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Oxidative and genetic responses induced by Δ-9-tetrahydrocannabinol (Δ-9-THC) to *Dreissena polymorpha*

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HIGHLIGHTS

- We investigated adverse effects of Δ-9-tetrahydrocannabinol (Δ-9-THC) to *Dreissena polymorpha*.
- Mussels were exposed under laboratory condition to two Δ-9-THC concentrations.
- 0.5 μg/L Δ-9-THC caused significant imbalances of bivalve oxidative status.
- Oxidative stress induced lipid peroxidation, protein carbonylation and DNA damage.
- Δ-9-THC can be considered a potentially harmful compound to freshwater mussels.

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ABSTRACT

Cannabis is the most used illicit substance worldwide and its main psychoactive compound, the Δ-9-tetrahydrocannabinol (Δ-9-THC), is detected in aquatic environments at measurable concentrations. Even though its occurrence is well documented, no information is available on its hazard to aquatic organisms. The aim of this study was to assess the adverse effects induced to zebra mussel (*Dreissena polymorpha*) specimens by 14 day exposures to environmentally relevant Δ-9-THC concentrations (0.05 μg/L and 0.5 μg/L) by means of the application of a biomarker suite. Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities, as well as the lipid peroxidation (LPO) and protein carbonyl content (PCC), were measured as oxidative stress indices. The single cell gel electrophoresis (SCGE) assay, the DNA diffusion assay and the micronucleus test (MN test) were applied to investigate DNA injuries, while the neutral red retention assay (NRRA) was used to assess Δ-9-THC cytotoxicity. The lowest treatment induced negligible adverse effects to bivalves, while 0.5 μg/L Δ-9-THC exposure caused remarkable alterations in *D. polymorpha* oxidative status, which lead to significant increase of lipid peroxidation, protein carbonylation and DNA damage.

1. Introduction

The newest World Drug Report has estimated that in 2010 between 153 million and 300 million people aged 15–64 (3.4–6.6% of the world's population in that age group) had used an illicit substance at least once in the previous year (UNODC, 2012). Although over the last three years the extent of illicit drug use has remained stable, the estimated 15.5 million–38.6 million problem drug users (almost 12% of illicit drug users) remain a particular concern (UNODC, 2012). Cannabis is the most widely used illicit substance worldwide, with an estimated annual prevalence use in 2010 ranging from 2.6 to 5% of the adult population (between 119 million and 224 million estimated users aged 15–64), followed by amphetamine-group substances, cocaine and opiates (UNODC, 2012). Besides being a social and health problem, the illicit drug issue has recently become also an environmental problem. Drugs of abuse, in fact, are the latest group of emerging pollutants identified in the aquatic environment demanding attention (Boleda et al., 2009; Kasprzyk-Hordern et al., 2010). Aquatic ecosystems are the ultimate destination for all these compounds following their metabolism in the human body and/or their accidental or deliberate disposal. Similarly to legal pharmaceuticals, a large proportion of the administered drug may be excreted as the parent compound and/or as metabolites, through human urine, feces, saliva and sweat, and discharged directly into the sewage system (Daughton, 2001; Roberts and Thomas, 2006; Castiglioni et al., 2006), contributing to the environmental contamination. An increasing number of monitoring surveys have showed the occurrence of cocaine (CO), amphetamines (AMP), Δ-9-tetrahydrocannabinol (Δ-9-THC), ecstasy (3,4-methylenedioxy-N-methylamphetamine; MDMA), opiates (heroin, morphine and codeine), as well as of their corresponding metabolites, in both surface and wastewaters worldwide in ng/L concentrations (Zuccato et al.,

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2008; Postigo et al., 2010; Castiglioni et al., 2011a,b), matching levels of common pharmaceuticals used for therapeutic purposes (Santos et al., 2010). Since these compounds may have potent pharmacological and biological activities, their presence in surface waters even at low concentrations, together with the residues of many therapeutic pharmaceuticals and other organic compounds, may lead to unexpected pharmacological interactions causing toxic effects to aquatic organisms. However, despite of the increasing knowledge on illicit drug residue distribution in the aquatic ecosystems, the information on their potential hazard to organisms is greatly inadequate. The first effort to fill this gap was carried out by Binelli et al. (2012), who showed that environmentally relevant cocaine concentrations induced a remarkable sub-lethal effect to the zebra mussel *Dreissena polymorpha*. Further studies showed that concentrations of the main cocaine metabolites, the benzoylecgonine (Parolini et al., 2013a) and the ecgonine methyl ester (Parolini and Binelli, in press), similar to those found in the aquatic system, induced alterations of antioxidant activity, as well as increases of lipid peroxidation, protein carbonylation and genetic damage to *D. polymorpha* specimens. Lastly, a redox proteomics study showed an increase in protein carbonylation in gills from this bivalve after 14 days of exposure to a 1 μg/L benzoylecgonine concentration, as well as oxidative modifications in different classes of proteins, such as those of the cytoskeleton, energetic metabolism and stress response (Pedriali et al., 2012). In spite of these findings, no data are available on the potential hazard of other illicit drugs towards aquatic organisms, neither for the main psychoactive chemical of the cannabis, the Δ-9-tetrahydrocannabinol (Δ-9-THC), even if its occurrence in aquatic environment and its toxicity to classical mammalian models are well documented. Many studies showed that Δ-9-THC concentration in European wastewater treatment plant influents and effluents was measured in the 11.3–127 ng/L and 13.0–39.2 ng/L range, respectively (Castiglioni et al., 2006; Boleda et al., 2007, 2009; Postigo et al., 2008a,b; Pal et al., 2012), while in surface waters it ranges between 0.3 and 24 ng/L (Boleda et al., 2007; Zuccato et al., 2008; Pal et al., 2012). Regarding Δ-9-THC toxicity, it has been reported that it can induce oxidative damage both in vitro (Sarafian et al., 1999) and in vivo (Mandal and Das, 2009) and, as a result of damaged cellular structures and oxidized macromolecules (DNA, lipids and enzymatic proteins) due to oxidative stress, it can eventually cause parenchymal liver necrosis (Kaplowitz, 2000). Hence, this research was aimed to evaluate the sub-lethal effects induced by the Δ-9-THC to the zebra mussel, considered an excellent biological model in freshwater ecotoxicology. 14 day exposures to two environmentally relevant Δ-9-THC concentrations (0.05 and 0.5 μg/L) were performed and adverse effects were evaluated by using an in vivo multi-biomarker approach. The end-points of ten different biomarkers were measured and their integrated response was used both in detecting sub-lethal Δ-9-THC effects and in supposing its possible mechanism of action in zebra mussel specimens. In detail, the activities of four defense enzymes, namely catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) were measured as oxidative stress indices. Furthermore, we measured also the lipid peroxidation (LPO) and protein carbonyl content (PCC) as preferential biomarkers to point out oxidative damage induced by the Δ-9-THC administration. The neutral red retention assay (NRRA), a simple indicator of general cellular stress in bivalves (Lowe et al., 1995), was applied to evaluate the cytotoxicity, while primary (DNA strand breaks) and fixed (apoptotic and micronucleated cell frequency) genetic damages were investigated by the single cell gel electrophoresis (SCGE) assay, the DNA diffusion assay and the micronucleus test (MN test), respectively.

