SciVerse ScienceDirect xxx (xxxx) 104163

Contents lists available at ScienceDirect

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/etap

Exposure to 3,4-methylenedioxymethamphetamine (MDMA) induced biochemical but not behavioral effects in *Daphnia magna*

Beatrice De Felice*, Marco Parolini

University of Milan, Department of Environmental Science and Policy, Via Celoria 26, I-20133 Milan, Italy

ARTICLE INFO

Dr. M.D. Coleman

Keywords:
Oxidative stress
Illicit drugs
Amphetamine like stimulants

ABSTRACT

Among amphetamine like stimulants (ATS), the 3,4-methylenedioxymethamphetamine (MDMA) is often detected in sewage and surface waters, representing a potential threat for organisms because of its peculiar mechanism of action (i.e., stimulatory and hallucinogenic). The present study aimed at investigating biochemical (i.e., oxidative stress and energetic biomarkers) and behavioral (i.e., swimming activity) effects induced by a 21-days exposure to two concentrations (50 ng/L and 500 ng/L) of MDMA towards *Daphnia magna*. The amount of reactive oxygen species (ROS), the activity of antioxidant (SOD, CAT, GPx) and detoxifying (GST) enzymes and lipid peroxidation were measured as oxidative stress-related endpoints. Total energy content was estimated from the measurement of protein, carbohydrate and lipid content to assess energy reserves. The modulation of swimming activity was assessed as behavioral endpoint. Slight effects of MDMA exposure on oxidative stress responses and energy reserves were observed, while no alterations of the swimming behavior was noted.

1. Introduction

Amphetamine-like stimulants (ATS) are a group of illicit drugs of synthetic origins, including amphetamine (AMPH), methamphetamine (METH), methcathinone (CATH) and the so-called ecstasy-group substances, i.e., 3,4-methylenedioxymethamphetamine (MDMA) and its analogs. As claimed by the World Drug Report 2022, the amount of ATS seized has been steadily increased since 2009, reaching in 2020 an amount of 525 tons(UNODC, World Drug Report, 2022) . Although METH is still the main ATS on the market, seizures of ecstasy and amphetamine have doubled over the past decade, confirming a notable increase in the use of these illicit drugs. Overall, in 2020 the number of past year users of ATS has been estimated to be 54 million people, with 34 million users for methamphetamine and amphetamine and 20 million users for ecstasy (UNODC, World Drug Report, 2022) . Considering that most of ecstasy tablets contain a predominant percentage of MDMA, the information on ecstasy reflects the usage scenario of MDMA (UNODC, World Drug Report, 2022). According to seizure data, since 2011 the amount of ecstasy globally marketed has almost quadrupled compared to 2020, reaching the 20 tons (UNODC, World Drug Report, 2022). After the use of a dose, MDMA is metabolized by the liver and the 65 % is excreted as unaltered parental compound, while the 35 % as metabolites via feces and urine (Xavier et al., 2008). Through the sewage MDMA reaches the wastewater treatment plants (WWTPs), where it has been detected in concentration up to 476 ng/L and 83 ng/L in inlet and outlet waters, respectively (Fontes et al., 2020). Since WWTPs are not able to efficiently remove MDMA from the sewage, it enters the surface waters in non-negligible concentrations (Fontes et al., 2020). In addition, the discarding of unused MDMA and the production by-products from illegal manufacturing laboratories contribute to the release of this molecule into the aquatic ecosystems (Fontes et al., 2020). Several monitoring studies have detected measurable concentrations of MDMA in surface waters worldwide ranging between 0.3 ng/L to 24.80 ng/L ((Fontes et al., 2020)(Yadav et al., 2017) Pal et al., 2013). Although current environmental levels can be considered as relatively low, the continuative use and subsequent environmental input confer MDMA a sort of pseudo-persistence (Fontes et al., 2020), evolving in a long-term exposure to non-target aquatic organisms. Some ecotoxicological studies have investigated the likely negative effects arisen by the exposure to different ATS towards aquatic organisms, including METH (De Felice et al., 2020; Hossain et al., 2019; Liao et al., 2015; Tashiro et al., 2014) and AMPH (Lee et al., 2016; Parolini et al., 2016). However, there is a dearth of knowledge on the toxicity of MDMA, even though toxicological studies have demonstrated its capability to induce both stimulating and hallucinogenic effects (Liechti et al., 2001), which could result in different or unpredictable outcomes in non-target organisms compared to other ATS. Indeed, MDMA interferes with diverse neurotransmitters' pathways, causing the release of catecholamines,

* Corresponding author.
E-mail address: beatrice.defelice@unimi.it (B. De Felice).

https://doi.org/10.1016/j.etap.2023.104163
Received 28 February 2023; Received in revised form 23 May 2023; Accepted 25 May 2023

mainly serotonin, dopamine and norepinephrine (Xavier et al., 2008), and inhibiting the activity of different enzymes involved in neurotransmitters biosynthesis or degradation, such as the tryptophan hydroxylase (Capela et al., 2009). To the best of our knowledge, only a couple of studies have investigated the effects of MDMA on non-target aquatic organisms. Stewart et al. (2011) have reported a reduction in bottom swimming and immobility in zebrafish exposed to high concentrations of MDMA (0.25 - 120 mg/L). Parolini et al. (2014) have shown an increase in cellular stress and the modulation of enzymatic antioxidant defenses in *Dreissena polymorpha* exposed to 50 ng/L and 500 ng/L of MDMA. Considering the limited knowledge regarding MDMA ecotoxicity and mechanism of action towards non-target aquatic organisms, this work aimed at investigating the biochemical and behavioral effects induced by 21-days exposure to two concentrations of MDMA (50 ng/L and 500 ng/L) towards the freshwater *Daphnia magna*. Oxidative stress-related endpoints were evaluated as previous research on several model organisms have shown that the exposure to ATS molecules, including MDMA, caused the onset of an oxidative stress situation (De Felice et al., 2020; Liao et al., 2015; Parolini et al., 2014). As oxidative stress-related biomarkers the amount of reactive oxygen species (ROS), as well as the activity of antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT and glutathione peroxidase, GPx) and the levels of lipid peroxidation were investigated. Moreover, considering that the activation of the antioxidant defense is an activity that requires the consumption of energy, the measurements of protein, carbohydrate and lipid content were integrated to calculate the total caloric content, a proxy of energy reserves in the organism. Lastly, considering that ATS exposure has induced behavioral modulation in aquatic organisms (Horký et al., 2021; Tashiro et al., 2014; Stewart et al., 2011), the effects on swimming activity of Cladocerans were assessed by a video tracking analysis. According to results from previous studies on different aquatic organisms, we expect similar responses on oxidative stress, energy reserves and behavioral endpoints in *D. magna*.

