode with a spray voltage of +5.5 kV and a source temperature of 400 °C. The Multiple Reaction Monitoring (MRM) mode was used for analysis, choosing the 2 most abundant fragmentation products of the protonated pseudo molecular ions of benzoylecgonine and its deuterated analog (benzoylecgonine-d3). Quantitation of BE was performed using the isotopic dilution method and calibration curves were made freshly before each analytical run. The method quantitation limit (MQL) was calculated as the concentration at which the signal-to-noise ratio was 10 and it was 0.8 ng/L.

2.3 Biomarker methods

The biomarker suite applied in the present study was performed on homogenates from a pool of all living *D. magna* individuals found in each beaker at the end of the exposure (0.5 µg/L and 1.0 µg/L BE, and negative control). Three independent experimental replicates (n = 15 individuals per each single replicate) were performed for each treatment. As it cannot be excluded that BE was removed from the outer carapax, *D. magna* individuals were washed trice before biochemical analyses with 0.5 mL of homogenization buffer to prevent potential bias caused by *in vitro* interactions. After the washes, individuals were homogenized using a motor pestle in a 100 mM potassium phosphate buffer (added with KCl 100 mM, EDTA 1 mM, protease inhibitors 1:100 v/v and dithiothreitol 1 mM, pH 7.4). The homogenates were centrifuged at 15,000 x g for 15 minutes at 4 °C, then the supernatant was collected and immediately processed to determine protein content, SOD, CAT, GPx, GST and AChE activity through spectrophotometric methods. All the enzymatic activities were measure in triplicate per each pool. Briefly, SOD activity was assessed by measuring the inhibition of cytochrome c (10 µM) reduction by the superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 µM) reaction for 1 min at λ = 550 nm. Results were expressed as SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction). The CAT activity was assessed by measuring the consumption of H_2O_2 (50 mM) in potassium phosphate buffer (100 mM; pH 7) for 1 min at λ = 240 nm. The GPx activity was assessed monitoring for 1 min the consumption of NADPH at λ = 340 nm using H_2O_2 (0.2 mM) as substrate in potassium phosphate buffer (50 mM; pH 7) including glutathione (2 mM), sodium azide (1 mM), glutathione reductase (2 U/mL), and NADPH (120 µM). The GST activity was measured by adding reduced glutathione (1 mM) in phosphate buffer (100 mM; pH 7.4) and using CDNB (1 mM) as substrate. The reaction was monitored for 1 min at λ = 340 nm. AChE activity was measured according to the method described by Jemec et al. (2007), with slight modifications. The reaction mixture (1.5 mL) was prepared in potassium phosphate buffer (100 mM, pH 7.4) containing acetylthiocholine

chloride (1 mM) and 5,5' dithiobis-2-nitrobenzoic acid (0.5 mM), then 100 µL of supernatant was added. The reaction was monitored for 15 min at λ = 412 nm and AChE activity was expressed as nmoles of acetylcholine chloride hydrolyzed min^{-1} mg $protein^{-1}$ (ε = 13,600 M^{-1} cm^{-1}). The activity of enzymes was normalized on protein concentration measured according to the Bradford method, using bovine serum albumin as a standard. ROS measurement was performed using dichlorofluorescein-diacetate (DCFH-DA), adapting the fluorescence method by Deng and co-workers (Deng et al., 2009). *D. magna* individuals (n = 15 per experimental replicate) were washed twice with homogenization buffer and homogenized in a 100 mM potassium phosphate buffer (see above). The homogenate was then centrifuged for 20 min at 15,000 × g at 4 °C. Twenty microliters of the homogenate was added to a 96-well plate and incubated for 5 min at room temperature. Then, 100 µL of PBS and 8.3 µL of DCFH-DA (10 mg/mL in DMSO) were added to each well and the plate was incubated at 37 °C for 30 min. The fluorescence intensity was measured by a microplate reader with excitation at λ = 485 and emission at λ = 536 nm, respectively. The ROS concentration was expressed in arbitrary units as AU DCF mg $protein^{-1}$. Lipid peroxidation was assessed by the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al, 1979), adapted to tissue homogenates of 8-day old individuals and expressed as nmol TBARS mg $protein^{-1}$.