2. Materials and methods

The Δ-9-tetrahydrocannabinol (Δ-9-THC) standard (CAS number 1972-08-3; purity >99%) was purchased from Alltech-Applied Science (State College, PA, USA), while all the reagents used for biomarker analyses were purchased from Sigma-Aldrich (Steinheim, Germany). We diluted the methanol stock solution (1 g/L) to 10 mg/L in bi-distilled water (working solution), which was then used to obtain the desired Δ-9-THC concentration in experimental aquaria.

2.1. Experimental design

Zebra mussel specimens were collected in March 2012 by a scuba diver at a depth of 4–6 m in Lake Lugano (Northern Italy), which is considered a reference site due to its low drug pollution (Zuccato et al., 2008). The mussels were gently cut off from the rocks, quickly transferred to the laboratory in bags filled with lake water and placed in 15 L glass-holding aquaria filled with tap and lake water (50:50 v/v) to avoid a drastic change in the chemical composition of the water and to guarantee a food supply for the mussels during the first 24 h of acclimation. Several specimens (200 per tank) having similar shell length (20 ± 2 mm) were maintained in aquaria filled with 10 L of tap and deionized water (50:50 v/v), previously de-chlorinated by aeration, under a natural photoperiod with constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (>90% of saturation). The bivalves were fed daily with lyophilized algae belonging to the genus *Spirulina* spp., and the water was regularly renewed every two days for 2 weeks to gradually purify the mollusks of any possible pollutants that had previously accumulated in their soft tissues. Only specimens that were able to re-form their byssi and reattach themselves to the glass sheet were used in the experiments. Mussel viability was checked daily by the Trypan blue exclusion method, whereas biomarker baseline levels were checked weekly. Mussels were exposed to Δ-9-THC concentrations only when target biomarker levels were comparable with baseline ones obtained in previous studies (Parolini et al., 2010, 2011a,b, 2013a,b). Exposure assays were performed under semi-static conditions for 14 days. Control and exposure aquaria were processed at the same time, and the whole water volume (10 L) was renewed on a daily basis. Mussels were exposed to 0.05 μg/L (0.16 nM, low) and 0.5 μg/L (1.6 nM, high) of Δ-9-THC. The first concentration was similar to the environmental levels found in the effluents of European wastewater treatment plants (13–39.2 ng/L; Boleda et al., 2007, 2009; Postigo et al., 2008a,b) and close to the concentration measured at the intake of a drinking water treatment plant in Llobregat (Spain; Boleda et al., 2007). The second one was the same tested in previous studies investigating the toxicity of cocaine metabolites (Parolini et al., 2013a; Parolini and Binelli, in press) in order to allow a comparison among drug toxicity. The concentration of the Δ-9-THC working solution was checked in LC–MS/MS by using a HCT Ultra (Bruker, Germany) after purification and concentration by SPE (HLB 1 cm^3, Waters). Quantification was made by a calibration curve (0.01–10 μg/L; $R^2 = 0.99$). The concentration of the Δ-9-THC working solution was quantified in triplicate and was 10.3 ± 0.9 mg/L. Exact volumes of working solution were carefully added daily to the exposure aquaria until reaching the chosen nominal concentrations. We also checked the concentration of Δ-9-THC in water from both the exposure tanks by using the method mentioned above. Unfortunately, we were not able to obtain reliable Δ-9-THC concentrations in water samples since LC–MS/MS analysis showed that the Δ-9-THC concentrations measured in water were below the instrumental detection limit (0.01 ppb). Probably, for low Δ-9-THC concentrations such as those expected in our samples, the method used for sample preparation may influence the chromatographic background level and can generate a matrix suppression effect (Vlase et al., 2010). In fact, the methods using an isolation step by SPE to eliminate the impurities and to increase the sensitivity can affect the recovery of this molecule (Vlase et al., 2010). For these reasons, the method used to measure low Δ-9-THC levels in water samples should need to be improved and standardized. Considering the troubles in measuring Δ-9-THC concentrations in water samples, the certification of the working solution was extremely important to guarantee the reliability of our exposures. Hence, even if small differences due to contamination operations and

loss by adsorption cannot be excluded, the careful addition of exact volumes of certificated working solution should warrant that the exposure concentrations are very close to the nominal ones. Moreover, considering the lack of information on Δ-9-THC half-life in water, the complete water and chemical changes should guarantee a constant solution concentration of Δ-9-THC over each 24-h period, preventing losses of contaminant (Binelli et al., 2009b) and warranting the trustworthiness of our experimental design, as previously observed in previous exposures to pharmaceutical and personal care products (Binelli et al., 2009b; Parolini et al., 2010, 2011a,b), as well as illicit drugs (Binelli et al., 2012). Specimens were fed 2 h before the daily change of water and chemicals to avoid the adherence of the drugs to food particles and to prevent the reduction of their bioavailability. Several specimens (n = 30) were collected every 3 days for 14 days from each aquarium to evaluate Δ-9-THC-induced sub-lethal effects. Bivalve hemolymph was withdrawn and cyto-genotoxicity was evaluated on hemocytes. After the withdrawal, the soft tissues of mussel were immediately frozen in liquid nitrogen and stored at −80 °C until LPO and PCC analyses. Lastly, the soft tissue of other 15 specimens was frozen in liquid nitrogen and stored at −80 °C until the enzymatic activity was measured.