2. Materials and methods

2.1. Chemicals and reagents

3,4-Methylenedioxymethamphetamine (MDMA; CAS number 42542–10-9, molecular weight 193 g/mol, certified reference material, Cerilliant®) analytical standard used in this work was purchased from Sigma-Aldrich (Steinheim, Germany), after earning the permission for laboratory storage and use for scientific purposes only by the Italian Ministry of Health (Decree n SP/177, 11/12/2017). MDMA standard solution (1 g/L in methanol) was diluted to obtain a 1 mg/L stock solution, in ultrapure water, to be used for the experiments. The reagents used in this study for biochemical analyses (i.e., oxidative stress and energetic biomarkers) were purchased from Merk (Merck KGaA, Darmstadt, Germany).

2.2. Experimental design

Daphnia magna individuals came from the husbandry of the University of Milan and were maintained following a procedure described in a previous work (De Felice et al., 2019, 2020). Briefly, adult *Daphnia magna* organisms were maintained in 400 mL beakers (30 organisms/L) filled with a commercial mineral water (San Benedetto®) and fed ad libitum with a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* and the yeast *Saccharomyces cerevisiae* every other day following the doses highlighted by the OECD guidelines. All the individuals were maintained in a thermostatic chamber with a temperature of 20.0 ± 0.5 °C and a photoperiod of 16 h light: 8 h dark.

Two different concentrations of MDMA, i.e., 50 ng/L (0.25 nM) and 500 ng/L (2.5 nM) were used for the exposure with daphnids (< 24 hrs.old). The lower concentration was similar to the mean levels of MDMA detected in the effluents of WWTPs worldwide, while the higher one reflected the concentration measured in the inlet of WWTPs (Fontes et al., 2020). In addition, both these concentrations were similar to those used in a previous study testing the toxicity of another ATS drug (i.e., methamphetamine; De Felice et al., 2020). Semi-static exposures were performed as described by De Felice et al. (2019); (2020). Briefly, *D. magna* individuals were exposed, with a daily renewal of the exposure conditions, to the two selected concentrations (i.e., 50 ng/L and 500 ng/L) for 7, 14 and 21 days. The renewal of the exposure medium should ensure a constant MDMA concentration.

over a 24-hrs period, as a previous study showed that in wastewater MDMA degradation does not begin up to or even longer time periods than 28 h (van Nuijs et al., 2012).

Individuals in the control group were maintained under the same experimental condition in a medium without MDMA. Although in the standard solution MDMA was diluted in methanol, no solvent control group was included in the experimental design because the estimated amount of methanol in the exposure beakers was negligible (< 0.03 %).

For each experimental group three independent replicates were performed, including twenty daphnids in beakers (100 mL) filled with the culture medium and a volume of the stock solution to reach the selected, nominal concentrations. Overall, 60 individuals per treatment for each time point were exposed. Organisms were maintained at husbandry conditions (i.e., fed ad libitum). In detail, organisms were fed with the algae *Pseudokirchneriella subcapitata* (8×10^6 cell/mL per organisms per day up to the 8-days of life and 16×10^6 cell/mL per organisms per day up to the 21-days of life) and the yeast *Saccharomyces cerevisiae* (0.5 g/L). After 7, 14 and 21 days of exposure, thirty organisms (ten from each exposure beakers) selected randomly from each experimental group were taken to perform the video tracking analysis (see Section 2.3 Analysis of swimming behavior). Then, all the individuals were stored at − 80 °C, in an Eppendorf tube, until biochemical analyses (see Section 2.2 Oxidative stress and energetic biomarkers).

2.3. Oxidative stress and energetic biomarkers

A suite of oxidative stress and energetic biomarkers were used on homogenates of a pool of all the alive individuals (17–20 individuals each replicate) collected at the end of each time point per each treatment. All the biomarkers' analyses were carried out in duplicate. Using a pestle, each pool was homogenized in 100 mM potassium phosphate buffer (pH 7.4), added with KCl (100 mM), EDTA (1 mM), specific protease inhibitors (Protease Inhibitor Cocktail from Sigma-Aldrich, Steinheim, Germany; 1:100 v/v) and dithiothreitol (DTT; 1 mM). After a centrifuge (15,000g for 20 min) the supernatant was immediately collected and transferred to a new Eppendorf tube. The amount of ROS was measured through the fluorometric methodology by Deng et al. (2009). The possible modulation in fluorescence of dichlorofluorescein-diacetate (DCFH-DA; 10 mg/mL in dimethyl sulfoxide) were measured through a multimode plate reader (EnSight, Perkin Elmer) using a wavelength of $\lambda = 485$ nm as excitation and of $\lambda = 536$ nm as emission. The ROS concentration was indicated as arbitrary units (i.e., AU DCF/mg protein). Enzyme activity, lipid peroxidation, protein, carbohydrates, and lipid content were assessed by spectrophotometric methods using a spectrophotometer (Genova Bio; Jenway) following the method described elsewhere (De Felice et al., 2019, 2020; Sancho et al., 2009). Briefly, the content in protein of supernatant was assessed through the Bradford method (Bradford, 1976), using as a standard the bovine albumin serum. The superoxide dismutase (SOD) activity was measured as the inhibition of cytochrome c (10 mM) reduction operated by the superoxide anion produced from the reaction between xanthine oxidase (1.87 mU/mL) and hypoxanthine (50 mM) at $\lambda = 550$ for 1 min nm. The results were displayed as SOD units (1 SOD unit = 50 % inhibition of the xanthine oxidase reaction). The catalase (CAT) activity was measured as the utilization of H_2O_2 (50 mM) in potassium phos-

phate buffer (66.7 mM; pH 7) at λ = 240 nm for 1 min. The glutathione peroxidase (GPx) activity was measured as the depletion of the reduced nicotinamide adenine dinucleotide phosphate (0.12 mM) by using the H_2O_2 (0.2 mM) as a substrate in a potassium phosphate buffer (50 mM), including glutathione (2 mM), sodium azide (1 mM) and glutathione reductase (2 U/mL) for 1 min at λ = 340 nm. The glutathione S-transferase (GST) activity was measured using 1-chloro-2,4 dinitrobenzene (CDNB; 1 mM) as a substrate, adding reduced glutathione (1 mM) in a potassium phosphate buffer (80 mM; pH 7.4) for 1 min at λ = 340 nm. CAT, GPx and GST activities were normalized on protein content and the results were expressed as μM/min/mg protein. Lipid peroxidation was assessed following the thiobarbituric acid reactive substances (TBARS) methodology developed by (Ohkawa et al., 1979) and results were displayed as nmol TBARS/mg protein.

