Biochemical and behavioral effects induced by cocaine exposure to *Daphnia magna*

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Abstract

Illicit drugs and their metabolites have been identified as emerging aquatic pollutants. Cocaine (COC) is one of the most used illicit drug worldwide. After human consumption, COC enters the aquatic ecosystems, where it is commonly detected in ng L^{-1} concentration range. Although a number of studies has shown that the exposure to environmental concentrations of COC can induce diverse biochemical, molecular and histological effects on aquatic organisms, the information of COC-induced behavioral alterations is scant. Thus, the present study aimed at exploring both biochemical and behavioral effects induced by the exposure to two environmental concentrations (50 ng L^{-1} and 500 ng L^{-1}) of COC on the freshwater cladoceran *Daphnia magna*. Specimens were exposed to selected COC concentrations for 21 days and the effects on the oxidative status, including the amount of reactive oxygen species and the activity of antioxidant (SOD, CAT and GPx) and detoxifying (GST) enzymes, and swimming activity were investigated after 7, 14 and 21 days of treatment, while effects on reproductive success was assessed after 21-days only.. Exposure to COC induced an overproduction of reactive oxygen species and a modulation of the activity of defense enzymes. Moreover, COC affected the swimming behavior and altered the reproductive success of treated specimens. Our results highlighted that environmental concentrations of COC can cause adverse effects at different levels of the biological hierarchy in a zooplanktonic species, confirming the potential threat due to this illicit drug for the aquatic community.

Keywords: behavioral ecotoxicology; biomarkers; cocaine; *Daphnia magna*

1. Introduction

Illicit drugs have been considered for a long time as a dramatic socio-economic and public health problem. However, only recently they have been identified as a serious environmental issue, attracting the interest of analytical and environmental chemistry (Zuccato and Castiglioni 2009), as well as ecotoxicology (Capaldo et al. 2018; Parolini et al. 2013; 2017). Among illicit drugs, cocaine (COC), a psychostimulant affecting human behavior and brain physiology by the alteration of dopamine release from dopaminergic neurons (Jeon et al. 2008), represents one of the most used illicit drugs worldwide (UNODC, 2018). Indeed, the recent World Drug report (2018) has estimated that the global amount of COC users ranges between 13.9 and 229 million people (age range 15-64), showing an increase of ~7 % compared with the previous years (UNODC, 2018). After the ingestion of a COC dose, this drug undergoes hepatic metabolism and is excreted through urine and feces as two main metabolites, namely benzoylecgonine (BE, 45% of the dose) and ecgonine methyl ester (EME, 40%), and limitedly (1-9%) as unchanged parental compound (Baselt 2004). Thus, COC continuatively enters the sewage, whereby it has been monitored in concentrations up to 420 ng L^{-1} in the inlet water of wastewater treatment plants (WWTPs; Pal et al. 2103 and references therein). Moreover, considering that WWTPs cannot efficiently remove COC from the sewage, it reaches surface waters, whereby it was detected in concentrations ranging between 0.4 and 44 ng L^{-1} (Pal et al. 2013 and the reference therein), although two recent monitoring surveys carried out in Brazilian surface waters reported concentrations up to 5,896 ng L^{-1} (Thomas et al. 2014; Pereira et al. 2016). Despite the low COC concentrations currently found in aquatic ecosystems, the risk for the aquatic communities cannot be neglected. Although the toxicity of COC was well-known on humans (Leri et al. 2003; Spronk et al. 2013) and murine organisms (Brami-Cherrier et al. 2005; Dixon et al. 2010), the information on aquatic organisms is still limited. A preliminary study showed that exposure to three increasing COC concentrations (range 40 ng L^{-1} - 10 µg L^{-1}) induced cytotoxic and genotoxic effects on the freshwater bivalve *Dreissena polymorpha* (Binelli et al. 2012). Similar cyto-genetic

effects have been found in 96 hours post fertilization (hpf) larvae of zebrafish (*Danio rerio*) exposed to COC (0.01 – 10 μg L^{-1} range) and have been caused by an overproduction of reactive oxygen species (ROS) that imbalanced the oxidative status of larvae (Parolini et al. 2017). A companion proteomic study has revealed that the exposure to 0.3 and 1 μg L^{-1} of COC modulated the protein profile of 96 hpf zebrafish larvae, changing the expression of several proteins belonging to different functional classes, including cytoskeleton, eye constituents, lipid transport, lipid and energy metabolism, and stress response (Parolini et al. 2018a). Cyto-genotoxicity has been observed on the brown mussel (*Perna perna*) after the exposure to crack COC (0.5, 5.0, and 50.0 μg L^{-1}; dos Santos Barbosa Ortega et al. 2018). Gay et al. (2013) have demonstrated that an environmental concentration of COC (20 ng L^{-1}) modulated the levels of brain dopamines, catecholamines and pituitary activity, and induced histological alteration in diverse tissues and organs (Capaldo et al. 2019,2018; Gay et al. 2016) in the European eel (*Anguilla anguilla*). Moreover, the neurotoxicity of COC has been highlighted on planarians (Pagan et al. 2013), while injections of COC (ranging from 2.5 to 10 mg/g body weight) affected the locomotor activity of the crayfish (*Orconectes rusticus*; Nathaniel et al. 2012).