2.2. NRRA, enzyme activity and oxidative stress biomarkers

The activities of SOD, CAT, GPx, and GST were measured in triplicate (n = 3) in the cytosolic fraction extracted from a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 100 mM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) using a Potter homogenizer. Specific protease inhibitors (1:10) were also added to the buffer: dithiothreitol (DTT, 100 mM), phenanthroline (Phe, 10 mM) and trypsin inhibitor (Try, 10 mg/mL). The homogenate was centrifuged at 15,000 g for 1 h at 4 °C. The sample was held in ice and immediately processed for the determination of protein and enzymatic activities. The total protein content of each sample was determined according to the Bradford (1976) method using bovine serum albumin as a standard. Enzymatic activities were determined spectrophotometrically as described by Orbea et al. (2002). Briefly, the CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 substrate in 67 mM potassium phosphate buffer (pH 7). The SOD activity was determined by measuring the degree of inhibition of cytochrome c (10 μM) reduction at 550 nm by the superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 μM) reaction. The activity is given in SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction). The GPx activity was measured by monitoring the consumption of NADPH at 340 nm using 0.2 mM H_2O_2 substrate in 50 mM potassium phosphate buffer (pH 7) containing additional glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL), and NADPH (120 μM). Lastly, the GST activity was measured by adding reduced glutathione (1 mM) and 1-chloro-2,4 dinitrobenzene in phosphate buffer (pH 7.4) to the cytosolic fraction; the resulting reaction was monitored for 1 min at 340 nm. Lipid peroxidation (LPO) and protein carbonyl content (PCC) were measured in triplicate (n = 3) from a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 50 mM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) containing 1 mM DTT and 1 mM PMSF using a Potter homogenizer. LPO level was assayed by the determination of thiobarbituric acid-reactive substances (TBARS) according to Ohkawa et al. (1979). The absorbance was read at 532 nm after removal of any fluctuated material by centrifugation. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of $1.56 * 10^5$ M/cm and expressed as nmol TBARS formed/g fresh weight. For carbonyl quantification the reaction with 2,4-dinitrophenylhydrazine (DNPH) was employed according to Mecocci et al. (1998). The carbonyl content was calculated from the absorbance measurement at 370 nm with the use of molar absorption coefficient of 22,000 mol/cm and expressed as nmol/(mg protein). The NRRA method followed the protocol proposed by Lowe and Pipe (1994) and was applied on mussel hemocytes. Slides were examined systematically thereafter at 15 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests finished when dye loss was evident in at least 50% of the hemocytes. The mean retention time was then calculated from five replicates.

2.3. Genotoxicity biomarkers

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

2.4. Statistical analysis

Data normality and homoscedasticity were verified using the Shapiro–Wilk and Levene's tests, respectively. To identify dose/effect and time/effect relationships a two-way analysis of variance (ANOVA) was performed using time and Δ-9-THC concentrations as variables, while biomarker end-points served as cases. The ANOVA was followed by a Fisher LSD post-hoc test to evaluate significant differences ($^*p < 0.05$; $^{**}p < 0.01$) between treated samples and related controls (time to time), as well as among exposures. The Pearson's correlation test was carried out on all measured variables in the exposure assays to investigate possible correlations between the different biological responses. Principal component analysis (PCA) was used to evaluate biomarker response accountability for the variance at 0.5 μg/L Δ-9-THC for each sampling time. All statistical analyses were performed using the STATISTICA 7.0 software package.

3. Results

3.1. Biomarker baseline levels

The 14-day average hemocyte viabilities of bivalves from control, 0.05 μg/L and 0.5 μg/L tanks were 89 ± 4%, 88 ± 5% and 85 ± 7%, respectively. Baseline levels of enzyme activity, cyto-genotoxic and oxidative stress biomarkers were similar to those obtained in previous laboratory studies (Binelli et al., 2009a,b; Parolini et al., 2010, 2011a,b, 2013a; Parolini and Binelli, 2012, in press) and fell within the physiological range of this bivalve species.

3.2. Enzyme activity and oxidative stress biomarker results

The trends of the antioxidant (SOD, CAT and GPx) and detoxifying enzymes (GST) are reported in Fig. 1. SOD activity showed an overall inhibition at both the tested concentrations, following significant time- ($F = 2.80$; $p < 0.05$) and concentration ($F = 21.22$; $p < 0.01$) dependencies. In contrast, no significant ($p > 0.05$) alterations with respect to controls were shown in CAT activity at the lowest Δ-9-THC treatment, while a typical bell-shaped trend was noticed at 0.5 μg/L,