The total carbohydrate content was measured following the anthrone methodology developed by (Jermyn, 1975) and results were expressed as mg sugars/individual. The lipid content was measured following the sulfo-phospho-vanillin method developed by (Frings et al., 1972) and results were expressed as mg lipids/individuals. The caloric content of *Daphnia magna* individuals per each pool was calculated following the formula reported by Sancho et al. (2009), using as conversion factors 4.10 cal/mg for carbohydrates, 5.65 cal/mg for proteins and 9.45 cal/mg for lipids. Results were expressed as mcal/individual.

2.4. Analysis of swimming activity

Modulation in *Daphnia magna* swimming activity were investigated on ten organisms for each experimental replicate (i.e., 30 organisms for each treatment and 30 for control) for each time-point (i.e., after 7, 14 and 21 days of exposure) through a video tracking approach (i.e., De Felice et al., 2019; 2020). Each single individual was randomly transferred into a well within a 12-well plate, called 'arena', filled with 3 mL of culture medium. Individuals were left 5 min to acclimate before the beginning of the video tracking analysis. Each individual was filmed for 30 s with an iPhone 6 place at a constant distance above the plate. The obtained videos of 1080p Full HD in quality (900 frames, 30 frames/s) were examined using AnimalTraker a plugin of the ImageJ software (Gulyás et al., 2016) to track the *D. magna* swimming activity. The analyzed parameters were the distance moved (mm) and the swimming speed (cm/sec).

2.5. Statistical analysis

The effects of MDMA treatment, time of exposure and their interaction on oxidative stress and energetic biomarkers were analyzed by the application of a two-way Analysis of the Variance (ANOVA), considering each biochemical endpoint as a dependent variable, while the treatment and the time of exposure as predictors. The effect of treatment, time of exposure, and their interaction on behavioral endpoints of *Daphnia magna* were investigated by Linear Mixed Models (LMM). Treatment and time of exposure were included in the models as fixed effect factors, while the exposure tank (i.e., experimental replicate) was included as a random factor. A Tukey' HSD post-hoc test was carried out to identify significant differences between treatments. Significance was set at $P < 0.05$ (*) and $P < 0.01$ (**). All the analyses were run in R 4.03 (R. Core TEAM, 2020).

3. Results

No mortality was observed in controls and in organisms exposed to the highest MDMA concentration, while a mortality accounting for 1.59 % of the total population was noted in individuals exposed to 50 ng/L of MDMA after 7 days of exposure. In the 14-day exposure tests, the mortality accounted for 1.59 %, 3.17 %, and 1.59 % for controls, 50 ng/L, and 500 ng/L treatment groups, respectively. Similarly, for the 21-day exposures, the mortality was 4.69 %, 6.25 % and 9.38 % for controls, 50 ng/L, and 500 ng/L treatment groups, respectively.

3.1. Oxidative stress related biomarkers

Results of the statistical analyses performed to assess the effects regarding oxidative stress biomarkers are displayed in Table S1. A significant effect of the exposure time was noted for all the biomarkers, except for CAT, showing a progressive increase of the response over the duration of exposure, and obviously according to the age of the individuals (Table S1; Fig. 1a-f).

No significant effects of treatment and time × treatment interaction were noted for ROS and lipid peroxidation levels, as well as for SOD and GPx activity (Table S1). However, an overall, significant effect of the treatment was observed only on CAT activity ($F_{2,18} = 4.833$; $P = 0.020$), which in individuals exposed to 500 ng/L of MDMA was significantly higher compared to that measured in individuals from the 50 ng/L ($P = 0.040$; +57.67 %) and the control group ($P = 0.035$; +60.26 %) (Fig. 1c). Although no significant effect of the treatment as fixed effect factor was noted on GST activity, a significant effect of time × treatment interaction ($F4,16 = 6.532$; $P < 0.001$) occurred. In detail, the results of this interaction showed that the activity of GST measured in individuals exposed to 500 ng/L of MDMA for 7 days was significantly lower compared to coeval individuals from the control group ($P = 0.045$; −29.48 %) and 50 ng/L group ($P = 0.005$; −44.93 %).

3.2. Energetic biomarkers

Results of statistical analyses regarding energetic biomarkers are displayed in Table S2. Overall, a significant effect of the exposure time was noted for all the selected biomarkers, showing an increase in the content of all the macromolecules and total energy content over the time. No significant effects of the treatment and time × treatment interaction were noted for all the energetic biomarkers (Table S2; Fig. 2a, c and d), except for carbohydrates content (Fig. 2b). Indeed, a significant effect of MDMA treatment on the content of carbohydrates ($F_{2,17} = 3.608$; $P = 0.049$) was noted, showing a slight but significant reduction in individuals exposed to 500 ng/L of MDMA compared to conspecifics from the 50 ng/L group ($P = 0.039$), while no differences occurred with respect to the control group.

3.3. Swimming activity

Results of the statistical analyses performed to assess the effects on swimming activity are displayed in Table S3. Overall, a significant effect of the exposure time was noted for both distance moved and swimming speed, showing an increase in the swimming activity over time (Table S3), whereby 21-days old individuals moved faster and for longer distances than 7-days old ones ($P < 0.001$). The same results were reported comparing the activity of 14-days old individuals with that of 7-days old ones ($P < 0.001$). The effect of treatment and time × treatment interaction was not significant for both the analyzed endpoints (Table S3; Fig. 3a -b).

4. Discussion

This work highlighted how the exposure to MDMA concentrations similar to those measured in freshwater ecosystems (i.e., 50 ng/L and 500 ng/L) induced slight effects on the oxidative status and energy reserves in the cladoceran *Daphnia magna*, while no behavioral changes were noted.