2.4 Video tracking and swimming activity analysis

We performed three independent replicates per treatment to assess changes in swimming activity induced by the exposure to the BE selected concentrations. Video tracking analyses were performed on all living individuals at the end of BE exposure into 24-well plates and tracked individually (each well contained 1 specimens and 3 mL of exposure medium). Video recordings were carried out by placing the 24-well plate (well dimension 25 mm × 25 mm × 10 mm) with 12-15 animals on a light panel, and the movement of each individual was tracked for 15 seconds for three times. We tracked the movement of at least 40 individuals per treatment. The three 1080p Full HD videos acquired for each specimens were analyzed using the software LoliTrack v.4 (Loligo Systems, Tjele, Denmark). This software was calibrated to measure the following endpoints: time of activity (%) and swimming velocity (mm/s). The tracking was based on differences in contrast between objects (animals) and background (water) without use of markers. When the object appeared against a contrasting background, the software assigned a coordinate pair (x, y) to the centroid of the contrasting object. Each well in the 24-well plates was defined as an arena, and each individual was considered as a single object. According to manufacturer manual, the lowest threshold for activity is defined as activity threshold (pixels; e.g. if the object moves a distance larger than this minimum

distance between frames, the object is scored as active). If the object is active, the speeds of movement(s) are calculated frame by frame.

2.5 Chronic toxicity test

Chronic toxicity evaluation at the two BE concentrations (0.5 µg/L and 1.0 µg/L) was performed in agreement with the standard 21 days chronic reproduction test (OECD, 2012). For each treatment group, 10 replicates of 1 specimens (< 24 h old) were used. The exposures were conducted in glass beakers containing 50 mL of test medium. The exposure was performed at 20.5 ± 0.5 °C under a 16 h light:8 h dark photoperiod. *D. magna* individuals were transferred every single day to clean glass beakers filled with freshly prepared medium and fed with *P. subcapitata* and *S. cerevisiae* (see above), to which was then added the exact amount on BE to reach the selected concentrations. Every day, the number of living, immobile or dead offspring were recorded, until the 21st day.

2.6 Statistical analysis

The effect of BE exposure on the amount of ROS, enzyme activity and swimming behavior of 8-day old *D. magna* individuals was investigated by using linear mixed models (LMM) including the treatment as fixed effect factor and the exposure tank as random effect. When a significant effect of treatment was found, a Fisher LSD post-hoc test was applied to point out significant differences among treatments. Significance was set at $p < 0.05$ (*) and $p < 0.01$ (**). Statistical analyses were performed using IBM SPSS Statistics 21.0 software package.

3. RESULTS

3.1 Concentration of BE in water and immobilization/mortality events

To check the reliability of the exposure we measured the concentration of BE in water from both control and exposure beakers. No residues of BE were found in control beakers, whereas no variation in BE concentration occurred neither in 0.5 µg/L nor in 1.0 µg/L treatment over the 24-h exposure. In detail, the mean (±standard deviation) BE concentration 1 h after the spike in water was 501.38±12.86 ng/L (0.5±0.01 µg/L) and 974.36±123.59 ng/L (0.9±0.1 µg/L), while after 24 hours of exposure it was 501.14±20.14 ng/L (0.5±0.02 µg/L) and 944.47 ±72.39 (0.9±0.1 µg/L) ng/L for 0.5 µg/L and 1.0 µg/L treatment, respectively.

Immobilization/mortality at the end of the short-term toxicity test (48 h) was below 10% in all treatments (0.5 µg/L, 1.0 µg/L, and control,) and for every replicates. Considering that 10% of immobilization/mortality is accepted in the control (OECD, 2004) for the standard *D. magna* acute toxicity test, we concluded that tested concentration were below acute toxicity range. Mortality (mean of all the independent replicates we performed in the present study; n = 14 replicates per treatment for a total of 210 individuals per treatment) of *D. magna* individuals found at the end of the 48-h exposure was 4.3 % in control, 6.7 % and 3.9 % in 0.5 and 1.0 µg/L beakers, respectively. No significant difference in *D. magna* individuals' mortality among treatments was found ($p > 0.05$).