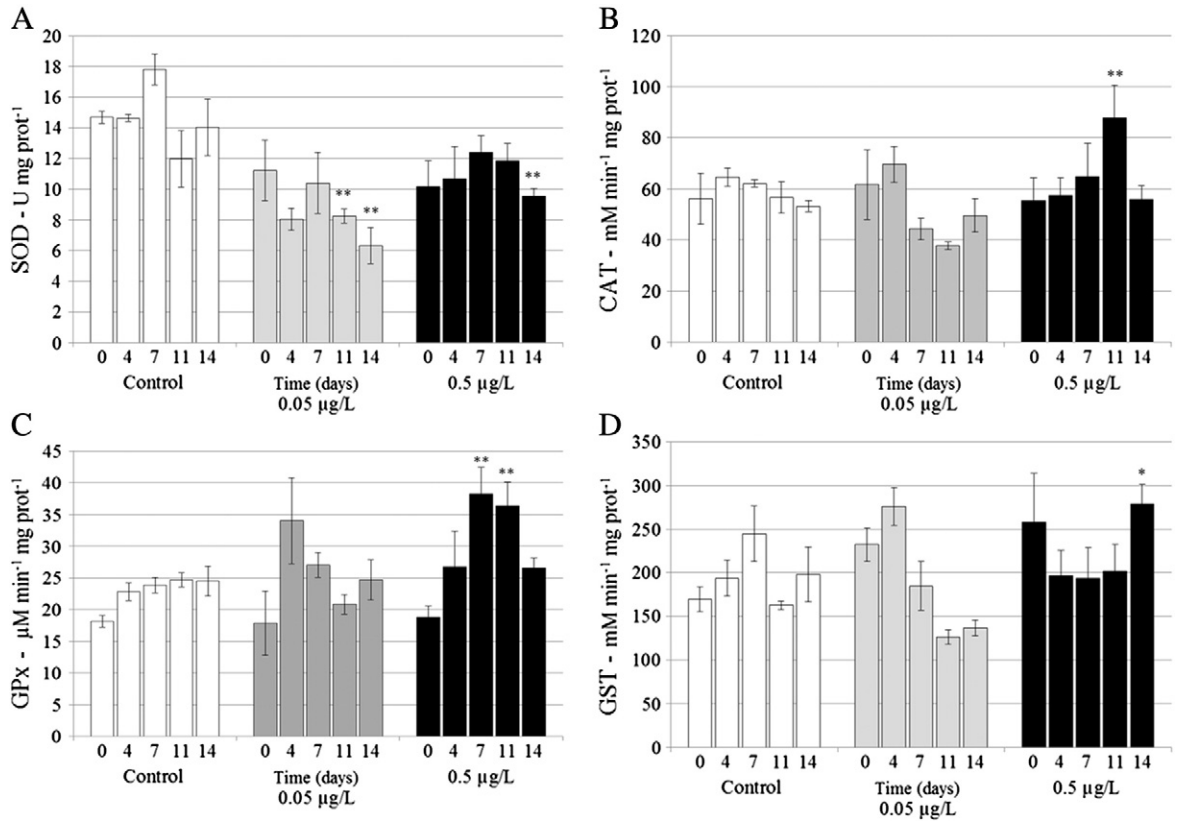

Fig. 1. Effects of Δ-9-THC treatments on the activity (mean ± SEM) of superoxide dismutase (SOD; A), catalase (CAT; B), glutathione peroxidase (GPx; C), and glutathione S-transferase (GST; D), measured in the whole soft tissue of zebra mussels (n = 3; pool of 3 specimens). Significant differences (two-way ANOVA, Fisher's LSD post-hoc test, *$p < 0.05$; **$p < 0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

with values 35% higher than control at t = 11 days. GPx showed a similar trend at both the concentrations, following significant time- ($F = 10.02$; $p < 0.01$) and concentration ($F = 8.45$; $p < 0.01$) relationships. In detail, a significant ($p < 0.01$) 40% increase of its activity compared to controls was noted after 7 days of exposure at 0.5 µg/L followed by a decline to baseline levels. No significant ($p > 0.05$) variations in GST activity with respect to baseline levels were noticed at the lowest Δ-9-THC concentration, while at 0.5 µg/L a significant ($p < 0.05$) increase was found at the end of the exposure, with values 30% higher than the corresponding control. The NRRT showed a decreasing trend at both the tested concentrations (Fig. 2a), according to significant time- ($F = 48.42$; $p < 0.01$) and dose-dependent ($F = 37.30$; $p < 0.01$) relationships. A significant ($p < 0.01$) increase of lysosome membrane destabilization in bivalves was observed after 11 days of treatment to both the Δ-9-THC concentrations, showing values 2- to 3-fold lower than the corresponding control at the end of the test, respectively. 14 days of exposure resulted in significantly time- ($F = 4.56$; $p < 0.01$) and concentration-dependent ($F = 11.37$; $p < 0.01$) increases in LPO levels (Fig. 2b), showing significant 26% increases ($p < 0.01$) of lipid peroxidation compared to baseline ones at the end of exposure to 0.5 µg/L. Similar time- ($F = 11.88$; $p < 0.01$) and concentration- ($F = 9.85$; $p < 0.01$) dependent increases of protein carbonylation were found after 14 days of exposure to 0.5 µg/L, reaching values 26% higher than the baseline levels (Fig. 2c).

3.3. Genetic biomarker results

No significant increases ($p > 0.05$) of LDR values compared to controls were found during the 14 days of exposure to both Δ-9-THC concentrations (Fig. 3a), while time- ($F = 6.59$; $p < 0.01$) and concentration- ($F = 15.48$; $p < 0.01$) dependent increases of the mean percentage of DNA in the comet tail (Fig. 3b) were found. A significant ($p < 0.01$) 36% increase of %DNA in tail in comparison to baseline levels was noticed after 11 days of exposure to 0.5 µg/L, reaching values 43% higher than controls at the end of the test. Time- ($F = 4.56$; $p < 0.05$) and concentration- ($F = 13.77$; $p < 0.01$) dependent increases of micronucleated cell frequency were found at the end of the 0.5 µg/L treatment, with values 3-fold higher than the corresponding control (Fig. 3c), while no significant ($p > 0.05$) increase in apoptotic cell frequency (Fig. 3d) was noticed at both the Δ-9-THC exposures.