Some studies with murine models have highlighted that one of the mechanisms of action (MoA) of MDMA relies on the induction of oxidative stress caused by the molecule itself or by its active metabolites, through an overproduction of ROS (Camarero et al., 2002; Chipana et

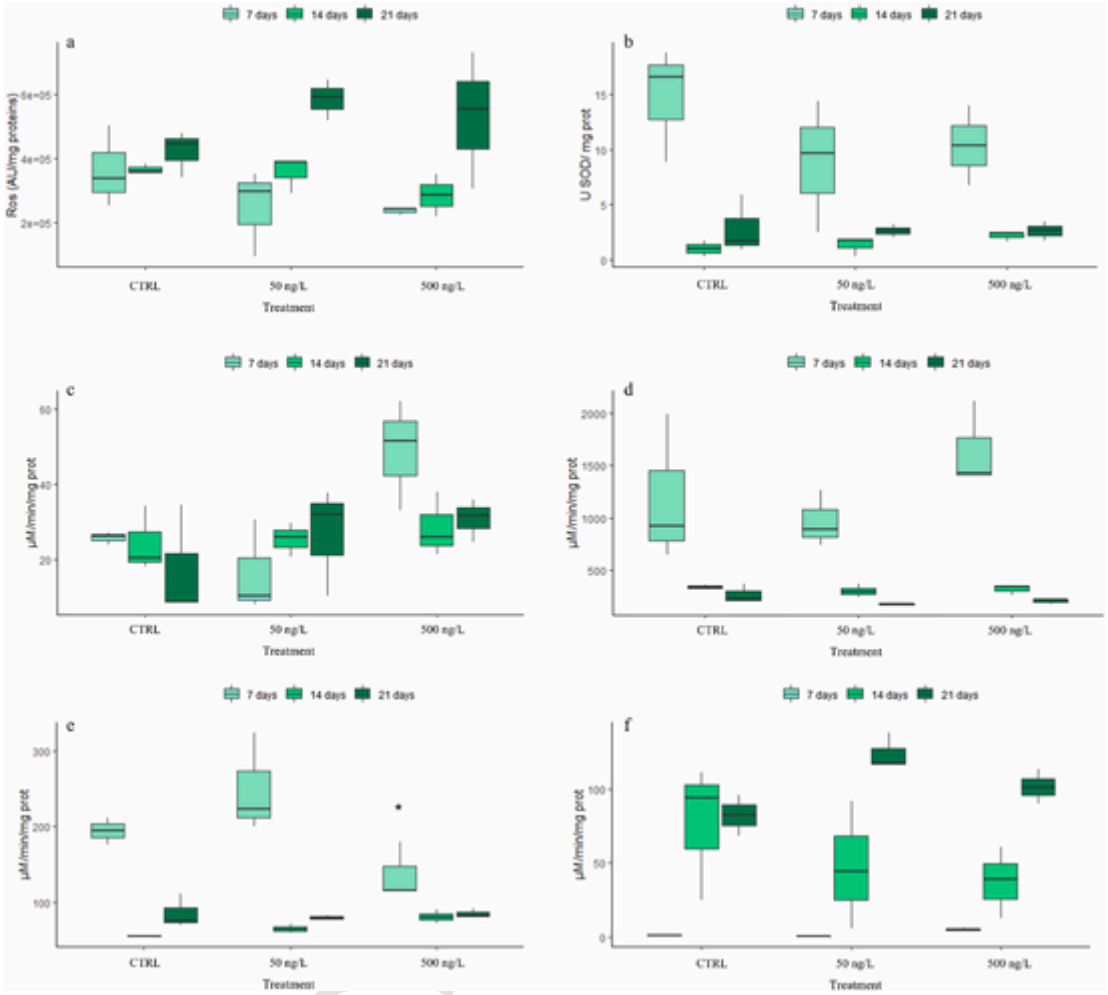

Fig. 1. Box and whiskers plots of oxidative stress biomarkers measured in *Daphnia magna* individuals collected after 7, 14 and 21-days of exposure to two environmentally relevant MDMA concentrations. ROS (a) SOD (b), CAT (c), GPx (d), GST (e) and LPO (f). Asterisks above the box and whiskers plots show significant differences between treated individuals and the corresponding control (per each time point; * $p < 0.05$).

al., 2006; Shenouda et al., 2009; Cerretani et al., 2008; Cerretani et al., 2011) and subsequent modulation of the antioxidant defenses (Cerretani et al., 2008; (Cerretani et al., 2011); Peraile et al., 2013; (Ninković et al., 2004)). Our findings highlighted that the exposure to MDMA induced only a slight modulation of *D. magna* oxidative status. Although no ROS overproduction was observed in treated individuals compared to conspecifics from the control group, a modulation of antioxidant enzyme defenses was noted. The lack of significant modulation of SOD activity (Fig. 1b) suggested that the exposure to MDMA did not cause an overproduction of O_2^-, and consequently of H_2O_2 (i.e., the final byproduct of SOD reaction), as confirmed by the unaltered activity of GPx in treated individuals compared to controls (Fig. 1d). However, the significant activation of CAT (Fig. 1c), which plays a complementary role of GPx in H_2O_2 degradation (Box et al., 2007; Oropesa et al., 2016), suggested an overproduction of H_2O_2, likely generated through pathways not directly related to SOD activity, such as the spontaneous dismutation of superoxide radicals (Gwoździński et al., 2010)) or processes catalyzed by peroxisome enzymes (Khessiba et al., 2005). The discrepancy in GPx and CAT response might depend on competition, different affinity (Pereira et al., 2013) and/or levels of the same substrate (i.e., H_2O_2; (Baud, 2004); Pereira et al., 2013). Our results were consistent with those obtained in the freshwater bivalve *Dreissena polymorpha* (Parolini et al., 2014), whereby the exposure to the same concentrations of MDMA did not modulate the activity of SOD and GPx, but induced the activation of CAT, suggesting a similar MoA in freshwater invertebrates. The exposure to MDMA also induced a modulation in the activity of the phase II detoxification enzyme GST (Fig. 1e). An early (i.e., after 7 days only), significant inhibition of GST was observed in individuals exposed to 500 ng/L MDMA concentration with respect to the other experimental groups. These results agreed those from a previous study on *Dreissena polymorpha* exposed to the same MDMA concentrations (Parolini et al., 2014), suggesting a potential depletion of glutathione for supporting detoxification processes of this illicit drug. In addition, this hypothesis was also supported by findings obtained on rat hepatocytes exposed to a wide range of MDMA concentrations (0.1 – 1.6 mM, corresponding to $0.019 \times 10^9 - 0.309 \times 10^9$ ng/L), showing a decrease in GST (and GPx) activity coupled with a GSH depletion (Carvalho et al., 2004). Alternatively, as the decrease in GST activity was observed only after 7-days of exposure, it might be due to the low levels of this enzyme synthetized by young individuals, whose detoxification defenses could result as more sensitive to MDMA compared to older conspecifics. Lastly, no studies have currently investigated the possible negative effects of MDMA exposure on the expression of the gene encoding for GST in invertebrates. However, we might speculate that the inhibition of GST activity could be due to a modulation at gene level, as observed in a previous study on zebrafish embryos exposed to other illicit drugs such as cocaine and its main metabolites (Parolini et al., 2017). Despite the slight imbalance of oxidative status and detoxification defense observed in MDMA-treated individuals, they did not suffer an oxidative stress condition, as no oxidative damage (i.e., changes

