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Exposure to crack cocaine causes adverse effects on marine mussels *Perna perna*

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ABSTRACT

Our study aimed to evaluate crack cocaine effects in different life stages of the marine mussel *Perna perna*. For this purpose, fertilization rate, embryo-larval development, lysosomal membrane stability and DNA strand breaks were assessed. Effect concentrations in gametes and in larval development were found after 1 h (IC_{50} = 23.53 mg·L^{-1}) and 48 h (IC_{50} = 16.31 mg·L^{-1}), respectively. The highest tested concentration showing no acute toxicity (NOEC) was 10 mg·L^{-1}, while the lowest observed effect concentration (LOEC) was 20 mg·L^{-1}. NOEC concerning embryo-larval development was 0.625 mg·L^{-1}, while the LOEC was 1.25 mg·L^{-1}. Cyto-genotoxic effects were evidenced in mussels exposed to crack cocaine concentrations ranging from 5 to 500 µg·L^{-1}. Our results report the first data on effects of an illicit drug to marine organisms and should encourage further ecotoxicological studies of these contaminants of emerging concern in coastal ecosystems.

Illicit drugs are a growing public health problem worldwide which cocaine and marijuana are the most consumed ones. The World Drug Report (UNODC, 2014) observed that the highest consumption of cocaine (including crack cocaine) occurs in the Americas. In North America, cocaine consumption has declined since 2006, partly due to a sustained shortage, although they are considered the largest consumers in the world. Illicit drug use has increased dramatically in the last decade in developing countries of South America (Johnson et al., 2013). The consumption and trafficking of cocaine in South America have become more prominent, particularly in Brazil, due to factors such as geographic location and the population increase clustered in urban centers. Brazilian cocaine users were estimated to be nearly 2 million in the 2004–2005 period, and this number rose to 3.35 million in 2012 (UNODC, 2014). Brazil has been reported as the world's largest market for crack cocaine (Laranjeira et al., 2012) and the main cocaine destination country in South America over the period 2010–2015 (UNODC, 2017).

The increase in illicit drugs consumption produces not only public health problems, but also induces potential environmental impacts since these contaminants of emerging concern were recently identified as toxic for aquatic organisms (Binelli et al., 2012; Parolini et al., 2013).

Such compounds are continually released into the environment via wastewater treatment plants (WWTPs). Concerning freshwater environments, many studies have been published about real concentrations of cocaine in surface waters (Hernández et al., 2015; Baker and Kasprzyk-Hordern, 2013; Baker et al., 2012; Castiglioni et al., 2011; Metcalfe et al., 2010; Van Nuijs et al., 2009). However, few studies have analyzed estuarine and marine environments. Klosterhaus et al. (2013) found cocaine in seawater (2.4 ng·L^{-1}), sediment (0.2 ng·g^{-1} dw) and mussels (0.3 ng·g^{-1} ww) from San Francisco Bay (USA). Recently, Pereira et al. (2016) found higher concentrations of cocaine and benzoylecgonine (537.0 ng·L^{-1} and 20.8 ng·L^{-1}, respectively) in seawater samples from Santos Bay (Brazil).

As pointed out by Binelli et al. (2012), although some researchers have focused on the development of reliable methodologies to quantify illicit drug concentrations in aquatic environments, few data are available on the acute or chronic effects of these compounds on the aquatic community. Cyto-genotoxic effects were detected in the zebra mussel (*Dreissena polymorpha*) exposed to environmentally relevant concentrations of cocaine (Binelli et al., 2012), benzoylecgonine

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(Parolini et al., 2013), or a realistic mixture of cocaine, benzoylecgonine, amphetamine, morphine and 3,4-methylenedioxymetham-phetamine (Parolini et al., 2015).

The occurrence of cocaine and its main human metabolite, benzoylecgonine, in coastal waters suggests the need for ecotoxicological studies in order to elucidate the mechanism of action and adverse effects of these compounds on marine biota. To the best of our knowledge, no data are available on possible damage produced by illicit drugs or their metabolites on marine organisms.

Among marine organisms, bivalves gained global importance as bioindicators and have been extensively used in biomonitoring programs of coastal waters (Maranho et al., 2015a, 2015b; Pereira et al., 2014; Aguirre-Martínez et al., 2013; Martín-Díaz et al., 2007). These organisms have broad geographical distribution, availability in the field and through aquaculture production, as well as suitability for laboratory and in situ experiments (Maranho et al., 2015c; Cajaraville et al., 2000).

Mussels *Perna perna* are found in natural deposits of the intertidal areas subjected to direct impact of waves and high hydrodynamics. They are filter feeders of phytoplankton and suspended particulate matter. This bioindicator was previously validated as recommended test organism for environmental quality assessments (Pereira et al., 2012, 2011, 2007; Abessa et al., 2005).

Crack cocaine is a very toxic by-product of cocaine, resulting from the addition of sodium bicarbonate to cocaine base paste (Falck et al., 2008). The most recent Brazilian national population-based survey was conducted in 2012 and revealed that two million Brazilians were using crack cocaine (Laranjeira et al., 2012). These data show that its consumption in Brazil is continuing to increase and might be considered as “epidemic”, mainly in metropolitan regions (Oliveira et al., 2014). This compound is able to induce genotoxicity in peripheral blood, liver and brain cells of mice (Yujra et al., 2016), as well as genomic damage in multiple organs of Wistar rats (Moretti et al., 2016).

In light of the high consumption of crack cocaine in the metropolitan coastal region of Santos (São Paulo, Brazil), the occurrence in seawater, and the potential toxicity for aquatic organisms, our study aimed to assess fertilization rate, embryotoxicity and cyto-genotoxicity of crack cocaine in different life stages of the marine mussel *Perna perna*.