4. Discussion

The involvement of pollutants in causing oxidative stress to aquatic organisms has been reported in several studies and has been associated with production of reactive oxygen species (ROS) (Vijayavel et al., 2004; Valavanidis et al., 2006). Toxic effects of some environmental contaminants, in fact, often depend on their capacity to increase the cellular levels of ROS, that can happen either by the straightforward activation of processes that leads to their synthesis or indirectly acting on enzymes and scavengers, decreasing cell defenses (Viarengo et al., 2007). In this sense, the antioxidant defense system is crucial in the neutralization processes of generated ROS by chemical redox reactivity (Regoli et al., 2002a,b). Indeed, the activities of SOD, CAT, GPx and GST are often modified in response to cellular oxidative stress and provide information on responses to pollutant-induced changed levels of ROS in different aquatic organisms (Viarengo et al., 2007), as also shown in previous studies on zebra mussel specimens exposed to different pharmaceuticals (Parolini et al., 2010, 2011a,b; Parolini and Binelli, 2012) and illicit drugs (Parolini et al., 2013a; Parolini and Binelli, in press). Overall, exposure to Δ-9-THC induced different variations in *D. polymorpha* defense enzyme activities depending on exposure concentrations. At the lowest treatment (0.05 µg/L), slight variations of enzymatic activity were found since, with the exception of a significant ($p < 0.05$) decrease of SOD

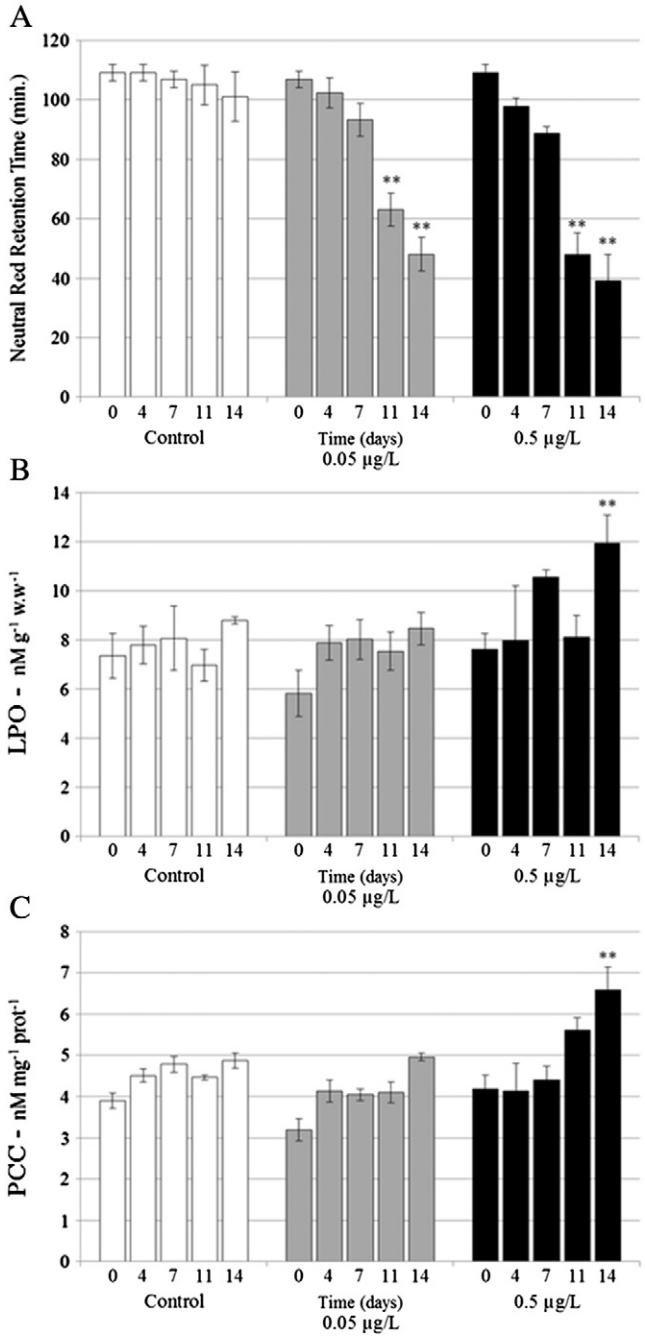

Fig. 2. Measure (mean ± SEM) of neutral red retention time (NRRT; A) in the hemocytes of zebra mussels (n = 5), lipid peroxidation (LPO; B) and protein carbonylation (PCC; C) in zebra mussel homogenates from pools of 3 specimens (n = 3) treated with two Δ-9-THC concentrations. Significant differences (two-way ANOVA, Fisher's LSD post-hoc test, *p < 0.05; **p < 0.01) were referred to the comparison between treated mussels and the corresponding control (time to time).