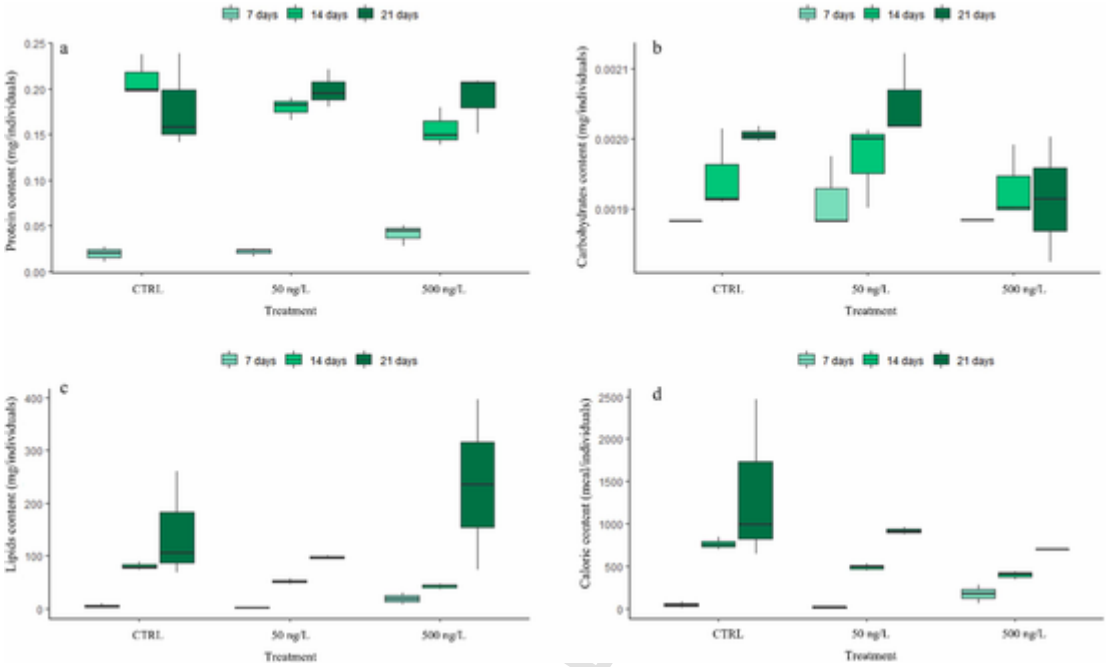

Fig. 2. Box and whiskers plots of the energetic biomarkers measured in *Daphnia magna* individuals collected after 7, 14 and 21-days of exposure to two environmentally relevant MDMA concentrations. Protein content (a), carbohydrates content (b), lipid content (c) and caloric content (d).

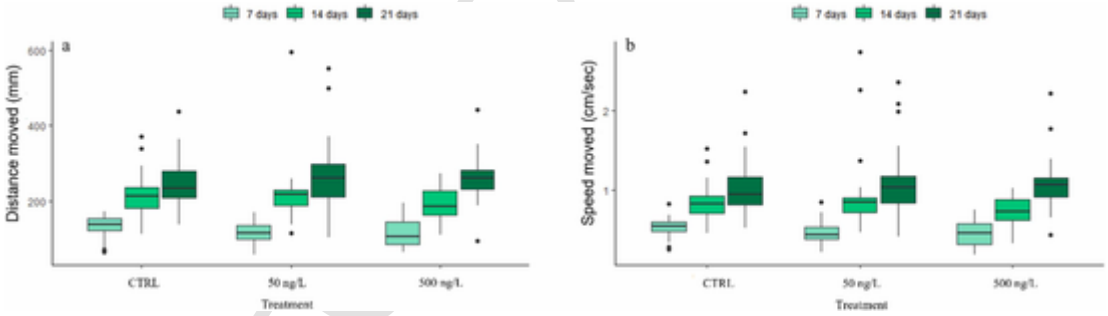

Fig. 3. Box and whisker plots of swimming behavior endpoints: distance moved (a) swimming speed (b), after 7, 14 and 21-days of exposure to two environmentally relevant MDMA concentrations.

in lipid peroxidation levels) raised (Fig. 1f). Although several studies performed on murine models showed that MDMA exposure led to the increase of oxidative damage such as lipid peroxidation (Miranda et al., 2007; Moon et al., 2008; Müller, 2009; Adeniyi et al., 2016), our results are consistent with a previous study performed on *D. polymorpha* in with the slight imbalance of oxidative status did not result in an increase of oxidative damage (Parolini et al., 2014). Our findings suggest that MDMA has a similar MoA in freshwater invertebrates and a limited toxicity compared to murine model organisms, at least at low concentrations similar to those measured in aquatic ecosystems.

