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Genotoxic effects induced by the exposure to an environmental mixture of illicit drugs to the zebra mussel

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ABSTRACT

Despite the growing interest on the presence of illicit drugs in freshwater ecosystems, just recently the attention has been focused on their potential toxicity towards non-target aquatic species. However, these studies largely neglected the effects induced by exposure to complex mixtures of illicit drugs, which could be different compared to those caused by single psychoactive molecules. This study was aimed at investigating the genetic damage induced by a 14-day exposure to a realistic mixture of the most common illicit drugs found in surface waters worldwide (cocaine, benzoylecgonine, amphetamine, morphine and 3,4-methylenedioxymethamphetamine) on the zebra mussel (*Dreissena polymorpha*). The mixture caused a significant increase of DNA fragmentation and triggered the apoptotic process and micronuclei formation in zebra mussel hemocytes, pointing out its potential genotoxicity towards this bivalve species.

1. Introduction

Illicit drugs, including opioids, cocaine, cannabis, amphetamine-type substances (ATSs) and ecstasy-group substances (UNODC, 2007) represent a global problem with significant direct and indirect adverse effects on human health and social welfare (EMCDDA, 2007). Because of their widespread diffusion in both surface and wastewaters worldwide as consequence of their metabolism in the human body and/or accidental or deliberate disposal (Pal et al., 2013), illicit drugs are considered emerging aquatic pollutants. Even if their current environmental levels are relatively low, similarly to pharmaceuticals their continuous use lead to a continuative input to aquatic environments, imparting them with 'pseudo-persistence' (Barcelò and Petrovic, 2007; Petrovic et al., 2009). Thus, this situation could result in long-term and multigenerational exposure to aquatic species. Since illicit drugs are biologically-active and neurologically-addictive substances, adverse effects to non-target aquatic organisms and the whole ecosystem are possible. Moreover, the continual but unnoticed injuries towards aquatic organisms are worrisome because these effects could accumulate so slowly that changes remain undetected until their cumulative level produces irreversible damage that could be attributed to natural adaptation (Daughton and Ternes, 1999). Lastly, their presence in aquatic ecosystems combined with the residues of many other therapeutics may lead to unexpected pharmacological interactions, causing unforeseeable toxic effects to organisms (Pal et al., 2013). In fact, in natural ecosystems, non-target organisms are exposed for their whole lifespan to complex mixtures of drugs, whose toxicity is typically higher than that of single therapeutics (Kortenkamp et al., 2009). Although many monitoring surveys pointed out the levels of illicit drugs in the aquatic ecosystems (Pal et al., 2013 and references therein), there is a dearth of studies investigating their possible adverse effects to non-target organisms. In addition, these few experiments have been generally focused on a single drug, while to date the diverse effects of these substances in a mixture are largely neglected. Our first studies showed than environmentally relevant concentrations of the main illicit drugs found in surface water worldwide, namely cocaine (COC), benzoylecgonine (BE), amphetamine (AMPH), 3,4-methylenedioxymethamphetamine (MDMA) and morphine (MOR), were able to imbalance the activity of defense enzymes in the zebra mussel (*Dreissena polymorpha*), which represents an excellent biological model in ecotoxicological studies (Binelli et al., 2015). However, neither significant primary nor fixed genetic damage was induced on hemocytes of the zebra mussel at low environmental concentrations (Binelli et al., 2012; Parolini et al., 2014; Magni et al., 2014), with the exception of BE, one of the main COC metabolite (Parolini et al., 2013). In addition, we first pointed out that the exposure to a mixture of the same illicit drugs, administered at realistic environmental concentrations, imbalanced the activity of antioxidant enzymes and caused

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an increase of protein carbonyl levels in zebra mussel homogenates at greater extent with respect to the same drugs administered singularly (Parolini et al., 2015). Since no other information is available on the potential toxicity of illicit drug mixtures to non-target aquatic organisms, this study was aimed to assess the genotoxic effects induced by a realistic mixture of COC, BE, AMPH, MDMA and MOR to the zebra mussel. Although in freshwater ecosystems other illicit drugs could be found at concentrations higher than those of the drugs considered in the present study (*i.e.*, methamphetamine concentrations in wastewater effluents are higher than those of AMPH; Subedi and Kannan, 2014), our mixture considered only the five drug residues mentioned above because their toxicity was previously tested singularly on the same biological model, and they are the most common psychoactive compounds found in surface water worldwide. Genetic damage was investigated on zebra mussel hemocytes after 14-day *in vivo* exposure to the selected mixture through the application of three specific biomarkers: the Single Cell Gel Electrophoresis (SCGE) assay was used to investigate the primary DNA fragmentation, while the DNA diffusion assay and the Micronucleus test were applied to show fixed injuries.

2. Materials and methods

2.1. Selection of exposure concentrations

Since the genotoxicity of these molecules to zebra mussel hemocytes was singularly investigated in our previous studies, to create the drug mixture we selected similar concentrations that are close to their current maximum levels measured in surface water worldwide (Pal et al., 2013). This allowed both the comparison of adverse effects among single chemicals and the mixture and, at the same time, to safeguard the ecological realism. Thus, mussels were exposed to a mixture of the main illicit drug residues found in surface waters, namely COC (50 ng/L), BE (300 ng/L), AMPH (300 ng/L), MDMA (50 ng/L) and MOR (100 ng/L).

