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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-h 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.

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, 2016). 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 7500 ng/L and 3425 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

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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., 2013). 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 et al. (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, 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 h of exposure, while effects on reproduction were evaluated following the reproductive cycle of single daphnids (younger than 24 h 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, 2004). 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 h 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

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