Thus, the present study was aimed to enlarge the knowledge of COC toxicity exploring biochemical and behavioral effects induced by a 21-days exposure to two environmental concentration of COC (50 ng L^{-1} and 500 ng L^{-1}) on the freshwater cladoceran *Daphnia magna*. Previous studies on both murine models (Muriach et al. 2010; Pomierny-Chamioło et al. 2013) and aquatic species have pointed out that COC exposure can induce an oxidative stress situation (Parolini et al. 2018a, 2017). Accordingly, we expect that COC can alter the oxidative status of cladocerans. Thus, a suite of oxidative stress-related biomarkers was evaluated: the amount of reactive oxygen species (ROS) and the activity of antioxidant (superoxide dismutase - SOD; catalase - CAT and glutathione peroxidase - GPx) and detoxifying (glutathione S-transferase - GST) enzymes. Moreover, as previous study on crustaceans have demonstrated that COC altered the locomotor activity of a crayfish species (Nathaniel et al. 2012), we expect changes in swimming activity of *D. magna,* which was investigated

by a video-tracking analysis. Effects of COC exposure on biochemical and swimming activity endpoints was investigated after 7, 14 and 21 days of exposures. Lastly, a 21-days reproduction test was performed to assess changes in the reproductive output of the model species and potential consequences at population level after 21 days of exposure. As no study has investigated the reproductive toxicity of COC on invertebrate species so far, we have no *a priori* expectation on the effects of this illicit drug on *D. magna* reproduction.

2. Materials and Methods

2.1 Chemicals and reagents

The analytical standard of cocaine (COC) was purchased from Sigma-Aldrich (Steinheim, Germany), after obtaining the permission for possession and use for scientific purposes by the Italian Ministry of Health (Decree n. SP/177, 11/12/2017). The deuterated analogue cocaine-*d3* (COC-*d3*), used as internal standard (IS), was acquired from Cerilliant Corporation (Round Rock, Texas, USA) as a solution of 0.1 mg/mL in acetonitrile (ACN). All the reagents used for biomarker analyses were purchased by Sigma-Aldrich (Steinheim, Germany). For chemical analysis, analytical grade methanol (MeOH) and hydrochloric acid (HCl, 37%) were purchased from Carlo Erba (Italy), ammonium hydroxide solution (25%) and acetic acid (AA) for LC-MS (>99%) from Fluka (Buchs, Switzerland) and ACN from Riedel de Haen (Seelze, Germany). HPLC grade Milli-Q water was obtained with a MILLI-RO PLUS 90 apparatus (Millipore, Molsheim, France). Solid phase extraction cartridges Oasis® MCX (60 mg, 3 cc) were purchased by Waters Corp. (Milford, MA, USA).

2.2 Daphnia magna *husbandry*

Daphnia magna individuals were cultured in 400 mL beakers (40 individuals L^{-1}) filled with a culture medium made of commercial mineral water (San Benedetto®) and fed *ad libitum* every other day with a suspension of the unicellular green algae *Pseudokirchneriella subcapitata* and the yeast

120 *Saccharomyces cerevisiae*. The culture was maintained at 20 ± 0.5 °C under a 16 hrs light:8 hrs dark
121 photoperiod to allow reproduction, which is parthenogenetic in this species. Details of husbandry
122 conditions are reported elsewhere (Parolini et al. 2018b).

123 *2.3 Experimental design*

124 We planned different exposures, aimed at exposing organisms to investigate biochemical and
125 swimming behavior effects, as well as reproductive alterations. First, a stock solution of COC (1 mg
126 L^{-1}; stock solution 1) was prepared by diluting a commercial standard solution (1 g L^{-1} in MeOH) in
127 the same commercial water used for the culture medium and used to perform exposures for
128 biochemical and swimming behavior analyses. Such exposures were performed in beakers filled with
129 100 mL of the culture medium to which 5 µL and 50 µL of stock solution 1 were added to reach the
130 selected exposure concentrations, 50 ng L^{-1} and 500 ng L^{-1} of COC, respectively. Moreover, a second
131 stock solution (100 µg L^{-1}; stock solution 2) was prepared to be used for chronic toxicity reproduction
132 test exposures, which were performed in 50 mL of culture medium to which 25 µL and 250 µL of the
133 stock solution 2 were added to reach 50 ng L^{-1} and 500 ng L^{-1} of COC, respectively. The concentration
134 of the stock solution was confirmed by liquid chromatography tandem mass spectrometry (LC-
135 MS/MS; see *2.4 Chemical analysis of COC in stock solution and exposure beakers*).
136 The 50 ng L^{-1} tested concentration was comparable with the maximum level of COC found in surface
137 waters worldwide, while the 500 ng L^{-1} reflected the value found in influents of WWTPs worldwide
138 (Pal et al. 2013). As we planned to investigate COC-induced effects on biochemical and swimming
139 activity endpoints every seven days and we could not perform repeated biomarker measures on the
140 same individuals, we planned three different exposures. In detail, we planned three experimental
141 groups (control, 50 ng L^{-1} and 500 ng L^{-1}), including three independent replicates (beakers) per
142 treatment, which lasted for 7, 14 or 21 days. All the exposures started at the same day and relied on
143 organisms born by the same mothers. Twenty daphnids less than 24 hours old were randomly selected
144 from husbandry beakers and seeded into beakers filled with 100 mL of culture medium and volumes

of the stock solution were added up to the selected concentrations (see above). As three beakers containing 20 daphnids each were prepared per treatment, including control, a total of 60 individuals per treatment for each time point (7, 14 or 21 days) were exposed. Overall, 540 daphnids were used to perform exposure for analysis of COC-induced effects on biochemical and swimming behavior endpoints. Moreover, in order to assess the effect of COC on *D. magna* reproduction, a 21-days chronic toxicity reproduction test was performed according to the OECD guidelines (OECD, 2004). Fifteen individuals (< 24 hours old) per experimental treatment, including control, were exposed individually into 50 mL glass beakers filled with culture medium to which 25 µL and 250 µL of the stock solution 2 were added to reach 50 ng L^{-1} and 500 ng L^{-1} of COC, respectively. The culture medium and the amount of COC were renewed every single day for 21 days, checking for the viability of individuals. The number of offspring born by each single individuals and the number of clutches over the 21 days of the exposure were recorded.