3.2 Biomarker results

BE exposure induced a significant ($F = 6.030$; $p < 0.01$) increase of ROS in response to the highest tested concentration, with a 13% increase with respect to control (Figure 1a). In spite of no significant effect of BE treatment on SOD ($F = 0.330$; $p > 0.05$) and CAT ($F = 0.877$; $p > 0.05$) activity (Figure 1b and 1d), a significant increase of GPx ($F = 4.172$; $p < 0.05$) was noted at the end of the exposure to the highest BE concentration, showing a 68% increase compared to control (Figure 1c). A significant ($F = 7.505$; $p < 0.01$) increase in GST activity was found at both the BE tested concentrations, with a 80% and 46% increase found at 0.5 µg/L and 1.0 µg/L with respect to control, respectively (Figure 1e). A significant increase of lipid peroxidation ($F = 10.442$; $p < 0.01$) was found after the exposure to the highest BE concentration, with values 2-fold higher than the control (Figure 1f). BE exposure had a significant effect ($F = 35.497$; $p < 0.01$) on AChE activity of *D. magna* individuals, showing a significant inhibition (-36%) at the end of the exposure to both the BE tested concentrations (Figure 2).

3.3 Swimming activity results

BE exposure caused a significant ($F = 18.041$; $p < 0.01$) reduction in the activity of *D. magna* individuals, showing a 5% decrease in treated specimens at the highest tested concentration compared to the control (Figure 3a). In contrast, the same treatment induced a significant increase of the swimming velocity ($F = 38.984$; $p < 0.01$) of treated specimens with respect to control (Figure 3b).

3.4 Chronic toxicity test results

A marginally significant effect of BE treatment ($F = 3.635$; $p = 0.049$) on the total number of offspring was noted, with a decrease of 43% and 39% caused by the exposure to 0.5 µg/L and 1.0 µg/L compared to control (Figure 4a). Accordingly, a significant reduction of the number of parthenogenetic cycles ($F = 4.533$; $p < 0.05$) was found, showing that reproductive events of specimens treated with 0.5 µg/L and 1.0 µg/L of BE were 45% and 21% lower compared to the control (Figure 4b). In contrast, no significant effects of BE treatments on the mean number of offspring per reproductive cycle ($F = 0.211$; $p > 0.05$) and on the mean number of days to the first reproduction event from the beginning of the experiment ($F = 0.454$; $p > 0.05$) was found (data not shown).

4. DISCUSSION

A number of studies showed that cocaine causes damage on structure and function of diverse organs due to dissimilar mechanisms of actions. For instance, some detrimental effects are caused by the overstimulation of the adrenergic system, while the most of the direct toxic effects are promoted by oxidative stress and mitochondrial dysfunction occurring during metabolism (Riezzo et al., 2012). However, the toxicity of cocaine involves also its main metabolites, such as the benzoylecgonine. Some studies pointed out the bioactivity of BE, showing that this molecule can induce both physiological and behavioral effects on murine models (Morishima et al., 2001), but also on terrestrial and aquatic non-target organisms (Parolini et al., 2013; García-Cambero et al., 2015; Parolini et al., 2017). Oxidative stress seems to be involved in the mechanism of action of BE, as demonstrated by the increased production of reactive oxygen (ROS) species in peritoneal macrophages isolated from treated mice, which can cause hepatic and cerebral toxicity (Vaz et al., 1994), as well as in the zebra mussel (Parolini et al., 2013; Pedriali et al., 2014) and in zebrafish embryos (Parolini et al., 2017). In addition, some studies showed that BE is neurotoxic for murine models (Nassogne et al. 1998; Bunney et al. 2001). Results from the present study showed that the

exposure to two concentrations of BE similar to those found in the aquatic system worldwide altered the oxidative status and inhibited *Daphnia magna* AChE activity, affecting swimming behavior and reproduction.