The imbalance of the oxidative status is often related with a modulation in the energy reserves of the organism (Yu et al., 2018), as reflected by alterations in total caloric content due to changes in the content of proteins, lipids and carbohydrates (Brey et al., 2010). The MDMA exposure did not induce a modulation in the total content of proteins, lipids and carbohydrates (Fig. 2a-c), and consequently no changes in total energy reserved (i.e., caloric content) were observed in treated individuals compared to control conspecifics (Fig. 2d). However, an overall reduction of carbohydrate content was observed in individuals treated with 500 ng/L of MDMA compared to conspecifics form the 50 ng/L treatment group, suggesting an increase in carbohydrate catabolism in individuals exposed to the higher MDMA concentration. According to results from previous investigations on murine models, the alteration of carbohydrate metabolism might be due to an enhancement of glycogenolysis (Bull et al., 2006; Soto-Montenegro et al., 2007) or an overproduction of catecholamines (Dangé, 1986; De Coen et al., 2001). However, as both these hypotheses were postulated on findings from experiments performed on vertebrates, they need to be validated in invertebrates such as *Daphnia magna*.

The lack of alterations in energy reserves of MDMA-treated individuals did not impair the swimming behavior of *D. magna*, neither swimming distance nor swimming speed (Fig. 3a and b, respectively). These results were in contrast with a study performed by Stewart et al. (2011) that highlighted a reduction in zebrafish (*Danio rerio*) bottom swimming and immobility after the exposure to a range of MDMA concentrations (0.25 - 120 mg/L). These discrepancies might be mainly related to differences in species sensitivity to MDMA, as well as to the concentrations administered, which were two orders of magnitude higher compared to those used in the present study.

Overall, our results suggest that the exposure to low, environmentally similar concentrations of MDMA can induce slight, negative effects at biochemical level in D. magna. Moreover, considering the continuative input of MDMA and its pseudo-persistence in freshwater ecosystems, Cladocerans can be exposed to this illicit drug for their whole lifespan, resulting in adverse consequences at individual, population and community levels. Thus, considering the key ecological role

of D. magna in freshwater ecosystems, the potential onset of effects at population level caused by prolonged exposure to MDMA could result in negative consequences at community level, modulating for instance prey-predator relationships.

5. Conclusions

The exposure to two low concentrations of MDMA (50 ng/L and 500 ng/L) similar to those measured globally in freshwaters induced slight alterations at biochemical level in *Daphnia magna*, but no behavioral effects. Although the exposure to MDMA caused an imbalance in the oxidative status of treated organisms, they did not suffer an oxidative stress condition and did not experience changes in energy reserves and impairments of swimming behavior. These results suggest that the concentrations of MDMA currently detected in freshwaters might not pose a threat for the health status of zooplanktonic species. However, considering the increasing trend of use predicted for MDMA and other synthetic drugs of the ATS family, concentrations in freshwaters are expected to increase, resulting in potentially worse effects than those pointed out in the present study. Moreover, further studies should be important to shed light on the mechanism(s) of toxic action of MDMA in invertebrates, as well as on other behavioral effects such as the reproduction and predator avoidance. Lastly, considering that in freshwaters MDMA occurs in complex mixture with other ATS drugs, such as amphetamine and methamphetamine, it should be interesting to explore and compare the toxicity of these drugs both individually and in mixture to identify the most toxic molecule of the ATS class towards freshwater organisms.

CRediT authorship contribution statement

Beatrice De Felice : Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Marco Parolini :** Conceptualization, Data curation, Methodology, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104163.

References

Adeniyi, P.A., Ishola, A.O., Laoye, B.J., Olatunji, B.P., Bankole, O.O., Shallie, P.D., Ogundele, O.M., 2016. Neural and behavioural changes in male periadolescent mice after prolonged nicotine-MDMA treatment. Metab. Brain Dis. 31 (1), 93–107. https://doi.org/10.1007/s11011-015-9691-z.

Baud, O., 2004. Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. J. Neurosci. 24 (7), 1531–1540. https://doi.org/10.1523/jneurosci.3989-03.2004.

Box, A., Sureda, A., Galgani, F., Pons, A., Deudero, S., 2007. Assessment of environmental pollution at Balearic Islands applying oxidative stress biomarkers in the mussel *Mytilus galloprovincialis*. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 146 (4), 531–539. https://doi.org/10.1016/j.cbpc.2007.06.006.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brey, T., Müller-Wiegmann, C., Zittier, Z.M., Hagen, W., 2010. Body composition in aquatic organisms – a global data bank of relationships between mass, elemental composition and energy content. J. Sea Res. 64 (3), 334–340. https://doi.org/10.1016/j.seares.2010.05.002.

Bull, E.J., Porkess, V., Rigby, M., Hutson, P.H., Fone, K.C.F., 2006. Pre-treatment with 3,4-methylenedioxymethamphetamine (MDMA) causes long-lasting changes in 5-HT2A receptor-mediated glucose utilization in the rat brain. J. Psychopharmacol. 20 (2), 272–280. https://doi.org/10.1177/0269881106059583.

Camarero, J., Sanchez, V., O'Shea, E., Green, A.R., Colado, M.I., 2002. Studies, using in vivo microdialysis, on the effect of the dopamine uptake inhibitor GBR 12909 on 3,4-methylenedioxymethamphetamine ("ecstasy")-induced dopamine release and free radical formation in the mouse striatum. J. Neurochem. 81 (5), 961–972. https://doi.org/10.1046/j.1471-4159.2002.00879.x.

Capela, J.P., Carmo, H., Remião, F., Bastos, M.L., Meisel, A., Carvalho, F., 2009. Molecular and cellular mechanisms of ecstasy-induced neurotoxicity: an overview. Mol. Neurobiol. 39 (3), 210–271. https://doi.org/10.1007/s12035-009-8064-1.

Carvalho, M., Remião, F., Milhazes, N., Borges, F., Fernandes, E., Carvalho, F., Bastos, M.L., 2004. The toxicity of N-methyl-α-methyldopamine to freshly isolated rat hepatocytes is prevented by ascorbic acid and N-acetylcysteine. Toxicology 200 (2–3), 193–203. https://doi.org/10.1016/j.tox.2004.03.016.

Cerretani, D., Riezzo, I., Fiaschi, A.I., Centini, F., Giorgi, G., D'Errico, S., Fiore, C., Karch, S.B., Neri, M., Pomara, C., Turillazzi, E., Fineschi, V., 2008. Cardiac oxidative stress determination and myocardial morphology after a single ecstasy (MDMA) administration in a rat model. Int. J. Leg. Med. 122 (6), 461–469. https://doi.org/10.1007/s00414-008-0262-2.