Although the COC standard solution was in MeOH, no solvent control treatment was planned. Considering the dilution performed to obtain the stock solution and to reach the selected concentrations in the test beakers, the estimated concentrations of MeOH in exposure beakers were expected to be negligible (maximum calculated amount of MeOH accounted for 0.03% of the final volume). Moreover, our preliminary analyses did not show significant differences between negative and solvent (MeOH) control for both biochemical and behavioral endpoints tested in the present study (unpublished data). Exposures were performed under semi-static conditions, renewing the culture medium and adding COC solution every day. Daphnids were fed ad libitum over the 21-days exposures as the exposure medium included a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (8×10^6 cells ind^{-1} day^{-1} until they were 8-days old; 16×10^6 cells ind^{-1} day^{-1} until they were 21-days old) and the yeast *Saccharomyces cerevisiae* (15×10^6 cells ind^{-1} day^{-1}). After 7, 14 or 21 days of exposure, individuals were video-tracked and then transferred to a 1.5 mL Eppendorf tube and stored at – 80° C until the biochemical analyses. Moreover, to check for the reliability of the exposure, the concentration of COC in exposure medium from control and

171 treatment beakers was measured. Water samples were stored at – 20 °C until the chemical analyses
172 were performed.

173 *2.4 Chemical analysis of COC in stock solution and exposure beakers*

174 The chemical analysis of water samples to check COC expected concentrations was carried out by
175 solid phase extraction (SPE) followed by liquid chromatography tandem mass spectrometry (LC-
176 MS/MS). A method published previously was adapted for these analysis (Castiglioni et al. 2011).
177 Different aliquots were prepared for extraction: 25 mL for samples spiked at 50 ng L^{-1} and 2.5 mL
178 for samples spiked at 500 ng L^{-1}. SPE was performed using mixed reverse-phase cation exchange
179 cartridges (Oasis® MCX). Before extraction, the pH of each aliquot was adjusted to 2.0 with 37%
180 HCl and was spiked with the IS (2 ng of COC-d_3). Cartridges were conditioned with 6 mL methanol,
181 3 mL Milli-Q water, and 3 mL Milli-Q water acidified to pH 2. Samples were passed manually
182 through the cartridges at a flow rate of 5 mL min^{-1}. Cartridges were then vacuum-dried for 10 min
183 and eluted with 2 mL of MeOH and 2 mL of a 2% ammonia solution in MeOH. SPE eluates were
184 pooled and dried under a gentle nitrogen stream. Dried samples were redissolved in 100 µL of Milli-
185 Q water, centrifuged for 2 min at 2500 rpm, and transferred into glass vials for LC injection. LC-
186 MS/MS analysis was performed using an Agilent HP-1200 Series LC system with a binary pump and
187 an autosampler (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 5500 triple
188 quadrupole mass spectrometer equipped with a turbo ion spray source (Applied Biosystems–Sciex,
189 Thornhill, Ontario, Canada). LC separation was performed at room temperature using an Atlantis T3
190 column (2.1 x 150 mm, 3 µm) from Waters and a mobile phase consisting of A (0.1% AA in Milli-Q
191 water) and B (ACN). The flow rate was 200 µL/min and the injection volume was 4 µL. The MS
192 analysis was done in the positive ion mode with a spray voltage of +5.5 kV and a source temperature
193 of 400 °C. The MS analysis was done in the positive ion mode using the Selected Reaction
194 Monitoring (SRM) acquisition mode. MS/MS parameters and retention time are shown in SM (Table
195 S1). Quantitation of COC was performed using the isotopic dilution method and a 6-point calibration

196 curve was made freshly before each analytical run. Method detection limit (MDL) and method
197 quantitation limit (MQL) are reported in Table S1.

198 *2.5 Biomarker methods*

199 The biomarkers suite applied in the present study was performed on homogenates from pools of all
200 alive specimens found in each exposure beaker at the end of the specific exposures. Three independent
201 experimental replicates (pool of n = 17 – 20 individuals per replicate) for each treatment were
202 performed. All the biochemical measurements were carried out in duplicate for each pool. According
203 to Parolini et al. (2018b), individuals were homogenized using a motor pestle in a 100 mM potassium
204 phosphate buffer (added with KCl 100 mM, EDTA 1 mM, protease inhibitors 1:100 v/v and
205 dithiothreitol 1 mM, pH 7.4) and centrifuged at 15.000 × g for 10 min. The supernatant was collected
206 and immediately processed to assess protein content and enzyme activity through spectrophotometric
207 methods, while the amount of ROS was assessed through a fluorimetric method. Details of all the
208 biomarker methods applied in the present study are reported by Parolini and co-authors (2018b).
209 Briefly, SOD activity was measured at λ = 550 nm as the inhibition of cytochrome c (10 µM)
210 reduction caused by the superoxide anion generated by the xanthine oxidase (1.87 mU mL^{-1})/hypoxanthine (50 µM) reaction, and expressed as SOD units (1 SOD unit = 50% inhibition of the
212 xanthine oxidase reaction). The CAT activity was determined by measuring the decrease of H_2O_2 (50
213 mM) in potassium phosphate buffer (66.7 mM at pH 7) at λ = 240 nm. The GPx activity was measured
214 by monitoring the consumption of NADPH (0.12 mM) at λ = 340 nm using 0.2 mM H_2O_2 as a
215 substrate in 50 mM potassium phosphate buffer, added with reduced glutathione (2 mM), sodium
216 azide (1 mM), glutathione reductase (2 U mL^{-1}). The GST activity was measured at λ = 340 by adding
217 reduced glutathione (1 mM) in 80 mM phosphate buffer (pH 7.4) and using CDNB (1 mM) as a
218 substrate. Spectrophotometric reading were performed by a Genova Bio spectrophotometer (Jenway).
219 The amount of ROS was assessed according to a fluorimetric method that relies on the change in
220 fluorescence of the dichlorofluorescein-diacetate (DCFH-DA; 10 mg mL^{-1} in DMSO) in presence of