2.2. Experimental design

Zebra mussel specimens were sampled in October 2013 in Lake Lugano, which is considered a reference site because of its negligible illicit drug contamination (Zuccato et al., 2008). Several hundreds bivalves were placed in glass aquaria (12 L) filled with tap and deionized water (50:50 v/v), previously de-chlorinated by aeration overnight, and maintained for 2 weeks under controlled conditions: temperature of 20 ± 1 °C, photoperiod of 16:8 light/night hours and oxygen concentration $> 90\%$. Water was completely changed every two days and zebra mussels were fed daily with an algal suspension of *Spirulina* spp. We selected only bivalves having a similar shell length (16 ± 3 mm), which were gently laid down on glass supports in 3 beakers (5 L) per treatment (control and illicit drug mixture, respectively) filled with 4 L of tap and deionized water (1:1 v/v), where they were acclimatized for one week at the same conditions described above. We put in each beaker 60 mussels for a total of 180 organisms per treatment. Hemocytes' viability, assessed by the Trypan Blue exclusion method, and the baseline levels of genetic biomarkers were checked at the end of this week to confirm the achievement of baseline levels for each investigated endpoint (see Parolini et al. (2014); Magni et al. (2014)). A 14-day exposure was performed under semi-static conditions, renewing the exposure water and adding the exact volume of each illicit drug working solution (see below) in water to reach the selected concentration every 24 h. Mussels were fed 1 h before the change of the whole exposure medium. A stock solution of each drug (10 mg/L for COC, BE and AMPH; 1 mg/L for MDMA and MOR) was prepared diluting the analytical standard (1 g/L in methanol; Alltech-Applied Science, State College, PA, USA) in ultrapure water. Exact volumes of each stock solution were daily added to the exposure tanks until reaching the selected nominal concentrations. The concentration of the illicit drug residues composing the mixture was measured in both control and exposure beakers to confirm that water concentrations were similar to the nominal ones, guaranteeing the exposure conditions. Water was sampled 1 h after the spiking of the stock solutions from the control and exposure beakers and integrated in a unique sample (100 mL) *per* treatment. Preliminary analyses showed that 1 h is a sufficient period of time allowing the homogenization of drug concentration into the exposure tank. The analysis was performed in triplicate using solid phase extraction (SPE) and high performance liquid chromatography tandem mass spectrometry analysis (HPLC-MS/MS), according to the method described in detail by Parolini et al. (2015). Every three days, four mussels were randomly collected from each tank ($n=12$ specimens *per* treatment), including controls. The hemolymph was withdrawn according to the procedure described by Parolini et al. (2010) and used for genetic biomarkers after checking hemocyte viability by the Trypan Blue exclusion method.

2.3. Genetic biomarkers

A detailed description of genetic biomarker methods and procedures was reported by Parolini et al. (2010). Briefly, the alkaline ($pH > 13$) Single Cell Gel Electrophoresis (SCGE) assay was performed on hemocytes in accordance to Buschini et al. (2003). To assess effectiveness of the electrophoretic conditions and the robustness of results we treated zebra mussel hemocytes for 1 h with H_2O_2 (0.3%) as positive control. Fifty cells per slide ($n=10$; each slide corresponds to a single specimens) were analyzed using the Comet Score® image analysis system. The tail length/nucleoid diameter ratio (LDR) and the percentage of DNA in the comet tail were chosen as DNA fragmentation end-point. The apoptotic cell frequency (%) was evaluated according to Singh (2000) adapted for zebra mussel hemocytes and 200 cells *per* slide ($n=6$; each slide corresponds to a single specimens) were analyzed. Lastly, the Micronuclei (MN) frequency (‰) was calculated according to Pavlica et al. (2000). We counted 400 cells *per* slide ($n=10$; each slide corresponds to a single specimen) and micronuclei were identified following the criteria proposed by Kirsch-Volders et al. (2000). The reliability of the all the applied genotoxicity biomarker methods for the model species was previously confirmed by *in vivo* exposure to increasing concentrations of the genotoxic compound benzo(α)pyrene (Binelli et al., 2008).

2.4. Statistical analysis

The effect of illicit drug mixture treatment on genetic endpoints was analyzed by means of two-way Analysis of Variance (ANOVA), after checking for normality and homoscedasticity of the data. The treatment and the exposure time was considered as fixed effect factors together with their two-way interactions. Fisher LSD *post-hoc* test was applied to point out significant differences ($^{*}p < 0.05$; $^{**}p < 0.01$) between treated specimens and corresponding control (time to time). The Pearson's correlation test was performed using all the measured endpoints, including those from oxidative stress biomarker to assess the correlations between all the investigated adverse effects. Statistical analyses were carried out using the STATISTICA 7.0 software package.

3. Results

No residues of illicit drugs composing the mixture were found