Although no increase of ROS was caused by the exposure to the lowest BE tested concentration, the highest one promoted a significant ROS overproduction (Figure 1a), in accordance with a previous study performed on zebrafish embryos exposed up to 96 hours post fertilization to similar BE concentrations (Parolini et al., 2017). Cocaine metabolites, including BE, may be involved in the activation of redox cycles, the depletion and/or the decrease of antioxidant enzymes, and the consequent overproduction of ROS, leading to oxidative stress situations, even if the exact mechanisms of toxicity are not fully understood (Neri et al., 2013). Accordingly to ROS measurements, no significant effects of BE on enzyme activity was found at the end of the exposure in 0.5 µg/L BE-treated individuals compared controls. However, the BE-induced increase of ROS levels due to 1.0 µg/L exposure modulated the antioxidant enzyme activity in treated *D. magna* specimens with respect to control. The lack of increase in SOD activity (Figure 1b) suggests that both BE concentrations did not cause and overproduction of superoxide anion, and consequently of hydrogen peroxide, the final product of $O_2^{\bullet -}$ dismutation. However, the significant induction of GPx after 1.0 µg/L BE treatment (Figure 1d) indicated that BE promoted the production of hydrogen peroxide, which could be produced by other cellular enzymes like those contained in peroxisomes (Khessiba and Roméo, 2005). In contrast, no significant effect of BE treatments on CAT activity was noted (Figure 1c). Although GPx and CAT play a concomitant role to counteract the toxicity of hydrogen peroxide, the discrepancy between their responses could be due to their competition for the same substrate (i.e. H_2O_2; Kappus, 1985) or, alternatively, to the activation of CAT exclusively when the concentration of H_2O_2 is extremely high (Pereira et al., 2013). These results are consistent with those found in our previous study performed on zebrafish embryos at 96 hours post fertilization, in which concentrations of BE similar to those we tested here caused and induction of GPx but not of CAT (Parolini et al., 2017). In contrast, a 21-day exposure to the same BE treatments showed an opposed response in the zebra mussel *Dreissena polymorpha*, depending on the tested concentration. Whilst 0.5 µg/L of BE caused a significant increase of SOD, GPx and CAT activity, the exposure to 1.0 µg/L determined a significant inhibition of the three antioxidant enzymes (Parolini et al., 2013). The contrasting results occurring among biological models exposed to the same BE treatments may be related to the sensitivity to this cocaine metabolite at different developmental stage and/or to the duration of the exposure. In fact, some studies have demonstrated that early-life stages are more sensitive than adults and show an early response to the exposure to environmental pollutants. Other studies showed that the activity of antioxidant enzymes can

increase when the organism is exposed to low concentrations of chemical or during short-term exposures, but it can decrease or be inhibited at high concentration or after prolonged exposure (Valavanidis et al., 2006; Wang et al., 2011). Lastly, the induction of GST found after the exposure to both the BE concentrations suggests the involvement of phase II detoxification enzymes in the metabolism of BE in *D. magna*. Overall, the increase of ROS coupled with the impairment of antioxidant defenses showed in individuals treated with 1.0 µg/L of BE may suggest an imbalance of the equilibrium between pro- and antioxidant molecules in favor to the former, leading to an oxidative stress situation that can negatively affect *D. magna* health status. In fact, when an organism undergoes oxidative stress, it can experience detrimental oxidative damage to cellular macromolecules, including lipids, proteins and DNA may occur, resulting in alteration of their structure and functionality, disruption of cellular activity and organ damage. The increase of lipid peroxidation in *D. magna* treated with the highest BE concentration (Figure 1f) supported our hypothesis regarding the oxidative stress situation experienced by *D. magna* individuals because it suggested that ROS were not totally scavenged by the antioxidant enzymes and promoted oxidative damage to lipids. These results are in accordance with those found in a previous study where zebra mussel specimens were exposed for 14 days to the same BE concentration (Parolini et al., 2013). In addition, a number of studies have demonstrated that oxidative stress plays a crucial role in both the regulation and activity of AChE. For instance, an *in-vitro* study by Schallreuter and co-authors (2004) showed that low hydrogen peroxide activated human recombinant AChE, while high concentrations inhibited the enzyme activity. Oxidative stress changed AChE activity *in vivo* during hypertension (De Carvalho Corrêa et al., 2008), while ROS production due to ethanol exposure alters the expression and the activity of AChE (Rico et al., 2007). Hydrogen peroxide also inhibited AChE activity in human erythrocyte membrane (Molochkina et al., 2005) and skin cells (Schallreuter and Elwary, 2007). In addition, reduced expression and activity of AChE were related to an increase of oxidative stress in zebrafish embryos treated with the pro-oxidant molecule t-butyl hydroperoxide (Rodríguez-Fuentes et al., 2015). A significant inhibition of AChE was found in *D. magna* exposed to both BE concentrations (Figure 2). Since no evidence for direct action of BE on AChE expression and/or activity was found in any species and the amount of ROS was not increased by the exposure to 0.5 µg/L of BE, the significant AChE inhibition at the lowest tested concentration was unexpected and we do not have any reasonable explanation to this result. In contrast, the ROS overproduction induced by the highest treatment of BE may affect the activity of this crucial enzyme that hydrolyzes the neurotransmitter acetylcholine in cholinergic synapses allowing the effective control and modulation of the neural transmission in both vertebrates and invertebrates (Oliveira et al., 2012). By a functional point of view, a number of studies showed that