(Fig. 1) after 11 and 14 days of exposure, no significant (p > 0.05) differences with respect to controls in CAT and GPx levels, as well as in GST, were noticed. In contrast, notable imbalances in all the enzyme activities were found at the highest treatment. A significant (p < 0.01) increase of GST was observed at the end of exposure to 0.5 µg/L, suggesting the involvement of phase II enzymes in the detoxification of the Δ-9-THC. Moreover, since the activity of GST as pro-oxidant has been shown in different aquatic organisms (Zhang et al., 2003; Lushchak and Bagnyukova, 2006), its enhancements might suggest both an increased ROS production and the activation of the antioxidant defense system via the xenobiotics (Elia et al., 2007), as confirmed by the imbalances of SOD, CAT and GPx. A significant (p < 0.01) inhibition of SOD was found at the end of exposure to 0.5 µg/L of Δ-9-THC. Considering its primarily role in the dismutation of superoxide anions into H_2O_2, its inhibition could be associated to a higher production of ROS (Gonzales-Rey and Bebianno, 2013) and it is indicative of increased accumulation of superoxide radical ($O_2^{\bullet -}$; Verlecar et al., 2008). However, the $O_2^{\bullet -}$ was not the only radical affecting the zebra mussel oxidative status. The reduced SOD activity, in fact, could be due to inhibition effect and/or negative feed-back by the product of its reaction, suggesting that it had already produced cytosolic H_2O_2, as proposed by Vlahogianni and Valavanidis (2007) in *M. galloprovincialis* specimens exposed to Cu. Moreover, both the spontaneous dismutation of superoxide radical by non-enzymatic ways (Gwoździński et al., 2010) and other cellular enzymes like those contained in peroxisomes (Khessiba and Roméo, 2005), can lead to the production of hydrogen peroxide. The significant (p < 0.01) activations of CAT and GPx after 11 and 7 days (Fig. 1b, c) respectively, confirmed the production of H_2O_2 and, at the same time, that these enzymes succeeded in counteracting its toxicity. However, the following decrease of their activities noticed at the end of the experiment may be due to an overwhelming excess of H_2O_2 that enzymes cannot counteract (Gonzalez-Rey and Bebianno, 2011). The bell-shaped trends of CAT and GPx are typical of enzymatic response to toxic chemicals, which shows an initial increase due to the activation of enzyme synthesis followed by a decrease in enzymatic activity due to the enhanced catabolic rate and/or a direct inhibitory action of toxic chemicals on the enzyme molecules (Viarengo et al., 2007). For instance, a similar bell-shape was found in gills from *Mytilus galloprovincialis* exposed to 250 ng/L of the non-steroidal anti-inflammatory drug (NSAID) ibuprofen (Gonzalez-Rey and Bebianno, 2011) and in zebra mussel specimens at the end of a 96 h-exposure to 8 µg/L of the same compound (Parolini et al., 2011a). For this reason, to confirm the oxidative stress situation that organisms suffer, enzyme assays should be used in association with other biomarkers, such as the lysosomal membrane stability, which following the development of the pollutant-induced stress syndrome may help to correctly interpret the "physiological meaning" of changes observed in antioxidant enzymatic activities (Viarengo et al., 2007). The stability of lysosome membranes in mussels, in fact, can be affected by the production of oxyradicals generated by the exposure to contaminants (Regoli et al., 1998) and alterations to these organelles have been related to the increase of peroxidative processes (Winston et al., 1996), common pathways of toxicity of several environmental pollutants also in the zebra mussel, including pharmaceuticals (Parolini et al., 2010, 2011a) and illicit drugs (Parolini et al., 2013a). The time-dependent decrease of NRRT found at both the administered concentrations (Fig. 2a) showed a progressive aggravation of the bivalve health status, confirming that treated specimens suffered a cellular stress situation likely linked to the induction of oxidative stress (Lowe et al., 1995), mainly at the highest concentration. This condition could lead to notable oxidative damage to different cellular macromolecules, such as lipids of membranes, proteins and DNA, as observed in Mediterranean mussels exposed to environmentally relevant levels of the pesticides chlorpyrifos and penoxsulam (Patetsini et al., 2013). The measure of lipid peroxidation, a traditional end-point of oxidative stress (Tedesco et al., 2010), and protein carbonyl content of tissues, the most general indicator of severe protein oxidative damage (Dalle-Donne et al., 2003), supported this hypothesis. On the basis of enzymatic responses, in fact, the accumulation of superoxide radical supposed by SOD inhibition in combination with H_2O_2 that CAT and GPx cannot efficiently counteract might give rise to the production of hydroxyl radicals by classic Haber–Weiss reaction, resulting in elevated LPO and carbonylated protein levels (Verlecar et al., 2008). In fact, while at the lowest treatment no increases of cellular damage were found, accordingly to slight imbalances of antioxidant enzyme activities, the significant increase (p < 0.01) of lipid peroxidation and protein carbonylation (Fig. 2c) noticed at the end of exposure to 0.5 µg/L Δ-9-THC exposure (Fig. 2b) confirmed that zebra mussel specimens suffered an