Cerretani, D., Bello, S., Cantatore, S., Fiaschi, A.I., Montefrancesco, G., Neri, M., Pomara, C., Riezzo, I., Fiore, C., Bonsignore, A., Turillazzi, E., Fineschi, V., 2011. Acute administration of 3,4-methylenedioxymethamphetamine (MDMA) induces oxidative stress, lipoperoxidation and TNFα-mediated apoptosis in rat liver. Pharmacol. Res. 64 (5), 517–527. https://doi.org/10.1016/j.phrs.2011.08.002.

Chipana, C., Camarasa, J., Pubill, D., Escubedo, E., 2006. Protection against MDMA-induced dopaminergic neurotoxicity in mice by methyllycaconitine: involvement of nicotinic receptors. Neuropharmacology 51 (4), 885–895. https://doi.org/10.1016/j.neuropharm.2006.05.032.

Dangé, A.D., 1986. Changes in carbohydrate metabolism in tilapia, *Oreochromis (Sarotherodon) mossambicus*, during short-term exposure to different types of pollutants. Environ. Pollut. Ser. A, Ecol. Biol. 41 (2), 165–177. https://doi.org/10.1016/0143-1471(86)90091-7.

De Coen, W.M., Janssen, C.R., Segner, H., 2001. The use of biomarkers in *Daphnia magna* toxicity testing V. In vivo alterations in the carbohydrate metabolism of *Daphnia magna* exposed to sublethal concentrations of mercury and lindane. Ecotoxicol. Environ. Saf. 48 (3), 223–234. https://doi.org/10.1006/eesa.2000.2009.

De Felice, B., Mondellini, S., Salgueiro-González, N., Castiglioni, S., Parolini, M., 2020. Methamphetamine exposure modulated oxidative status and altered the reproductive output in *Daphnia magna*. Sci. Total Environ. 721, 137728. https://doi.org/10.1016/j.scitotenv.2020.137728.

Deng, J., Yu, L., Liu, C., Yu, K., Shi, X., Yeung, L.W.Y., Lam, P.K.S., Wu, R.S.S., Zhou, B., 2009. Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. Aquat. Toxicol. 93 (1), 29–36. https://doi.org/10.1016/j.aquatox.2009.03.001.

Fontes, M.K., Maranho, L.A., Pereira, C.D.S., 2020. Review on the occurrence and biological effects of illicit drugs in aquatic ecosystems. Environ. Sci. Pollut. Res. 27 (25), 30998–31034. https://doi.org/10.1007/s11356-020-08375-2.

Frings, C.S., Fendley, T.W., Dunnn, R.T., Queen, C.A., 1972. Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clin. Chem. 18, 673–674.

Gulyás, M., Bencsik, N., Pusztai, S., Liliom, H., Schlett, K., 2016. AnimalTracker: an ImageJ-based tracking API to create a customized behaviour analyser program. Neuroinformatics 14 (4), 479–481. https://doi.org/10.1007/s12021-016-9303-z.

Gwoździński, M. Gonciarz, E. Kilańczyk, A. Kowalczyk, A. Pieniążek, & G.. Brichon, 2010. Antioxidant enzyme activities and lipid peroxidation in Mytilus galloprovincialis from the French Mediterranean coast. Oceanological and Hydrobiological Studies, Vol. 39, No.4, 33–43. https://www.infona.pl/resource/bwmeta1.element.baztech-article-BUS8–0005-0044.

Horký, P., Grabic, R., Grabicová, K., Brooks, B.W., Douda, K., Slavík, O., Hubená, P., Sancho Santos, E.M., Randák, T., 2021. Methamphetamine pollution elicits addiction in wild fish. J. Exp. Biol. 224 (13). https://doi.org/10.1242/jeb.242145.

Hossain, M.S., Kubec, J., Grabicová, K., Grabic, R., Randák, T., Guo, W., Buřič, M., 2019. Environmentally relevant concentrations of methamphetamine and sertraline modify the behavior and life history traits of an aquatic invertebrate. Aquat. Toxicol. 213, 105222. https://doi.org/10.1016/j.aquatox.2019.105222.

Jermyn, M.A., 1975. Increasing the sensitivity of the anthrone method for carbohydrate. Anal. Biochem. 68 (1), 332–335.

Khessiba, A., Roméo, M., Aïssa, P., 2005. Effects of some environmental parameters on catalase activity measured in the mussel (*Mytilus galloprovincialis*) exposed to lindane. Environ. Pollut. 133 (2), 275–281. https://doi.org/10.1016/j.envpol.2004.05.035.

Lee, S.S., Paspalof, A.M., Snow, D.D., Richmond, E.K., Rosi-Marshall, E.J., Kelly, J.J., 2016. Occurrence and potential biological effects of amphetamine on stream communities. Environ. Sci. Technol. 50 (17), 9727–9735. https://doi.org/10.1021/acs.est.6b03717.

Liao, P.-H., Hwang, C.-C., Chen, T.-H., Chen, P.-J., 2015. Developmental exposures to waterborne abused drugs alter physiological function and larval locomotion in early life stages of medaka fish. Aquat. Toxicol. 165, 84–92. https://doi.org/10.1016/j.aquatox.2015.05.010.

Liechti, M.E., Vollenweider, F.X., 2001. Which neuroreceptors mediate the subjective effects of MDMA in humans? A summary of mechanistic studies. Hum. Psychopharmacol.: Clin. Exp. 16 (8), 589–598. https://doi.org/10.1002/hup.348.

Miranda, M., Bosch-Morell, F., Johnsen-Soriano, S., Barcia, J., Almansa, I., Asensio, S.,

Romero, F.J., 2007. Oxidative stress in rat retina and hippocampus after chronic MDMA ('ecstasy') administration. Neurochem. Res. 32 (7), 1156–1162. https://doi.org/10.1007/s11064-007-9285-1.

Moon, K.H., Upreti, V.V., Yu, L.R., Lee, I.J., Ye, X., Eddington, N.D., Song, B.J., 2008. Mechanism of 3, 4–methylenedioxymethamphetamine (MDMA, ecstasy)-mediated mitochondrial dysfunction in rat liver. Proteomics 8 (18), 3906–3918. https://doi.org/10.1002/pmic.200800215.

Müller, M. , 2009. 3, 4-Methylenedioxymethamphetamine (MDMA," Ecstasy"): studies on MDMA metabolism and pharmacokinetics in humans and squirrel monkeys. SciDok - Der Wissenschaftsserver der Universität des Saarlandes. http://dx.doi.org/10.22028/D291–22593.