pro-oxidant molecules. The fluorescence intensity was measured by an Infinite® 200 PRO microplate reader (TECAN Life Sciences) with $\lambda = 485$ nm as excitation and $\lambda = 536$ nm as emission wavelength, respectively.

2.6 Video tracking analysis

To assess changes in swimming activity induced by the exposure to COC, video tracking analyses were performed. At the end of the exposures lasted 7, 14 or 21 days, ten individuals per treatment for each experimental replicate (n = 30 individuals for each treatment) were transferred individually into a 12-well plate (11.5 cm x 8 cm x 1.5 cm), called 'arena', filled with 3 mL of culture medium (without food) and were filmed with an iPhone 6 for 30 seconds (900 frames, 30 frames per second), obtaining 1080p Full HD videos. Videos were analyzed using the ImageJ plugin AnimalTracker, a tracking application specifically designed to support animal behavioral analyses. We relied on a module that processed the video recordings and provided the observed object's XY coordinates in each frame (see Gulyàs et al. 2016 for details). AnimalTracker returned the swimming activity of *D. magna* individuals, in terms of distance moved (mm) and swimming speed (cm s^{-1}) of each individual.

2.7 Statistical analysis

The effects of COC treatment, the exposure time and their interaction on the amount of ROS, enzyme activities of *D. magna* individuals were investigated using general linear models (GLM), while the effects on swimming behavior were assessed by Linear mixed models (LMM), including the identity of the test beaker in the models as a random factor to account for the so-called 'tank effect'. In the models the effect of beaker identity was tested by likelihood ratio test, by comparing the log-likelihood value of the model including or excluding the random effect of beaker identity. The effect of COC on chronic toxicity reproduction test endpoints was analyzed by Generalized linear models, assuming a Poisson distribution of data. Fisher' LSD post-hoc test was applied to point out significant differences among treatments, exposure time and treatment × time interactions. Significance was set

245 at $p < 0.05$ (*) and $p < 0.01$ (**). Statistical analyses were performed using IBM SPSS Statistics 25.0
246 software package.

247

248 # 3. Results

249 *3.1 Concentration of COC in stock solution and exposure beakers*

250 The concentration of COC in the stock solution (nominal concentration: 1 mg L^{-1}) was 1.19 mg L^{-1}
251 (accuracy: 119 %). No COC residues were found in control beakers, while concentrations of COC in
252 culture medium from beakers spiked with the lowest (50 ng L^{-1}) and highest (500 ng L^{-1}) tested
253 concentrations were respectively 67 ng L^{-1} (accuracy: 134 %) and 634 ng L^{-1} (accuracy: 127 %).

254 *3.2 COC-induced effects on oxidative stress-related endpoints*

255 Over the 21 days of the exposure, a mortality of 2.7 (± 0.57) %, 6.7 (± 0.95) % and 4 (± 0.95) %
256 occurred in the control, 50 ng L^{-1} and 500 ng L^{-1} experimental groups, respectively. These results
257 agreed the OECD guidelines (OECD, 2004), which indicate that the mortality in the control group
258 should not exceed the 10 % in order to consider the tests with *Daphnia magna* as valid. Moreover,
259 no significant ($p > 0.05$) differences between treated and control groups occurred.

260 Results of statistical analyses are reported in Table 1. A significant effect of the time of exposure was
261 noted for all the considered biochemical endpoints, with the exception of GST. It is interesting to note
262 that that both biochemical endpoints and swimming activity changes at different ages of the
263 individuals (Figures 1-3). For this reason, the effect of COC on these endpoints was highlighted by
264 comparing the responses obtained after the exposure to both the COC concentrations with the
265 corresponding temporal control. A significant effect of COC treatment and time × treatment
266 interaction on the amount of ROS was found. Independently of the time of the exposure, the amount
267 of ROS in 50 ng L^{-1} and 500 ng L^{-1} specimens were 46 % and 79% higher compared to controls.
268 Moreover, a significant ROS overproduction was noted after 7 days of exposure to 500 ng L^{-1} (3.6-

269 fold higher) and after 21 days of treatment to 50 ng L^{-1} and 500 ng L^{-1} (1.8-fold higher in both the
270 cases) compared to the corresponding control (Figure 1). A significant effect on SOD activity was
271 induced by COC treatment, showing a significant 20% activity decrease in specimens exposed to 500
272 ng L^{-1} COC compared to the control group ($p < 0.001$). Moreover, SOD changed according to a
273 significant time × treatment interaction, with a 40 % and 21 % decrease in enzyme activity measured
274 after 7 and 21 days of exposure at 500 ng L^{-1}, respectively, with respect to the corresponding controls
275 (Figure 2a). Although no significant effect of COC on CAT activity was noted, the significant time
276 × treatment interaction showed a decrease of activity at the end of the exposure to 50 ng L^{-1} (-33%)
277 and after 7 days (-31%) to 500 ng L^{-1} compared to the corresponding controls, as well as an increase
278 after 14 days at 500 ng L^{-1} (+ 19 %) (Figure 2b). Despite a significant effect of COC treatment on the
279 GPx activity, whereby a significant activity increase measured in specimens from 50 ng L^{-1} (+ 28%)
280 and to 500 ng L^{-1} (+ 23%) tested concentration compared to the controls ($p < 0.047$ in both the cases)
281 was found, the time × treatment interaction was not significant (Figure 2c). A significant increase of
282 GST activity (Figure 2d) was noted, showing an activation (+ 13%) in specimens exposed to 500 ng
283 L^{-1} COC compared to control ($p = 0.015$; Table 1).