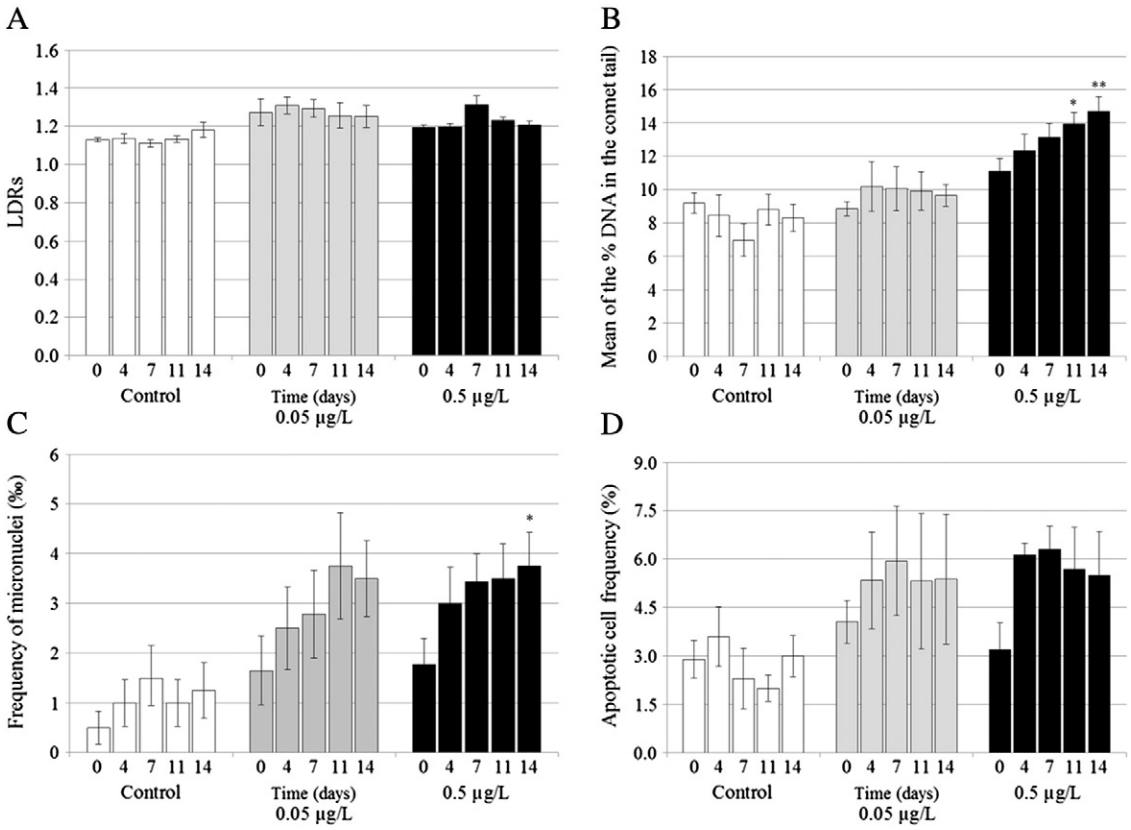

Fig. 3. Results (mean ± SEM; standard error of the mean) of the SCGE assay expressed by the length/diameter ratio (A) and the mean percentage of tail DNA (B). Measurements were carried out on zebra mussel hemolymph samples (n = 10) exposed to Δ-9-THC concentrations. Frequency of micronucleated hemocytes (C) (n = 10; mean ± SEM) and percentages of apoptotic hemocytes (n = 5; mean ± SEM) measured by the DNA Diffusion assay (D) observed in *D. polymorpha* specimens exposed to EME treatments. Significant differences (two-way ANOVA, Fisher's LSD post-hoc test, *$p < 0.05$; **$p < 0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

oxidative stress situation, which might have led to the disruption of cell membrane integrity (Yajima et al., 2009) and loss of protein structural and functional efficiencies (Dalle-Donne et al., 2003, 2006), respectively. DNA is the other target biomolecule of cellular oxidative injury and many studies have highlighted that the increase of pollutant-induced ROS could undermine its integrity in different aquatic organisms (Regoli et al., 2002a,b; Mamaca et al., 2005), including zebra mussel (Parolini et al., 2010, 2011a; Parolini and Binelli, 2012). Even if LDR parameter did not show any significant ($p > 0.05$) variation compared to controls (Fig. 3a) at both the tested concentrations, 0.5 µg/L Δ-9-THC exposure induced DNA fragmentation in treated bivalves, as shown by the significant ($p < 0.01$) increase of %DNA in tail after 11 days of exposure (Fig. 3b). On the other hand, this end-point is generally considered more sensitive than LDR (Binelli et al., 2008). In addition, covalent binding to certain breakdown products of lipid hydroperoxides, such as malondialdehyde (MDA), can result in DNA strand breaks and crosslinks (Cheung et al., 2002), while it is well-known that carbonyl compounds are toxic due to their carcinogenic properties (Labieniec and Gabryelak, 2004). The significant positive correlation ($r = 0.88$; $p < 0.05$) between primary DNA damage and protein carbonylation found in our study suggested the involvement of carbonylated proteins in Δ-9-THC genotoxicity and agreed data by Patetsini et al. (2013), who found an increase of DNA fragmentation linked to protein carbonyl content in *M. galloprovincialis* specimens treated with two pesticides. Although several studies showed that DNA strand breaks are apoptosis-inducing factor in zebra mussels (Binelli et al., 2009a,b; Parolini and Binelli, 2012) and the main contributor to micronuclei induction (Van Goethem et al., 1997), only a significant increase ($p < 0.01$) of micronucleated cell frequency (Fig. 3c) was found, while the apoptotic process was not triggered (Fig. 3d). Accordingly, the significant ($r = 0.94$; $p < 0.05$) positive correlation between %DNA in tail and ‰MN confirmed the strict relationship between primary and fixed genetic damages. The integration of all the responses into a principal component analysis (PCA; Fig. 4) supported our hypotheses on the evolution of the oxidative damage induced by Δ-9-THC to zebra mussel specimens. The PCA analysis indicated that the original variable set could be narrowed down to three new variables of factors, which explained 94.03% of the total variance. The first factor accounted for 49.35% of the variance, while the second and the third factors, accounted respectively for 33.16 and 11.52% of the variance. Since the F1 and F2 scores explained most of the total variance, they were plotted in a two dimensional way to produce an integrated view of obtained biomarker responses, allowing to follow the evolution of adverse effects due to Δ-9-THC exposure towards zebra mussel specimens over time. By considering the projection of variables on the factor plane (Fig. 4a) PCA distinguished two different groups. The first one included antioxidant responses and revealed their activation to counterbalance the ROS production until the 11th day of exposure. The second one grouped responses of biomarkers of effect, indicating that Δ-9-THC induced oxidative (LPO and PCC) and genetic damages (% DNA in tail and ‰ MN) to zebra mussel specimens at the end of treatment because defense enzymes cannot balance the excess of pro-oxidant molecules, as confirmed by the projection of cases on factor plane (Fig. 4b). In spite of notable adverse effects caused by a low Δ-9-THC concentration to an invertebrate biological model, our findings disagreed with those from a previous study by Pinto et al. (2010), which investigated the effects to mice treated with two daily intraperitoneal injections of Δ-9-THC (a total daily dose of 10 mg/kg body weight) for a period of 10 days. Hepatic levels of lipid peroxidation, protein carbonylation and DNA damage measured in treated mice were not significantly different from controls, while just slight alterations in antioxidant and detoxifying enzymes were found (Pinto et al., 2010). Moreover, several