Ninković, M., Maličević, Ž., Selaković, Vesna, M., Simić, I., Vasiljević, I.D., 2004. N-Methyl-3,4-methylenedioxyamphetamine-induced hepatotoxicity in rats: Oxidative stress after acute and chronic administration. Vojnosanit. Pregl. 61 (2), 125–131. ⟨http://www.doiserbia.nb.rs/Article.aspx?ID=0042-84500402125N#.YVIkYbgzZPY⟩.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95 (2), 351–358. https://doi.org/10.1016/0003-2697(79)90738-3.

Oropesa, A.L., Novais, S.C., Lemos, M.F.L., Espejo, A., Gravato, C., Beltrán, F., 2016. Oxidative stress responses of *Daphnia magna* exposed to effluents spiked with emerging contaminants under ozonation and advanced oxidation processes. Environ. Sci. Pollut. Res. 24 (2), 1735–1747. https://doi.org/10.1007/s11356-016-7881-9.

Pal, R., Megharaj, M., Kirkbride, K.P., Naidu, R., 2013. Illicit drugs and the environment — a review. Sci. Total Environ. 463–464, 1079–1092. https://doi.org/10.1016/j.scitotenv.2012.05.086.

Parolini, M., Magni, S., Binelli, A., 2014. Environmental concentrations of 3,4-methylenedioxymethamphetamine (MDMA)-induced cellular stress and modulated antioxidant enzyme activity in the zebra mussel. Environ. Sci. Pollut. Res. 21 (18), 11099–11106. https://doi.org/10.1007/s11356-014-3094-2.

Parolini, M., Magni, S., Castiglioni, S., Binelli, A., 2016. Amphetamine exposure imbalanced antioxidant activity in the bivalve *Dreissena polymorpha* causing oxidative and genetic damage. Chemosphere 144, 207–213. https://doi.org/10.1016/j.chemosphere.2015.08.025.

Parolini, M., Ghilardi, A., Della Torre, C., Magni, S., Prosperi, L., Calvagno, M., Binelli, A., 2017. Environmental concentrations of cocaine and its main metabolites modulated antioxidant response and caused cyto-genotoxic effects in zebrafish embryo cells. Environ. Pollut. 226, 504–514. https://doi.org/10.1016/j.envpol.2017.04.046.

Peraile, I., Granado, N., Torres, E., Gutiérrez-López, M.D., Moratalla, R., Colado, M.I., O' Shea, E., 2013. Cocaine potentiates MDMA-induced oxidative stress but not dopaminergic neurotoxicity in mice: implications for the pathogenesis of free radical-induced neurodegenerative disorders. Psychopharmacology 230 (1), 125–135. https://doi.org/10.1007/s00213-013-3142-5.

Pereira, L., Fernandes, M.N., Martinez, C.B.R., 2013. Hematological and biochemical alterations in the fish *Prochilodus lineatus* caused by the herbicide clomazone. Environ. Toxicol. Pharmacol. 36 (1), 1–8. https://doi.org/10.1016/j.etap.2013.02.019.

R. Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria: Available at: https://www.Rproject.org/.

Sancho, E., Villarroel, M.J., Andreu, E., Ferrando, M.D., 2009. Disturbances in energy metabolism of *Daphnia magna* after exposure to tebuconazole. Chemosphere 74 (9), 1171–1178. https://doi.org/10.1016/j.chemosphere.2008.11.076.

Shenouda, S.K., Varner, K.J., Carvalho, F., Lucchesi, P.A., 2009. Metabolites of MDMA induce oxidative stress and contractile dysfunction in adult rat left ventricular myocytes. Cardiovasc. Toxicol. 9 (1), 30–38. https://doi.org/10.1007/s12012-009-9034-6.

Soto-Montenegro, M.L., Vaquero, J.J., Arango, C., Ricaurte, G., García-Barreno, P., Desco, M., 2007. Effects of MDMA on blood glucose levels and brain glucose metabolism. Eur. J. Nucl. Med. Mol. Imaging 34 (6), 916–925. https://doi.org/10.1007/s00259-006-0262-8.

Stewart, A., Riehl, R., Wong, K., Green, J., Cosgrove, J., Vollmer, K., Kyzar, E., Hart, P., Allain, A., Cachat, J., Gaikwad, S., Hook, M., Rhymes, K., Newman, A., Utterback, E., Chang, K., Kalueff, A.V., 2011. Behavioral effects of MDMA ("ecstasy") on adult zebrafish. Behav. Pharmacol. 22 (3), 275–280. https://doi.org/10.1097/fbp.0b013e328345f758.

Tashiro, N., Nishimura, K., Daido, K., Oka, T., Todo, M., Toshikawa, A., Kitamura, Y., 2014. Pharmacological assessment of methamphetamine-induced behavioral hyperactivity mediated by dopaminergic transmission in planarian *Dugesia japonica*. Biochem. Biophys. Res. Commun. 449 (4), 412–418. https://doi.org/10.1016/j.bbrc.2014.05.059.

UNODC, World Drug Report 2022 (United Nations publication, 2021).

van Nuijs, A.L., Abdellati, K., Bervoets, L., Blust, R., Jorens, P.G., Neels, H., Covaci, A., 2012. The stability of illicit drugs and metabolites in wastewater, an important issue for sewage epidemiology. J. Hazard. Mater. 239, 19–23. https://doi.org/10.1016/j.jhazmat.2012.04.030.

Xavier, C.A.C., Lobo, P.L.D., de Franca Fonteles, M.M., de Vasconcelos, S.M.M., de Barros Viana, G.S., de Sousa, F.C.F., 2008. Ecstasy (MDMA): pharmacological and toxic effects, mechanism of action and clinical management. Rev. De. Psiquiatr. Clínica 35 (3), 96–104.

Yadav, M.K., Short, M.D., Aryal, R., Gerber, C., van den Akker, B., Saint, C.P., 2017. Occurrence of illicit drugs in water and wastewater and their removal during wastewater treatment. Water Res. 124, 713–727. https://doi.org/10.1016/j.watres.2017.07.068.

Yu, P., Liu, Z., Wu, D., Chen, M., Lv, W., Zhao, Y., 2018. Accumulation of polystyrene microplastics in juvenile *Eriocheir sinensis* and oxidative stress effects in the liver. Aquat. Toxicol. 200, 28–36. https://doi.org/10.1016/j.aquatox.2018.04.015.