284 *3.3 COC-induced effects on swimming behavior*

285 Log-likelihood ratio test did not show any significant effect of exposure beaker identity for both the
286 considered variables ($\chi^2_1 = 0.00$; $P = 1$ for both the cases). The COC treatment induced a significant
287 effect on the distance moved by *D. magna* specimens. Moreover, a significant effect of the time of
288 the exposure and time × treatment interaction (Figure 3a) was found, suggesting that swimming
289 activity changed at different ages of the individuals. In detail, the exposure to 500 ng L^{-1} COC caused
290 a significant decrease of the distance moved compared to the corresponding control, accounting for
291 the 19% and 11% after 14 and 21 days of exposure, respectively. Conversely, the exposure to 50 ng
292 L^{-1} COC induced an increase (13.5%) in the distance moved after 21-day exposure compared to
293 control. Although no significant effect of the COC treatment on the swimming speed was found, the

294 significant effect of time × treatment interaction revealed that specimens exposed for 21 days to 50
295 ng L^{-1} COC were 20% quicker than the corresponding controls, while a slowing down was noted in
296 14-days old specimens treated with 500 ng $L^{-1}$with respect to the corresponding control (Figure 3b).

297 *3.4 Chronic toxicity test results*

298 COC treatment induced a significant decrease on the total number of offspring (Wald $\chi^2_{2,31}$= 49.417;
299 $p < 0.001$), with a 38% ($p = 0.005$) and 28% ($p = 0.033$) fecundity reduction compared to the control
300 group in specimens exposed to 50 ng L^{-1} and 500 ng L^{-1}, respectively (Figure 4). In contrast, no
301 significant effect (Wald $\chi^2_{2,31} = 0.833$; $p = 0.660$) on the number of clutches between treated and
302 control specimens was noted (data not shown).

303

304 ## 4. Discussion

305 The present study showed that the exposure to environmental concentrations of cocaine (50 ng L^{-1}
306 and 500 ng L^{-1}) imbalanced the oxidative status and negatively affected the swimming activity and
307 reproductive effort of *D. magna*.

308 Many studies have shown that COC exposure can damage the structure and the function of diverse
309 organs through different mechanisms of actions, whereby the majority of the direct toxic effects is
310 mediated by the onset of oxidative stress and mitochondrial dysfunction occurring during the
311 metabolism of this illicit drug (Riezzo et al. 2012 and references therein). Our findings showed that
312 the exposure to COC induced an overproduction of ROS at both the tested concentrations. These
313 results agreed with a previous study on zebrafish larvae (96 h post fertilization), which showed a
314 significant increase in ROS levels after a short-term exposure to increasing COC concentrations,
315 ranging between 0.1 and 1 μg L^{-1} (Parolini et al. 2017). Such ROS overproduction modulated the
316 activity of the *D. magna* antioxidant enzymatic shield, which relies on a cascade mechanism of three
317 main enzymes, namely SOD, CAT and GPx (Lushchak 2011). The significant decrease of SOD

activity found at 500 ng L^{-1} COC might be related to a ROS overproduction (Gonzales-Rey and Bebianno, 2014) and suggests the accumulation of superoxide anion ($O_2^{\bullet -}$) within the organism (Verlecar et al. 2008). Alternatively, the decrease of SOD activity might be due to the inhibition and/or negative feed-back mechanism related to the byproducts of SOD reaction, suggesting the production of cytosolic hydrogen peroxide (Vlahogianni and Valavanidis 2007). Moreover, the spontaneous dismutation of superoxide anion by non-enzymatic pathways (Gwoździński et al. 2010) and other cellular enzymes, such as those contained in the peroxisomes (Khessiba et al. 2005), might boost the production of hydrogen peroxide. Despite no activation of CAT, the increase of GPx activity found at 500 ng L^{-1} COC, independently of the time of exposure, supported the hypothesis of the H_2O_2 production. Although GPx and CAT play a complementary role in metabolizing hydrogen peroxide, the divergence in their activity response could be explained by a competition for the same substrate (Kappus 1985) or, alternatively, by the levels of H_2O_2 that the organism has to face. In fact, while GPx acts at low H_2O_2 levels, CAT is activated only at high concentrations of this pro-oxidant molecule (Pereira et al., 2013). Similar trends of the antioxidant enzymes were found in zebrafish larvae exposed to COC and its main metabolites (Parolini et al. 2017), as well as in *D. polymorpha* specimens exposed to ibuprofen (Parolini et al. 2011) and Δ-9-tetrahydrocannabinol (Δ-9-THC; Parolini and Binelli 2014). Lastly, the increase of GST activity observed in specimens exposed to the highest treatment suggested the involvement of this phase II enzyme in detoxification processes of COC. These results were in accordance with previous studies showing an increase of GST in murine models exposed to COC (e.g., Devi and Chan 1997; Uys et al. 2011), as well as in the brown mussel *Perna perna* treated with crack COC (dos Santos Barbosa Ortega et al. 2018).