452 the decrease and/or the inhibition of AChE activity altered diverse behavioral endpoints, which may
453 subsequently affect fitness and survival of the exposed organisms (Beauvais et al., 2000; Castro et
454 al., 2004; Cooper and Bidwell, 2006; Sismeiro-Vivas et al., 2007). For instance, the exposure to
455 diverse contaminants can alter the swimming and filtering activity of *D. magna*; indeed, some
456 studies showed that the swimming activity was reduced by the exposure to heavy metals or organic
457 pollutants in diverse aquatic organisms (e.g. Little et al., 1990; Kavitha and Venkateswara Rao,
458 2007; 2008), including *D. magna* (e.g., Dodson et al., 1995; Baillieul and Blust, 1999; Untersteiner
459 et al., 2003; Cerbin et al., 2010). Whilst, in accordance to biomarker results, no effects on the
460 parameters chosen to assess effects of BE on locomotor activity were induced by the exposure to
461 the lowest tested BE concentration, individuals treated with 1.0 µg/L of BE showed significant
462 changes in both the considered endpoints (Figure 3). In fact, in spite of a significant decrease of
463 swimming activity caused by the highest BE treatment (Figure 3a), an unexpected significant
464 increase of swimming velocity (Figure 3b) was noted. These findings are surprising because usually
465 both the parameters are strictlycorrelated in *D. magna* (Untersteiner et al., 2003). In addition, many
466 studies showed that swimming velocity is reduced in response to the exposure to some toxic
467 chemicals (e.g., Baillieul and Blust, 1999; Untersteiner et al., 2003). This discrepancy may be
468 related to the different mechanism of action occurring between diverse toxic molecules. Although
469 the exposure to 1.0 µg/L BE decreased the total swimming activity of *D. magna* individuals, this
470 molecule could promote high velocity jerky movements, which can results in an overall increase of
471 individuals' velocity. This hypothesis was supported by previous studies on young rats, which
472 showed seizures (Erickson et al., 1990), behavioral activation characterized by jumping, jerking and
473 various degrees of tonic seizures after direct intraventricular injection of BE (Konkol et al.,
474 1992a,b). Since the locomotion of *D. magna* depends on a continuative, high energy demanding,
475 muscular activity, the decrease of swimming activity under BE exposure may be due to the high
476 energy demand of the organism to support essential physiological processes to counteract the
477 toxicity of the chemical. Thus, as swimming behavior derives from the integration of physiological,
478 sensorial, nervous and muscular systems (Charoy et al., 1995), our results should indicate a general
479 impairment of the health status of BE-treated *D. magna*, which could lead to adverse effects on
480 fitness and survival of the organism. The reduced performance in swimming we found could
481 negatively affect the filtering activity and, consequently, the food uptake of treated individuals,
482 which can lead to a drastic reduction in growth and reproduction (Baillieul, 1997), being food
483 uptake one of the main driving forces of the latter (Enserink et al., 1993). According to this
484 expectation, the exposure to the highest BE concentration reduced the number of parthenogenetic
485 cycles and the total number of offspring (Figure 4a and 4b, respectively). In addition, changes in