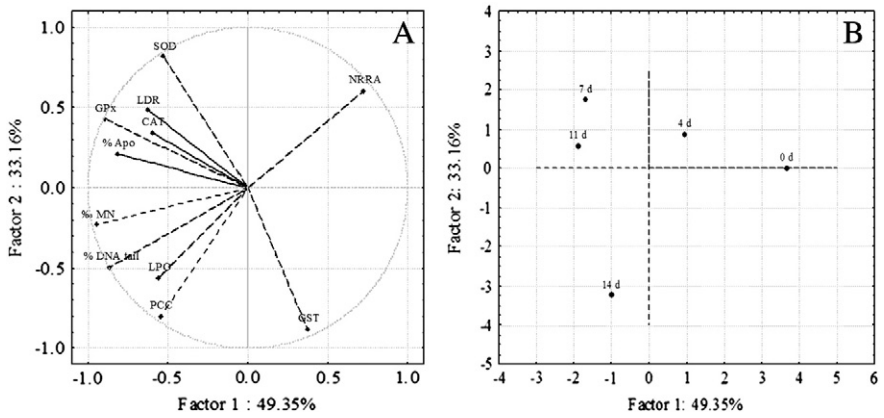

Fig. 4. Projection of variables (A) and cases (B) on the factor plane after application of principal component analysis (PCA) using all biomarker responses obtained during the 14 days of exposure to 0.5 μg/L Δ-9-THC concentration.

studies have reported that Δ-9-THC has a cannabinoid receptor-independent protective effect against increased oxidative stress conditions (Hampson et al., 1998; Chen and Buck, 2000; Marsicano et al., 2002) in mammalian biological models, probably due to its high resemblance to the antioxidant vitamin E (Chen and Buck, 2000). Even if differences in Δ-9-THC effects could be mainly ascribable to the difference in physiology/metabolism of vertebrate and invertebrate biological models, as well as time and method of exposure, it is important to highlight that Δ-9-THC could be potentially considered a hazardous compound towards bivalves and further research should be necessary to confirm its risk to aquatic communities. This is particularly true if we compare the sub-lethal toxicity of 0.5 μg/L Δ-9-THC to zebra mussel specimens with that of the same concentration of other two illicit drug residues, the cocaine metabolites benzoylecgonine (BE; Parolini et al., 2013a) and ecgonine methyl ester (EME; Parolini and Binelli, in press), which was found to cause remarkable adverse effects to *D. polymorpha* specimens. In order to rank the chronic toxicity of these compounds to this bivalve species, we integrated the whole biomarker dataset obtained at the common concentration of 0.5 μg/L for the three molecules into a biomarker response index (BRI), according to the procedures previously described in detail by Parolini et al. (2013b). The application of approaches integrating all biomarker data into a synthetic index, in fact, could help to minimize variation of responses (Hagger et al., 2006) and to allow an easier interpretation of complex ecotoxicological data (Moore et al., 2004; Sforzini et al., 2011). Since changes in individual biomarker values over a stress gradient yield characteristic trends (increasing, decreasing or bell-shaped; Hagger et al., 2010), we calculated the percentage alteration level (AL) of each biomarker for the exposure time, compared to corresponding controls. To calculate the BRI, similarly to Dagnino et al. (2007) and Parolini et al. (2013b) we gave a specific score to each response depending on their AL, and each biomarker was weighted according to its level of biological organization according to Hagger et al. (2010). In spite of the notable differences in chemical–physical features and in biomarker responses among drugs, the comparison of BRI values (Fig. 5) showed that, at the same concentration, Δ-9-THC toxicity to *D. polymorpha* specimens seems slightly higher than BE and EME, confirming its potential hazard to aquatic organisms.

5. Conclusions

Our findings showed that Δ-9-THC exposure could induce adverse effects to a freshwater bivalve species, highlighting its possible hazard to freshwater communities. Even if current environmental Δ-9-THC levels did not cause any deleterious effect to bivalves, 14 day experiments to 0.5 μg/L concentration caused an oxidative stress situation in treated specimens leading to damage to different macromolecules, such as lipids of biological membranes, proteins and DNA. However, in spite of these evidences, the Δ-9-THC mechanism of action in zebra mussel based on biomarker results should be studied in-depth by using powerful techniques, such as the so-called "omic techniques". Notwithstanding, our findings represent a warning signal of the potential Δ-9-THC hazard towards aquatic organisms. Even if current environmental levels are low and seem to not pose a serious hazard to bivalves, the scenario pointed out by our 14-day experiment must not be absolutely underestimated since in the environment organisms are exposed to Δ-9-THC concentrations for their whole life-span, resulting in possibly even higher toxicity. Moreover, since the Δ-9-THC is the main psychoactive chemical of the cannabis, the most used illicit drug worldwide, its steady usage could lead to its continuous input to freshwaters, increasing the environmental levels. In conclusion, considering the extensive use of cannabis , the Δ-9-THC occurrence in aquatic environments and its potential hazard to bivalves pointed out by the comparison with other illicit drug residues, in-depth investigations on its toxicity should be a priority in environmental risk assessment, in order to enhance knowledge on their possible sub-lethal effects, the mechanism of action for aquatic organisms and effects on population dynamics, which should clarify its true ecological hazard for the biocenosis.

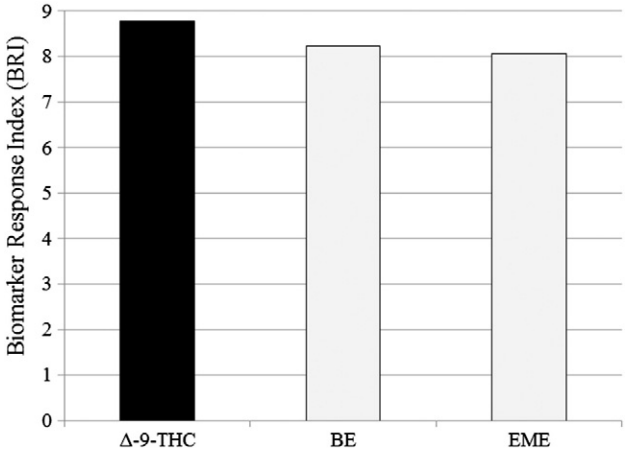

Fig. 5. Comparison of Δ-9-THC, benzoylecgonine (BE) and ecgonine methyl ester (EME) sub-lethal toxicity by integrating biomarker responses into a biomarker response index (BRI). BE and EME BRI values (gray bars) were from Parolini and Binelli (2013).

Conflict of interest

The authors declare to have not conflict of interest in performing this research and in writing the present manuscript.

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