Overall, our findings suggested that an imbalance in the oxidative status of *D. magna* treated specimens occurred, which could lead to the onset of oxidative stress. Such situation often results in detrimental behavioral effects at individual level (e.g., Hedgespeth et al. 2014, Rivetti et al. 2016). COC exposure caused significant alterations in swimming activity of cladocerans, in terms of both

distance moved and swimming speed. Opposite responses were found at either treatment concentrations, whereby on one hand, the exposure to 50 ng L^{-1} COC induced an increase of distance moved and swimming speed, while on the other hand the highest tested concentration negatively affected both the endpoints. This discrepancy suggests a different mechanism of action of COC in *D. magna*, which depends on the administered concentration. In fact, at low concentrations COC might act as a stimulant molecule, boosting the swimming activity, while at high concentrations COC becomes toxic and impairs the swimming behavior. Alternatively, as COC is a psychomotor stimulant drug, at low doses it increases locomotor activity whereas, when the dose increases, the locomotor activity decreases (Grilly and Salamone 2011). Our interpretation is supported by results from a previous study by Nielsen and Roslev (2018), showing that the exposure to high concentrations (1 - 10 µg mL^{-1}) of two psychotropic drugs, namely fluoxetine and propranolol, stimulated the swimming activity of *D. magna*, whereas very high concentrations (> 100 µg mL^{-1}) inhibited it. However, as fluoxetine is an anti-depressant and propranolol is a β-blocker, their mechanisms of action in modulating swimming activity could be different from that of COC. Changes in the swimming activity can be related to an increased energy demand of the organism to complete the physiological processes needed to counteract COC toxicity. As the swimming behavior integrates physiological, sensorial, nervous and muscular responses (Charoy et al. 1995), our results suggest an overall impairment of the health status of treated *D. magna* specimens, with potentially detrimental consequences to fitness and survival of the organism. In fact, the alteration of the swimming performance could affect the filtering activity and therefore the food uptake, leading to an impairment of reproduction (Baillieul 1997). Both COC treatments caused a significant decrease in the total number of offspring with respect to the control, while no change in the number of clutches was recorded (data not shown). The decrease in reproductive success of individuals exposed to 500 ng L^{-1} COC was a plausible consequence considering the impairment of swimming performance. These findings agree with those from previous studies that demonstrated changes of *D. magna* reproductive success in response to the exposure to different emerging contaminants, including pesticides (e.g.,

Villarroel et al. 2009,2003), pharmaceuticals (de Oliveira et al. 2016) and illicit drugs (Parolini et al. 2018b). In contrast, the adverse effects caused by the exposure to 50 ng L^{-1} COC was unforeseen. In fact, considering that COC boosted the swimming activity, null or positive effects on reproduction was expected. Thus, we may speculate that the decreased reproductive success of individuals treated with the lowest COC concentration depended on a different use of energy obtained by the food uptake, which was diverted to support swimming rather than reproduction, or alternatively on direct, yet unknown, reproductive effects. Overall, our findings suggest that environmental COC concentrations could negatively affect the population dynamic of *D. magna*, with potential detrimental consequences on the whole trophic chain because of the pivotal role of this species in freshwater ecosystems.

5. Conclusion

Our findings showed that the exposure to low concentrations of COC can alter the oxidative status and affect both the swimming and the reproductive behavior of the cladoceran *D. magna*. As the concentrations tested in the present study were similar to those measured in aquatic ecosystems worldwide, our results cannot be underestimated. Moreover, the uninterrupted use of COC and the consequent input in the sewage confer to COC a sort of pseudo-persistence. Thus, aquatic organisms might be exposed to similar or higher COC concentrations for their whole life-span, resulting in potentially worst adverse effects with respect to those we found in our laboratory exposures. Further studies are therefore recommended to shed light on the toxicity at different level of the ecological hierarchy and on the mechanisms of action of COC in aquatic species and to formulate an accurate risk assessment of this illicit drugs for freshwater ecosystems.

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526

Table and figure captions

Table 1: Effects due to treatment, time of exposure and their interactions on biochemical (SOD, CAT, GPx, and GST) swimming activity and reproduction variables in *D. magna*. Details of statistical approach used to analyse each single variable are reported in *2.7 Statistical analysis* section. Significant effects are reported in bold.

Biochemical effects	F	df	P
ROS			
Time	184.110	2,18	**<0.001**
Treatment	25.021	2,18	**<0.001**
Time × treatment	16.573	4,18	**<0.001**
SOD			
Time	217.367	2,18	**<0.001**
Treatment	12.828	2,18	**<0.001**
Time × treatment	4.687	4,18	**0.009**
CAT			
Time	4.783	2,18	**0.022**
Treatment	0.806	2,18	0.462
Time × treatment	6.644	4,18	**0.002**
GPx			
Time	4.790	2,18	**0.021**
Treatment	4.024	2,18	**0.045**
Time × treatment	0.733	4,18	0.581
GST			
Time	2.941	2,18	0.078
Treatment	3.809	2,18	**0.042**
Time × treatment	2.494	4,18	0.080
Swimming activity	F	df	P
Distance moved			
Time	8.381	2,258	**<0.001**
Treatment	3.074	2,258	**0.048**
Time × treatment	4.799	4,258	**0.001**
Swimming speed			
Time	8.807	2,259	**<0.001**
Treatment	2.295	2,259	0.103
Time × treatment	5.149	4,259	**0.001**
Reproduction	Wald χ^2	df	P
Number of offspring			
Treatment	49.417	2,31	**<0.001**
Number of clutches			
Treatment	0.833	2,31	0.660

Figure 1: Mean (± standard deviation) of the amount of reactive oxygen species (ROS) measured in *D. magna* specimens after 7, 14 and 21 days of exposure to 50 ng L^{-1} and 500 ng L^{-1} of COC. Asterisks above the histograms show significant differences between treated individuals and the corresponding control (** $p < 0.01$).