locomotor activity may affect prey-predator relationship, causing a potential alteration to the trophic interactions occurring between phyto- and zooplankton, as well as between zooplankton and fish (Uttieri et al., 2014). *D. magna* is one of the most important phytoplankton grazers in freshwater systems, thus changes in population dynamics of this cladoceran could result in serious consequences also on phytoplankton species. At the same time *D. magna* represents one of the major dietary components of diverse fish species (Dodson and Hanazato, 1995) and its swimming behavior is a pivotal component of prey selection and predator avoidance (Schmidt et al., 2005). Some studies showed that a reduced movement of the zooplankton could diminish the ability of the predator to locate its prey, decreasing the risk of predation (Zaret, 1980), while irregular movements may increase visibility to predators and predation risk (Strickler et al., 2005). For instance, Brewer and Coughlin (1996) showed that virtual *D. magna* with a higher hopping frequency were more vulnerable to attack by a predator such as the bluegill *Lepomis macrochirus*. Similar results were obtained by O'Keefe and co-authors (1998), which reported that faster swimming *D. magna* individuals were preferentially predated by the bluegill. Thus, the contrasting alteration of the considered swimming parameters suggests that exposure to BE may positively or negatively affect the predation risk of *D. magna* specimens by a fish predator. Due to the complexity of the obtained results and the ecological relevance of this issue, further study should be necessary to understand the role of BE in altering the ecological relationships between aquatic species.

5. CONCLUSIONS

Our findings showed that the exposure to environmentally relevant concentrations of BE may induce notable adverse effects to *Daphnia magna* specimens at different level of biological organization. The exposure to 1.0 µg/L of BE induced an oxidative stress situation in *D. magna*, leading to behavioral and reproductive effects. The effects on *D. magna* reproduction may result particularly worrisome because they can negatively affect the population dynamic of this cladoceran species and, consequently, food web interactions. In addition, these results highlighted the linkage between biochemical, behavioral and reproductive endpoints, confirming the potential of biomarker techniques as early predictors of toxicant-induced alterations also at higher hierarchical level. Then, our findings confirmed the reliability of a suite of biomarkers to suppose the possible mechanisms of action of an emerging pollutant and the usefulness of behavioral and reproductive endpoints to clarify the eventual ecological hazard of a single focal chemical. Our results are particularly alarming because BE is the main illicit drug residue found in the aquatic system worldwide and the concentrations we tested in the present study are similar to those currently measured in aquatic

environments. Moreover, considering the uninterrupted use of COC worldwide and the human metabolism, an incessant BE input in freshwater and its consequent increase in concentration is expected. This trend can confer to BE a sort of pseudo-persistence, representing a critical aspect for the environmental risk assessment of this drug residue; indeed, aquatic organisms are exposed to BE, as well as to other drugs, for their whole life-span. This may result in possibly higher toxic effects than those we pointed out here. Considering these noteworthy issues, further investigations on the adverse effects of BE to aquatic organisms at different level of the ecological hierarchical scale should be a priority in order to shed light on its true ecological hazard for freshwater ecosystem.

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721

722 Figure captions

723

724 **Figure 1:** mean (±SD) of the amount of reactive oxygen species (ROS; a) SOD (b), GPx (c), CAT
725 (d), GST (e) and lipid peroxidation (f) measured in *D. magna* specimens after 48-h treatment with
726 0.5 µg/L and 1.0 µg/L of BE. Asterisks above the histograms show significant differences between
727 treated and control specimens (* $P < 0.05$; ** $P < 0.01$).

728

729 **Figure 2**: mean (±SD) acetylcholinesterase (AChE) activity measured in *D. magna* specimens after
730 48-h treatment with 0.5 µg/L and 1.0 µg/L of BE. Asterisks above the histograms show significant
731 differences between treated and control specimens (* $P < 0.05$; ** $P < 0.01$).

732

733 **Figure 3**: mean (±SD) swimming activity (a) and velocity (b) measured in *D. magna* specimens
734 after 48-h treatment with 0.5 µg/L and 1.0 µg/L of BE. Asterisks above the histograms show
735 significant differences between treated and control specimens (* $P < 0.05$; ** $P < 0.01$).

736

737 **Figure 4**: mean number (±SD) of offspring (a) and parthenogenetic cycles (b) of *D. magna*
738 specimens after 21-day exposure to 0.5 µg/L and 1.0 µg/L of BE. Asterisks above the histograms
739 show significant differences between treated and control specimens (* $P < 0.05$; ** $P < 0.01$).