An aliquot of crack cocaine (100 mg), as well as the highest concentration of the biomarker's assay (500 $\mu g \cdot L^{-1}$) were analyzed by LC-MS/MS to quantify cocaine. Samples were analyzed by an HPLC Agilent 1260 (Agilent Technologies, CA, USA) combined with a 3200 QTRAP hybrid triple quadrupole/LIT (linear ion trap) mass spectrometer Sciex, Ontario (Canada), according procedure described by Shihomatsu (2015) and employed by Pereira et al. (2016). The cocaine standard used was bought from Cerilliant (FE07271503).

The conditions used for the LC separation were as follows: An injection volume of 10 µL of each sample was analyzed by an Agilent Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm column at 25 °C. The eluent flow rate was 0.7 $mL \cdot min^{-1}$, and the mobile phase for positive mode analysis was 0.1% formic acid (Sigma-Aldrich LC-MS Grade) in water (solvent A) and acetonitrile (J.T. Baker LC-MS Grade) (solvent B). A linear gradient of 0.7 $mL \cdot min^{-1}$ was used, starting with a mixture of 95% solvent A and 5% solvent B. The solvent A percentage decreased linearly from 95% to 5% over the course of 5 min and this condition was maintained for 1 min. The mixture was then returned to the initial condition over the course of 2 min. Cocaine was detected and quantified using ESI ionization and Multiple Reaction Monitoring (MRM) mode, with the selection of a precursor ion and two ion products to quantify and qualify the compound. Data were recorded and processed using Analyst® 1.5.2 (Sciex, Ontario, Canada).

MRM parameters, limit of detection (LOD) and limit of quantification (LOQ) are shown in Table 1.

Adult mussels *Perna perna* were acquired from a long-line shellfish-farming zone located at the Toque-Toque beach, São Sebastião (São Paulo, Brazil). Individuals (average 74.5 × 36.2 mm) were transported to the laboratory where they were kept in a 300 L aquarium filled with seawater for 24 h prior to the assays, under controlled temperature (25 °C) and salinity (35 ppt).

Table 1
Parameters of multiple reactions monitoring for the positive ion mode, limit of detection, limit of quantification and retention time.

Compound	Q1	Q3	DP (V)	CE (V)	CXP (V)	LOD ($ng \cdot L^{-1}$)	LOQ ($ng \cdot L^{-1}$)	RT (min)
Cocaine	304.2	182.2 105.1	36 36	27 39	4 4	3.0	12.0	3.90

Q1 (first quadrupole); Q3 (last quadrupole); DP (Declustering potential); CE (Collision Energy); CXP (Collision Exit Potential); LOD (Limits of detection); LOQ (Limits of quantification); RT (Retention Time). In Q3, in the upper cell is the quantifier ion and in the lower cell is the qualifier ion.

Natural seawater employed in the bioassays was obtained in the mussel farm area, previously selected based on the results of the environmental monitoring performed by the São Paulo Environmental Agency, which has detected aquatic contaminants below threshold levels in this area (CETESB, 2016). Seawater was previously filtered through a cellulose membrane of 0.22 µm for the acute and chronic assays. For the biomarker assays, natural seawater was filtered (150 µm) and maintained under aeration during the assays.

In order to assess the fertilization rate and larvae development, bioassays were performed following the protocol recommended for mussels by ASTM (1992), with minor adaptations proposed by Zaroni et al. (2005). For the fertilization rate (n = 5) and the larvae development assays (n = 5), adult individuals were induced to spawn by thermal stimulation, and gametes from at least three males and three females were collected separately and transferred to glass beakers. Four replicates were used for concentrations ranged from 0 to 100 $mg \cdot L^{-1}$, including the solvent control (seawater plus DMSO 1:500 v/v).

The fertilization rate was assessed by adding a solution of sperm (density around 5×10^7 spermatozoa/mL) to glass tubes containing crack cocaine concentrations. After 40 min, oocytes were added to the glass tubes. After 60 min of exposure, formaldehyde was added to each vial and the first 100 fertilized eggs from each replicate were analyzed with the aid of a Sedgwick-Rafter under an optical microscope. Fertilization was checked for the presence of polar body or start of cleavages.

For the embryo-larval development assay, the fecundation was first attained by adding 2 mL of sperm solution to 200 mL of ovules solution. With the aid of a Sedgwick-Rafter chamber, the density of fertilized eggs was estimated and about 500 embryos were transferred to glass tubes containing crack cocaine concentrations for a period of 48 h at 25 °C and salinity of 35 ppt. Four replicates were used for each group, including solvent control (seawater plus DMSO 1:500 v/v). After the exposure period, formaldehyde was added to each vial and the first 100 larvae from each replicate were analyzed. Larvae developed to D-phase were considered normal, whereas those presenting delay and/or morphological anomalies (irregular shape and extravasation of contents) were considered abnormal (Pereira et al., 2014; Cortez et al., 2012; Zaroni et al., 2005).

The mean percentage of normal fertilization rate and embryo-larval development was calculated for each tested concentration. Through these bioassays were obtained (i) the mean concentration of crack cocaine that causes fertilization rate and embryo-larval development inhibition to 50% of the exposed organisms (IC_{50}; 48 h); (ii) the highest tested concentrations showing no adverse effects (No Observable Effect Concentration - NOEC); and (iii) the lowest tested concentrations showing significant toxicity (Lowest Observable Effect Concentration - LOEC).

Mussels *P. perna* were exposed for 48 h to four crack cocaine concentrations in a way to evaluate cytotoxicity (lysosomal membrane