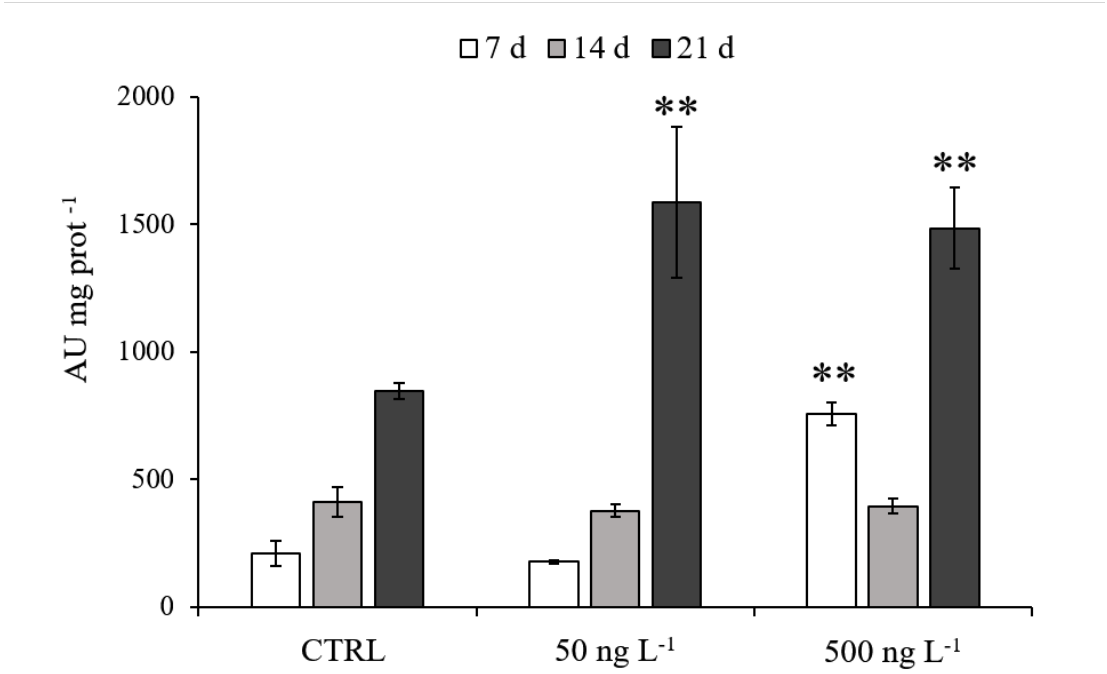


Figure 2: Mean (± standard deviation) of SOD (a), CAT (b), GPx (c) and GST (d) activity measured in *D. magna* specimens after 7, 14 and 21 days of exposure to 50 ng L^{-1} and 500 ng L^{-1} of COC. Asterisks above the histograms show significant differences between treated individuals and the corresponding control (* $p < 0.05$; ** $p < 0.01$).

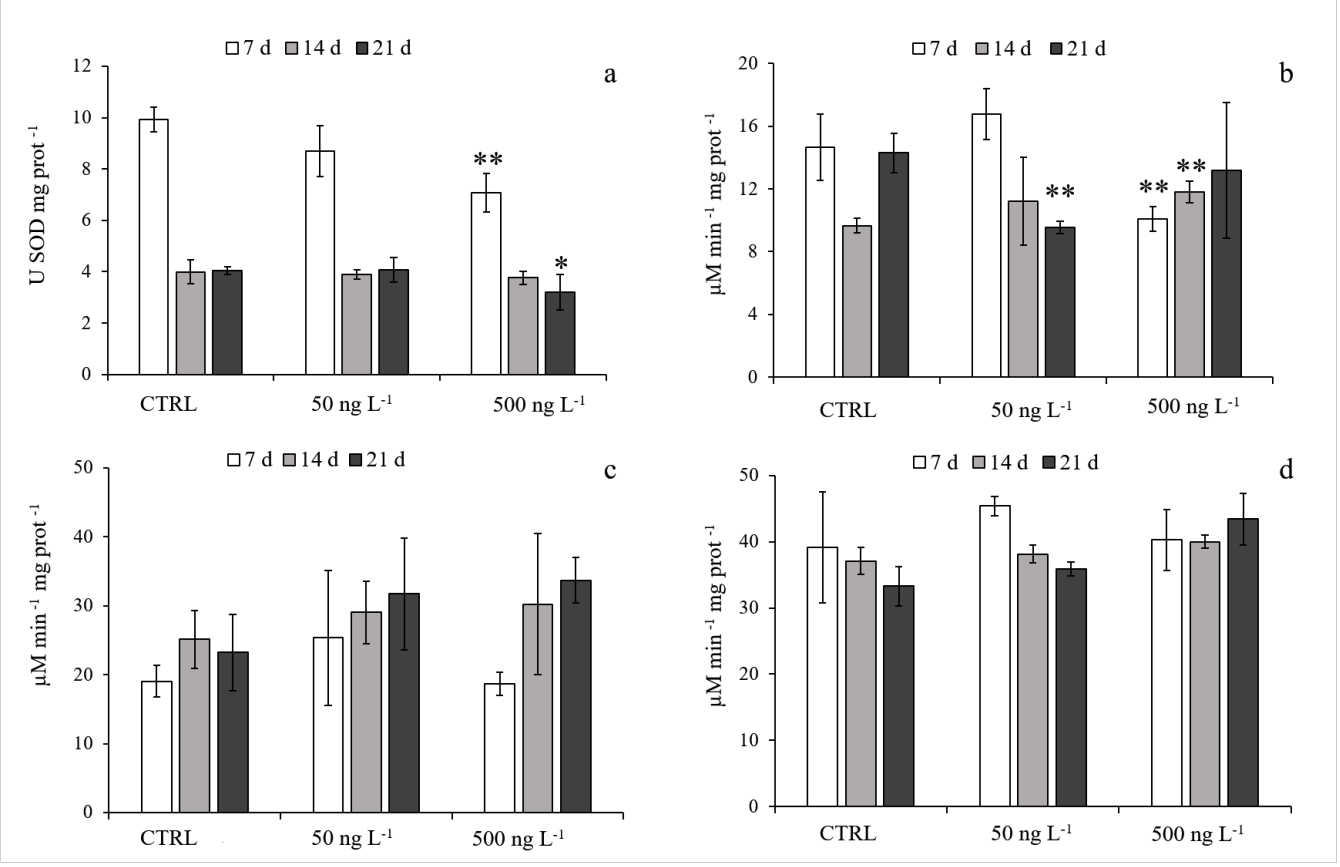
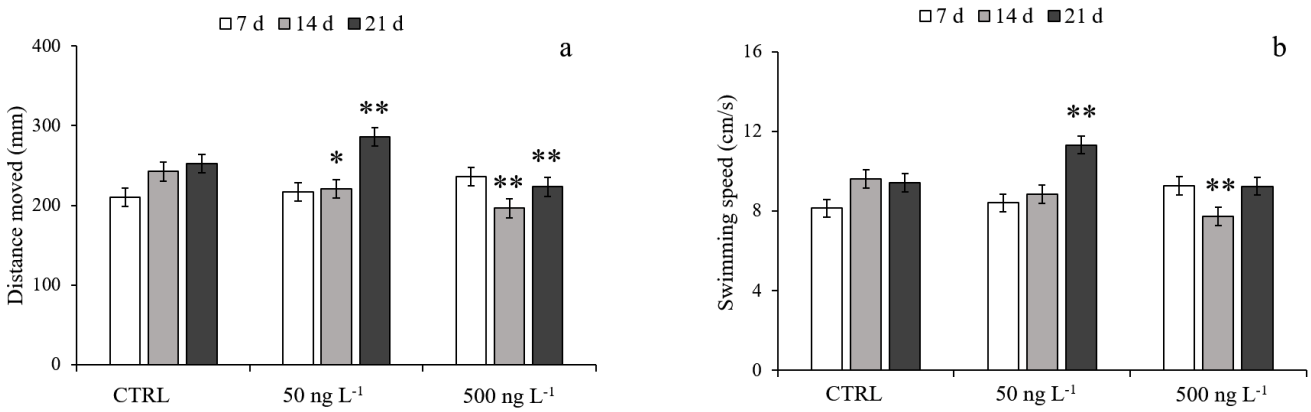


Figure 3: Mean (± standard deviation) of distance moved (a) and swimming speed (b) measured in *D. magna* specimens after 7, 14 and 21 days of exposure to 50 ng L^{-1} and 500 ng L^{-1} of COC. Asterisks above the histograms show significant differences between treated individuals and the corresponding control (* $p < 0.05$; ** $p < 0.01$).



574 **Figure 4**: Mean (± standard deviation) number of offspring of *D. magna* specimens after 21 days of
575 exposure to 50 ng L^{-1} and 500 ng L^{-1} of COC. Asterisks above the histograms show significant
576 differences between treated individuals and the corresponding control (** $p < 0.01$).

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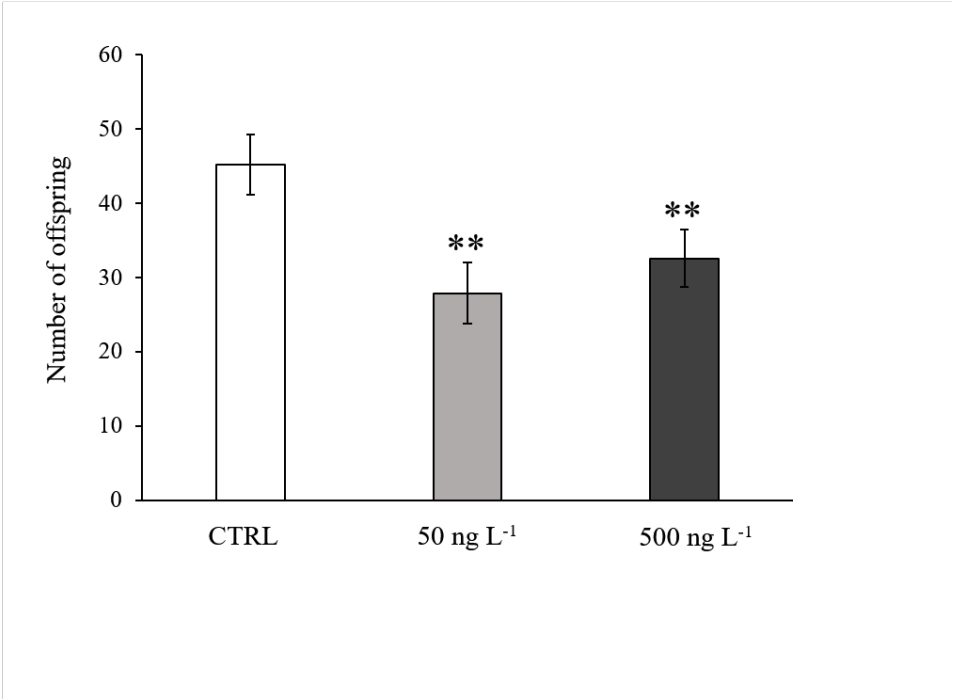

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